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(71) Applicant: YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL/IL]; At the Weizmann Institute of Science, P.O. Box 95, 7610002 Rehovot (IL).

(72) Inventors: CHEN, Alon; 3 Ashbal Street, The Weizmann Institute of Science, 7610002 Rehovot (IL). ISSLER, Orna; c/o Yeda Research and Development Co. Ltd., At the Weizmann Institute of Science, P.O. Box 95, 7610002 Rehovot (IL).

(74) Agents: EHRLICH, Gal et al.; G. E. Ehrlich (1995) Ltd., 11 Menachem Begin Road, 5268104 Ramat Gan (IL).

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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:** A method of treating a bipolar disorder in a subject in need thereof is disclosed. The method comprising administering to the subject a therapeutically effective amount of a miR-135, a precursor thereof or a nucleic acid molecule encoding said miR-135 or said precursor thereof, thereby treating the bipolar disorder. Methods of diagnosing a mood disorder in a human subject and of monitoring treatment of an anti-depressant drug or a medicament for the treatment of a mood disorder are also disclosed.

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

5 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.

10 Mood disorders, such as major depression, and anxiety disorders 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.

15 The current dogma regarding the etiology of depression is of a complex interaction between environmental factors and genetic predisposition, suggesting a mechanistic role for epigenetic processes.

20 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 dysregulated 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 25 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.

22 MicroRNAs (miRs) are a subset of endogenous small (approximately 22 nucleotide) noncoding RNA molecules that repress gene expression post-30 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' 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 10 normal process such as plasticity [Kosik KS. Nat Rev Neurosci (2006) 7:911-20]. Several miR-screening studies have reported that miR levels in various adult rodents or human brain structures are affected by a range of behavioral and pharmacological manipulations [O'Connor R. M. et al., Mol Psychiatry (2012) 17: 359-376]. Additionally, it has been suggested that miRs play a role in psychiatric disorders such as 15 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 20 related disorders.

U.S. Patent Application No. 20100222413 (to Stoffel M. et al.) discloses chemically modified oligonucleotides 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.

25 SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of treating a bipolar disorder in a subject in need thereof, the method comprising administering to the subject therapeutically effective amount of a miR-135, a precursor thereof or a nucleic acid molecule encoding the miR-135 or the precursor 30 thereof, thereby treating the bipolar disorder.

According to an aspect of some embodiments of the present invention there is provided a use of a therapeutically effective amount of a miR-135, a precursor thereof or a nucleic acid molecule encoding the miR-135 or the precursor thereof for the manufacture of a medicament identified for treating a bipolar disease in a subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a packaging material packaging an agent selected from the group consisting of a miR-135, a precursor thereof and a nucleic acid molecule encoding the miR-135 or the precursor thereof and a medicament for the treatment of a bipolar disorder.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring treatment of an anti-depressant drug or a medicament for the treatment of a mood disorder, the method comprising: (a) treating a human subject in need thereof with an anti-depressant drug or a medicament for the treatment of a mood disorder; and (b) measuring an expression level of a miR-135 in biological sample of the human subject prior to and following the treatment, wherein a higher expression level of the miR-135 following the treatment by the anti-depressant drug or the medicament for the treatment of the mood disorder as compared to the expression level of the miR-135 prior to the treatment by the anti-depressant drug or the medicament for the treatment of the mood disorder 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 mood disorder in a human subject in need thereof, the method comprising measuring an expression level of a miR-135 in a biological sample of the human subject, wherein a lower expression level of the miR-135 as compared to that in a biological sample of a healthy human subject is indicative of the mood disorder.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a miR-135 wherein the miR-135 comprises a modification selected from the group consisting of a locked nucleic acid (LNA) and a 2'-Fluoroarabinooligonucleotides (FANA).

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a modified miR135 selected from the group consisting of SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 203, SEQ ID NO: 204, SEQ ID NO: 205, SEQ ID NO: 206, SEQ ID NO: 207, SEQ ID NO: 208 and SEQ ID NO: 209.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a microRNA selected from the group consisting of miR-335, miR-181, miR-182, miR-26, miR-27, miR-15 and miR-19 wherein the microRNA comprises a modification selected from the group consisting of a locked nucleic acid (LNA) and a 2'-Fluoroarabino-oligonucleotides (FANA).

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising the isolated polynucleotide of some embodiments of the invention, and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the present invention there is provided a method of upregulating an expression of a gene selected from the group consisting of adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-

activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); 5 myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, 10 cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent 15 mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syne1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming 20 growth factor, beta receptor II (Tgfb2); thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and zinc finger, BED domain containing 4 (Zbed4) in a neuroglia cell, the method comprising: (a) downregulating an activity or expression of a miR-135 or a precursor thereof in the neuroglia cell; and (b) measuring an expression of the gene in the 25 neuroglia cell, thereby upregulating the expression of the gene.

According to an aspect of some embodiments of the present invention there is provided a method of downregulating an expression of a gene selected from the group consisting of adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, 30 alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent,

L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syne1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfb2); thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and

zinc finger, BED domain containing 4 (Zbed4) in a neuroglia cell, the method comprising: (a) upregulating an activity or expression of a miR-135 or a precursor thereof in the neuroglia cell; and (b) measuring an expression of the gene in the neuroglia cell, thereby downregulating the expression of the gene.

5 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 the subject an agent capable of downregulating an activity or expression of a miR-135 target selected from the group consisting of adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell 10 adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a 15 glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor 20 coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent 25

kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related 5 Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier 10 family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syne1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfbr2); thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, 15 subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and zinc finger, BED domain containing 4 (Zbed4), wherein said agent is not miR-135, thereby treating the medical condition.

According to some embodiments of the invention, the medical condition is selected from the group consisting of a bipolar disorder, a depression, a major depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, 20 a compulsive behavior, an addiction, a social phobia, a schizophrenia, a sleep disorder, an eating disorder, a growth disorder and a reproduction disorder.

According to some embodiments of the invention, the miR-135 is selected from the group consisting of miR-135a and miR-135b.

According to some embodiments of the invention, the miR-135 is as set forth in 25 SEQ ID NO: 58-62.

According to some embodiments of the invention, the miR-135 comprises miR-135\* as set forth in SEQ ID NO: 192-193.

According to some embodiments of the invention, the miR-135 comprises a modification selected from the group consisting of a modified sugar-phosphate backbone 30 and a modified base.

According to some embodiments of the invention, the miR-135 comprises a modification in both a sugar and an internucleoside linkage.

According to some embodiments of the invention, the modification is selected from the group consisting of a phosphorothioate, a chiral phosphorothioate, a phosphorodithioate, a phosphotriester, an aminoalkyl phosphotriester, a methyl phosphonate, an alkyl phosphonate, a chiral phosphonate, a phosphinate, a phosphoramidate, an aminoalkylphosphoramidate, a thionophosphoramidate, a thionoalkylphosphonate, a thionoalkylphosphotriester, a boranophosphate, a phosphodiester, a 2'-O-methoxyethyl, a 2'-O-methyl, a 2'-fluoro, a locked nucleic acid (LNA), a peptide nucleic acid (PNA) and a 2'-Fluoroarabinooligonucleotides (FANA).

According to some embodiments of the invention, the miR-135 is as set forth in  
10 SEQ ID NO: 194-209.

According to some embodiments of the invention, the bipolar disorder is selected from the group consisting of Bipolar I, Bipolar II, Rapid-cycling bipolar disorder, Cyclothymia and Bipolar Disorder Not Otherwise Specified (BD-NOS).

According to some embodiments of the invention, the miR-135 is selected from  
15 the group consisting of SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61 and SEQ ID NO: 62.

According to some embodiments of the invention, the miR-135 comprises miR-135\* selected from the group consisting of SEQ ID NO: 192 and SEQ ID NO: 193.

According to some embodiments of the invention, the miR-135 is selected from  
20 the group consisting of SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 203, SEQ ID NO: 204, SEQ ID NO: 205, SEQ ID NO: 206, SEQ ID NO: 207, SEQ ID NO: 208 and SEQ ID NO: 209.

According to some embodiments of the invention, the medicament for the  
25 treatment of a bipolar disorder is selected from the group consisting of a lithium, an antipsychotic medicament and a mood stabilizer medicament.

According to some embodiments of the invention, the method further comprises  
(c) treating the human subject when a higher expression level of the miR-135 is observed in step (b).

According to some embodiments of the invention, the method further comprises  
30 obtaining a biological sample from the human subject prior to the treating.

10

According to some embodiments of the invention, the mood disorder comprises a bipolar disorder.

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

According to some embodiments of the invention, the medicament for the treatment of the mood disorder is selected from the group consisting of a lithium, an antipsychotic medicament and a mood stabilizer medicament.

According to some embodiments of the invention, the biological sample is  
10 selected from the group consisting of a whole blood, a serum, a plasma and white blood cells.

According to some embodiments of the invention, the mood disorder is selected from the group consisting of a bipolar disorder, a depression, a major depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive  
15 behavior, an addiction, a social phobia, a schizophrenia, a sleep disorder and an eating disorder.

According to some embodiments of the invention, the miR-135 is selected from the group consisting of miR-135a or miR-135b.

According to some embodiments of the invention, the subject is a human subject.  
20 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  
30 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  $\pm$  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 with 5HT 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  $\pm$  s.e.m. \*P = 0.014, \*\*\*P = 0.0002, for miR-16 #p < 0.0535, for miR-27 #P = 0.0967; Figure 2D

## 12

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 5 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 10 miR-135b targets Htr1a via both the seed matches and miR-135a only by seed 2. \*\*\*P < 0.0001.

FIGs. 3A-F 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  $\pm$  s.e.m. (n=8 in the 0 group, n=10 in the 90 15 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 20 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 25 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.

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  $\pm$  s.e.m. (n=5 30

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 5 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 10 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  $\pm$  SEM n= 7 controls and 18 mice in social defeat group, # 15 = 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 20 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. 25 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 30 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

15

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 5 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 10 brain (adapted from Allen Brain Atlas [www.mousebrain-map.org/](http://www.mousebrain-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 15 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  $\beta$ -adrenoreceptors, increasing the consolidation of emotionally-aversive memories.

FIGs. 17A-B illustrate Ago2 in the RISC complex. Figure 17A is a schematic 20 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 novel ICR mouse was placed in the chamber 30 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. 27-29 depict results of a luciferase reporter assay indicating that miR182 targets DSCAM, L1CAM and TSNAX 3'UTR. Figure 27 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 28) and Tsna (Figure 29) 3' UTRs blocked the represoric 5 effect of miR182. Bars represent mean  $\pm$  s.e.m. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

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 10 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 15 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 20 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 25 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.

30 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 5 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 10 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 15 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.

FIGs. 35A-D illustrate microRNA “fingerprint” of 5HT neurons. (Figure 35A) Schematic illustration of the experimental design for determining 5HT neuronal 20 microRNA “fingerprint”. ePet-EYFP mouse embryo hindbrains were dissected and FACS sorted to 5HT-YFP positive and YFP negative cells. miR expression from the two populations was compared using the Agilent microRNA microarray; (Figures 35B-35D) Validation of cell phenotype by real time PCR indicating that YFP (Figure 35B) and TPH2 (Figure 35C) were significantly enriched in 5HT compared to non-5HT cells and that GAD67, a GABAergic marker, was significantly higher in non-5HT cells (Figure 25 35D). Bars represent mean  $\pm$  s.e.m. \*\*\*P<0.001.

FIGs. 36A-C illustrate evolutionary conservation of miR-135 variants. (Figures 36A-C) miR-135a-1 (Figure 36A), miR-135a-2 (Figure 36B) and miR-135b (Figure 36C) are highly conserved through evolution while the mature miR sequences, highlighted in color, are almost perfectly conserved. Data modified from UCSC genome 30 browser (Kent WJ, 2002).

FIGs. 37A-G illustrate that miR-135 is upregulated in adult mouse RN following antidepressant treatment. (Figure 37A) Alignment between mature miR-135a and miR-

135b indicating one nucleotide difference; (Figure 37B) Expression levels of miR-135a and miR-135b in the mouse RN; (Figure 37C) Expression profile of several miRs in adult mouse RN indicating miR-135a is approximately 5 times less abundant than miR-124, and 2.5 fold less than miR-16. Bars represent means  $\pm$  s.e.m; (Figure 37D) Mice 5 exposed to social defeat demonstrated increased social avoidance unless treated with chronic imipramine. Interaction ratio is calculated as time spent in the zone near the non-familiar mouse divided by the time spent in the same zone during habituation multiplied by 100. Bars represent means  $\pm$  s.e.m. \*P<0.05; (Figures 37E and 37F) miR-135a levels were upregulated in the RN following chronic (Figure 37E) or acute (Figure 37F) 10 imipramine administration and were unchanged following exposure to chronic social defeat protocol. Bars represent means  $\pm$  s.e.m. \*\*P<0.01; (Figure 37G) SSRI and not NRI or saline, following either acute or chronically administration, caused a significant 15 increase in miR-135a levels in the RN. Bars represent means  $\pm$  s.e.m. \*\*\*P<0.001.

FIGs. 38A-M illustrate that overexpression of miR-135 specifically in 5HT 15 neurons cause behavioral resiliency to social defeat. (Figure 38A) Schematic illustration of miR-135 conditional overexpression mouse model. Transgenic mice with floxed transcriptional STOP sequence upstream to miR-135a and GFP sequences were crossed with the ePet-Cre recombinase mice. Double transgenic mice overexpress miR-135a 20 specifically in 5HT-positive cells. Littermate mice carrying only the transgene for miR-135a and not the ePet-Cre transgene served as controls; (Figure 38B) miR-135a expression levels in the RN were upregulated by approximately 2 fold in miR-135 overexpressing (OE) mice compared to controls; (Figures 38C and 38D) miR-135 target 25 genes, Slc6a4 (Figure 38C) and Htr1a (Figure 38D) mRNA were downregulated in miR-135OE mice compared to control littermates. Bars represent means  $\pm$  s.e.m. # P<0.1; \*P<0.05; (Figures 38E-38G) In the dark-light transfer test miR-135OE mice and their 30 control littermates were tested either under 'basal' stress conditions or following chronic social defeat. No differences were observed between the genotypes under 'basal' conditions, however, following chronic social defeat miR-135OE mice spent more time in light (Figure 38E), visited the lit compartment more frequently (Figure 38F) and traveled a longer distance in light (Figure 38G); The behavioral performance of the miR-135OE mice did not significantly differ following the social defeat protocol. In contrast, control mice demonstrated significant increase in anxiety like-behaviors in all measured

20

parameters of the dark-light test following social defeat. Bars represent means  $\pm$  s.e.m. \*P<0.05, \*\*\*P<0.001; (Figures 38H-38J) In the elevated plus-maze test, control mice that were exposed to social defeat spent less time (Figure 38H), had a smaller number of visits (Figure 38I) and traveled less distance (Figure 38J) in the open arms, compared to 5 control mice tested under ‘basal’ conditions. No significant differences between ‘basal’ and stress conditions were observed in the miR-135OE group. Bars represent means  $\pm$  s.e.m. \*\*P<0.01, \*\*\*P<0.001; (Figures 38K and 38L) In the forced swim test no significant differences were observed between the groups when tested under ‘basal’ stress conditions (Figure 38K), however, when tested following chronic social defeat 10 miR-135OE mice demonstrated decreased immobility compared to control littermates (Figure 38L). Line graphs represent means  $\pm$  s.e.m. \*\*\*p<0.001; (Figure 38M) No differences in locomotion activity were observed between the miR-135OE and control littermates as measured by total distance traveled in the open-field test. Bars represent means  $\pm$  s.e.m.

15 FIGs. 39A-N illustrate that knockdown of miR-135 in the RN of adult mice caused increased anxiety-like behavior and attenuated response to antidepressants. (Figure 39A) Schematic illustration of “miR-135 capture” structure; (Figure 39B) Schematic illustration of miR-135 KD and control viral vectors; (Figure 39C) miR-135 KD lentiviruses infection increased Htr1a and Slc6a4 mRNA expression levels in 20 RN46a cells that endogenously express these genes and miR-135; (Figure 39D) Brain section map showing the site of injection, adapted from the Paxinos and Franklin mouse brain atlas (Paxinos, 1997) (left panel) and GFP immunostaining in DRD of adult mice infected with miR-135KD lentiviruses; (Figure 39E-39H) In the dark light transfer test, miR-135KD mice spent less time (Figure 39E) had fewer visits (Figure 39F) and traveled less distance (Figures 39G and 39H) in the light compartment, compared to control KD injected mice. Bars represent means  $\pm$  s.e.m. \*P<0.05; (Figures 39I-39L) In 25 the elevated plus-maze test miR-135KD mice demonstrated tendency to spend less time (Figure 39I) had fewer visits (Figure 39J) and travel significantly less distance (Figures 39K and 39L) in the open arms. Bars represent means  $\pm$  s.e.m. # P<0.1, \*P<0.05; (Figure 39M) In the forced swim test, miR-135KD mice did not differ in their 30 immobility time from control mice when tested under ‘basal’ conditions, however when tested 30 minutes following SSRI administration, miR-135KD mice demonstrated

increased immobility time, indicating attenuated response to antidepressants. Bars represent means  $\pm$  s.e.m. \*p<0.05; \*\*\*p<0.001; (Figure 39N) No significant differences in locomotor activity between miR-135KD and control mice were observed as measured by total distance traveled in the open-field test. Bars represent means  $\pm$  s.e.m.

5 FIGs. 40A-O illustrate that overexpression of miR-135 in 5HT neurons alters 5HT levels across the brain and blocks social defeat induced 5HT reduction. (Figures 40A, 40D, 40G, 40J, 40M) Schematic illustration of microdissection sites from miR-135OE 5HT and control mouse brains under basal conditions or following chronic social defeat. PrL - prelimbic cortex, BLA - basolateral amygdala, CA1V - hippocampus CA1 10 ventral, DRV - dorsal raphe nucleus, ventral part, MnR - median raphe nucleus. Section map adopted from (Paxinos, 1997); (Figures 40B, 40E, 40H, 40K, 40N) 5HT levels as measured by HPLC in different brain sites revealed decreased 5HT levels in miR-135OE mice compared to controls in basal stress conditions. Additionally, 5HT levels were downregulated in control mice exposed to social defeat compared to basal stress 15 conditions, an effect not observed in miR-135OE 5HT mice; (Figures 40C, 40F, 40I, 40L, 40O) 5HT metabolism calculated as the ratio between the levels of the metabolite 5HIAA to 5HT levels was upregulated in miR-135OE 5HT mice compared to controls in basal stress conditions. Furthermore, 5HT metabolism was reduced in the BLA, CA1V, 20 DRV and MnR in miR-135OE 5HT exposed to chronic social defeat compared to control mice from the same genotype. In the PrL, DRV and MnR 5HT metabolism was upregulated in control mice exposed to social defeat compared to basal conditions. Bars represent means  $\pm$  s.e.m. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

FIGs. 41A-E illustrates lower levels of miR-135 in the blood of human depressed patients. (Figure 41A) miR-135a levels in total blood of depressed human patients are 25 robustly reduced compared to those of healthy controls; (Figure 41B) miR-16 blood levels do not differ significantly between the groups. Bars represent means  $\pm$  s.e.m. \*\*\*P<0.001; (Figure 41C) Depressed patients treated for 3 months with cognitive behavioral therapy (CBT) showed a significant increase in total blood miR-135a levels; (Figure 41D) miR-16 levels were similar in all groups of patients. Bars represent means 30  $\pm$  s.e.m. \*P<0.05; (Figure 41E) Schematic representation of a suggested model describing the involvement of miR-135 in regulating serotonergic synapse components under normal condition (upper panel), depression (middle panel) and antidepressant

administration (lower panel).

FIGs. 42A-L illustrate 5HT levels in RN structures of miR-135OE mice under 'basal' conditions and following chronic stress conditions. (Figures 42A, 42D, 42G, 42J) Schematic illustration of RN microdissection sites from miR-135OE 5HT and control mouse brains under basal conditions or following chronic social defeat. DRD - dorsal raphe nucleus, dorsal part, DRI - dorsal raphe nucleus, interfascicular part, DRVL - dorsal raphe nucleus, ventrolateral part, VLPAG - ventrolateral periaqueductal gray; DRC - dorsal raphe nucleus, caudal part. Section map adopted from (Paxinos G., 1997); (Figures 42B, 42E, 42H, 42K) 5HT levels in all the brain areas illustrated apart from the DRVL were decreased in miR-135OE-5HT mice compared to controls in basal stress conditions. Additionally, 5HT levels were decreased in control mice exposed to social defeat compared to basal conditions, an effect missing in the miR-135OE 5HT mice; (Figures 42C, 42F, 42I, 42L) 5HT metabolism calculated as the ratio between the levels of the metabolite 5HIAA to 5HT levels, was increased in miR-135OE 5HT mice compared to controls in basal stress conditions. Furthermore, 5HT metabolism was reduced in all the areas described in miR-135OE 5HT exposed to chronic social defeat compared to mice from the same genotype tested under basal conditions. Bars represent means  $\pm$  s.e.m. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

FIGs. 43A-L illustrates 5HT levels in brain structures of miR-135OE mice under 'basal' conditions and following chronic stress conditions. (Figures 43A, 43D, 43G, 43J) Schematic illustration of microdissection sites from miR-135OE 5HT and control mouse brains under basal conditions or following chronic social defeat. IL - infralimbic cortex, BNST - bed nucleus of stria terminalis, CeA - central amygdala, S -subiculum. Section map adopted from (Paxinos G., 1997); (Figures 43B, 43E, 43H, 43K) 5HT levels in all the brain areas illustrated were decreased in miR-135OE-5HT mice compared to controls in basal stress conditions. Additionally, 5HT levels were decreased in control mice exposed to social defeat compared to basal conditions, an effect missing in the miR-135OE 5HT mice; (Figures 43C, 43F, 43I, 43L) 5HT metabolism calculated as the ratio between the levels of the metabolite 5HIAA to 5HT levels was increased in miR-135OE 5HT mice compared to controls in basal stress conditions. Furthermore, 5HT metabolism was reduced in all the areas described in miR-135OE 5HT exposed to chronic social defeat compared to mice from the same genotype tested under basal

23

conditions. Bars represent means  $\pm$  s.e.m. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

FIG. 44 illustrates modifications to miR-135 oligonucleotides. 5'Ph illustrates a 5' phosphorylation while bold underline illustrates a 2' O-methylation.

#### DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

5 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.

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

15 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.

20 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.

25 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.

30 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 identify 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 5 illustrated that miR-135 expression levels are altered in the RN following acute stress (Figures 3A-D) and following treatment with antidepressants (Figures 3E-F). *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, 10 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 cannabinoid receptor 1 (CB1) by miR-19. miR-19 over-expression repressed Adrb1 15 (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 20 same, such as miR-135, miR-335, miR-181, miR-182, miR-26, miR-27, miR-15 and miR-19, as therapeutic modalities.

While further reducing the present invention to practice, the present inventor has uncovered that miR135 can be used as a potent therapeutic agent for the treatment of bipolar disorder, which affects as much as 4 % of people.

25 While further reducing the present invention to practice, the present inventors have uncovered that miR-135 is significantly down-regulated in the blood of human depressed patients (as compared to healthy controls) and is upregulated following improvement in the patients' psychiatric score. In fact, miR-135 was found to be an essential regulatory element responsible for maintaining intact serotonergic tone under 30 normal conditions, and essential for the brain response to antidepressants (see schematic model in Figure 41E).

Increased levels of miR-135 were found to repress both Slc6a4 and presynaptic Htr1a levels, causing an increase in 5HT in the synaptic cleft, which is associated with decreases in depressive symptoms. Further bioinformatic analysis conducted by the present inventors, predicted new targets for miR135 which are associated with 5 neuropsychiatric disorders including bipolar affective disorder or lithium action. These targets may thus be used as targets for therapeutic intervention in neuropsychiatric disorders.

The present assay further provides a non-invasive test for both screening patients for psychiatric conditions and monitoring treatment. Together, these results place miR-10 135 as a pivotal tool for the diagnosis and management of psychiatric conditions such as mood disorders in which continuous monitoring of patients psychological balance is critical.

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 15 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 20 miR-135, miR-335, miR-26 and miR-182.

According to one aspect of the present invention there is provided a method of treating a bipolar disorder in a subject in need thereof, the method comprising administering to the subject therapeutically effective amount of a miR-135, a precursor thereof or a nucleic acid molecule encoding the miR-135 or the precursor thereof, 25 thereby treating the bipolar disorder.

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 30 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 5 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 10 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 15 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.

20 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 25 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” or “subject in need thereof” includes 30 mammals, such as human beings, male or female, at any age which suffers from the pathology or 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).

5 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 10 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.

15 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).

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

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

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

30 As used herein the term "bipolar disorder", also known as bipolar affective disorder, manic-depressive disorder, or manic depression, refers to the mental illness classified as a mood disorder. Typically, individuals with bipolar disorder experience one or more episodes of an abnormally elevated state, clinically referred to as mania.

Mania may occur with different levels of severity. At milder levels of mania, or "hypomania", individuals may appear energetic, excitable, and may be highly productive. As mania becomes more severe, individuals begin to behave erratically and impulsively, often making poor decisions due to unrealistic ideas about the future, and 5 may have great difficulty with sleep. At the most severe level, individuals may experience psychosis. The manic episodes typically alternate with episodes or symptoms of depression, or mixed episodes which present features of both mania and depression. Such episodes are normally separated by periods of normal state. Manic and depressive episodes may last from a few days to several months, but in some patients 10 depression and mania may rapidly alternate, referred to as rapid cycling.

Bipolar disorder according to some embodiments of the present invention encompasses any type of bipolar disorder and any form and/or subform of bipolar disorder, including but not limited to, mania, acute mania, severe mania, hypomania, depression, moderate depression, dysthymia, severe depression, episodes of mania 15 and/or depression, psychosis/psychotic symptoms (e.g. hallucinations, delusions), mixed bipolar state, bipolar I disorder, bipolar II disorder, rapid-cycling bipolar disorder, Cyclothymia and/or Bipolar Disorder Not Otherwise Specified (BD-NOS).

According to one embodiment, treating a bipolar disorder may be effected by administering to the subject a therapeutically effective amount of a miR-135, a precursor 20 thereof or a nucleic acid molecule encoding the miR-135 or the precursor thereof.

According to one embodiment, treating a bipolar disorder may be further effected by administering to the subject a medicament for the treatment of a bipolar disorder.

Exemplary medicaments for the treatment of a bipolar disorder which may be used in accordance with the present teachings include, but are not limited to, lithium, 25 antipsychotic medicaments and mood stabilizer medicaments as described in further detail below.

According to one embodiment, there is provided a use of a therapeutically effective amount of a miR-135, a precursor thereof or a nucleic acid molecule encoding the miR-135 or the precursor thereof for the manufacture of a medicament identified for 30 treating a bipolar disease in a subject in need thereof.

Thus, according to one embodiment, when the medical condition is a mood disorder, e.g. bipolar disease, depression or anxiety, the microRNA is miR-135.

It will be appreciated that the mood disorder, e.g. bipolar disease, depression or anxiety, may not necessarily be related to serotonin.

According to one embodiment, 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 the subject an agent capable of downregulating an activity or expression of a miR-135 target gene selected from the group consisting of adenylyl cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylyl cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1

30

(Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 5 (choline transporter), member 7 (Slc5a7); solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syne1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfbr2); thyroid hormone receptor, beta 10 (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and zinc finger, BED domain containing 4 (Zbed4).

According to a specific embodiment, the agent is not miR-135.

Agents which can be used in accordance with the present teachings (e.g., capable 15 of downregulating an activity or expression of a miR-135 target gene) are described in detail below.

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 20 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 25 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 and arrhythmia), 30 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 5 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 10 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 15 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, 20 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)].

25 The medical condition in which a low glutamate receptor level is therapeutically beneficial may comprise, for example, seizures (e.g. epilepsy), Huntington's 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 30 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 5 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. According to one embodiment, this term encompasses any type of micoRNA including 5 prime (i.e. miR) or 3 prime (i.e. miR\*) and their precursors.

10 As used herein, the term "miR-135 or a precursor thereof" is meant to encompass any type of miR-135 including miR-135a and miR-135b 5 prime (i.e. miR-135) or 3 prime (i.e. miR-135\*) and their precursors. Exemplary precursor miR-135 include, but are not limited to, miR-135a-1 as set forth in Accession No. MI0000452, ENTREZGENE 406925 and SEQ ID NO: 58; miR-135a-2 as set forth in Accession No. 15 MI0000453, ENTREZGENE: 406926 and SEQ ID NO: 59; and miR-135b as set forth in Accession No. MI0000810, ENTREZGENE: 442891 and SEQ ID NO: 60. Exemplary mature miR-135 include, but are not limited to, miR-135a as set forth in Accession No. MIMAT0000428 (SEQ ID NO: 61) and miR-135b as set forth in Accession No. MIMAT0000758 (SEQ ID NO: 62). Exemplary mature miR-135\* include, but are not 20 limited to, miR-135a\* as set forth in Accession No. MIMAT0004595 (SEQ ID NO: 192) and miR-135b\* as set forth in Accession No. MIMAT0004698 (SEQ ID NO: 193).

It will be appreciated that the microRNAs of the present teachings (e.g. miR-135) 25 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 30 other molecules e.g. FK506 binding protein 5 (FKBP5), cannabinoid receptor 1 (CB1), Down Syndrome Cell Adhesion Molecule (Dscam), Translin-associated protein X (Tsnax) and Cell adhesion molecule L1 (L1cam); as well as other targets associated with

stress-related neuropsychiatric disorders (e.g. bipolar disorder) including those listed in Table 1, below.

Table 1: Putative targets of miR-135 associated with stress-related neuropsychiatric disorders

5

| Human ortholog | Gene name   |
|----------------|---|
| Adcyap1        | adenylate cyclase activating polypeptide 1 (also PACAP)                 |
| Adcyap1r1      | adenylate cyclase activating polypeptide 1 receptor 1                   |
| Adra2a         | adrenergic receptor, alpha 2a   |
| Ank3           | ankyrin 3, epithelial   |
| Arc            | activity-regulated cytoskeleton-associated protein                      |
| Arhgap6        | Rho GTPase activating protein 6   |
| Atf3           | activating transcription factor 3                                       |
| Bace1          | beta-site APP cleaving enzyme 1   |
| Cacna1d        | calcium channel, voltage-dependent, L type, alpha 1D subunit            |
| Cadm3          | cell adhesion molecule 3  |
| Cplx1          | complexin 1   |
| Cplx2          | complexin 2   |
| Csmd1          | CUB and Sushi multiple domains 1  |
| Csnk1g1        | casein kinase 1, gamma 1  |
| Dcx            | doublecortin  |
| Diras2         | DIRAS family, GTP-binding RAS-like 2                                    |
| Dlg2           | discs, large homolog 2 (Drosophila)                                     |
| Elk1           | ELK1, member of ETS oncogene family                                     |
| Frk            | fyn-related kinase  |
| Fut9           | fucosyltransferase 9 (alpha (1,3) fucosyltransferase)                   |
| Gabrb2         | gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2               |
| Gata3          | GATA binding protein 3  |
| Ghsr           | growth hormone secretagogue receptor                                    |
| Gpr3           | G protein-coupled receptor 3  |
| Gria3          | glutamate receptor, ionotropic, AMPA3 (alpha 3)                         |
| Grik3          | glutamate receptor, ionotropic, kainate 3                               |
| Grk5           | G protein-coupled receptor kinase 5                                     |
| Gsk3b          | glycogen synthase kinase 3 beta   |
| Hcn1           | hyperpolarization activated cyclic nucleotide-gated potassium channel 1 |
| Hcn2           | hyperpolarization-activated, cyclic nucleotide-gated K+ 2               |
| Htr1a          | 5-hydroxytryptamine (serotonin) receptor 1A                             |
| Impa1          | inositol (myo)-1(or 4)-monophosphatase 1                                |

|         |   |
|---------|---|
| Kalrn   | kalirin, RhoGEF kinase  |
| Kcnn3   | potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 |
| Kpna3   | karyopherin alpha 3 (importin alpha 4)  |
| Myt1l   | myelin transcription factor 1-like  |
| Ncoa2   | nuclear receptor coactivator 2  |
| Ndrg4   | N-Myc Downstream-Regulated Gene 4   |
| Nos1ap  | nitric oxide synthase 1 (neuronal) adaptor protein  |
| Nr3c2   | nuclear receptor subfamily 3, group C, member 2   |
| Ntng1   | netrin G1   |
| Nucks1  | nuclear casein kinase and cyclin-dependent kinase substrate 1                             |
| Pde1a   | phosphodiesterase 1A, calmodulin-dependent  |
| Pde4a   | phosphodiesterase 4A, cAMP specific   |
| Pde8b   | phosphodiesterase 8B  |
| Plcb1   | phospholipase C, beta 1   |
| Prlr    | prolactin receptor  |
| Rab1b   | RAB1B, member RAS oncogene family   |
| Rap2a   | Ras-Related Protein Rap-2a  |
| Rorb    | Retinoid-Related Orphan Receptor Beta   |
| Sirt1   | sirtuin 1 (silent mating type information regulation 2, homolog) 1                        |
| Slc12a6 | solute carrier family 12, (potassium/chloride transporters) member 6                      |
| Slc5a7  | solute carrier family 5 (choline transporter), member 7                                   |
| Slc6a4  | solute carrier family 6 (neurotransmitter transporter, serotonin), member 4               |
| Sp1     | trans-acting transcription factor 1   |
| Sv2b    | synaptic vesicle glycoprotein 2 b   |
| Syne1   | Synaptic nuclear envelope 1 (encodes nesprin-1)   |
| Syt1    | synaptotagmin I   |
| Syt2    | synaptotagmin II  |
| Syt3    | synaptotagmin III   |
| Tgfbr2  | transforming growth factor, beta receptor II  |
| Thrb    | thyroid hormone receptor, beta  |
| Trpc6   | transient receptor potential cation channel, subfamily C, member 6                        |
| Vamp2   | vesicle-associated membrane protein 2   |
| Wnt3    | wingless-related MMTV integration site 3  |
| Zbed4   | zinc finger, BED domain containing 4  |

Table 1, cont.

It will be appreciated that the microRNAs of the present invention can be identified via various databases including for example the micro-RNA registry ([www.sanger.ac.uk/Software/Rfam/mirna/index.html](http://www.sanger.ac.uk/Software/Rfam/mirna/index.html)).

The methods of the present invention may be effected by administering to the subject a microRNA (e.g. miR-135) or an effector thereof or expressing in a cell of the subject an exogenous nucleic acid molecule (i.e. polynucleotide) encoding the microRNA (e.g. miR-135) or the precursor thereof. 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. Such modified or substituted oligonucleotides may be preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

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 and 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, 5 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.

10 It will be appreciated that a polynucleotide comprising an RNA molecule can be produced biologically using an expression vector as is further described hereinbelow. Alternatively, a polynucleotide comprising an RNA molecule can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides as described below.

15 According to one embodiment, the polynucleotide of the present invention is a modified polynucleotide. Polynucleotides can be modified using various methods known in the art.

20 Thus, the polynucleotides of the invention can be synthesized to include a modification that imparts a desired characteristic. For example, the modification can improve stability, hybridization thermodynamics with a target nucleic acid, targeting to a particular tissue or cell-type, or cell permeability, e.g., by an endocytosis-dependent or - independent mechanism. Modifications can also increase sequence specificity, and consequently decrease off-site targeting. Chemical modifications are described in greater detail below.

25 According to one embodiment, the polynucleotides comprise a single modification. According to another embodiment, the polynucleotides comprise two, three, four, five or more modifications.

30 For example, the oligonucleotides or polynucleotides of the present invention may comprise heterocyclic 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 (e.g. sugar-phosphate 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.

5 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; 10 5,587,361; 5,625,050 and 8,017,763; as well as in U.S. Pat. Applic. No. 20100222413, incorporated herein by reference.

According to one embodiment, the polynucleotide comprises a phosphorus-modified internucleotide linkage at the 5' or 3' end of the nucleotide sequence.

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; 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'; and boron phosphonate. Various salts, mixed salts, and free acid forms of the above modifications can also be used.

According to one embodiment, the modified polynucleotide comprises a phosphorothioate at the internucleotide linkage at the 5' or 3' end of the nucleotide sequence.

According to one embodiment, the modified polynucleotide comprises a boranophosphate at the internucleotide linkage at the 5' or 3' end of the nucleotide sequence.

30 According to one embodiment, the modified polynucleotide comprises a methyl phosphonate at the internucleotide linkage at the 5' or 3' end of the nucleotide sequence.

According to one embodiment, the modified polynucleotide comprises a

phosphodiester at the internucleotide linkage at the 5' or 3' end of the nucleotide sequence.

According to one embodiment, the polynucleotide comprises a sugar modification (e.g. ribose modification).

5 According to one embodiment, the polynucleotide comprises a modification corresponding to position 2 of the ribose.

According to one embodiment, the modified polynucleotide comprises at least one 2'-modified nucleotide, e.g., a 2'-deoxy, 2'-fluoro, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl 10 (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), 2'-Fluoroarabinooligonucleotides (FANA), or 2'-O--N-methylacetamido (2'-O-NMA).

According to one embodiment, the modified polynucleotide comprises at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides 15 of the modified polynucleotide include a 2'-O-methyl modification.

According to one embodiment, the modified polynucleotide comprises a modified internucleotide linkage and a sugar backbone modification.

According to one embodiment, the modified polynucleotide comprises a phosphorus-modified internucleotide linkage and a sugar backbone modification (e.g. 2'-modified nucleotide).

Exemplary modified miR-135 polynucleotides include, but are not limited to, SEQ ID NOs: 194-209.

Alternatively, modified oligonucleotide or polynucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short-chain alkyl 25 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 30 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<sub>2</sub> 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 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 10 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 15 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.

According to one embodiment, the polynucleotides of the invention can have a 20 chemical modification on a nucleotide in an internal (*i.e.*, non-terminal) region having noncomplementarity with the target nucleic acid. For example, a modified nucleotide can be incorporated into the region of a miRNA that forms a bulge. The modification can include a ligand attached to the miRNA, *e.g.*, by a linker. The modification can, for example, improve pharmacokinetics or stability of the polynucleotide, or improve 25 hybridization properties (*e.g.*, hybridization thermodynamics) of the polynucleotide to a target nucleic acid.

In some embodiments, the orientation of a modification or ligand incorporated into or tethered to the bulge region of a polynucleotide is oriented to occupy the space in the bulge region. For example, the modification can include a modified base or sugar on 30 the nucleic acid strand or a ligand that functions as an intercalator. These are preferably located in the bulge. The intercalator can be an aromatic, *e.g.*, a polycyclic aromatic or heterocyclic aromatic compound. A polycyclic intercalator can have stacking

capabilities, and can include systems with 2, 3, or 4 fused rings. In some embodiments, the orientation of a modification or ligand incorporated into or tethered to the bulge region of the polynucleotide is oriented to occupy the space in the bulge region. This orientation facilitates the improved hybridization properties or an otherwise desired 5 characteristic of the polynucleotide.

In one embodiment, the polynucleotide can include an aminoglycoside ligand, which can cause the polynucleotide to have improved hybridization properties or improved sequence specificity. Exemplary aminoglycosides include glycosylated polylysine; galactosylated polylysine; neomycin B; tobramycin; kanamycin A; and 10 acridine conjugates of aminoglycosides, such as Neo-N-acridine, Neo-S-acridine, Neo-C-acridine, Tobra-N-acridine, and KanaA-N-acridine. Use of an acridine analog can increase sequence specificity. For example, neomycin B has a high affinity for RNA as compared to DNA, but low sequence-specificity. In some embodiments the guanidine 15 analog (the guanidinoglycoside) of an aminoglycoside ligand is tethered to a polynucleotide agent. In a guanidinoglycoside, the amine group on the amino acid is exchanged for a guanidine group. Attachment of a guanidine analog can enhance cell permeability of the polynucleotide.

A polynucleotide can be designed and synthesized to include a region of noncomplementarity (e.g., a region that is 3, 4, 5, or 6 nucleotides long) flanked by 20 regions of sufficient complementarity to form a duplex (e.g., regions that are 7, 8, 9, 10, or 11 nucleotides long) with a target RNA.

For increased nuclease resistance and/or binding affinity to the target, the polynucleotides of the invention can include 2'-O-methyl, 2'-fluorine, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-amino, and/or phosphorothioate linkages. Inclusion 25 of locked nucleic acids (LNA), e.g. inclusion of nucleic acid analogues in which the ribose ring is “locked” by a methylene bridge connecting the 2'-O atom and the 4'-C atom, ethylene nucleic acids (ENA), e.g., 2'-4'-ethylene-bridged nucleic acids, and certain nucleobase modifications such as 2-amino-A, 2-thio (e.g., 2-thio-U), G-clamp modifications, can also increase binding affinity to the target. The inclusion of pyranose 30 sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage.

A polynucleotide can be further modified by including a 3' cationic group, or by inverting the nucleoside at the terminus with a 3'-3' linkage. In another alternative, the

3'-terminus can be blocked with an aminoalkyl group, e.g., a 3' C5-aminoalkyl dT. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3' end of the oligonucleotide.

5 Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

The 5'-terminus can be blocked with an aminoalkyl group, e.g., a 5'-O-alkylamino substituent. Other 5' conjugates can inhibit 5'-3' exonucleolytic cleavage. While not being bound by theory, a 5' conjugate, such as naproxen or ibuprofen, may 10 inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 5' end of the oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

In one embodiment, the polynucleotide includes a modification that improves 15 targeting, e.g. a targeting modification described herein. Examples of modifications that target single-stranded oligonucleotide agents to particular cell types include carbohydrate sugars such as galactose, N-acetylgalactosamine, mannose; vitamins such as folates; other ligands such as RGDs and RGD mimics; and small molecules including naproxen, ibuprofen or other known protein-binding molecules.

20 The polynucleotide of the invention can be constructed using chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. For example, the polynucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the 25 polynucleotide and target nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used.

Oligonucleotides or polynucleotides of the present invention may also include 30 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 5 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 °C (Sanghvi, Y. S. et al. (1993), "Antisense Research and Applications," pages 276-278, CRC Press, Boca Raton), and are presently preferred base substitutions, 10 even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

According to one embodiment, the modified polynucleotide is further modified so as to be attached to a ligand that is selected to improve stability, distribution or cellular uptake of the agent, e.g., cholesterol.

Thus, the polynucleotide may be modified to include a non-nucleotide moiety, 25 e.g., a cholesterol moiety. The non-nucleotide moiety (e.g. cholesterol moiety) can be attached, e.g., to the 3' or 5' end of the polynucleotide.

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).

30

**Table 1A: miRNA polynucleotide sequences**

| <i>Sequence</i>   | <i>miRNA</i> |
|-------------------|--------------|
| SEQ ID NOs: 77-80 | miR-15       |

| <i>Sequence</i>   | <i>miRNA</i> |
|-------------------|--------------|
| 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 5 miRNA precursor molecule.

According to a specific embodiment, the miR-135 comprises miR-135a or miR-135b.

According to a specific embodiment, the precursor miR-135 polynucleotide of the present invention has a nucleic acid sequence as set forth in SEQ ID NOs: 58-60.

10 According to a specific embodiment, the mature miR-135 polynucleotide of the present invention has a nucleic acid sequence as set forth in SEQ ID NOs: 61-62.

According to a specific embodiment, the mature miR-135\* polynucleotide of the present invention has a nucleic acid sequence as set forth in SEQ ID NOs: 192-193.

15 Also contemplated are sequences homologous 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 20 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), 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), miR-135 (e.g. GenBank accession nos. NR\_029677.1, NR\_029678.1, NR\_029893.1), 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).

Such precursor polynucleotide agents are typically administered to the target cells (e.g. neuroglia cells, choroid plexus (CP) cells, stem cells or differentiated stem 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, CP cells, stem cells or differentiated stem cells) in a constitutive or inducible manner.

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.

An exemplary neuroglia cell specific promoter includes glial fibrillary acidic protein (GFAP) promoter.

Examples of choroid plexus specific promoters include, but are not limited to a  $\beta$  splice variant of the type 2 corticotrophin releasing factor receptor (CRFR2 $\beta$ ) promoter, a G protein-coupled receptor 125 (GPR125) promoter and a transthyretin promoter.

According to one embodiment, the promoter sequence (e.g. choroid plexus specific promoter) is placed 3' to the polynucleotide sequence (e.g. miR-135 polynucleotide sequence) on a nucleic acid construct such that expression thereof is constitutive, but tissue specific.

According to another embodiment, the choroid plexus specific promoter sequence is situated relative to the polynucleotide sequence (e.g. miR-135 polynucleotide sequence) on a nucleic acid construct such that expression thereof is tissue specific, but also may be controlled in an exogenously regulatable fashion.

In order to ensure that the polynucleotide of interest is expressed both specifically in cells of the choroid plexus and in an exogenously controllable fashion, a nucleic acid construct may be designed such that it comprises a polynucleotide encoding a transactivator under control of the choroid plexus specific promoter. The 5 polynucleotide may be inserted in the same nucleic acid construct or in an additional construct under control of an inducible promoter. The transactivator in combination with an inducer act to regulate expression from the inducible promoter.

Inducible promoters suitable for use with the present invention are preferably response elements capable for directing transcription of the polynucleotide sequence. A 10 suitable response element can be, for example, a tetracycline response element (such as described by Gossen and Bujard (Proc. Natl. Acad. Sci. USA 89:5547-551, 1992); an ectysone-inducible response element (No D *et al.*, Proc Natl Acad Sci U S A. 93:3346-3351, 1996) a metal-ion response element such as described by Mayo *et al.* (Cell. 29:99-108, 1982); Brinster *et al.* (Nature 296:39-42, 1982) and Searle *et al.* (Mol. Cell. Biol. 15 5:1480-1489, 1985); a heat shock response element such as described by Nouer *et al.* (in: Heat Shock Response, ed. Nouer, L., CRC, Boca Raton, Fla., pp167-220, 1991); or a hormone response element such as described by Lee *et al.* (Nature 294:228-232, 1981); Hynes *et al.* (Proc. Natl. Acad. Sci. USA 78:2038-2042, 1981); Klock *et al.* (Nature 329:734-736, 1987); and Israel and Kaufman (Nucl. Acids Res. 17:2589-2604, 1989). 20 Preferably the response element is an ectysone-inducible response element, more preferably the response element is a tetracycline response element.

Examples of cardiac cell specific promoters include, but are not limited to, cardiac NCX1 promoter and  $\alpha$ -myosin heavy chain ( $\alpha$ MHC) promoter.

The expression constructs of the present invention may also include additional 25 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, enhancers) 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 30 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 5 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.

10 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 moiety. 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 15 AAUAAA, located 11-30 nucleotides upstream of the site. Termination and polyadenylation signals suitable for the present invention include those derived from SV40.

20 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 25 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.

30 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<sup>+</sup>, pMTO10/A<sup>+</sup>, pMAMneo-5 and baculovirus pDSVE.

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  $\psi$  (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).

Various methods can be used to introduce the nucleic acid constructs of the present invention into mammalian cells. Such methods are generally described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang *et al.*, Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega *et al.*, Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa *et al.* [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, 5 electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. 10 Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

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 15 or viral particles.

In order to circumvent the blood brain barrier, the constructs of the present invention may be administered directly into the brain (via the ventricle), into the olfactory bulb (via an intranasal administration), via the spinal cord (e.g. by an epidural catheter) or by expression in the choroid plexus, as further detailed herein.

20 Alternatively, lipid-based systems may be used for the delivery of these constructs into the target cells (e.g. brain cells such as neuroglia cells) of the present invention.

Liposomes include any synthetic (i.e., not naturally occurring) structure 25 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, 30 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. Optionally, the liposomes do not comprise sterically shielded lipids such as ganglioside-GM<sub>1</sub> 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 (miR-135 or the precursor thereof) into a liposome. For example, the micro-RNA polynucleotide agent (miR-135 or the precursor thereof) 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<sup>th</sup> 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 may cross the blood barriers. Thus, according to an embodiment the liposomes of the present invention do not comprise a blood barrier targeting polysaccharide (e.g. mannose) in their membrane portion. Optionally, 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, dendrimers and Gagomers.

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 5 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.

10 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).

15 For *ex vivo* therapy, cells (e.g. neuroglia cells such as oligodendrocytes, CP cells, stem cells and/or differentiated stem cells) are preferably treated with the agent of the present invention (e.g., a microRNA, e.g. miR-135, or a precursor thereof or a polynucleotide encoding the microRNA e.g. miR-135, or a precursor thereof), following which they are administered to the subject in need thereof.

20 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 25 individual using intravenous, intra-kidney, intra-gastrointestinal track, and/or intraperitoneal administration.

The cells of the present invention (e.g. neuroglia cells such as oligodendrocytes, CP cells, stem cells, differentiated stem cells and/or cardiac cells) can be derived from either autologous sources or from allogeneic sources such as human cadavers or donors. 30 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-phenoxy cinnamylidene-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 methacrylate (HEMA), methacrylic acid (MAA), and methyl methacrylate (MMA), resulting in a capsule thickness of 2-5  $\mu$ m. Such microcapsules can be further encapsulated with an additional 2-5  $\mu$ m 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  $\mu$ m (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 5 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 10 (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 15 (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, 20 ketorolac, meclofenamate, naproxen, nabumetone, phenylbutazone, piroxicam, sulindac, tolmetin, acetaminophen, ibuprofen, Cox-2 inhibitors and tramadol.

For *in vivo* therapy, the agent (e.g., microRNA, e.g. miR-135, a precursor thereof or a polynucleotide encoding the microRNA or the precursor thereof) is 20 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) 25 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., 30 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 5 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.

10 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.

15 Herein the term "active ingredient" refers to the peptide accountable for the biological effect (e.g. miroRNA, e.g. miR-135, a precursor thereof, or a polynucleotide encoding the microRNA or the precursor thereof).

20 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.

25 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.

30 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, intraperitoneal, 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.

5 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.

10 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 15 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.

20 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., 25 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.

30 The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous 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 (e.g. miRNA) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., mood disorder such as bipolar disease) 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 5 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 10 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 15 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 20 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 25 (CMS) model, social defeat stress (SDS) model and maternal deprivation model and sleep deprivation model may be used. For example, animal models of bipolar disease include, for example, transgenic mice with neuron-specific expression of mutant Polg (D181A) [as taught by Kato et al., *Neuroscience and Biobehavioral Reviews* (2007) 6 (31):832–842, incorporated herein by reference], as well as the well established mania 30 rat models of Amphetamine-induced hyperactivity [taught e.g. in US Patent No. 6,555,585] and Ketamine-induced hyperactivity [taught e.g. in Ghedim et al., *Journal of Psychiatric Research* (2012) 46: 1569-1575], incorporated by reference, may be used.

60

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 5 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 10 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.

15 It will be appreciated that the therapeutic compositions of the invention may comprise, in addition to the micro-RNA (e.g. miR-135 or polynucleotide encoding same), 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 20 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.

25 According to one embodiment, the therapeutic composition of the invention comprises, in addition to the micro-RNA (e.g. miR-135 or polynucleotide encoding same), a medicament for the treatment of a bipolar disorder. Any medicament or any combination of medicaments for the treatment of a bipolar disorder may be used in accordance with the present teachings, including but not limited to, lithium (e.g. Lithium carbonate, Lithium citrate, Lithium sulfate), antipsychotic medicaments (e.g. typical antipsychotics and atypical antipsychotics, as detailed below), mood stabilizer 30

medicaments (e.g. Valproic acid (VPA, Valproate), minerals, anticonvulsants, antipsychotics) and anti-depressants.

Exemplary typical antipsychotic medicaments which may be used in accordance with the present teachings, include but are not limited to, Low potency medicaments: 5 Chlorpromazine (Largactil, Thorazine), Chlorprothixene (Truxal), Thioridazine (Mellaril), Mesoridazine and Levomepromazine; Medium potency medicaments: Loxapine (Loxapac, Loxitane), Molindone (Molan), Perphenazine (Trilafon) and Thiothixene (Navane); High potency medicaments: Haloperidol (Haldol, Serenace), Fluphenazine (Prolixin), Droperidol, Zuclopentixol (Clopixol), Flupentixol (Depixol), 10 Prochlorperazine and Trifluoperazine (Stelazine). In addition, Prochlorperazine (Compazine, Buccastem, Stemetil) and Pimozide (Orap) may be used.

Exemplary atypical antipsychotic medicaments (also referred to as second generation antipsychotics) which may be used in accordance with the present teachings, include but are not limited to, Amisulpride (Solian), Aripiprazole (Abilify), Asenapine 15 (Saphris), Blonanserin (Lonasen), Bitopertin (RG1678), Brexpiprazole (OPC-34712), Caripramine (Prazinil), Clozapramine (Clofekton), Clozapine (Clozaril), Cariprazine (RGH-188), Iloperidone (Fanapt), Lurasidone (Latuda), LY2140023, Melperone (Buronil), Mosapramine (Cremin), Olanzapine (Zyprexa), Paliperidone (Invega), Perospirone (Lullan), Pimavanserin (ACP-103), Quetiapine (Seroquel), Remoxipride 20 (Roxiam), Risperidone (Risperdal), Sertindole (Serdolect), Sulpiride (Sulpirid), Vabicaserin (SCA-136), Ziprasidone (Geodon), Zotepine (Nipolept) and Zicronapine (Lu 31-130).

Exemplary mood stabilizers which may be used in accordance with the present teachings, include but are not limited to, minerals (e.g. lithium); anticonvulsant mood 25 stabilizers including Valproic acid (Depakine), divalproex sodium (Depakote), and sodium valproate (Depacon, Epilim), Lamotrigine (Lamictal), Carbamazepine (Tegretol), Oxcarbazepine (Trileptal), Topiramate (Topamax), Riluzole (Rilutek) and Gabapentin (Neurontin); antipsychotics (as described above); and food supplements (e.g. omega-3 fatty acids).

30 Exemplary anti-depressants which may be used in accordance with the present teachings, include but are not limited to, Selective serotonin reuptake inhibitors (SSRIs, such as Citalopram, Escitalopram, Fluoxetine, Fluvoxamine, Paroxetine and Sertraline);

62

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);

5 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).

10 According to one embodiment, the anti-depressant drug comprises selective serotonin reuptake inhibitors (SSRI), tricyclic antidepressants and noradrenaline reuptake inhibitors (NRI).

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

15 It will be appreciated that additional non-pharmaceutical therapeutic strategies may be employed in combination with the present teachings, including but not limited to, clinical psychology, electroconvulsive therapy, involuntary commitment, light therapy, psychotherapy, transcranial magnetic stimulation and cognitive behavioral therapy.

20 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).

25 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 Adrb1 is 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 5 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 10 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 cannabinoid receptor 1 (CB1) gene in a neuroglia cell, the method 15 comprising modulating an activity or expression of a miR-19 or a precursor thereof.

As used herein, the term "cannabinoid 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 20 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, 25 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, 5 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.

10 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.

15 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.

20 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.

25 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.

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

According to one embodiment, there is provided a method of downregulating an expression of a gene selected from the group consisting of adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier

family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syn1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfbr2); 5 thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and zinc finger, BED domain containing 4 (Zbed4) in a neuroglia cell, the method comprising: (a) upregulating an activity or expression of a miR-135 or a precursor thereof in the neuroglia cell; and (b) 10 measuring an expression of the gene in the neuroglia cell, thereby downregulating the expression of the gene.

According to a specific embodiment, downregulating the expression of the gene is effected by upregulating an activity or expression of a microRNA or a precursor thereof which is not miR-135.

15 According to a specific embodiment, downregulating the expression of the miR-135 target gene is effected by administering to the subject an agent capable of downregulating an activity or expression of the miR-135 target gene.

20 Downregulation of a gene (e.g. miR-135 target gene) or gene product 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 (e.g., antisense, siRNA, shRNA, micro-RNA), Ribozyme, DNAzyme and a CRISPR system (e.g. CRISPR/Cas)], or on the protein level using e.g., antagonists, enzymes that cleave 25 the polypeptide and the like.

Following is a list of agents capable of downregulating expression level and/or 25 activity of a gene or gene product of some embodiments of the invention.

One example, of an agent capable of downregulating an activity of a polypeptide gene product is an antibody or antibody fragment capable of specifically binding the gene product (i.e. protein). Such inhibition is valuable for extracellular, cell surface or secreted polypeptides in particular. As used herein, the term "epitope" refers to any 30 antigenic determinant on an antigen to which the paratope of an antibody binds.

Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, and Fv that are capable of binding to macrophages. 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; and (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.

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).

Downregulation of a gene (e.g. miR-135 target gene) can be also achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of specifically inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g., the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

According to an embodiment of the invention, the RNA silencing agent is specific to the target RNA (e.g., target gene) and does not cross inhibit or silence a gene or a splice variant which exhibits 99% or less global homology to the target gene, e.g., less than 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81% global homology to the target gene.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an

endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

Accordingly, some embodiments of the invention contemplates use of dsRNA to downregulate protein expression from mRNA.

According to one embodiment, the dsRNA is greater than 30 bp. The use of long dsRNAs (i.e. dsRNA greater than 30 bp) has been very limited owing to the belief that these longer regions of double stranded RNA will result in the induction of the interferon and PKR response. However, the use of long dsRNAs can provide numerous advantages in that the cell can select the optimal silencing sequence alleviating the need to test numerous siRNAs; long dsRNAs will allow for silencing libraries to have less complexity than would be necessary for siRNAs; and, perhaps most importantly, long dsRNA could prevent viral escape mutations when used as therapeutics.

Various studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects - see for example [Strat et al., Nucleic Acids Research, 2006, Vol. 34, No. 13 3803–3810; Bhargava A et al. Brain Res. Protoc. 2004;13:115–125; Diallo M., et al., Oligonucleotides. 2003;13:381–392; Paddison P.J., et al., Proc. Natl Acad. Sci. USA. 2002;99:1443–1448; Tran N., et al., FEBS Lett. 2004;573:127–134].

The invention according to some embodiments thereof also contemplates introduction of long dsRNA specifically designed not to induce the interferon and PKR pathways for down-regulating gene expression. For example, Shinagwa and Ishii [Genes & Dev. 17 (11): 1340-1345, 2003] have developed a vector, named pDECAP, to express long double-strand RNA from an RNA polymerase II (Pol II) promoter. Because the transcripts from pDECAP lack both the 5'-cap structure and the 3'-poly(A) tail that facilitate ds-RNA export to the cytoplasm, long ds-RNA from pDECAP does not induce the interferon response.

Another method of evading the interferon and PKR pathways in mammalian systems is by introduction of small inhibitory RNAs (siRNAs) either via transfection or endogenous expression.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 basepairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that 5 chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

10 It has been found that position of the 3'-overhang influences potency of a siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

15 The strands of a double-stranded interfering RNA (e.g., a siRNA) may be connected to form a hairpin or stem-loop structure (e.g., a shRNA). Thus, as mentioned the RNA silencing agent of some embodiments of the invention may also be a short hairpin RNA (shRNA).

20 The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number 25 between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-3' (Brummelkamp, T. R. et al. (2002) *Science* 296: 550) and 5'-UUUGUGUAG-3' (Castanotto, D. et al. (2002) *RNA* 8:1454). It will be recognized 30 by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

Synthesis of RNA silencing agents suitable for use with some embodiments of the invention can be effected as follows. First, the target gene mRNA sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential 5 siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be 10 effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (www.ambion.com/techlib/tn/91/912.html).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the 15 BLAST software available from the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be 20 more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the 25 genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

It will be appreciated that the RNA silencing agent of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

30 In some embodiments, the RNA silencing agent provided herein can be functionally associated with a cell-penetrating peptide." As used herein, a "cell-penetrating peptide" is a peptide that comprises a short (about 12-30 residues) amino

acid sequence or functional motif that confers the energy-independent (i.e., non-endocytotic) translocation properties associated with transport of the membrane-permeable complex across the plasma and/or nuclear membranes of a cell. The cell-penetrating peptide used in the membrane-permeable complex of some embodiments of the invention preferably comprises at least one non-functional cysteine residue, which is either free or derivatized to form a disulfide link with a double-stranded ribonucleic acid that has been modified for such linkage. Representative amino acid motifs conferring such properties are listed in U.S. Pat. No. 6,348,185, the contents of which are expressly incorporated herein by reference. The cell-penetrating peptides of some embodiments of the invention preferably include, but are not limited to, penetratin, transportan, pIsI, TAT(48-60), pVEC, MTS, and MAP.

mRNAs to be targeted using RNA silencing agents include, but are not limited to, those whose expression is correlated with an undesired phenotypic trait. Exemplary mRNAs that may be targeted are those that encode truncated proteins i.e. comprise deletions. Accordingly the RNA silencing agent of some embodiments of the invention may be targeted to a bridging region on either side of the deletion. Introduction of such RNA silencing agents into a cell would cause a down-regulation of the mutated protein while leaving the non-mutated protein unaffected.

Another agent capable of downregulating a gene (e.g. miR-135 target gene) is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of the target gene. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl. Acad. Sci. USA 1997;94:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl. Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S.

Pat. No. 6,326,174 to Joyce et al. DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in vivo (Itoh et al, 20002, Abstract 409, Ann Meeting Am Soc Gen Ther www.asgt.org). In another 5 application, DNAzymes complementary to bcr-ab1 oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Downregulation of a gene (e.g. miR-135 target gene) can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA 10 transcript encoding the gene.

Design of antisense molecules which can be used to efficiently downregulate a gene must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which 15 specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

Another agent capable of downregulating a gene (e.g. miR-135 target gene) is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding a gene. Ribozymes are being increasingly used for the sequence-specific inhibition of 20 gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific 25 somatic mutations in genetic disorders [Welch et al., Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized 30 ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as

other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

5 Another agent capable of downregulating a gene (e.g. miR-135 target gene) is a RNA-guided endonuclease technology e.g. CRISPR system.

As used herein, the term "CRISPR system" also known as Clustered Regularly Interspaced Short Palindromic Repeats refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated 10 genes, including sequences encoding a Cas gene (e.g. CRISPR-associated endonuclease 9), a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat) or a guide sequence (also referred to as a "spacer") including but not limited to a crRNA sequence or a sgRNA sequence (i.e. single guide 15 RNA).

In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III CRISPR system. In some embodiments, one or more elements of a CRISPR system (e.g. Cas) is derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*, *Neisseria meningitidis*, *Streptococcus thermophilus* or *Treponema denticola*.

In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system).

25 In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence (i.e. guide RNA) is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. Thus, according to some embodiments, 30 global homology to the target sequence may be of 50 %, 60 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 % or 99 %. A target sequence may comprise any polynucleotide, such as

DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

Thus, the CRISPR system comprises two distinct components, a guide RNA (gRNA) that hybridizes with the target sequence, and a nuclease (e.g. Type-II Cas9 protein), wherein the gRNA targets the target sequence and the nuclease (e.g. Cas9 protein) cleaves the target sequence or silences target genes. The guide RNA may comprise a combination of an endogenous bacterial crRNA and tracrRNA, i.e. the gRNA combines the targeting specificity of the crRNA with the scaffolding properties of the tracrRNA (required for Cas9 binding). Alternatively, the guide RNA may comprise a single guide RNA (sgRNA) capable of directly binding Cas.

Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. This results in disruption of the gene of interest (i.e. target sequence) e.g. via insertions or deletions.

According to one embodiment, the Cas protein (e.g. Cas9) has no nuclease activity (i.e. is catalytically inactive) and is said to be 'dead' (dCas9). Catalytically inactive Cas9 protein can be used in accordance with the present teachings to bind to DNA (based on guide RNA specificity), this typically results in blockage of RNA polymerase binding or elongation, leading to suppression of transcription. Thus, dCas9 can be used for transcription repression.

As mentioned, a tracrRNA sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracrRNA sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence (e.g. crRNA).

In some embodiments, the tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of a CRISPR complex. As with the target sequence, a complete complementarity is not needed, provided there is sufficient to be functional. In some embodiments, the tracr sequence has at least 50 %,

60 %, 70 %, 80 %, 90 %, 95 % or 99 % of sequence complementarity along the length of the tracr mate sequence when optimally aligned.

Introducing CRISPR/Cas into a cell may be effected using one or more vectors driving expression of one or more elements of a CRISPR system such that expression of 5 the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracrRNA sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single 10 vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite 15 strand of the coding sequence of a second element, and oriented in the same or opposite direction. A single promoter may drive expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracrRNA sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one 20 intron, or all in a single intron).

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.

25 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), as discussed above.

Methods of downregulating microRNA expression are known in the art.

30 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 10 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 15 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 20 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 25 the target gene and introduced into the 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.

30 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.

80

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 suitable promoter.

In other embodiments of the invention, synthetic single stranded nucleic acids 5 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 10 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.

In other embodiments of the invention, downregulation of microRNA can be effected using an antisense polynucleotide capable of specifically hybridizing with a 15 microRNA or with a precursor thereof.

It will be appreciated that the microRNA antisense agents (e.g. anti-miRNA oligos) of the present invention may also comprise chemical modifications, molecular modifications and/or the addition of moieties, e.g. a cholesterol moiety (e.g. antagomirs).

20 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 25 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 another specific embodiment, there is provided a method of 30 upregulating an expression of a gene selected from the group consisting of an adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an

ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syne1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming

growth factor, beta receptor II (Tgfbr2); thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3) and zinc finger, BED domain containing 4 (Zbed4) in a neuroglia cell, the method 5 comprising: (a) downregulating an activity or expression of a miR-135 or a precursor thereof in the neuroglia cell; and (b) measuring an expression of the gene in the neuroglia cell, thereby upregulating the expression of the gene.

According to a specific embodiment, upregulating the expression of any of these genes is effected by a method other than downregulating an activity or expression of a 10 microRNA (e.g. miR-135) or a precursor thereof. Thus, upregulating an activity or expression of a gene may be affected by overexpressing the gene or a target thereof.

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 15 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 20 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 30 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, Grk3, Grm5, Grk2, Grm7, Gria2, Dscam, L1cam, Tsna, Sgk1, Stx1a, Adcyap1, Adcyap1r1, Adra2a, Ank3, Arc, Arhgap6, Atf3, Bace1, Cacna1d, Cadm3, Cplx1, Cplx2, Csm1, Csnk1g1, Dcx, Diras2, Dlg2, Elk1, Frk, Fut9, Gabrb2, Gata3, Ghsr, Gpr3, Gria3, Grk5, Gsk3b, Hcn1, Hcn2, Impa1, Kalrn, Kcnn3, Kpna3, Myt1l, Ncoa2, Ndrg4, Nos1ap, Nr3c2, Ntng1, Nucks1, Pde1a, Pde4a, Pde8b, Plcb1, Prlr, Rab1b, Rap2a, Rorb, Sirt1, Slc12a6, Slc5a7, Sp1, Sv2b, Syne1, Syt1, Syt2, Syt3, Tgfb2, Thrb, Trpc6, Vamp2, Wnt3 and/or Zbed4) 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. 5 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; 10 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; Tsanax as set forth in e.g. 15 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, Adcyap1 as set forth in e.g. NM\_001117.4, NM\_001099733.1 or a portion thereof, Adcyap1r1 as set forth in e.g. NM\_001118.4, NM\_001199635.1, NM\_001199636.1, NM\_001199637.1 or a portion 20 thereof, Adra2a as set forth in e.g. NM\_000681.3 or a portion thereof, ANK3 as set forth in e.g. NM\_001149.3, NM\_001204403.1, NM\_001204404.1 or NM\_020987.3, or a portion thereof, Arc as set forth in e.g. NM\_015193.4 or a portion thereof, Arhgap6 as set forth in e.g. NM\_001287242.1, NM\_006125.2, NM\_013423.2, NM\_013427.2 or a portion thereof, Atf3 as set forth in e.g. NM\_001030287.3, NM\_001040619.2, 25 NM\_001206484.2, NM\_001206486.2, NM\_001206488.2, NM\_001674.3 or a portion thereof, Bace1 as set forth in e.g. NM\_001207048.1, NM\_001207049.1, NM\_012104.4, NM\_138971.3, NM\_138972.3, NM\_138973.3 or a portion thereof, Cacna1d as set forth in e.g. NM\_000720.3, NM\_001128839.2, NM\_001128840.2 or a portion thereof, Cadm3 as set forth in e.g. NM\_001127173.1, NM\_021189.3 or a portion thereof, Cplx1 30 as set forth in e.g. NM\_006651.3 or a portion thereof, Cplx2 as set forth in e.g. NM\_001008220.1, NM\_006650.3 or a portion thereof, Csmd1 as set forth in e.g. NM\_033225.5 or a portion thereof, Csnk1g1 as set forth in e.g. NM\_022048.3 or a

portion thereof, Dcx as set forth in e.g. NM\_000555.3, NM\_001195553.1, NM\_178151.2, NM\_178152.2, NM\_178153.2 or a portion thereof, Diras2 as set forth in e.g. NM\_017594.3 or a portion thereof, Dlg2 as set forth in e.g. NM\_001142699.1, NM\_001142700.1, NM\_001142702.1, NM\_001206769.1, NM\_001364.3 or a portion thereof, Elk1 as set forth in e.g. NM\_001114123.2, NM\_001257168.1, NM\_005229.4 or a portion thereof, Frk as set forth in e.g. NM\_002031.2 or a portion thereof, Fut9 as set forth in e.g. NM\_006581.3 or a portion thereof, Gabrb2 as set forth in e.g. NM\_000813.2, NM\_021911.2 or a portion thereof, Gata3 as set forth in e.g. NM\_001002295.1, NM\_002051.2 or a portion thereof, Ghsr as set forth in e.g. NM\_004122.2, NM\_198407.2 or a portion thereof, Gpr3 as set forth in e.g. NM\_005281.3 or a portion thereof, GRIA3 as set forth in e.g. NM\_000828.4, NM\_001256743.1, NM\_007325.4 or a portion thereof, Grk5 as set forth in e.g. NM\_005308.2 or a portion thereof, GSK3B as set forth in e.g. NM\_001146156.1, NM\_002093.3 or a portion thereof, Hcn1 as set forth in e.g. NM\_021072.3 or a portion thereof, Hcn2 as set forth in e.g. NM\_001194.3 or a portion thereof, IMPA1 as set forth in e.g. NM\_001144878.1, NM\_001144879.1, NM\_005536.3 or a portion thereof, Kalm as set forth in e.g. NM\_001024660.3, NM\_003947.4, NM\_007064.3, or a portion thereof, KCNN3 as set forth in e.g. NM\_001204087.1, NM\_002249.5, NM\_170782.2 or a portion thereof, Kpna3 as set forth in e.g. NM\_002267.3 or a portion thereof, Myt11 as set forth in e.g. NM\_015025.2 or a portion thereof, Ncoa2 as set forth in e.g. NM\_006540.2 or a portion thereof, Ndrg4 as set forth in e.g. NM\_020465.3, NM\_022910.3, NM\_001130487.1, NM\_001242836.1 or a portion thereof, NOS1AP as set forth in e.g. NM\_001126060.1, NM\_001164757.1, NM\_014697.2 or a portion thereof, Nr3c2 as set forth in e.g. NM\_000901.4, NM\_001166104.1 or a portion thereof, Ntng1 as set forth in e.g. NM\_001113226.1, NM\_001113228.1, NM\_014917.2 or a portion thereof, Nucks1 as set forth in e.g. NM\_022731.4 or a portion thereof, Pde1a as set forth in e.g. NM\_005019.4, NM\_001003683.2, NM\_001258312.1 or a portion thereof, Pde4a as set forth in e.g. NM\_001111307.1, NM\_001111308.1, NM\_001111309.1 or a portion thereof, Pde8b as set forth in e.g. NM\_003719.3, NM\_001029851.2, NM\_001029852.2 or a portion thereof, Plcb1 as set forth in e.g. NM\_015192.3, NM\_182734.2 or a portion thereof, Prlr as set forth in e.g. NM\_000949.5, NM\_001204315.1, NM\_001204316.1 or a portion thereof, Rab1b as set

forth in e.g. NM\_030981.2 or a portion thereof, Rap2a as set forth in e.g. NM\_021033.6 or a portion thereof, Rorb as set forth in e.g. NM\_006914.3 or a portion thereof, Sirt1 as set forth in e.g. NM\_001142498.1, NM\_012238.4 or a portion thereof, Slc12a6 as set forth in e.g. NM\_133647.1, NM\_005135.2, NM\_001042495.1 or a portion thereof,  
5 Slc5a7 as set forth in e.g. NM\_021815.2 or a portion thereof, Sp1 as set forth in e.g. NM\_138473.2, NM\_003109.1, NM\_001251825.1 or a portion thereof, Sv2b as set forth in e.g. NM\_001167580.1, NM\_014848.4 or a portion thereof, Syne1 as set forth in e.g. NM\_033071.3, NM\_182961.3 or a portion thereof, Syt1 as set forth in e.g. NM\_001135805.1, NM\_001135806.1, NM\_005639.2 or a portion thereof, Syt2 as set  
10 forth in e.g. NM\_001136504.1, NM\_177402.4 or a portion thereof, Syt3 as set forth in e.g. NM\_001160328.1, NM\_001160329.1, NM\_032298.2 or a portion thereof, Tgfbr2 as set forth in e.g. NM\_001024847.2, NM\_003242.5 or a portion thereof, Thrb as set forth in e.g. NM\_000461.4, NM\_001128176.2, NM\_001128177.1, NM\_001252634.1 or a portion thereof, Trpc6 as set forth in e.g. NM\_004621.5 or a portion thereof, Vamp2 as  
15 set forth in e.g. NM\_014232.2 or a portion thereof, Wnt3 as set forth in e.g. NM\_030753.4 or a portion thereof and/or Zbed4 as set forth in e.g. NM\_014838.2 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.

20 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.

25 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.

30 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 heterocyclic nucleosides consisting of purines and the pyrimidines bases, bonded in a 3' to 5' phosphodiester linkage.

Preferably used oligonucleotides are those modified in a backbone (e.g. sugar-phosphate backbone), internucleoside linkages and/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.,  $^{32}\text{P}$ - $\gamma$ -ATP or  $^{32}\text{P}$ - $\alpha$ -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<sup>TM</sup> (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, Stx1a, Adcyap1, Adcyap1r1, Adra2a, Ank3, Arc,

Arhgap6, Atf3, Bace1, Cacna1d, Cadm3, Cplx1, Cplx2, Csmnd1, Csnk1g1, Dcx, Diras2, Dlg2, Elk1, Frk, Fut9, Gabrb2, Gata3, Ghsr, Gpr3, Gria3, Grk5, Gsk3b, Hcn1, Hcn2, Impa1, Kalrn, Kcnn3, Kpna3, Myt1l, Ncoa2, Ndrg4, Nos1ap, Nr3c2, Ntng1, Nucks1, Pde1a, Pde4a, Pde8b, Plcb1, Prlr, Rab1b, Rap2a, Rorb, Sirt1, Slc12a6, Slc5a7, Sp1, 5 Sv2b, Syne1, Syt1, Syt2, Syt3, Tgfbr2, Thrb, Trpc6, Vamp2, Wnt3 and/or Zbed4) 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].

10 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.

15 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 20 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 25 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 30 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).

5       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 10 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 sulphydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' 15 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 20 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 25 (1972)]. 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 30 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.

5 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  
10 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  
15 (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  
20 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.,  
25 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  
30 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.,  $^{125}\text{I}$ , 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-Grk3 antibody; anti-Grm5 antibody; anti-Grk2 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 5 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-10 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 15 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 20 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 Concanavalin A (GE Healthcare Bio-Sciences, Uppsala, Sweden) which may be also conjugated to beads, followed by Western blot analysis specific 25 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 30 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-  
5 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  
10 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  
15 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  
25 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  
30 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, 5 fluorescently labeled immunohistochemistry (using a fluorescent dye conjugated to an antibody), radiolabeled immunohistochemistry (using radiolabeled e.g., <sup>125</sup>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 10 substrates as described hereinabove.

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

15 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.

20 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.

25 According to one embodiment, the method comprises measuring an expression of the MaoA gene following 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.

30 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.

As mentioned hereinabove, the present inventors have further realized that the level of miR-135 is significantly down-regulated in blood samples of human subjects 5 suffering from mood disorders including depression, anxiety, bipolar disorder and stress (as compared to healthy human subjects) and is upregulated in blood samples of human subjects being treated with an anti-depressant therapy (as compared to the same subject prior to initiation of treatment or untreated human subjects).

Thus, there is provided a method of diagnosing a mood disorder in a human 10 subject in need thereof, the method comprising measuring an expression level of a miR-135 in a biological sample of the human subject, wherein a lower expression level of the miR-135 as compared to that in a biological sample of a healthy subject is indicative of the mood disorder.

Any mood disorder may be diagnosed in accordance with the present invention, 15 including but not limited to, a bipolar disorder, a depression, a major depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a schizophrenia, a sleep disorder and an eating disorder (e.g. Anorexia nervosa, Bulimia nervosa, Eating disorders not otherwise specified, Binge eating disorder (BED) or Pica eating disorder).

20 Measuring the expression level of miR-135 (e.g. miR-135a) is typically effected in a biological sample.

According to a specific embodiment, the term "biological sample" refers to body fluids such as fresh whole blood, fractionated whole blood, blood plasma, blood serum, cerebrospinal fluid (CSF), urine, lymph fluids, and various external secretions of the 25 respiratory, intestinal and genitourinary tracts, tears, saliva, milk as well as white blood cells including mononuclear cells (e.g. lymphocytes, monocytes, dendritic cells).

Typically, whole blood and fractionated whole blood (i.e. blood sample fractionated, e.g. by centrifugation, into separate components) comprises all of the blood components including blood plasma, leukocytes, platelets and erythrocytes. Blood serum 30 is the blood component that is neither a blood cell nor a clotting factor, it is the blood plasma with the fibrinogens removed. Serum includes proteins not used in blood clotting (coagulation) and the electrolytes, antibodies, antigens, hormones, and any exogenous

substances (e.g., drugs and microorganisms). Furthermore, blood plasma comprises all of the components of the serum along with clotting factors.

According to a specific embodiment, the biological sample is a whole blood sample.

5 According to a specific embodiment, the biological sample is a serum or plasma sample.

According to a specific embodiment, the biological sample is a mononuclear cell sample).

According to a specific embodiment, the cell sample is devoid of erythrocytes.

10 According to a specific embodiment the biological sample is at least 90 % white blood cells (e.g., at least 90 % mononuclear cells).

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 blot analysis, RNase protection assay, and PCR (e.g. real-time PCR).

15 As mentioned, a lower expression level of the miR-135 as compared to that in a biological sample of a healthy human subject (i.e. a subject not being affected by a mood disorder) is indicative of the mood disorder.

According to one embodiment, a lower expression level of the miR-135 as compared to that in a biological sample of a healthy human subject is statistically 20 significant.

The expression level of miR-135 in a human subject having a mood disorder may be lower by about 5 %, 10 %, 15 %, 20 %, 25 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or 100 % as compared to that of a healthy human subject.

25 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 disorder (mood disorder including depression or bipolar disorder). Standardized questionnaires can be helpful for diagnosis of depression, such as the Hamilton Rating Scale for Depression, and the Beck Depression Inventory.

30 Diagnosis of bipolar disorder can be further assessed using typical criteria. The most widely used criteria for diagnosing bipolar disorder are from the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (e.g.

version DSM-IV-TR), and the World Health Organization's International Statistical Classification of Diseases and Related Health Problems (e.g. ICD-10).

Furthermore, tests may be carried out to exclude medical illnesses such as hypo- or hyperthyroidism, metabolic disturbance, a systemic infection or chronic disease, and 5 syphilis or HIV infection. An EEG may be used to exclude epilepsy, and a CT scan of the head to exclude brain lesions.

The present inventors have further shown that blood levels of miR-135 (e.g. miR-135a) are upregulated in human subjects following an antidepressant therapy (see Example 16 of the Examples section which follows).

10 Thus, according to another embodiment of the present invention, there is provided a method of monitoring treatment of an anti-depressant drug or a medicament for the treatment of a mood disorder (e.g. bipolar disorder), the method comprising: (a) treating a human subject in need thereof with an anti-depressant drug or a medicament for the treatment of a mood disorder; and (b) measuring an expression level of a miR-15 135 in a biological sample of the human subject prior to and following the treatment, wherein a higher expression level of the miR-135 following the treatment by the anti-depressant drug or the medicament for the treatment of the mood disorder as compared to the expression level of the miR-135 prior to the treatment by the anti-depressant drug or the medicament for the treatment of the mood disorder is indicative of the efficient 20 treatment.

According to one embodiment, the method further comprises (c) treating the human subject when a higher expression level of the miR-135 is observed in step (b) to improve treatment.

25 The biological sample (e.g. fresh whole blood, fractionated whole blood, blood plasma or blood serum) is typically obtained from the human subject following to treatment with an anti-depressant drug or with a medicament for the treatment of a mood disorder, however, a blood sample may also be obtained from the subject prior to treatment for further comparison of miR-135 levels.

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

30 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 5 (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 10 antidepressants and Secondary amine tricyclic antidepressants); and Monoamine oxidase inhibitor (MAOIs).

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

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

As used herein, the term "a medicament for the treatment of a mood disorder" refers to any medicament or any combination of medicaments used for the treatment of a mood disorder including bipolar disorder. Exemplary medicaments include, but not 20 limited to, lithium (e.g. Lithium carbonate, Lithium citrate, Lithium sulfate), antipsychotic medicaments (e.g. typical antipsychotics and atypical antipsychotics, as detailed hereinabove) and mood stabilizer medicaments (e.g. Valproic acid (VPA, Valproate), minerals, anticonvulsants, antipsychotics, as detailed hereinabove).

Other treatment options which are encompassed by the present methods (alone or 25 in combination with the above) include non-pharmaceutical therapeutic strategies, including but not limited to, clinical psychology, electroconvulsive therapy, involuntary commitment, light therapy, psychotherapy, transcranial magnetic stimulation and cognitive behavioral therapy.

An efficient anti-depressant/mood disorder treatment is determined when a 30 significantly higher expression level of the miR-135 is obtained following to the treatment as compared to the miR-135 expression level prior to the treatment.

100

The expression level of miR-135 in a subject following treatment may be higher by about 5 %, 10 %, 15 %, 20 %, 25 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or 100 % as compared to that of the subject prior to the anti-depressant or mood disorder treatment.

5 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.

10 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  $\pm 10\%$ .

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

15 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.

20 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.

25 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 30 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

101

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.

25

## 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

102

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.

103  
**EXAMPLE 1****Differential expression of miRs in serotonin neurons****MATERIALS AND EXPERIMENTAL PROCEDURES**5 ***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 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***

30 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

104

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.

5 ***Animals and housing***

Adult C57BL/6J male mice, 10 weeks old (Harlan, Jerusalem, Israel) were housed in a temperature-controlled room ( $22 \pm 1$  °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 10 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***

15 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 20 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***

25 Mice received i.p. injection of tricyclic- 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***

30 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.

105

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***

5 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 10 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          | TATGGCTTTTATTCCCTATGTGA | miR135a |
| 2          | TATGGCTTTCATTCCTATGTGA  | miR135b |
| 3          | TTTGTTCGTTCGGCTCGCGTGA  | miR375  |
| 4          | GATGACACGCAAATTCTGTGAA  | U6      |
| 5          | TAAGGCACCGCGGTGAATGCC   | miR124  |

**Table 1C: Primers sequences used for molecular cloning**

| Primer Sequence                              | Orientation | Product size           | Gene                    |           |
|--|-------------|------------------------|-------------------------|-----------|
| AGTTCTGCCGCTGATGATG<br>(SEQ ID NO: 6)        | sense       | 2600 <sup>with 2</sup> | Htr1a 3' UTR            | <b>1</b>  |
| GCACAAAATGGAGAGTCTGATT<br>AAA (SEQ ID NO: 7) | antisense   |                        | Htr1a 3' UTR            | <b>2</b>  |
| TGCCTTTAACGAAACAGC<br>(SEQ ID NO: 8)         | sense       | 2000 <sup>with 4</sup> | MaoA 3'UTR              | <b>3</b>  |
| CCAAGTTTACAACCATCAAGC<br>A (SEQ ID NO: 9)    | antisense   |                        | MaoA 3'UTR              | <b>4</b>  |
| ATCCGCATGAATGCTGTGTA<br>(SEQ ID NO: 10)      | sense       | 760 <sup>with 6</sup>  | Slc6a4 3'UTR            | <b>5</b>  |
| GTGGGTGGTGGAAAGAGACAC<br>(SEQ ID NO: 11)     | antisense   |                        | Slc6a4 3'UTR            | <b>6</b>  |
| CCTACACCGCAGAGCATTGAA<br>(SEQ ID NO: 12)     | sense       | 870 <sup>with 8</sup>  | Tph2 3' UTR             | <b>7</b>  |
| ACATCCCTGTGGGATTGAG<br>(SEQ ID NO: 13)       | antisense   |                        | Tph2 3' UTR             | <b>8</b>  |
| TGTCTGCTTATATTCTCAGT<br>AG (SEQ ID NO: 14)   | sense       | 320 <sup>with 6</sup>  | Slc6a4 3'UTR<br>mutated | <b>9</b>  |
| GAAAATAAGCAAGACATCC<br>CTGTT (SEQ ID NO: 15) | antisense   | 440 <sup>with 5</sup>  | Slc6a4 3'UTR<br>mutated | <b>10</b> |

106

|  |           |                         |                                |           |
|--|-----------|-------------------------|--------------------------------|-----------|
| AAAGATCCCTTCCCCAATG<br>(SEQ ID NO: 16)               | sense     | 1400 <sup>with 12</sup> | Htr1a 3' UTR<br>short          | <b>11</b> |
| CAGTGCCTCTTCTCCACAGA<br>(SEQ ID NO: 17)              | antisense |                         | Htr1a 3' UTR<br>short          | <b>12</b> |
| ATAAGCAAGGCCAAAGGA<br>AGA (SEQ ID NO: 18)            | sense     | 1300 <sup>with 12</sup> | Htr1a 3' UTR<br>mutated seed 1 | <b>13</b> |
| TTTGCCCCCTGCTTATAAGT<br>CC (SEQ ID NO: 19)           | antisense | 120 <sup>with 11</sup>  | Htr1a 3' UTR<br>mutated seed 1 | <b>14</b> |
| CTGCCCTGCCACATGTGTTTT<br>AT (SEQ ID NO: 20)          | sense     | 170 <sup>with 12</sup>  | Htr1a 3' UTR<br>mutated seed 2 | <b>15</b> |
| TAACAAATAAAACACATGTG<br>GCA (SEQ ID NO: 21)          | antisense | 1260 <sup>with 11</sup> | Htr1a 3' UTR<br>mutated seed 2 | <b>16</b> |
| ACCGGTCATATGATTCCCCAGT<br>TTCCTGCTTT (SEQ ID NO: 22) | sense     | 199 <sup>with 18</sup>  | Pre-mmu-<br>miR135b            | <b>17</b> |
| ACCGGTCCCTGTGGCTGGTCC<br>TTAG (SEQ ID NO: 23)        | antisense |                         | Pre-mmu-<br>miR135b            | <b>18</b> |

***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 stereotaxic instrument and a motorized nanoinjector (Angle Two™ Stereotaxic Instrument, myNeurolab). As previously

107

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 5 injected at a rate of 0.2  $\mu$ l 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  $\mu$  slices in order to confirm the preciseness of the injection site, using immunohistochemistry.

10 ***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 15 (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.

20 ***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.

25 ***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 °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 30 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 5 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 10 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 tests* were used when necessary to reveal statistical significance. Differences between groups were considered significant 15 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, 20 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 25 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 30 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

109

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  $\pm 1.5$  in the 5HT neurons 5 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*    |

10

**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-4661   |
| -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    |

| 110   |                |
|-------|----------------|
| -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).

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

112

(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 5 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 10 on Slc6a4 and Htr1a 3'UTR. 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

### 15 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. 20 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.

### 25 *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) 30 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

113

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 5 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). 10 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 15 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 bioinformatically 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 20 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 25 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 30 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

114

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 5 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, 10 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 15 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 20 sequences on ADRb1-3'UTR.

### EXAMPLE 3A

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

#### **25 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 30 room ( $22 \pm 1$  °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.

5 Lentiviruses are generated for both *in-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***

10 In order to generate mice lacking miR-19 in the forebrain, inventors are breeding 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 15 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***

20 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 25 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**

30 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

116

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

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#### miRNA-19 and cannabinoid receptor 1 (CB1)

### MATERIALS AND EXPERIMENTAL PROCEDURES

#### *Animals and housing*

As described in Example 3A, above.

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#### *Generating lentiviruses for miRNA-19b manipulation in adult brain*

As described in Example 3A, above.

### RESULTS

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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.

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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).

117

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) 86:249-5. 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 10 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

15 **Identification of differentially expressed miRNAs in mice subjected to chronic  
stress**

#### MATERIALS AND EXPERIMENTAL PROCEDURES

##### *Immunoprecipitation of Ago2 protein*

20 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 25 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 30 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 5 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.

**10 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).

15 In order to identify a genuine connection between miRNAs and their target gene’s 3` UTR following the social defeat paradigm, an immunoprecipitation (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 20 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 25 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 30 nonspecific antibody. A specific Ago2 band was present only in the Ago2 precipitate (Figure 17B, lanes 3, 4).

119

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.

5        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.

10       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 15 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 20 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, 25 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 30 collected 8 days after the social avoidance test from the amygdala, BNST, PFC, dorsal raphe and hippocampus along with trunk blood.

120

Pools of 3 amygdala punches obtained from 3 different mice were combined and the immunoprecipitation 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 5 “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 inventors expected 10 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 15 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                  |

121

|                   |      |      |
|-------------------|------|------|
| 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)

122

\*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 5 “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                  |

123

|                   |      |      |
|-------------------|------|------|
| 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<br>(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 |

124

|        |      |      |
|--------|------|------|
| 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.

5

**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                 |
| Khl38         | -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.

10

**EXAMPLE 4A****miR-15a and miR-15b as regulators of the stress response****MATERIALS AND EXPERIMENTAL PROCEDURES*****Total RNA extraction***

15

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 20 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. 25 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***

30 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

126

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

10 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

#### 15 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 20 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 25 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 30 polyadenylation sites and is statistically associated with a higher rate of depressive disorders [Binder et al. *supra*].

127

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 5 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 amygdala, 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-10 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 15 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).

20

#### EXAMPLE 4C

##### miR-181 regulates glutamate receptors

#### MATERIALS AND EXPERIMENTAL PROCEDURES

##### *Cloning of 3' UTRs into Psicheck2 luciferase expression plasmid*

25 3'UTR sequences of Grm1, Grk3, Grm5, Grk2 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 30 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. 5 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.

10 **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, 15 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 20 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:

25

**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 30 miR-181, there are 6 glutamate receptors (Grm1, Grik3, Grm5, Gria2, Grik2 and Grm7).

129

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 5 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**

#### 10 **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 15 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: 20 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 25 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. 30 During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. Perforated clear plexiglass dividers were then placed between

130

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***

5 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***

10 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 15 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***

20 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 25 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***

30 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  $\mu$ m-pore cellulose acetate filters and concentrated by ultracentrifugation.

## RESULTS

To date miR-182 was reported mainly in cancer related studies such as human 5 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 10 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].

15 Bioinformatical 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 Htr1a 3'UTR by miR-182 (Figure 8). Two conserved seed matches sequence for miR-182 appeared in Htr1a mouse 3' UTR.

20 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.

25 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 30 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

132

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.

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

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## EXAMPLE 6

### Regulation of miR182 expression levels by acute stress

## MATERIALS AND EXPERIMENTAL PROCEDURES

As described in Example 5A, hereinabove

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## 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 20 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.

### miR-target interaction assay for miR182 predicted target genes

Using a luciferase assay, eleven predicted target genes of miR182, chosen after 25 extensive bioinformatics, were examined (Figure 27). 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 30 miR182 as in luciferase assay (Figure 27). When testing the 3'UTR of the listed above target gene of miR182 a conserved seed match sequence for miR182 was observed both

133

in TsnaX, L1cam and Dscam, suggesting this miR-target interaction had a functional role (data not shown).

Next, the direct repressive effect of miR182 on these three genes was verified. Therefore, the 3'UTRs was mutated to remove miR182 seed match sequence and 5 compared the regular 3'UTRs to the mutated one *in vitro* by luciferase assays. miR182 repressive effect on L1cam 3'UTR was abolished when mutated its seed match sequence (Figure 28), and similarly the effect of miR182 on TsnaX was abolished in the mutated 3'UTR (Figure 29) indicating miR182 targeted this gene directly. Similar verification for Dscam and Htr1a with mutated 3'UTR are performed.

10 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.

15

## EXAMPLE 7

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

#### **MATERIALS AND EXPERIMENTAL PROCEDURES**

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##### ***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 30 behaviors, a plasmid based miR135b inhibitor was utilized and its efficiency was tested in a luciferase assay. In this assay, HEK293T cells were co-transfected with miR135OE, miR135KD and 3'UTR plasmids, and the ability of miR135bKD plasmid to block the

134

repressing effect of miR135 on Slc6a4 and Htr1a 3' UTR was tested. miR135b repressoric 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 5 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 10 (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**

#### 15 **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**

20 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 25 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 30 (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

135

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 their 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 known 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 litter 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

137

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 forced swim test (P<0.05, Figure 34E).

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## MATERIALS AND EXPERIMENTAL PROCEDURES FOR MIR-135 SECTION

### *MicroRNA microarray of 5HT neurons*

Hindbrain cells from embryonic day 12 of ePet-EYFP mice were FACS sorted to distinguish 5HT YFP-positive neurons from surrounding non-5HT YFP-negative 10 cells as previously described (Wylie et al., 2010). Total RNA, including the miRNA population, with 3 biological repeats from each cell type 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 the manufactures instructions. The microarrays were scanned and the data were extracted 15 and processed using the Feature Extraction Software (Agilent Technologies). Following scanning, intensity output data of the GeneView.txt files were analyzed to quantify differential relative expression of microRNAs using the Partek<sup>®</sup> Genomics Suite (Partek Inc., St. Louis, MO). The data were log2 transformed, quantile normalized and filtered according to the flag “gIsGeneDetected” in the GeneView file. Of 666 murine 20 miRs, 198 remained for further analysis after this filtering step. Differentially expressed miRs were then identified using a threshold of a 1.5 fold change.

### *Cloning of target transcripts 3' UTRs into psiCHECK-2 luciferase expression plasmid*

3' UTRs sequences of Slc6a4 and Htr1a were PCR amplified from mouse 25 genomic DNA. 3'UTRs PCR fragments were ligated into pGEM-T easy vector (Promega) according to the manufacturer's guidelines, and further sub-cloned into a single *NotI* site at the 3' end of luciferase in the psiCHECK-2 reporter plasmid (Promega). Mutated 3' UTR sequences, lacking miR-135 seed sequences, were synthesized with primer overhangs across the seed match sequence. Cloning orientation 30 was verified by diagnostic cuts and sequencing.

**Table 7: Oligonucleotide primers used for cloning**

|    | Gene                        | Product size           | Orientation | Primer Sequence                               |
|----|-----------------------------|------------------------|-------------|---|
| 1  | Slc6a4 3'UTR                | 760 <sup>with 2</sup>  | sense       | ATCCGCATGAATGCTGTGTA<br>(SEQ ID NO: 10)       |
| 2  | Slc6a4 3'UTR                |                        | antisense   | GTGGGTGGTGGAAAGAGACAC<br>(SEQ ID NO: 11)      |
| 3  | Htr1a 3' UTR                | 2600 <sup>with 4</sup> | sense       | AGTTCTGCCGCTGATGATG<br>(SEQ ID NO: 6)         |
| 4  | Htr1a 3' UTR                |                        | antisense   | GCACAAATGGAGAGTCTGATTAAA<br>(SEQ ID NO: 7)    |
| 5  | Slc6a4 3'UTR mutated        | 320 <sup>with 2</sup>  | sense       | TGTCTTGCTTATATTTCTCAGTAG<br>(SEQ ID NO: 14)   |
| 6  | Slc6a4 3'UTR mutated        | 440 <sup>with 1</sup>  | antisense   | GAAAATATAAGCAAGACATCCCTGTT<br>(SEQ ID NO: 15) |
| 7  | Htr1a 3' UTR short          | 1400 <sup>with 8</sup> | sense       | AAAGATCCCTTCCCCAATG<br>(SEQ ID NO: 16)        |
| 8  | Htr1a 3' UTR short          |                        | antisense   | CAGTGCCTCTCTCCACAGA<br>(SEQ ID NO: 17)        |
| 9  | Htr1a 3' UTR mutated seed 1 | 1300 <sup>with 8</sup> | sense       | ATAAGCAAGGGCCAAAGGAAGA<br>(SEQ ID NO: 18)     |
| 10 | Htr1a 3' UTR mutated seed 1 | 120 <sup>with 7</sup>  | antisense   | TTTGCGCCCTTGCTTATAAGTCC<br>(SEQ ID NO: 19)    |
| 11 | Htr1a 3' UTR mutated seed 2 | 170 <sup>with 8</sup>  | sense       | CTGCCCTGCCACATGTGTTTTAT<br>(SEQ ID NO: 20)    |

***Transfections and luciferase assays***

HEK293T cells were grown on poly-L-lysine in a tissue culture plate to 70-85 % confluence and transfected using polyethyleneimine with the following plasmids: psiCHECK-2 plasmid containing the wild type or mutated 3' UTR and the overexpressing vector for a specific miRNA (miR-135a as set forth in SEQ ID NO: 210 or miR-135b as set forth in SEQ ID NO: 211), or empty overexpression plasmids. Twenty-four hours following transfection cells were lysed and luciferase reporter activities were assayed as previously described (Kuperman et al., 2011). Renilla

139

luciferase values were normalized to control firefly luciferase levels, transcribed from the same vector but not affected by 3' UTR, then tested and averaged across six repetitions per condition.

***Animals and housing***

5        Adult C57BL/6 male mice (Harlan, Jerusalem, Israel) were used for the *in vivo* lentiviral experiments. ePet-Cre mice expressing Cre recombinase specifically in serotonergic neurons were used as previously described (Scott et al., 2005). Transgenic mice carrying a conditional cassette for miR-135 overexpression were also used. Mice were housed in a temperature-controlled room ( $22 \pm 1$  °C) on a reverse 12 h light/dark  
10      cycle. Food and water were available *ad libitum*. All experimental protocols were performed on male mice and were approved by the Institutional Animal Care and Use Committee of The Weizmann Institute of Science.

**Amphetamine-induced hyperactivity rat model**

15      In these experiments rats are equally divided into 4 treatment groups. Rats are pretreated with miR-135, lithium, valproate, or saline (control) and then half of the rats in each group are administered amphetamine (0.5 mg/kg subcutaneously (s.c.)) and the other half is given saline (s.c.).

20      Alternatively, the rats are first administered amphetamine (0.5 mg/kg subcutaneously (s.c.)) or saline (s.c.) followed by treatment with miR-135, lithium, valproate, or saline (control).

Ten minutes later all rats are placed in the activity meter and the activities of rats pretreated or treated with miR-135 are compared to non treated rats (control group) and to rats pretreated or treated with lithium or with valproate. One week later the procedure is repeated.

25      **Ketamine-induced hyperactivity rat model**

In these experiments rats are equally divided into 4 treatment groups: control, lithium, valproate and miR-135 treated rats and are treated as follows:

30      Rats are pretreated (for 14 days) with miR-135, lithium (47.5 mg/kg, i.p., twice a day), valproate (200 mg/kg, i.p., twice a day), or with saline (i.p., twice a day) as a control. Between days 8 and 14, these rats are treated with ketamine (25 mg/kg, i.p.) or saline.

In a reversal protocol, rats are administered ketamine (25 mg/kg, i.p.) or saline

140

first followed by administration of miR-135, lithium, valproate, or saline for 7 days. Then, rat activity is monitored as discussed herein.

#### ***Chronic Social defeat***

Mice were subjected to a social defeat protocol as previously described (Elliott 5 et al., 2010). Briefly, 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 Plexiglas 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 10 then repeated with an unfamiliar ICR mouse for 10 consecutive days.

#### ***Antidepressant treatment***

Mice received i.p. injection of the tricyclic, imipramine, the SSRI, fluoxetine, or the NRI, reboxetine (20 mg/kg in saline), or saline. Chronic injections were carried out for 18-21 consecutive days, and acute injection was performed 24 hours prior to brain 15 microdissection. For behavioral testing, mice were injected with 20 mg/kg SSRI i.p. 30 minutes prior to the test.

#### ***Brain microdissection***

Brain samples were taken from mice raphe nuclei (RN) using an acryl brain matrix (Stoelting). Slices were taken using standard razor blades based on designated 20 anatomical markers. Blunted 14G syringes were used to extract the raphe region from 2 mm slices and the tissue was kept at -70 °C until RNA purification.

#### ***microRNA purification and quantitative real time PCR expression analysis***

mRNAs including microRNAs were isolated using miRNeasy mini kit (Qiagen) according to the manufacturer instructions, and treated using miScript Reverse 25 transcription kit to generate 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 an internal control. For mRNA quantification specific primers were designed for each transcript using the 30 software "primer express 2" and expression was tested using real time PCR as previously described (Haramati et al., 2011).

**Table 8: Oligonucleotide primers used for microRNA real time PCR**

| Gene     | Primer sequence                         |
|----------|---|
| miR-135a | TATGGCTTTTATTCTATGTGA (SEQ ID NO: 1)    |
| miR-135b | TATGGCTTTCTTCATTCTATGTGA (SEQ ID NO: 2) |
| miR-375  | TTTGTTCGTTGGCTCGCGTGA (SEQ ID NO: 3)    |
| U6       | GATGACACGCAAATTCGTGAA (SEQ ID NO: 4)    |
| miR-124  | TAAGGCACGCGGTGAATGCC (SEQ ID NO: 5)     |
| miR-16   | TAGCAGCACGTAATATTGGCG (SEQ ID NO: 158)  |

**Table 9: Oligonucleotide primers used for mRNA real time PCR**

| Gene   | Sense Primer                           | Antisense Primer                       |
|--------|--|--|
| Slc6a4 | GGGTTGGATAGTACGTTCGCA (SEQ ID NO: 159) | CATACGCCCTCCTGATGTC (SEQ ID NO: 160)   |
| Htr1a  | GTGCACCATCAGCAAGGACC (SEQ ID NO: 161)  | GCGCCGAAAGTGGAGTAGAT (SEQ ID NO: 162)  |
| Tph2   | AGTATTTGTGGATGTGCCATG (SEQ ID NO: 163) | TGGGAATGGGCTGACCATATT (SEQ ID NO: 164) |
| YFP    | CATGCCCGAAGGCTACGT (SEQ ID NO: 165)    | CGATGCCCTTCAGCTCGAT (SEQ ID NO: 166)   |
| GAD-67 | TCATGTCCCGGAAGCACC (SEQ ID NO: 167)    | AATTGCCCTTCTATGCCG (SEQ ID NO: 168)    |

5 *Cloning of miR-135 knockdown lentiviral constructs, production of lentiviruses and in vitro verifications*

miR-135 knockdown (KD) plasmid pEZX-H1-miR-135KD-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 containing 10 *NheI* flanking site and were ligated to pGEM-T Easy vector. The H1-135 KD fragment was subcloned into the p156-pRRL-CMV-GFP lentiviral construct using the *NheI* restriction site resulting in p156-pRRL-H1-miR-135bKD-CMV-GFP and p156-pRRLH1-control KD-CMV-GFP lentiviral constructs, which were further confirmed by DNA sequencing. Recombinant lentiviruses were produced by transient transfection in 15 HEK293T cells, as described previously (Tiscornia et al., 2006). 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. For miR-135 KD efficiency verification rat raphe cell line (RN46A) were infected with either miR-135 KD or control KD lentiviruses, and 48 later mRNA were harvested and expression 20 levels of Htr1a and Slc6a4 were tested using real time PCR.

***Intracerebral injections of lentiviruses***

For stereotaxic surgery and lentiviral delivery, computer-guided a stereotaxic instrument and a motorized nanoinjector were used (Angle Two<sup>TM</sup> Stereotaxic Instrument, myNeurolab) as previously described (Lebow et al., 2012). Mice were 5 placed on a stereotaxic apparatus under general anesthesia, and the lentiviral preparation was delivered to coordinates determined as defined by the Franklin and Paxinos atlas to the DR: ML 0 mm; AP - 4.6 mm; DV -3.9 mm in 300 tilt. Injections were performed at a rate of 0.2  $\mu$ l / 1 min. Following two weeks recovery period, mice were subjected to behavioral and physiological studies. Following phenotyping, mice 10 were anesthetized and perfused with phosphate buffered 4 % paraformaldehyde. The fixed brains were serially sectioned to 30 mm slices in order to confirm the location of the injection site, using immunohistochemistry.

***Immunohistochemistry***

The procedure used for immunohistochemistry is as described previously 15 (Regev et al., 2011). For GFP immunostaining, biotinylated anti-GFP antibody was used raised in goat as primary antibody (Abcam, Cambridge, UK), and streptavidin conjugated Cy2 as secondary antibody (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA).

***Behavioral assessments***

20 All behavioral assessments were performed during the dark phase following habituation to the test room for 2 hours before each test. Mice were tested for anxiety-like behavior using the elevated plus-maze, dark-light transfer and open-field tests, and for depression-like behavior using the forced swim test.

Open-field test: The open-field test was performed in a 50 x 50 x 22 cm white 25 box, lit to 120 lux. The mice were placed in the box for 10 minutes. Mice locomotion in the box was quantified using a video tracking system (VideoMot2; TSE Systems, Bad Hamburg, Germany).

Dark-light transfer test: The dark-light transfer test apparatus consisted of a 30 polyvinyl chloride box divided into a black dark compartment (14 x 27 x 26 cm) and a connected white 1200 lx illuminated light compartment (30 x 27 x 26 cm). During the 5 minute test, time spent in the light compartment, distance traveled in light, and number of light-dark transitions were quantified with a video tracking system (VideoMot2; TSE

Systems, Bad Hamburg, Germany).

Elevated plus-maze test: This test apparatus has the shape of a plus sign and contains 2 barrier walls and 2 lit (6 lux) open arms. During the 5 minute test, number of entries, distance traveled and the time spent in the open arms was automatically scored using a video tracking system (VideoMot2; TSE Systems, Bad Hamburg, Germany).

Modified forced swim test: The forced swim test was performed as previously described (Krishnan et al., 2007). The apparatus is a plastic bucket, with a diameter of 18 cm, filled with 25 °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 in the 3-6 minutes of the test was automatically scored using EthoVision XT (Noldus, Wageningen, Netherlands).

Social avoidance test: In the social avoidance test, mice were placed in a 15 cm x 35 cm arena with a small 15 cm x 8 cm neighboring chamber separated by a divider with small open slits allowing full sensory contact. The tested mice were allowed to habituate to the arena for three minutes, and then an unfamiliar ICR mouse was placed in the neighboring chamber for another three minutes. Time spent near the divider was quantified using video tracking by Ethovision software (Noldus, Wageningen, Netherlands). Interaction ratio was calculated by dividing the time the mice spent in a defined area near the unfamiliar ICR to the time the mice spent in the same area during habituation, and multiplied by 100.

#### ***Microdissections, sample preparation and HPLC-ED analysis of 5HT and 5-HIAA concentrations***

Microdissections were performed as previously described (Neufeld-Cohen et al., 2010a) using the Palkovits microdissection technique (Palkovitz, 1988). Coronal brain sections (300 µm) were taken using a Leica CM1950 cryostat (North Central Instruments, USA). The sections were mounted onto glass slides and microdissected on a cold plate at -10 °C under a stereomicroscope using microdissection needles with varying inner diameters. Microdissections were each put in a tube containing 100 µL of acetate buffer (3.0 g/L sodium acetate, 4.3 mL/L glacial acetic acid; pH adjusted to 5.0). Next, samples were homogenized and centrifuged at 4 °C and 13,000 rpm for 3 min. The pellet was reconstituted with 175 µL of 0.2 M NaOH for protein content, and 50 µL of the supernatant was used for detection of 5HT and 5-hydroxyindoleacetic acid

144

(HIAA) using high performance liquid chromatography with electrochemical detection (HPLC-ED) as previously described (Evans et al., 2008). Samples were placed in an ESA model 542 autosampler injecting the samples into the HPLC chromatographic system (ESA, Chelmsford, MA, USA). The HPLC system also consisted of an ESA 5 Model 582 Solvent Delivery Module to pump the mobile phase (9.53 g/L potassium dihydrogen orthophosphate dihydrate, 300 mg/L octanesulphonic acid, 35 mg/L EDTA, 920 mL/L HPLC grade H<sub>2</sub>O and 80 mL/L HPLC grade methanol; pH adjusted to 3.4 using orthophosphoric acid) through the chromatographic system. The stationary phase, where chromatographic separation occurred, consisted of an integrated 10 precolumn/column system (Ultrasphere-XL 3 µm Octyl Guard Cartridge, 5/70 x 4.6 mm; MAC-MOD Analytical, USA). Electrochemical detection was accomplished using an ESA Model 5200A Coulochem II detector with dual potentiostats connected to an ESA 5021 Conditioning Cell with the electrode potential set at 0 mV and an ESA 5014B Microdialysis Cell with the channel 1 and channel 2 electrode potentials set at 15 25 mV and 250 mV, respectively. For each run, the average peak heights of known concentrations of 5HT and 5-HIAA were determined manually using chromatography analysis software (EZChrom Elite for Windows, Version 2.8; Agilent Technologies, USA) and used to calculate the concentration of the unknown samples. Tissue concentrations of 5HT and 5-HIAA were standardized to the amount of protein.

20 ***Human sample studies***

**Case-Control study:** Eleven male patients diagnosed with major depression (N=9) or bipolar disorder (N=2) and twelve healthy male controls were selected from a study described in detail in (Menke et al., 2012). Briefly, patients were recruited from the Max-Planck Institute of Psychiatry and blood draws for RNA were performed 25 within the first five days of in-patient admission for a depressive syndrome. The mean HRDS score at admission was 24.3 (SD: 5.3 and range 17-32). Controls were screened for the absence of lifetime psychiatric disorders using the Composite International Diagnostic Interview (CIDI) (Wittchen HU, 1999) and for absence of current psychiatric symptoms using the HRDS. Whole blood was collected using a PAXgene 30 (PreAnalytiX GmbH, Hombrechtikon, Switzerland) whole blood RNA collection tube after 2 hours of fasting at 6 pm. Total RNA was isolated from PAXgene whole blood samples using the PAXgene Blood RNA Kit with the Qiagen method for column

145

purification of nucleic acids (PreAnalytiX GmbH, Hombrechtikon, Switzerland). The quality, concentration and purity of the RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and 260 nm UV absorption (Nanophotometer, Germany).

5        Cognitive behavioral therapy (CBT): Patients for this analysis were derived from a randomized clinical trial designed to identify neuroimaging and other biological predictors of remission to cognitive behavioral therapy (CBT). All patients provided written informed consent prior to participation in the study. The study was conducted in accordance with the Declaration of Helsinki and its amendments, and approved by  
10 Emory's Institutional Review Board. The study design is described in detail in (Dunlop et al., 2012). Briefly, eligible participants were adult outpatients between 18 and 60 years of age who met the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)* criteria for a primary diagnosis of MDD without psychotic features. Patients received 16 sessions of CBT. The CBT provided followed a  
15 standardized protocol (Beck et al., 1979). All patients were assessed for symptom change weekly for the first 6 weeks using the Hamilton Depression Rating Scale (HRDS) and then every other week for the remaining six weeks. All patients had an HRDS score of 15 or greater at randomization. From this study, 12 patients who had blood drawn for RNA at baseline, week 2 (N = 16) and week 12 were selected. No  
20 current drug treatment was allowed at the baseline blood draw. 75 % of patients were women, and 87 % of European Ancestry. The mean age in the CBT group was 42.4 (SD: 9.6) years. Whole blood was collected into Tempus RNA tubes (Applied Biosystems) and extracted using the Tempus Spin RNA Isolation Reagent kit (Applied Biosystems). RNA quality and quantity were measured using the Agilent 2100  
25 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and photometric methods (Nanophotometer, Implen, Munich, Germany), respectively.

#### *Statistical analysis*

30 Data was 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 for the microarray real time PCR data validation. One way ANOVA was used for comparison between multiple groups such as between the different treatments in the luciferase assay. Two way ANOVA was used in cases of two independent variables, such as the

146

SSRI or NRI injections both in acute or chronic administration. Repeated measure analysis was used when needed. Post hoc *t* tests were used when necessary to reveal statistical significance. Statistical analysis was performed using Jmp7 software, and differences between groups were considered significant when *p* < 0.05.

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## EXAMPLE 10

### *microRNA “fingerprint” of 5HT neurons*

5HT neurons were isolated from the RN of ePet-EYFP embryos, and their miR expression profile was compared to non-5HT cells, obtained from the same brain area, using miR microarray (Figure 35A). Cell sorting validation was performed by 10 comparing the mRNA expression levels of relevant marker genes. YFP, the fluorescent marker for the ePET-positive neurons was significantly enriched in the 5HT population (Figure 35B), tryptophan hydroxylase 2 (TPH2), a key enzyme in the production of 5HT, was robustly expressed in the 5HT cells (Figure 35C) and glutamate decarboxylase 67 (GAD67), the enzyme catalyzing synthesis of GABA, a common non-5HT 15 neurotransmitter in the RN, was abundant in the non-5HT cells (Figure 35D). The miR “fingerprint” obtained from the microarray (Figure 1A) contained fourteen (Table 10, below) and twenty-seven (Table 11, below) miRs that were expressed 2-fold more or less, respectively, in 5HT neurons compared to the non-5HT neurons. Representative validation of the array results was performed using real time PCR for miRs highly 20 expressed in 5HT neurons such as miR-375 (Figure 1B), and for miRs expressed at lower levels in 5HT neurons such as miR-135a (Figure 1C).

**Table 10: MicroRNA microarray results - list of microRNAs expressed at least two-fold higher in 5HT neurons compared to non-5HT cells of the mouse RN**

| microRNA name   | Fold change |
|-----------------|-------------|
| mmu-miR-375     | 20.72       |
| mmu-miR-376c    | 11.73       |
| mmu-miR-7a      | 4.44        |
| mmu-miR-137     | 2.87        |
| mghv-miR-M1-2   | 2.79        |
| mmu-miR-709     | 2.61        |
| mmu-miR-291b-5p | 2.51        |
| mmu-miR-1224    | 2.40        |
| mmu-miR-1892    | 2.37        |
| mmu-miR-702     | 2.31        |

|                |      |
|----------------|------|
| mmu-miR-139-3p | 2.25 |
| mmu-miR-762    | 2.24 |
| mmu-miR-671-5p | 2.10 |
| mmu-miR-483*   | 2.04 |

**Table 11: MicroRNA microarray results - list of microRNAs expressed at least two-fold lower in 5HT neurons compared to non-5HT cells of the mouse RN**

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

5 In order to further study the potential role of miRs as modulators of 5HT neurons, extensive bioinformatic analysis was performed in a hypothesis-driven manner. Targeting prediction of known 5HT-related genes expressed in serotonergic neurons that have been previously demonstrated to be associated with psychopathologies, were bioinformatically crossed with the microarray results. miR targeting predictions for these 10 genes were performed using two different web-based algorithms: Target Scan ([www.targetscan.org](http://www.targetscan.org)) and MiRanda ([www.microrna.org](http://www.microrna.org)) and were crossed with the list of 91 miRs altered by at least  $\pm 1.5$  fold in the 5HT neuron miR

148

array, compared to non-5HT cells. Two protein coding target genes expressed in 5HT neurons in the RN were selected: the serotonin transporter, responsible for 5HT reuptake (also known as SERT or *Slc6a4*) and serotonin inhibitory receptor 1a (also known as *Htr1a*). Based on the miR array data and the bioinformatic analysis, seven miRs were 5 chosen for further *in vitro* validation (Figures 1D-E).

### EXAMPLE 11

#### *miR-135 targets Htr1a and Slc6a4 transcripts*

*In vitro* luciferase assays were performed to test the miR-target interaction 10 between the 3'UTR of the tested 5HT-related genes and the miRs predicted to putatively target these transcripts. miR-135 targeting of *Slc6a4* 3'UTR (Figures 2A, 2C) and *Htr1a* 3'UTR (Figures 2B, 2D) resulted in robust repression of translation of these transcripts. Additionally, significant repression of *Htr1a* 3'UTR was mediated by miR-335, miR- 15 181c and miR-26a (Figure 2D). Due to the strong effect of miR-135 on both *Htr1a* and *Slc6a4* the present studies further focused on this miR-target interaction. Further 20 bioinformatic analysis revealed that miR-135 has 3 highly conserved variants, miR-135a-1, miR-135a-2 and miR-135b (Figure 36A-C). In addition, miR-135 seed match sequences in the *Slc6a4* 3'UTR is highly conserved (Figure 2E), and in one out of the two seed matches in the *Htr1a* 3'UTR strong conservation was observed (Figure 2F). 25 Mutation studies on the 3'UTR of the *Slc6a4* transcript, in which the miR-135 seed match sequence was removed, revealed that both miR-135a and miR-135b targeting of *Slc6a4* was mediated via its seed match sequence, since 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 together, revealed that miR-135a represses 30 *Htr1a* 3'UTR via the distal and not the proximal seed match while miR-135b acts via both predicted sites (Figure 2H).

### EXAMPLE 12

#### *RN-miR-135 levels are upregulated by antidepressants*

30 Since both *Htr1a* (Savitz et al., 2009) and *Slc6a4* (Murphy et al., 2008) have been previously associated with depression and antidepressant cellular machinery, the present inventor sought to examine the regulation of miR-135 expression in response to

149

antidepressant treatment. The mature miR-135a and miR-135b differ by only one nucleotide (Figure 37A), yet are differentially expressed in the RN as observed in real time PCR results conducted on cDNA obtained from microdissected RN of adult wild type mice (Figure 37B). miR 135b was expressed approximately 10 fold less than miR-5 135a, while the later is relatively highly expressed, only 5-fold less than miR-124, the most abundant miR in the brain and 2.5 fold less than miR-16 that was previously shown to have a role in controlling 5HT functions (Figure 37C). Considering miR-135a is expressed at higher levels in the RN than miR-135b, and was also the variant differently altered in the 5HT microarray, the present inventor focused on regulation studies on this 10 form. Next, the levels of miR-135a was tested in mice exposed to a chronic social defeat model (an environmental model used for the induction of depression-like behaviors) and to chronic treatment with the tricyclic antidepressant imipramine.

Using the social avoidance test, it was verified that social defeat can cause social avoidance and antidepressant administration can reverse this (Figure 37D). Indeed, only 15 mice exposed to social defeat and injected with saline and not those who received imipramine developed social avoidance as implied by an interaction ratio lower than 100 % (Figure 37D). Interestingly, chronic social defeat stress did not alter miR-135a levels in the RN, however, imipramine administered chronically (Figure 37E) or acutely (Figure 37F), both in stressed and non-stressed mice, significantly increased miR-135a 20 expression levels in the RN. Since imipramine is not a specific 5HT reuptake inhibitor, the effects of both acute and chronic administration of the selective serotonin reuptake inhibitor (SSRI), fluoxetine, and the noradrenaline reuptake inhibitor (NRI), reboxetine, was further tested, a robust increase in miR-135a levels in the RN following both acute 25 and chronic SSRI treatment was found, yet no differences were observed following the NRI treatment (Figure 37G).

### EXAMPLE 13

#### *miR-135 overexpression specifically in 5HT neurons reduces anxiety and depression-like behaviors following social defeat*

30 To further explore the role of 5HT-miR-135 *in vivo*, a mouse model was established that specifically overexpresses miR-135 in 5HT neurons of the RN (miR-135OE). Mice expressing Cre recombinase specifically in the RN 5HT-positive neurons

150

(ePet-Cre) were crossbred with a transgenic mouse line carrying a conditional miR-135a cassette (Figure 38A). As controls, mice positive for the miR-135 overexpression transgene and negative for the ePet-Cre were used. miR-135a expression level in the RN of mice over expressing miR-135a specifically in 5HT neurons was tested by real time 5 PCR, and was demonstrated to be overexpressed by approximately 2-fold compared to control mice (Figure 38B). Overexpression levels of miR-135 in this mouse model were similar to those measured in the RN of mice following SSRI administration. Additionally, the levels of miR-135 target transcripts Slc6a4 (Figure 38C) and Htr1a (Figure 38D) were reduced in the RN of miR-135OE mice compared to control mice, 10 demonstrating *in vivo* repression of miR-135 target genes.

The miR-135OE and their littermate controls were behaviorally characterized in tests for anxiety and depression-like behaviors, either under ‘basal’ conditions or following the chronic social defeat protocol. Under ‘basal’ conditions no differences were observed between miR-135OE and control mice in tests for anxiety and 15 depression-like behaviors (Figure 38E-J, left bars and 38K). However, miR-135OE mice demonstrated a significant resiliency to the effects of chronic social defeat. In the dark-light transfer test miR-135OE mice exposed to social defeat spent more time in light (Figure 38E), visited the lit compartment more frequently (Figure 38F) and traveled longer distance in light (Figure 38G) relative to control mice. The behavioral 20 performance of the miR-135OE mice did not significantly differ following the social defeat protocol. In contrast, control mice demonstrated significant increases in anxiety like-behaviors in all measured parameters of the dark-light test following social defeat (Figure 38E-G). Similar results were observed in the elevated plus-maze test as control mice that were exposed to social defeat spent less time (Figure 38H), had fewer visits 25 (Figure 38I) and traveled less distance (Figure 38J) in the open arms compared to miR-135OE mice tested under the same conditions. No significant differences between ‘basal’ and stress conditions were observed in the miR-135OE group. Similar results were observed in tests assessing depression-like behaviors. While no differences were observed under ‘basal’ conditions (Figure 38K), when tested following chronic social 30 defeat, miR-135OE mice exhibited significantly less immobility time in the forced swim test compared to controls (Figure 38L), which is interpreted as decreased depression-like behavior. These differences could not account for changes in locomotor activity since

151

the distance traveled in the open-field was similar in both genotypes (Figure 38M). Taken together, overexpression of miR-135 specifically in 5HT neurons protected against the adverse effect of chronic stress on anxiety and depression-like behaviors.

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#### EXAMPLE 14

##### *Knockdown of miR-135 in adult mice RN increased anxiety like-behaviors and decreased the response to antidepressants*

To determine the importance of miR-135 endogenous levels in mediating depression-like behaviors in response to antidepressant treatment and anxiety-like 10 behaviors under 'basal' conditions, a lentiviral-based system was established to specifically knockdown (KD) the endogenous levels of miR-135 in the RN of wild-type mice. Expression plasmid containing a miR-135 inhibitor (miRNA capture, Figure 39A) or control sequence were subcloned into a lentiviral construct containing the H1 promoter and the GFP reporter (Figure 39B) to allow constitutive expression of the miR- 15 135 capture sequence. The efficiency of the lentiviruses, produced from these constructs, to suppress the expression of the target genes Htr1a and Slc6a4 was tested *in vitro* by infecting RN46A cells, which endogenously expressing both Htr1a, Slc6a4 and miR- 135. RN46A cell infected with miR-135 KD lentiviruses expressed significantly higher 20 levels of Htr1a and Slc6a4 mRNA as tested by real time PCR compared to cells infected by the KD control lentiviruses (Figure 39C).

Wild-type adult mice RN were infected with either miR-135KD or control lentiviruses, and following a recovery period mice behaviors were assessed using tests for anxiety and depression-like behaviors. Infection accuracy was subsequently verified using GFP immunohistochemistry (Figure 39D). In the dark-light transfer test the miR- 25 135KD mice demonstrated a significant increase in anxiety-like behavior compared to control-injected mice (Figure 39E-H). The miR-135 KD mice spent less time (Figure 39E), visited less (Figure 39F) and walked shorter distances in the lit compartment (Figure 39G). Similarly, in the elevated plus-maze test the miR-135 KD mice demonstrated increased anxiety-like behaviors compared to control-injected mice. The 30 miR-135 KD mice showed a tendency to spend less time (Figure 39I), visit less (Figure 39J) and travel significantly less distance (Figure 39K) in the open arms of the maze (Figure 39I-L).

152

Depression-like behaviors of the miR-135 KD mice were tested both under 'basal' conditions and following SSRI treatment. In the forced swim test no differences were observed between the groups under 'basal' conditions (Figure 39M). However, following SSRI administration, miR-135 KD mice were significantly more immobile 5 compared to control-injected mice (Figure 39M), suggesting an important role for endogenous RN-miR-135 levels in mediating SSRI-induced antidepressant effects. Reduced levels of miR-135 in the RN did not affect the locomotor activity of these mice (Figure 39N).

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## EXAMPLE 15

### *miR-135 overexpression altered 5HT levels and metabolism*

To evaluate whether changes in miR-135 levels are also reflected in the tissue concentrations of central 5HT and its turnover, the RN subdivisions were microdissected and the brain regions innervated by these areas from the miR-135 OE mouse model and 15 control littermates. Figures 40A-40O, Figures 42A-L and Figures 43A-L depict tissue concentrations of 5HT and the 5HT metabolism (5HIAA/5HT ratio) in these mice under 'basal' conditions and following the social defeat protocol.

Tissue concentrations of 5HT and the 5HT metabolism within an anxiety and depression-related neural circuit were influenced by the miR-135 genotype, as well as 20 the social defeat manipulation miR-135 OE decreased tissue 5HT concentrations, and increased serotonin metabolism, a pattern consistent with increased serotonin turnover, in brain regions implicated in regulation of anxiety-related behavior and stress resilience, such as the prelimbic cortex (PrL), infralimbic cortex (IL), basolateral amygdala (BLA), CA1 region of the ventral hippocampus (CA1V), subiculum (S), bed nucleus of the stria 25 terminalis (BNST), central nucleus of the amygdala (CeA), dorsal, ventral, caudal and interfascicular parts of the dorsal raphe nucleus (DRD, DRV, DRC, DRI), and median raphe nucleus (MnR) (Figures 40A-40O, Figures 42A-L and Figures 43A-L). These results are in line with miR-135 OE decrease of Htr1a and Slc6a4 expression (Figures 38C-D) under 'basal' conditions, effects that would be expected to result in increased 30 serotonergic neuronal firing rates and serotonergic signaling, respectively.

Social defeat decreased tissue 5HT concentrations and increased 5HT metabolism in anxiety-related brain regions in control mice, a pattern consistent with

153

increased serotonin turnover, including the PrL and BNST (Figures 40A-40O and Figures 42A-L), persistent with previous studies demonstrating social defeat-induced activation of anxiety-related subsets of serotonergic neurons in the DRD and DRC. These effects of social defeat were prevented in miR-135OE mice, suggesting a 5 mechanistic explanation to the behavioral resiliency to chronic stress observed in these mice.

#### EXAMPLE 16

*miR-135a levels in the blood are downregulated in depressed patients and upregulated 10 by therapy*

Since circulating miRs levels were shown to correlate with disease states, the present inventor tested whether blood miR-135 levels are altered in depressed human patients. Relative levels of miR-135a and miR-16 were tested in two sets of human 15 blood samples. One compared depressed patients to matched healthy controls, the other changes in miRNA levels over time within depressed patient receiving 3 months of cognitive behavioral therapy (CBT). miR-135a levels were robustly reduced in currently depressed patients (mean Hamilton Depression Rating Scale (HRDSS)= 24.3 (SD: 5.3), i.e. with moderate to severe depressive symptoms) compared to controls (Figure 41A), 20 while no significant changes in miR-16 were observed (Figure 41B). Comparing miR-135a blood levels in depressed patients prior to and three months following treatment revealed a significant increase in miR-135a levels following CBT (Figure 41C). No effect was observed in the same blood samples for miR-16 levels (Figure 41D). These 25 results suggest miR-135a levels in human blood as a possible biomarker for depression state and response to treatment.

#### EXAMPLE 17

*Anti-bipolar effect of miR-135 in amphetamine-induced hyperactivity rat model*

To evaluate the anti-bipolar effect of miR-135 preclinically, an *in vivo* 30 amphetamine-induced hyperactivity model of mania in rats is used which is relevant to the manic phase of bipolar disorder. This model focuses on an induced increase in the activity level of the animal (hyperactivity) as a parallel to the hyperactivity of the manic

154

patient. The reversal of the induced hyperactivity in rodents, by pretreatment with a drug indicates the possible efficacy of this drug in the treatment of human mania. The most consistent finding with lithium (the standard drug for mania) is the reduction in rearing. Rearing is followed in the models by observing the vertical activity of the animals.

5 Accordingly, pretreatment of rats with miR-135 prior to amphetamine-induced hyperactivity is being tested for the treatment of bipolar disease. Moreover, treatment of rats with miR-135 following amphetamine-induced hyperactivity is being tested for the treatment of bipolar disease.

10 Rats are housed under a 12 hr light/dark cycle and behavioral testing is conducted in the light phase. Specifically, rat activities are followed in an activity meter (Elvicom, Israel) based on 2 levels laser beams and equipped with a computerized system that can count the vertical movements of rats (rearing). Activities are recorded for 30 min for each session, and the resultant appropriate movement is reported per 30 min.

15 The activities of rats pretreated or treated with miR-135 are compared to non treated rats (control group) and to rats pretreated or treated with the standard drugs for mania, i.e. lithium or valproate. Rats are followed before and after being challenged with amphetamine. The statistical analyses are conducted using a two way ANOVA.

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## EXAMPLE 18

### *Anti-bipolar effect of miR-135 in ketamine-induced mania rat model*

To evaluate the anti-bipolar effect of miR-135 preclinically, an *in vivo* ketamine-induced hyperactivity model of mania in rats is used. Ketamine induces hyperlocomotion of treated rats, which can be monitored as discussed above.

25 Rats are pretreated (for 14 days) with miR-135 or with the standard drugs for mania, i.e. lithium (47.5 mg/kg, i.p., twice a day) or valproate (200 mg/kg, i.p., twice a day), or with saline (i.p., twice a day) as a control. Between days 8 and 14, these rats are treated with ketamine (25 mg/kg, i.p.) or saline.

30 In a reversal protocol, rats first receive ketamine (25 mg/kg, i.p.) or saline followed by administration of miR-135, lithium, valproate, or saline for 7 days.

The activities of rats pretreated or treated with miR-135 are compared to non treated rats (control group) and to rats pretreated or treated with the standard drugs for

155

mania, i.e. lithium or valproate. Rats are followed before and after being challenged with ketamine. The statistical analyses are conducted using a two way ANOVA.

## DISCUSSION

5 In the current study the role of specific miR was elucidated in regulating central 5HT system activity, under 'basal' and challenged conditions. The unique "fingerprint" of miR expression was determined in serotonergic neurons and several 5HT-linked target genes were bioinformatically identified. *In-vitro* luciferase assays and mutation studies revealed a strong repressive effect for miR-135 on both Slc6a4 and Htr1a  
10 transcripts. Intriguingly, miR-135 levels in the RN were robustly upregulated following acute or chronic SSRI administration. Genetically modified mice models, expressing higher or lower levels of miR-135 demonstrated major alternations in anxiety and depression-like behaviors, 5HT levels and metabolism, and behavioral response to antidepressant treatment. Finally, miR-135 levels in blood of depressed human patients  
15 and response to treatment were presented.

The use of the ePet-EYFP mouse model for the isolation of 5HT and non-5HT cells from the mouse RN allowed to determine for the first time the specific miR profile of serotonergic neurons. While this approach was successful and informative, yet, in order to efficiently sort the 5HT positive neurons from the mouse RN embryonic and not  
20 adult brain tissue was used, which means that at least part of the miRs presented in the 5HT miRs profile may be relevant to developmental processes and not adult 5HT neuronal functions. Nevertheless, serotonergic signaling during specific developmental periods is known to affect adult anxiety phenotypes (Gross et al., 2002). Interestingly, miR-375, commonly associated with pancreatic beta cell differentiation, was robustly  
25 expressed in 5HT neurons compared to control, supporting the suggested common developmental path of these tissues.

Bioinformatic analysis suggested several putative miR-target interactions between the serotonin receptor 1A (Htr1a) and the serotonin transporter (SERT or Slc6a4) 3'UTRs and miRs differentially expressed in the 5HT microarray. Htr1a and  
30 Slc6a4 have been shown to play a major role in the serotonergic system function, in depression and anxiety disorders, and in the response to antidepressants (Savitz et al., 2009) and (Murphy et al., 2008). Htr1a is an inhibitory G protein-coupled receptor that

156

is expressed as an autoreceptor on 5HT producing cells and post-synaptically across the brain of 5HT projection sites. Stimulation of Htr1a autoreceptors inhibits serotonergic neuronal firing and the release of serotonin in nerve terminals and has been postulated to be one of the causes for the therapeutic lag that is commonly reported for most 5 serotonergic antidepressants such as SSRIs. Slc6a4 is a plasma membrane transporter that terminates serotonin action by recycling it from the synaptic cleft into presynaptic neurons, in a sodium-dependent manner. Slc6a4 is the direct target of most commonly used antidepressants, either the former generation of tricyclic antidepressants that inhibit different monoamine reuptake transporter activities including Slc6a4, or the more 10 specific SSRIs. Decreased activity of both Slc6a4 and the presynaptic Htr1a would be expected to increase 5HT levels in the synapse, which are consistent with antidepressant action and decreases in depressive symptoms. Luciferase assays confirmed miR-135 variants as significant repressors of both Slc6a4 and Htr1a transcripts. Mutation studies further demonstrated the importance of miR-135 seed binding sites in the Htr1a and 15 Slc6a4 3'UTRs in mediating the observed miR-135 repressive effects. Single nucleotide polymorphisms (SNPs) in the 3'UTR of human Slc6a4 and Htr1a, previously reported for these genes (Piva et al., 2010), are not within miR-135 seed match sequences.

miR-135 was previously reported to be mainly involved in cancer-related pathologies and developmental processes. miR-135 was demonstrated to target the 20 adenomatous polyposis coli gene and as a consequence to promote colorectal cancer, to modify chemotherapy resistance, and to regulate JAK2 in classic Hodgkin lymphoma. The role of miR-135 in developmental processes was demonstrated in megakaryocytopoiesis, porcine brain development, and mineralization in osteogenic differentiation by regulating Smad5, a key transducer of the BMP2 osteogenic signal. 25 Additionally, miR-135 was suggested to be involved in regulating blood pressure by suppressing NR3C2 and to have a potential role in heart failure.

Several microRNA screening studies have reported that microRNA levels in 30 various adult rodents or rat brain structures are affected by a range of behavioral and pharmacological manipulations (Kye et al., 2011). Stressful challenges were shown to alter miR expression in different brain sites using different paradigms (Smalheiser et al., 2011). The present inventor has recently demonstrated the involvement of miR-34 in the regulation of anxiety-like behaviors (Haramati et al., 2011), while miR-22, miR-138-2,

157

miR-148a, and miR-488 were associated with panic disorder (Muinos-Gimeno et al., 2011). Studies using mice, presented in the current manuscript, revealed a clear upregulation of miR-135 following antidepressant administration. Further comparison of SSRI and NRI antidepressants demonstrated an SSRI- and not NRI-specific effect, 5 further suggesting a role for miR-135 in the biology of 5HT neurons. While chronic stress is associated with increased susceptibility for the development of depression, surprisingly, chronic stress conditions did not affect miR-135 levels in the RN. Chronic SSRI treatment was reported to promote reduction in Slc6a4 and Htr1a protein levels but not mRNA [Slc6a4 (Benmansour et al., 2002), Htr1a (Savitz et al., 2009)] suggesting the 10 possible involvement of a post-transcriptional mechanism in mediating SSRI activity. miR-16 was shown to target Slc6a4 and to have a role in antidepressant response (Baudry et al., 2010), while lithium administration was shown to alter miRs expression (Creson et al., 2011). An association was found between variants in miR-182 (Saus et al., 2010) and miR-30e (Xu et al., 2010b) in major depression patients, and miRs 15 expression was altered in the prefrontal cortex of patients with suicidal depression (Smalheiser et al., 2012). Additionally, miR448 was shown to control expression of several 5HT receptors as part of adipose tissue development, and polymorphism in serotonin receptor 1B moderates the regulation by miR96 and associates with aggressive behaviors (Jensen et al., 2009). Finally, the miR510 targeting site of serotonin receptor- 20 type 3E was shown to play a role in irritable bowel syndrome.

Antidepressant treatment was reported to activate the cAMP signal transduction pathway and to promote CREB binding to CRE sites. Promoter analysis of miR-135 5' flanking regions identified several putative CRE sites, suggesting a potential mechanism for the observed miR-135 upregulation by SSRI. Taken together, upregulation of miR- 25 135 expression levels in the RN following antidepressant administration, along with the results demonstrating miR-135 targeting of Htr1a and Slc6a4, suggests that miR-135 is an endogenous antidepressant.

To further support a role for miR-135 as an endogenous antidepressant, a series of experiments were conducted in which miR-135 levels were manipulated *in vivo* and 30 the effects on animal behavior was assessed. The transgenic mouse model that overexpresses miR-135 specifically in 5HT neurons, in levels equivalent to those observed following antidepressant treatment, showed a strong protective effect from the

158

adverse behavioral effects of chronic social defeat. These results resembled the effect observed when Htr1a (Bortolozzi et al., 2012) or Slc6a4 (Thakker et al., 2005) were knocked down using siRNA approaches, showing reduced depression-like behaviors, or when Htr1a autoreceptor levels were increased using an elegant transgenic mice model, 5 leading to elevation in anxiety and depression-like behaviors and reduced response to antidepressants (Richardson-Jones et al., 2010). In contrast, the developmental knockout mouse models for Htr1a (Savitz et al., 2009) and Slc6a4 (Holmes et al., 2003) showed paradoxical increases in anxiety and depression-like behaviors, which were suggested to be mediated by developmental compensatory changes. In addition to Htr1a and Slc6a4, 10 it is also likely that miR-135a affects other genes in serotonin neurons that may possibly contribute to the observed phenotypes.

Using a complementary approach, the levels of miR-135 were knocked down specifically in the RN of adult wild-type mice using lentiviruses and behavior in mice was assessed under 'basal' (unstressed) conditions and following SSRI administration. 15 In contrast to the behaviors observed by the mice that overexpress miR-135, reduced levels of this miR caused a robust increase in anxiety-like behavior and attenuated response to antidepressants. These results support an important role for miR-135 in maintaining intact responses to challenge under 'basal' conditions and its essential role in the mechanism of antidepressant action. These findings are in agreement with the 20 published literature, which shows that higher levels of Htr1a are associated with increased behavioral despair and blunted responses to antidepressants (Richardson-Jones et al., 2010). Furthermore, polymorphism in the human Htr1a gene was associated with higher Htr1a autoreceptor binding and increased anxiety and depression (Fakra et al., 2009). In contrast, lower expression levels of Slc6a4, due to a shorter promoter variant, 25 were reported to be associated with increased anxiety and depression and reduced responses to antidepressants (Homberg and Lesch, 2011).

Further support for a role of miR-135 in 5HT circuits emerged from the HPLC data indicating a robust alteration in 5HT levels and its metabolism across the brain of the miR-135OE mice model. 5HT levels were lower, while 5HT metabolism was higher, 30 in the miR-135OE mice compared to controls under 'basal' stress conditions both in sub-nuclei of the raphe where 5HT is synthesized, and in the projection sites important for controlling anxiety and depression-like behaviors. This pattern of change in 5HT

159

levels and 5HT metabolism is consistent with increased serotonergic neuronal firing and increased serotonergic signaling in miR-135OE mice. These differences could be a result of compensatory changes associated with the overexpression of miR-135 from development through adulthood. However, despite the low 'basal' 5HT levels, the mice 5 demonstrate normal behaviors under 'basal' conditions, probably due to more active 5HT system as can be depicted by their higher 5HT metabolism at 'basal' conditions. Conceivably, lower expression levels of Slc6a4 and Htr1a that function as inhibitors of 5HT secretion in the RN enable the mice to function normally with lower levels of 5HT. Interestingly, chronic stress caused a decrease in 5HT levels accompanied by an increase 10 in 5HT metabolism in some brain areas of control mice as expected, while in the miR-135OE mice these effects were not observed. These changes may provide a mechanistic explanation for the behavioral resiliency to chronic stress observed in the miR-135OE mice.

The possible use of circulating miRs as a non-invasive biomarker for 15 pathological conditions is a rising field, which is promoted by relatively high levels and stability of miRs in the circulation. While little is known about the role and origin of the extracellular miRs, circulating miRs have been associated with pathophysiological states, such as different types of cancer, heart diseases, oxidative liver injury, sepsis, pregnancy and more. In the current study, the levels of miR-135 in the total blood of 20 depressed patients were determined and a robust decrease in miR-135 levels in the blood of depressed patients, compared to match controls, was observed. These findings are in line with previous data from animal models indicating miR-135 to be an endogenous antidepressant, and suggest miR-135 as a possible biomarker for depression state and possibly for response to treatment.

25 To conclude, the present inventor proposes that miR-135, expressed by 5HT neurons, is an essential regulatory element responsible for maintaining intact serotonergic tone under normal conditions, and essential for the brain response to antidepressants (see schematic model in Figure 41E). Increased levels of miR-135 repress both Slc6a4 and presynaptic Htr1a levels, causing an increase in 5HT in the 30 synaptic cleft, which is associated with decreases in depressive symptoms.

Further bioinformatic analysis conducted by the present inventors, predicted the following miR135 targets to be associated with stress-related neuropsychiatric disorders

160

including bipolar affective disorder or lithium action: adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K+ 2 (Hcn2), inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalm); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syne1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfbr2);

161

thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3) and zinc finger, BED domain containing 4 (Zbed4).

Together, these data suggest that miR-135a may play a critical role in the  
5 pathophysiology of bipolar affective disorder, its treatment and diagnosis.

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  
10 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  
15 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.

162  
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163

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## WHAT IS CLAIMED IS:

1. A method of treating a bipolar disorder in a subject in need thereof, the method comprising administering to the subject therapeutically effective amount of a miR-135, a precursor thereof or a nucleic acid molecule encoding said miR-135 or said precursor thereof, thereby treating the bipolar disorder.

2. Use of a therapeutically effective amount of a miR-135, a precursor thereof or a nucleic acid molecule encoding said miR-135 or said precursor thereof for the manufacture of a medicament identified for treating a bipolar disease in a subject in need thereof.

3. The method of claim 1, or use of claim 2, wherein said miR-135 is selected from the group consisting of miR-135a and miR-135b.

4. The method of claim 1, or use of claim 2, wherein said miR-135 is as set forth in SEQ ID NO: 58-62.

5. The method of claim 1, or use of claim 2, wherein said miR-135 comprises miR-135\* as set forth in SEQ ID NO: 192-193.

6. The method of claim 1, or use of claim 2, wherein said miR-135 comprises a modification selected from the group consisting of a modified sugar-phosphate backbone and a modified base.

7. The method of claim 1, or use of claim 2, wherein said miR-135 comprises a modification in both a sugar and an internucleoside linkage.

8. The method or use of claim 6 or 7, wherein said modification is selected from the group consisting of a phosphorothioate, a chiral phosphorothioate, a phosphorodithioate, a phosphotriester, an aminoalkyl phosphotriester, a methyl phosphonate, an alkyl phosphonate, a chiral phosphonate, a phosphinate, a

168

phosphoramidate, an aminoalkylphosphoramidate, a thionophosphoramidate, a thionoalkylphosphonate, a thionoalkylphosphotriester, a boranophosphate, a phosphodiester, a 2'-O-methoxyethyl, a 2'-O-methyl, a 2'-fluoro, a locked nucleic acid (LNA), a peptide nucleic acid (PNA) and a 2'-Fluoroarabinooligonucleotides (FANA).

9. The method or use of claim 6 or 7, wherein said miR-135 is as set forth in SEQ ID NO: 194-209.

10. The method of claim 1, or use of claim 2, wherein said bipolar disorder is selected from the group consisting of Bipolar I, Bipolar II, Rapid-cycling bipolar disorder, Cyclothymia and Bipolar Disorder Not Otherwise Specified (BD-NOS).

11. An article of manufacture comprising a packaging material packaging an agent selected from the group consisting of a miR-135, a precursor thereof and a nucleic acid molecule encoding said miR-135 or said precursor thereof and a medicament for the treatment of a bipolar disorder.

12. The article of manufacture of claim 11, wherein said miR-135 is selected from the group consisting of SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61 and SEQ ID NO: 62.

13. The article of manufacture of claim 11, wherein said miR-135 comprises miR-135\* selected from the group consisting of SEQ ID NO: 192 and SEQ ID NO: 193.

14. The article of manufacture of claim 11, wherein said miR-135 comprises a modification selected from the group consisting of a modified sugar-phosphate backbone and a modified base.

15. The article of manufacture of claim 11, wherein said miR-135 comprises a modification in both a sugar and an internucleoside linkage.

16. The article of manufacture of claim 14 or 15, wherein said modification is

169

selected from the group consisting of a phosphorothioate, a chiral phosphorothioate, a phosphorodithioate, a phosphotriester, an aminoalkyl phosphotriester, a methyl phosphonate, an alkyl phosphonate, a chiral phosphonate, a phosphinate, a phosphoramidate, an aminoalkylphosphoramidate, a thionophosphoramidate, a thionoalkylphosphonate, a thionoalkylphosphotriester, a boranophosphate, a phosphodiester, a 2'-O-methoxyethyl, a 2'-O-methyl, a 2'-fluoro, a locked nucleic acid (LNA), a peptide nucleic acid (PNA) and a 2'-Fluoroarabinooligonucleotides (FANA).

17. The article of manufacture of claim 14 or 15, wherein said miR-135 is selected from the group consisting of SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 203, SEQ ID NO: 204, SEQ ID NO: 205, SEQ ID NO: 206, SEQ ID NO: 207, SEQ ID NO: 208 and SEQ ID NO: 209.

18. The article of manufacture of claim 11, wherein said medicament for the treatment of a bipolar disorder is selected from the group consisting of a lithium, an antipsychotic medicament and a mood stabilizer medicament.

19. A method of monitoring treatment of an anti-depressant drug or a medicament for the treatment of a mood disorder, the method comprising:

(a) treating a human subject in need thereof with an anti-depressant drug or a medicament for the treatment of a mood disorder; and

(b) measuring an expression level of a miR-135 in biological sample of said human subject prior to and following said treatment, wherein a higher expression level of said miR-135 following said treatment by said anti-depressant drug or said medicament for said treatment of said mood disorder as compared to said expression level of said miR-135 prior to said treatment by said anti-depressant drug or said medicament for said treatment of said mood disorder is indicative of the efficient treatment.

20. The method of claim 19, further comprising (c) treating the human subject when a higher expression level of said miR-135 is observed in step (b).

170

21. The method of claim 19, further comprising obtaining a biological sample from said human subject prior to said treating.

22. The method of claim 19, wherein said mood disorder comprises a bipolar disorder.

23. The method of claim 19, wherein said anti-depressant drug is selected from the group consisting of selective serotonin reuptake inhibitors (SSRI), tricyclic antidepressants and noradrenaline reuptake inhibitors (NRI).

24. The method of claim 19, wherein said medicament for said treatment of said mood disorder is selected from the group consisting of a lithium, an antipsychotic medicament and a mood stabilizer medicament.

25. A method of diagnosing a mood disorder in a human subject in need thereof, the method comprising measuring an expression level of a miR-135 in a biological sample of the human subject, wherein a lower expression level of said miR-135 as compared to that in a biological sample of a healthy human subject is indicative of the mood disorder.

26. The method of claim 19, 21 or 25, wherein said biological sample is selected from the group consisting of a whole blood, a serum, a plasma and white blood cells.

27. The method of claim 25, wherein said mood disorder is selected from the group consisting of a bipolar disorder, a depression, a major depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a schizophrenia, a sleep disorder and an eating disorder.

28. The method of claim 25, wherein said miR-135 is selected from the group consisting of miR-135a or miR-135b.

29. The method of claim 1, or use of claim 2, wherein said subject is a human subject.

30. An isolated polynucleotide comprising a miR-135 wherein said miR-135 comprises a modification selected from the group consisting of a locked nucleic acid (LNA) and a 2'-Fluoroarabinooligonucleotides (FANA).

31. An isolated polynucleotide comprising a modified miR135 selected from the group consisting of SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 203, SEQ ID NO: 204, SEQ ID NO: 205, SEQ ID NO: 206, SEQ ID NO: 207, SEQ ID NO: 208 and SEQ ID NO: 209.

32. An isolated polynucleotide comprising a microRNA selected from the group consisting of miR-335, miR-181, miR-182, miR-26, miR-27, miR-15 and miR-19 wherein said microRNA comprises a modification selected from the group consisting of a locked nucleic acid (LNA) and a 2'-Fluoroarabinooligonucleotides (FANA).

33. A pharmaceutical composition comprising the isolated polynucleotide of claim 30, 31 or 32, and a pharmaceutically acceptable carrier.

34. A method of upregulating an expression of a gene selected from the group consisting of adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3)

fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntn1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syn1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfb2); thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and zinc finger, BED domain containing 4 (Zbed4) in a neuroglia cell, the method comprising:

- (a) downregulating an activity or expression of a miR-135 or a precursor thereof in said neuroglia cell; and
- (b) measuring an expression of said gene in said neuroglia cell,

thereby upregulating the expression of the gene.

35. A method of downregulating an expression of a gene selected from the group consisting of adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); an ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Caenca1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3); glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntn1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2,

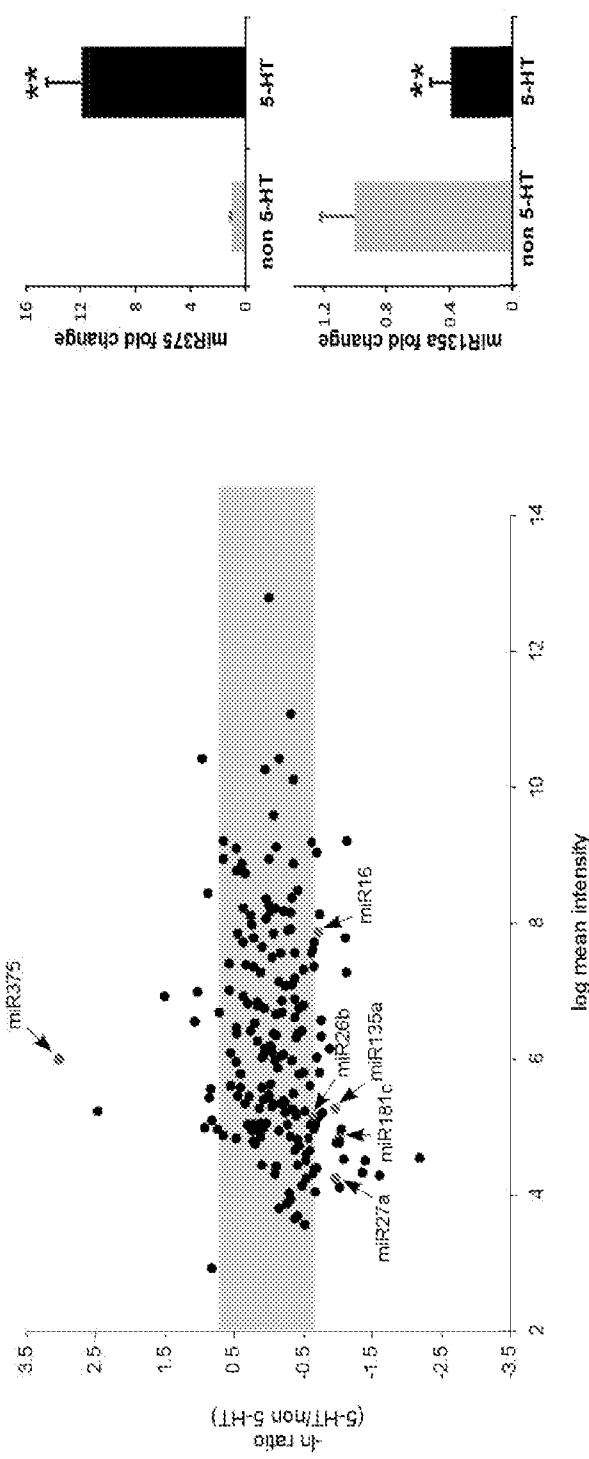
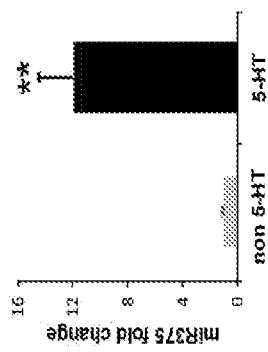
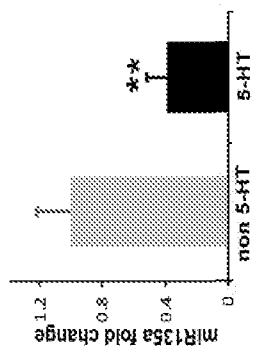
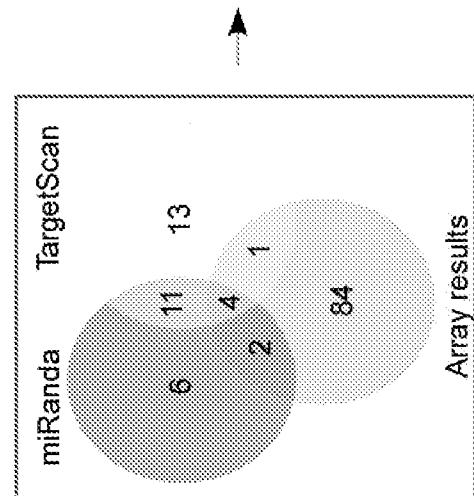
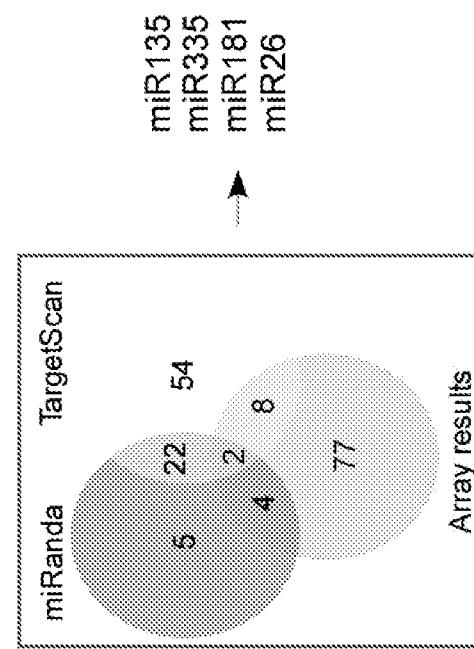
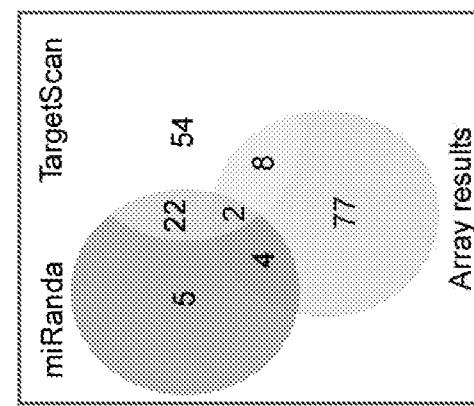
homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syn1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfb2); thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and zinc finger, BED domain containing 4 (Zbed4) in a neuroglia cell, the method comprising:

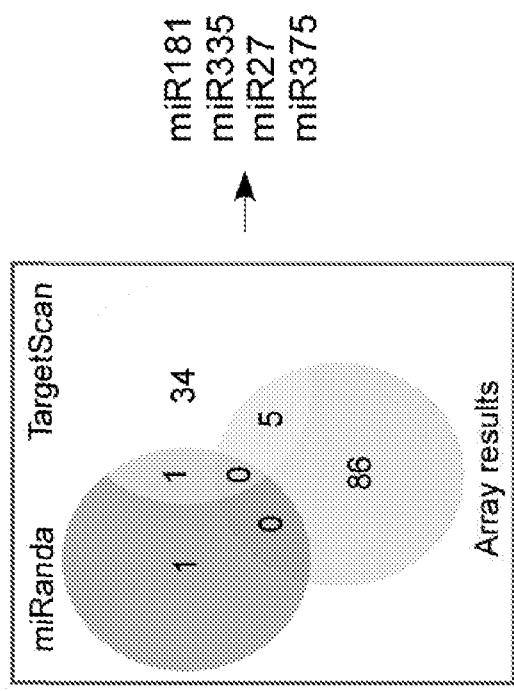
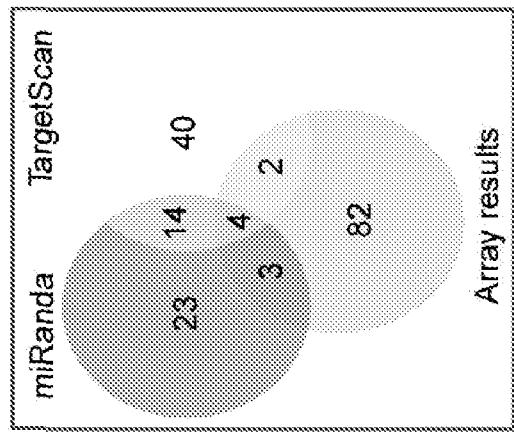
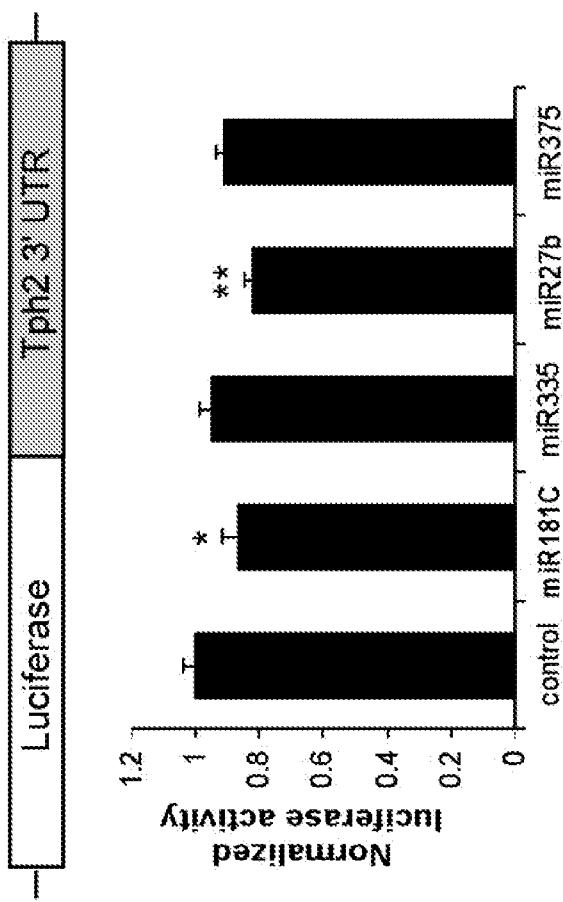
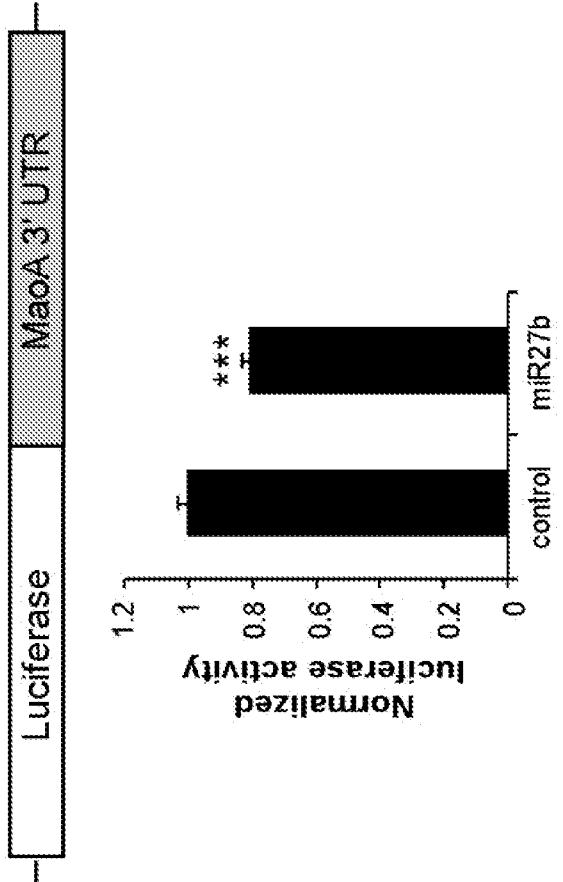
- (a) upregulating an activity or expression of a miR-135 or a precursor thereof in said neuroglia cell; and
- (b) measuring an expression of said gene in said neuroglia cell, thereby downregulating the expression of the gene.

36. 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 the subject an agent capable of downregulating an activity or expression of a miR-135 target selected from the group consisting of adenylate cyclase activating polypeptide 1 (Adcyap1 or PACAP); adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1); adrenergic receptor, alpha 2a (Adra2a); ankyrin 3 (ANK3); activity-regulated cytoskeleton-associated protein (Arc); Rho GTPase activating protein 6 (Arhgap6); activating transcription factor 3 (Atf3); beta-site APP cleaving enzyme 1 (Bace1); calcium channel, voltage-dependent, L type, alpha 1D subunit (Cacna1d); cell adhesion molecule 3 (Cadm3); complexin 1 (Cplx1); complexin 2 (Cplx2); CUB and Sushi multiple domains 1 (Csmd1); casein kinase 1, gamma 1 (Csnk1g1); doublecortin (Dcx); DIRAS family, GTP-binding RAS-like 2 (Diras2); discs, large homolog 2 (Drosophila) (Dlg2); ELK1, member of ETS oncogene family (Elk1); fyn-related kinase (Frk); fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (Fut9); gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (Gabrb2); GATA binding protein 3 (Gata3); growth hormone secretagogue receptor (Ghsr); G protein-coupled receptor 3 (Gpr3); a glutamate receptor, ionotropic AMPA3 (alpha 3) (GRIA3);

glutamate receptor, ionotropic, kainate 3 (Grik3); G protein-coupled receptor kinase 5 (Grk5); a glycogen synthase kinase-3beta (GSK3B); hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1), hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 2 (Hcn2), 5-hydroxytryptamine (serotonin) receptor 1A (Htr1a); inositol monophosphatase (IMPA1), kalirin, RhoGEF kinase (Kalrn); a potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3); karyopherin alpha 3 (importin alpha 4) (Kpna3); myelin transcription factor 1-like (Myt1l); nuclear receptor coactivator 2 (Ncoa2); N-Myc Downstream-Regulated Gene 4 (Ndrg4); a nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP); nuclear receptor subfamily 3, group C, member 2 (Nr3c2); netrin G1 (Ntng1); nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1); phosphodiesterase 1A, calmodulin-dependent (Pde1a); phosphodiesterase 4A, cAMP specific (Pde4a); phosphodiesterase 8B (Pde8b); phospholipase C, beta 1 (Plcb1); prolactin receptor (Prlr); RAB1B, member RAS oncogene family (Rab1b); Ras-Related Protein Rap-2a (Rap2a); Retinoid-Related Orphan Receptor Beta (Rorb); sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1); solute carrier family 12, (potassium/chloride transporters) member 6 (Slc12a6); solute carrier family 5 (choline transporter), member 7 (Slc5a7); solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4); trans-acting transcription factor 1 (Sp1); synaptic vesicle glycoprotein 2 b (Sv2b); Synaptic nuclear envelope 1 (encodes nesprin-1) (Syne1); synaptotagmin I (Syt1); synaptotagmin II (Syt2); synaptotagmin III (Syt3); transforming growth factor, beta receptor II (Tgfbr2); thyroid hormone receptor, beta (Thrb); transient receptor potential cation channel, subfamily C, member 6 (Trpc6); vesicle-associated membrane protein 2 (Vamp2); wingless-related MMTV integration site 3 (Wnt3); and zinc finger, BED domain containing 4 (Zbed4), wherein said agent is not miR-135, thereby treating the medical condition.

37. The method of claim 36, wherein said medical condition is selected from the group consisting of a bipolar disorder, a depression, a major depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a schizophrenia, a sleep disorder, an eating disorder, a growth disorder and a reproduction disorder.

**FIG. 1A****FIG. 1B****FIG. 1C****FIG. 1D****FIG. 1E****FIG. 1A**

**FIG. 1F** *Tph2***FIG. 1G** *MaoA***FIG. 1H****FIG. 1I**

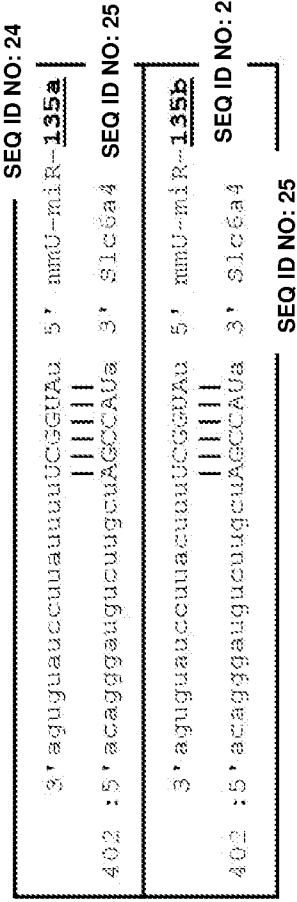
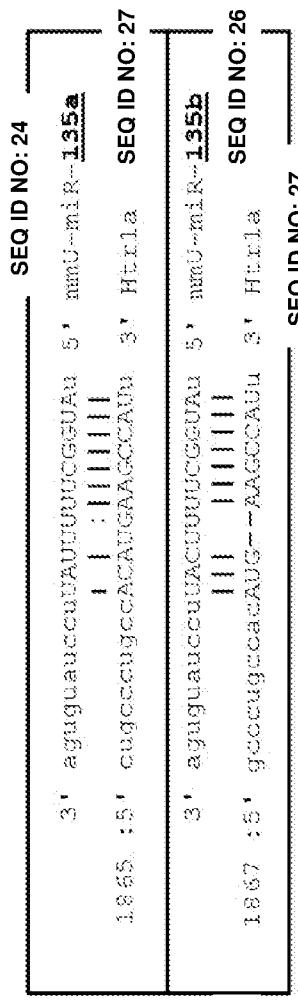
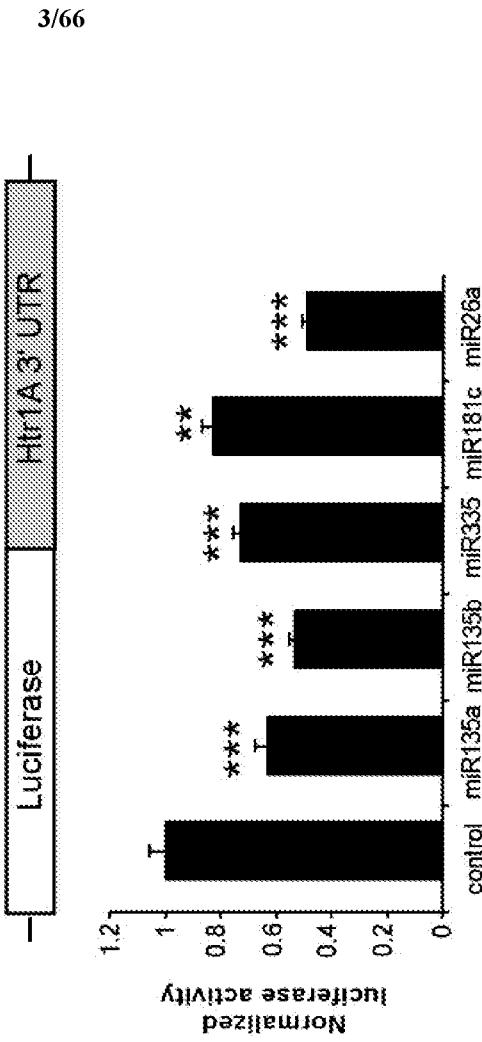
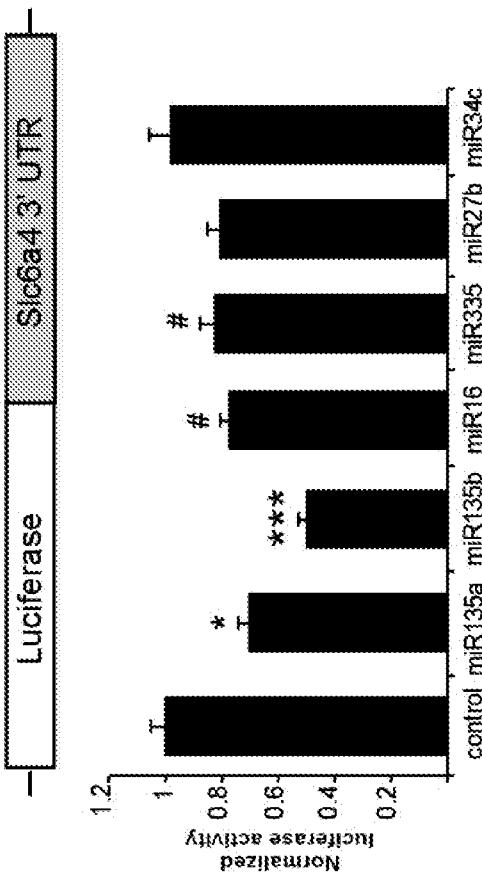
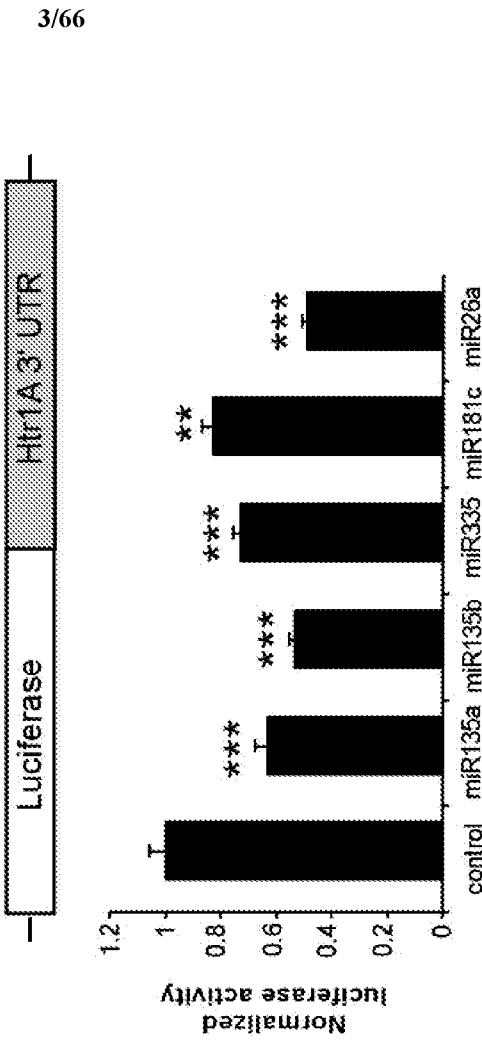
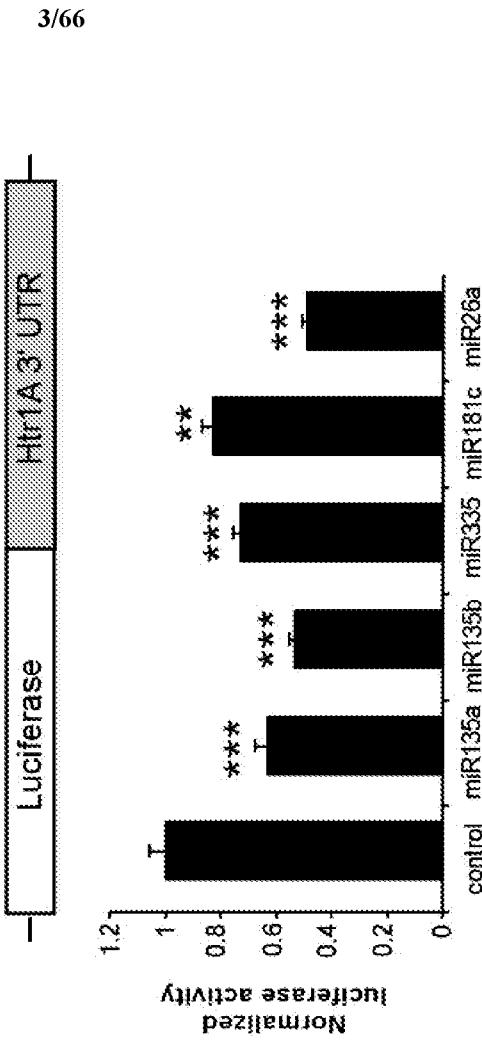
**FIG. 2A****FIG. 2B****FIG. 2C****FIG. 2D**

FIG. 2E

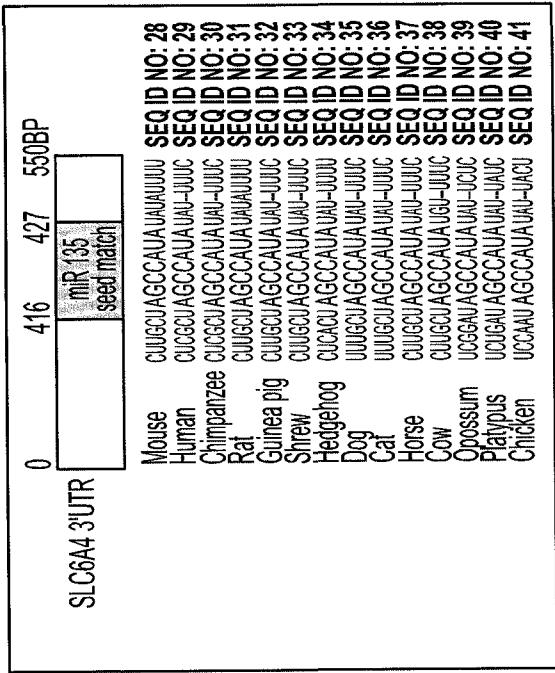


FIG. 2F

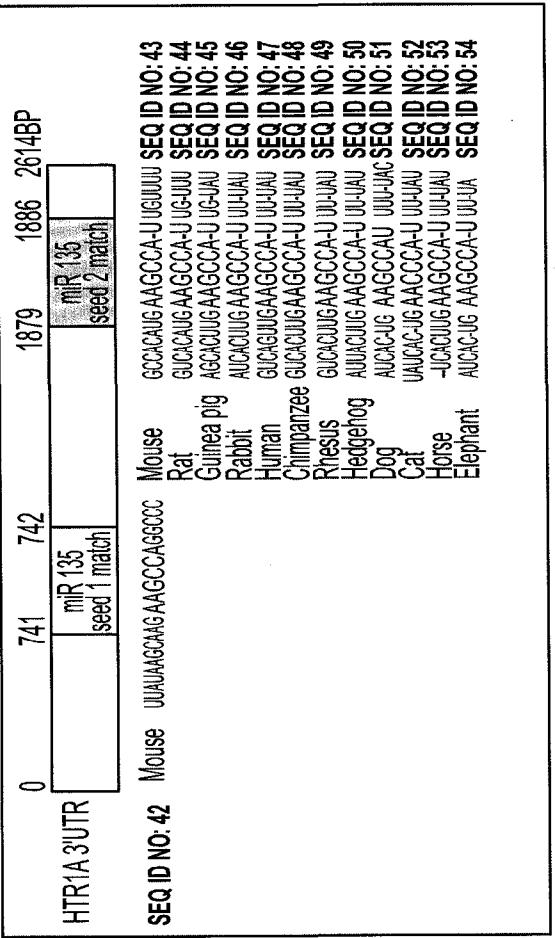


FIG. 2G

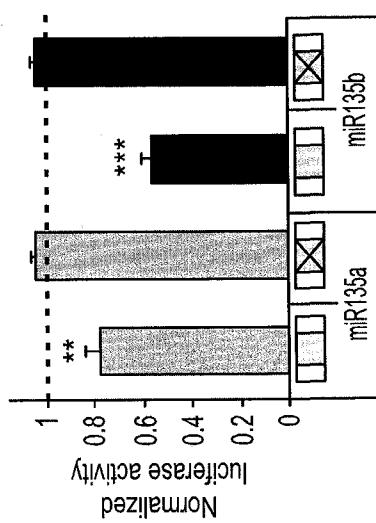
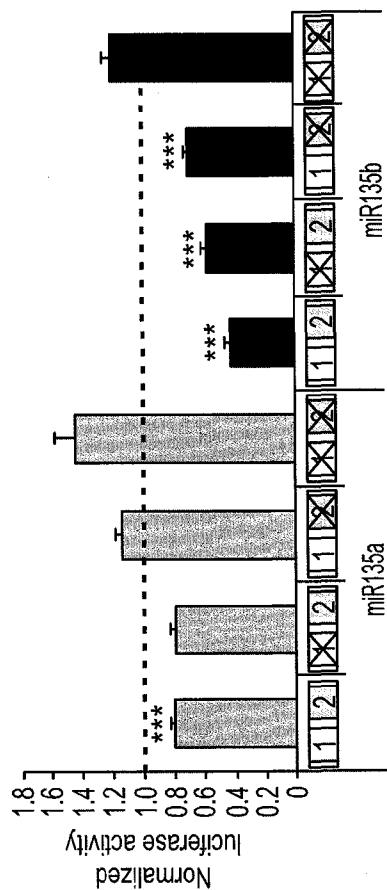


FIG. 2H



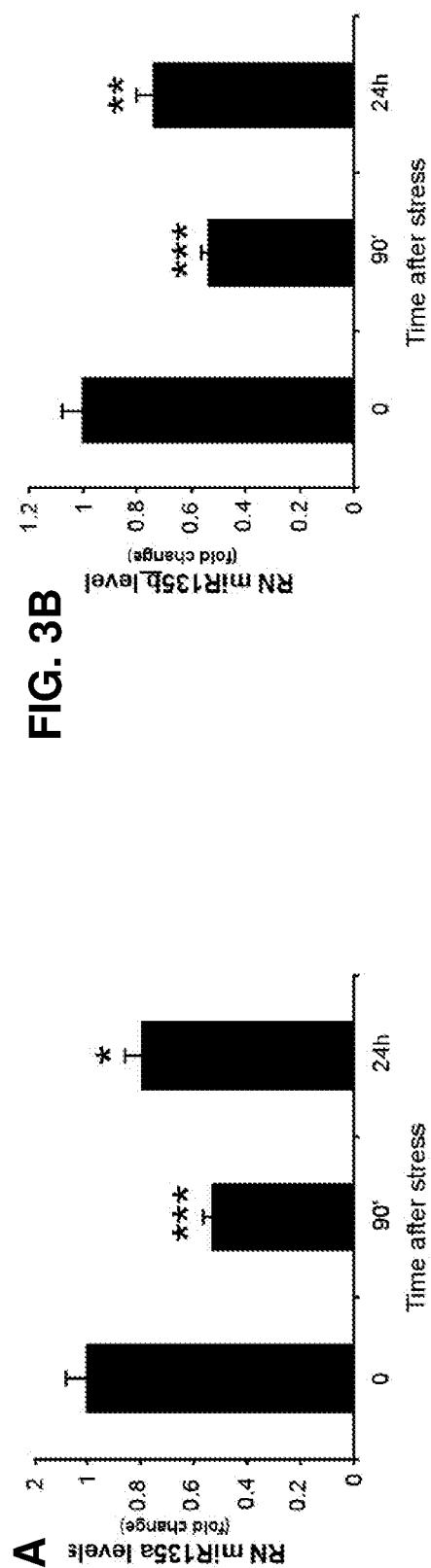
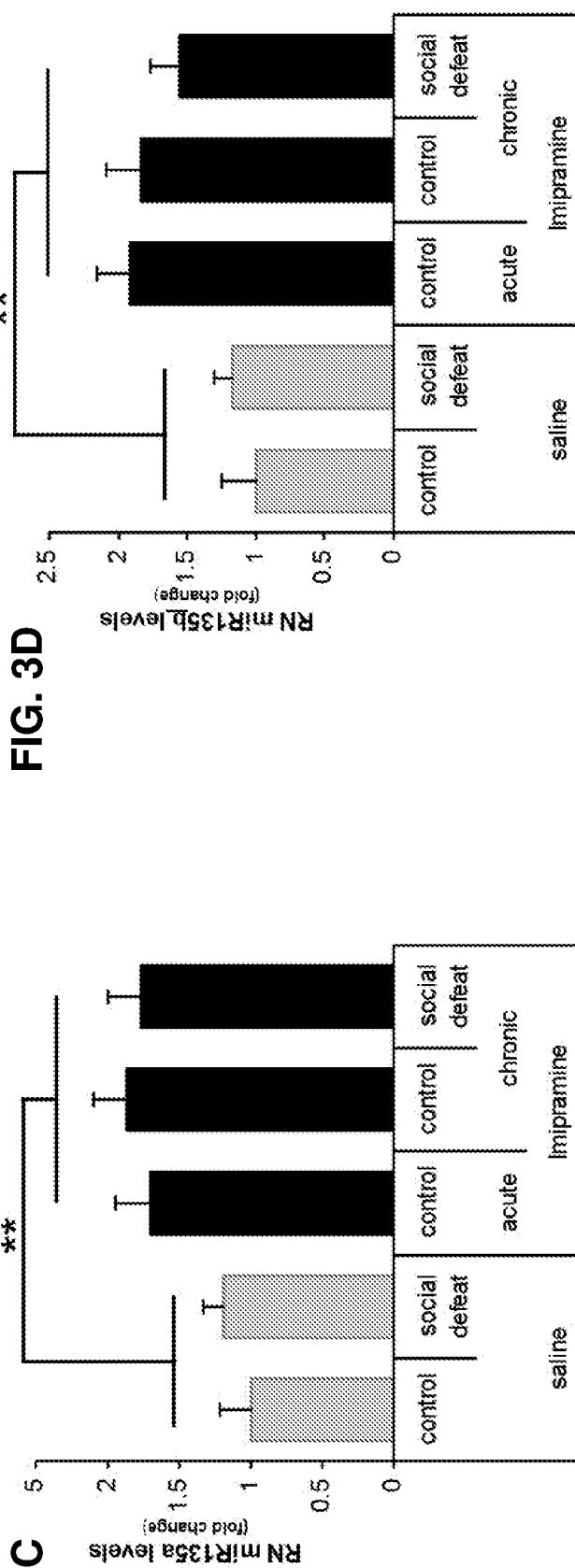
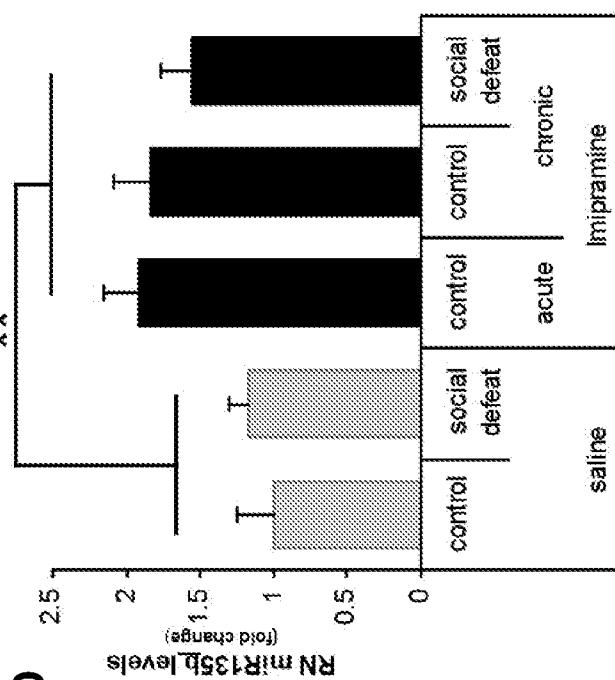
**FIG. 3A****FIG. 3C****FIG. 3D****FIG. 3B**

FIG. 3E

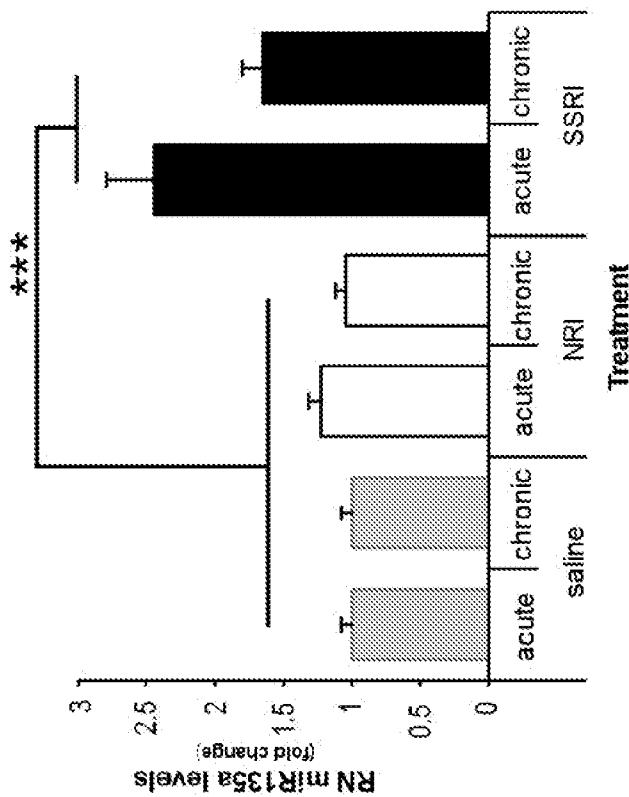


FIG. 3F

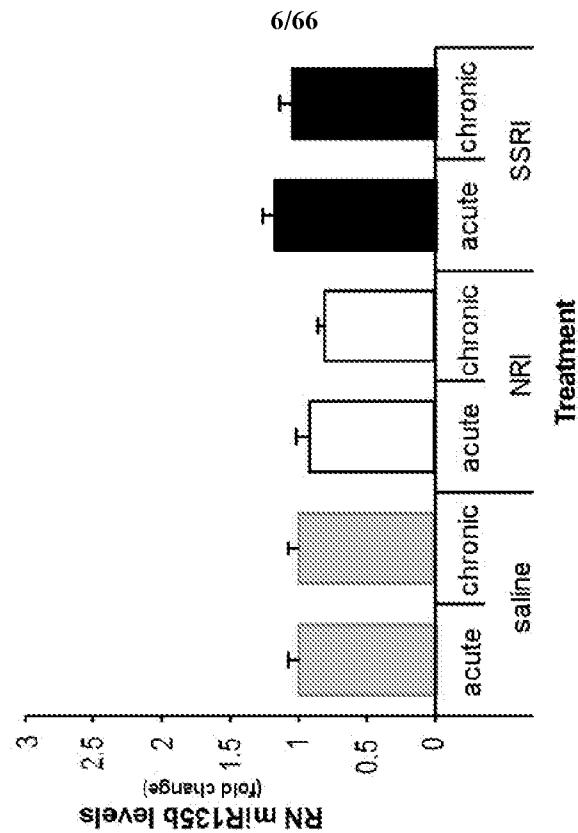


FIG. 4B

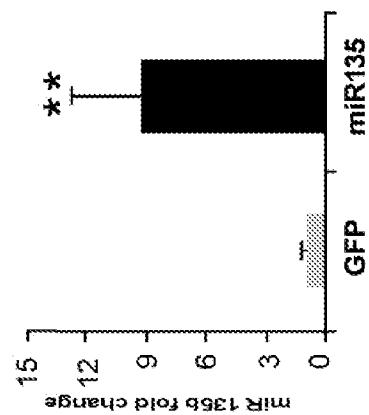
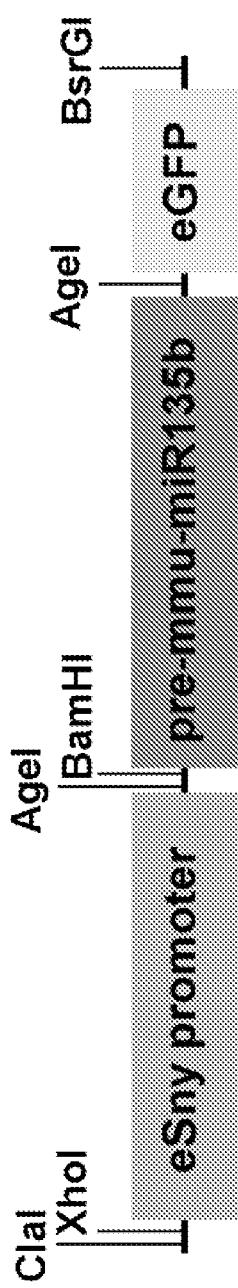


FIG. 4A



**FIG. 4C** **FIG. 4D**

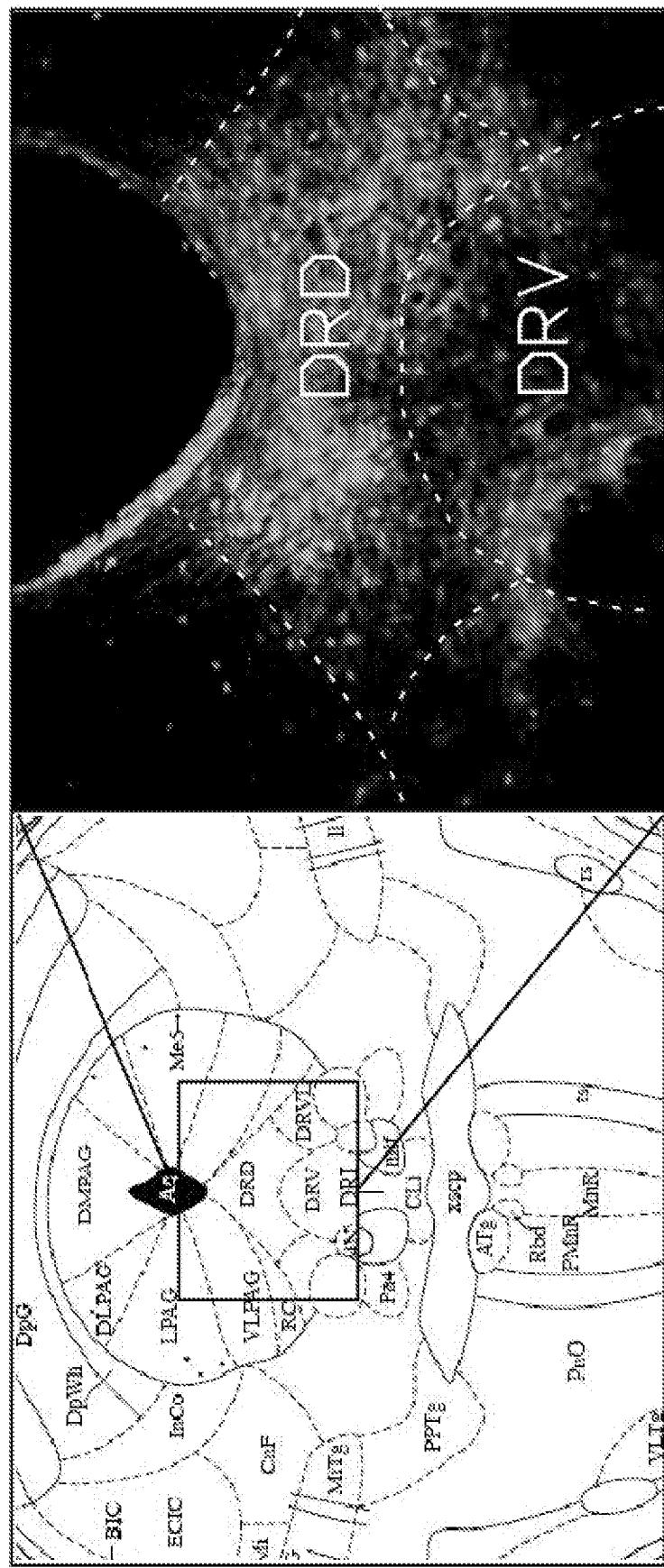


FIG. 4E

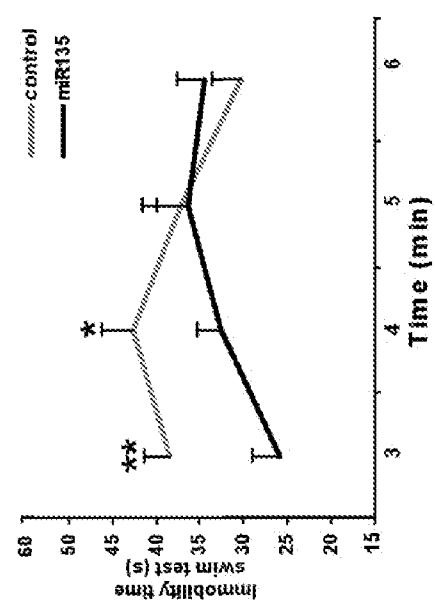


FIG. 4F

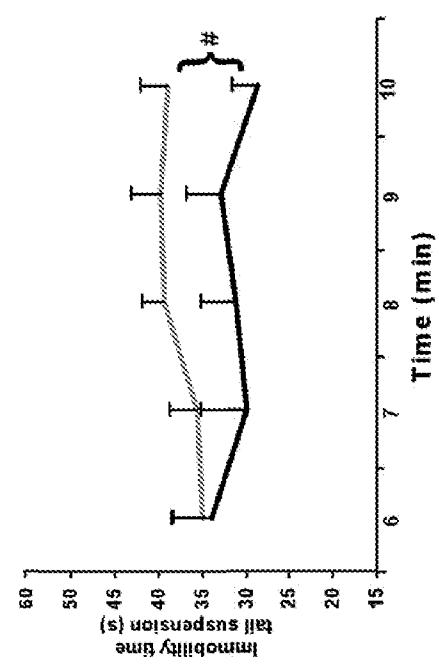


FIG. 4G

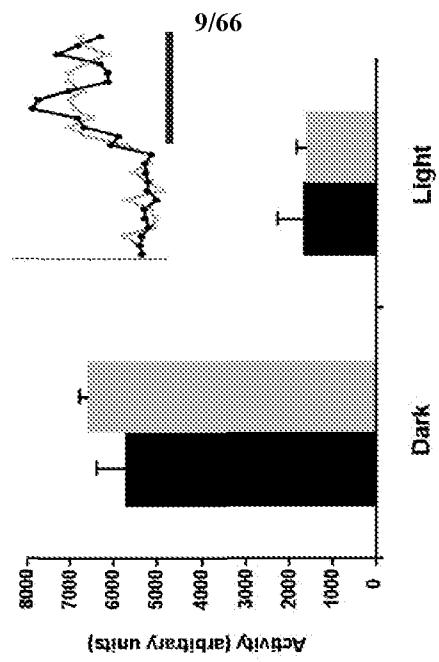
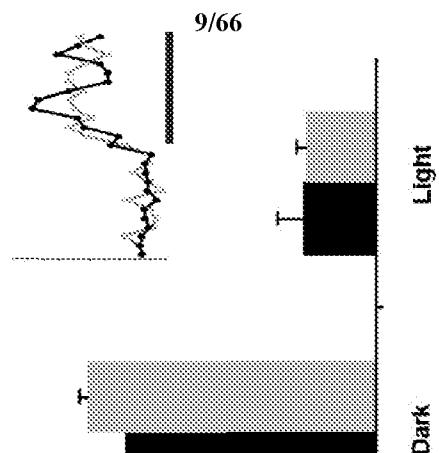
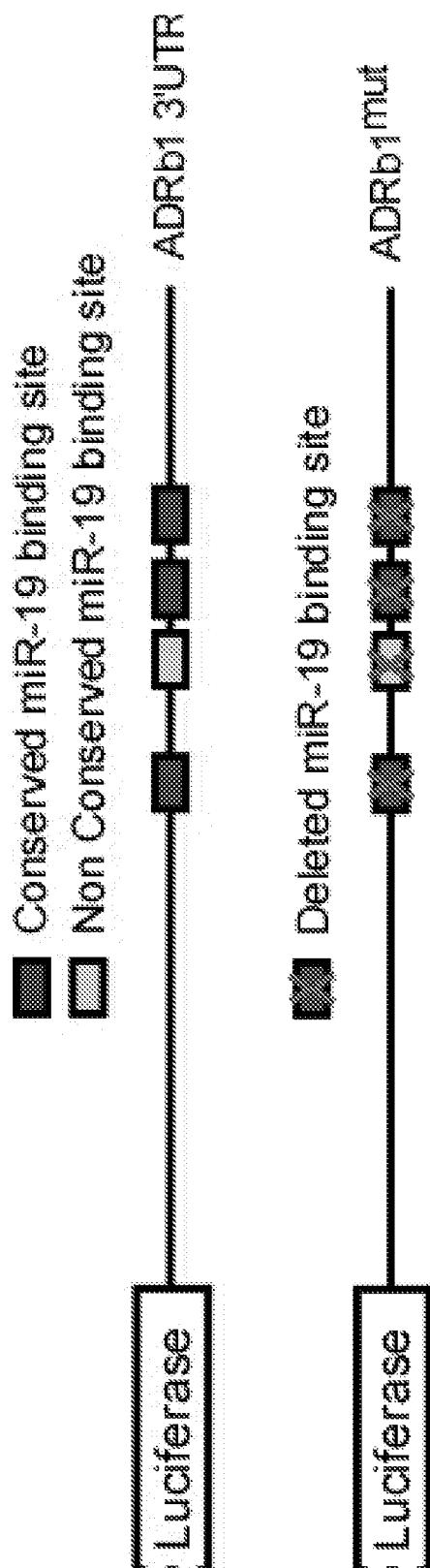
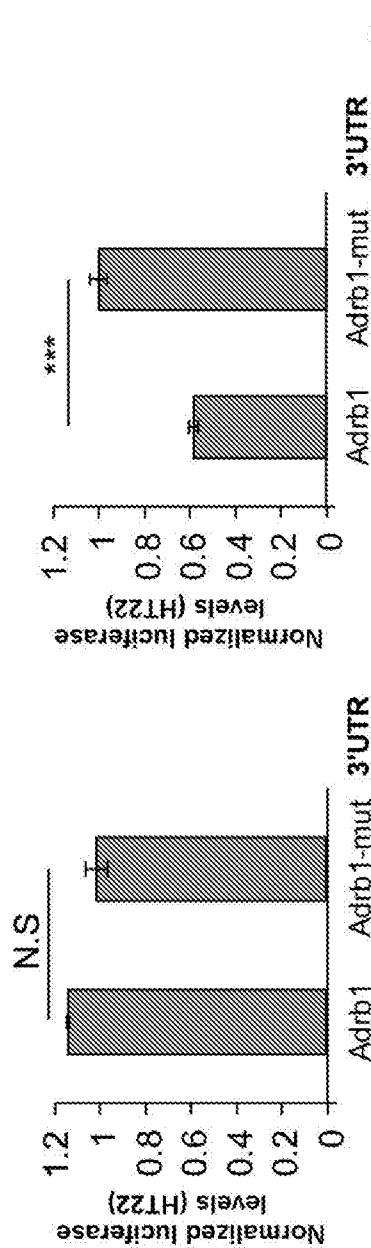
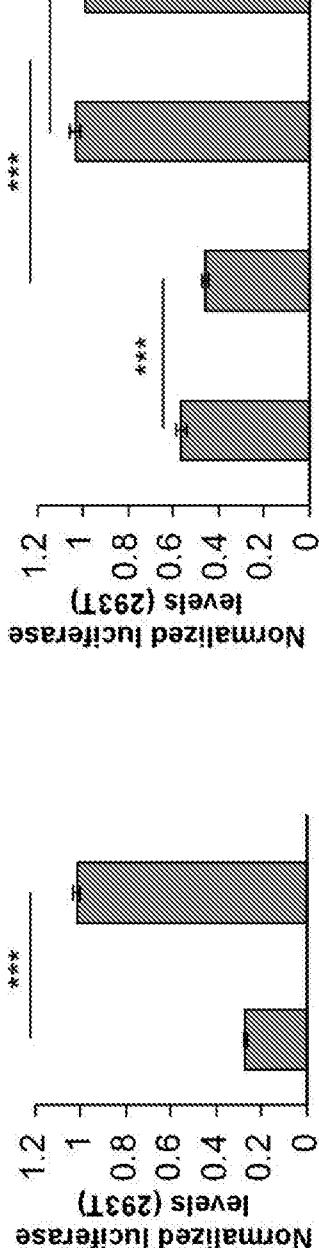
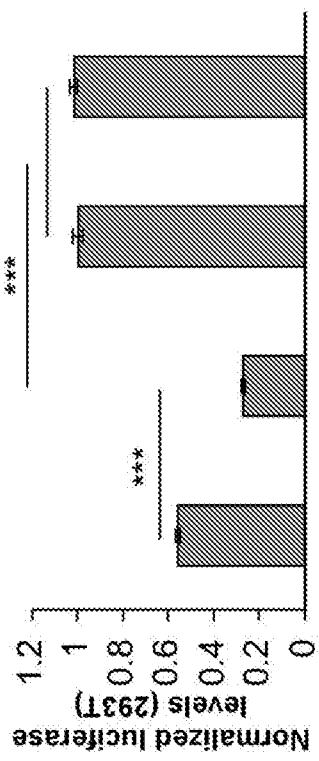
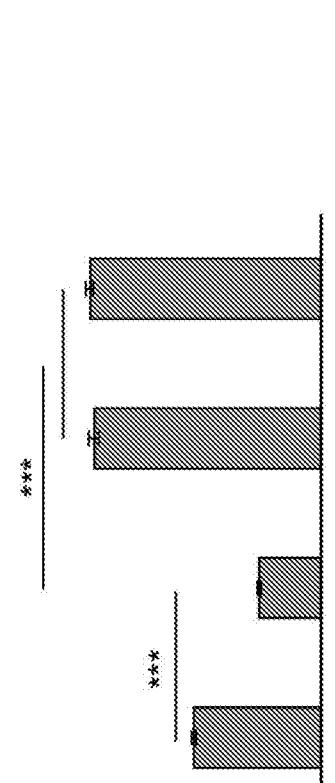


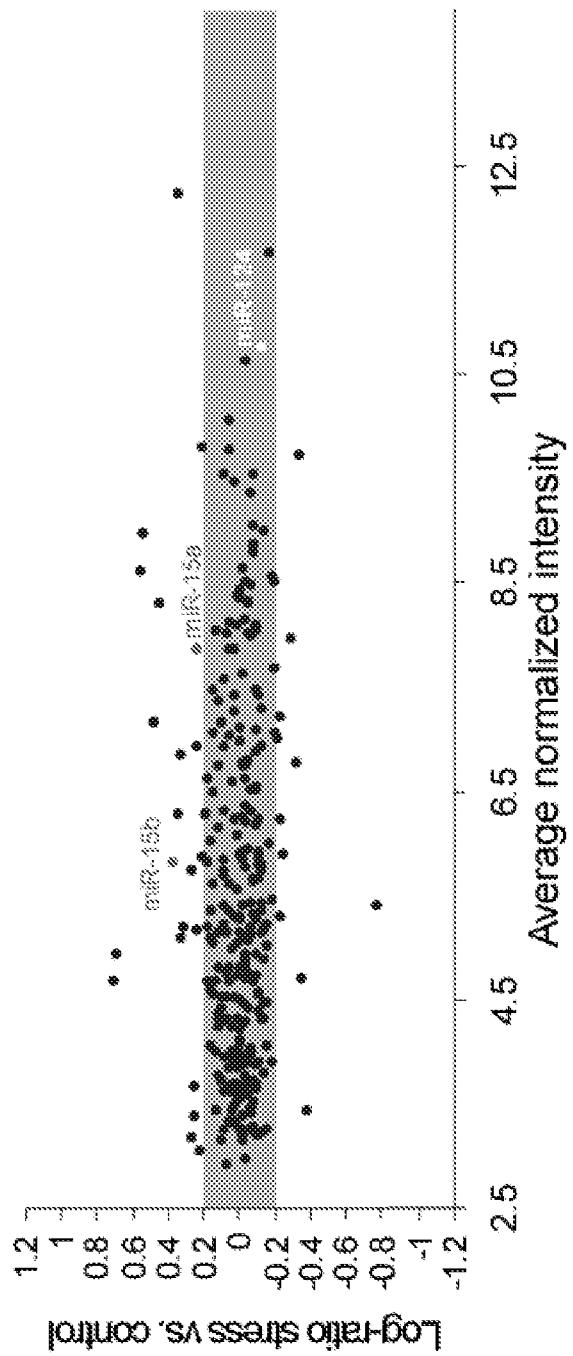
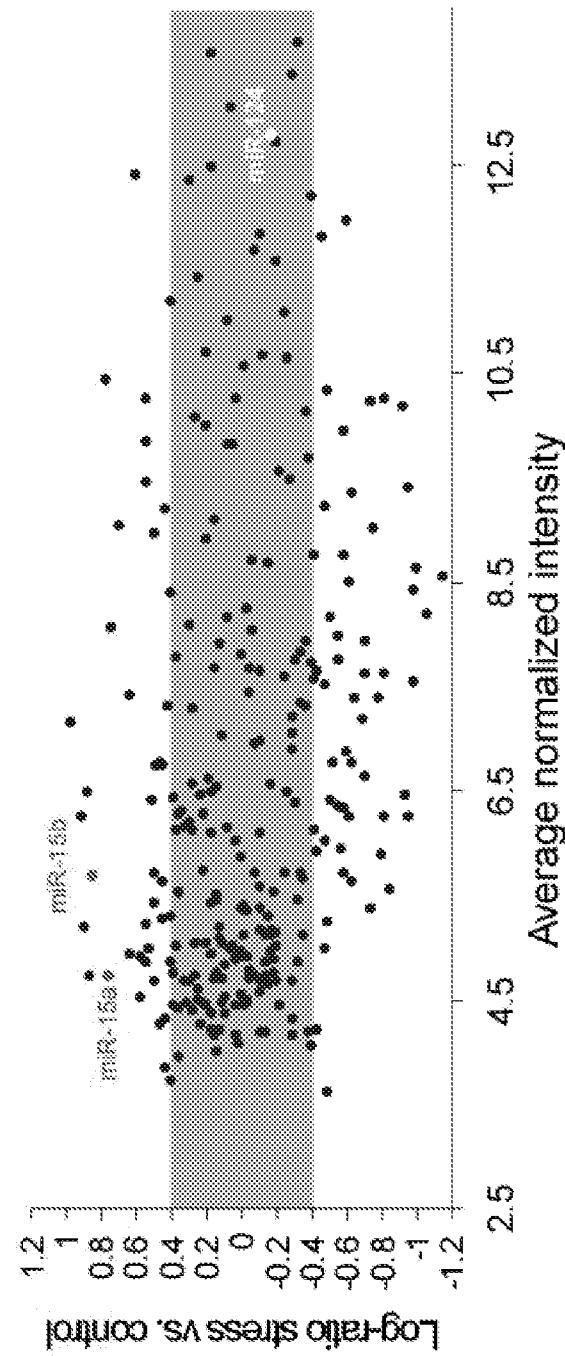
FIG. 4H



**FIG. 5**

**FIG. 6A****FIG. 6C****FIG. 6E****FIG. 6D**

Adrb1 Adrb1 Adrb1-mut Adrb1-mut 3'UTR  
19b KD Control KD 19b KD Control KD 3'UTR  
miArrest plasmid

**FIG. 7A****FIG. 7B**

|                                  |    |                        |               |
|----------------------------------|----|------------------------|---------------|
| Position 554-560 of Crhr1 3' UTR | 5' | ...GCGGCGCTTGGCTU...   | SEQ ID NO: 55 |
| mmu-mir-15a                      | 3' | GUUUCUUAACGAGAU...     | SEQ ID NO: 56 |
| Position 554-560 of Crhr1 3' UTR | 5' | ...UGGAAGCCUUGCUUCU... | SEQ ID NO: 55 |
| mmu-mir-15b                      | 3' | ICAUUUGGUACUACCGAACAU  | SEQ ID NO: 57 |

FIG. 7C

FIG. 7D

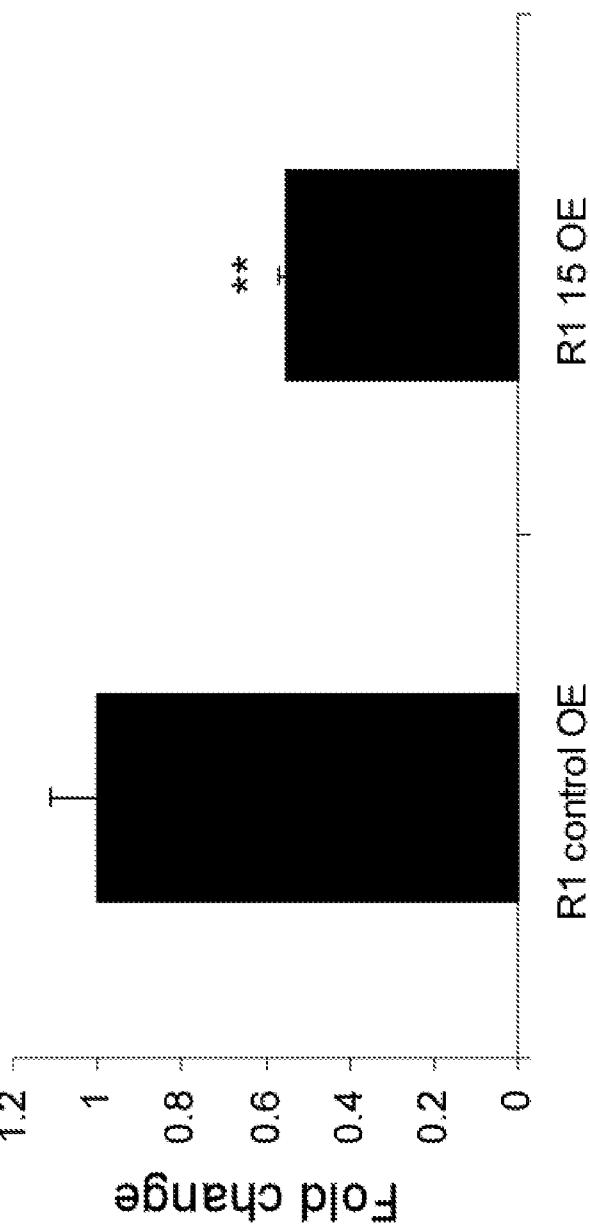


FIG. 8

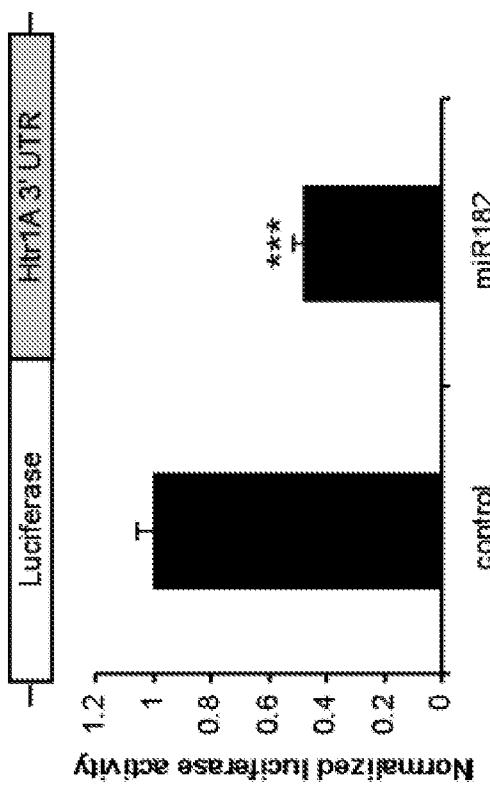
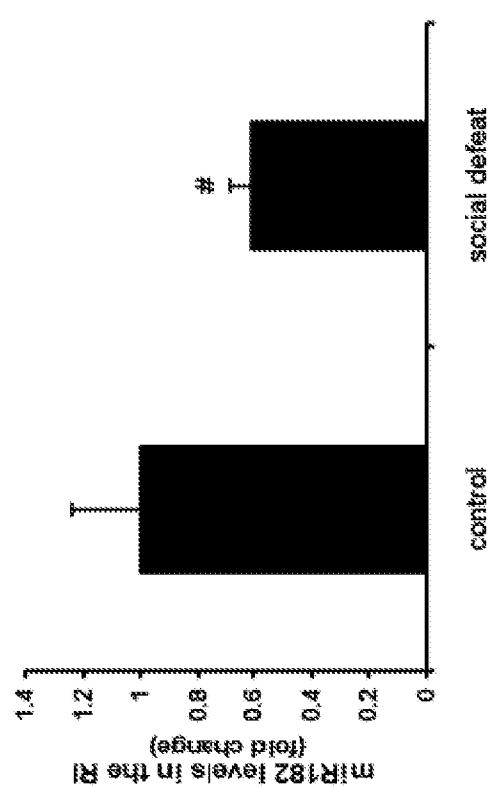


FIG. 9



## FIG. 10

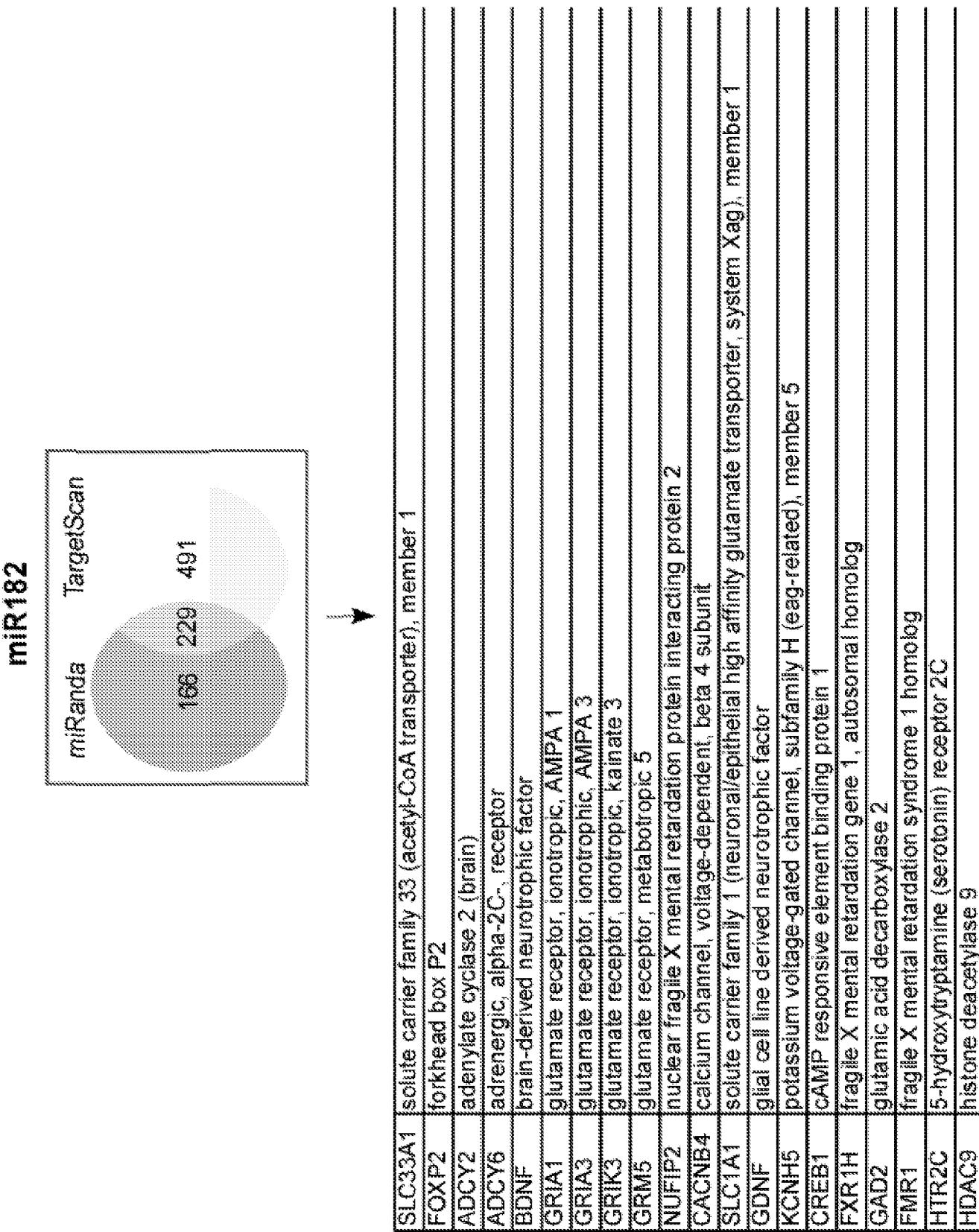


FIG. 11A

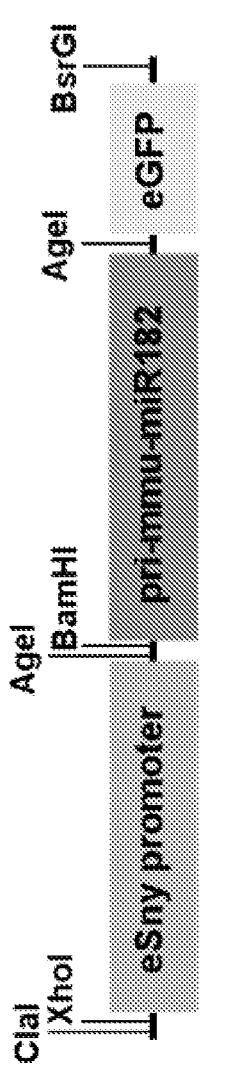


FIG. 11B

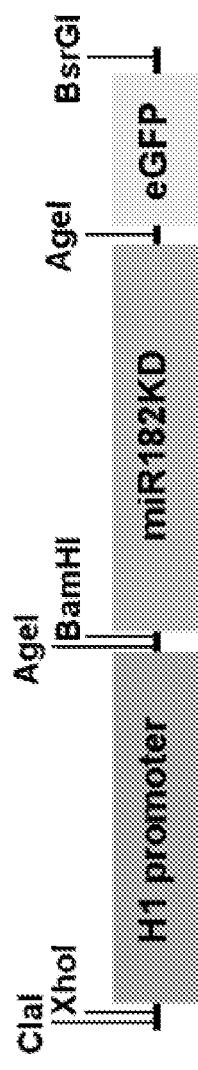
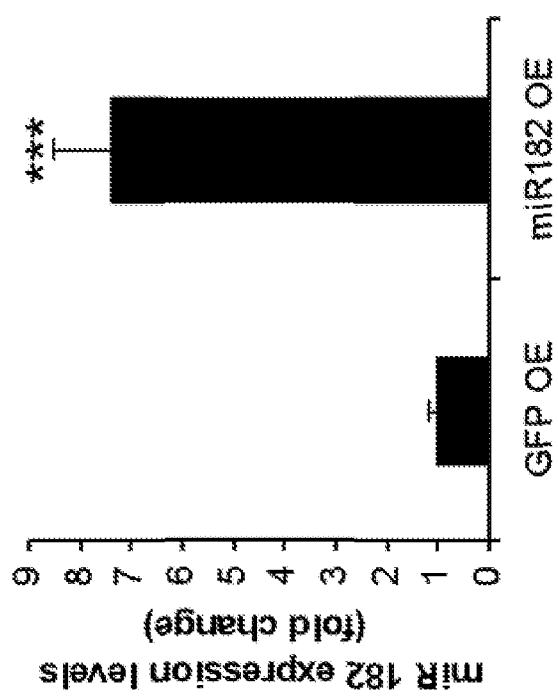
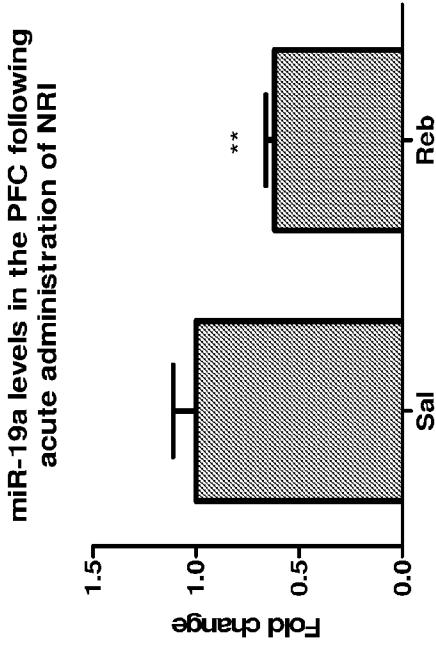
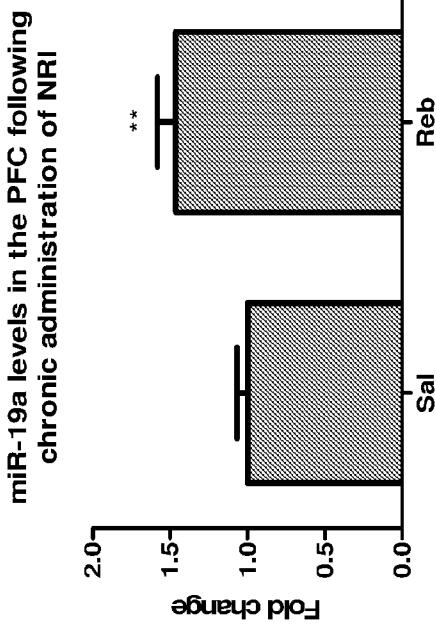
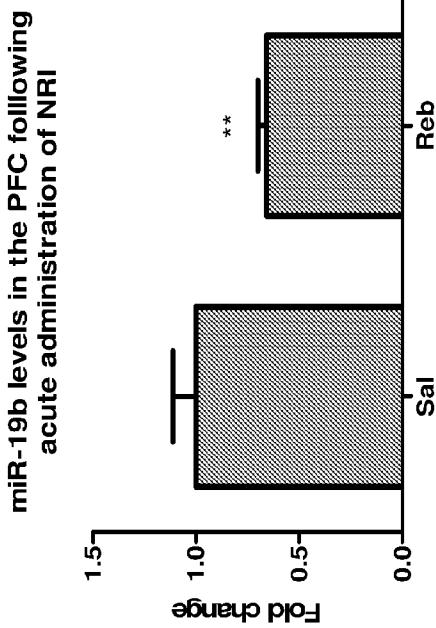
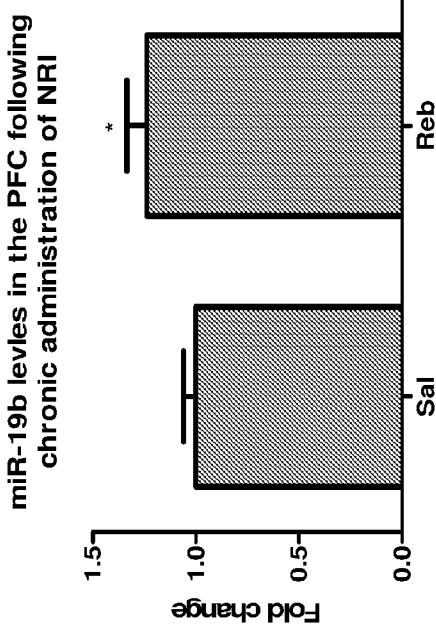


FIG. 11C

**FIG. 12A****FIG. 12B****FIG. 12C****FIG. 12D**

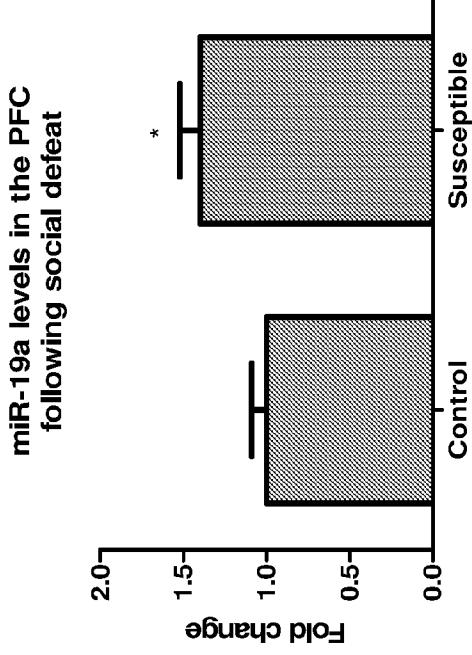
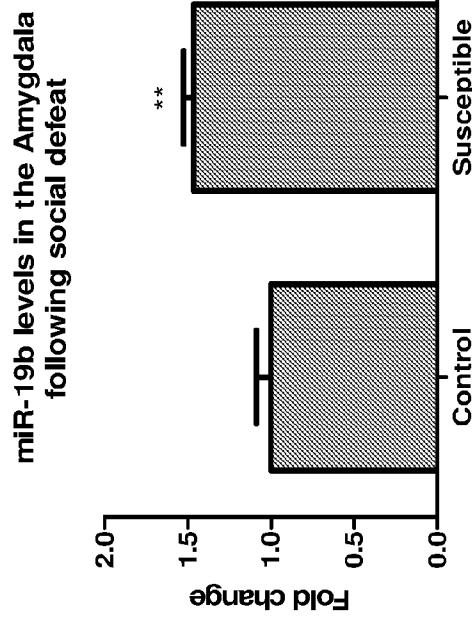
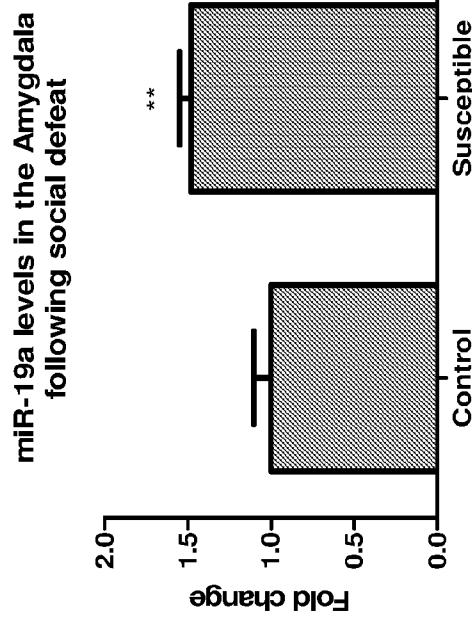
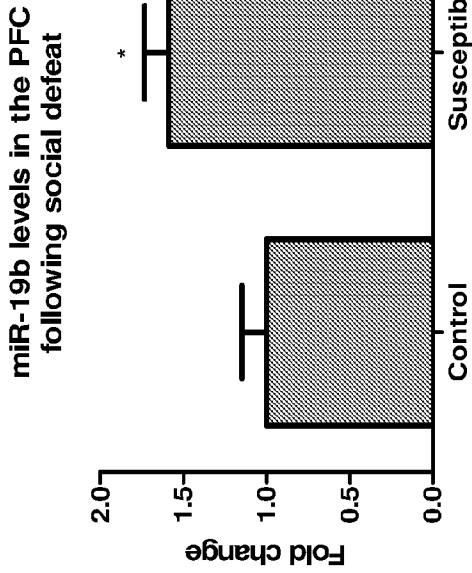
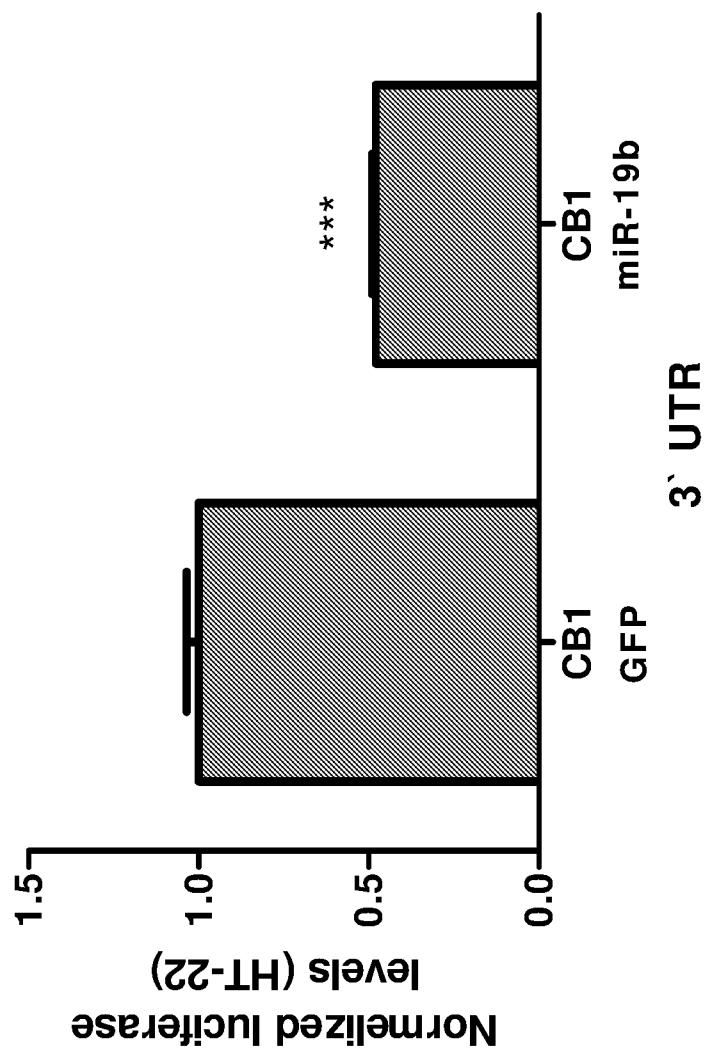
**FIG. 13A****FIG. 13B****FIG. 13C****FIG. 13D**

FIG. 14



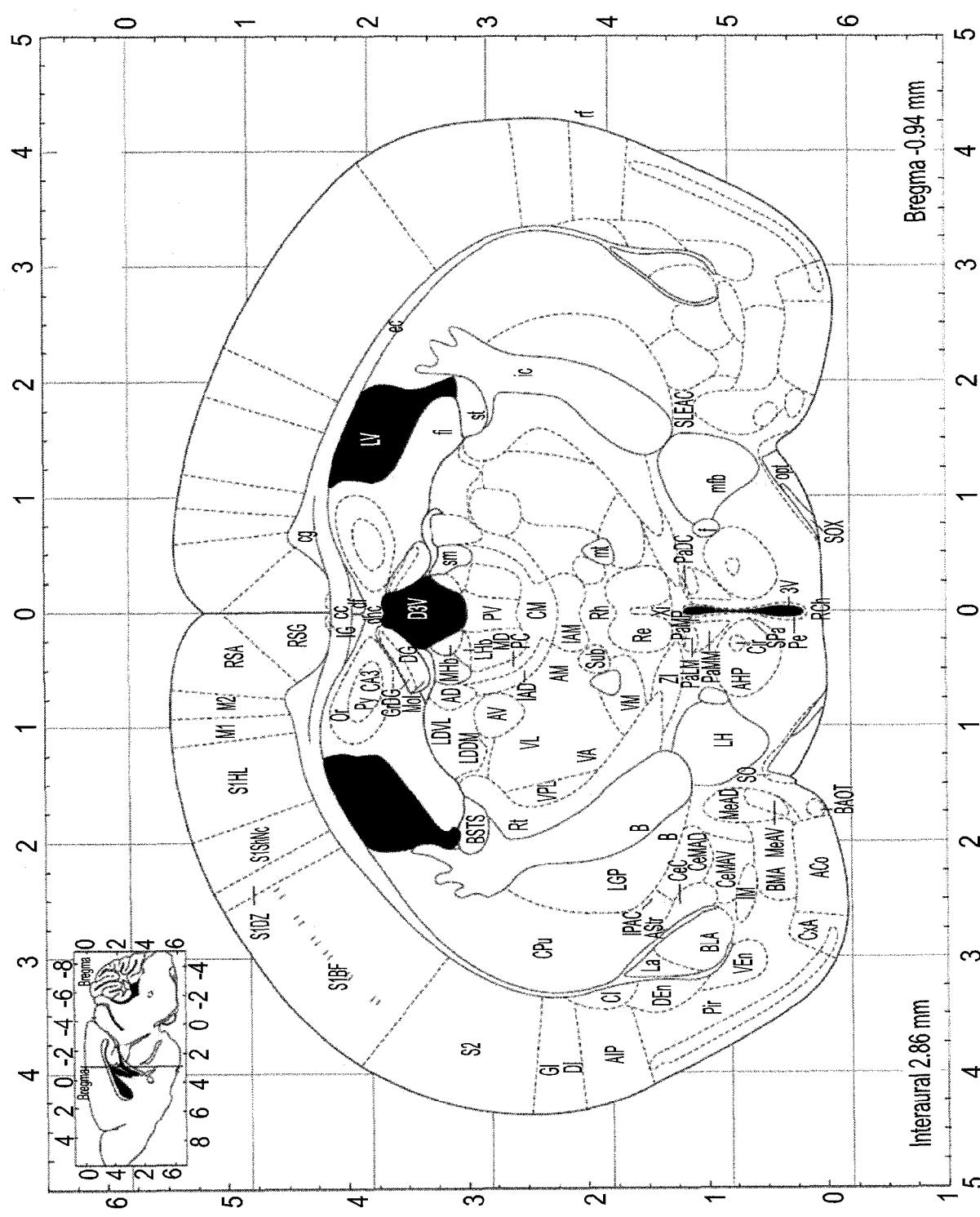


FIG. 15A

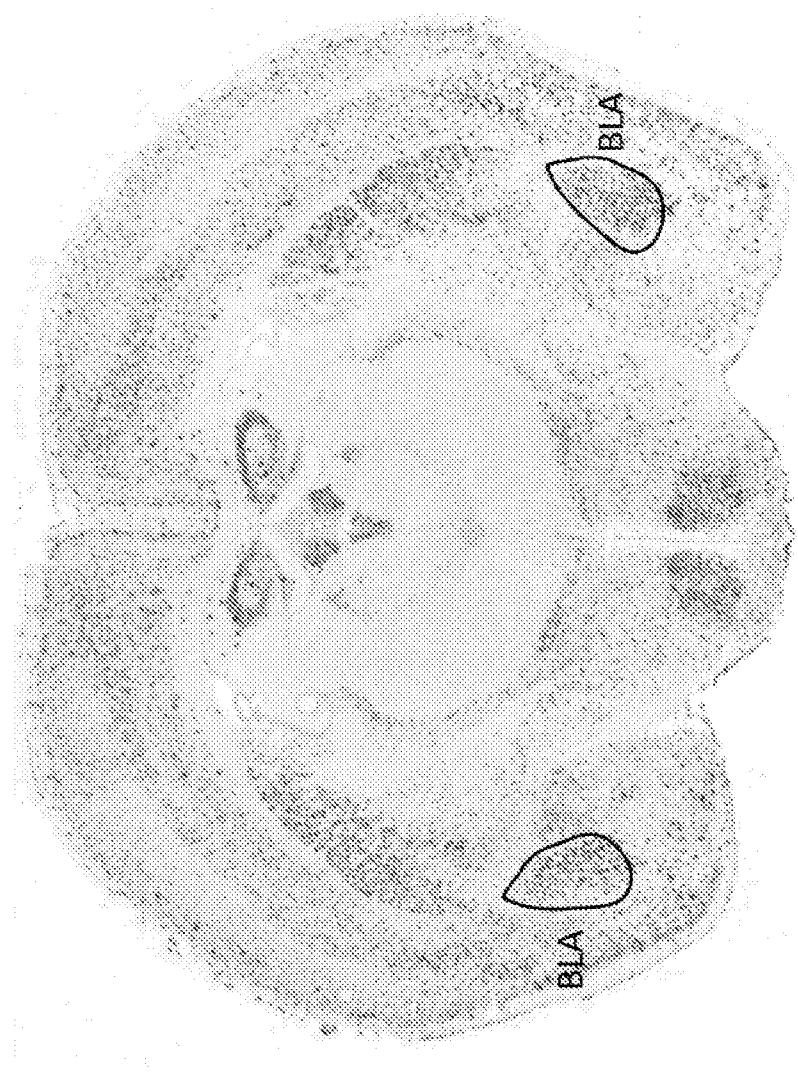
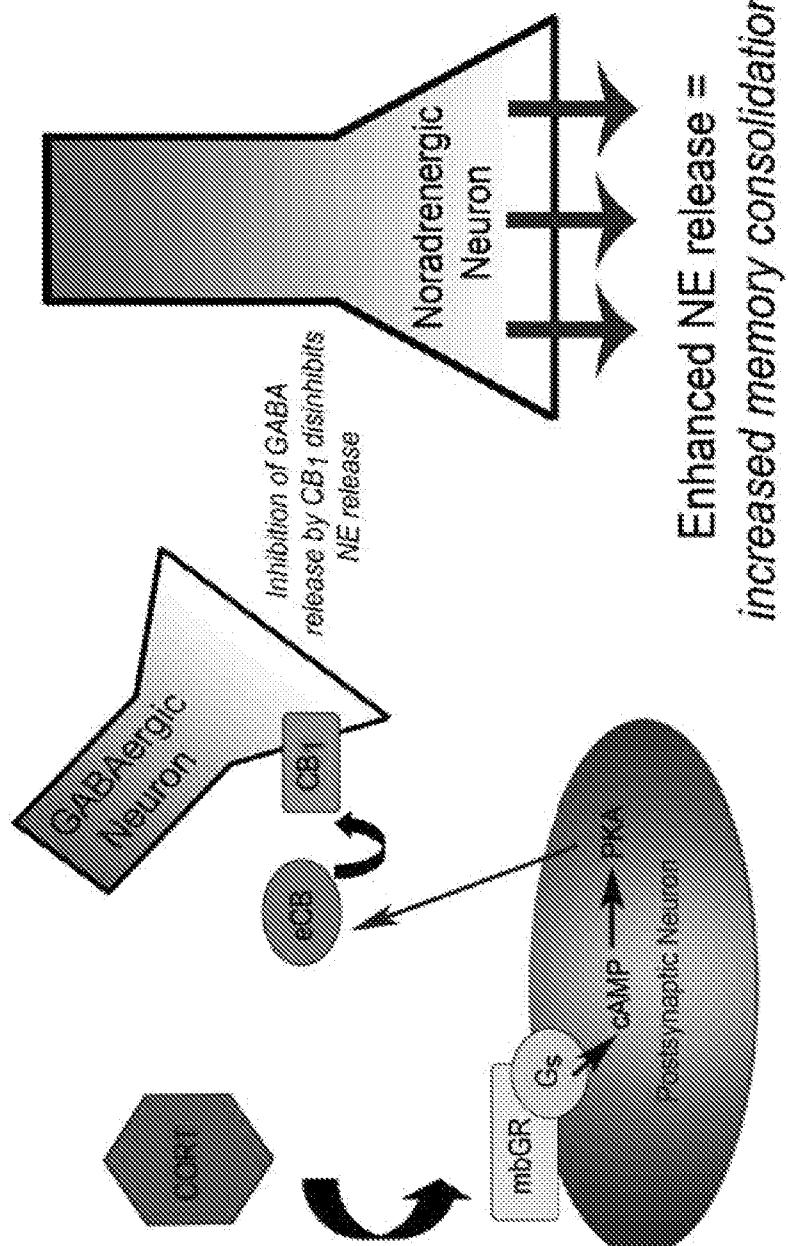
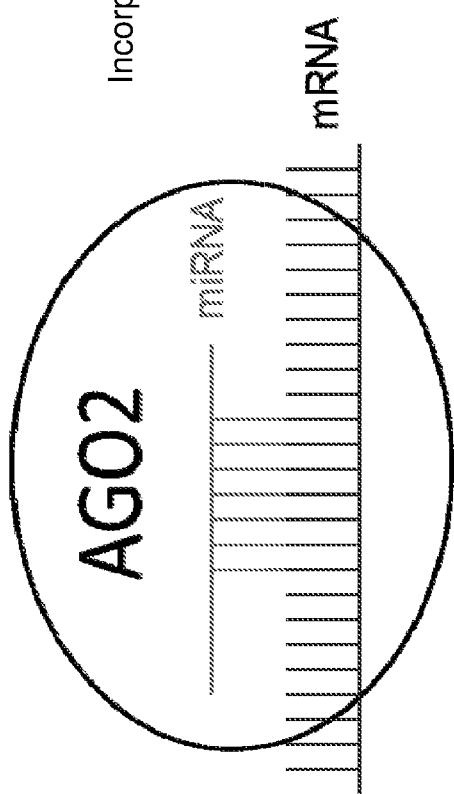
**FIG. 15B**

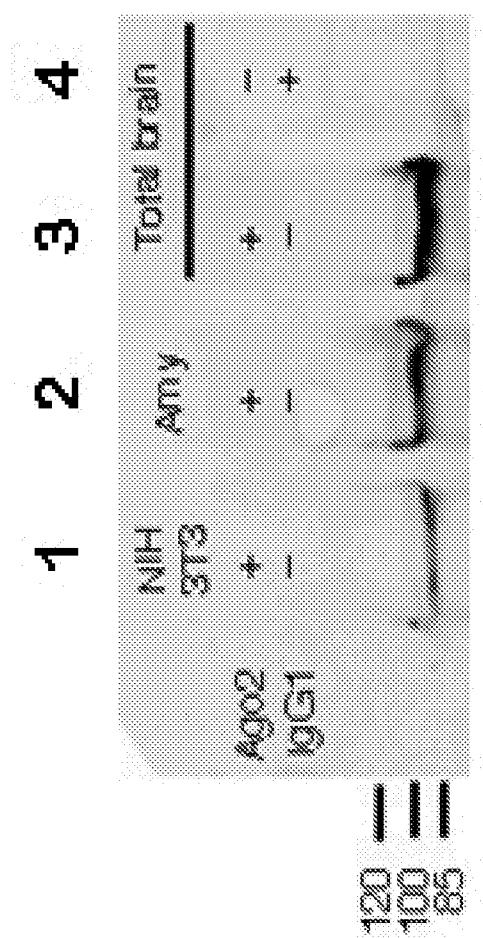
FIG. 16



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

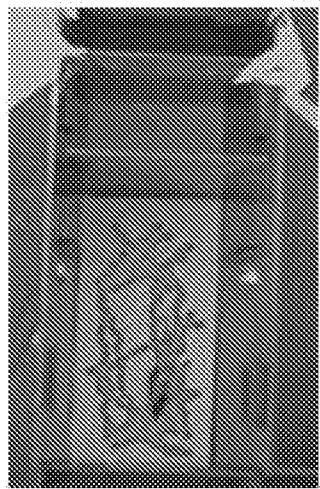
**FIG. 17A**

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

**FIG. 17B**

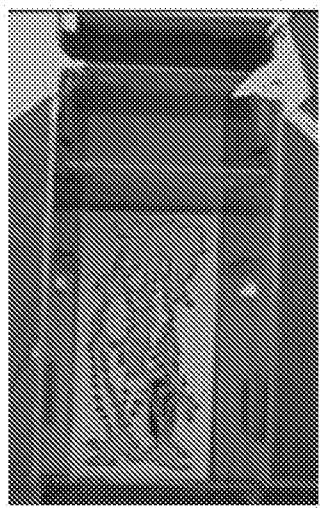
**FIG. 18A**

Susceptible



Habituation

Resilient

**FIG. 18B**

Unfamiliar  
mouse

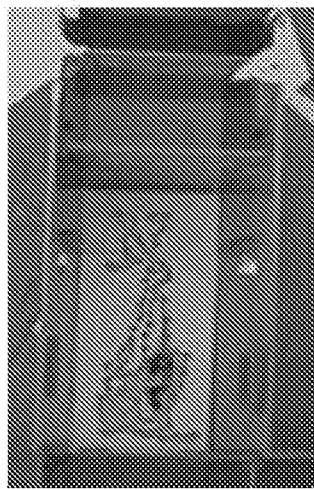
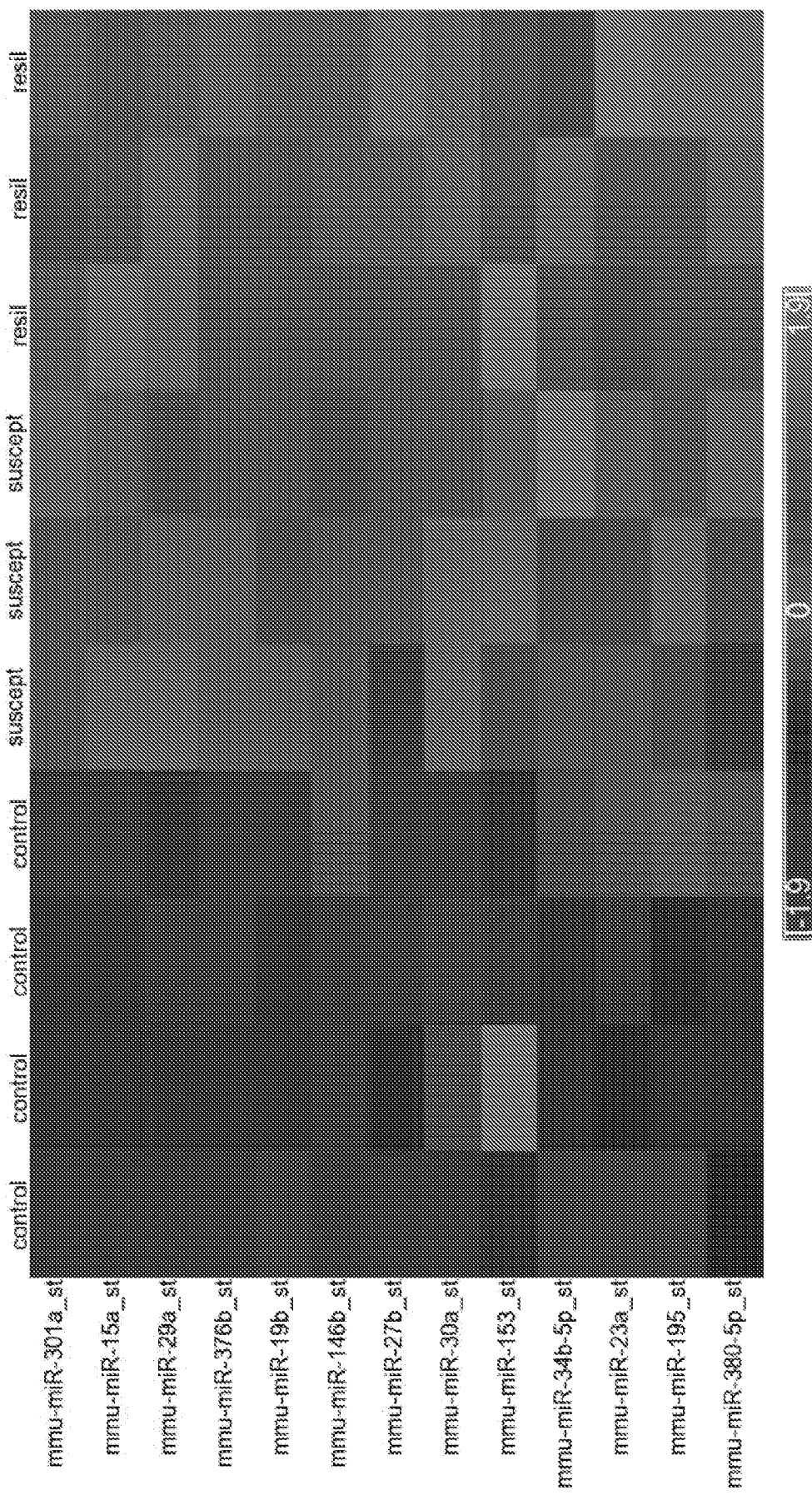
**FIG. 18C****FIG. 18D**

FIG. 19A



**FIG. 19B**

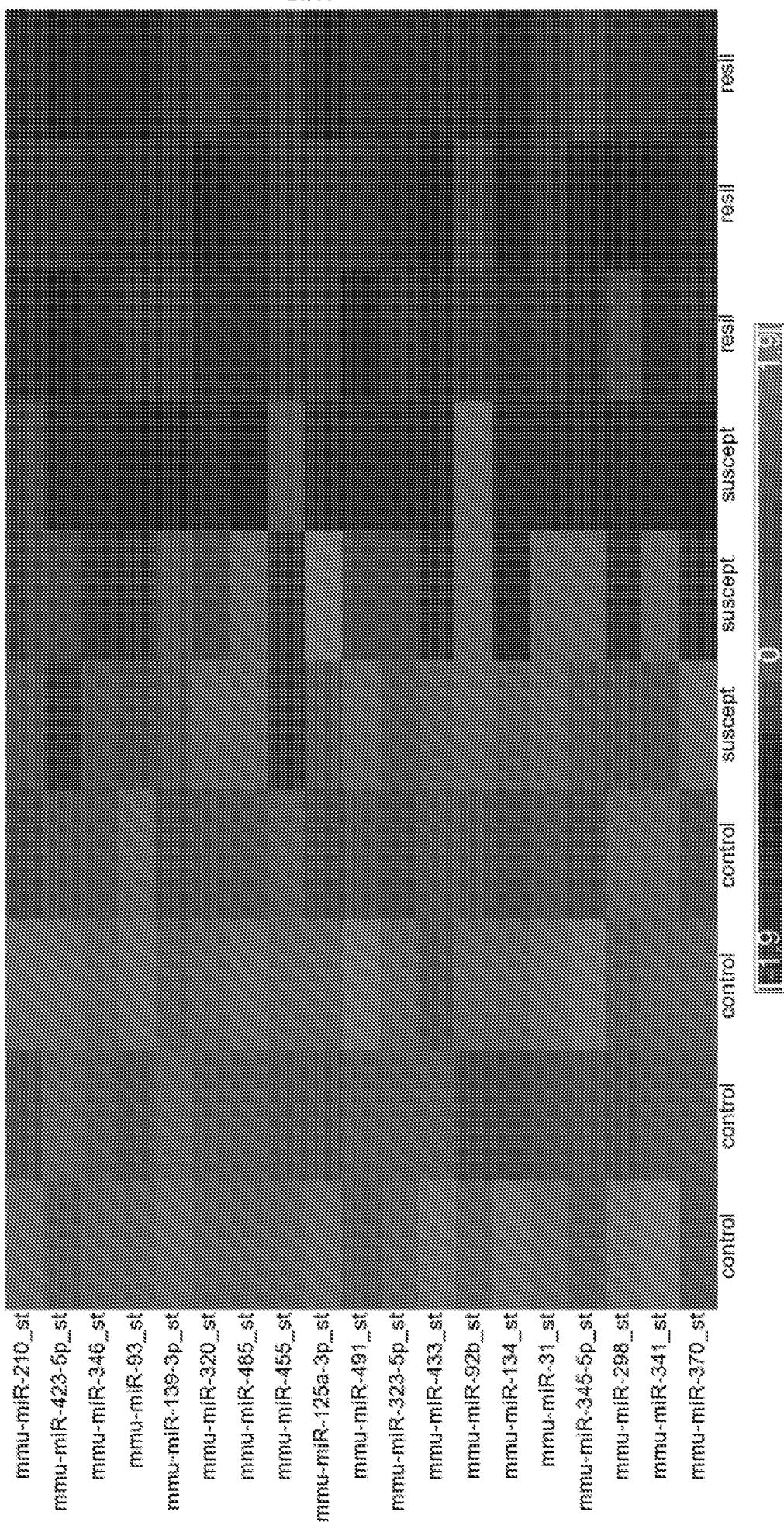
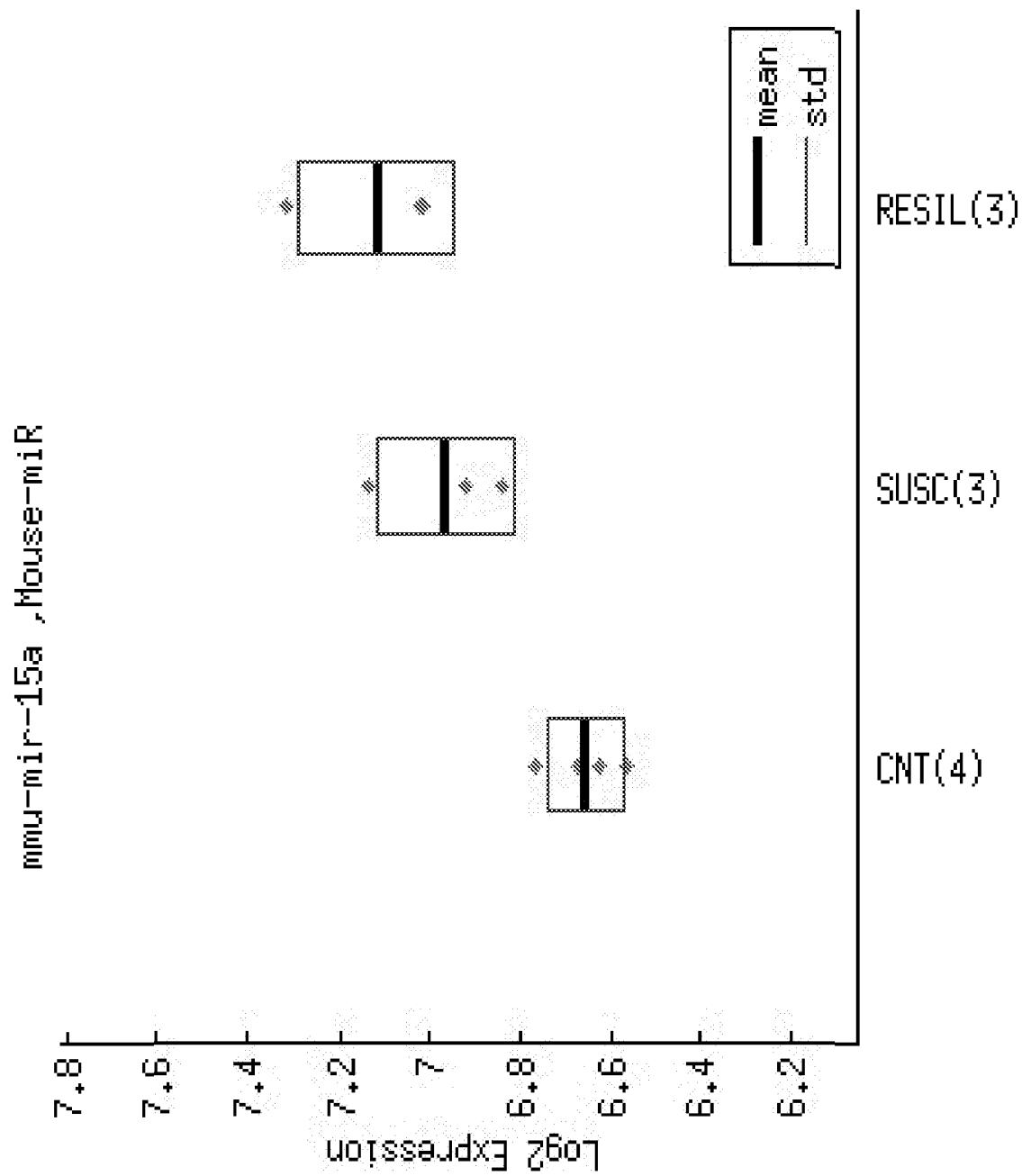


FIG. 20A



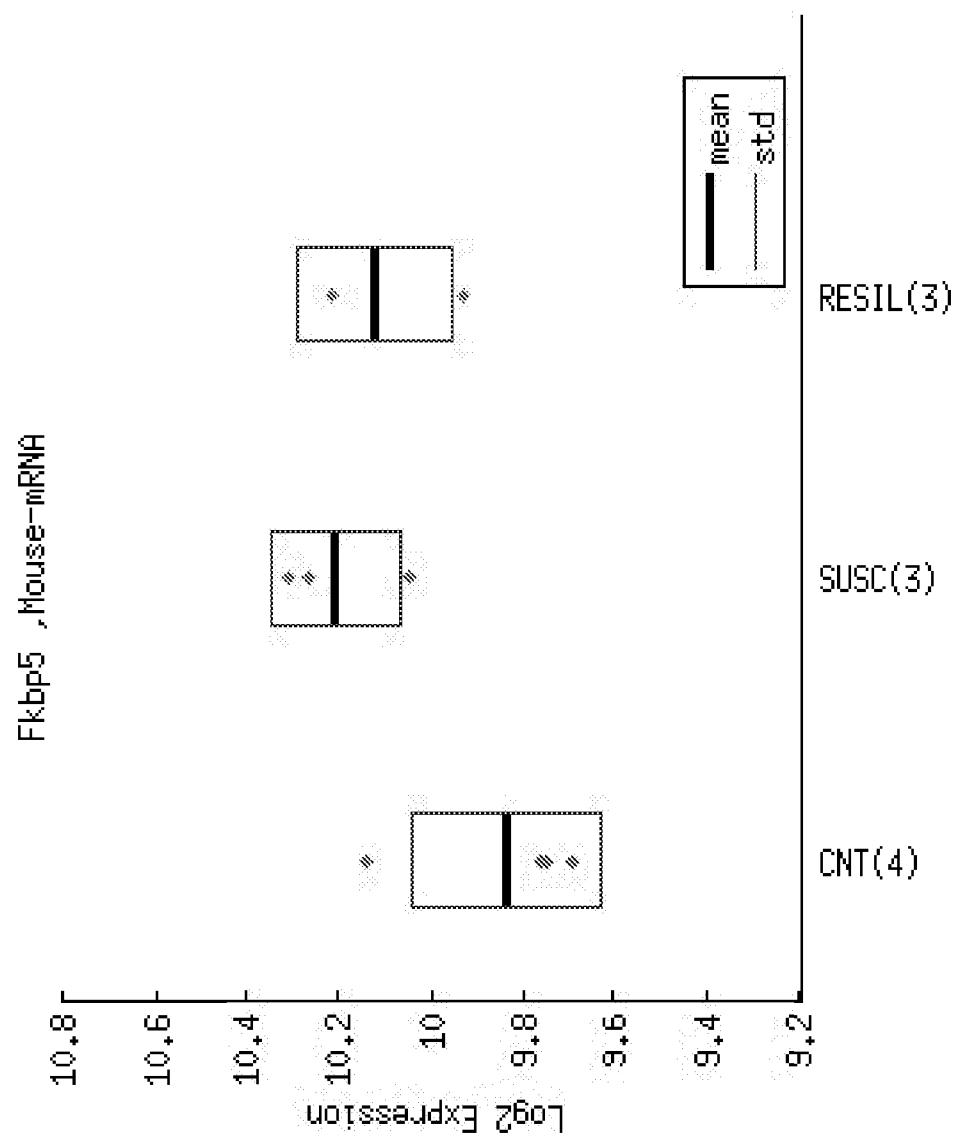
**FIG. 20B**

FIG. 20C

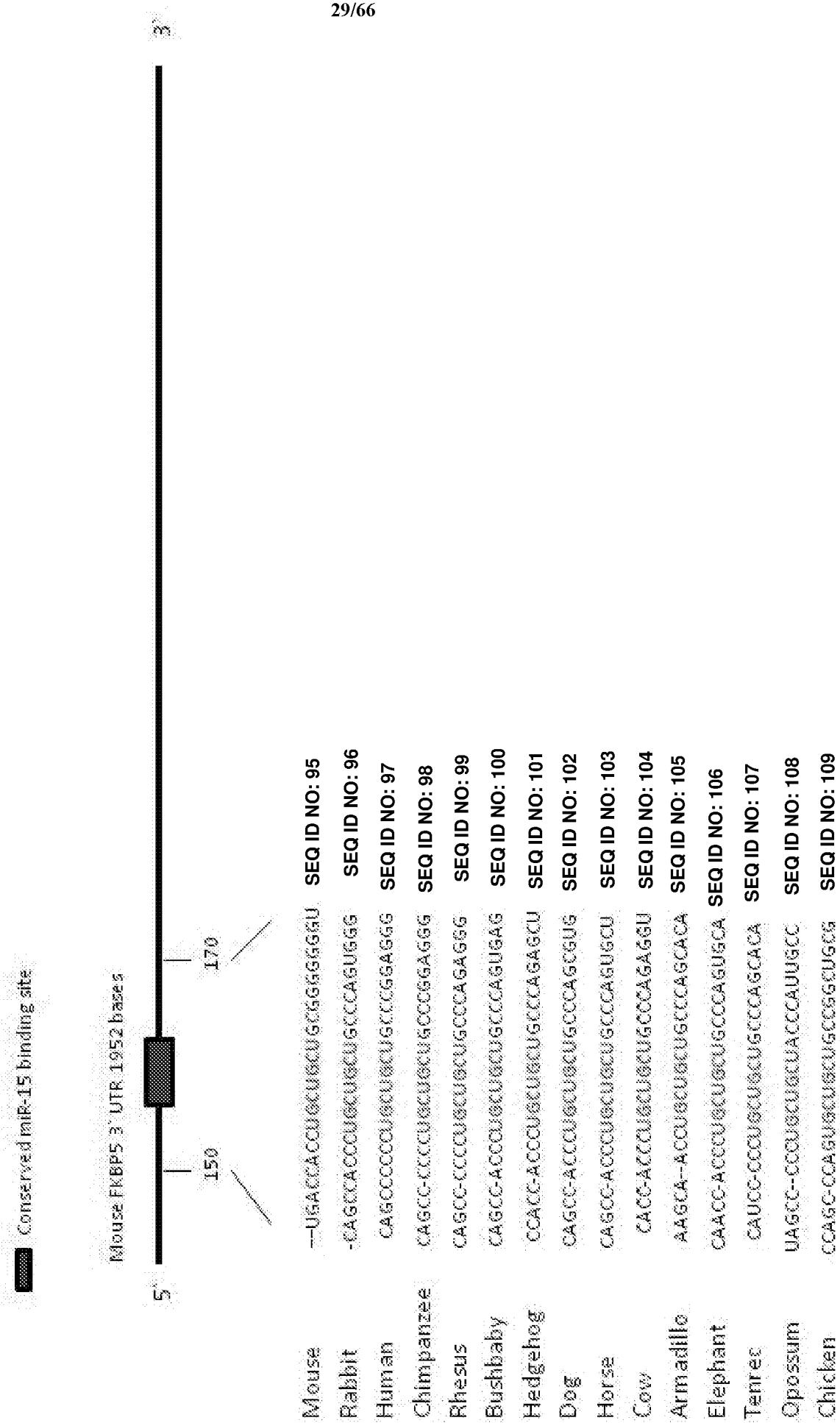


FIG. 21A

miR-15a levels in susceptible mice relative to control following social defeat

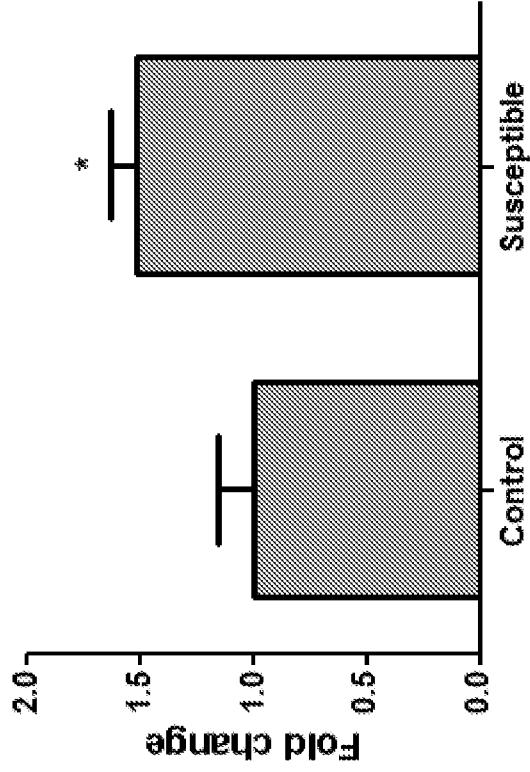


FIG. 21B

FKBP6 levels in susceptible mice relative to control following social defeat

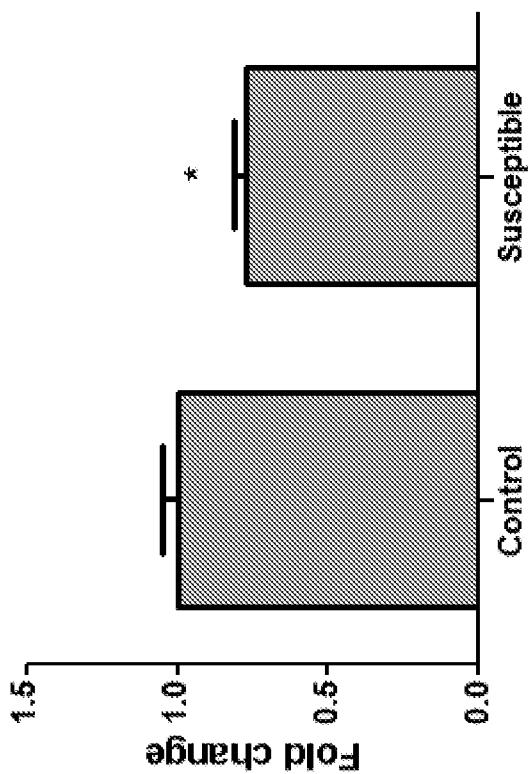


FIG. 22

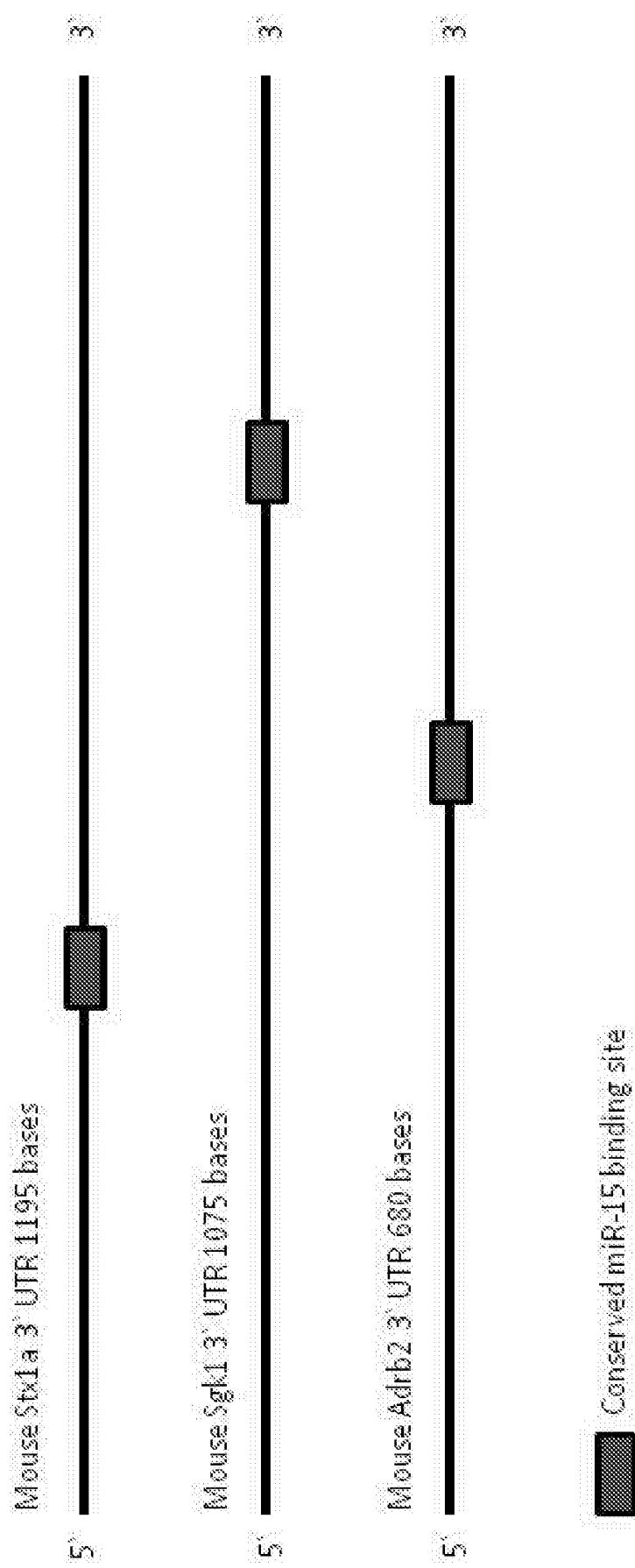


FIG. 23

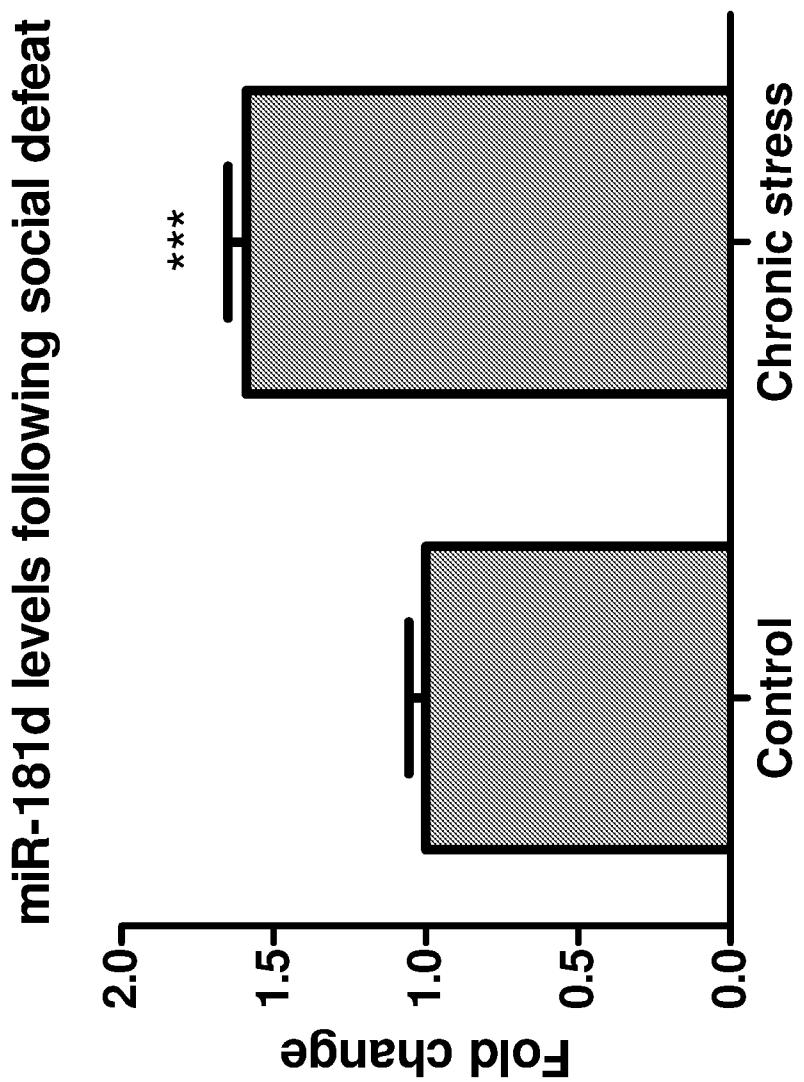


FIG. 24

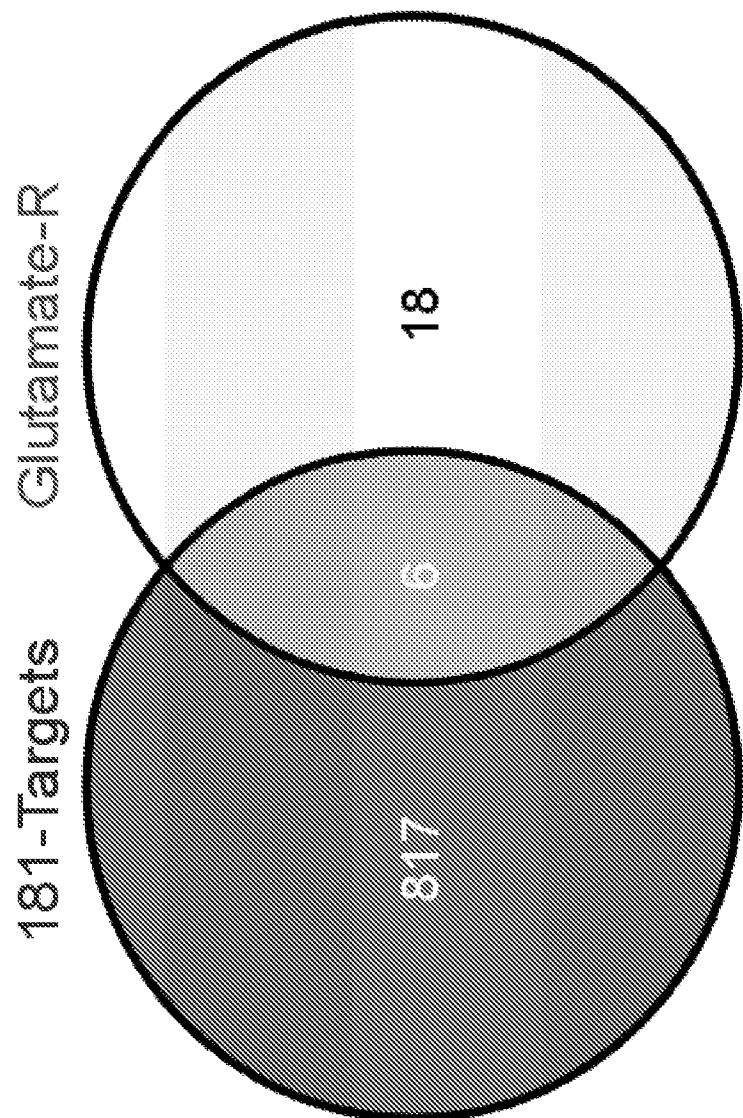


FIG. 25

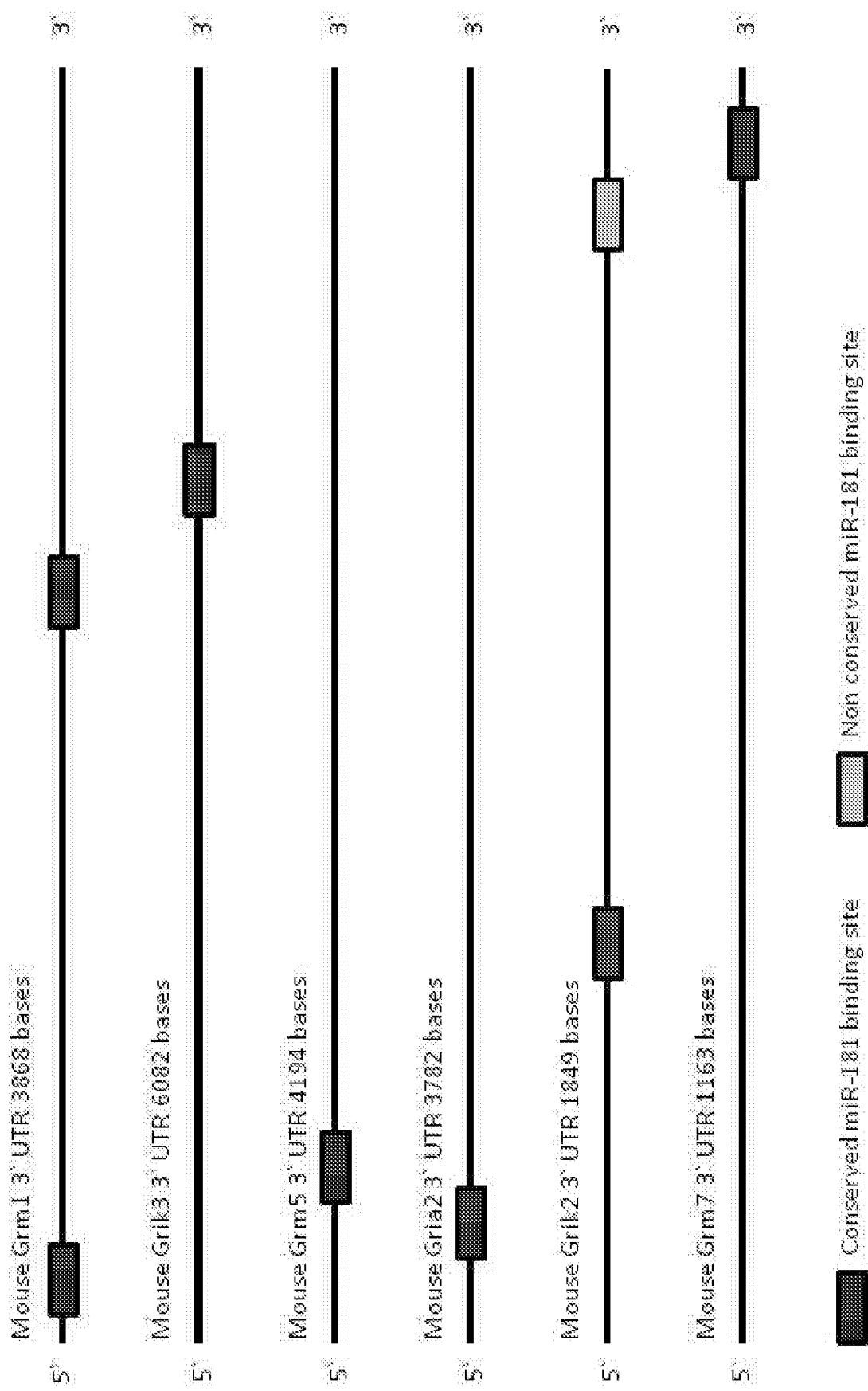


FIG. 26

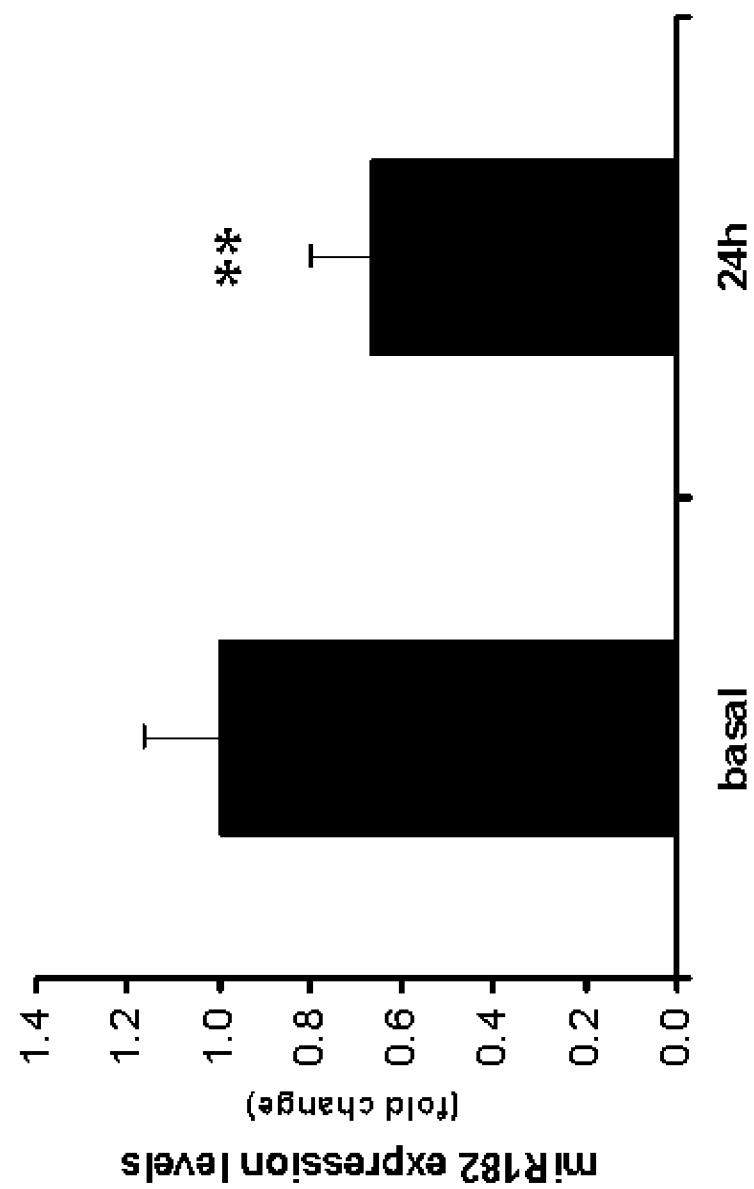


FIG. 27

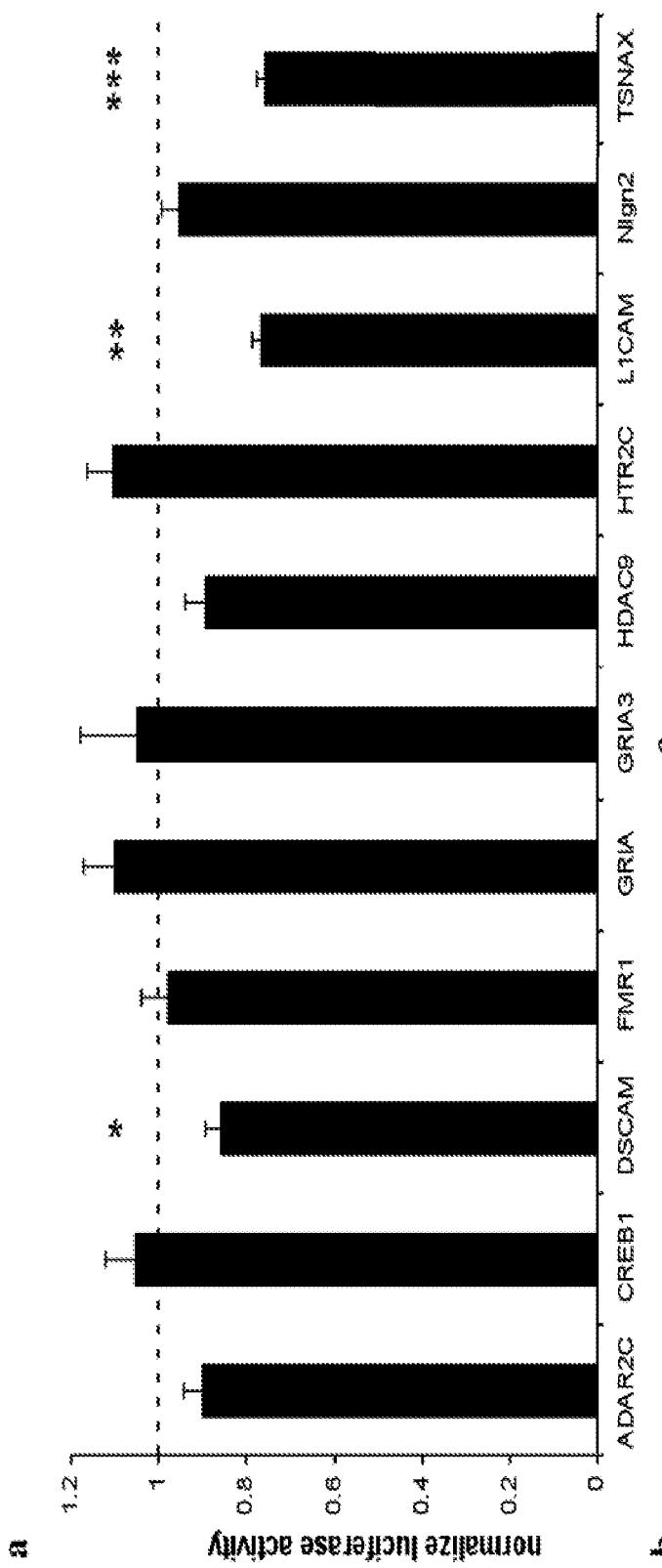


FIG. 28

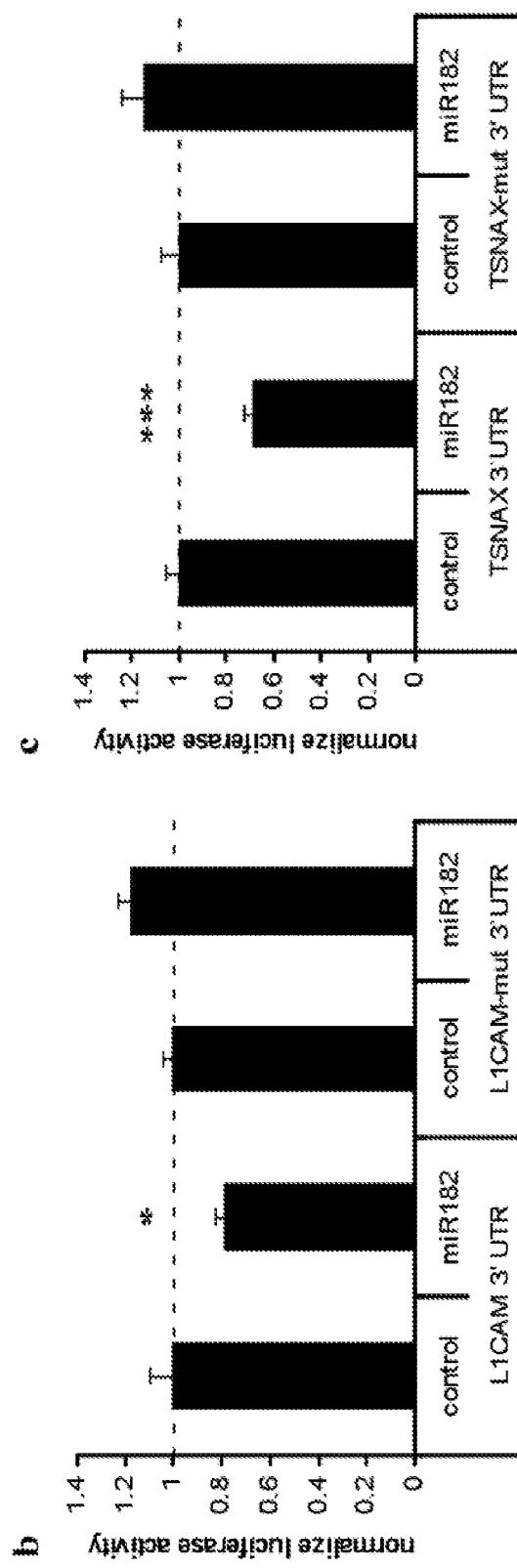


FIG. 29

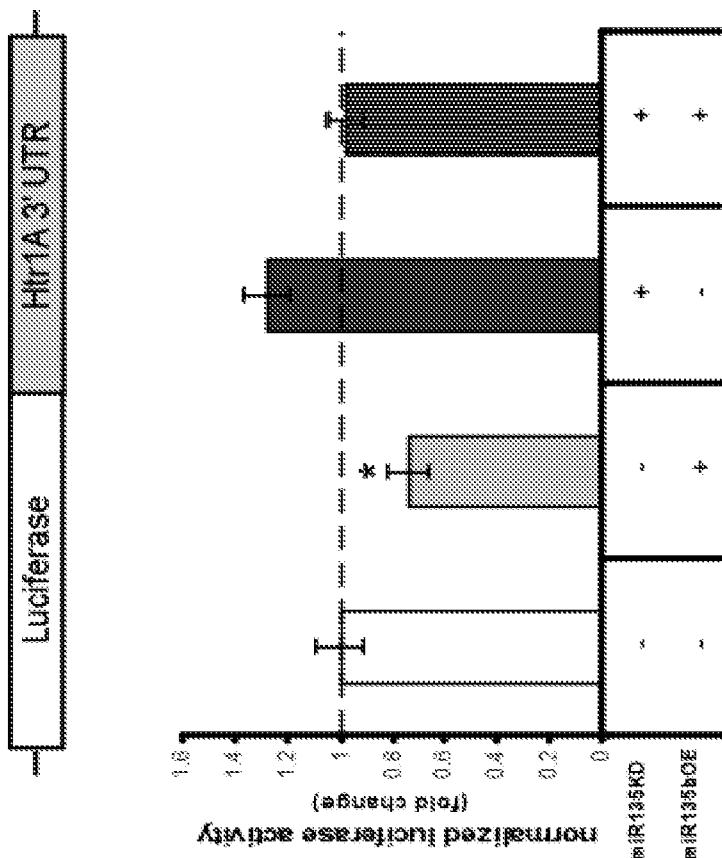
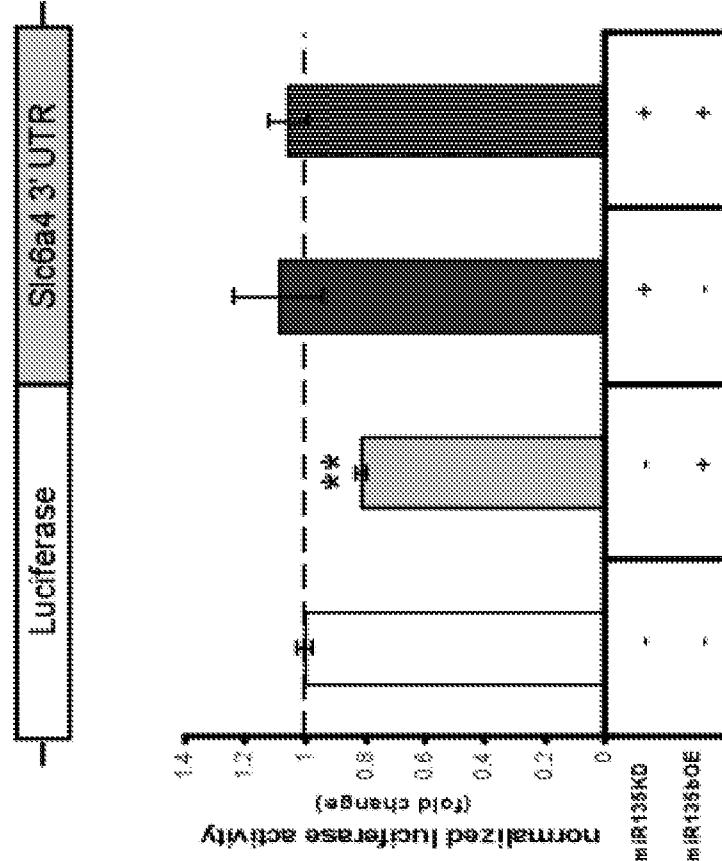
**FIG. 30A****FIG. 30B**

FIG. 30C

```

graph LR
    H1[H1] --- controlKD[control KD]
    H1[H1] --- miR135KD[miR135 KD]
    controlKD --- CMV[CMV]
    miR135KD --- CMV[CMV]
    CMV --- eGFP[eGFP]
  
```

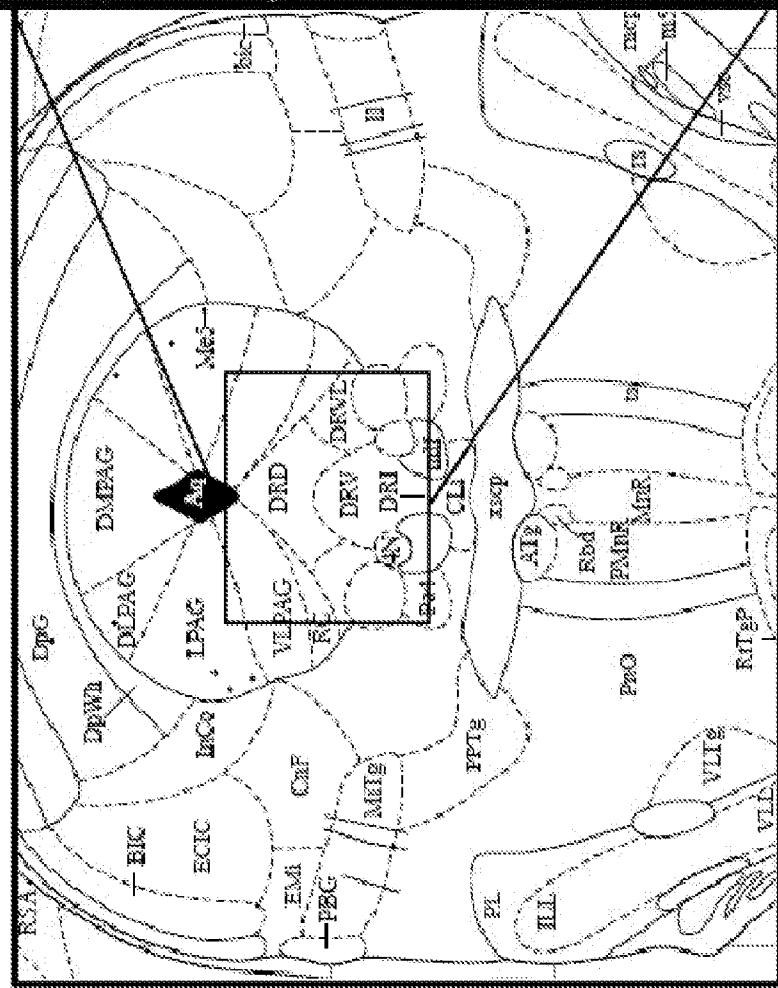


FIG. 30D

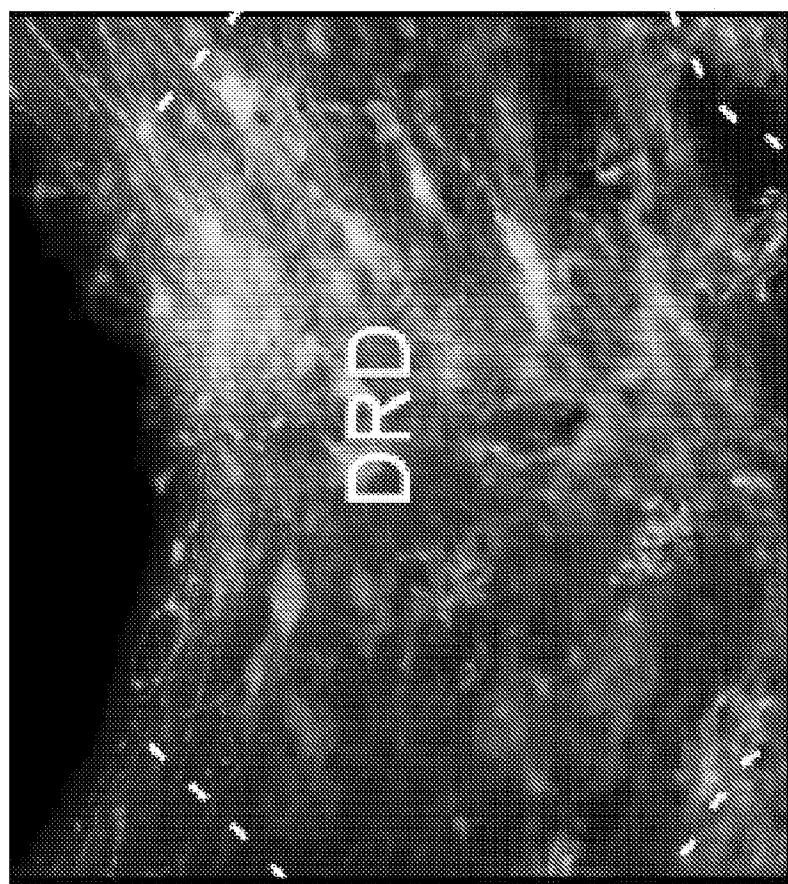


FIG. 30E

FIG. 31A

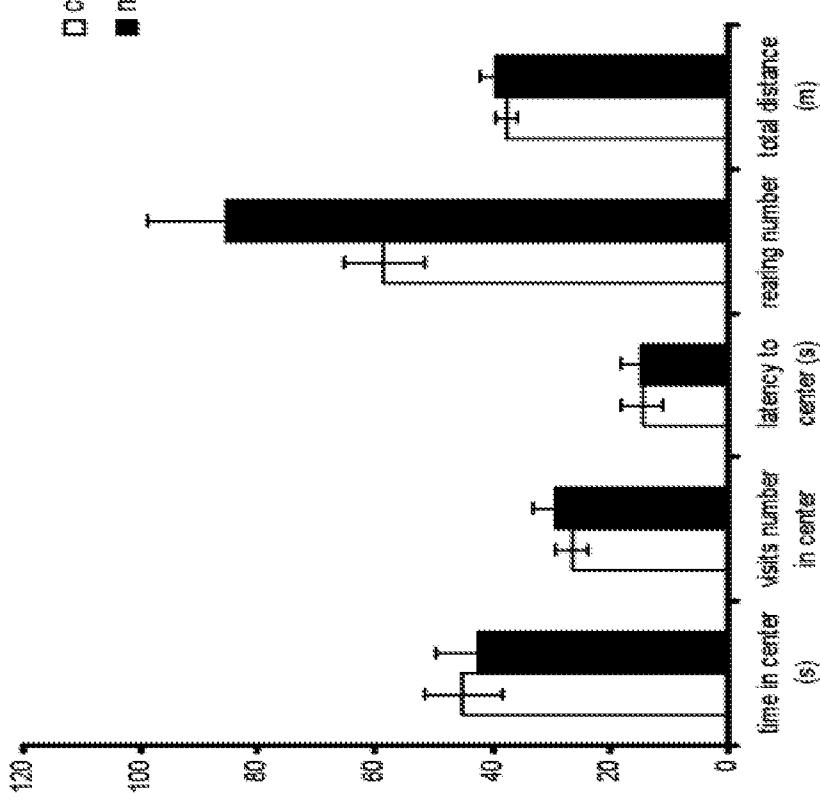


FIG. 31B

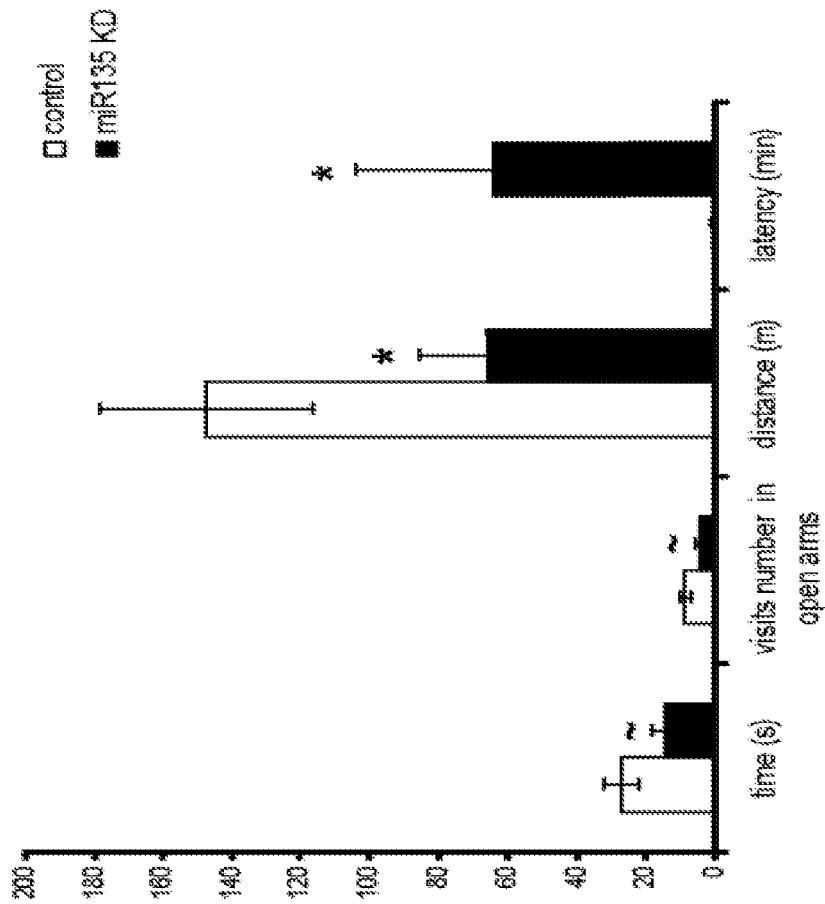


FIG. 31C

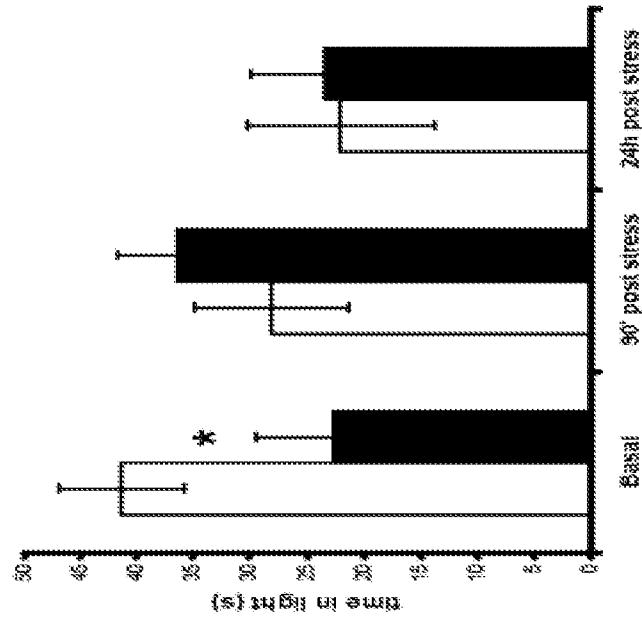


FIG. 31D

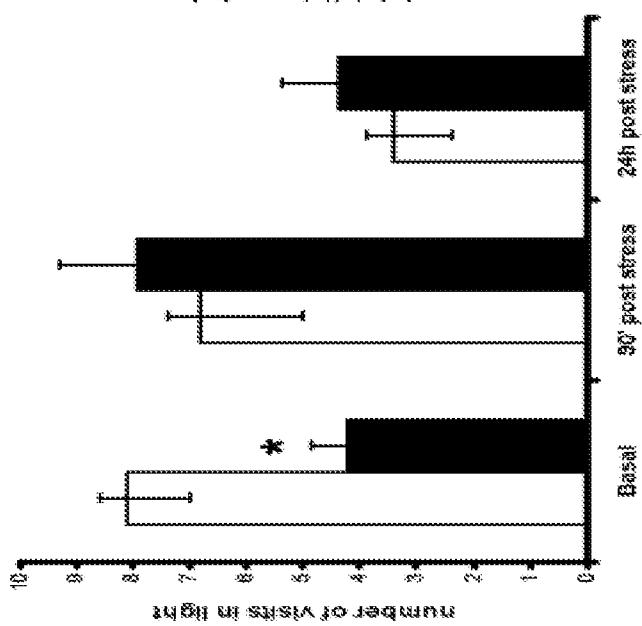
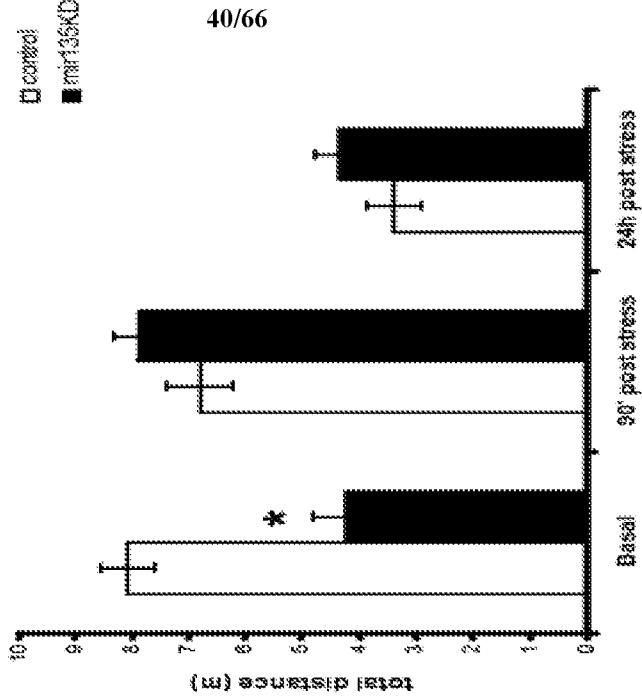


FIG. 31E



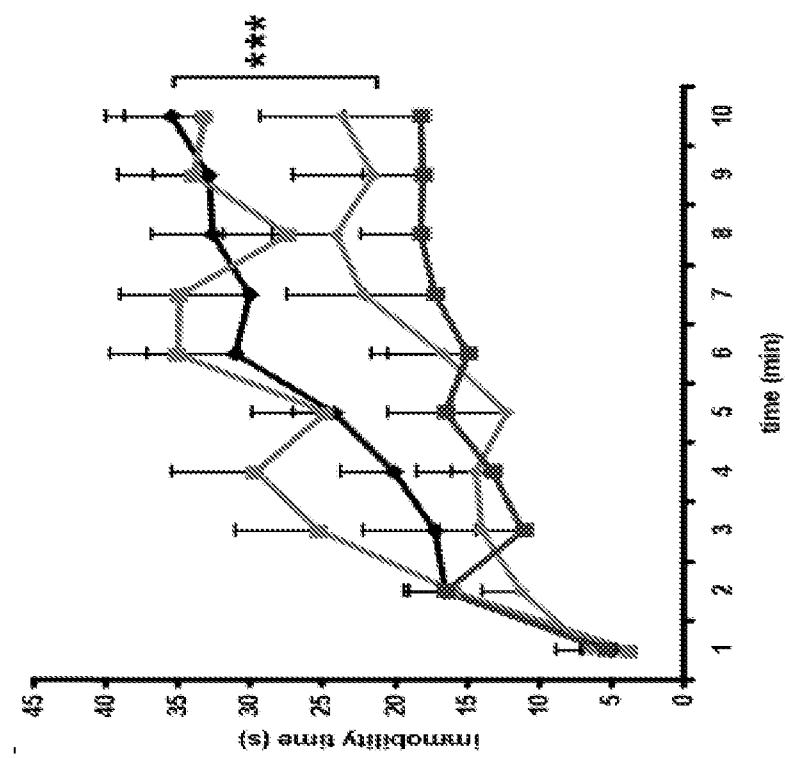
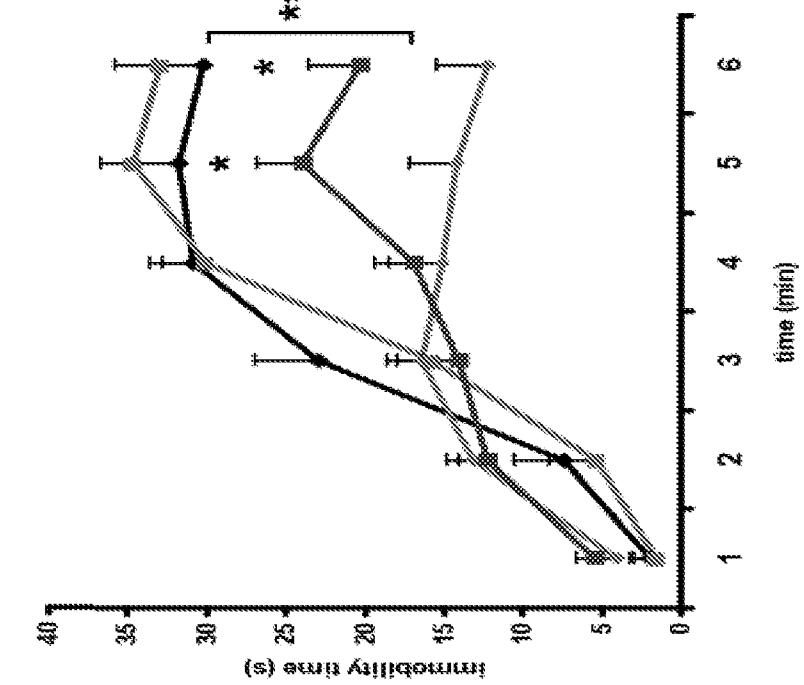
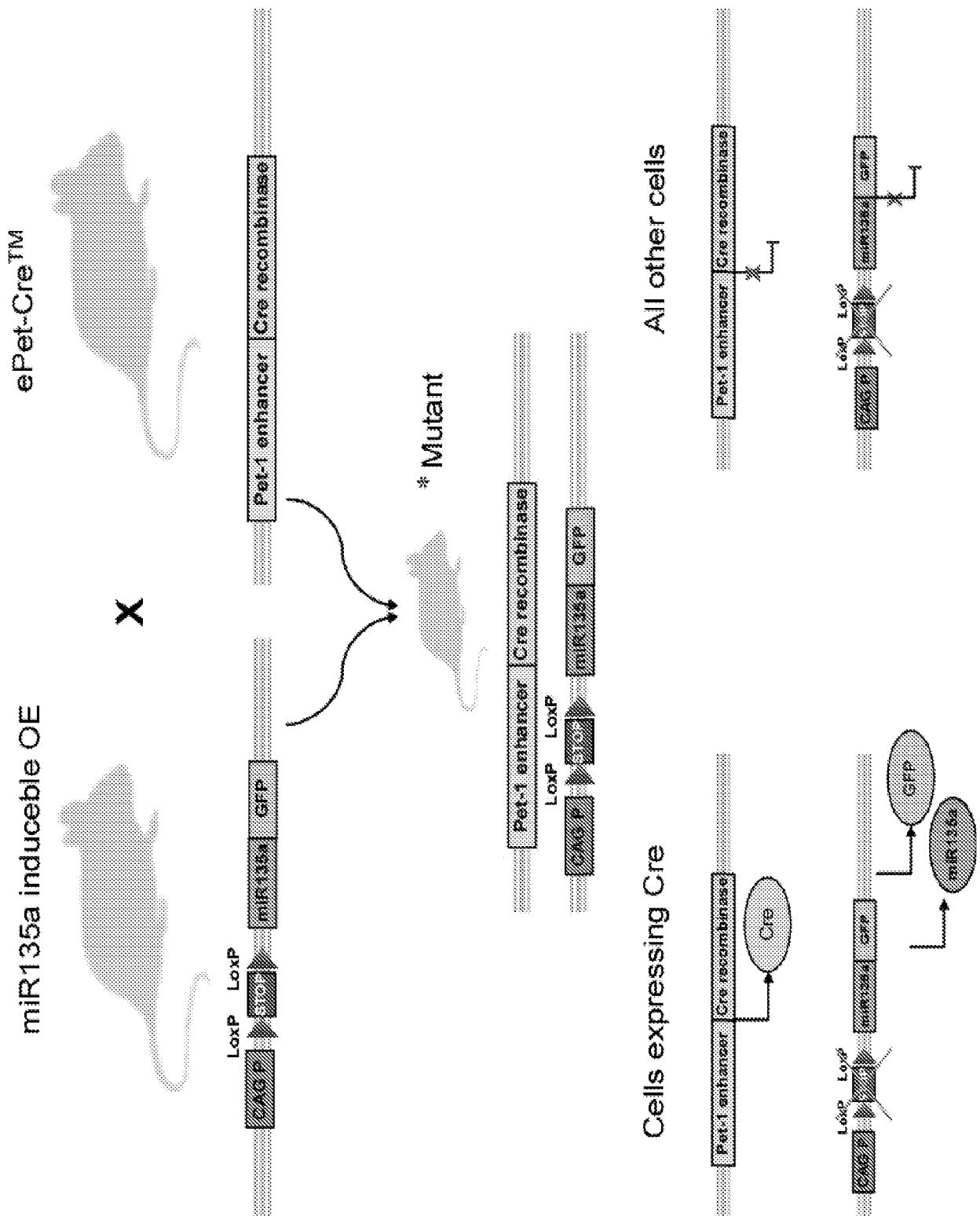
**FIG. 31F****FIG. 31G**

FIG. 32



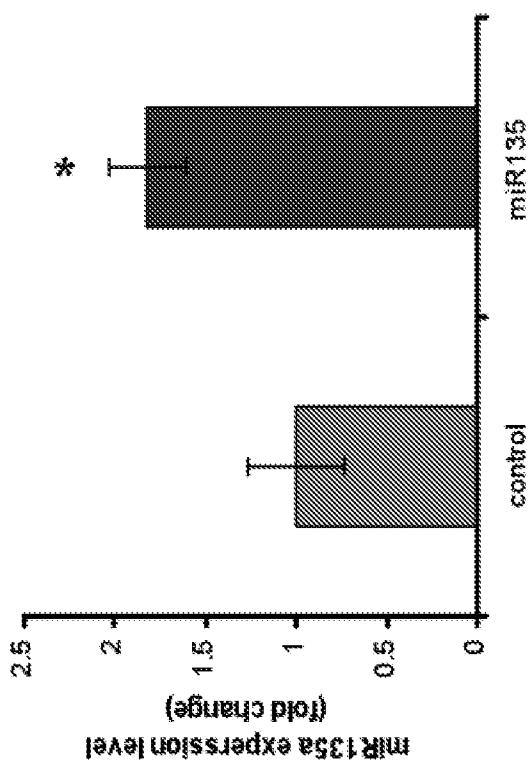
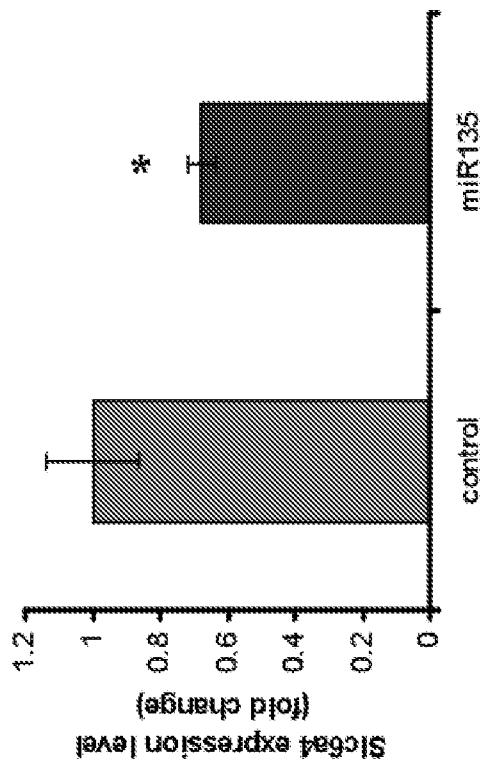
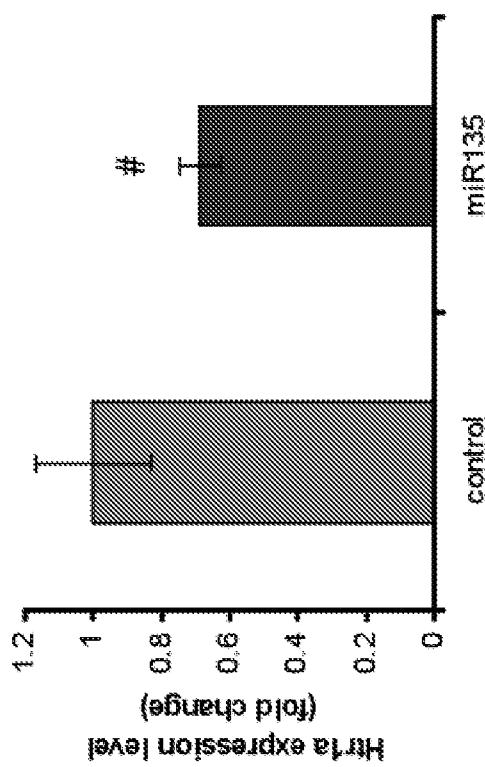
**FIG. 33A****FIG. 33B****FIG. 33C**

FIG. 34A

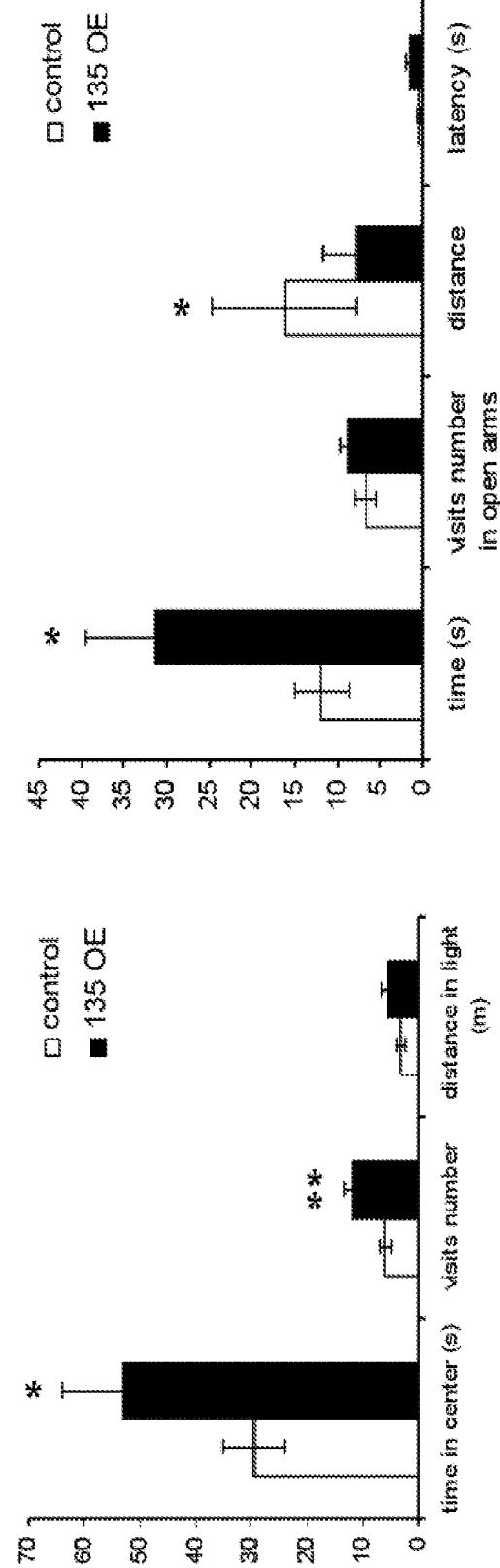
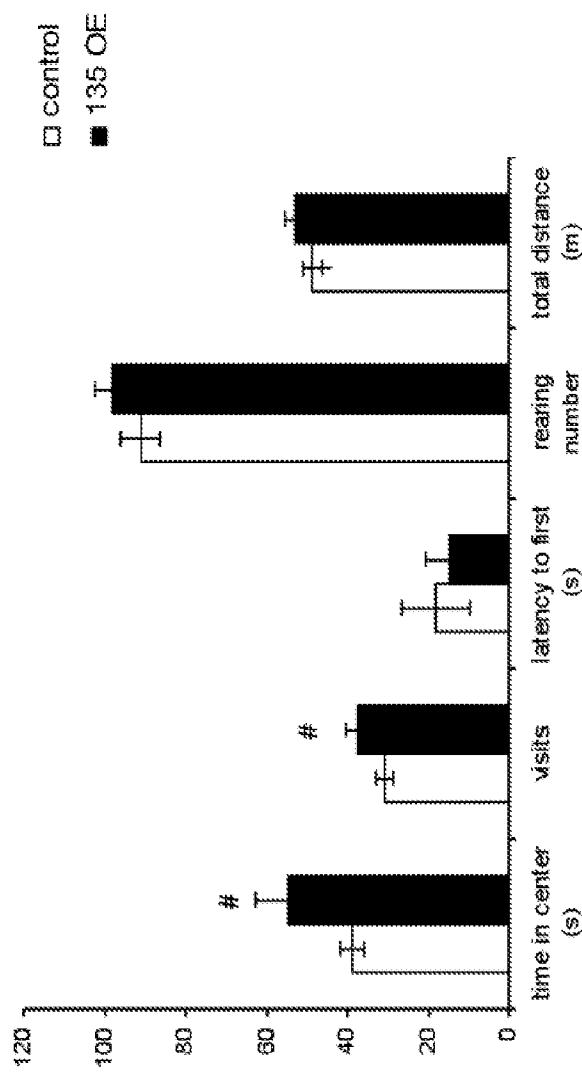


FIG. 34B

FIG. 34C

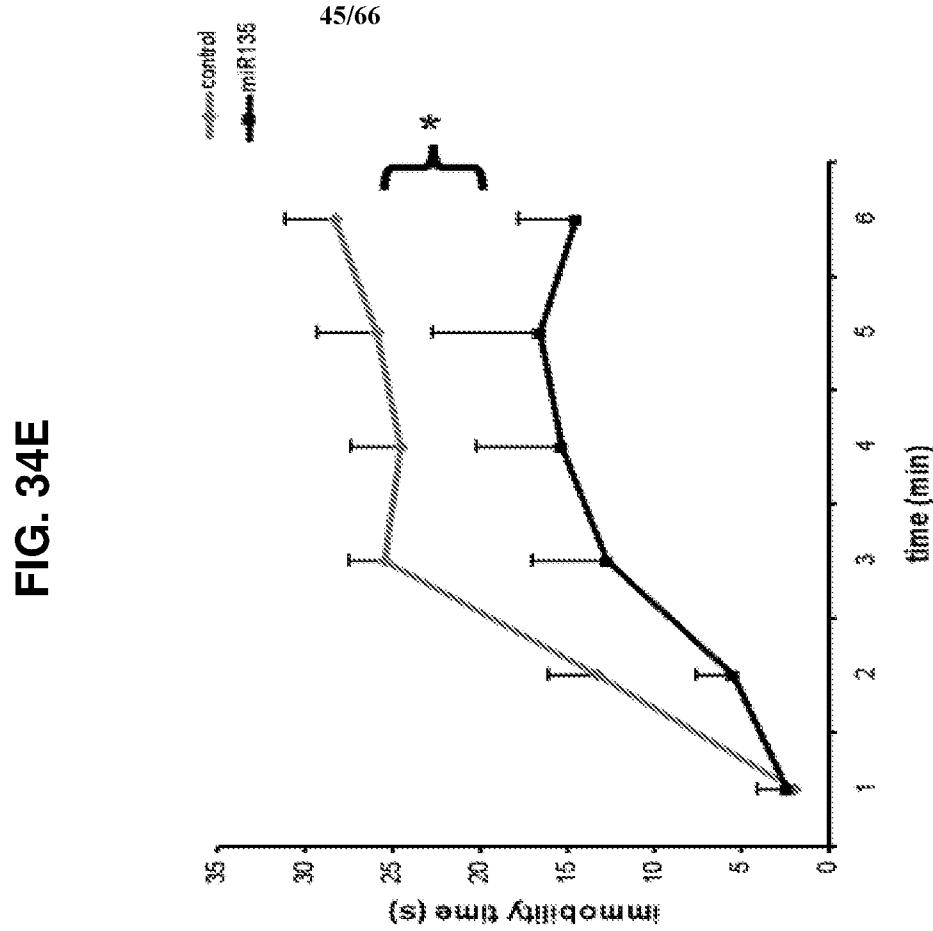


FIG. 34D

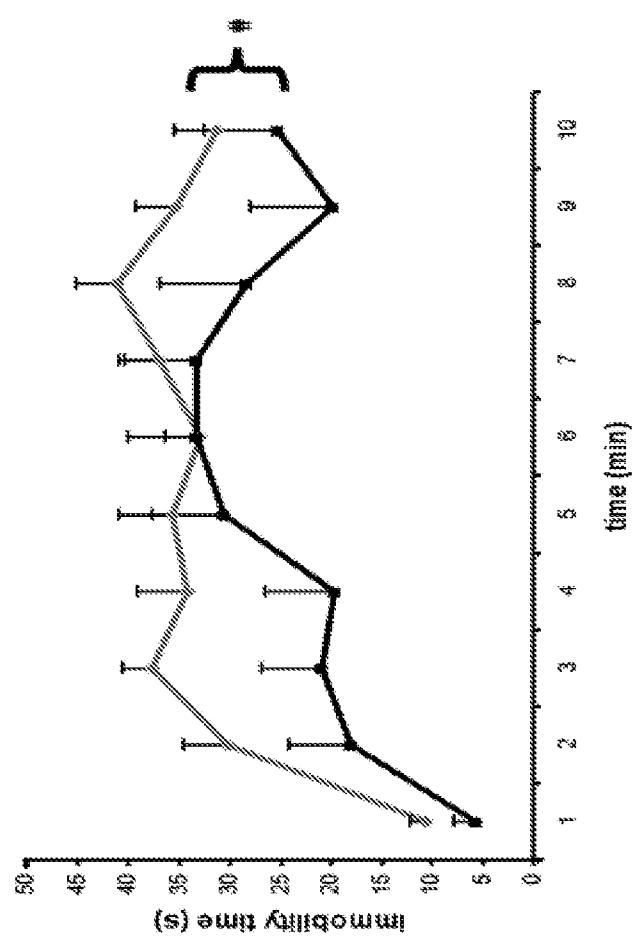
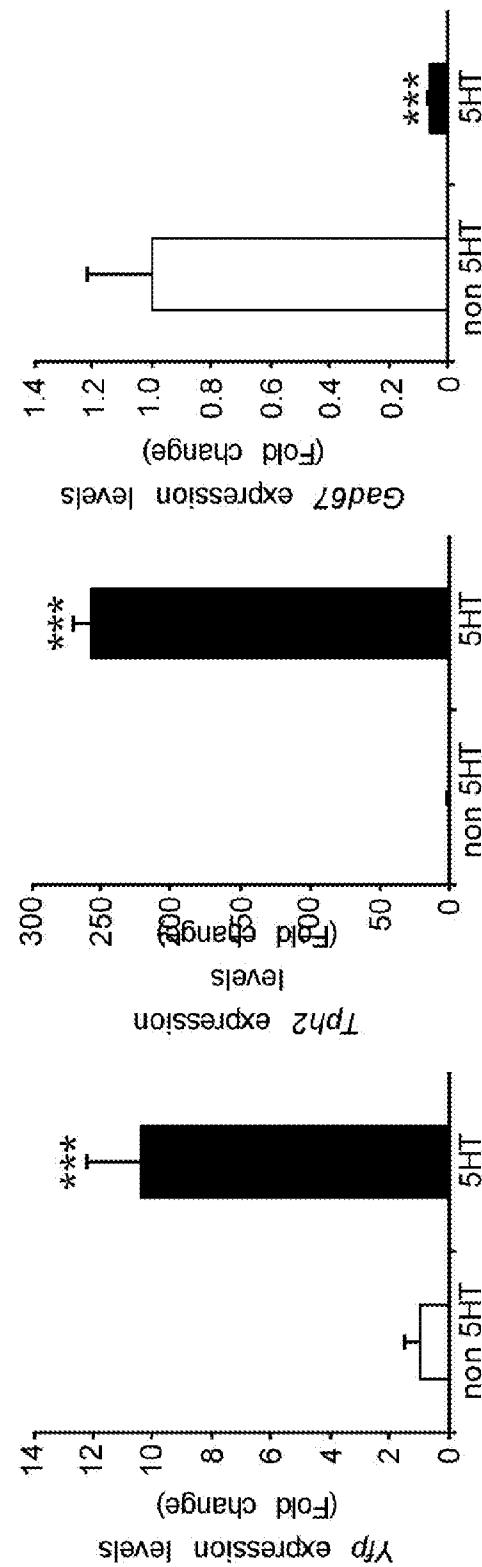
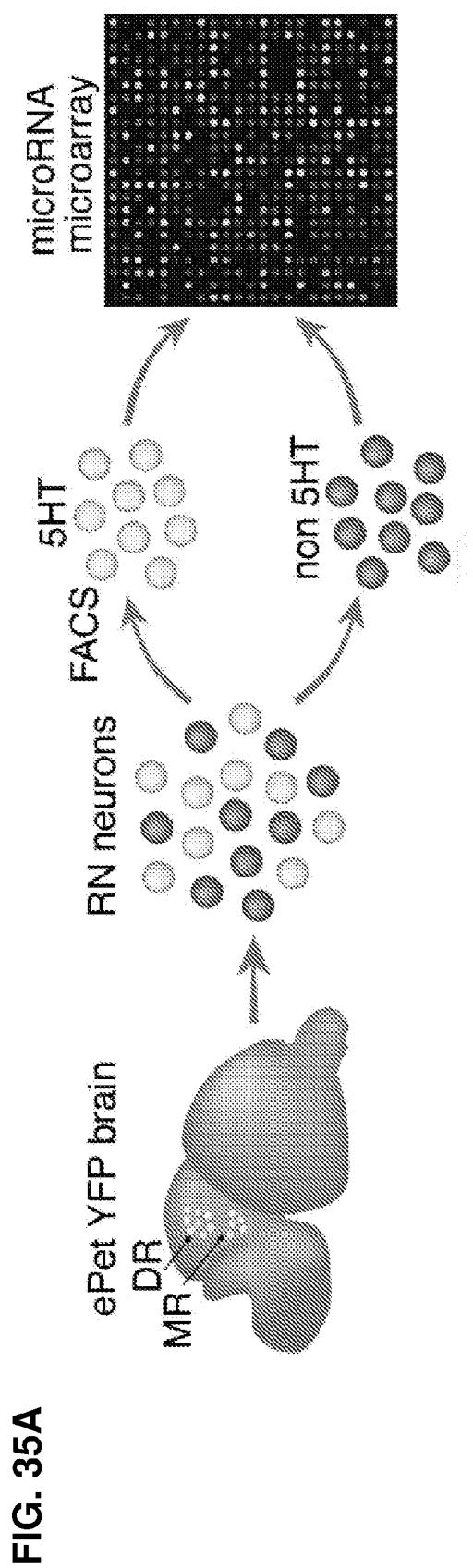


FIG. 34E



**FIG. 35C**  
**FIG. 35D**

|            |      |           |
|------------|------|-----------|
| SEQ ID NO: | 169– | mouse     |
| SEQ ID NO: | 170– | human     |
| SEQ ID NO: | 171– | orangutan |
| SEQ ID NO: | 172– | dog       |
| SEQ ID NO: | 173– | horse     |
| SEQ ID NO: | 174– | opossum   |
| SEQ ID NO: | 175– | chicken   |

FIG. 36A

pre-miR135a-1, mice chromosome 9:106,056,455-106,056,544

**FIG. 36B**

pre-miR135a-2, mice chromosome 10:91,543,831-91,534,930

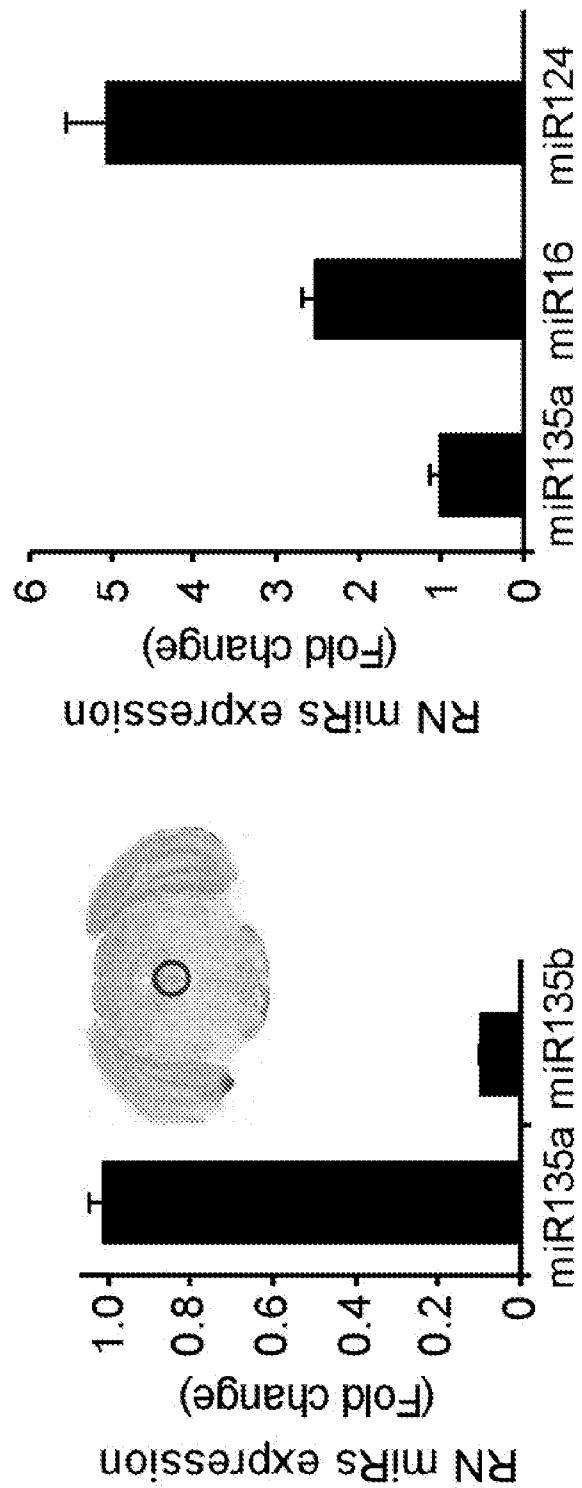
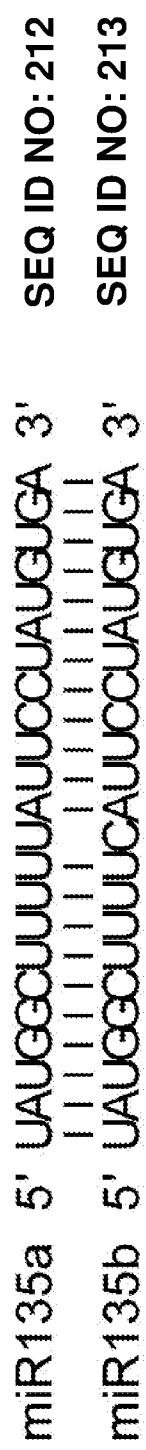
Mouse  
Rat  
Human  
Orangutan  
Dog  
Horse

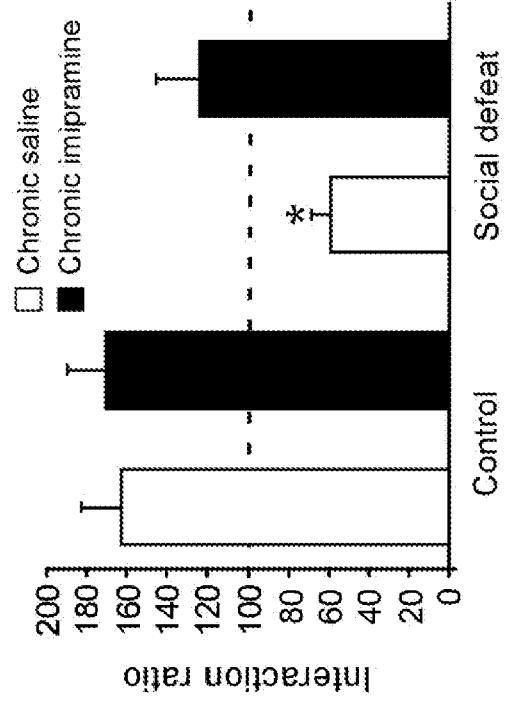
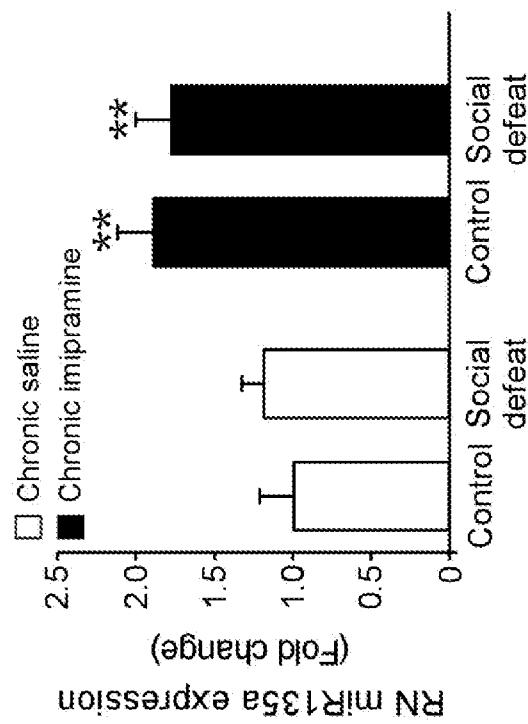
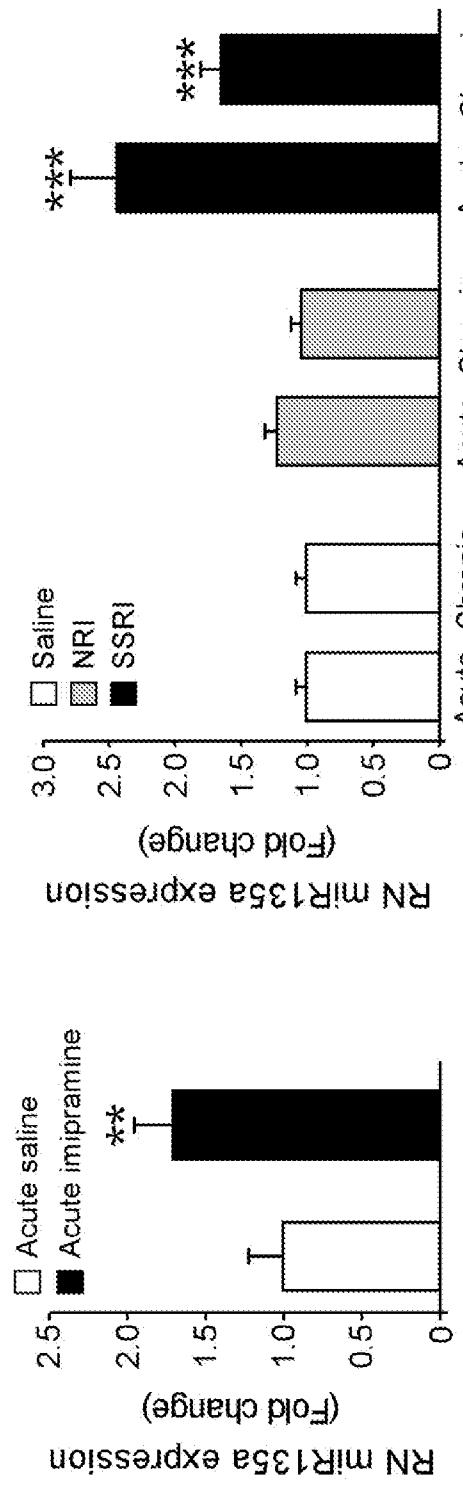
|                           |                                     |
|---------------------------|-------------------------------------|
| SEQ ID NO: 176– mouse     | SEQ ID NO: 180– dog                 |
| SEQ ID NO: 177– rat       | SEQ ID NO: 181– horse               |
| SEQ ID NO: 178– human     | SEQ ID NO: 182– opossum (not shown) |
| SEQ ID NO: 179– orangutan | SEQ ID NO: 183– chicken (not shown) |

FIG. 36C

pre-miR135b, mice chromosome 1:134,094,665-134,094,761

|            |                |            |              |
|------------|----------------|------------|--------------|
| SEQ ID NO: | 184– mouse     | SEQ ID NO: | 188– dog     |
| SEQ ID NO: | 185– rat       | SEQ ID NO: | 189– horse   |
| SEQ ID NO: | 186– human     | SEQ ID NO: | 190– opossum |
| SEQ ID NO: | 187– orangutan | SEQ ID NO: | 191– chicken |

**FIG. 37A****FIG. 37B****FIG. 37C**

**FIG. 37D****FIG. 37E****FIG. 37F****FIG. 37G**

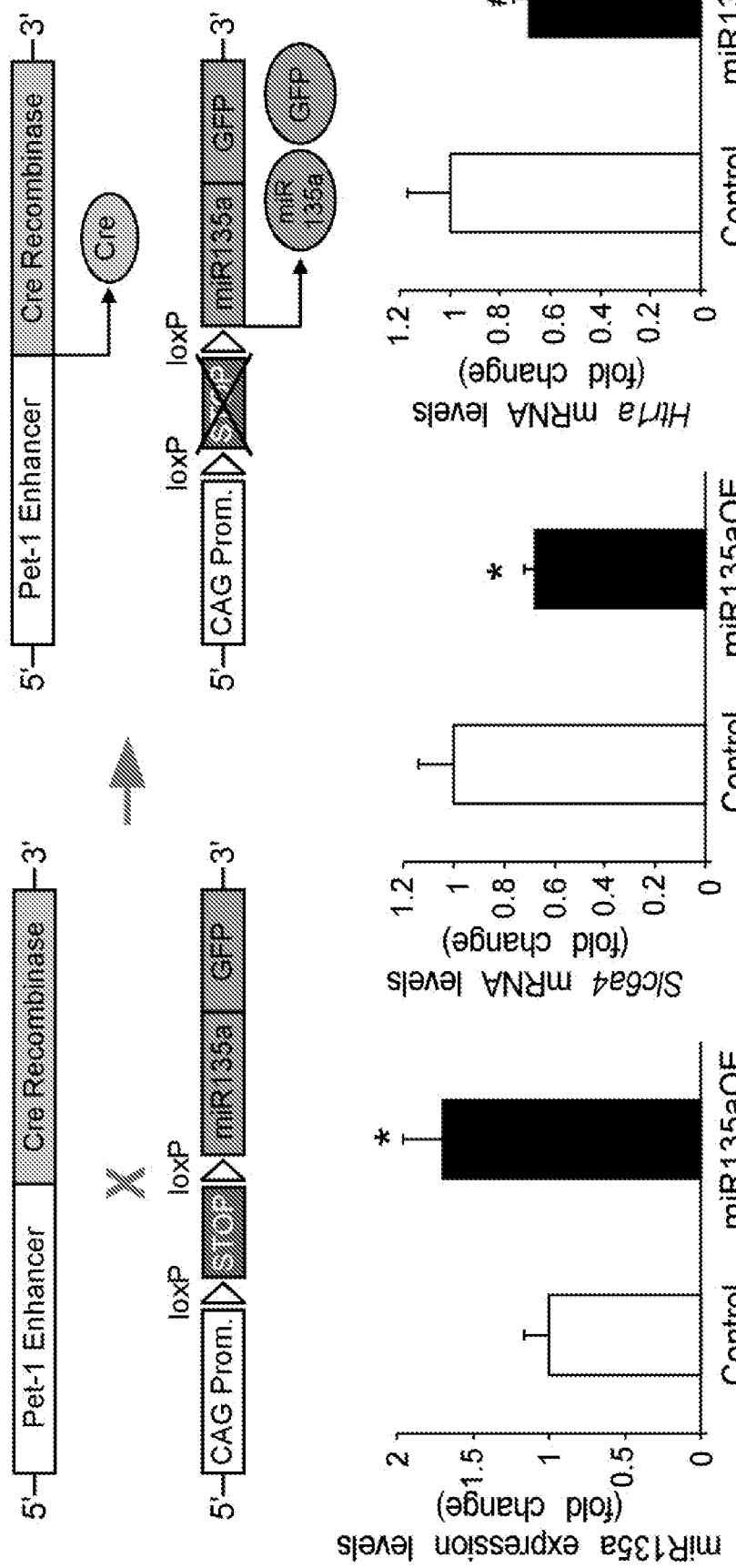
**FIG. 38A****FIG. 38B****FIG. 38C****FIG. 38D**

FIG. 38E

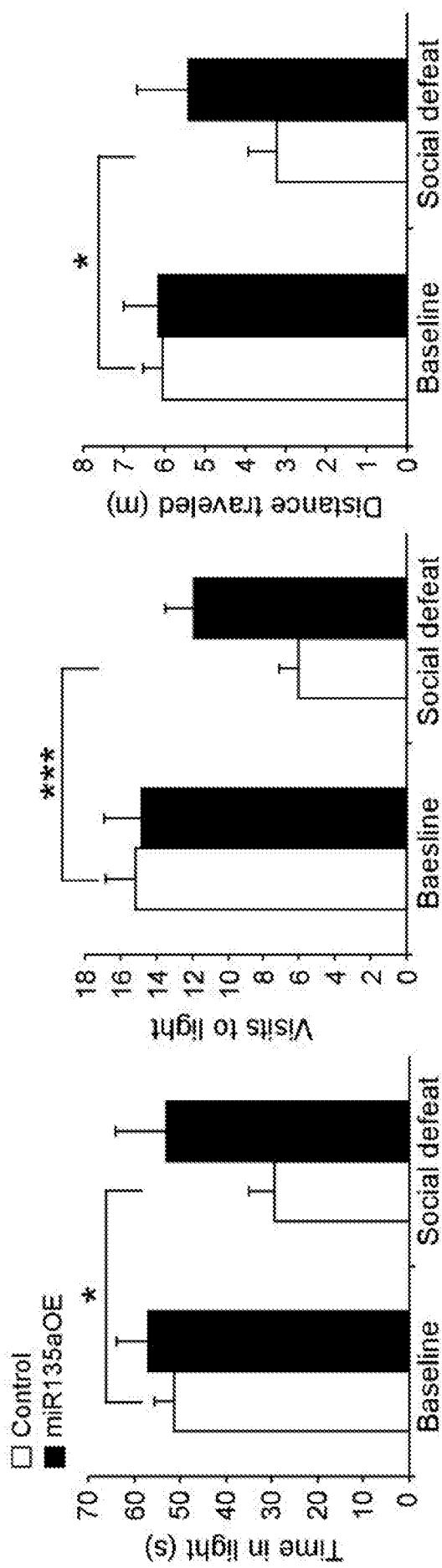


FIG. 38F

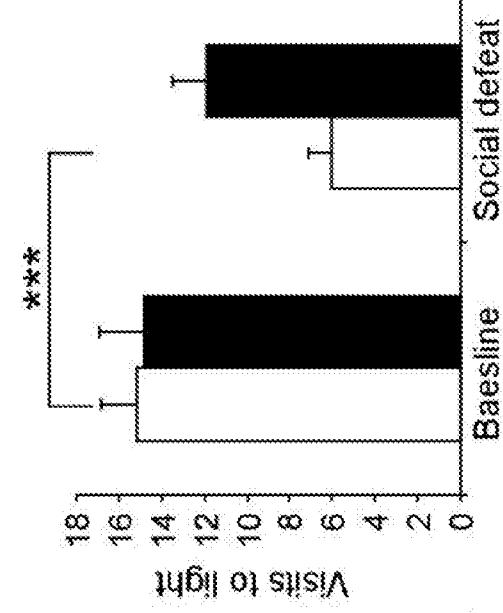


FIG. 38G

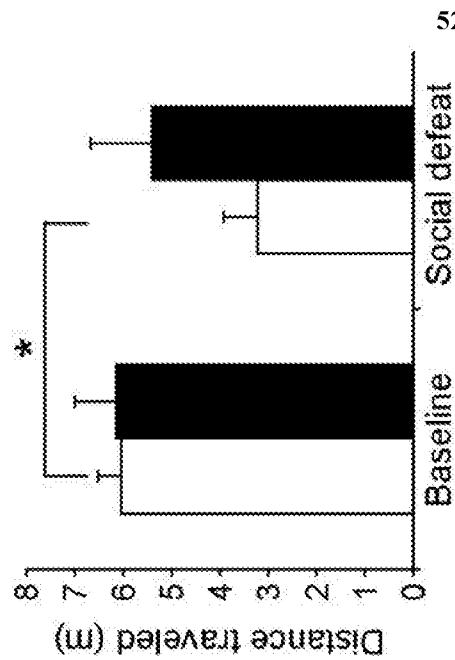


FIG. 38H

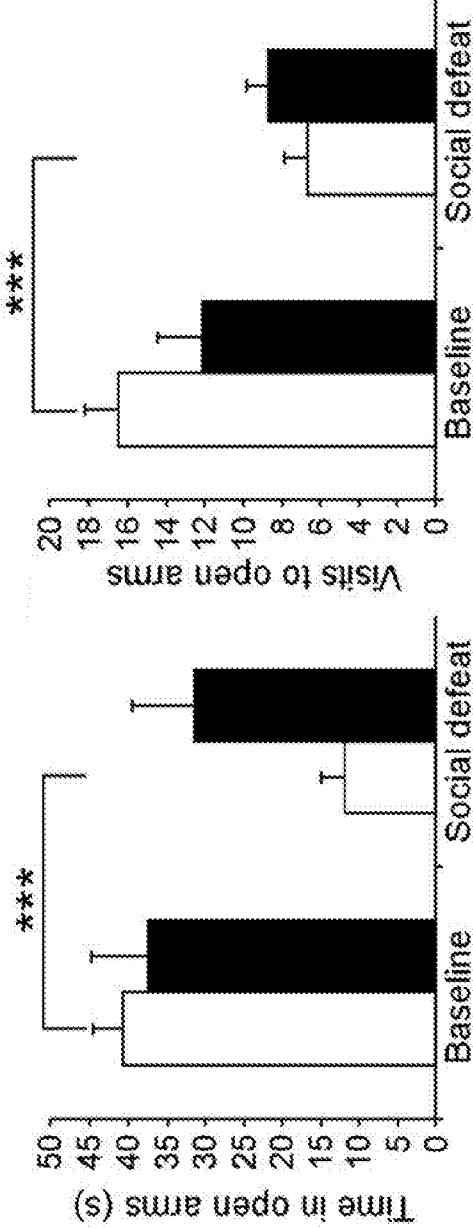


FIG. 38I

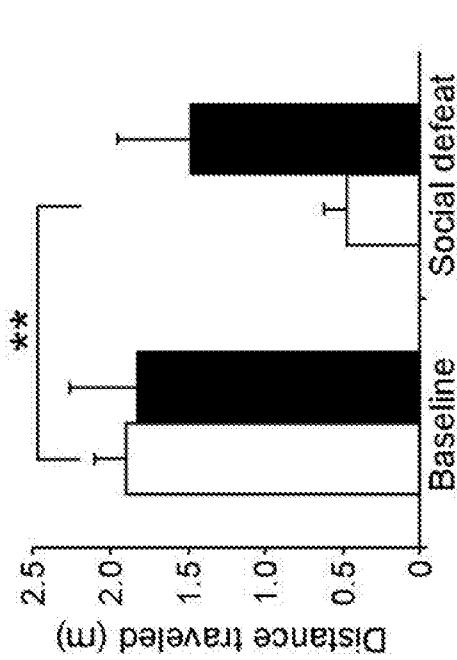


FIG. 38J

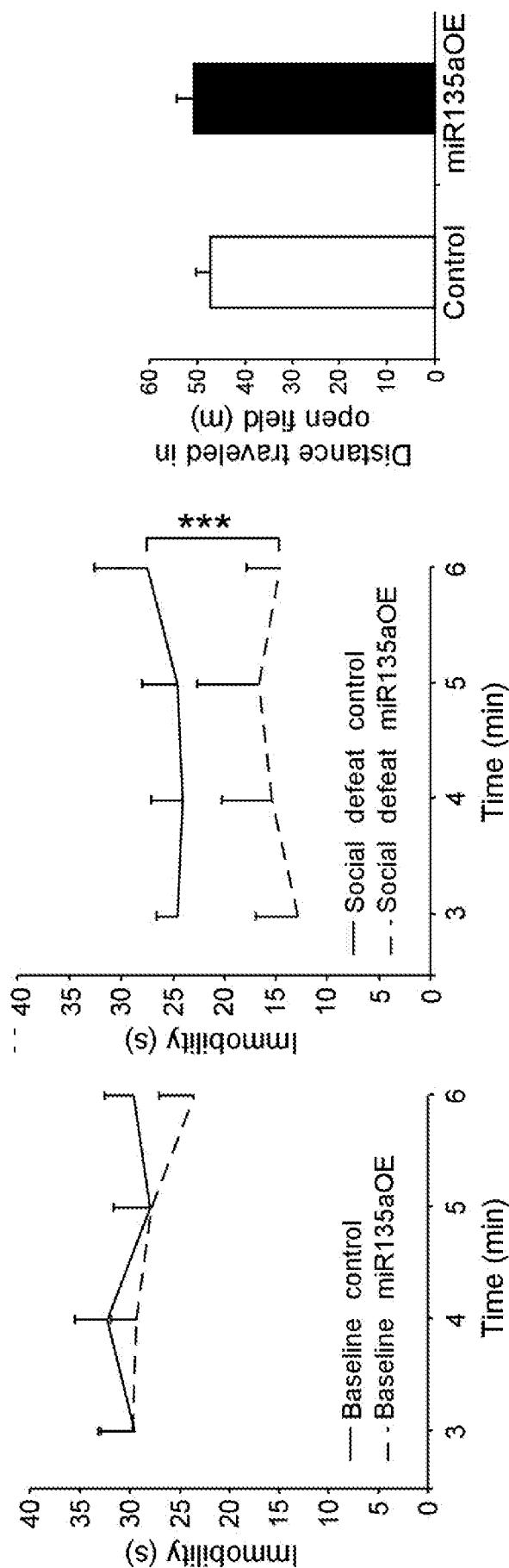


FIG. 38K

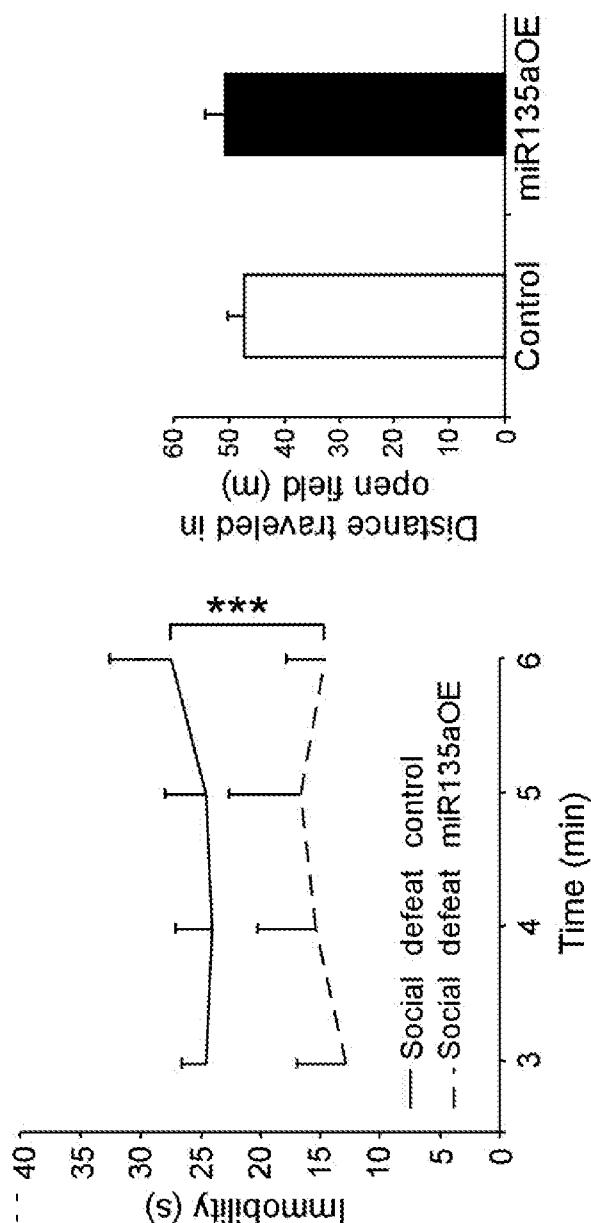


FIG. 38L



FIG. 38M

FIG. 39A

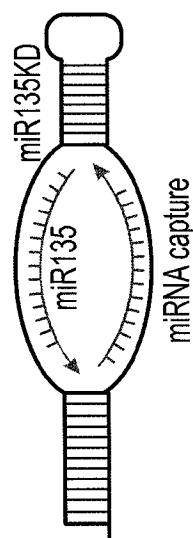


FIG. 39B

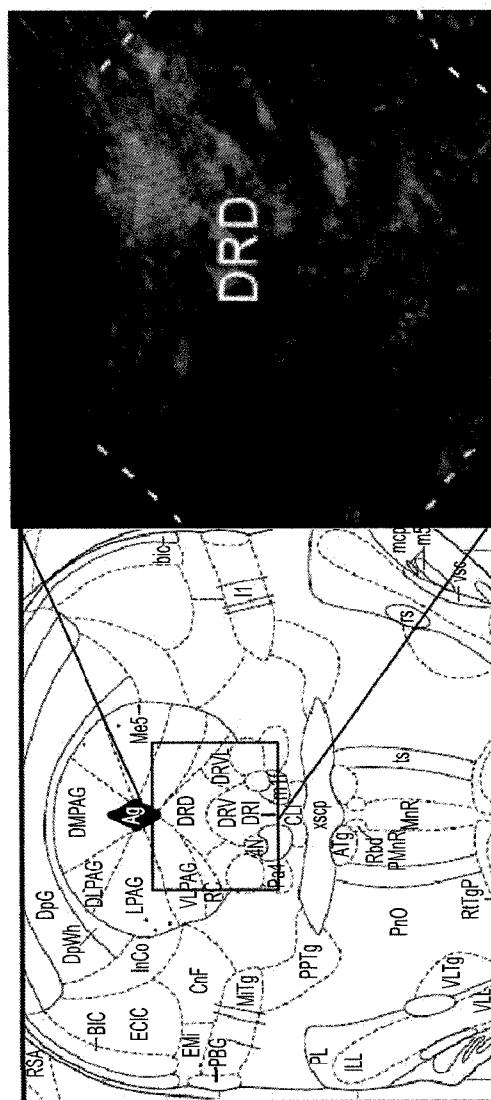
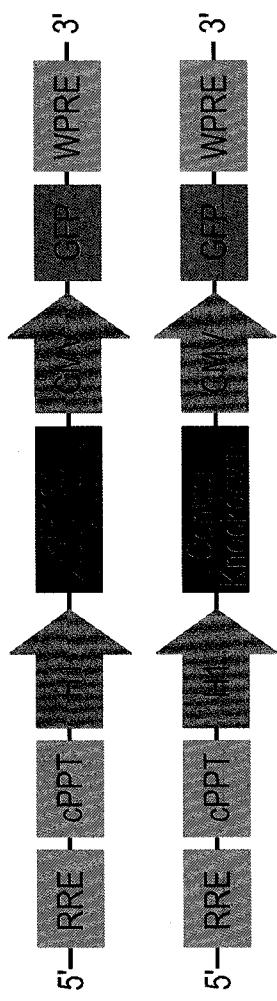


FIG. 39C

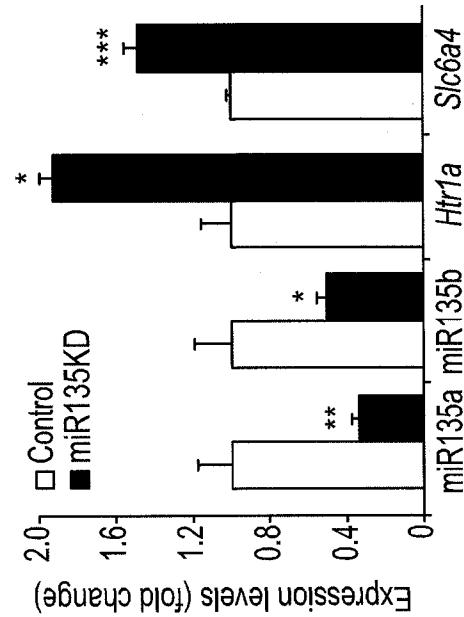


FIG. 39E

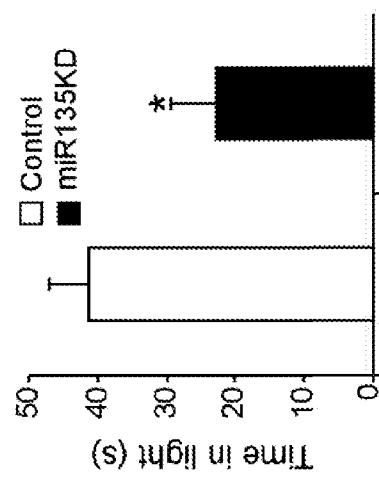


FIG. 39F

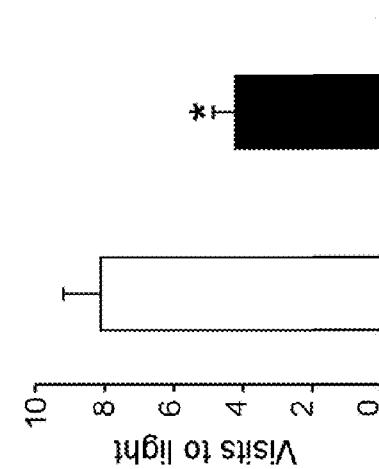


FIG. 39G

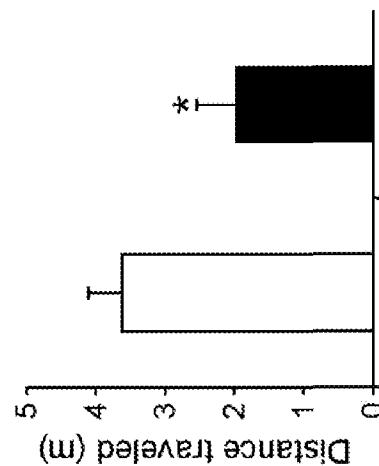
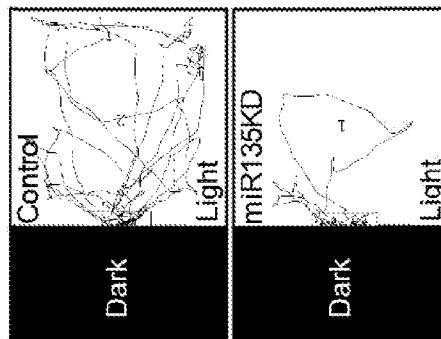


FIG. 39H



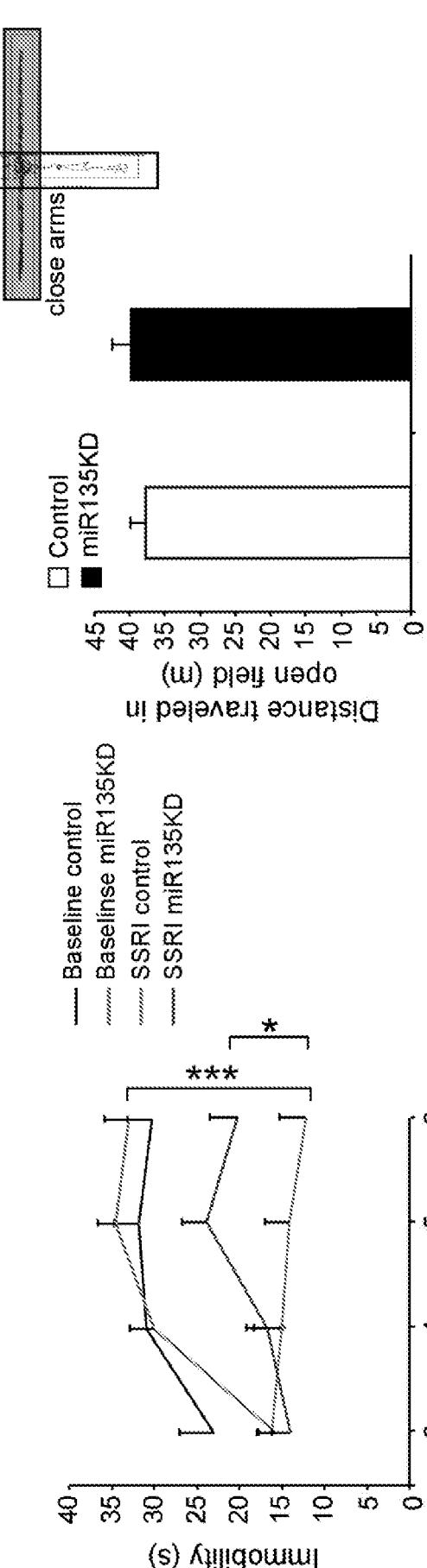
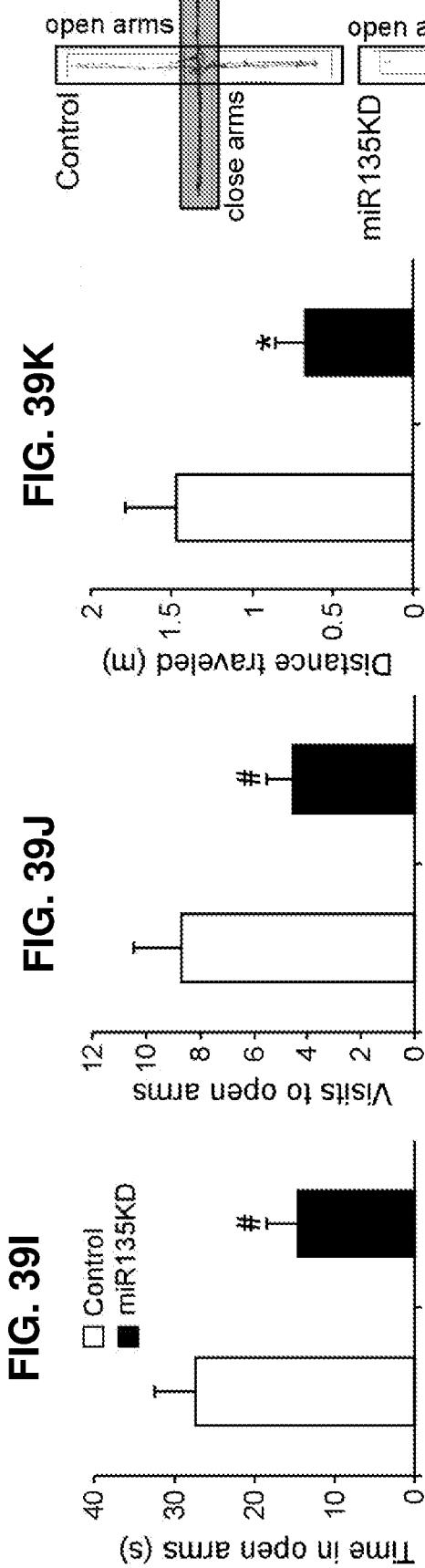
**FIG. 39I****FIG. 39J****FIG. 39K****FIG. 39N****FIG. 39M**

FIG. 40C

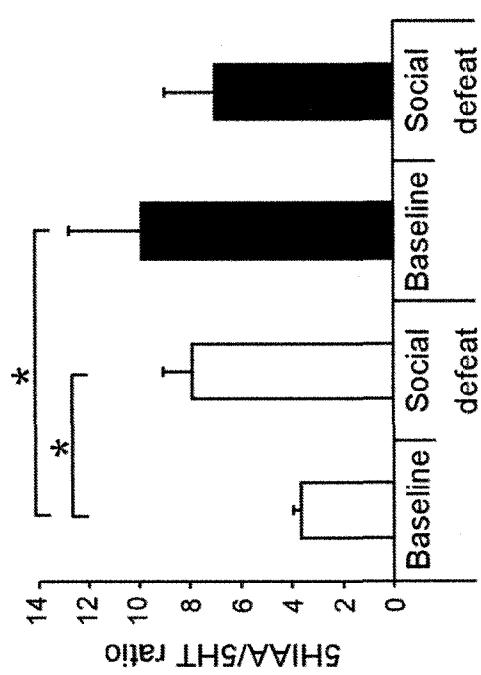


FIG. 40B

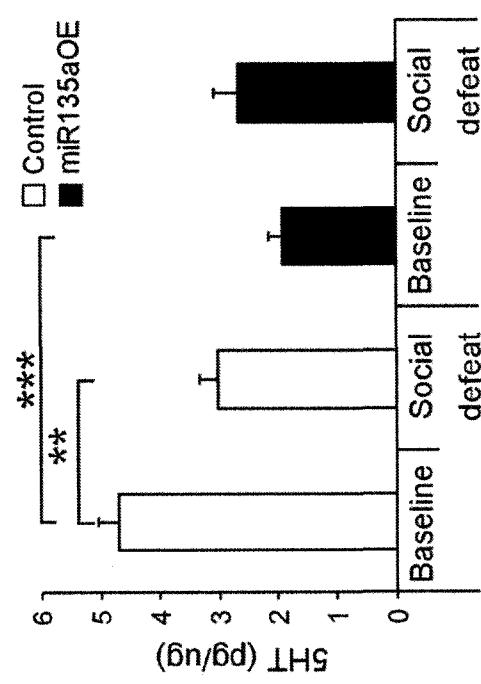


FIG. 40A

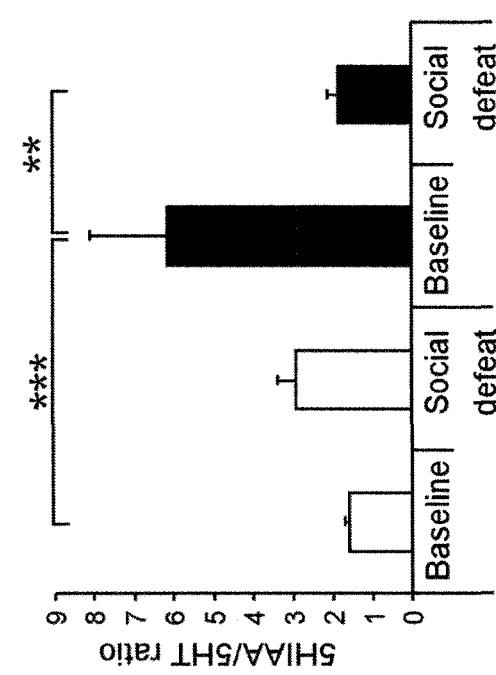
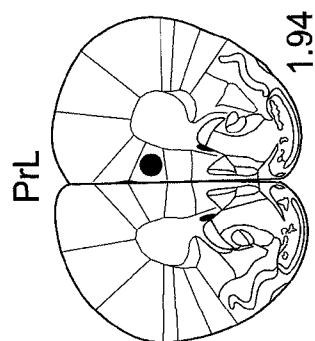


FIG. 40F

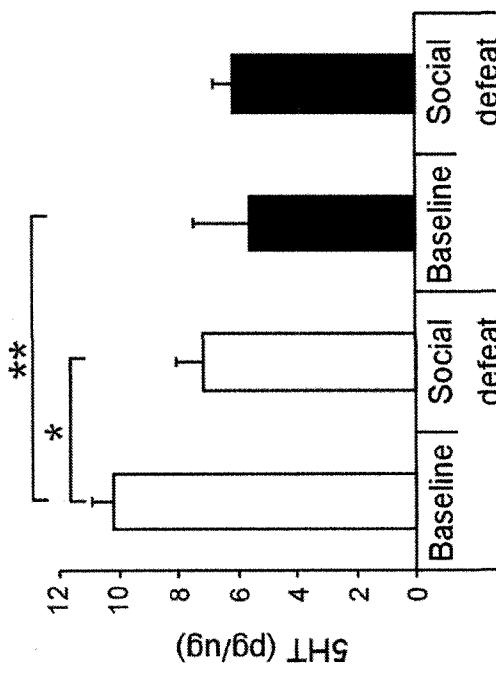


FIG. 40E

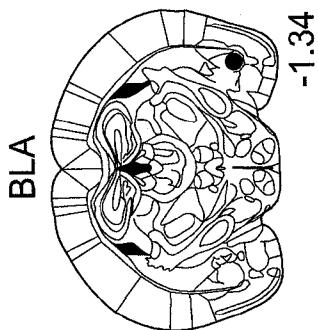


FIG. 40D

FIG. 40I

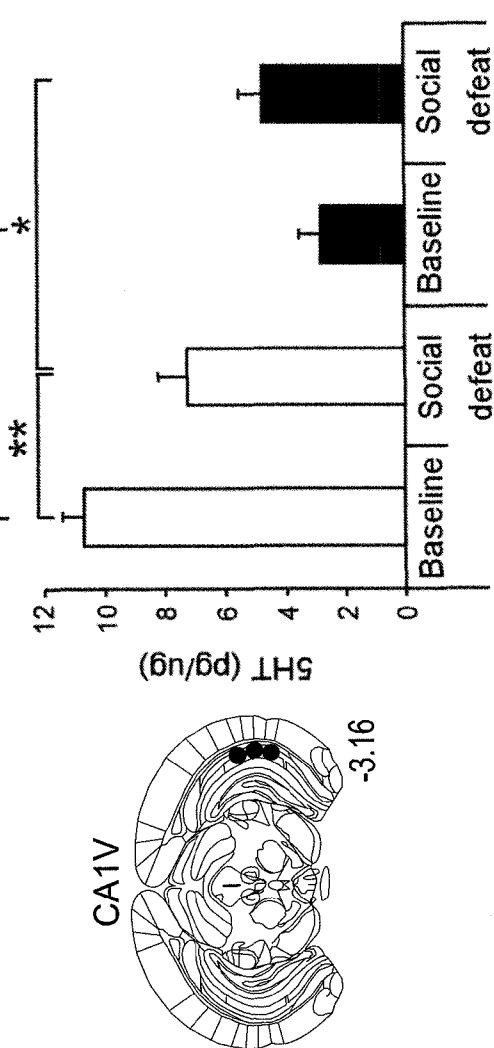


FIG. 40G

FIG. 40H

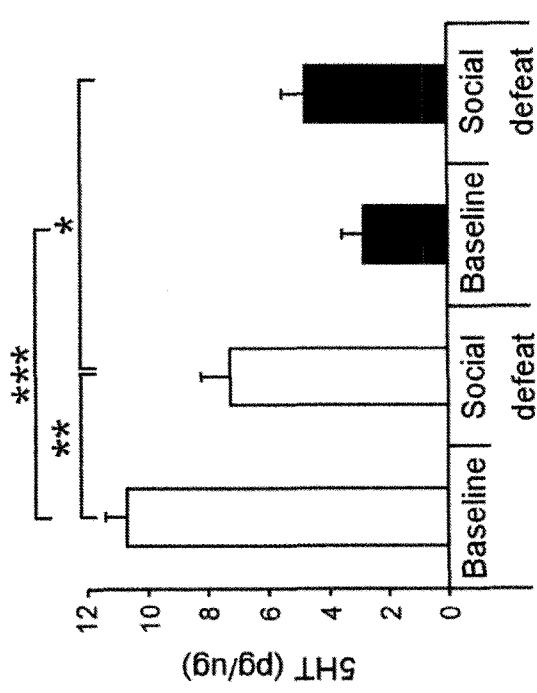


FIG. 40H

FIG. 40I

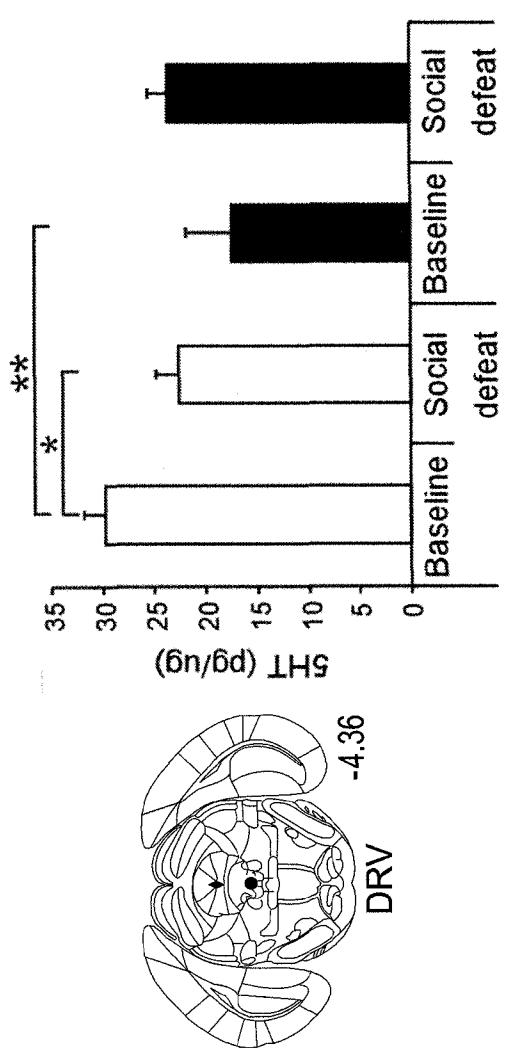


FIG. 40J

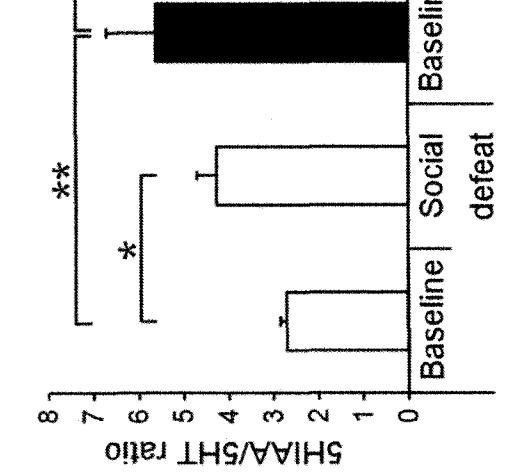


FIG. 40L

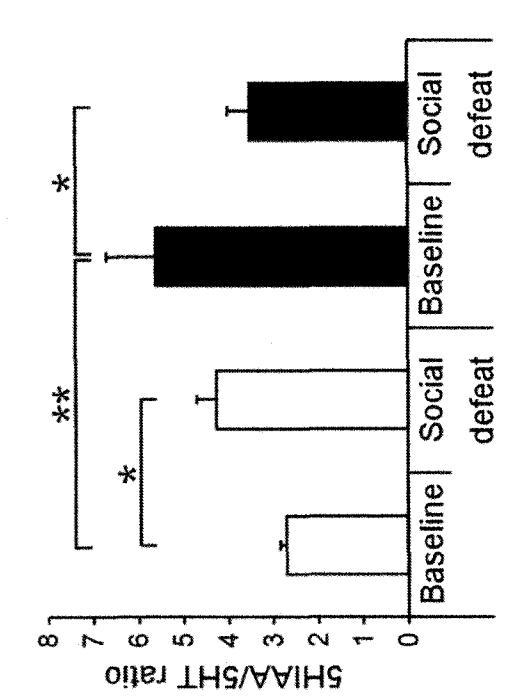


FIG. 40L

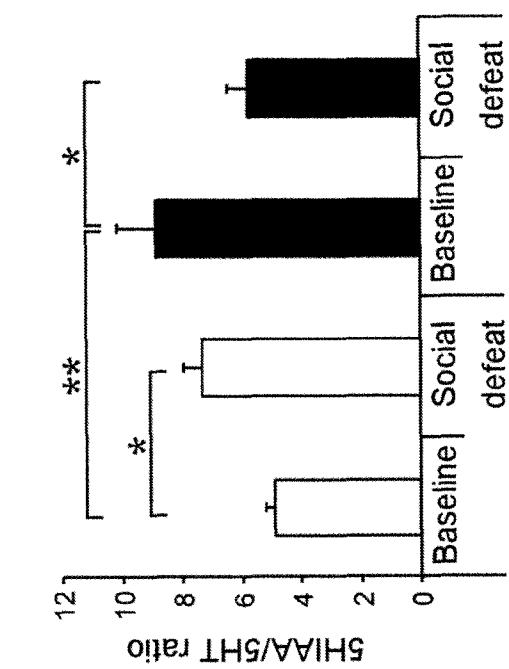


FIG. 400

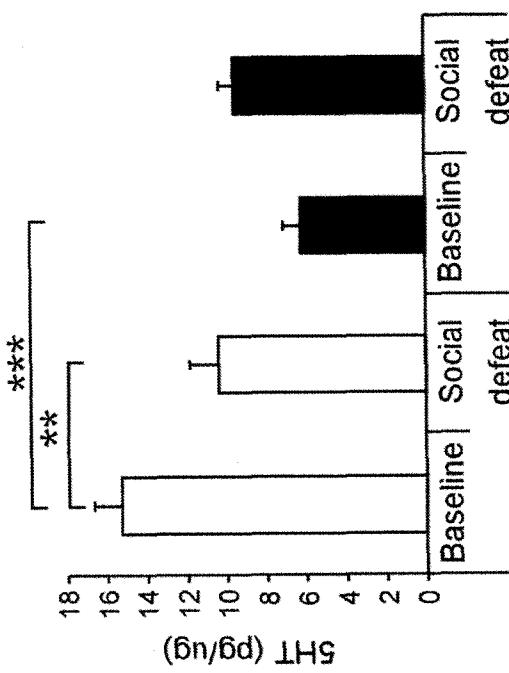


FIG. 40N

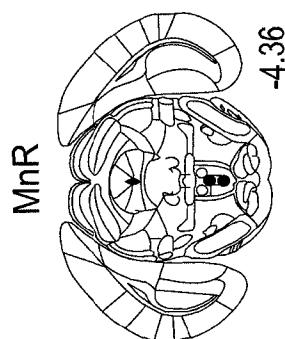


FIG. 40M

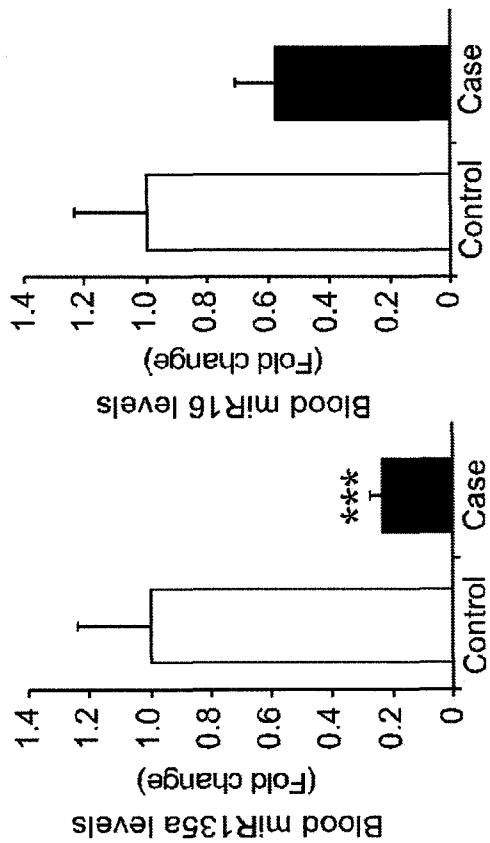
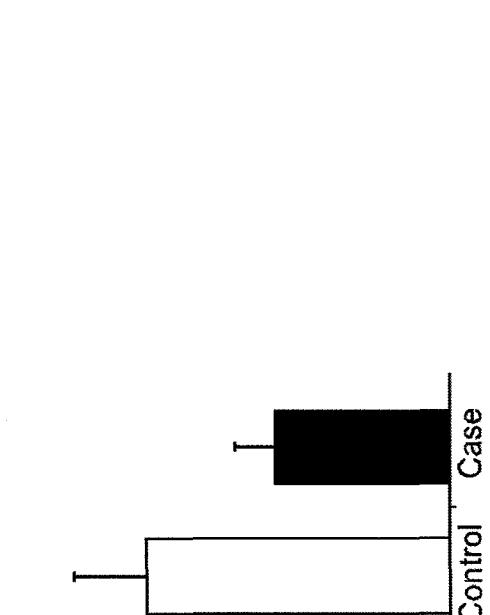
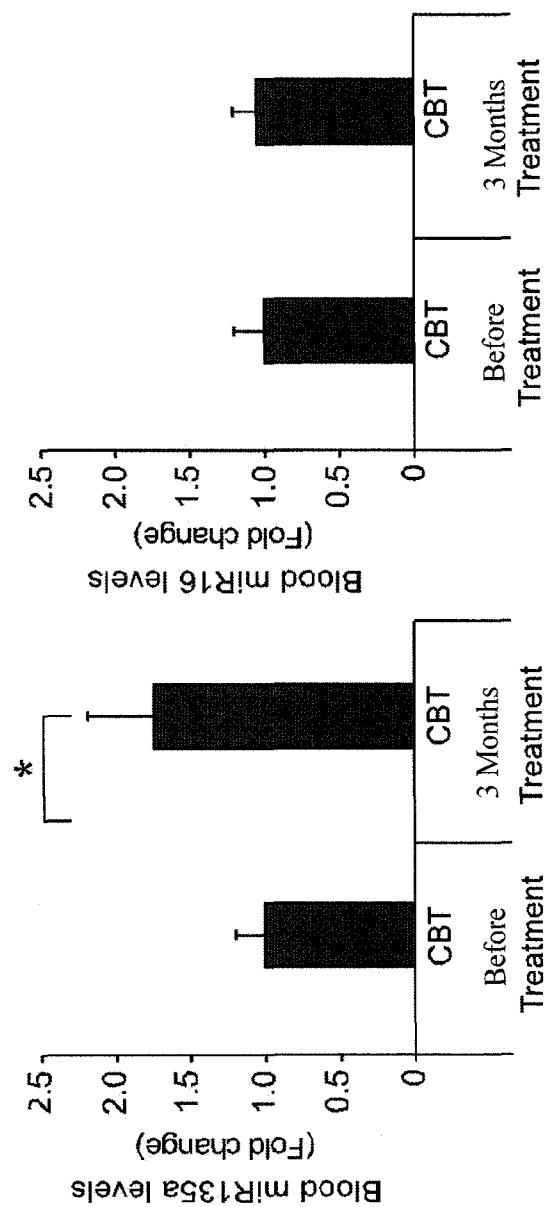
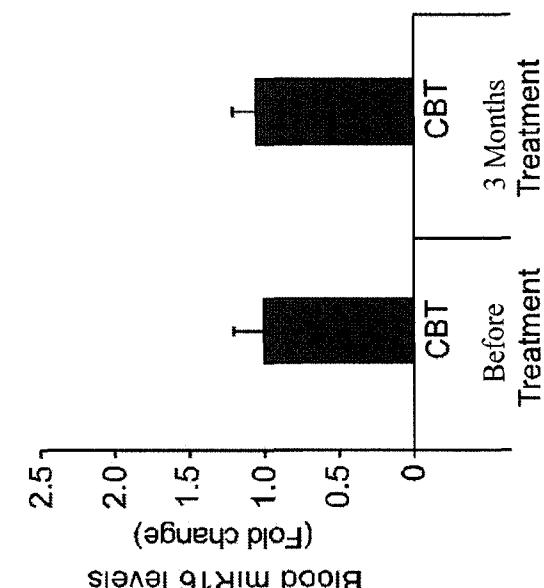
**FIG. 41A****FIG. 41B****FIG. 41C****FIG. 41D**

FIG. 41E

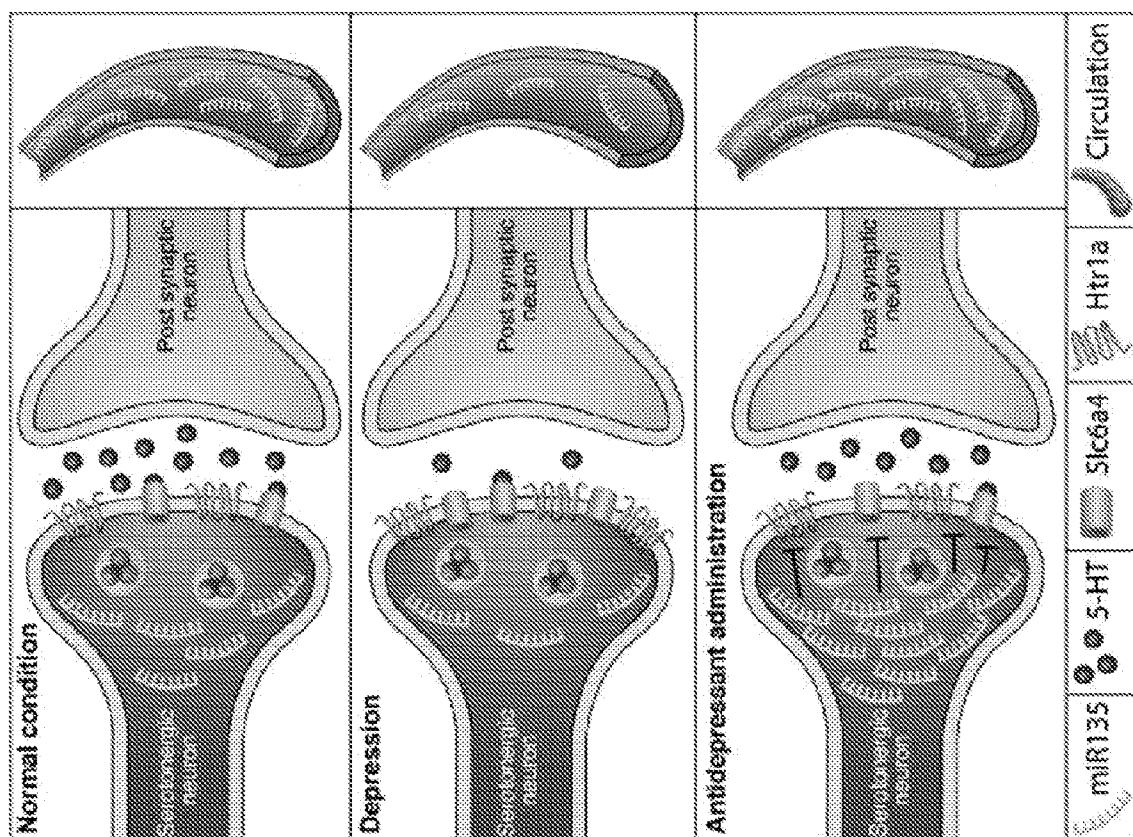


FIG. 42A

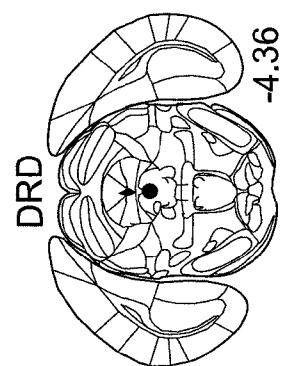


FIG. 42B

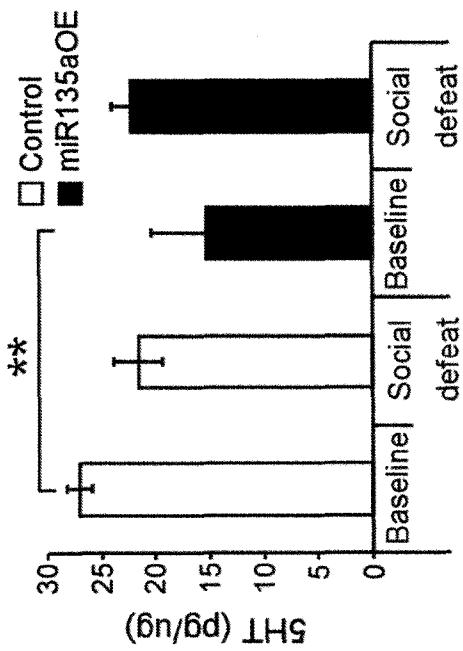


FIG. 42C

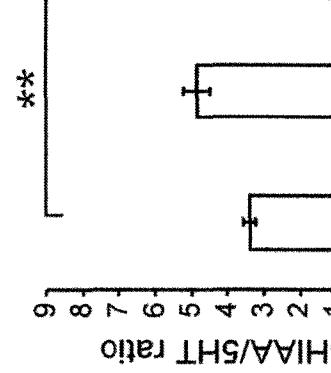
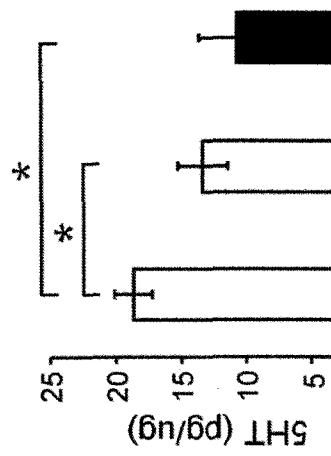
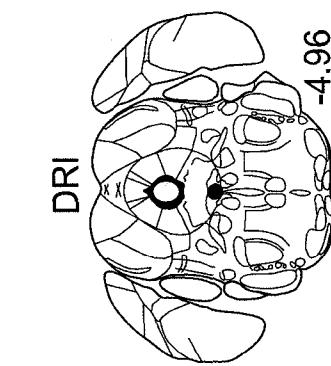
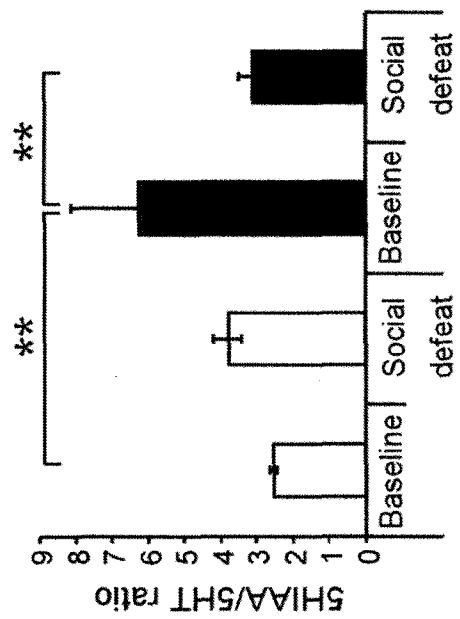


FIG. 42D

FIG. 42E

FIG. 42F

FIG. 42I

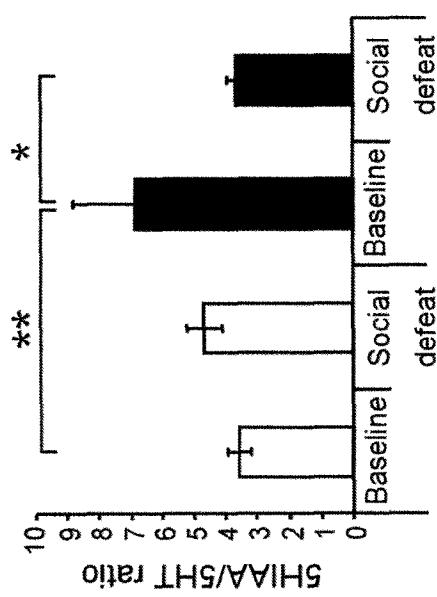


FIG. 42H

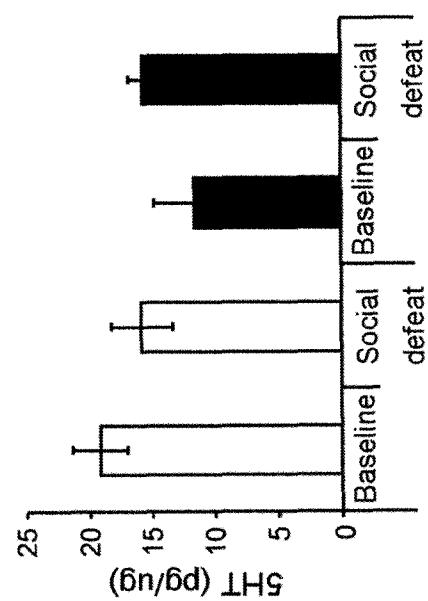


FIG. 42G

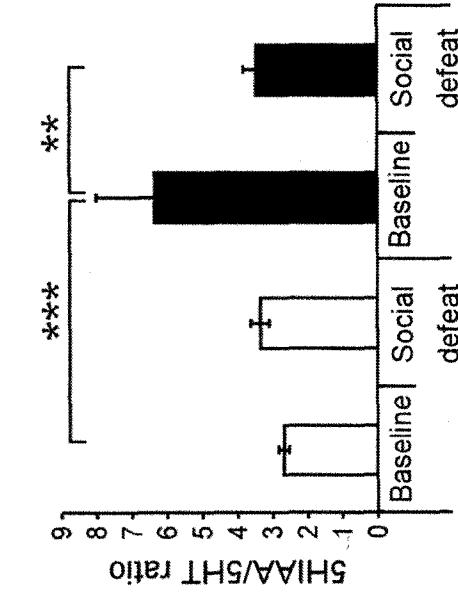
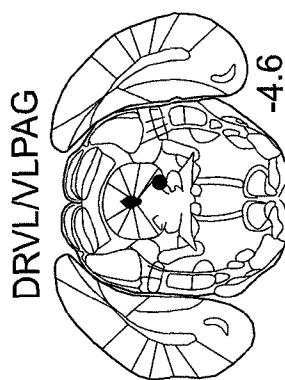


FIG. 42L

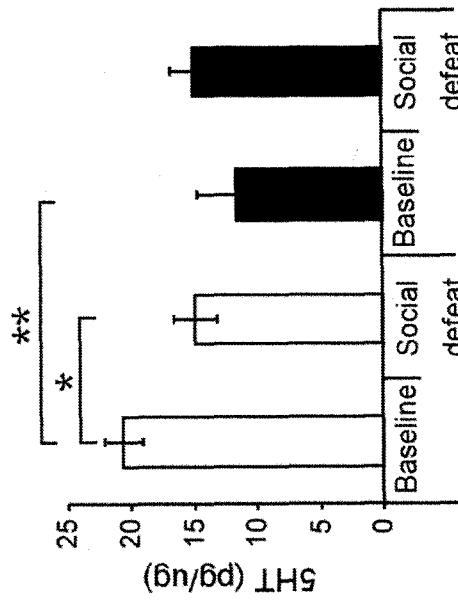


FIG. 42K

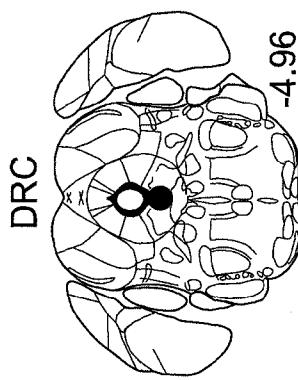


FIG. 42J

FIG. 43C

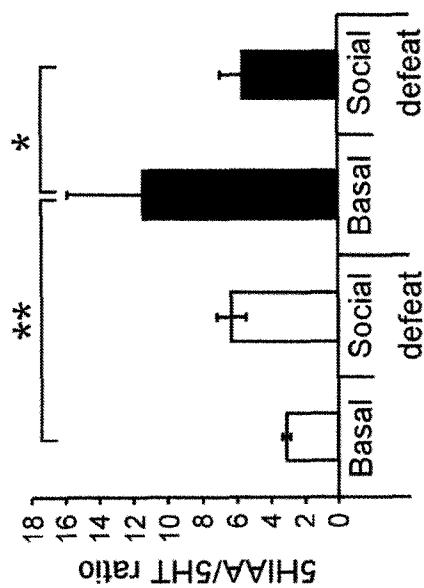


FIG. 43B

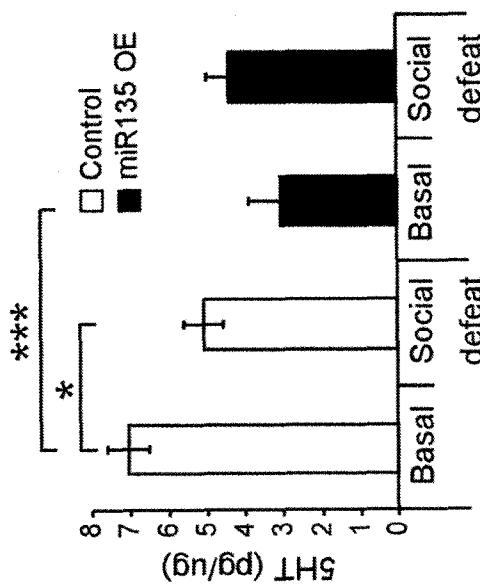


FIG. 43A

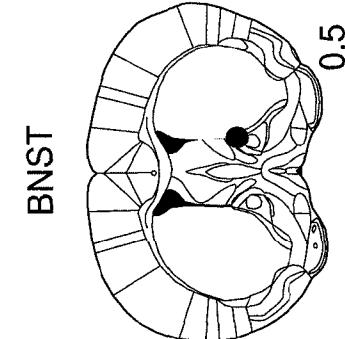
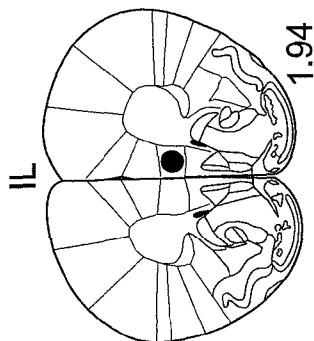


FIG. 43D

FIG. 43F

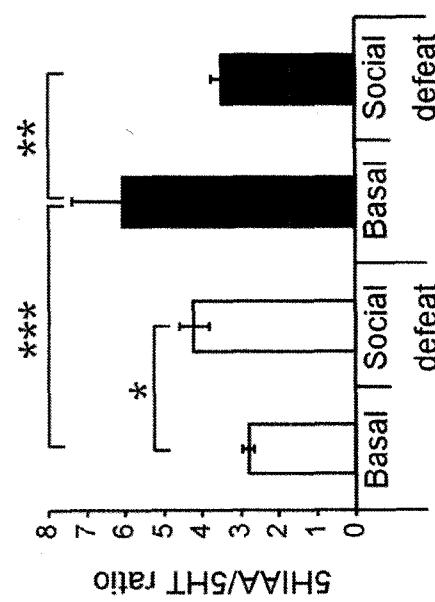


FIG. 43E

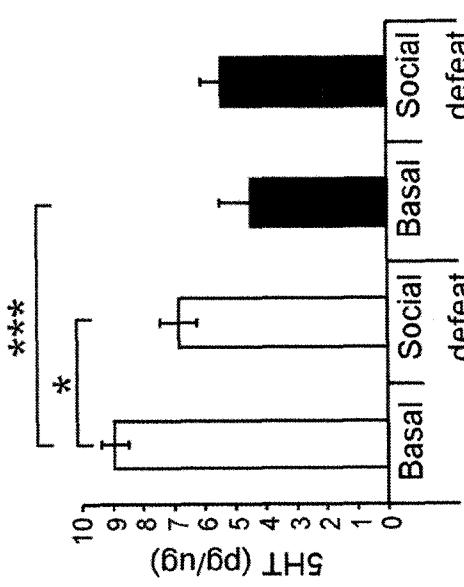


FIG. 43I

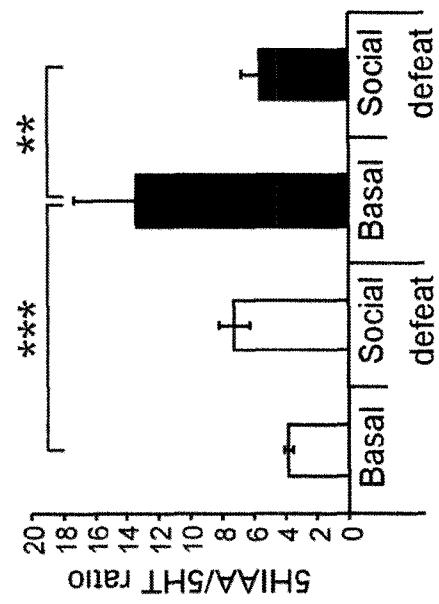
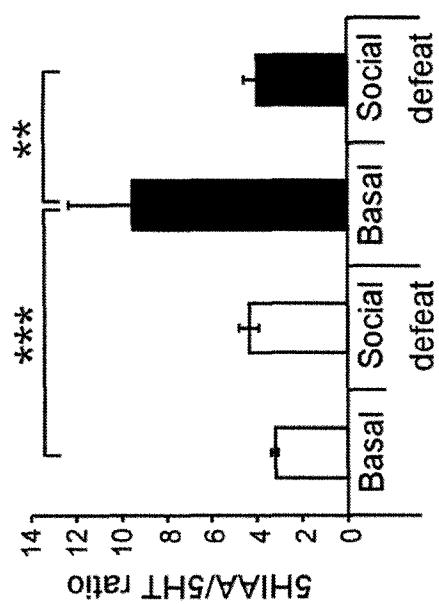


FIG. 43L

FIG. 43H

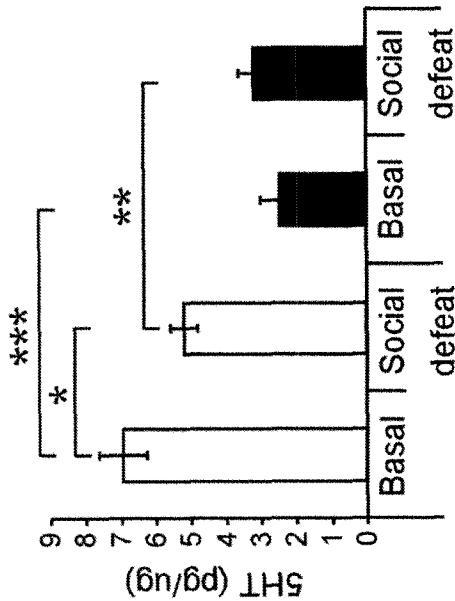
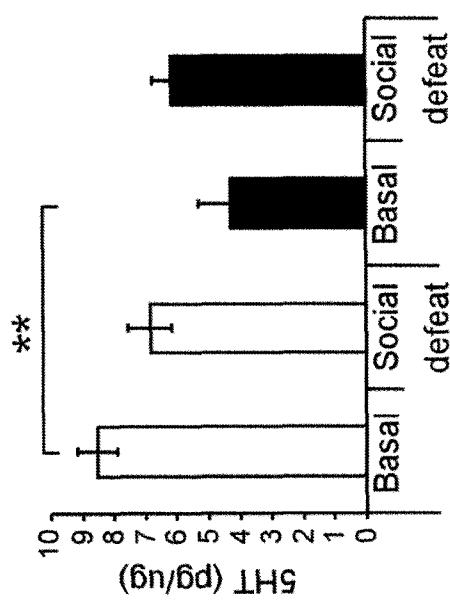


FIG. 43K

FIG. 43G

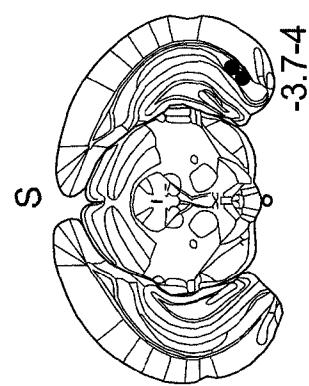
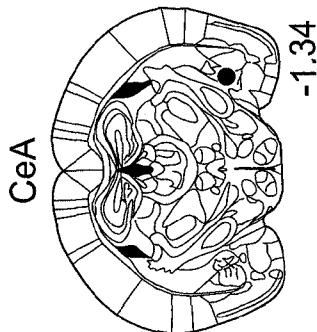


FIG. 43J

FIG. 44

| oligo no    | Sequence   | SEQ ID NO |
|-------------|--|-----------|
| OLG-135-001 | 5'Ph/UAUGGCCUUUUUUUUUUUAUGUGA <u>A</u>                   | 194       |
| OLG-135-002 | <b>U</b> AUAGGGAUUUGGAGGCCGGGG <u>G</u>                  | 195       |
| OLG-135-003 | <b>U</b> CAUAUAGGGAUUUGGAGGCCGG <u>G</u>                 | 196       |
| OLG-135-004 | <u>U</u> CACAUAGGAUUAAAAGCCAU <u>A</u>                   | 197       |
| OLG-135-005 | 5'Ph/UAUGGCCUUUU <u>U</u> UUUCCUAUGUGA <u>A</u>          | 198       |
| OLG-135-006 | <b>U</b> AUAGGGAUU <u>G</u> GGAGGCCGGGG <u>G</u>         | 199       |
| OLG-135-007 | 5'Ph/ <b>U</b> A <u>G</u> GUUUUUUUUUUUCCUAUGUGA <u>A</u> | 200       |
| OLG-135-008 | 5'Ph/ <b>U</b> AA <u>U</u> UUAAAGCUUCUUUGUUCUG <u>G</u>  | 201       |
| OLG-135-009 | <u>C</u> CAGAACAAAGAAGCUUUAAA <u>A</u>                   | 202       |
| OLG-135-010 | 5'Ph/UAUGGCCUUUUCAUUCCUAUGUGA <u>A</u>                   | 203       |
| OLG-135-011 | <u>A</u> UGUAGGGCUAAAAGGCCAU <u>GG</u>                   | 204       |
| OLG-135-012 | <u>U</u> CAUGUAGGGCUAAAAGCCAU <u>G</u>                   | 205       |
| OLG-135-013 | <u>U</u> CACAUAGGAUGAAAAGCCAU <u>A</u>                   | 206       |
| OLG-135-014 | 5'Ph/UAUGGCCUUUU <u>C</u> AUUCCUAUGUGA <u>A</u>          | 207       |
| OLG-135-015 | <u>A</u> UGUAGGG <u>C</u> UUAAAAGGCCAU <u>GG</u>         | 208       |
| OLG-135-016 | 5'Ph/ <b>U</b> A <u>G</u> GUUUUUCAUUCCUAUGUGA <u>A</u>   | 209       |

5'Ph  
**bold underline**  
 5' phosphorylation  
 2' O-Methyl