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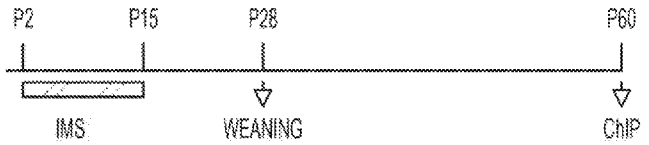

FIG. 1A

(57) Abstract: The present invention relates to methods and treatments for improving cognitive functioning in patients in need thereof. In certain aspects, the methods comprise administering at least one brain-derived neurotrophic factor (Bdnf)-tropomyosine kinase B (TrkB) inhibitor.

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BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)-TROPOMYOSINE KINASE B (TRKB) INHIBITION FOR IMPROVING COGNITIVE DEFICITS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Patent Application Ser. No. 62/241,362 filed October 14, 2015, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 10, 2016, is named 01001-004974-WO0_SL.TXT and is 1,273 bytes in size.

GRANT INFORMATION

This invention was supported in part with government support under NIH grant number R21 MH099251 awarded by National Institutes of Health. The United States Government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to methods and treatments for improving cognitive functioning in patients in need thereof. In certain aspects, the methods comprise administering at least one brain-derived neurotrophic factor (Bdnf)-tropomyosine kinase B (TrkB) inhibitor.

BACKGROUND OF THE INVENTION

Studies showed that histone deacetylase (HDAC) inhibitors can reverse cognitive deficits found in neurodegenerative disorders and age-related memory decline. However, the role of HDACs in stress-induced cognitive deficits has not been investigated. Deficits in executive cognitive functions occur in 20 to 30% of patients with major depression (McIntyre et al., 2013; Millan et al., 2012) as well as in a large subset of patients with unipolar depression (Trivedi and Greer, 2014). Such deficits include widely replicated findings of deficits in working memory (WM) and attention, i.e., deficits that are principal mediators of psychosocial impairment, including impaired workforce performance (McIntyre et al., 2013). The observations that patients in remission from depression still exhibit cognitive deficits, and that the presence of such deficits contributes to relapse, indicates that the cognitive deficits are core deficits rather than an epiphenomenon of depression (Millan et al., 2012;

McIntyre et al., 2013). The causes of these cognitive deficits are not known and, at present, no mechanisms have been identified that could explain their emergence.

Thus, there is an urgent need for therapeutics and treatments for stress-induced and related cognitive deficits.

SUMMARY OF THE INVENTION

In certain embodiments, the present invention relates to a method of treating cognitive defects in a mood disorder in a patient in need thereof, comprising administering a therapeutically effective amount of at least one brain-derived neurotrophic factor (Bdnf)-tropomyosine kinase B (TrkB) inhibitor to the patient.

In additional embodiments, the present invention relates to a method of improving cognitive functioning in a patient in need thereof comprising administering a therapeutically effective amount of at least one brain-derived neurotrophic factor (Bdnf)-tropomyosine kinase B (TrkB) inhibitor to the patient.

In certain embodiments, the treating comprises at least a 10% improvement of cognitive functioning, compared with a baseline cognitive functioning reference or control.

In additional embodiments, the cognitive functioning improvement is greater than about 50%.

In additional embodiments, the TrkB inhibitor is a TkrB antagonist.

In further embodiments, the TrkB inhibitor is administered by mouth, topically, rectally, or intravenously.

In additional embodiments, the mood disorder is an anxiety disorder or stress-induced disorder including early life stress, or a combination thereof.

In additional embodiments, the mood disorder is a panic disorder, an obsessive compulsive disorder, a post-traumatic stress disorder, or any combination thereof.

In additional embodiments, the patient is an adolescent or young adult.

In additional embodiments, the TkrB inhibitor comprises ANA-12.

In additional embodiments, the method further comprises administering at least one selective serotonin reuptake inhibitor (SSRI) selected from the group consisting of Citalopram (Celexa); Escitalopram (Lexapro, Cipralex); Paroxetine (Paxil, Seroxat); Fluoxetine (Prozac); Fluvoxamine (Luvox); Sertraline (Zoloft, Lustral), and combinations thereof.

In certain embodiments, the patient exhibits a mood disorder comprising an anxiety disorder or stress-induced disorder including early life stress, or a combination thereof.

In additional embodiments, the patient exhibits a mood disorder comprising a panic disorder, an obsessive compulsive disorder, a post-traumatic stress disorder, or any combination thereof.

In additional embodiments, the patient is a mammal. In additional embodiments, the patient is a human. In additional embodiments, the patient exhibits a cognitive deficit in one or more of the following features: working memory or attention set-shifting.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-C are diagrams showing the experimental design and dosing timeline for the model mice. Fig. 1A is a timeline showing that mice exclusively used for HDAC1 and HDAC3 ChIPs were exposed to the IMS from P2 and P15, weaned at P28 and raised to adulthood (P60). At P60, their brains were removed. SFR mice raised in parallel served as controls. Fig. 1B is a timeline showing how other groups of mice were chronically treated with either Ana-12 (IMS Balb/c mice) or with MS-275 (SFR Balb/c mice) from postnatal age P35 until P59. At the end of treatment, mice were tested in the ASST followed by training and testing in the WM test. One hour post completion of the WM test at 20 s delay (typically around P84), brains were collected for real-time RT-PCR and ChIP experiments. Age-and sex-matched vehicle-treated mice served as controls. Fig. 1C shows a timeline of the Forster IMS C57Bl/6 or foster IMS Balb/c mice that were raised by Balb/c mothers during IMS exposure, weaned at P28, and tested in the ASST in adulthood (at P66). One hour post completion of the EDS phase of the ASST, brain were collected for real-time PCR and ChIP experiments. Age- and sex-matched non-tested mice served as controls.

Figs. 2A-D are graphs showing reduced HDAC1 levels at promotor III of the *Bdnf* gene in IMS Balb/c mice. Fig. 2A is a graph illustrating comparison of the levels of HDAC1 and HDAC3 at promotors III and IV of the *Bdnf* gene and the promotors of the *Egr2* and *Fos* genes in SFR and IMS Balb/c mice. Data are mean ± sem (n = 8/group for the HDAC3 *Bdnf* PIII ChIP (4 males and 4 females) and n = 6/group for all other groups (3 males and 3 females)) and were compared by Student's t-test. SFR and IMS Balb/c mice differ significantly in their levels of HDAC1 associated with *Bdnf* promotor III. Fig. 2B shows Bdnf transcript variant III expression in non-tested and WM-tested (20 s delay) SFR and IMS Balb/c mice. Data (mean ± sem; n = 6/group) were compared by 2-way ANOVA (see Results) and statistical differences were resolved post hoc (Tukey HSD test) as indicated. Fig. 2C shows a comparison of the levels of Pol II association at *Bdnf* promotor III between non-tested and WM-tested IMS Balb/c mice. Data (mean ± sem; n = 6/group) were compared using two-tailed Student's t test. Fig. 2D shows Egr2 mRNA expression levels in non-tested

and WM-tested SFR and IMS Balb/c mice. Data (mean ± sem; n = 6/group) were compared by 2-way ANOVA that revealed no significant differences.

Figs. 3A-B are graphs showing the effects of adolescent ANA-12 treatment of IMS and SFR Balb/c mice on cognitive task performance in adulthood. Fig. 3A is a graph showing performance of SFR, IMS and Ana-12-treated SFR and IMS Balb/c mice in the ASST. Data are mean ± sem of 8 animals per group (4 males and 4 females) and were compared by 2-way ANOVA (see Results). Statistical differences were resolved post hoc (Tukey HSD tests) as indicated. Compared to SFR mice, IMS Balb/c mice exhibited significant deficits in the EDS phase of the ASST, a deficits that is abolished in Ana-12-treated IMS mice. In contrast, Ana-12 treatment of SFR Balb/c mice led to the same deficits found in IMS mice. Fig. 3B is a graph illustrating the performance of the same groups of mice in the WM test. Data were compared by 2-way ANOVA (see Results) and significant differences resolved post hoc (Tukey HSD tests) are indicated. When tested at 20 s inter-trial delays, the percentages of correct arm entries of IMS Balb/c and Ana-12-treated SFR mice were significantly lower compared to non-treated SFR and Ana-12-treated IMS mice.

Figs. 4A-D are graphs illustrating the effect of adolescent MS-275 treatment of SFR Balb/c mice on cognitive task performance and expression of Bdnf and Egr2 mRNA. Fig. 4A is a graph quantifying the performance of SFR Balb/c mice treated with MS-275 in the ASST. Data are mean ± sem (n = 8/group). Significant differences between non-treated and MS-275-treated SFR mice were found only for the EDS phase of the ASST (Student's t test). Fig. 4B is a graph illustrating the performance of the same group of mice shown in Fig. 4A in the spatial WM test. At 20 s delay, significant differences were found between the two groups (Student's t test). Fig. 4C is a graph of the real-time RT-PCR measures of Bdnf transcript variant III expression. Data are mean ± sem (n = 6/group). Significant differences revealed by one-way ANOVA ($F(2,16) = 4.56$; $p = 0.02$) were resolved post hoc (Tukey-Kramer multiple comparisons test) only for tested MS-275-treated mice that exhibited significantly increased expression of Bdnf mRNA. Fig. 4D is a graph for the same group of mice shown in Fig. 4C, quantifying the real-time RT-PCR measures of Egr2 mRNA and revealed no significant differences between the three groups (ANOVA, $F(2,16) = 0.5$; $p = 0.62$).

Figs. 5A-B are graphs showing that Foster IMS C57Bl/6 mice have deficits in the ASST and reduced HDAC1 levels at the promotor of the *Egr2* gene. Fig. 5A is a graph illustrating ASST performance of SFR and IMS C57Bl/6 mice raised by their biological mothers and foster IMS C57Bl/6mice raised by Balb/c mothers. Data are mean ± sem (n=7/group) and were compared by 2-way ANOVA (see Examples). Statistical differences

were resolved post hoc (Tukey HSD tests) as indicated. Fig. 5B is a graph comparing the levels of HDAC1 and HDAC3 at promotors III and IV of the *Bdnf* gene and the promotors of the *Egr2* and *Fos* genes in SFR and IMS C57Bl/6 mice raised by their biological mothers and foster IMS C57l/6 mice raised by Balb/c mothers. Data are mean ± sem (n=6/group). One-way ANOVA revealed significant differences between groups only for the levels of HDAC1 at the *Egr2* gene promotor ($F(2,14)=9.12$; $p=0.005$). Post hoc Tukey-Kramer multiple comparisons resolved these differences for foster IMS C57Bl/5 mice that had significantly lower levels of HDAC1 compared to SFR and IMS C57Bl/6 mice.

Figs. 6A-D are graphs showing that Forster IMS C57Bl/6 mice exhibit increased Egr2 mRNA expression after ASST testing and increased histone H3 acetylation at the *Egr2* promotor. Fig. 6A is a comparison of Bdnf transcript variant III and Fig. 6B shows Egr2 mRNA levels between non-tested and ASST-tested foster IMS C57Bl/6 and Balb/c mice. Data (mean ± sem; n=6/group) were compared by 2-way ANOVA (see Results). Significant differences resolved post hoc (Tukey HSD tests) are indicated. Whereas foster IMS Balb/c mice exhibited increased Bdnf mRNA expression after ASST testing, ASST tested foster IMS C57Bl/6 mice expressed significantly higher levels of Egr2 mRNA. *$p < 0.05$ in (Fig. 6A) and $p < 0.01$ in (Fig. 6B). Fig. 6C shows the comparison of the levels of Pol II association with the *Egr2* promotor between non-tested and ASST-tested foster IMS C57Bl/5 mice. Data (mean ± sem; n = 6/group) were compared using two-tailed Student's t test. Fig. 6D shows the ratio of acH3K9 over H3K9me3 levels associated with the *Egr2* promotor of SFR and IMS C57Bl/6 and Balb/c mice compared to foster IMS C57Bl/6 mice. Data are mean ± sem (n = 5/group). One-way ANOVA revealed significant differences between groups ($F(4,19) = 3.9$; $p = 0.02$) that were resolved post hoc for foster IMS C57Bl/6 mice that exhibited a significantly higher acH3K9/H3K9me3 ratio. *$p < 0.05$ compared to all other groups.

DETAILED DESCRIPTION

In the stress-susceptible mouse strain Balb/c, early life stress triggers a persistent decrease in HDAC expression in the forebrain neocortex, including reduced expression of class I HDACs. The same mice show pronounced cognitive deficits in adulthood, namely deficits in working memory and attention set-shifting. The present data illustrate that these mice also exhibit reduced association of HDAC1 with promotor III of the brain-derived neurotrophic factor (*Bdnf*) gene, and that cognitive testing leads to abnormally increased Bdnf mRNA expression. A pharmacological reduction of Bdnf-tropomyosine kinase B receptor signaling effectively reverses the cognitive deficits, indicating that enhanced

transcriptional activation of the *Bdnf* gene contributes to their emergence. In contrast to Balb/c mice, C57Bl/6 mice only develop attention set-shifting deficits when raised by Balb/c foster mothers during the time the pups are exposed to early life stress. HDAC1 levels at *Bdnf* promotor III are unaltered in such C57Bl/6 mice, although they exhibit decreased levels of HDAC1 at the promotor of the early-growth response gene 2 (*Egr2*) and abnormally increased Egr2 mRNA expression after cognitive testing. Hence, contrary to the beneficial effects of HDAC inhibition in neurodegenerative diseases, the reduced HDAC1 levels at promotors of distinct plasticity-associated genes predispose animals exposed to early life stress to enhanced expression of these genes upon cognitive challenge, an effect that negatively influences cognitive task performance.

One prominent risk factor for depression is early life stress (ELS) as described in Pechtel and Pizzagalli, 2011. Mouse models have been utilized as correlating with human developmental learning, and models of human emotional behavior for many years (*See* Millan, M. J. 2008 and Schmauss et al., 2014). For example, P1-P28 (mouse pup day 1-mouse pup day 28) mice correspond developmentally to early human childhood, with P21-P28 as the typical weaning time for the mouse pups. Mice beyond P28 to P60 correspond to human adolescence (*See* Millan, M. J. 2008). Mice that are beyond P60 (mouse pup day 60), and in particular beyond P66 are considered to correspond to human adults.

Numerous studies on animal models of early life stress have demonstrated persistent changes in emotional behavior that are also found in human depression. For example, in the inbred mouse strain Balb/c a widely employed rodent paradigm of early life stress, infant maternal separation (IMS), elicits not only increased anxiety and depression-like behavior in adulthood, but also pronounced deficits in spatial WM and attention set-shifting (Mehta and Schmauss, 2011). Moreover, several of the gene expression changes found in IMS Balb/c mice are found in the forebrain neocortex, and they are attributable to a histone-based epigenetic response to IMS exposure, namely increased acetylation of histone H4 protein that is due to decreased activity of several class I/II HDACs, including class I HDACs 1 and 3 (Levine et al., 2012). This epigenetic phenotype emerges during adolescent development and persists into adulthood. While it ameliorates the severity of the emotional phenotype in adulthood (Levine et al., 2012) and increases responsiveness to antidepressant treatment (Schmauss, 2015), only a pharmacological treatment that activated class I HDACs 1 and 3 during adolescent development of IMS Balb/c mice was able to abolish their cognitive deficits (Schmauss et al., 2014). This suggests that reduced HDAC1 and/or HDAC3 activity contributes to the emergence of cognitive deficits, a hypothesis tested in the present study.

Contrary to the pronounced deficits in WM and attention set shifting found in IMS Balb/c mice, these cognitive functions are unaffected after IMS exposure of the more resilient strain C57Bl/6 (Mehta and Schmauss, 2011). However, C57Bl/6 foster pups raised by Balb/c mothers during IMS exposure also develop deficits in attention set-shifting, but no deficits in WM (Schmauss et al., 2014). Whether or not the comparatively milder cognitive deficits of foster IMS C57Bl/6 mice are also functionally related to reduced HDAC1 and/or HDAC3 activity, is another hypothesis addressed in the present study.

The data provided herein illustrate that both IMS Balb/c mice and foster IMS C57Bl/6 mice exhibit reduced HDAC1 association with promotors of distinct plasticity-associated genes, and that cognitive test exposure triggers an abnormally increased expression of these genes. However, the affected genes differed between the two strains: In IMS Balb/c mice, reduced HDAC1 levels are found on promotor III of the *Bdnf* gene, and increased expression Bdnf and hence, increased Bdnf-tropomyosine kinase B (TrkB) receptor signaling can explain the cognitive phenotype of IMS Balb/c mice. Moreover, a pharmacological reduction of HDAC1 activity during adolescent development of non-stressed Balb/c mice precisely recapitulates this effect. In contrast, in IMS C57Bl/6 mice fostered by Balb/c mothers, reduced HDAC1 levels were detected at the promotor of the early growth response gene 2 (*Egr2*), and cognitive task exposure also enhanced the expression of the *Egr2* gene above normal levels. Hence, while reduced HDAC1 association at promotors of distinct plasticity-associated genes is a common denominator contributing to early-life stress-triggered cognitive deficits, the genes affected are determined by the genetic backgrounds.

The present data using validated mouse models for these early stress conditions and corresponding cognitive deficits illustrate that in certain embodiments pharmacological treatment of a patient with a TrkB inhibitor such as ANA-12 will lead to improvement of the cognitive deficits and improvement in cognitive functioning (executive cognitive functions such as working memory and attention set-shifting), which may also serve to benefit certain emotional phenotypes or conditions such as MDD (major depressive disorder) without any additional pharmacological intervention. In certain alternative instances, it is expected that in addition to treating a patient with a TrkB inhibitor such as ANA-12, that treating with one or more SSRI (including any combination of: Citalopram (Celexa); Escitalopram (Lexapro, Cipralex); Paroxetine (Paxil, Seroxat); Fluoxetine (Prozac); Fluvoxamine (Luvox); Sertraline (Zoloft, Lustral)) would result in improvement to both the cognitive deficits and emotional phenotype e.g., depressive condition/s as described herein.

Abbreviations

ASS= attention set-shifting;

ASST= attention set-shifting test;

CD= compound discrimination;

EDS=extradimensional shift of attention;

ELS= early life stress (for example events during childhood that exceed the child's coping resources including physical, sexual, emotional and verbal abuse, neglect, social deprivation, disaster, household dysfunctions such as witnessing violence, criminal activity, parental separation parental death or illness, poverty, and substance abuse. See, Pechtel and Pizzagalli 2011);

IDS= intradimensional shift of attention;

IMS= infant maternal separation;

MDD= major depressive disorder;

SD=simple discrimination, for example between odor (scented terra cotta pots) or texture (different digging media);

WM = working memory.

TrkB Inhibitors

ANA-12 is a selective, small-molecule non-competitive antagonist of TrkB (Kd = 10 nM and 12 μM for the high- and low-affinity sites, respectively), the main receptor of brain-derived neurotrophic factor (BDNF/Bdnf) (Cazorla 2011). The compound crosses the blood-brain-barrier and exerts central TrkB blockade, producing effects as early as 30 minutes (~400 nM) and as long as 6 hours (~10 nM) following intraperitoneal injection in mice (Cazorla 2011). It blocks the neurotrophic actions of BDNF without compromising neuron survival (Cazorla 2011). ANA-12 produces rapid antidepressant- and anxiolytic-like effects in animal models, the former of which have been elucidated to be mediated by blockade of BDNF signaling in the nucleus accumbens. (Zhang JC 2015 and Shirayama Y 2015).

MS-275 is also known as Entinostat, or SNDX-275 and is a benzamide histone deacetylase inhibitor undergoing clinical trials for treatment of various cancers. Entinostat inhibits class I HDAC1 and HDAC3 with IC_{50} of 0.51 μM and 1.7 μM, respectively. It has the formula: $C_{21}H_{20}N_4O_3$.

Examples of additional exemplary TrkB inhibitor compounds include LOX0-101; N-T04; AZ623 and Entrectinib. It is expected that any TrkB inhibitor that can penetrate the blood brain barrier will be suitable for the methods described herein. For example, LOX0-101 is expected to be a suitable inhibitor and is described in: Cancer Discovery 5:1049-1057,

2015. Robert C. Doebele *et al.* An Oncogenic NTRK Fusion in a Patient with Soft-Tissue Sarcoma with Response to the Tropomyosin-Related Kinase Fusion Inhibitor LOXO-101.

The term "effective amount" of a compound is a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, for example, an amount which results in the alleviation, prevention of, or a decrease in the symptoms associated with a disease that is being treated.

"Activation," "stimulation," and "treatment," as it applies to cells or to receptors, may have the same meaning, e.g., activation, stimulation, or treatment of a cell or receptor with a ligand, unless indicated otherwise by the context or explicitly. "Ligand" encompasses natural and synthetic ligands, e.g., cytokines, cytokine variants, analogues, muteins, and binding compounds derived from antibodies. "Ligand" also encompasses small molecules, e.g., peptide mimetics of cytokines and peptide mimetics of antibodies. "Activation" can refer to cell activation as regulated by internal mechanisms as well as by external or environmental factors. "Response," e.g., of a cell, tissue, organ, or organism, encompasses a change in biochemical or physiological behavior, e.g., concentration, density, adhesion, or migration within a biological compartment, rate of gene expression, or state of differentiation, where the change is correlated with activation, stimulation, or treatment, or with internal mechanisms such as genetic programming.

"Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity; to the ability to stimulate gene expression or cell signaling, differentiation, or maturation; to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" can also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], concentration in a biological compartment, or the like. "Activity" may refer to modulation of components of the innate or the adaptive immune systems.

"Homology" refers to sequence similarity between two polynucleotide sequences or between two polypeptide sequences when they are optimally aligned. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology is the number of homologous positions shared by the two sequences divided by the total number of positions compared ×100. For example, if 6 of 10 of the positions in two sequences are matched or

homologous when the sequences are optimally aligned then the two sequences are 60% homologous. Generally, the comparison is made when two sequences are aligned to give maximum percent homology.

"Isolated nucleic acid molecule" means a DNA or RNA of genomic, mRNA, cDNA, or synthetic origin or some combination thereof which is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, or is linked to a polynucleotide to which it is not linked in nature. For purposes of this disclosure, it should be understood that "a nucleic acid molecule comprising" a particular nucleotide sequence does not encompass intact chromosomes. Isolated nucleic acid molecules "comprising" specified nucleic acid sequences may include, in addition to the specified sequences, coding sequences for up to ten or even up to twenty or more other proteins or portions or fragments thereof, or may include operably linked regulatory sequences that control expression of the coding region of the recited nucleic acid sequences, and/or may include vector sequences.

The phrase "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to use promoters, polyadenylation signals, and enhancers.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that not all progeny will have precisely identical DNA content, due to deliberate or inadvertent mutations. Mutant

progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

As used herein, "polymerase chain reaction" or "PCR" refers to a procedure or technique in which specific nucleic acid sequences, RNA and/or DNA, are amplified as described in, *e.g.*, U.S. Pat. No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is used to design oligonucleotide primers. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers can coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. *See generally* Mullis *et al.* (1987) *Cold Spring Harbor Symp. Quant. Biol.* 51:263; Erlich, ed., (1989) PCR TECHNOLOGY (Stockton Press, N.Y.) As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

With respect to cells, the term "isolated" refers to a cell that has been isolated from its natural environment (e.g., from a tissue or subject). The term "cell line" refers to a population of cells capable of continuous or prolonged growth and division in vitro. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants. As used herein, the terms "recombinant cell" refers to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active polypeptide or production of a biologically active nucleic acid such as an RNA, has been introduced.

The term "vector" includes any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. In some embodiments, useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic

machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "operatively linked," "under control," or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In some embodiments, expression includes transcription of the nucleic acid, for example, to generate a biologically-active polypeptide product or inhibitory RNA (e.g., shRNA, miRNA) from a transcribed gene.

"Inhibitors" and "antagonists," or "activators" and "agonists," refer to inhibitory or activating molecules, respectively, e.g., for the activation of, e.g., a ligand, receptor, cofactor, a gene, cell, tissue, or organ. A modulator of, e.g., a gene, a receptor, a ligand, or a cell, is a molecule that alters an activity of the gene, receptor, ligand, or cell, where activity can be activated, inhibited, or altered in its regulatory properties. The modulator may act alone, or it may use a cofactor, e.g., a protein, metal ion, or small molecule. Inhibitors are compounds that decrease, block, prevent, delay activation, inactivate, desensitize, or down regulate, e.g., a gene, protein, ligand, receptor, or cell. Activators are compounds that increase, activate, facilitate, enhance activation, sensitize, or up regulate, e.g., a gene, protein, ligand, receptor, or cell. An inhibitor may also be defined as a compound that reduces, blocks, or inactivates a constitutive activity. An "agonist" is a compound that interacts with a target to cause or promote an increase in the activation of the target. An "antagonist" is a compound that opposes the actions of an agonist. An antagonist prevents, reduces, inhibits, or neutralizes the activity of an agonist. An antagonist can also prevent, inhibit, or reduce constitutive activity of a target, e.g., a target receptor, even where there is no identified agonist.

To examine the extent of inhibition, for example, samples or assays comprising a given, e.g., protein, gene, cell, or organism, are treated with a potential activator or inhibitor and are compared to control samples without the inhibitor. Control samples, i.e., samples not treated with antagonist, are assigned a relative activity value of 100%. Inhibition is achieved when the activity value relative to the control is about 90% or less, typically 85% or less, more typically 80% or less, most typically 75% or less, generally 70% or less, more generally 65% or less, most generally 60% or less, typically 55% or less, usually 50% or less, more usually 45% or less, most usually 40% or less, preferably 35% or less, more preferably 30% or less, still more preferably 25% or less, and most preferably less than 25%. Activation is achieved when the activity value relative to the control is about 110%, generally at least

120%, more generally at least 140%, more generally at least 160%, often at least 180%, more often at least 2-fold, most often at least 2.5-fold, usually at least 5-fold, more usually at least 10-fold, preferably at least 20-fold, more preferably at least 40-fold, and most preferably over 40-fold higher.

Endpoints in activation or inhibition can be monitored as follows. Activation, inhibition, and response to treatment, e.g., of a cell, physiological fluid, tissue, organ, and animal or human subject, can be monitored by an endpoint. The endpoint may comprise a predetermined quantity or percentage of, e.g., indicia of inflammation, oncogenicity, or cell degranulation or secretion, such as the release of a cytokine, toxic oxygen, or a protease. The endpoint may comprise, e.g., a predetermined quantity of ion flux or transport; cell migration; cell adhesion; cell proliferation; potential for metastasis; cell differentiation; and change in phenotype, e.g., change in expression of gene relating to inflammation, apoptosis, transformation, cell cycle, or metastasis (see, e.g., Knight (2000) *Ann. Clin. Lab. Sci.* 30:145-158; Hood and Cheresh (2002) *Nature Rev. Cancer* 2:91-100; Timme, *et al.* (2003) *Curr. Drug Targets* 4:251-261; Robbins and Itzkowitz (2002) *Med. Clin. North Am.* 86:1467-1495; Grady and Markowitz (2002) *Annu. Rev. Genomics Hum. Genet.* 3:101-128; Bauer, *et al.* (2001) *Glia* 36:235-243; Stanimirovic and Satoh (2000) *Brain Pathol.* 10:113-126).

An endpoint of inhibition is generally 75% of the control or less, preferably 50% of the control or less, more preferably 25% of the control or less, and most preferably 10% of the control or less. Generally, an endpoint of activation is at least 150% the control, preferably at least two times the control, more preferably at least four times the control, and most preferably at least ten times the control.

"Small molecule" is defined as a molecule with a molecular weight that is less than 10 kDa, typically less than 2 kDa, preferably less than 1 kDa, and most preferably less than about 500 Da. Small molecules include, but are not limited to, inorganic molecules, organic molecules, organic molecules containing an inorganic component, molecules comprising a radioactive atom, synthetic molecules, peptide mimetics, and antibody mimetics. As a therapeutic, a small molecule may be more permeable to cells, less susceptible to degradation, and less apt to elicit an immune response than large molecules. Small molecules, such as peptide mimetics of antibodies and cytokines, as well as small molecule toxins, have been described (see, e.g., Casset, *et al.* (2003) *Biochem. Biophys. Res. Commun.* 307:198-205; Muyldermans (2001) *J. Biotechnol.* 74:277-302; Li (2000) *Nat. Biotechnol.* 18:1251-1256; Apostolopoulos, *et al.* (2002) *Curr. Med. Chem.* 9:411-420; Monfardini, *et al.* (2002) *Curr. Pharm. Des.* 8:2185-2199; Domingues, *et al.* (1999) *Nat. Struct. Biol.* 6:652-

656; Sato and Sone (2003) *Biochem. J.* 371:603-608; U.S. Patent No. 6,326,482 issued to Stewart, *et al*).

"Treat" or "treating" refers to administering a therapeutic agent, such as a composition containing a TrkB inhibitor (e.g., ANA-12) or combinations with one or more SSRI inhibitor (e.g., Citalopram (Celexa); Escitalopram (Lexapro, Cipralex); Paroxetine (Paxil, Seroxat); Fluoxetine (Prozac); Fluvoxamine (Luvox); Sertraline (Zoloft, Lustral), or similar compositions described herein, internally or externally to a subject or patient having one or more disease symptoms, or being suspected of having a disease or being at elevated at risk of acquiring a disease, for which the agent has therapeutic activity. Typically, the agent is administered in an amount effective to alleviate one or more disease symptoms in the treated subject or population, whether by inducing the regression of or inhibiting the progression of such symptom(s) by any clinically measurable degree. The amount of a therapeutic agent that is effective to alleviate any particular disease symptom (also referred to as the "therapeutically effective amount") may vary according to factors such as the disease state, age, and weight of the patient, and the ability of the drug to elicit a desired response in the subject Whether a disease symptom has been alleviated can be assessed by any clinical measurement typically used by physicians or other skilled healthcare providers to assess the severity or progression status of that symptom. While an embodiment of the present invention (e.g., a treatment method or article of manufacture) may not be effective in alleviating the target disease symptom(s) in every subject, it should alleviate the target disease symptom(s) in a statistically significant number of subjects as determined by any statistical test known in the art such as the Student's t-test, the chi^2-test, the U-test according to Mann and Whitney, the Kruskal-Wallis test (H-test), Jonckheere-Terpstra-test and the Wilcoxon-test.

"Treatment," as it applies to a human, veterinary, or research subject, refers to therapeutic treatment, prophylactic or preventative measures, to research and diagnostic applications. "Treatment" as it applies to a human, veterinary, or research subject, or cell, tissue, or organ, encompasses combination treatments including: one or more TrkB inhibitors, optionally in combination with one or more SSRI inhibitor (described herein), or related methods described herein as applied to a human or animal subject, a cell, tissue, physiological compartment, or physiological fluid.

Pharmaceutical Compositions and Administration

Formulations of therapeutic and diagnostic agents may be prepared by mixing with acceptable carriers, excipients, or stabilizers in the form of, *e.g.*, lyophilized powders, slurries, aqueous solutions or suspensions (see, *e.g.*, Hardman, *et al.* (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, NY; Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, Lippincott, Williams, and Wilkins, New York, NY; Avis, *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Marcel Dekker, NY; Weiner and Kotkoskie (2000) *Excipient Toxicity and Safety*, Marcel Dekker, Inc., New York, NY).

Toxicity and therapeutic efficacy of the therapeutic compositions, administered alone or in combination with another agent, can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index (LD_{50}/ ED_{50}). In particular aspects, therapeutic compositions exhibiting high therapeutic indices are desirable. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration.

In an embodiment of the invention, a composition of the invention is administered to a subject in accordance with the Physicians' Desk Reference 2003 (Thomson Healthcare; 57th edition (November 1, 2002)).

The mode of administration can vary. Suitable routes of administration include oral, rectal, transmucosal, intestinal, parenteral; intramuscular, subcutaneous, intradermal, intramedullary, intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, intraocular, inhalation, insufflation, topical, cutaneous, transdermal, or intra-arterial.

In particular embodiments, the composition or therapeutic can be administered by an invasive route such as by injection (see above). In further embodiments of the invention, the composition, therapeutic, or pharmaceutical composition thereof, is administered intravenously, subcutaneously, intramuscularly, intraarterially, intra-articularly (e.g. in arthritis joints), intratumorally, or by inhalation, aerosol delivery. Administration by non-

invasive routes (*e.g.*, orally; for example, in a pill, capsule or tablet) is also within the scope of the present invention.

Compositions can be administered with medical devices known in the art. For example, a pharmaceutical composition of the invention can be administered by injection with a hypodermic needle, including, e.g., a prefilled syringe or autoinjector.

The pharmaceutical compositions of the invention may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 6,620,135; 6,096,002; 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

As used herein, "inhibit" or "treat" or "treatment" includes a postponement of development of the symptoms associated with a disorder and/or a reduction in the severity of the symptoms of such disorder. The terms further include ameliorating existing uncontrolled or unwanted symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result has been conferred on a vertebrate subject with a disorder, disease or symptom, or with the potential to develop such a disorder, disease or symptom.

In certain embodiments, the present methods are suitable for treating adolescent or young adult patients (with typical age ranges from 10-22; but including those as young as 5-10). In certain embodiments, patients in need of treatment will include those that have experienced ELS (early life stress, as described for example in Pechtel and Pizzagalli 2011). In certain embodiments, it is expected that the patient will exhibit a cognitive deficit in one or more of the following features: working memory and attention set-shifting.

In additional embodiments, it is expected that the patient will exhibit improvement in at least one of these features including working memory and attention set-shifting, during and following treatment with an effective amount of at least one TrkB inhibitor, when compared to normal baseline or a control patient value. Additionally, in certain instances and based on the present results in the model data, treatment should continue until improvements are observed, at which time treatment can be stopped. The benefits of the treatment are expected to endure following treatment, as shown in the model data provided herein.

In certain embodiments, patients in need of the present treatments will also benefit from receiving an effective amount of one or more of a selective serotonin reuptake inhibitor (SSRI's), such as one or more of the following:

- Citalopram (Celexa)
- Escitalopram (Lexapro, Cipralex)

- Paroxetine (Paxil, Seroxat)
- Fluoxetine (Prozac)
- Fluvoxamine (Luvox)
- Sertraline (Zoloft, Lustral)

In certain embodiments, the TrkB inhibitor (e.g. ANA-12) and desired SSRI may be combined into one formulation for ease of patient delivery and compliance.

The pharmaceutical compositions may also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991).

Kits

The present invention also provides kits comprising the components of the combinations of the invention in kit form. A kit of the present invention includes one or more components including, but not limited to, one or more TrkB inhibiting compounds (such as Ana-12) as discussed herein, in association with one or more additional components including, but not limited to a pharmaceutically acceptable carrier and/or an SSRI agent, as discussed herein. Kits may also include primers, buffers, and probes along with instructions for determining elevated levels of nucleic acid, proteins, or protein fragments of a desired target.

In one embodiment, a kit includes additional compounds/composition of the invention or a pharmaceutical composition thereof in one container (*e.g.*, in a sterile glass or plastic vial) and a second pharmaceutical composition in another container (*e.g.*, in a sterile glass or plastic vial).

In another embodiment, the kit comprises a combination of the invention, including one or more TrkB inhibitors (e.g. ANA-12) in combination with one or more SSRI inhibitors (as described herein) along with a pharmaceutically acceptable carrier, optionally in

combination with one or more additional therapeutic agent components formulated together, optionally, in a pharmaceutical composition, in a single, common container.

If the kit includes a pharmaceutical composition for parenteral administration to a subject, the kit can include a device for performing such administration. For example, the kit can include one or more hypodermic needles or other injection devices as discussed above. The kit can include a package insert including information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding a combination of the invention may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references, manufacturer/distributor information and patent information.

EXAMPLES

Example 1. Reduced levels of HDAC1 at Bdnf promotor III and increased Bdnf mRNA expression in IMS Balb/c mice exposed to cognitive tests.

The finding that activation of HDAC1 and HDAC3 during adolescent development abolished the WM and attention set-shifting deficits of IMS Balb/c mice (Schmauss et al., 2014) suggests that reduced activity of these HDACs is functionally linked to the emergence of these deficits. To test this, we first performed chromatin immunoprecipitation (ChIP) experiments to compare the levels of HDAC1 and HDAC3 at promotors of selected plasticity-associated genes between standard facility-reared (SFR) Balb/c controls and IMS Balb/c mice at postnatal age P60 (see Fig. 1A). To this end, we focused on the promotors of the *Bdnf* gene because of the proposed role of Bdnf expression in modulating synaptic plasticity in the prefrontal cortex (PFC), the anatomic regions that governs the cognitive functions affected by early life stress exposure (Lewis et al., 2005). We further targeted the promotors of two immediate-early genes that encode transcription factors known to participate in the modulation of synaptic plasticity in response to cognitive challenges. One of the targeted genes is *Egr2*. It was previously shown with immunocytochemical studies on C57Bl/6 mice performing an attention-set shifting task (ASST) that Egr2 immunoreactivity was specifically increased in the ventrolateral orbital frontal cortex and the pre- and infralimbic subregions of the medial prefrontal cortex (mPFC) during the associative learning phases of the ASST, and that infralimbic Egr2 expression further increased when mice performed the set-shifting phases of the ASST (DeSteno and Schmauss, 2008). In contrast to

ASST-tested mice, mice that performed a spatial WM test did not exhibit induced *Egr2* gene expression (DeSteno and Schmauss, 2008). This differs from the other gene targeted in this study, namely the *Fos* gene. We previously showed that stimulation-induced levels of *Fos* gene expression in the anterior cingulate and prelimbic subregions of the mPFC positively correlate with the performance of mice in the spatial WM test. Moreover, exposure to the ASST leads to induction of *Fos* gene expression in all subregions of the mPFC, and the magnitude of this induction is critical for optimal ASST performance (Glickstein et al., 2002; Glickstein et al.,2005). In contrast to *Egr2* gene induction, ASST-tested mice also exhibit *Fos* gene induction in other brain regions, including orbital, motor and somatosensory cortices, hippocampus, dorsal striatum, nucleus accumbens, thalamus, and hypothalamus (Glickstein et al., 2005).

In the present studies, forebrain neocortical tissue that includes the entire mPFC was used to determine the levels of HDAC1 and HDAC3 on promotors of the *Bdnf*, *Egr2*, and *Fos* genes. As shown in **Fig. 2A**, in SFR Balb/c mice the highest levels of HDAC1 were found at *Bdnf* promotor III, and HDAC1 association with the same promotor was significantly reduced in IMS Balb/c mice. In contrast, the levels of HDAC1 at *Bdnf* promotor IV as well as the HDAC1 levels at the promotors of the *Egr2* and *Fos* genes were indistinguishable between SFR and IMS mice. Moreover, no significant differences were found for HDAC3 associations with any of the promotors examined.

The finding of reduced HDAC1 association with *Bdnf* promotor III in IMS mice suggests increased acetylation of histones marks of open chromatin and facilitated gene transcription, thus allowing increased gene transcription from this promotor either at baseline or in response to stimulation. The later option is supported by previous results showing, for example, that enhanced serotonergic stimulation led to increased enrichment of acetylated histone H4K12 (acH4K12) and RNA Polymerase II (Pol II) at *Bdnf* promotor III along with increased expression of Bdnf mRNA transcribed from this promotor in IMS Balb/c mice (Schmauss, 2015). Here we compared the expression of Bdnf transcript variant III mRNA in non-tested and WM-tested SFR and IMS Balb/c mice. In this study, tested mice had completed the WM test at 20 s delay one hour prior to brain collection (**see Fig. 1B**). Age- and sex matched non-tested SFR and IMS mice served as controls. The results of real-time RT-PCR experiments are shown in **Fig. 2B**. A 2-way ANOVA revealed a significant main effect of rearing groups (SFR and IMS; $F(1,20) = 21.9$; $p < 0.0001$), a significant main effect of treatment (tested versus non tested; $F(1,20) = 30.4$; $p = 0.0001$), and a significant interaction between these main effects ($F(1,20) = 18.5$, $p = 0.0004$). Post hoc Tukey HSD tests

resolved these differences for WM-tested IMS Balb/c mice that expressed significantly more Bdnf mRNA compared with non-tested IMS mice as well as tested and non-tested SFR mice. This enhanced transcriptional activation of *Bdnf* promotor III is consistent with results of ChIP experiments demonstrating significantly increased density of the actively elongating form of RNA polymerase II (Pol II) at *Bdnf* promotor III in WM-tested compared with non-tested IMS Balb/c mice (**Fig. 2C**). The levels of HDAC1 at *Bdnf* promotor III of WM-tested mice did not differ from the baseline levels determined for IMS mice at P60 ($1/2^{\Delta Ct}$: 2.7±1.1 (IMS at P60) and 2.5 ±0.9 (WM-tested IMS at P84)).

In contrast to the large increase in Bdnf mRNA expression in WM tested IMS Balb/c mice, Egr2 mRNA levels did not significantly differ between WM-tested and non-tested SFR and IMS Balb/c mice (**Fig. 2D**), and WM test exposure did also not lead to increased recruitment of Pol II to the *Egr2* promotor (not shown). Finally, both SFR and IMS mice exhibited a similar test-induced induction of c-Fos mRNA expression.

Example 2. Reducing Bdnf-TrkB signaling during adolescent development improves the cognitive functions of IMS Balb/c.

Next we asked whether overexpression of the *Bdnf* gene contributes to the cognitive deficits of IMS Balb/c mice. Since Bdnf protein mediates its effect through TrkB receptors (Kaplan and Miller, 2000), we took a pharmacological approach to reduce Bdnf-TrkB-receptor activation using the low-molecular weight TrkB antagonist Ana-12. Ana-12 has been shown to penetrate the blood-brain barrier, to bind selectively to TrkB receptor, and to prevent TrkB-receptor activation by Bdnf with high potency. Ana-12 does not to affect TrkA and TrkC receptor function (Cazorla et al., 2011). In the study shown in **Fig. 3**, we treated both SFR and IMS Balb/c mice with Ana-12. Treatment was initiated at postnatal day 35 (P35; the developmental time point at which reduced HDAC levels were firmly established in the forebrain neocortex of IMS mice (Levine et al., 2012)), and it continued until P59 (**see Fig. 1B**). At the end of this treatment, SFR and IMS mice and their non-treated IMS and SFR controls were tested in an attention set-shifting test (ASST), followed by training and testing in the spatial WM test.

Fig. 3A summarizes results obtained from the ASST. In this test, mice proceed through 4 consecutive test phases, beginning with a simple discrimination (SD) between two different odors or textures to find a food reward, and followed by a compound discrimination (CD) in which an additional stimulus property (one new odor or texture) is added that does not guide correct response selection. Then, a completely new set of odors and textures is presented, but the stimulus property (odor or texture) that associated with correct response

selection in the CD still guides correct response selection. This is the intradimensional set shifting phase (IDS) of the ASST. Finally, another set of new odors and textures is presented, but in this extradimensional set-shifting task (EDS), the previously irrelevant stimulus dimension (odor or texture) is now guiding correct response selection. For all groups of mice, Repeated Measures ANOVA and post hoc Tukey-Kramer multiple comparisons showed that the number of trials to criterion was significantly higher for the EDS phase compared with all other test phases, including the IDS (SFR: $p < 0.001$; SFR-Ana12: $p < 0.001$); (IMS: $p < 0.001$; IMSAna12: $p < 0.01$). Moreover, 2-way ANOVA revealed a significant main effect of treatment ($F(3,108) = 7.2$, $p = 0.0002$), a significant main effect of test phase ($F(3,108)=75.8$; $p < 0.0001$), and a significant interaction between these main effects ($F(9,108)= 6$, $p < 0.0001$). Post hoc Tukey HSD tests resolved these differences for the EDS phase of the ASST: Consistent with our previous findings (Mehta and Schmauss, 2011), compared to non-treated SFR mice, non-treated IMS Balb/c mice required significantly more trials to criterion to complete the EDS phase (**Fig. 3A**). This deficit, however, was not detected in IMS Balb/c mice that were treated with the TrkB antagonist Ana-12 during adolescence (**Fig. 3A**). Strikingly, Ana-12 treatment of SFR mice significantly impaired their performance in the EDS phase. These findings indicate that both hypo- and hyperactivation of TrkB-receptor can cause deficits in this test.

A similar result was found in a delayed alternation spatial WM test (T maze). Here, 2-way ANOVA revealed a significant main effect of treatment ($F(3,67) = 3.4$, $p = 0.02$), a significant main effect of delay periods ($F(3,67) = 19.4$, $p < 0.0001$), and a significant interaction between these main effects ($F(6,67) = 2.4$, $p = 0.03$). Post hoc Tukey HSD tests resolved these differences for the groups of mice tested at 20 s inter-trial delay. At this delay, non-treated IMS mice had a significantly lower percentage of correct arms entries compared to non-treated SFR mice, but Ana-12 treated IMS mice did not differ from non-treated SFR mice (**Fig. 3B**). Moreover, similar to the effect of Ana-12 treatment of SFR mice on the performance in the EDS phase of the ASST, Ana-12 treatment of SFR mice also impaired significantly their WM performance when tested at 20 s delay, again indicating that both hypo- and hyperactivation of the Bdnf-TrkB signaling pathway can cause cognitive deficits.

Example 3: Reducing HDAC 1 activity during adolescent development of SFR Balb/c mice also leads to cognitive deficits.

The results shown above indicate that, in IMS Balb/c mice, enhanced Bdnf-TrkB signaling plays a significant role in the emergence of their executive cognitive deficits. To further test the role of reduced activity of HDAC1 in this process, we conducted additional

studies on SFR Balb/c mice that were treated during adolescence (P35 to P59) with MS-275, an inhibitor of class I HDACs with nanomolar affinity to HDAC1, a 100-fold lower affinity to HDAC3, and no affinity to other HDACs (Hu et al.,2003; Khan et al., 2008). As shown in **Fig. 4A**, compared to non-treated SFR mice, MS-275-treated SFR mice exhibit deficits in the EDS phase of the ASST that are similar to those found in IMS mice. Moreover, they exhibit a significant deficit in the spatial WM test at 20 s inter-trial delay (**Fig. 4B**). Next we compared the levels of mRNA transcribed from *Bdnf* promotor III and the *Egr2* promotor between SFR Balb/c mice and non-tested and WM-tested SFR Balb/c mice that were treated with MS-275 using real-time RT-PCR. The tested MS-275-treated mice completed the spatial WM test (20s delay) one hour prior to brain collection (see **Fig. 1B**). **Fig. 4C** illustrates that, although the levels of Bdnf mRNA did not differ between SFR and non-tested MS-275-treated Balb/c mice, tested MS-275-treated Balb/c mice expressed significantly more Bdnf transcript variant III mRNA. In contrast, Egr2 mRNA levels did not significantly differ between the three groups of mice (**Fig. 4D**). Hence, MS-275 treatment during adolescent development of SFR mice leads to the same cognitive deficits detected in IMS Balb/c mice and the same test-induced increase of Bdnf transcript variant III mRNA expression.

Example 4: Cognitive deficits of C57Bl/6 mice fostered by Balb/c mothers during IMS exposure.

It was previously shown that C57Bl/6 mice raised by Balb/c mothers and exposed to the IMS paradigm do not develop WM deficits but strikingly, they do exhibit deficits in the EDS phase of the ASST (Schmauss et al., 2014). We replicated this finding in the present study in which C57Bl/6 mothers raised either their own pups with and without IMS exposure, and Balb/c mothers fostered C57Bl/6 pups during IMS exposure (**see Fig. 1C**).

For the ASST performance of SFR and IMS C57Bl/6 mice raised by their biological mothers, Repeated Measures ANOVA and post hoc Tukey-Kramer multiple comparisons revealed that their numbers of trials to criterion were significantly higher for the EDS phase compared to all other test phases, including the IDS phases ($p < 0.01$) (**Fig. 5A**). The EDS performance of foster IMS C57Bl/6 mice raised by Balb/c mothers also differed significantly from all other test phases, including the IDS ($p < 0.001$) (**Fig. 5A**). A 2-way ANOVA further revealed a significant main effect of rearing groups ($F(2,76) = 10.46$, $p = 0.0001$), a significant main effect of test phase ($F(3,76) = 34.31$, $p < 0.0001$), and a significant interaction between these main effects ($F(6,76) = 5.0$, $p = 0.0002$). Post hoc Tukey HSD tests resolved these differences for foster IMS C57Bl/6 mice that required a significantly higher number of trials to complete the EDS phase of the ASST (**Fig. 5A**).

In order to test whether foster IMS C57Bl/6 mice also exhibit decreased HDAC1 association with promotors of any of the three plasticity associated genes studied here at baseline (P60; see **Fig. 1A**), we performed HDAC1 and HDAC3 ChIPs on forebrain neocortical tissues of SFR and IMS C57Bl/6mice raised by their biological mothers and on foster IMS C57Bl/6 mice that were raised by Balb/c mothers. No significant differences were found between the three groups for the levels of HDAC1 and HDAC3 at *Bdnf* promotors III and IV and the *Fos* promotor (**Fig. 5B**). A different result was obtained for the HDAC1 ChIP targeting the *Egr2* promotor: Compared with SFR and IMS C57Bl/6 mice, the levels of HDAC1 (but not HDAC3) were significantly lower in foster IMS C57Bl/ 6 mice (**Fig. 5B**).

We further measured Bdnf transcript variant III and Egr2 mRNA in ASST-tested and non-tested foster IMS C57Bl/6 mice by real-time RTPCR and compared these results to non-tested and ASST-tested IMS Balb/c mice. In these experiments, all pups raised by Balb/c mothers were foster pups, and brains of tested animals were collected 1 h after completion of the EDS phase of the ASST (**see Fig. 1C**). For Bdnf mRNA expression, 2-way ANOVA revealed a significant main effect of test exposure ($F(1,16)=4.1$, $p = 0.05$) and a significant interaction between strain and test exposure ($F(1,16) =4.1$, $p=0.05$). Post hoc Tukey HSD tests resolved these differences for ASST-tested foster IMS Balb/c mice that exhibited significantly increased Bdnf mRNA expression compared to all other groups of mice (**Fig. 6A**). (The magnitude of this increase, however, is only ~2.5-fold compared to the ~10-fold increase detected in WM-tested IMS Balb/c mice (see **Fig. 2B**)).

For Egr2 mRNA expression, 2-way ANOVA also revealed significant main effects of test exposure ($F(1,18) = 6.55$, $p = 0.02$)) and strain ($F(1,18) = 4.4$, $p = 0.05$)), and a significant interaction between them ($F = 4.36$, $p = 0.05$). Post hoc Tukey HSD tests showed that Egr2 mRNA levels were significantly elevated in tested foster IMS C57Bl/6 mice compared with all other groups of mice (**Fig. 6B**), a finding consistent with the increased levels of Pol II at the Egr2 promotor in ASST-tested foster IMS C57Bl/6 mice relative to non-tested foster IMS C57Bl/6 mice (**Fig. 6C**). The HDAC1 levels at the *Egr2* promotor of ASST-tested foster IMS C57Bl/6 mice, however, did not differ from corresponding baseline levels measured at P60 ($1/2^{\Delta Ct}$: 0.42 ± 0.09 (foster IMS C57Bl/6 at P60) and 0.37 ± 0.03 (ASST-tested foster IMS C57Bl/6 at P66)). In summary, foster IMS C57Bl/6 mice that have only deficits in the ASST differ from IMS Balb/c mice by exhibiting reduced HDAC1 levels at the *Egr2* promotor rather than promotor III of the *Bdnf* gene, and they exhibit increased Egr2 mRNA expression rather than increased Bdnf mRNA expression after ASST testing.

Finally, there are also differences in histone modifications associated with *Bdnf* promotor III and the *Egr2* promotor between IMS Balb/c and foster IMS C57Bl/6 mice. Whereas IMS Balb/c mice have increased acH4K12 levels associated with *Bdnf* promotor III compared with SFR Balb/c mice (Schmauss, 2015), the levels of acH4K12 at this promotor did not differ between SFR C57Bl/6 controls and foster IMS C57Bl/6 mice ($1/2^{\Delta Ct}$: 3.01 ± 1.03 (SFR C57Bl/6) and 3.18 ± 0.9 (foster IMS C57Bl/6) (p=0.9)). For the *Egr2* promotor, however, we observed a significant reduction of the levels of a repressive mark of gene transcription, namely H3K9me3, in foster IMS C57Bl/6 mice. Since reduced levels of acH3K9me3 were not found in IMS Balb/c mice, we conducted additional ChIPs targeting the *Egr2* promotor using an antibody directed against acH3K9, a marker of active gene transcription, and we expressed these results as the ratio of acH3K9/H3K9me3 levels associated with the Egr2 promotor. As shown in **Fig. 6D**, foster IMS C57Bl/6 mice (raised by Balb/c mothers) had a significantly higher acH3K9/H3K9me3 ratio compared to SFR and IMS C57Bl/6 and Balb/c mice (raised by their biological mothers). Hence, although reduced promotor-associated levels of HDAC1 are a common denominator for Balb/c and C57Bl/6 mice raised by Balb/c mothers during IMS exposure, the genes affected and the histone modification profiles at the promotors of the affected genes are also influenced by the genetic backgrounds of the pups.

Example 5: Treatments for Adolescent and Young Adults with Stress-Induced/Related Cognitive Impairment

The below outline is illustrative of a method for improving cognitive functioning in a patient in need thereof comprising administering a therapeutically effective amount of at least one brain-derived neurotrophic factor (Bdnf)-tropomyosine kinase B (TrkB) inhibitor to the patient.

A population of patients that will benefit from the present treatment methods include the following: an adolescent (typically 10-18) or young adult patient (18-24) with a mood/emotional disorder which can be an anxiety disorder or stress- induced disorder, including early life stress, or a combination thereof. Additionally, the mood disorder can also be a panic disorder, an obsessive compulsive disorder, a post-traumatic stress disorder, or any combination thereof. In certain embodiments, it is expected that adults could also benefit from treatments described herein.

The patient will be treated with an effective amount of a TrkB inhibitor that is capable of crossing the blood brain barrier.

It is expected, based upon the model mouse data shown herein, that the treated human patient will exhibit at least a 10% improvement of cognitive functioning, compared with a baseline cognitive functioning reference or control. In certain instances it is expected that the cognitive functioning improvement will be greater than about 50% following treatment with an effective amount of a TrkB inhibitor such as ANA-12. It is also expected that the treated human patient will exhibit an improvement in one or more of the typical indicators of cognitive functioning including working memory and attention set-shifting. The treatment will be continued for a period of time until these improvements are observed and maintained and this time can vary from several weeks, to several months. It is expected that these treated patients will be less likely to experience a relapse.

In additional instances, the patient will be evaluated (either simultaneously or prior to TrkB inhibitor treatment, or subsequent to TrkB inhibitor treatment) for symptoms that would indicate treatment with an SSRI would be indicated (See Millan et al. 2012 and McIntyre et al. 2013). In these instances where treatment with one or more SSRI are indicated during treatment with at least one TrkB inhibitor, the patient will be treated with at least one selective serotonin reuptake inhibitor (SSRI) selected from the group consisting of Citalopram (Celexa); Escitalopram (Lexapro, Cipralex); Paroxetine (Paxil, Seroxat); Fluoxetine (Prozac); Fluvoxamine (Luvox); Sertraline (Zoloft, Lustral), or any combination thereof. It is expected that for patients treated with both a TrkB inhibitor and at least one SSIR, that both their cognitive and emotional phenotypes will exhibit improvement and will be less likely to experience a relapse. The treatment will be continued for a period of time until these improvements are observed and maintained and this time can vary from several weeks, to several months.

Discussion

Optimal cognitive task performance depends upon a finely-tuned balance between neuronal activation and inhibition, a balance that is maintained by tightly regulated gene induction and repression (Rao et al., 2000; Millan et al., 2012; Pezze et al., 2014). Since HDACs are in a strong position to modulate the expression of plasticity-related genes implicated in the control of cognitive functions, the present study asked whether the cognitive deficits found in mice exposed to early life stress can be explained by reduced expression of class I HDACs 1 and/or 3 that we previously detected in IMS Balb/c mice (Levine et al., 2012). We found that IMS Balb/c mice exhibit reduced association of HDAC1 (but not HDAC3) specifically with *Bdnf* promotor III, and that cognitive task exposure leads to increased density of the transcriptionally active form of Pol II at this promotor, a finding

consistent with the large increase in Bdnf transcript variant III expression found after cognitive testing. Moreover, when non-stressed Balb/c mice were treated during adolescence with the HDAC1-preferring inhibitor MS-275, they exhibit the same cognitive deficits found in IMS mice as well as the same enhancement of test-induced *Bdnf* gene expression. The results of these pharmacological studies indicate clearly that the cognitive deficits and the associated change in *Bdnf* gene expression are due to reduced HDAC1 activity.

Our finding that blunting the Bdnf-TrkB receptor signaling during adolescence abolishes both the WM and attention set-shifting deficits in IMS Balb/c mice indicates that overexpression of the Bdnf gene contributes substantially to the emergence of these deficits. However, we found that SFR mice treated with the TrkB receptor antagonist Ana-12 also exhibit deficits in spatial WM and the EDS phase of the ASST. While this result was unexpected, it is consistent with the prevalent view that both hypo- and hyperactivation can cause cognitive deficits (Rao et al., 2000; Pezze et al., 2014), i.e., both reduced and enhanced Bdnf-TrkB receptor signaling can have detrimental effects on cognitive functions.

Strikingly, in IMS Balb/c mice, the same HDAC1-dependent increase in Bdnf-TrkB signaling has been shown to enhance the antidepressant efficacy of adolescent treatment with fluoxetine (Schmauss, 2015). However, the present findings can explain why adolescent treatment of IMS Balb/c mice with fluoxetine (an exemplary SSRI), although effectively improving their emotional phenotype, does not improve their cognitive deficits (Schmauss et al., 2014). This raises the intriguing question whether antidepressant drug treatments that enhance the Bdnf/TrkB-signaling effects benefit those patients that also have cognitive deficits, a question particularly germane to findings that patients in remission from depression that continue to have cognitive deficits are more likely to relapse (Millan et al., 2012; McIntyre et al., 2013; Trivedi and Greer, 2014).

Although studies illustrated that the effect of IMS exposure on pups is due to the IMS-triggered alteration in maternal care (Huot et al., 2004; Schmauss et al., 2014), we found that IMS-triggered cognitive deficits as well as the reduced HDAC1 association with promotors of distinct plasticity-related genes are influenced by the genetic backgrounds of the pups. IMS C57Bl/6 mice raised by Balb/c mothers during IMS exposure exhibit deficits in attention set-shifting, but no WM deficits. Moreover, while their levels of HDAC1 at Bdnf promotor III are unaltered, they exhibit decreased HDAC1 levels at the promotor of the *Egr2* gene, a gene selectively induced in the ventrolateral orbital frontal cortex and the pre- and infralimbic subregions of the mPFC during ASST (but not WM) exposure (DeSteno and Schmauss, 2008). Indeed, only foster IMS C57Bl/6 mice exhibit a robust (~5 fold) increase

of transcription of the *Egr2* gene upon ASST exposure. Since we previously showed that mice with enhanced ASST performance expressed lower levels of Egr2 than those with diminished ASST performance (DeSteno and Schmauss, 2008), the present finding lends further support for the conclusion that overexpression of the *Egr2* gene associates specifically with ASST-selective cognitive deficits.

Compared with IMS Balb/c mice, foster IMS C57Bl/6 mice also exhibit a different histone modification phenotype at the *Egr2* promotor that is characterized by an increased ratio of acetylated H3K9 (a histone mark of open chromatin and active gene transcription) over H3K9me3 (a marker of gene repression).While IMS Balb/c mice exhibit increased acetylation of histone H4 protein, especially histone H4K12 acetylation at *Bdnf* promotor III (Schmauss, 2015), increased acetylation of histone H3 protein was not detected in these mice (Levine et al., 2012). Hence, IMS Balb/c and foster IMS C57Bl/6 mice do not only exhibit reduced HDAC1 levels at promotors of different genes, the promotors of the affected genes also differ in their histone modification profiles. Nevertheless, despite the cognitive task-induced enhanced transcription of different plasticity-related genes in both strains of mice, reduced HDAC1 association with the respective promotors is a common epigenetic phenotype. Importantly, just as ongoing cognitive experience has been shown to regulate the molecular consequences of HDAC inhibition (Sewal et al., 2015), the present study shows that the effect of reduced promotor-associated HDAC1 levels on gene expression is potently unmasked by cognitive challenge. Moreover, in mice exposed to early life stress, reduced HDAC1 levels are not found on promotors of all plasticity-associated genes examined here, and cognitive challenge exerts different, gene-specific effects in different genetic backgrounds. This finding points to a sensitive interaction between genomic sequence variations (acting in cis) and the stress-modulated epigenome, an interaction unlikely to be mimicked with studies on genetically modified mice harboring a conditional HDAC1 deficiency.

Contrary to the present study that uncovered a role of reduced HDAC1 activity in the emergence of early-life stress-triggered cognitive deficits that are largely governed by the PFC, several other studies have demonstrated that HDAC inhibitors can ameliorate age-related cognitive decline (Peleg et al., 2010) or memory impairments in neurodegenerative disorders, i.e., functions that are primarily governed by the hippocampus (Fischer et al., 2007; Kilgore et al., 2010; Gräff et al., 2012). For example, in an animal model of inducible severe neurodegeneration and synapse loss, the non-selective HDAC inhibitor sodium butyrate increased histone H3 and H4 acetylation in the hippocampus, promoted associative and

spatial learning, and increased expression of synaptic proteins in remaining neurons (Fischer et al., 2007). Similarly, in an animal model of Alzheimer's Disease with early onset contextual memory impairment, three non-selective HDAC inhibitors (sodium butyrate, sodium valproate, and suberoylanilide hydroxamic acid (SAHA)) rescued memory deficits and led to stably maintained consolidated memory for two weeks (Kilgore et al., 2010). Yet, the study of Kilgore et al. (2010) also showed with biochemical assays that all three HDAC inhibitors share in common a potent inhibition of class I HDACs 1–3, and other studies pointed to a specific role of HDAC2 in the modulation of distinct cognitive functions. For example, studies on HDAC1 and HDAC2 overexpressing mice revealed that, although both lines of mice exhibit decreased acetylation of histone H4K12 in the hippocampus, only HDAC2 overexpressing mice have deficits in associative spatial learning (a hippocampal function) and WM (a PFC function) (Guan et al., 2009). Conversely, HDAC2 knockout mice with increased histone H4 and H2B acetylation in the hippocampus exhibit increased associative learning and improved WM, and they have increased hippocampal spine density and synaptic terminals (Guan et al., 2009). HDAC2 deficient mice also exhibit increased acetylation of histone H3 and H4 at *Bdnf* promotor I/II, *Egr2*, and *Fos*, and they are refractory to the effect of SAHA on synaptic plasticity and learning (Guan et al., 2009).

A specific role of HDAC2 in memory impairment is further supported by findings of increased HDAC2 expression in the hippocampal CA1 subfield and the PFC in an animal model of severe neurodegeneration. In this model, shRNA-mediated HDAC2 knockdown lead to increased acetylation of histone H4K12 at promotors of plasticity-associated genes along with increased expression of these genes, re-instated morphological and synaptic plasticity of surviving neurons, and improved associative and spatial memory (Gräff et al., 2012).

Finally, studies also identified specific roles for HDAC1 and HDAC3 in distinct hippocampal-dependent cognitive processes. One study showed that virus-mediated overexpression of HDAC1 in the hippocampus did not affect WM and short- and long-term memory. However, it facilitated the extinction of fear memories and, conversely, hippocampal injection of MS-275 or knockdown of HDAC1 with siRNA significantly impaired fear extinction. The authors linked these effects to binding of HDAC1 to *Fos* and *Egr2* promotors where it affects histone H3K9 acetylation (Bahari-Javan et al., 2012). Another study on mice with a focal deletion of dorsal hippocampal HDAC3 and increased histone H4K8 acetylation (that correlated with increased *Fos* and *Nr4a2* gene expression) revealed a cognitive phenotype of facilitated long-term memory (McQuown et al., 2011).

It is evident from the studies summarized above that class I HDACs1–3 play distinct roles in several cognitive functions. However, while reducing the activity of hippocampal HDACs1–3 (and possibly also prefrontal cortical HDAC2) can exert a plethora of pro-cognitive effects, the present study revealed that early-life stress-triggered cognitive deficits arise primarily from reduced HDAC1 association with promotors of distinct plasticity-associated genes. Whether reduced HDAC1 activity in mice exposed to early life stress only affects executive cognitive function governed by the PFC, or whether hippocampal-related functions are also affected by reduced HDAC1 (or HDAC3 activity) remains to be determined. However, since the HDAC-dependent epigenetic response of IMS Balb/c mice is only firmly established by mid-adolescence (P35) (Levine et al., 2012) it is conceivable that it predominantly affects functions governed by late-maturing brain regions such as the PFC (Pechtel and Pizzagalli, 2011).

Regardless of this physiological determination, as the present data using validated mouse models for these early stress conditions and corresponding cognitive deficits illustrate, that pharmacological treatment of a patient with a TrkB inhibitor such as ANA-12 will lead to improvement of the cognitive deficits and improvement in cognitive functioning (including improvement to working memory and attention set-shifting), which may also serve to benefit certain emotional phenotypes or conditions such as MDD (major depressive disorder) without any additional pharmacological intervention. In certain alternative instances, it is expected that in addition to treating a patient with a TrkB inhibitor such as ANA-12, that treating with one or more SSRI (including any combination of: Citalopram (Celexa); Escitalopram (Lexapro, Cipralex); Paroxetine (Paxil, Seroxat); Fluoxetine (Prozac); Fluvoxamine (Luvox); Sertraline (Zoloft, Lustral)) would result in improvement to both the cognitive deficits and emotional phenotype e.g., depressive condition/s.

Conclusions

The present results demonstrate for the first time that an epigenetic response to early life stress exposure contributes the emergence of working memory and attention set-shifting deficits, i.e. deficits that often accompany mental disorders and that are largely resistant to treatment. The results show that reduced histone deacetylase 1 levels at promotors of distinct plasticity-related genes allow for abnormally increased expression of these genes upon cognitive testing, an effect that influences cognitive task performance negatively. The genes affected by reduced HDAC1 activity differ in genetically different strains of mice, indicating that genomic sequence variations and their differential interaction with the stress-modulated

epigenome contribute to and determine the outcome of early-life stress-triggered cognitive impairment.

GENERAL METHODS

Standard methods in molecular biology are described Sambrook, Fritsch and Maniatis (1982 & 1989 2nd Edition, 2001 3rd Edition) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sambrook and Russell (2001) *Molecular Cloning, 3rd ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, CA). Standard methods also appear in Ausbel, *et al.* (2001) *Current Protocols in Molecular Biology, Vols.1-4*, John Wiley and Sons, Inc. New York, NY, which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4).

Methods for protein purification including immunoprecipitation, chromatography, electrophoresis, centrifugation, and crystallization are described (Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 1*, John Wiley and Sons, Inc., New York). Chemical analysis, chemical modification, post-translational modification, production of fusion proteins, glycosylation of proteins are described (see, *e.g.*, Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 2*, John Wiley and Sons, Inc., New York; Ausubel, *et al.* (2001) *Current Protocols in Molecular Biology, Vol. 3*, John Wiley and Sons, Inc., NY, NY, pp. 16.0.5-16.22.17; Sigma-Aldrich, Co. (2001) *Products for Life Science Research*, St. Louis, MO; pp. 45-89; Amersham Pharmacia Biotech (2001) *BioDirectory*, Piscataway, N.J., pp. 384-391). Production, purification, and fragmentation of polyclonal and monoclonal antibodies are described (Coligan, *et al.* (2001) *Current Protcols in Immunology, Vol. 1*, John Wiley and Sons, Inc., New York; Harlow and Lane (1999) *Using Antibodies*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Harlow and Lane, *supra*). Standard techniques for characterizing ligand/receptor interactions are available (see, *e.g.*, Coligan, *et al.* (2001) *Current Protocols in Immunology, Vol. 4*, John Wiley, Inc., New York).

Materials and Methods

Animals

Balb/cJ and C57Bl/6J mice were housed in a temperature- and light controlled barrier facility with free access to food and water. All experiments involving the animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees

at Columbia University and the New York State Psychiatric Institute.

Infant maternal separation (IMS)

In these experiments, Balb/c mothers either raised their own pups or fostered Balb/c or C57Bl/6 pups from other litters from the day of birth. All pups exposed to the IMS paradigm were separated from their mothers daily for three hours (from 1:00 to 4:00 PM), starting at postnatal age P2 and ending at P15. They were weaned at P28 and group housed by sex (see Fig. 1A). Other pups of Balb/c or C57Bl/6 mothers were left undisturbed with their mothers and were also weaned at P28. These mice are referred to as standard-facility reared (SFR). Housing and husbandry conditions were identical for SFR and IMS mice.

Drug treatments

Drugs were administered to mice (males and females) starting at P35 and ending at P59 (see **Fig. 1B**). All drugs were administered via the drinking water. Drug intake was monitored daily and drugs were replenished every 48 h (MS-275; Sigma Aldrich, St. Louise, MO) or 24 h (Ana-12; Calbiochem; Billerica, MA). MS-275 is also known as Entinostat, or SNDX-275 and is a benzamide histone deacetylase inhibitor undergoing clinical trials for treatment of various cancers. Entinostat inhibits class I HDAC1 and HDAC3 with IC_{50} of 0.51 μM and 1.7 μM, respectively, and is capable of penetrating the blood brain barrier weakly (See, Hooker J.M., et al., Histone deacetylase inhibitor, MS-275, exhibits poor brain penetration: PK studies of [C]MS-275 using Positron Emission Tomography. ACS Chem Neurosci. 2010;1(1):65-73). It has the formula: $C_{21}H_20N_4O_3$.

ANA-12 is a selective, small-molecule non-competitive antagonist of TrkB (Kd = 10 nM and 12 μM for the high- and low-affinity sites, respectively), the main receptor of brain-derived neurotrophic factor (BDNF/Bdnf) (Cazorla 2011). The compound crosses the blood-brain-barrier and exerts central TrkB blockade, producing effects as early as 30 minutes (~400 nM) and as long as 6 hours (~10 nM) following intraperitoneal injection in mice (Cazorla 2011). It blocks the neurotrophic actions of BDNF without compromising neuron survival (Cazorla 2011). ANA-12 produces rapid antidepressant- and anxiolytic-like effects in animal models, the former of which have been elucidated to be mediated by blockade of BDNF signaling in the nucleus accumbens. (Zhang JC 2015 and Shirayama Y 2015). It has also been found to alleviate methamphetamine-induced depression-like behavior (including anhedonia), behavioral sensitization, and nucleus accumbens neuroplasticity changes with subchronic (14-day) administration in mice, whereas the TrkB agonist 7,8-dihydroxyflavone was ineffective in doing so (Ren Q 2015).

IMS Balb/cmice that received Ana-12 consumed 1 mg/kg/day, and SFR mice that received MS-275 consumed 15 μM/day. We previously showed that neither treatment altered the emotional behavior of these mice when tested at the end of this treatment (Schmauss, 2015). However, at the dose administered, Ana-12 effectively blocked the antidepressant effects of fluoxetine in IMS Balb/c mice (Schmauss, 2015) and, in SFR Balb/c mice, MS-275 increased the levels of acH4K12 and Pol II at Bdnf promotor III only when co-administered with fluoxetine (Schmauss, 2015).

Cognitive tests

To avoid litter effects, one male and one female mouse of a total of at least 4 litters per treatment group were assigned to cognitive testing. Similar to a previous study (Mehta and Schmauss, 2011), no significant sex differences in cognitive task performance of SFR and IMS mice were found. Hence, males and females are equally represented in the groups of mice studied here. Starting at postnatal age P60, mice were food restricted such that they gradually (over the period of 4 to 5 days) lost 10–15% of their free-feeding body weights. Prior to ASST testing, mice learned to dig for food buried deeply in unscented terra cotta pots filled with familiar bedding medium. Then, they went through four consecutive test phases of the ASST, beginning with a simple discrimination (SD) between odor (scented terra cotta pots) or texture (different digging media), and followed by a compound discrimination (CD) in which another stimulus property (a second odor or texture) was introduced that was not a reliable predictor of food reward. The third phase required an intradimensional shift of attention (IDS), i.e., both relevant and irrelevant stimulus properties were changed, but the relevant stimulus dimension used in the SD and CD (odor or texture) remained the same. Finally, the formerly irrelevant stimulus dimension became relevant and required an extradimensional shift of attention (EDS). In all test phases, animals had to reach a criterion of 6 consecutive correct trials, and the number of trials to criterion was referred to as response accuracy.

Some of the Balb/c mice that completed the ASST were further trained for alternate arm entries in the T-maze until they reached more than 70% correct arm entries (in 10 trials per day) on 2 consecutive days with 5 s inter-trial delay periods. Then, mice performed the test with 2 longer inter-trial delays (15 and 20 s, each tested on 2 consecutive days) to test for their working memory. (All groups of mice did not maintain spatial WM at 30 s inter-trial delay). Only correct arm entries were rewarded with food, and the percentage of correct arm entries in the total number of 10 trials per delay period was taken as a measure of response accuracy.

RNA extraction and real-time PCR

One hour after completion of either the ASST or the WM test, forebrain neocortical tissue was dissected from male and female mice using the mesodiencephalic junction as the anatomic landmark for the caudal border of the forebrain. ASST-tested mice were 66 days old (see Fig. 1C), and their non-tested controls were matched for age and sex. The age of WM-tested mice and their non-tested controls (also derived from at least 4 different litters per group) ranged from P81 to P84 (see Fig. 1C). Total RNA was extracted using guanidine/cesium chloride ultracentrifugation. First-strand cDNA was synthesized using Murine Moloney Leukemia Virus reverse transcriptase (USB, Cleveland, OH) in conjunction with oligo dT15 primers. Real time PCR was performed using the iQ Real Time PCR detection System (Bio-Rad, Hercules, CA) and SYBR Green (Bio-Rad). Bdnf mRNA was amplified using the primers targeting Bdnf transcript variant III reported by Tsankova et al. (2006). Egr2 mRNAwas amplified using the primer pair 5′-ATGAACGGAGTGGCGGGA-3′ (SEQ ID NO:1); and 5′-AGTAGAGGTGGTCCAGTT-3′ (SEQ ID NO:2) and c-Fos mRNA was amplified using the primer pair 5′-ATGATGTTCTCGGGTTTGAA-3′ (SEQ ID NO:3) and 5′CACCGTGGGGATAAAGTTGG-3′ (SEQ ID NO:4). Cycle thresholds (Ct) of amplification (normalized to those obtain for β-actin) were expressed as $1/2^{\Delta Ct}$ values so that higher numbers reflect higher expression.

Chromatin immunoprecipitations (ChIP)

ChIP was performed on forebrain neocortical tissue of groups of male and female mice (selected from different litters as described above) either at P60 (baseline) or after cognitive testing with and without drug treatment (see Fig. 1). Dissected tissue was fixed with 1% paraformaldehyde, dounce-homogenized and sonicated using the Microson ultrasonic cell disrupter (Misonix, Farmingdale, NY) to an average DNA length of 200 to 400 base pairs. Samples were centrifuged at 15,000 ×g, and 50 µl aliquots of the supernatant were immunoprecipitated overnight with 20 µl protein A magnetic beads (Millipore, Temecula, CA) and ChIP-grade antibodies directed either against HDAC1 (Millipore), HDAC3 (Abcam Inc., Cambridge, MA), RNA Polymerase II CTD repeat YSPTSPS (Abcam), trimethyl-histone H3K9 (Millipore), or acetyl-histone H3K9 (Millipore). Beads were treated according to the instruction of the manufacturer. Immunoprecipitated DNA and a serial dilution of 1% input were analyzed

by SYBR-Green real-time PCR using primers targeting Bdnf promotor III (original nomenclature) adopted from Tsankova *et al.* (2006) and the promotors of the Egr2 and Fos genes adopted from Bahari-Javan *et al.* (2012).

Statistical analyses

Repeated Measures ANOVA and one-way ANOVA were performed using GraphPad Software (InStat; La Jolla, CA). Two-way ANOVA was performed using the VassarStats Statistical Computation website (American Library Association, February 27, 2012).

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INCORPORATION BY REFERENCE

All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The entire disclosure

of each of the patent documents, including certificates of correction, patent application documents, scientific articles, governmental reports, websites, and other references referred to herein is incorporated by reference herein in its entirety for all purposes. In case of a conflict in terminology, the present specification controls.

The invention can be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are to be considered in all respects illustrative rather than limiting on the invention described herein. Further, it should be understood that the order of steps or order for performing certain actions is immaterial so long as the invention remains operable. Moreover, two or more steps or actions can be conducted simultaneously.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In the case of conflict, the present specification will control.

WHAT IS CLAIMED IS:

1. A method of treating cognitive defects in a mood disorder in a patient in need thereof, comprising administering a therapeutically effective amount of at least one brain-derived neurotrophic factor (Bdnf)-tropomyosine kinase B (TrkB) inhibitor to the patient.

2. The method of claim 1, wherein the treating comprises at least a 10% improvement of cognitive functioning, compared with a baseline cognitive functioning reference or control.

3, The method of claim 2, wherein the cognitive functioning improvement is greater than about 50%.

4. The method of claim 1, wherein the TrkB inhibitor is a TkrB antagonist.

5. The method of claim 1, wherein the TrkB inhibitor is administered by mouth, topically, rectally, or intravenously.

6. The method of claim 1, wherein the mood disorder is an anxiety disorder or stress-induced disorder including early life stress, or a combination thereof.

7. The method of claim 1, wherein the mood disorder is a panic disorder, an obsessive compulsive disorder, a post-traumatic stress disorder, or any combination thereof.

8. The method of claim 1, wherein the patient is an adolescent or young adult.

9. The method of claim 1, wherein the TkrB inhibitor comprises ANA-12.

10. The method of claim 1, further comprising administering at least one selective serotonin reuptake inhibitor (SSRI) selected from the group consisting of Citalopram (Celexa); Escitalopram (Lexapro, Cipralex); Paroxetine (Paxil, Seroxat); Fluoxetine (Prozac); Fluvoxamine (Luvox); Sertraline (Zoloft, Lustral), and combinations thereof.

11. The method of claim 1, wherein the patient is a mammal.

12. The method of claim 11, wherein the patient is a human.

13. The method of claim 1, wherein the patient exhibits a cognitive deficit in one or more of the following features: working memory or attention set-shifting.

14. A method of improving cognitive functioning in a patient in need thereof comprising administering a therapeutically effective amount of at least one brain-derived neurotrophic factor (Bdnf)-tropomyosine kinase B (TrkB) inhibitor to the patient.

15. The method of claim 14, wherein the improvement comprises at least a 10% improvement of cognitive functioning, compared with a baseline cognitive functioning reference or control.

16. The method of claim 15, wherein the improvement is greater than about 50%.

17. The method of claim 14, wherein the TrkB inhibitor is a TkrB antagonist.

18. The method of claim 14, wherein the TrkB inhibitor is administered by mouth, topically, rectally, or intravenously.

19. The method of claim 14, wherein the patient exhibits a mood disorder comprising an anxiety disorder or stress-induced disorder including early life stress, or a combination thereof.

20. The method of claim 14, wherein the patient exhibits a mood disorder comprising a panic disorder, an obsessive compulsive disorder, a post-traumatic stress disorder, or any combination thereof.

21. The method of claim 14, wherein the patient is an adolescent or young adult.

22. The method of claim 14, wherein the TkrB inhibitor comprises ANA-12.

23. The method of claim 14, further comprising administering at least one selective serotonin reuptake inhibitor (SSRI) selected from the group consisting of Citalopram (Celexa); Escitalopram (Lexapro, Cipralex); Paroxetine (Paxil, Seroxat); Fluoxetine (Prozac); Fluvoxamine (Luvox); Sertraline (Zoloft, Lustral), and combinations thereof.

24. The method of claim 14, wherein the patient exhibits a cognitive deficit in one or more of the following features: working memory or attention set-shifting.

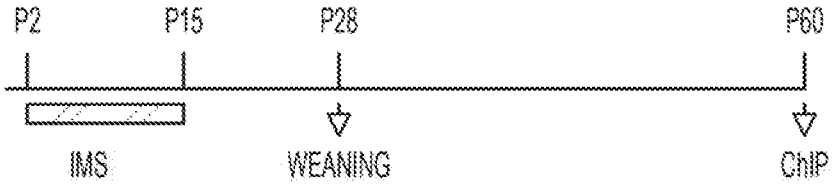


FIG. 1A

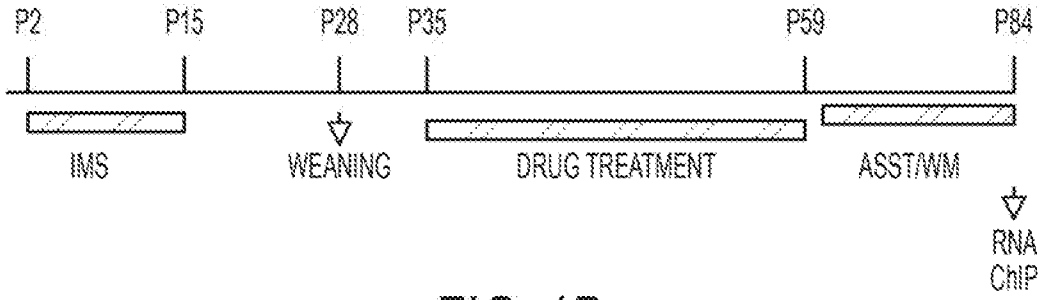


FIG. 1B

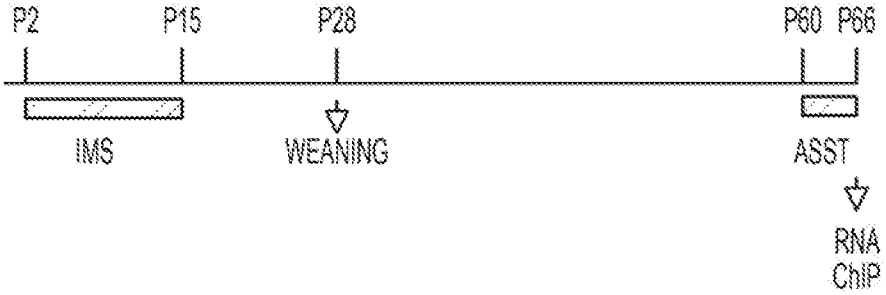


FIG. 1C

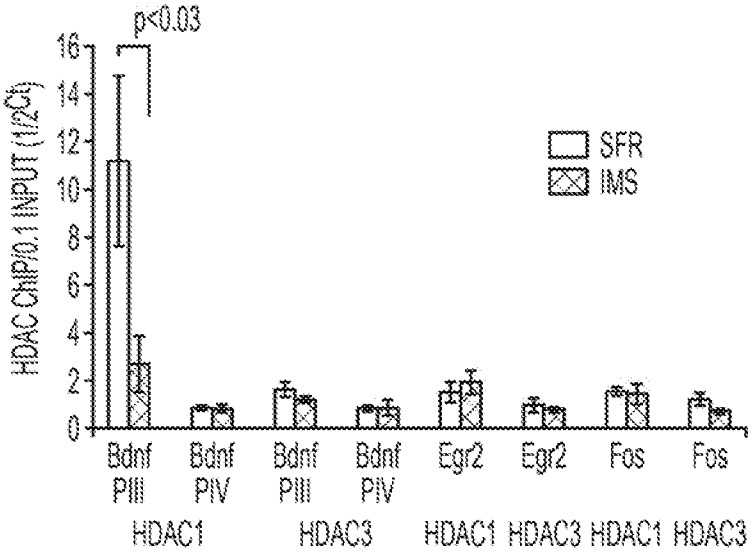


FIG. 2A

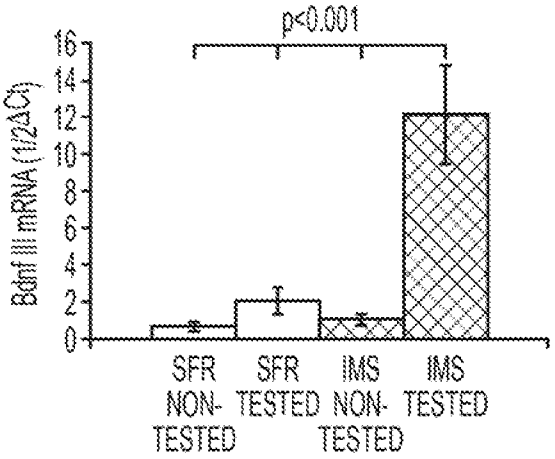
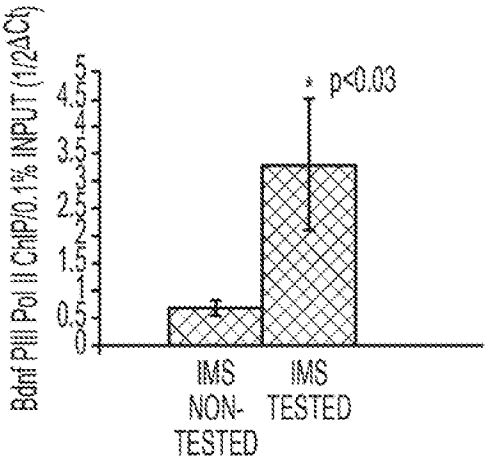


FIG. 2B



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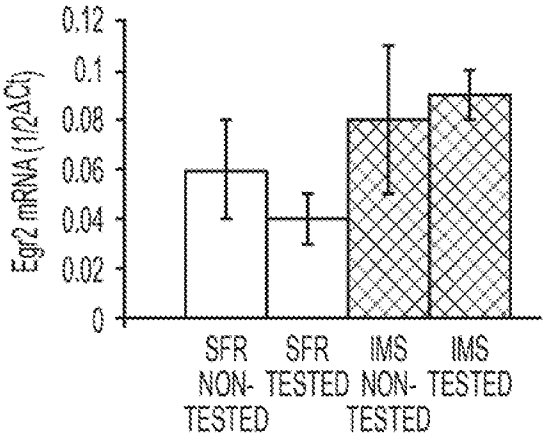

FIG. 2D

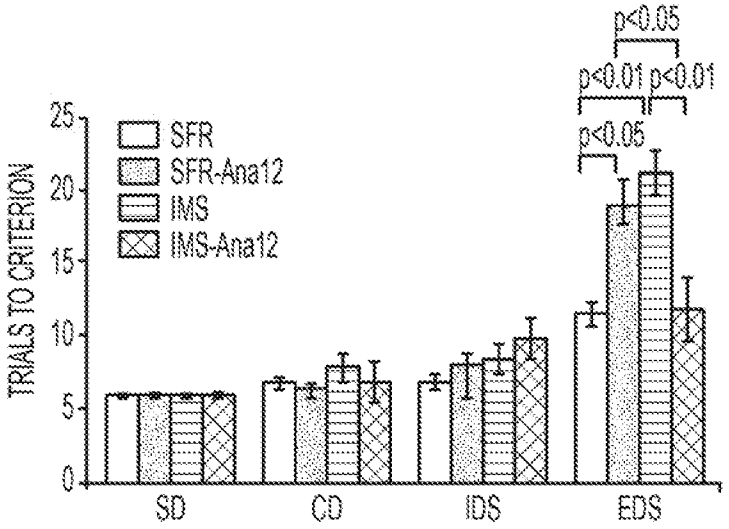
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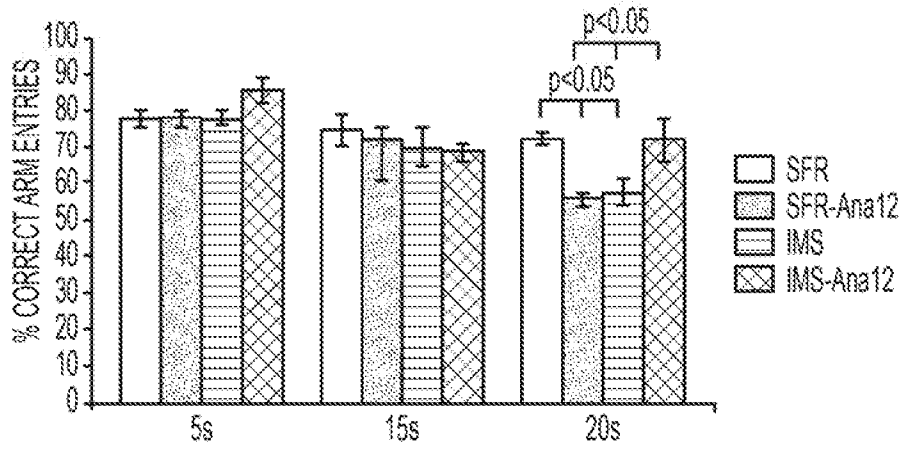
FIG. 3A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IMS
IMS-Ana12

FIG. 3B

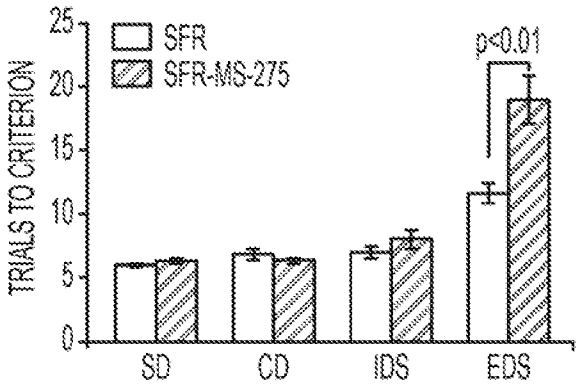

FIG. 4A

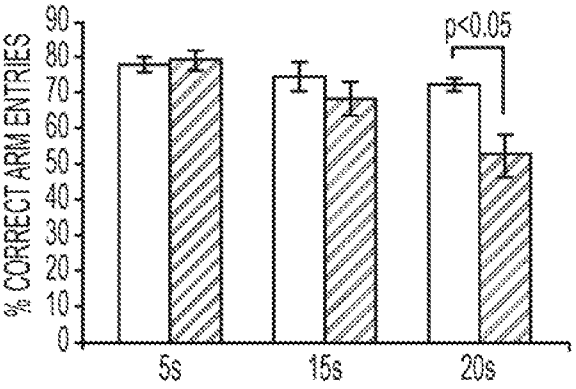

FIG. 4B

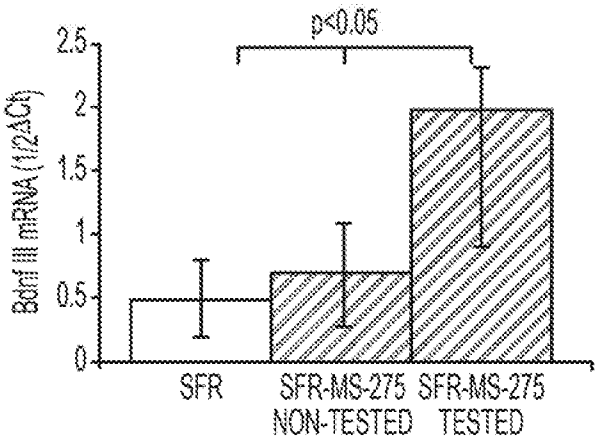

FIG. 4C

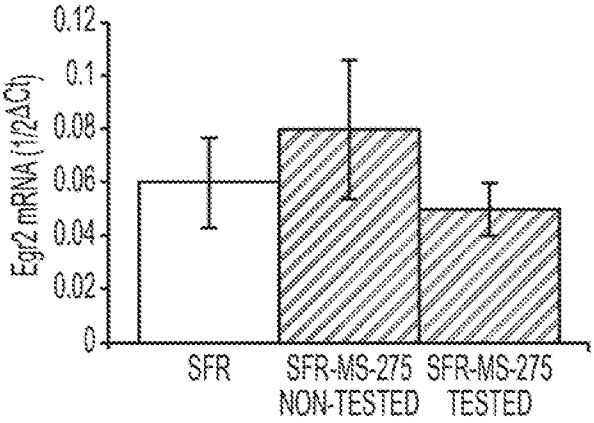

FIG. 4D

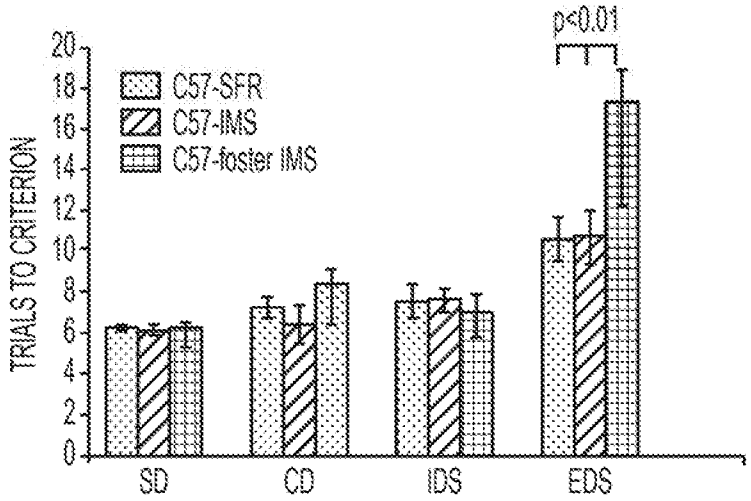
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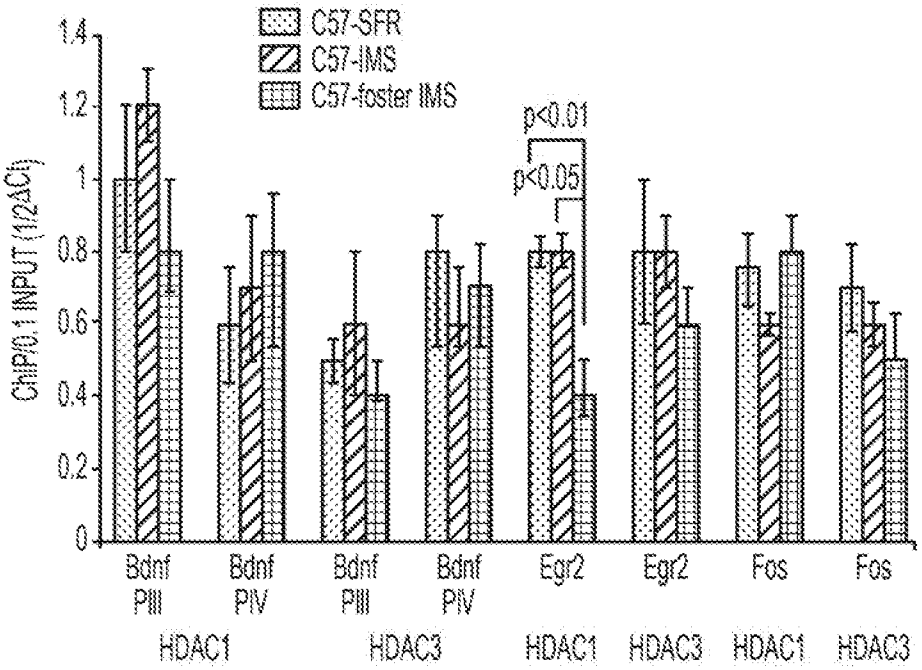
FIG. 5A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Bdnf PIV
Bdnf PIII
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FIG. 5B

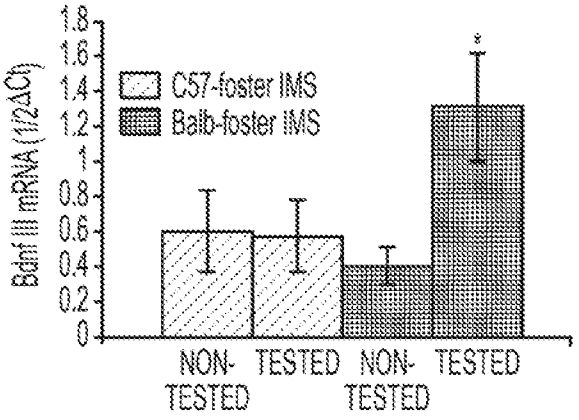

FIG. 6A

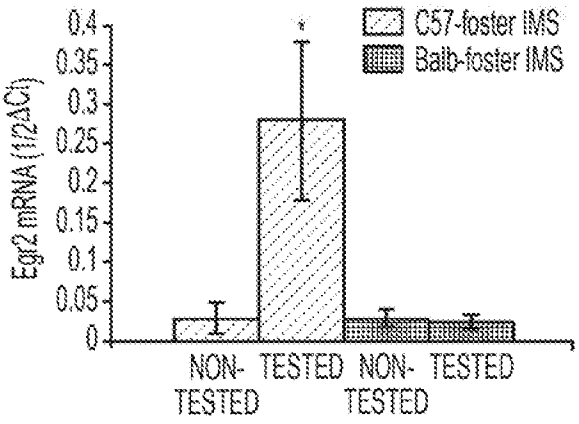

FIG. 6B

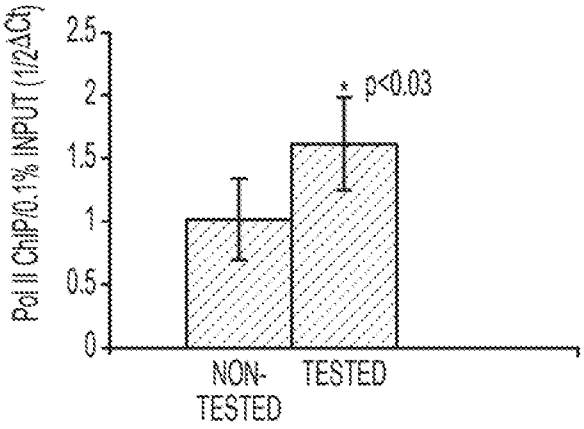

FIG. 6C

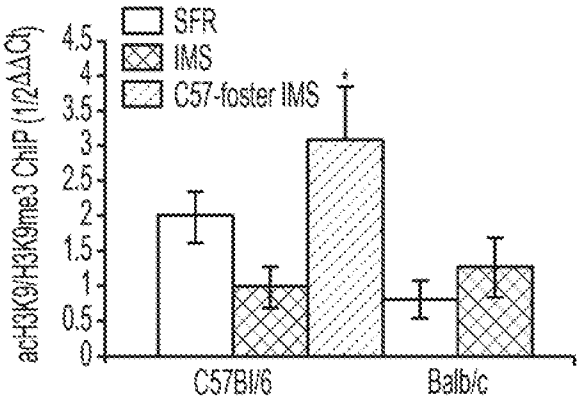

FIG. 6D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/056822

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/056822

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/00; A61P 29/00; A61P 35/00; A61P 43/00; A61P 9/00; A61P 9/10 (2016.01)
CPC - A61K 31/00; A61K 38/00; A61K 38/04; A61K 38/1787 (2016.11)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - A61K 31/00; A61P 29/00; A61P 35/00; A61P 43/00; A61P 9/00; A61P 9/10
CPC - A61K 31/00; A61K 38/00; A61K 38/04; A61K 38/1787

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/143.1; 435/7.1; 436/501 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, PubMed
Search terms used: ((tropomyosin receptor kinase B) OR TrkB OR (tyrosine receptor kinase B) OR NTRK2 OR (neurotrophic receptor tyrosine kinase "2") OR (neurotrophic tyrosine kinase receptor type "2") OR (BDNFNT "3" growth factors receptor) OR ("Trk" WF1 "B") ("Trk" WF1 "B") W8 (inhibit* OR antagonist% OR interfer*) (working memory) OR (task switching) OR (set shifting) OR (cognitive flexibility)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X – Y	US 2011/0236371 A1 (MCNAMARA et al) 29 September 2011 (29.09.2011) entire document	1-8, 11, 12, 14-21 – 9, 22
X	WO 2015/039334 A1 (MERCK SHARP & DOHME CORP. et al) 26 March 2015 (26.03.2015) entire document	1, 10, 14, 23
Y	CAZORLA et al. "Identification of a low–molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice," The Journal of Clinical Investigation, 02 May 2011 (02.05.2011), Vol. 121, No. 5, Pgs. 1846-1857. entire document	9, 22
P, X	ALDER et al. "Cognitive deficits triggered by early life stress: The role of histone deacetylase 1," Neurobiology of Disease, 31 May 2016 (31.05.2016), Vol. 94, Pgs. 1-9. entire document	1-24
A	WO 2010/000675 A1 (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE et al) 07 January 2010 (07.01.2010) entire document	1-24
A	ZHANG et al. "Antidepressant Effects of TrkB Ligands on Depression-Like Behavior and Dendritic Changes in Mice After Inflammation," International Journal of Neuropsychopharmacology, 31 October 2014 (31.10.2014), Vol. 18, Iss. 4, Pgs. 1-12. entire document	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 December 2016	19 DEC 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774