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Gutmann et al.

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(45) **Date of Patent:** **Aug. 10, 2021**

(54) **NEUROFIBROMIN/DOPAMINE SIGNALING AS A BIOMARKER FOR COGNITIVE AND BEHAVIORAL PROBLEMS IN CHILDREN WITH NEUROFIBROMATOSIS TYPE 1 (NF1)**
)

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 patent is extended or adjusted under 35
 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **16/593,316**

(22) Filed: **Oct. 4, 2019**

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Related U.S. Application Data

(63) Continuation of application No. 15/187,298, filed on
 Jun. 20, 2016, now abandoned.

(51) **Int. Cl.**
G01N 33/53 (2006.01)
G01N 33/68 (2006.01)
C12Q 1/6883 (2018.01)

(52) **U.S. Cl.**
 CPC **G01N 33/6893** (2013.01); **C12Q 1/6883**
 (2013.01); **C12Q 2600/156** (2013.01); **C12Q**
2600/158 (2013.01); **G01N 2333/4704**
 (2013.01); **G01N 2440/14** (2013.01); **G01N**
2800/30 (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — James Martinell

(74) *Attorney, Agent, or Firm* — Stinson LLP

(57) **ABSTRACT**

The present disclosure is generally related to neurofibroma-
 tosis type 1. More particularly, disclosed herein are methods
 for detecting behavioral disorders, methods for detecting
 cognitive impairment, and methods for detecting brain neu-
 rofibromin-dependent dopaminergic signaling associated
 with neurofibromatosis type 1.

19 Claims, 15 Drawing Sheets
(5 of 15 Drawing Sheet(s) Filed in Color)

Specification includes a Sequence Listing.

FIG. 1A

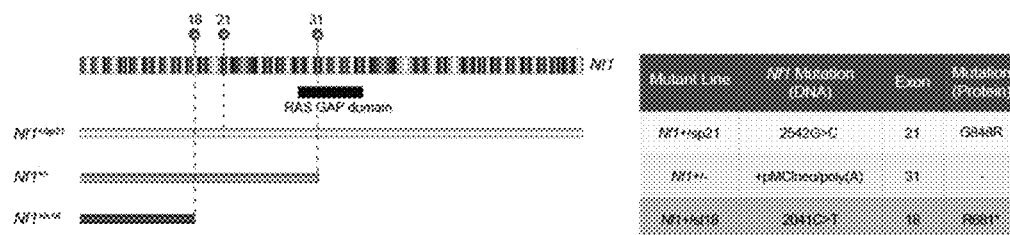


FIG. 1B

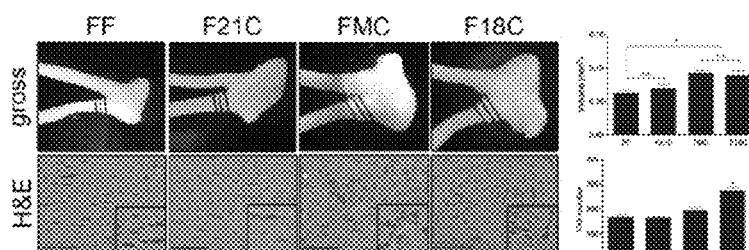


FIG. 1C

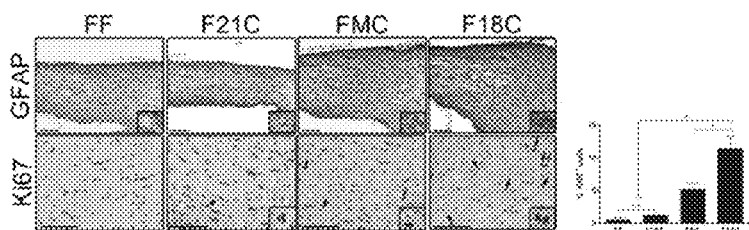


FIG. 1D

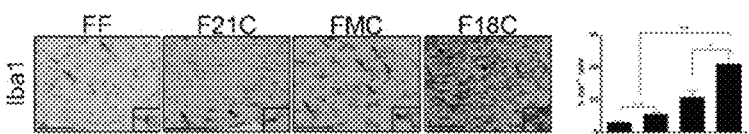


FIG. 1E

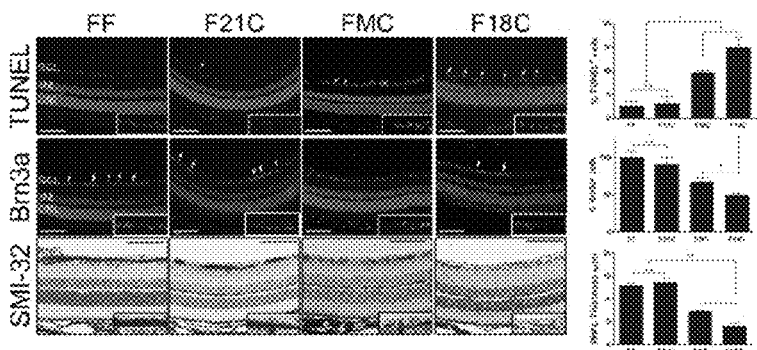


FIG. 2A

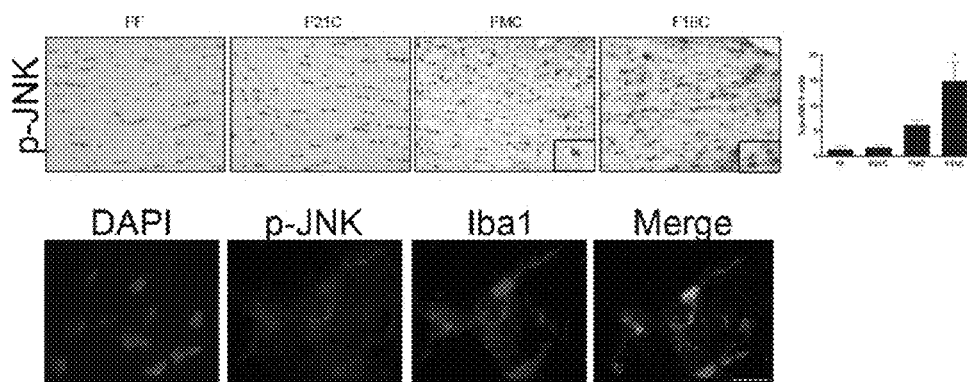


FIG. 2B

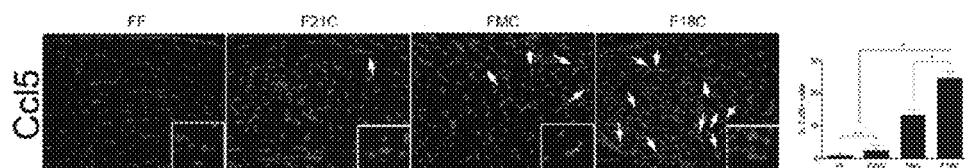


FIG. 2C

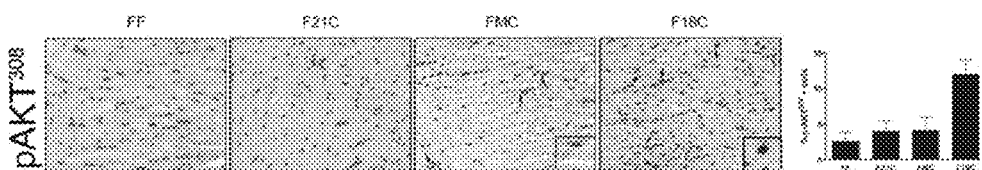


FIG. 2D

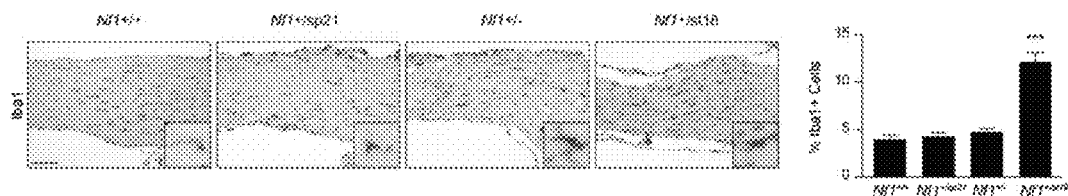


FIG. 3A



FIG. 3B

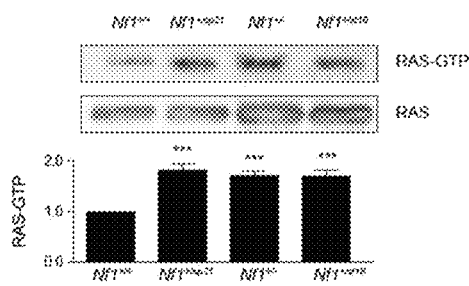


FIG. 3C

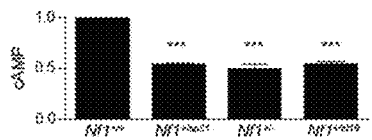


FIG. 4A

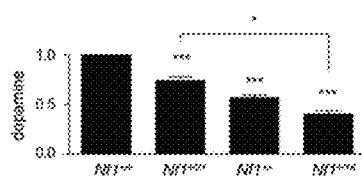


FIG. 4B

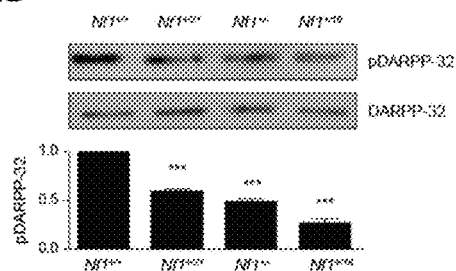


FIG. 4C

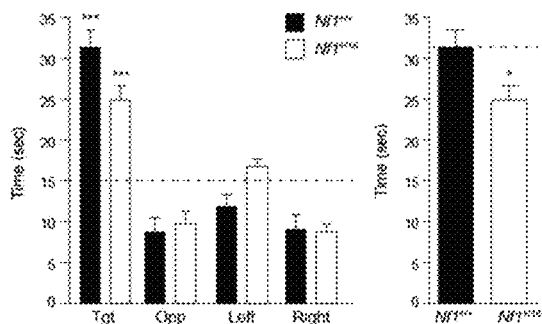


FIG. 4D

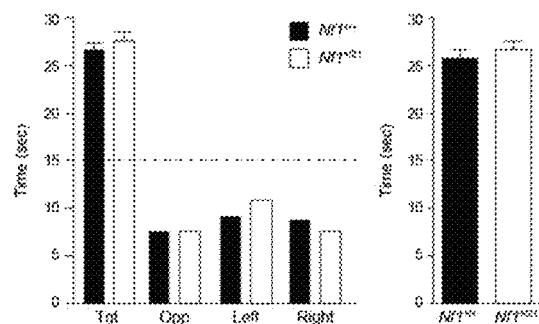


FIG. 4E

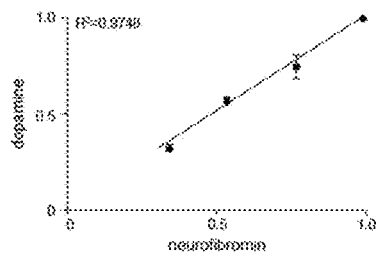


FIG. 4F

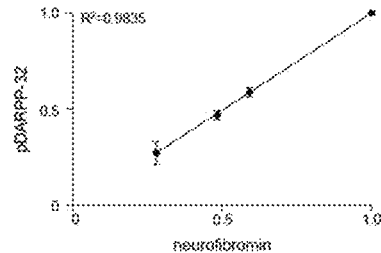


FIG. 5A

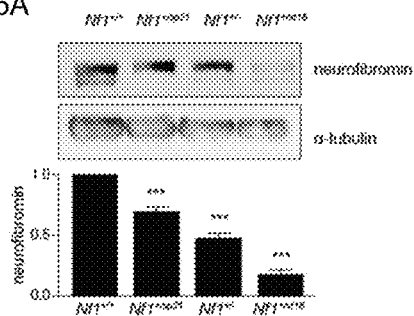


FIG. 5B

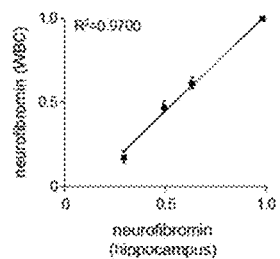


FIG. 5C

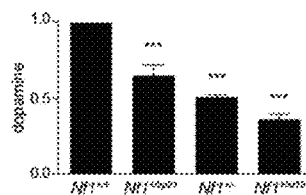


FIG. 5D

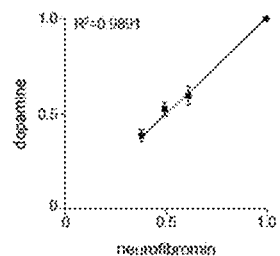


FIG. 5E

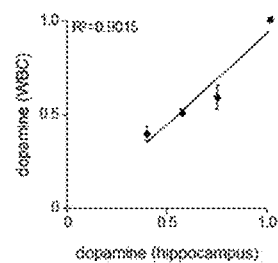


FIG. 5F

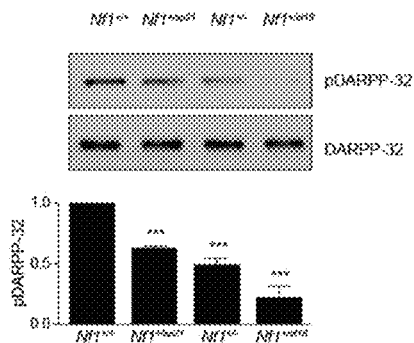


FIG. 5G

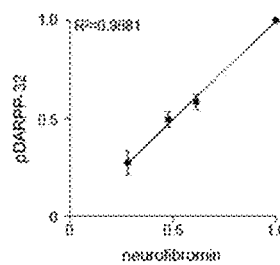
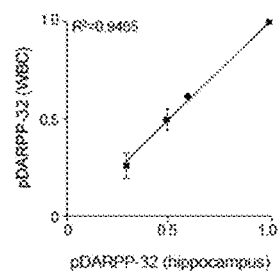


FIG. 5H



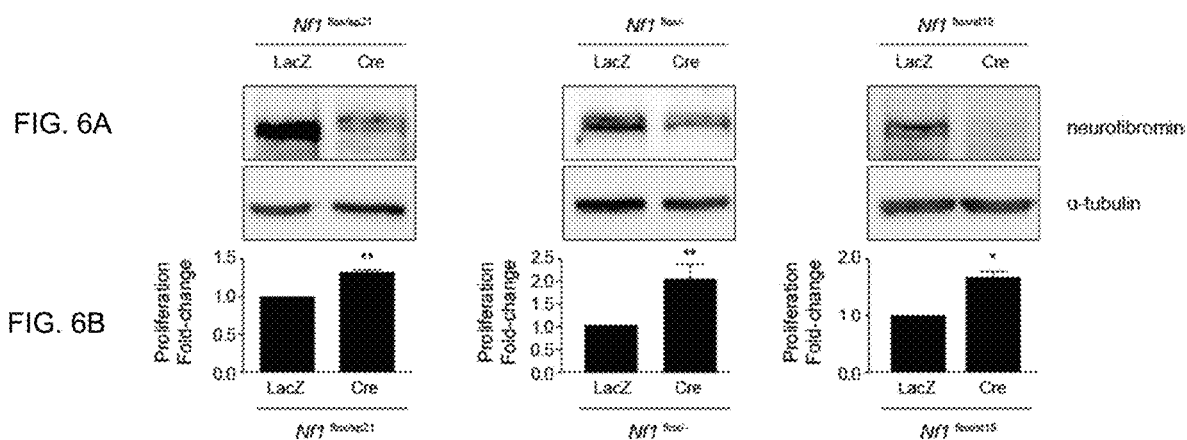


FIG. 7A

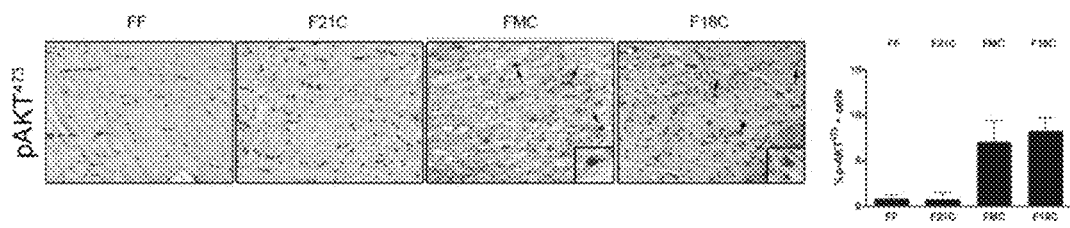


FIG. 7B

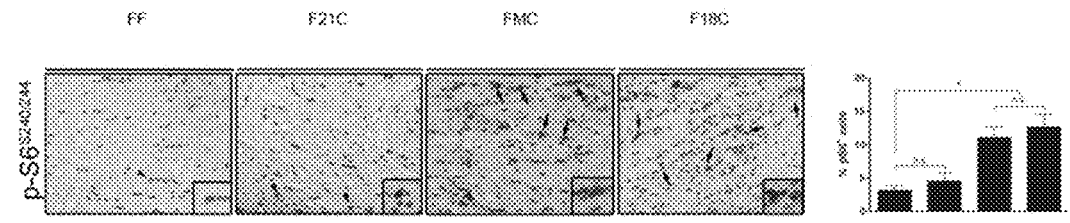


FIG. 8

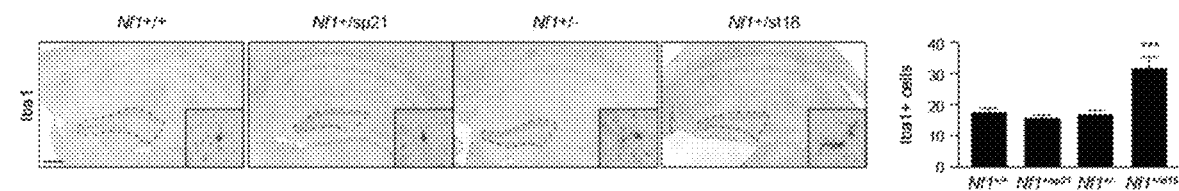


FIG. 9A

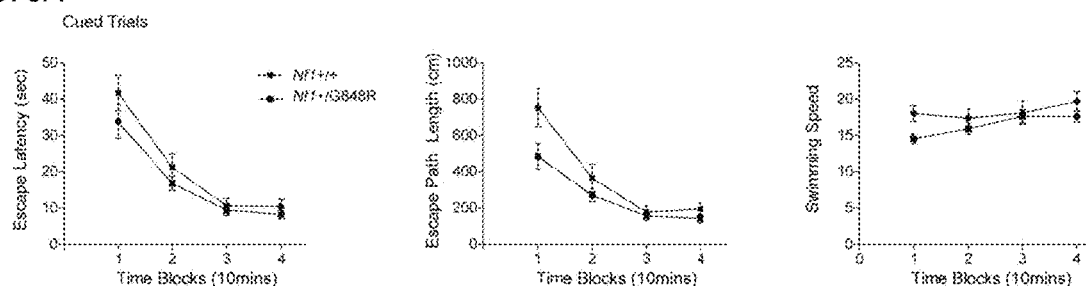


FIG. 9B

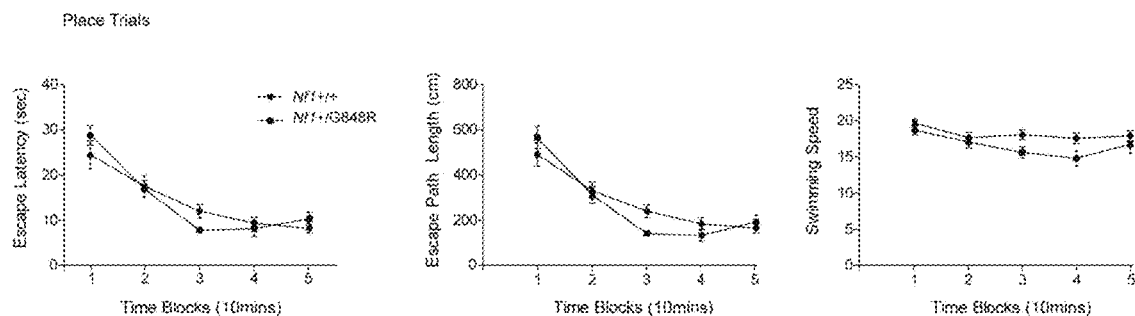


FIG. 9C

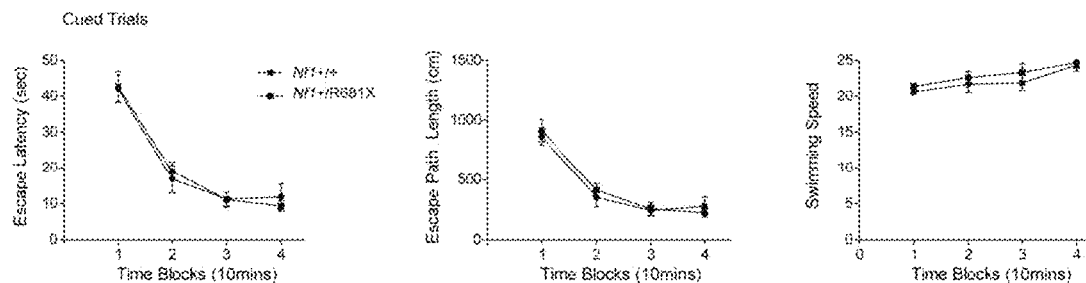


FIG. 9D

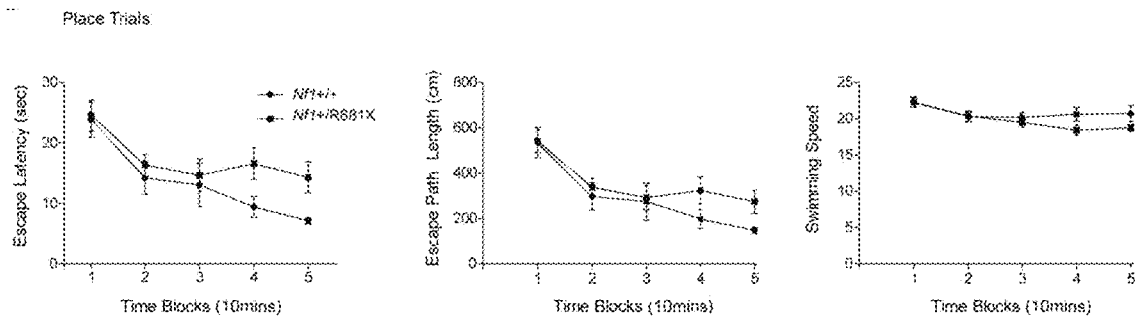


FIG. 10A

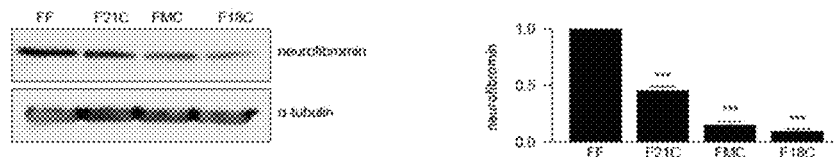


FIG. 10B

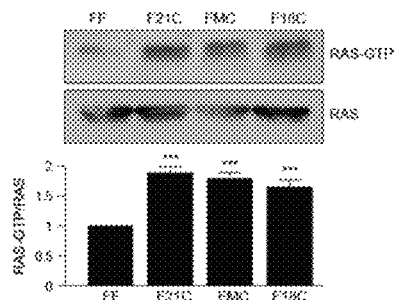


FIG. 10C

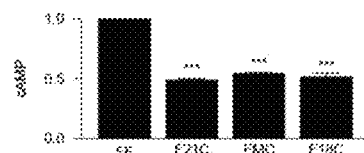


FIG. 10D

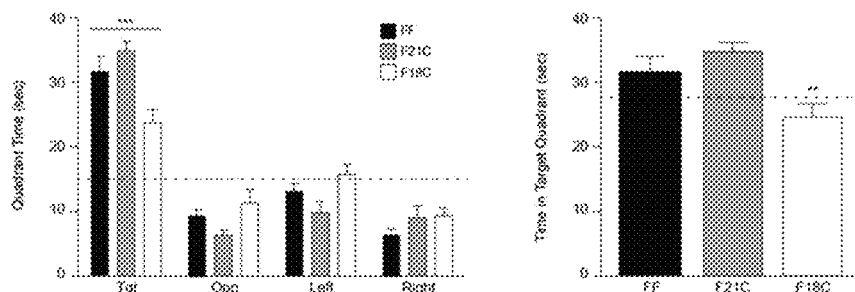


FIG. 10E

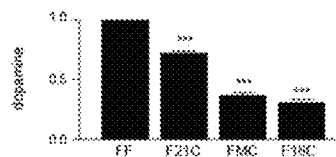


FIG. 10F

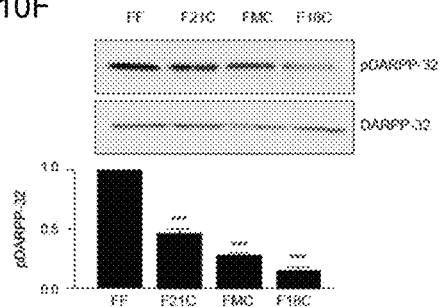


FIG. 11A

Cued Trials

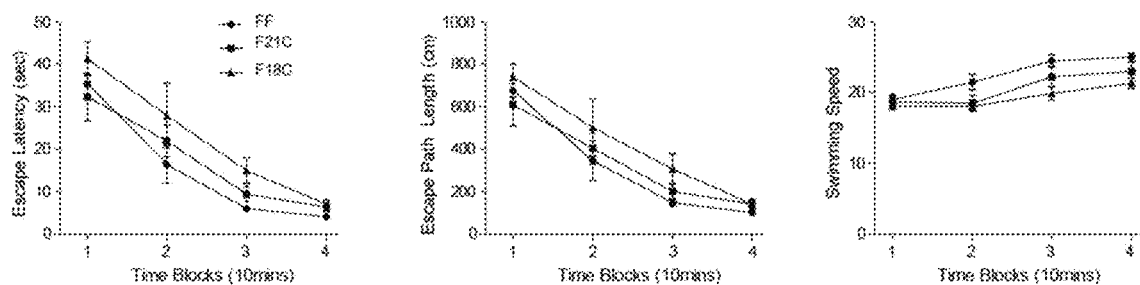


FIG. 11B

Place Trials

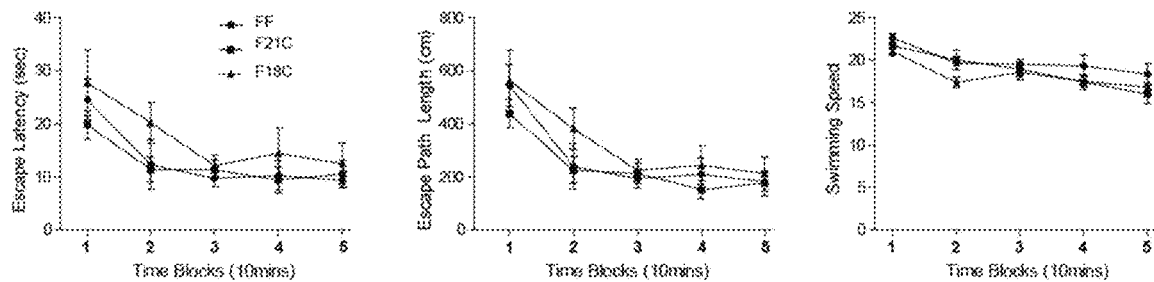


FIG. 12A

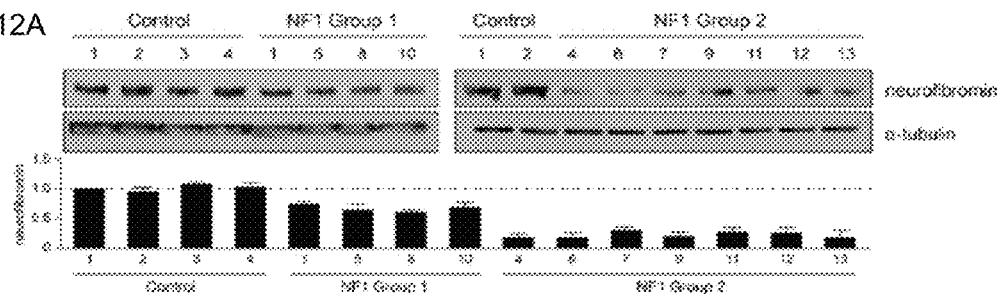


FIG. 12B

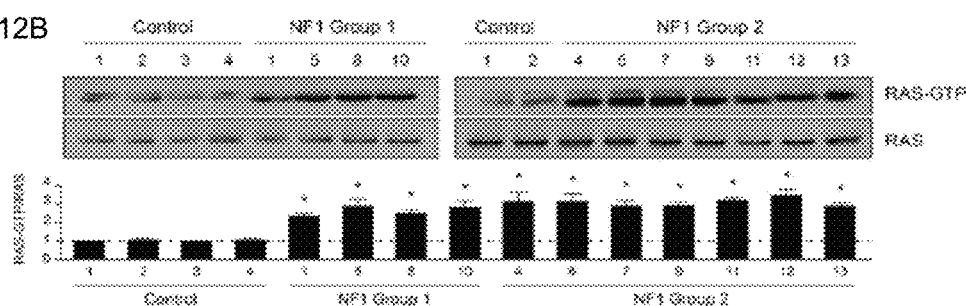


FIG. 12C

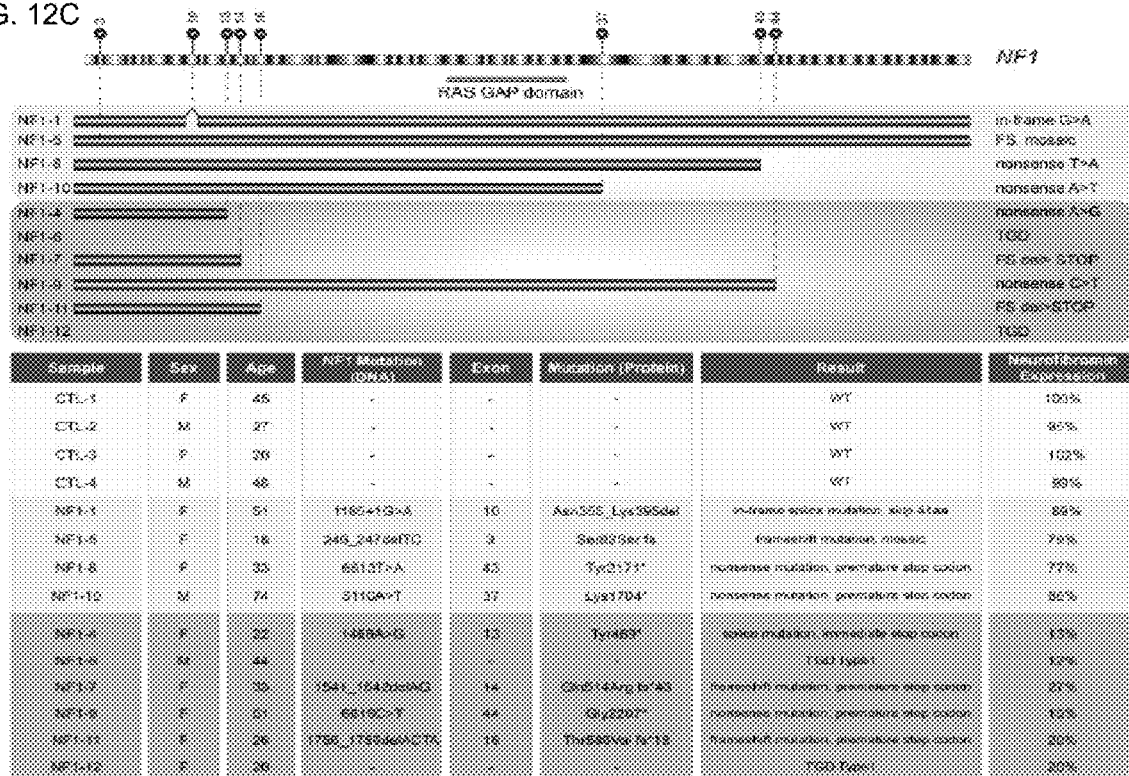


FIG. 13A

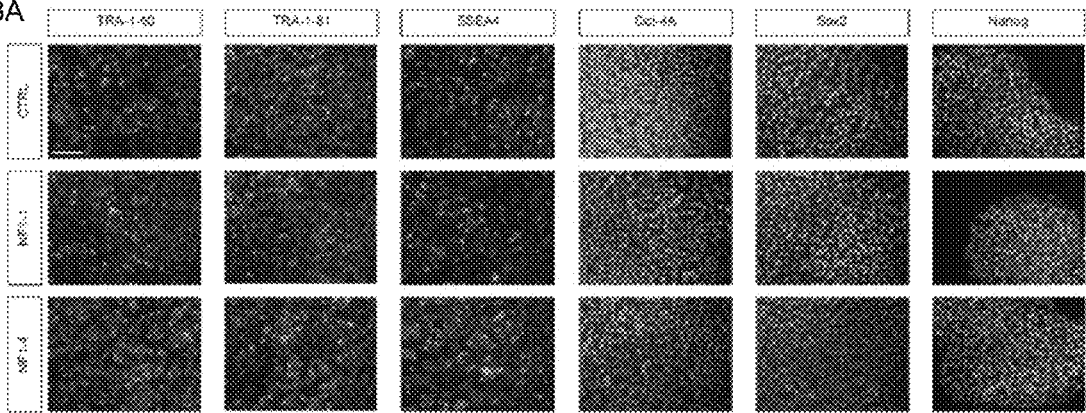


FIG. 13B

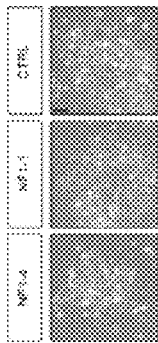


FIG. 13C

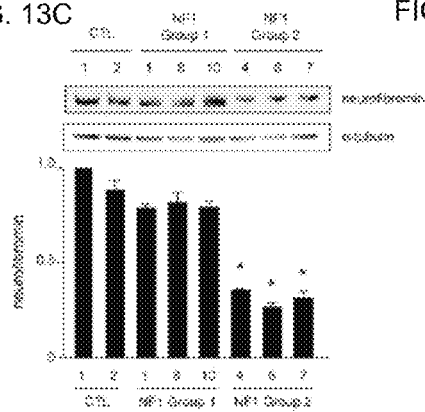


FIG. 13D

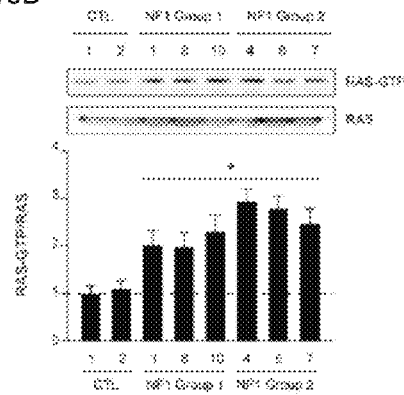


FIG. 14A

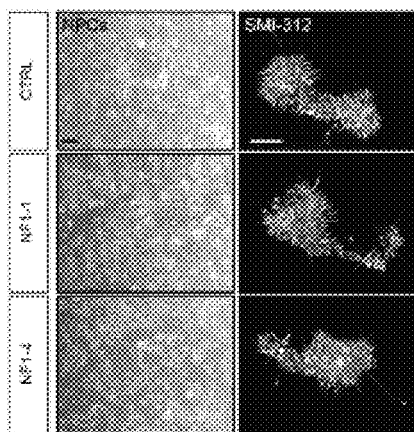


FIG. 14B

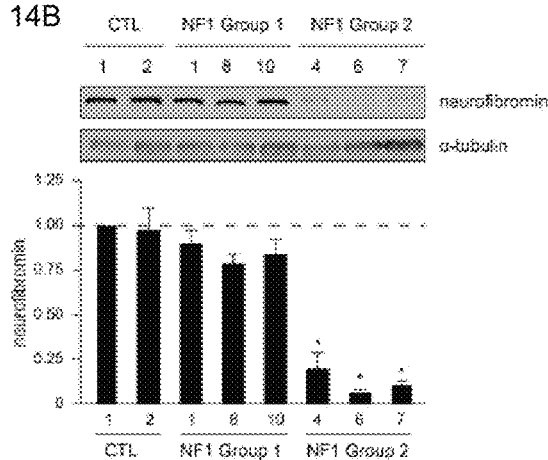


FIG. 14C

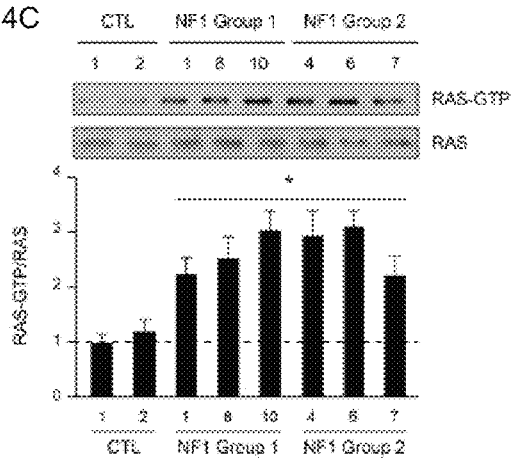


FIG. 14D

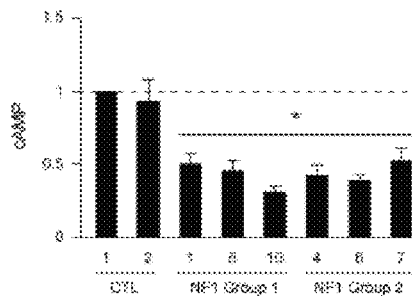


FIG. 15A

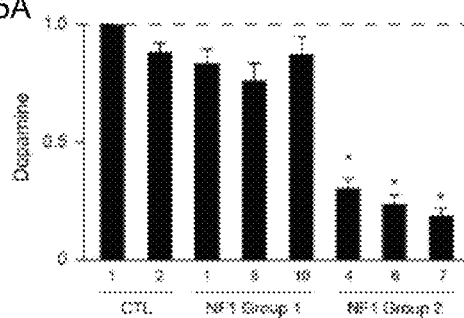


FIG. 15B

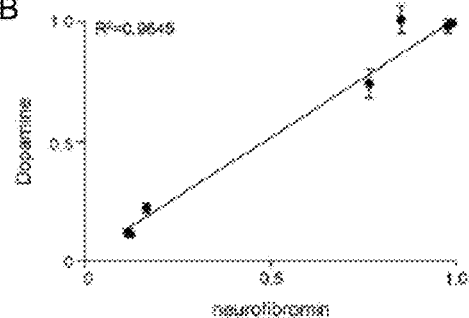


FIG. 15C

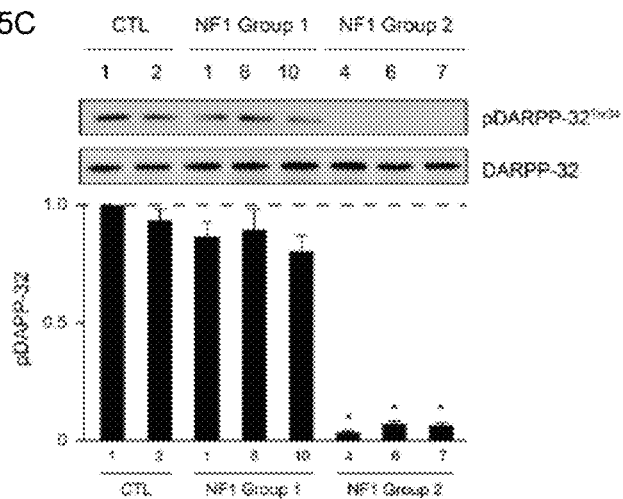
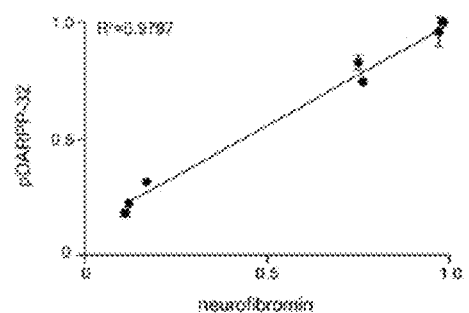


FIG. 15D



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NEUROFIBROMIN/DOPAMINE SIGNALING AS A BIOMARKER FOR COGNITIVE AND BEHAVIORAL PROBLEMS IN CHILDREN WITH NEUROFIBROMATOSIS TYPE 1 (NF1)

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation application of U.S. Patent Publication No. 2016/0370383, filed on Jun. 20, 2016 (abandoned), which claims priority under 35 U.S.C § 119(e) to U.S. Provisional Application No. 62/181,949, filed on Jun. 19, 2015, each of which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under 5-T32-EY013360, NS007205, and CA195692 awarded by The National Institutes of Health. The Government has certain rights in the invention.

INCORPORATION OF SEQUENCE LISTING

A paper copy of the Sequence Listing and a computer readable form of the Sequence Listing containing the file named "WUSTL015422_5 T25.txt", which is 82,262 bytes in size (as measured in MICROSOFT WINDOWS® EXPLORER), are provided herein and are herein incorporated by reference. This Sequence Listing consists of SEQ ID NO:1-4.

BACKGROUND OF THE DISCLOSURE

The present disclosure relates generally to neurofibromatosis type 1. More particularly, the present disclosure is directed to methods for detecting behavioral disorders, methods for detecting cognitive impairment, and methods for detecting neurofibromin-dependent dopaminergic signaling associated with neurofibromatosis type 1.

Neurofibromatosis type 1 (NF1) is a monogenic neurodevelopmental disorder affecting ~1 in 2500 individuals worldwide. Greater than 50% of individuals with NF1 exhibit cognitive deficits, which affect scholastic abilities and impact quality of life. These cognitive impairments include specific learning disabilities, attention deficits, autistic-like behaviors and visuospatial learning/memory problems. The specific cognitive symptoms as well as their severity vary greatly among individuals with NF1. Some children with NF1 can have problems with reading or mathematic achievement, while others exhibit deficits in visual perception or response inhibition. There may be cellular and molecular etiologies for these cognitive delays, which may explain why a small number of children respond to targeted therapeutic interventions.

Neurofibromin functions as a negative regulator of RAS and a positive regulator of dopamine homeostasis. High levels of RAS activation and low levels of dopamine have been reported in the brains of Nf1 genetically-engineered mice. Treatments such as lovastatin (a RAS inactivator) or dopamine uptake blockers can ameliorate the spatial learning and memory deficits in mice.

A challenge to the management of children with NF1 is the lack of predictive markers to identify individuals with a risk for specific morbidities. Evidence from early-phase NF1

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gene mutation studies has suggested that some types of germline NF1 gene mutations may be associated with certain clinical features. Cognitive problems in neurological disorders can be difficult to identify, especially in non-verbal or young children. Accordingly, there exists a need to identify predictive markers for disease phenotypes in NF1 patients.

BRIEF DESCRIPTION OF THE DISCLOSURE

The present disclosure is generally related to neurofibromatosis type 1. More particularly, the present disclosure is directed to methods for detecting behavioral disorders, methods for detecting cognitive impairment, and methods for detecting neurofibromin-dependent dopaminergic signaling associated with neurofibromatosis type 1.

In one aspect, the present disclosure is directed to a method for detecting a behavioral disorder in a subject having or suspected of having neurofibromatosis type 1 (NF1). The method comprises obtaining an expression level of neurofibromin in a sample obtained from the subject having or suspected of having neurofibromatosis type 1 (NF1); obtaining a reference expression level of neurofibromin from a healthy subject; identifying a difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin, wherein the difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin indicates a memory defect in a subject having or suspected of having neurofibromatosis type 1 (NF1).

In another aspect, the present disclosure is directed to a method for detecting a cognitive impairment in a subject having or suspected of having neurofibromatosis type 1 (NF1). The method comprises: obtaining an expression level of neurofibromin in a sample obtained from the subject having or suspected of having neurofibromatosis type 1 (NF1); obtaining a reference expression level of neurofibromin from a healthy subject; identifying a difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin, wherein the difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin indicates a cognitive impairment in a subject having or suspected of having neurofibromatosis type 1 (NF1).

In another aspect, the present disclosure is directed to a method for detecting altered brain neurofibromin-dependent dopaminergic signaling in a subject having or suspected of having neurofibromatosis type 1 (NF1). The method comprises: obtaining an expression level of neurofibromin in a sample obtained from the subject having or suspected of having neurofibromatosis type 1 (NF1); obtaining a reference expression level of neurofibromin from a healthy subject; identifying a difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin, wherein the difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin indicates a memory defect in a subject having or suspected of having neurofibromatosis type 1 (NF1).

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application

publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

The disclosure will be better understood, and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIGS. 1A-1E depict the influence of Nf1 germline mutations on tumor formation. FIG. 1A is a schematic illustration of the mouse Nf1 gene and summary of the engineered Nf1 mouse germline mutations with genotyping results. Alternate exons and the RAS-GAP domain are indicated. The position of the three introduced germline mutations is depicted above exons 18, 21 and 31. FIG. 1B (top panel) are photographs showing increased optic nerve volumes in FMC and F18C optic nerves compared to controls while no significant changes were detected between F21C and controls; (bottom panel) Hematoxylin and eosin staining of optic nerves reveal hypercellularity, increased mitotic figures and nuclear atypia in FMC and F18C with a significant increase in cell number in F18C mice. FIG. 1C depicts increased GFAP (top) and Ki67 (bottom) immunoreactivity in F18C mice compared to FMC mice. FIG. 1D depicts immunostaining with Iba1 demonstrating a significantly increased number of microglia in the optic nerve of FMC and F18C mice compared to controls. No significant difference was shown in microglia number between F21C and WT controls. Furthermore, F18C mice had significantly more microglia than FMC mice. FIG. 1E depicts an increase in the percentage of TUNEL⁺ cells (top panel), lower percentage of Brn3a⁺ cells (middle panel) and thinning of RNFL (SMI-32 immunostaining) in F18C mice as compared to FMC mice as well as F21C and controls. Scale bar, 100 μ m. All data are represented as means $\pm$ s.e.m.

FIG. 2A-2D depict the regulation of microglial activation and infiltration by Nf1 germline mutation. FIG. 2A (top panel) depicts increased pJNK immunoreactivity in FMC and F18C optic nerves compared to FF controls and F21C optic nerves; (bottom panel) depicts immunofluorescent staining revealing pJNK staining in Iba1 positive cells. FIG. 2B depicts increased CCL5 immunostaining in FMC and F18C optic nerves. FIG. 2C depicts elevation of pAkt.308 only in F18C optic nerves compared to F21C, FMC optic nerves and FF controls. FIG. 2D depicts immunostaining with Iba1 revealing significantly increased number of microglia in Nf1^{+/-st18} optic nerves compared to WT (Nf1^{+/+}), Nf1^{+/-sp21} and Nf1^{+/-} mice. Scale bar 100 μ m. Data are represented as means $\pm$ s.e.m. (***P<0.0001; One-way ANOVA with Bonferroni post-test).

FIGS. 3A-3C depict NF1 germline mutation dependence of neuronal neurofibromin expression. FIG. 3A depicts immunoblot analysis of neurofibromin in hippocampi of wild-type (Nf1^{+/+}; WT) and heterozygous mutant Nf1 mice: Nf1^{+/-sp21}, Nf1^{+/-} and Nf1^{+/-st18}. Neurofibromin expression was significantly reduced by 39%, 50% and 76% in Nf1^{+/-sp21}, Nf1^{+/-} and Nf1^{+/-st18} mice, respectively, compared to controls. α -tubulin was used as an internal loading control. FIG. 3B depicts elevation of RAS activity (RAS-GTP) in Nf1^{+/-sp21}, Nf1^{+/-} and Nf1^{+/-st18} hippocampi compared to controls. Total RAS was used as a loading control. FIG. 3C depicts reduction in cAMP levels in Nf1^{+/-sp21/+}, Nf1^{+/-} and Nf1^{+/-st18} hippocampi compared to WT controls.

FIGS. 4A-4D depict elicitation of memory deficit in Nf1^{+/-st18} mice by Nf1 germline mutation. FIG. 4A depicts the decrease in dopamine levels in all Nf1 mutant mice. Dopamine was reduced by 38%, 50% and 64% in Nf1^{+/-sp21}, Nf1^{+/-} and Nf1^{+/-st18} hippocampi, respectively, compared to

WT controls. FIG. 4B depicts the decrease in DARPP-32 activity (phosphorylation) by 44% in Nf1^{+/-sp21}, 51% in Nf1^{+/-} and 77% in Nf1^{+/-st18} hippocampi, compared to controls. FIGS. 4C & 4D. Left panel: All mice preferentially occupied the target (Tgt) quadrant of the water maze. The dashed line represents chance occupancy of any quadrant. Right panel: Nf1^{+/-st18} mice spent significantly less time in the target quadrant compared to their WT littermate controls (Nf1^{+/+}). Nf1^{+/-sp21} mice showed no memory deficit in the Morris water maze. FIGS. 4E & 4F depict the correlation of relative neurofibromin expression and DA levels (R²=0.9748) (FIG. 4E) or pDARPP-32 levels (R²=0.9835) (FIG. 4F) in Nf1^{+/-} hippocampi. All data are represented as means $\pm$ s.e.m. (FIGS. 4A-4B: ***P<0.0001; *P<0.01; One-way ANOVA with Bonferroni post-test. FIG. 4C: ***P<0.0001; Two-way ANOVA with Bonferroni post-test; *P<0.01; Student's t-test).

FIGS. 5A-5H depict prediction of brain dopaminergic signaling with blood biomarkers. FIG. 5A depicts a Neurofibromin immunoblot revealing a reduction in neurofibromin expression in white blood cells (WBC) of Nf1^{+/-sp21} (40%), Nf1^{+/-} (50%) and Nf1^{+/-st18} (70%) mice compared to WT controls (Nf1^{+/+}). FIG. 5B depicts the correlation of relative neurofibromin expression detected in hippocampi and WBC (R²=0.9700). FIG. 5C depicts the decrease of dopamine (DA) levels in Nf1^{+/-sp21} (40%), Nf1^{+/-} (50%) and Nf1^{+/-st18} (60%) WBC compared to WT controls. FIG. 5D depicts the correlation of relative neurofibromin expression and DA levels in WBC samples (R²=0.9881). FIG. 5E depicts the correlation of relative DA levels detected in hippocampi and WBC (R²=0.9015). FIG. 5F depicts the decrease of DARPP-32 phosphorylation in Nf1^{+/-sp21} (40%), Nf1^{+/-} (50%) and Nf1^{+/-st18} (70%) WBC compared to wild-type controls. FIG. 5G depicts the correlation of relative neurofibromin expression and DARPP-32 phosphorylation in WBC (R²=0.9891). FIG. 5H depicts the correlation of relative DARPP-32 phosphorylation detected in hippocampi and WBC (R²=0.9162). Data are represented as means $\pm$ s.e.m. (***P<0.0001; One-way ANOVA with Bonferroni post-test).

FIGS. 6A & 6B depict the effect of all germline Nf1 mutations on astrocyte proliferation. FIG. 6A depicts immunoblot analysis of neurofibromin. FIG. 6B are graphs depicting fold change in proliferation. Astrocyte proliferation was increased upon Nf1 gene inactivation (Cre) in all germline mutation bearing astrocytes compared to heterozygous control astrocytes (LacZ). Data are represented as means $\pm$ s.e.m. (**P<0.001; *P<0.005; Student's t-test).

FIGS. 7A & 7B depict activation of Nf1-mTOR pathway in FMC and F18C optic gliomas. FIG. 7A depicts immunostaining with pAkt^{Ser473} (images in left panel) and percent p-AKT^{Ser473+} cells (graph in right panel) revealing significantly higher immunoreactivity in optic nerves of FMC and F18C animals relative to F21C and FF controls. FIG. 7B depicts immunostaining with p-S6^{Ser240-244} (images in left panel) and percent p-S6^{Ser240-244+} cells (graph in right panel) revealing significantly higher immunoreactivity in optic nerves of FMC and F18C animals relative to F21C and FF controls. Data are represented as means $\pm$ s.e.m. (**P<0.001; *P<0.01; One-way ANOVA with Bonferroni post-test).

FIG. 8 depicts higher microglia infiltration in Nf1^{+/-st18} hippocampi. Immunostaining (images in left panel) and Iba1⁺ cells (graph in right panel) with Iba1 reveals significantly higher number of Iba1 positive microglia in the hippocampi of Nf1^{+/-st18} mice compared to Nf1^{+/+} (WT),

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Nf1^{+/-sp21} and Nf1^{+/-} mice. Data are represented as means±s.e.m. (**P<0.001; *P<0.01; One-way ANOVA with Bonferroni post-test).

FIGS. 9A-9D depict behavioral analysis of Nf1^{+/-sp21} and Nf1^{+/-st18} WT mice, Nf1^{+/-st18} mice, WT mice and Nf1^{+/-sp21} mice. Nf1^{+/-st18} WT mice and Nf1^{+/-st18} mice (FIGS. 9A & 9B), WT mice and Nf1^{+/-sp21} mice (FIGS. 9C & 9D) spent similar time swimming, covered a similar total distance, and exhibited swimming speeds during the cued (FIGS. 9A & 9C) and place (FIGS. 9B & 9D) training trials.

FIGS. 10A-10F depict the effect of Nf1 germline mutation on Neurofibromin/pDARPP-32 dependent spatial memory. FIG. 10A depicts immunoblot of neurofibromin in hippocampi of wild-type (FF), heterozygous null Nf1^{GFAP} flox/sp21, (F21C) and Nf1^{GFAP} flox/st18 (F18C) and null Nf1^{GFAP} flox/- (FMC) mice normalized to α -tubulin. Neurofibromin expression was significantly reduced in F21C, FMC and F18C mice hippocampi, compared to controls. FIG. 10B depicts elevation of RAS-GTP levels in F21C, FMC and F18C hippocampi, compared to WT controls. There was no significant difference between F21C, FMC and F18C hippocampi. FIG. 10C depicts reduced cAMP levels in F21C, FMC and F18C hippocampi compared to WT controls. There was no significant difference between F21C, FMC and F18C hippocampi. FIG. 10D (left panel) depicts the occupation of the target quadrant of the water maze compared to quadrants opposite (Opp) from, to the left of, and to the right of the target quadrant. All mice preferentially occupied the target (Tgt) quadrant of the water maze compared to the quadrants opposite (Opp), left and right to it. The dashed line represents chance occupancy of any quadrant (15 sec). FIG. 10D (right panel) depicts the time mice spent in the target quadrant. F18C mice spent significantly less time in the target quadrant compared to F21C and FF controls. The dashed line represents time spent in the target quadrant by FMC mice. FIG. 10E depicts the reduction in dopamine levels in F21C, FMC and F18C hippocampi compared to controls. FIG. 10F depicts the decrease in DARPP-32 phosphorylation in F21C, FMC and F18C hippocampi compared to controls. All data are represented as means±s.e.m. (***P<0.0001; **P<0.001; One-way/two-way ANOVA with Bonferroni post-test).

FIG. 11A & 11B depict the behavioral analysis of Nf1 CKO mice. WT, F21C and F18C mice spent similar time swimming, covered a similar total distance and exhibited similar swimming speeds during the cued (FIG. 11A) and place (FIG. 11B) training trials.

FIG. 12A-12C depict that germline NF1 gene mutations result in differences in neurofibromin expression in NF1-patient fibroblasts. FIG. 12A is a neurofibromin immunoblot of NF1-patient fibroblasts demonstrating two groups showing reductions of neurofibromin expression. Group 1 patients have a <25% reduction in neurofibromin expression (n=4) and Group 2 patients have a >70% reduction in neurofibromin expression (n=7) when analyzed versus sex- and age-matched control individuals. Tubulin was used as an internal protein loading control. FIG. 12B depicts elevation of RAS activity in all NF1-patient fibroblasts. FIG. 12C is a schematic representation of the NF1 gene with the identified NF1-patient mutations and summary of NF1-patient germline NF1 gene mutations, demographic information and genotyping results. The number of exon mutated is indicated above the mutation location. Light and dark boxes indicate alternate exons. Horizontal bars represent the length of the predicted transcribed mRNA. TGD, total genomic deletion;

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FS, frame shift mutation. All data are represented as means±SEM (*P<0.0001, one-way ANOVA with Bonferroni post-test).

FIG. 13A-13D depict that germline NF1 gene mutations promote differential neurofibromin expression in NF1-patient-derived iPSCs. FIG. 13A depicts immunostaining of iPSC cultures with stem cell-specific antibodies confirming the pluripotent nature of the cultured iPSCs. Scale bar, 50 μ m. FIG. 13B depicts bright-field images of representative iPSCs. Scale bar, 100 μ m. FIG. 13C depicts the reduction of neurofibromin expression in Group 2 NF1-patient-derived iPSCs. FIG. 13D depicts the elevated RAS activity levels (RAS-GTP) in all NF1-patient-derived iPSCs relative to control iPSC lines. All data are represented as means±SEM (*P<0.0001, one-way ANOVA with Bonferroni post-test).

FIG. 14A-14D depict that germline NF1 gene mutations promote differential neurofibromin expression in NF1-patient-derived NPCs. FIG. 14A are bright-field images and immunostaining of NPC cultures with the SMI-312 pan-axonal (neuronal) antibody. Scale bar left panel, 100 μ m. Scale bar right panel, 50 μ m. FIG. 14B depicts that neurofibromin expression was only significantly different in Group 2 NF1-patient samples relative to control NPCs. FIG. 14C depicts the elevated RAS activity levels in all NF1-patient NPCs. FIG. 14D depicts the decreased cAMP levels in all NF1-patient NPCs relative to control NPCs.

FIG. 15A-15D depict that germline NF1 gene mutations regulate dopamine signaling in a gene dose-dependent manner in NF1-patient NPCs. FIG. 15A depicts the reduction of dopamine (DA) levels in Group 2 NF1-patient samples relative to control NPCs. FIG. 15B depicts the relationship between neurofibromin expression and DA levels. FIG. 15C depicts the reduction of DARPP-32 activity (phosphorylation) only in Group 2 NF1-patient samples relative to control NPCs. FIG. 15D depicts the relationship between neurofibromin expression and DARPP-32 phosphorylation. All data are represented as means±SEM (*P<0.0001, one-way ANOVA with Bonferroni post-test).

DETAILED DESCRIPTION

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 the disclosure belongs. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred methods and materials are described below.

Disclosed are methods for detecting behavioral disorders, methods for detecting memory defects, and methods for detecting hippocampal neurofibromin-dependent dopaminergic signaling associated with neurofibromatosis type 1.

As used herein, "a subject in need thereof" refers to a subject having, susceptible to or at risk of a specified disease, disorder, or condition. More particularly, in the present disclosure the methods of screening biomarkers is to be used with a subset of subjects who have, are susceptible to or are at an elevated risk for experiencing behavioral disorders, memory defects, and/or altered brain neurofibromin-dependent dopaminergic signaling associated with neurofibromatosis type 1. Such subjects can be susceptible to or at elevated risk for behavioral disorders, memory defects, and/or altered brain neurofibromin-dependent dopaminergic signaling due to family history, age, environment, and/or lifestyle.

Based on the foregoing, because some of the method embodiments of the present disclosure are directed to spe-

cific subsets or subclasses of identified subjects (that is, the subset or subclass of subjects “in need” of assistance in addressing one or more specific conditions noted herein), not all subjects will fall within the subset or subclass of subjects as described herein for certain diseases, disorders or conditions.

As used herein, “susceptible” and “at risk” refer to having little resistance to a certain disease, disorder or condition, including being genetically predisposed, having a family history of, and/or having symptoms of the disease, disorder or condition.

As used herein, “expression level of a biomarker” refers to the process by which a gene product is synthesized from a gene encoding the biomarker as known by those skilled in the art. The gene product can be, for example, RNA (ribonucleic acid) and protein. Expression level can be quantitatively measured by methods known by those skilled in the art such as, for example, northern blotting, amplification, polymerase chain reaction, microarray analysis, tag-based technologies (e.g., serial analysis of gene expression and next generation sequencing such as whole transcriptome shotgun sequencing or RNA-Seq), Western blotting, enzyme linked immunosorbent assay (ELISA), and combinations thereof.

As used herein, “a reference expression level of a biomarker” refers to the expression level of a biomarker established for a subject without neurofibromatosis type 1, expression level of a biomarker in a normal/healthy subject without neurofibromatosis type 1 as determined by one skilled in the art using established methods as described herein, and/or a known expression level of a biomarker obtained from literature. The reference expression level of the biomarker can also refer to the expression level of the biomarker established for any combination of subjects such as a subject without neurofibromatosis type 1, expression level of the biomarker in a normal/healthy subject without neurofibromatosis type 1, and expression level of the biomarker for a subject without neurofibromatosis type 1 at the time the sample is obtained from the subject, but who later exhibits without neurofibromatosis type 1. The reference expression level of the biomarker can also refer to the expression level of the biomarker obtained from the subject to which the method is applied. As such, the change within a subject from visit to visit can indicate an increased or decreased risk for neurofibromatosis type 1. For example, a plurality of expression levels of a biomarker can be obtained from a plurality of samples obtained from the same subject and used to identify differences between the pluralities of expression levels in each sample. Thus, in some embodiments, two or more samples obtained from the same subject can provide an expression level(s) of a blood biomarker and a reference expression level(s) of the blood biomarker.

In one aspect, the present disclosure is directed to a method for detecting a behavioral disorder in a subject having or suspected of having neurofibromatosis type 1 (NF1). The method includes: obtaining an expression level of neurofibromin in a sample obtained from the subject having or suspected of having neurofibromatosis type 1 (NF1); obtaining a reference expression level of neurofibromin from a healthy subject; identifying a difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin, wherein the difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the

neurofibromin indicates a behavioral disorder in a subject having or suspected of having neurofibromatosis type 1 (NF1).

Suitably, the expression level of the neurofibromin in the sample obtained from the subject is reduced when analyzed against the reference expression level. In an embodiment where the expression level of neurofibromin is obtained from a hippocampal sample as detected by Western blot analysis, the expression level of neurofibromin can be reduced by greater than 70% versus a reference expression level of neurofibromin obtained from a hippocampal sample obtained from a healthy subject. In another embodiment where the expression level of neurofibromin is obtained from white blood cells from a blood sample as detected by Western blot analysis, the expression level of neurofibromin can be reduced by greater than 70% versus a reference expression level of neurofibromin obtained from white blood cells from a blood sample obtained from a healthy subject.

The method can further include identifying an expression level of dopamine. Suitably, the expression level of the dopamine in the sample obtained from the subject is reduced when analyzed against the reference expression level. In an embodiment where the expression level of dopamine is obtained from a hippocampal sample as detected by Western blot analysis, the expression level of dopamine can be reduced by greater than 70% versus a reference expression level of dopamine obtained from a hippocampal sample obtained from a healthy subject. In an embodiment where the expression level of dopamine is obtained from white blood cells from a blood sample as detected by Western blot analysis, the expression level of dopamine can be reduced by greater than 70% versus a reference expression level of dopamine obtained from white blood cells from a blood sample obtained from a healthy subject.

The method can further include identifying phosphorylation of dopamine and cAMP regulated neuronal phosphoprotein (DARPPP-32). Suitably, the phosphorylation the DARPPP-32 in the sample obtained from the subject is reduced when analyzed against phosphorylation of DARPPP-32 obtained from a hippocampal sample obtained from a healthy subject. In an embodiment where the expression level of neurofibromin is obtained from a hippocampal sample as detected by Western blot analysis, the expression level of DARPPP-32 can be reduced by greater than 70% versus a reference expression level of DARPPP-32 obtained from a hippocampal sample obtained from a healthy subject. In an embodiment where the phosphorylation of DARPPP-32 is obtained from white blood cells from a blood sample as detected by Western blot analysis, the phosphorylation of DARPPP-32 can be reduced by greater than 70% versus a phosphorylation of DARPPP-32 obtained from white blood cells from a blood sample obtained from a healthy subject.

In another embodiment, the method can further include detecting a point mutation in an exon of the Nf1 gene. The Nf1 gene can be a human Nf1 gene (see, Accession No. NM_00104292.2) and a mouse Nf1 gene (see, Accession No. NM_010897.2). Suitable exons in which a point mutation can be detected can be exon 18, exon 21, and combinations thereof. A particularly suitable point mutation to be detected in exon 18 is c.2041C>T of the human Nf1 gene (see, Accession No. NM_00104292.2). A particularly suitable point mutation to be detected in exon 21 is c.2542G>C of the human Nf1 gene (see, Accession No. NM_00104292.2).

Suitable samples can be brain, hippocampi, peripheral white blood cells, skin, and combinations thereof.

Suitable subjects can be any vertebrate species. Particularly suitable subjects can be humans and rodents. Suitable humans can be children. Particularly suitable human children include children having or suspected of having neurofibromatosis type 1 (NF1). Particularly suitable rodents include mice.

In another aspect, the present disclosure is directed to a method for detecting a cognitive impairment in a subject having or suspected of having neurofibromatosis type 1 (NF1). The method includes: obtaining an expression level of neurofibromin in a sample obtained from the subject having or suspected of having neurofibromatosis type 1 (NF1); obtaining a reference expression level of neurofibromin from a healthy subject; identifying a difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin, wherein the difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin indicates a cognitive impairment in a subject having or suspected of having neurofibromatosis type 1 (NF1).

Suitably, the expression level of the neurofibromin in the sample obtained from the subject is reduced when analyzed against the reference expression level as described herein.

The method can further include identifying an expression level of dopamine. Suitably, the expression level of the dopamine in the sample obtained from the subject is reduced when analyzed against the reference expression level as described herein.

The method can further include identifying phosphorylation of dopamine and cAMP regulated neuronal phosphoprotein (DARPPP-32). Suitably, the phosphorylation of DARPPP-32 in the sample obtained from the subject is reduced when analyzed against the reference expression level as described herein.

The method can further include detecting a point mutation in an exon of the *Nf1* gene. The *Nf1* gene can be a human *Nf1* gene (see, Accession No. NM_00104292.2) and a mouse *Nf1* gene (see, Accession No. NM_010897.2). Suitable exons in which a point mutation can be detected can be exon 18, exon 21, and combinations thereof. A particularly suitable point mutation to be detected in exon 18 is c.2041C>T of the human *Nf1* gene (see, Accession No. NM_00104292.2). A particularly suitable point mutation to be detected in exon 21 is c.2542G>C of the human *Nf1* gene (see, Accession No. NM_00104292.2).

Suitable samples can be brain, hippocampi, peripheral white blood cells, skin, and combinations thereof.

Suitable subjects can be any vertebrate species. Particularly suitable subjects can be humans and rodents as described herein. Particularly suitable human children include children having or suspected of having neurofibromatosis type 1 (NF1).

Cognitive impairments can be selected from learning disabilities, attention deficits, autistic-like behaviors, visuospatial learning/memory problems and combinations thereof.

In another aspect, the present disclosure is directed to a method for detecting brain neurofibromin-dependent dopaminergic signaling in a subject having or suspected of having neurofibromatosis type 1 (NF1). The method includes: obtaining an expression level of neurofibromin in a sample obtained from the subject having or suspected of having neurofibromatosis type 1 (NF1); obtaining a reference expression level of neurofibromin from a healthy subject; identifying a difference between the expression

level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin, wherein the difference between the expression level of the neurofibromin in the sample obtained from the subject and the reference expression level of the neurofibromin indicates a memory defect in a subject having or suspected of having neurofibromatosis type 1 (NF1).

In one embodiment, the present disclosure is directed to a method for detecting hippocampal neurofibromin-dependent dopaminergic signaling in a subject having or suspected of having neurofibromatosis type 1 (NF1).

Suitably, the expression level of the neurofibromin in the sample obtained from the subject is reduced when analyzed against the reference expression level as described herein.

The method can further include identifying an expression level of dopamine. Suitably, the expression level of the dopamine in the sample obtained from the subject is reduced when analyzed against the reference expression level as described herein.

The method can further include identifying phosphorylation of dopamine and cAMP regulated neuronal phosphoprotein (DARPPP-32). Suitably, the phosphorylation of DARPPP-32 in the sample obtained from the subject is reduced when analyzed against the reference expression level as described herein.

The method can further include detecting a point mutation in an exon of the *Nf1* gene. The *Nf1* gene can be a human *Nf1* gene (see, Accession No. NM_00104292.2) and a mouse *Nf1* gene (see, Accession No. NM_010897.2). Suitable exons in which a point mutation can be detected can be exon 18, exon 21, and combinations thereof. A particularly suitable point mutation to be detected in exon 18 is c.2041C>T of the human *Nf1* gene (see, Accession No. NM_00104292.2). A particularly suitable point mutation to be detected in exon 21 is c.2542G>C of the human *Nf1* gene (see, Accession No. NM_00104292.2).

Suitable samples can be brain, hippocampi, peripheral white blood cells, skin, and combinations thereof.

Suitable subjects can be any vertebrate species. Particularly suitable subjects can be humans and rodents as described herein. Particularly suitable human children include children having or suspected of having neurofibromatosis type 1 (NF1).

EXAMPLES

Materials and Methods

Mice.

All animals were maintained on an inbred C57BL/6 background using a 12 hour light/dark cycle with ad libitum access to food and water. Heterozygous *Nf1* mice were generated with one wild-type copy of the *Nf1* gene and one copy containing either a missense mutation in exon 21 (corresponding to the human c 2542G>C *NF1* gene mutation; p.G848R) (*Nf1*^{+/sp21}), a nonsense mutation in exon 18 (corresponding to the human c 2041C>T *NF1* gene mutation; p.R681X) (*Nf1*^{+/st18}) or a null inactivating allele created by the insertion of a neomycin cassette within exon 31 (*Nf1*^{+/neo31}, *Nf1*^{-/-}). Conditional knockout mice were generated with the *sp21*, *neo31* or *st18* mutation as the germline *Nf1* allele, with somatic *Nf1* gene inactivation resulting from Cre-mediated excision of an *Nf1*^{flox} allele in neuroglial progenitor cells. The resulting strains included *Nf1*^{GFAP} *flox/sp21* (*Nf1* *flox/sp21*; GFAP-Cre, F21C), *Nf1*^{GFAP} *flox/-* (*Nf1* *flox/neo31*; GFAP-Cre, FMC and *Nf1*^{GFAP} *flox/st18* (*Nf1* *flox/st18*; GFAP-Cre, F18C). Littermate *Nf1*^{+/+} and *Nf1*^{flox-flox} (FF) mice were used as controls. All experiments

were performed on 3-month-old animals, unless otherwise stated, under active Animal Studies Committee protocols at the Washington University School of Medicine.

White Blood Cell Isolation.

1-3 mL of fresh mouse peripheral blood was collected from 3-month-old mice by retro-orbital bleed into EDTA-coated vials. An equal volume of PBS was added to the blood before the solution was layered over 3 mL of Ficoll-Paque and fractionated by centrifugation to isolate the white blood cell (WBC) layer. WBCs were further washed by centrifugation, and the resulting cell pellet was snap-frozen and stored at -80°C . A minimum of 10 animals per genotype was used for WBC protein analysis.

Western Blotting, Immunohistochemistry and Immunofluorescence.

Western blotting was performed on snap-frozen hippocampi or WBC pellets, lysed in RIPA buffer supplemented with protease inhibitors as previously described (Brown et al, 2010) using appropriate primary antibodies (Table 1), secondary horseradish peroxidase-conjugated antibodies (Sigma, St. Louis, Mo.) and ECL (Fisher) chemiluminescence. Western signal band intensity was quantified using ImageJ Software (National Institutes of Health, USA). Immunohistochemistry and immunofluorescence were performed as previously described (Brown et al, 2010), on mice transcardially perfused with 4% PFA (Sigma) in 0.1 M sodium phosphate buffer (pH 7.4) and post-fixed in 4% PFA prior to paraffin embedding. For some immunofluorescence experiments (Bmn3a, Ccl5), amplification of the antibody signal was performed using the TSA Cy3 Plus system (Perkin Elmer) as previously described (Kaul, Toonen 2014). A minimum of 5 animals per genotype was used for these analyses.

TABLE 1

Primary Antibodies.				
Antibody	Source	Host animal	Dilution	Application
Neurofibromin	Santa Cruz (sc-67)	Rabbit	1:100	WB
Phospho-DARPP-32 (Thr34)	Santa Cruz	Goat	1:100	WB
DARPP-32	Cell Signaling	Rabbit	1:500	WB
RAS (Clone RAS10)	Millipore	Mouse	1:1,000	WB
α -Tubulin	Life Technologies	Mouse	1:20,000	WB
Iba1	Wako	Rabbit	1:1,000	IHC
Phospho-Akt (Thr308)	Cell Signaling	Rabbit	1:1000	IHC
Phospho-Akt (Ser473)	Cell Signaling	Rabbit	1:1000	IHC
phospho-S6 S240/244	Cell Signaling	Rabbit	1:1000	IHC
Phospho-SAPK/JNK	Cell Signaling	Rabbit	1:1000	IHC
Ccl5	Lifespan Biosciences	Rabbit	1:150	IF
GFAP	Invitrogen	Rat	1:200	IHC
Bmn3a	Santa Cruz	Mouse	1:500	IF
SMI-32	Covance	Mouse	1:500	IHC
cAMP	Abcam	Mouse	1:1000	IF
Ki67				IHC

RAS Activity, cAMP and Dopamine Assays.

Snap-frozen adult mouse hippocampi were used to perform RAS activity (Millipore), dopamine (Rocky Mountain Diagnostics) and cAMP (Enzo Life Sciences) assays. Active Ras (Ras-GTP) was detected by Raf1-RBD immunoprecipitation using the RAS activation kit according to the manufacturer's instructions. Samples processed for cAMP and dopamine ELISA assays were homogenized in ice-cold

0.1M HCl pH7.4 and levels quantitated as previously reported (Anastasaki et al., 2015 PMID: 25788518, Diggs-Andrews et al., 2014).

Behavioral Testing.

For Morris Water Maze testing, mice underwent trial sessions for two consecutive days, where they were trained in a water maze with a visibly-marked, but variably-placed, platform (cued trials). Additionally, mice were trained for 5 consecutive days in a water maze with a hidden platform that had a static location (place trials). Memory retrieval was analyzed in the same water maze 1 hour after the end of the last place trial on the third and fifth days, when the hidden platform was removed. The escape path length (distance traveled to platform) as well as the swimming speed and latency (swimming time to platform) were recorded for all training and probe trials. The time spent in the water maze quadrant containing the former location of the platform (target quadrant) and the spatial bias for the target quadrant (time in target versus other quadrants) were used as readouts for the probe trials. Ten animals per genotype were used for these analyses.

Primary Astrocyte Culture and Proliferation Assay. Primary astrocytes were generated from the brainstems of postnatal day 0-1 mouse pups and maintained in astrocyte growth medium (Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 0.01% penicillin/streptomycin). To inactivate the conditional Nfl^{fllox} allele, astrocytes (passage 1) were infected with adenovirus type 5 (Ad5) containing Cre recombinase (Ad5-Cre) (University of Iowa Gene Transfer Vector Core, Iowa City, Iowa). Control infections employed Ad5 containing β -galactosidase (Ad5-LacZ). 4 days post-infection, astrocytes were passaged and serum-starved for 48 h prior to Western blotting and proliferation analysis. Astrocyte proliferation was assessed using the BrdU Cell Proliferation ELISA kit (Roche) following manufacturer's instructions. Briefly, 6,000 serum-starved astrocytes were labeled with BrdU for 18 hours followed by a 2-hour incubation in peroxidase-conjugated anti-BrdU antibody. Proliferating astrocytes were identified using a colorimetric substrate reaction measured at 450 nM on a spectrophotometer (Bio-Rad).

Statistical Analysis.

All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software). Unpaired two-tailed Student's T-tests were used for experiments analyzing data between two groups. One-way or two-way ANOVA with Bonferroni post-test correction analyses were employed for multiple comparisons.

Optic Nerve Volume Measurements.

Optic nerves with an intact chiasm were microdissected following transcardial perfusion. Optic nerves were photographed and diameters measured at the chiasm (~150, ~300, and ~450 microns anterior to the chiasm) to generate optic nerve volumes. A minimum of five animals per genotype was employed for the measurement of optic nerves.

Retinal Nerve Fiber Layer Measurements.

Retinal nerve fiber layer (RNFL) thickness was quantitated using the average of 15 measurements of SMI-32-stained axons 0-250 μm proximal to the optic nerve head (ImageJ software). A minimum of five animals per genotype was used for this analysis.

Example 1

In this Example, the effect of specific Nfl germline mutations on optic glioma formation was analyzed.

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C57/B16 mice that harbor germline point mutations in exons 21 (sp21) and 18 (st18) were generated. Employing the GFAP-Cre driver line, we generated two new conditional knockout (CKO) mice that have a single germline mutation in all cells and an additional somatic inactivating mutation in neuroglial cells: 2542G>C (exon 21; Nf1 flox/21 GFAP-Cre; F21C) and 2041C>T (exon 18; Nf1 flox/18 GFAP-Cre; F18C) (FIG. 1A). Direct volume measurements of optic nerves revealed a 43% increase in optic nerve volumes of F18C mice, similar to Nf1^{flox/-} GFAP-Cre (FMC; Bajenaru et al, 2003 PMID: 14695164) mice (48% increase), whereas there was no significant increase in optic nerve volume of F21C mice compared to FF controls (FIG. 1B). Additionally, hematoxylin and eosin staining revealed hypercellularity (1.6 fold increase in cell number), mitotic figures and atypical cells in F18C optic nerves, whereas F21C optic nerves were indistinguishable from controls (FIG. 1B). Similarly, an increase in GFAP immunoreactivity was only found in FMC and F18C optic nerves compared to FF controls (FIG. 1C). GFAP immunoreactivity was significantly elevated in F18C compared to FMC mice. Together, these data suggest that tumor formation is Nf1 germline mutation-dependent.

Most children with NF1-OPG come to clinical attention due to vision loss. In fact, up to half of children with NF1-OPG succumb to loss of visual acuity. Previous studies from have shown differential loss of retinal ganglion cells (RGCs) due to tumor aggressiveness (Kaul et al, 2014 PMID 25246427). That the increased tumor growth in F18C mice may have worsened retinal dysfunction was determined. In that respect, a 1.5 fold increase in TUNEL⁺ cells and a 27%±6.5 reduction of Brn3a⁺ retinal ganglion cells in F18C retinae was found as compared to FMC and FF controls (FIG. 1E). Moreover, optical coherence tomography (OCT) has been a useful, non-invasive clinical tool to demonstrate retinal nerve fiber layer (RNFL) thinning in NF1 patients with loss of visual acuity. RNFL thickness was visualized by axon immunostaining for SMI-32 in F18C mice compared to FMC, F21C and controls. Thinning was identified only in FMC and F18C mice (FIG. 1E). F18C had further RNFL thinning compared to FMC mice. Taken together, these data suggest that visual acuity is highly sensitive to Nf1 germline mutation.

Ki67 immunostaining revealed a significant increase in proliferation in F18C mice compared to FF controls (2.2-fold) as well as to FMC mice (2.17-fold) (FIG. 1C). Conversely, there was no significant increase in Ki67⁺ cells in F21C optic nerves. To further determine if astrocyte proliferation was impacted by sp21 and st18 germline mutations upon Nf1 inactivation in a cell-autonomous manner, primary astrocytes were cultured from heterozygous Nf1^{flox/sp21}, Nf1^{flox/-}, Nf1^{flox/st18} brainstem tissue and were either infected with a control virus containing β -galactosidase (LacZ) or Cre recombinase (Cre) (LacZ) (FIG. 6). Upon bi-allelic mutation of Nf1 (F21C; FMC; F18C), astrocyte proliferation was similarly increased in all three groups (1.3-2-fold). Collectively, these data suggest that the specific heterozygous glioma microenvironment is responsible for the observed increase in proliferation in F18C mice in vivo compared to control and F21C mice.

Example 2

In this Example, the effect of Nf1 germline mutation on microglial infiltration and signaling was analyzed.

Non-neoplastic cells in the optic glioma microenvironment, such as Nf1 microglia, contribute to tumor formation,

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growth and maintenance by promoting astrocyte proliferation. In accordance with these findings, an increase in F18C optic nerve microglia (97%±7) was found, but not in F21C optic nerves compared to FMC mice (FIG. 1D) indicating that the specific Nf1 germline mutation contributes to microglia activation and infiltration. Since inactivation of the second Nf1 allele promoted higher proliferation in all astrocyte cultures irrespective if the Nf1 germline mutation (FIG. 6), whether optic nerve tumor formation in FMC and F18C mice is dependent on microglial infiltration and activation rather than astrocyte proliferation was analyzed. Previous studies have revealed that JNK pathway activation (phospho JNK) in microglia is critical for optic nerve formation and maintenance. Immunohistochemical staining showed increased levels of pJNK in optic nerve gliomas of FMC and F18C animals compared to controls (FIG. 2A). Similar to Iba1 staining, F18C optic nerves exhibited significantly higher JNK activation than FMC mice. Additionally, recently published work revealed that tumor associated microglia secrete CCL5 in order to promote glioma formation. As such, significantly elevated CCL5 (FIG. 2B) was observed in F18C when compared to FMC optic nerve gliomas. Finally, CCL-5 can promote activation of Akt through phosphorylation of Ser308. Since this site is phosphorylated independently of the RAS-mTOR pathway which is activated in FMC optic gliomas (FIG. 7), activation of pAkt^{Ser308} in optic nerves of the three mouse models was examined. pAkt^{Ser308} was only significantly elevated (2.9-fold) in F18C optic nerves (FIG. 2C), indicating that the Nf1 St18 mutation promotes tumor formation and increased microglia infiltration in an Nf1-pJNK-CCL5-pAkt^{Ser308}-dependent manner.

Finally, since microglia are affected by the specific Nf1 germline mutation, microglial infiltration of the optic nerve of mice that harbor only a germline Nf1 mutation: Nf1^{+/sp21}, Nf1^{+/+} and Nf1^{+/st18} mice were examined. Immunohistochemical staining with Iba1 revealed a significant increase in microglia numbers in the optic nerves (FIG. 2D) only of Nf1^{+/st18} animals compared to the other two Nf1 mutant mice and wild-type controls. Moreover, a significantly ($P<0.0001$) higher number of microglia were observed in the hippocampi of the same animals (FIG. 8). Collectively, these data suggest that microglia infiltration is highly sensitive to the specific Nf1 germline mutation.

Example 3

In this Example, the dependence of Nf1 germline mutation on Neurofibromin expression levels were analyzed.

Similarly to microglia, neurons are highly sensitive to Nf1 germline mutation. As such, NF1 germline mutation impacts neuronal signaling both in human NPCs and mouse hippocampal neurons. To determine the effect of specific Nf1 germline mutations on mouse neurons, neurofibromin expression levels were assayed in hippocampi of Nf1^{+/sp21}, Nf1^{+/+} and Nf1^{+/st18} animals (FIG. 3A). Western blotting revealed that neurofibromin expression was decreased by 39% in Nf1^{+/sp21}, 50% in Nf1^{+/+} and 76% in Nf1^{+/st18} hippocampi.

To further assess whether the specific germline mutation differentially impacted neurofibromin GAP activity in the three Nf1 models RAS activity (RAS-GTP) in adult mouse hippocampi were assayed. RAS activity was increased by 1.8-fold in Nf1^{+/sp21}, 1.7-fold in Nf1^{+/+} and 1.7-fold in Nf1^{+/st18} hippocampi (FIG. 3B). Since RAS negatively regulates cAMP in Nf1-depleted neurons, cAMP levels were measured in the three mouse models. cAMP levels were

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decreased by 49% in Nf1+/sp21, 51% in Nf1+/- and 52% in Nf1+/st18 hippocampi (FIG. 3C). Thus, similar to human iPSC-NPs, where RAS-cAMP regulation is NF1 dosage-independent, Nf1-RAS-dependent signaling in CNS mouse neurons was not sensitive to neurofibromin expression levels.

Example 4

In this Example, regulation of spatial memory in mice by neurofibromin was analyzed.

Unlike RAS-driven phenotypes, dopamine (DA) signaling, phosphorylation of dopamine and cAMP regulated neuronal phosphoprotein (DARPP-32) is regulated by neurofibromin levels. To this end, DA levels in all Nf1+/- mice were assayed and established that DA was decreased by 38% in Nf1+/sp21, 50% in Nf1+/- and 64% in Nf1+/st18 hippocampi (FIG. 4A), correlating with neurofibromin levels ($R^2=0.9748$) (FIG. 4E). In addition, DARPP-32 activity (pDARPP-32) was decreased by 44%, in Nf1+/sp21, 51% in Nf1+/- and 77% in Nf1+/st18 hippocampi (FIG. 4B), highly correlating with respective neurofibromin expression ($R^2=0.9835$) (FIG. 4F).

Since neurofibromin-dependent dopamine homeostasis and signaling are particularly important in regulating hippocampal-based memory, the performance of Nf1 mutant mice was tested in the Morris water maze (FIGS. 4C & 4D). All animals were able to identify the location of a hidden platform and performed similarly during the "cued" and "place" trials, indicating their intact ability to learn the spatial task (FIG. 9). Moreover, during the "probe" trials of the water maze, Nf1+/sp21 showed no memory deficits compared to WT controls (FIG. 4D). However, Nf1+/st18 mice spent significantly less time in the target quadrant, indicating a deficit in memory retrieval compared to control mice.

Consistent with the findings in hemizygous Nf1+/- mice, inactivation of the second Nf1 allele reduced neurofibromin levels in hippocampi of F21C, FMC and F18C mice by 52%, 73% and 82% respectively compared to FF controls (FIG. 10A). Similarly, DA levels as well as pDARPP-32 were decreased in a neurofibromin dose-dependent manner (FIGS. 10E & 10F). As such, pDARPP-32 levels were decreased by 52% in F21C, 74% in FMC and 83% in F18C hippocampi (FIG. 10F). Moreover, only F18C mice exhibited a spatial memory deficit in the Morris water maze (FIG. 10D), further underscoring the importance of Nf1 germline mutation-driven neurofibromin levels in eliciting behavioral phenotypes.

Example 5

In this Example, Neurofibromin, DA and pDARPP-32 were analyzed as biomarkers of brain neuronal function.

To assess whether peripheral neurofibromin, DA and pDARPP-32 can be used as biomarkers of brain neurofibromin-dependent dopaminergic signaling, white blood cells (WBC) were isolated from peripheral blood of Nf1 heterozygous mice (FIG. 5A). Upon neurofibromin immunoblotting, a significant correlation ($R^2_{Nf1}=0.9700$) between WBC and hippocampal neurofibromin levels was observed (FIG. 5B). Similarly, DA concentration in WBC correlates (FIG. 5C) with WBC neurofibromin ($R^2=0.9891$) (FIG. 5D) and hippocampal DA ($R^2=0.9015$) (FIG. 5E). Finally, DARPP-32 phosphorylation in leukocytes (FIG. 5F) correlates with WBC neurofibromin ($R^2_{Nf1}=0.9881$) (FIG. 5G) and is highly predictive of hippocampal pDARPP-32

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($R^2=0.9465$) (FIG. 5H). Collectively these data establish neurofibromin, DA and pDARPP-32 as novel blood biomarkers of memory defects in Nf1 models.

Example 6

In this Example, neurofibromin levels were analyzed in patients diagnosed with NF1.

Thirteen adult patients diagnosed with NF1 using NIH Consensus Development Conference diagnostic criteria who receive their medical care in the Washington University in St. Louis Children's Hospital Neurofibromatosis Clinical Program were randomly selected and underwent a skin punch biopsy under an approved Human Studies Protocol at the Washington University School of Medicine. A collection of primary fibroblast lines was established using unrelated male (n=5) and female (n=8), as well as from four sex- and age-matched control individuals with no known neurological problems. The specific germline NF1 gene mutation was identified following mutational analysis of DNA and RNA extracted from primary skin fibroblasts using an RNA-core assay complemented with dose analysis by multiplex ligation-dependent probe amplification (MLPA) as described in Messiaen, L. M. W. (In Monogr. Hum. Genet. Karger, Basel, D. K. (ed.), 2008, 16:63-77). NF1 gene nucleotide numbering is based on GenBank reference sequence NM_000267.3 and protein numbering based on NP_000258.1. Exon numbering was assigned according to the NCBI reference sequence along with the known legacy numbering in parenthesis. Nomenclature of the mutations follows the recommendations of the Human Genome Variation Society.

Primary fibroblasts were isolated and cultured from plated skin biopsies collected from patients with NF1 and control individuals for ~3 weeks. Established lines were reprogrammed into iPSCs using Cyto-Tune technology (Invitrogen). Confluent fibroblasts were infected with a Sendai virus carrying four stem cell reprogramming factors (OCT4, KLF4, SOX2, C-MYC). Six weeks later, iPSC colonies were isolated and their pluripotency confirmed by morphological assessment and the expression of stem cell markers (Nanog, SOX2, OCT4, SSEA-4, TRA-1-60, TRA-1-81). Chromosomal analysis demonstrated normal karyotypes in all lines. Two separate clones from each iPSC line were cultured in Neural Induction Medium (NIM; STEMCELL Technologies) for 5 days. Embryoid body aggregates were plated in NIM on adhesive plates pre-coated with poly-ornithine/laminin. Once neural rosettes formed, gentle dissociation and replating facilitated their differentiation into NPCs. A portion of NPCs were differentiated into dopaminergic neurons.

Western blotting was performed using appropriate primary antibodies, secondary horseradish peroxidase-conjugated antibodies (Sigma) and CL (Fisher) chemiluminescence. Neurofibromin (C) antibody (sc-67) was used instead of neurofibromin (N) (sc-68) unless otherwise specified. Immunocytochemistry was performed on 4% paraformaldehyde (PFA) fixed cultured cells.

Analysis of neurofibromin levels revealed two distinct subgroups (FIG. 12A): those with minor reductions (Group 1; <25% following normalization to α -tubulin) and those with >70% reductions (Group 2) in neurofibromin expression. This differential neurofibromin expression pattern was observed using both carboxyl- and amino-terminal neurofibromin antibodies and was not related to NF1 RNA expression levels nor to patient sex, age or the in vitro growth conditions employed (with or without serum; data not

shown). In all NF1-patient fibroblasts, there was increased RAS activity (FIG. 12B) relative to control patient fibroblasts.

To determine whether these differences in neurofibromin protein expression reflected the underlying germline NF1 gene mutation, all NF1-patient fibroblast samples were analyzed (FIG. 12C). In these individuals, 10 distinct mutations were conclusively identified. The mutations in Group 1 causing mild reductions in neurofibromin expression were frameshift (NF1-1, NF1-5) or nonsense mutations (NF1-8, NF1-10) and the mutations in Group 2 with more dramatic reductions in neurofibromin expression included nonsense mutations (NF1-4, NF1-7, NF1-9, NF1-11) and large microdeletions of the NF1 locus (NF1-6, NF1-12). These data indicate that there is no apparent correlation between the location or nature of the germline NF1 mutation and the level of neurofibromin expression.

Leveraging induced pluripotent stem cell (iPSC) technology and integration-free Sendai virus infection, primary skin fibroblasts were reprogrammed into iPSCs (FIG. 13A). Following immunoblot analysis, the same differential pattern of neurofibromin expression in fibroblasts was observed in the derivative iPSCs (FIG. 13C). Consistent with its established role as negative RAS regulator, the levels of active RAS (RAS-GTP) were elevated in all NF1-iPSCs, irrespective of neurofibromin levels (FIG. 13D), similar to the results in NF1-patient fibroblasts.

To examine the effect of the germline NF1 gene mutation on central nervous system lineage cells, iPSC were directed differentiate into neural progenitor cells (NPCs) (FIG. 14A). The neurofibromin expression observed similarly matched the patterns found in the parental fibroblasts and iPSCs (FIG. 14B). Previous work revealed that neurofibromin positively controls cyclic AMP (cAMP) in a RAS-dependent manner in brain neurons. Consistent with this mechanism, all NF1-NPCs exhibited 2-3-fold elevated RAS-GTP levels (FIG. 14C), irrespective of the germline NF1 gene mutation. Similarly all NF1-NPCs showed significantly reduced cAMP (>50%) levels relative to control NPCs (FIG. 14D).

In contrast, neurofibromin control of DA homeostasis in the striatum and hippocampus is RAS-independent. As such, Group 1 NF1-NPCs exhibited <25% reductions in DA levels, while Group 2 NF1-NPCs had ~75% reductions (FIG. 15A). Importantly, phosphorylation of the DA downstream effector, DA and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), was only significantly reduced in Group 2 NF1-NPCs (FIG. 15C). Together, these findings establish a strong relationship between neurofibromin expression and DA homeostasis/signaling (FIGS. 15B and 15D).

In the Examples provided herein, a combination of NF1-patient primary fibroblasts as well as derivative iPSCs and NPCs were employed to explore the mechanistic relation-

ship between the germline NF1 gene mutation and neurofibromin expression/function. The deployment of these unique NF1-patient bio specimens along with Nf1 genetically-engineered mouse strains provided complementary evidence relevant to disease heterogeneity, biomarker implementation and risk assessment in this common neurogenetic condition. Germline NF1 gene mutations result in dramatically different effects on neurofibromin expression in primary cells from individuals with NF1. One group of individuals with NF1 harbored >70% reductions in neurofibromin expression after a single germline mutation regardless of the cell lineage. The clinical importance of differential neurofibromin expression caused by a single germline mutation is especially germane to the interpretation of NF1 GEM studies that focus on phenotypes dictated by NF1 gene heterozygosity, such as behavior and learning. All NF1-patient fibroblasts, iPSCs and NPCs exhibited high levels of RAS activity, regardless of the level of neurofibromin expression. This indicates that the RAS-GAP activity of neurofibromin is it is comparably impaired in all individuals with NF1, irrespective of the nature or location of the germline NF1 gene mutation, and is thus highly sensitive to NF1 gene mutation. Low hippocampal DA levels and downstream signaling, as measured by reduced DARPP-32 phosphorylation, are associated with impaired spatial learning in mice.

Disclosed herein are methods for diagnosing cognitive and behavioral disorders in subjects with neurofibromatosis type 1 (NF1). In particular methods are disclosed for assessing neurofibromin expression, dopamine expression and phosphorylation of DARPP-32. Reduction in expression of these biomarkers results in impaired memory and behavioral disorders. Since cognitive problems in neurological disorders can be difficult to identify, particularly in non-verbal or young children, predictive molecular biomarkers including neurofibromin, dopamine and phosphorylated DARPP-32 provide especially useful for the early diagnosis of these problems and the appropriate treatment of affected individuals.

In view of the above, it will be seen that the several advantages of the disclosure are achieved and other advantageous results attained. As various changes could be made in the above methods without departing from the scope of the disclosure, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

When introducing elements of the present disclosure or the various versions, embodiment(s) or aspects thereof, the articles "a", "an", "the" and "said" are intended to mean that there are one or more of the elements. The terms "comprising", "including" and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

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<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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Val	Glu	Gln	Thr	Ile	Ala	Ile	Met	Lys	Asn	Leu	Leu	Asp	Asn	His	Thr	
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			980					985					990			
Leu	Asn	Leu	Val	Arg	Tyr	Val	Arg	Val	Leu	Gly	Asn	Met	Val	His	Ala	
		995					1000					1005				
Ile	Gln	Ile	Lys	Thr	Lys	Leu	Cys	Gln	Leu	Val	Glu	Val	Met	Met		
	1010					1015					1020					
Ala	Arg	Arg	Asp	Asp	Leu	Ser	Phe	Cys	Gln	Glu	Met	Lys	Phe	Arg		
	1025					1030					1035					
Asn	Lys	Met	Val	Glu	Tyr	Leu	Thr	Asp	Trp	Val	Met	Gly	Thr	Ser		
	1040					1045					1050					
Asn	Gln	Ala	Ala	Asp	Asp	Asp	Val	Lys	Cys	Leu	Thr	Arg	Asp	Leu		
	1055					1060					1065					
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	1070					1075					1080					
Pro	Leu	Gln	Pro	Glu	Glu	Gly	Asp	Gly	Val	Glu	Leu	Met	Glu	Ala		
	1085					1090					1095					
Lys	Ser	Gln	Leu	Phe	Leu	Lys	Tyr	Phe	Thr	Leu	Phe	Met	Asn	Leu		
	1100					1105					1110					

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1130						1135					1140			
Cys	Thr	Val	Leu	Ala	Met	Ser	Asn	Leu	Leu	Asn	Ala	Asn	Val	Asp
1145						1150					1155			
Ser	Gly	Leu	Met	His	Ser	Ile	Gly	Leu	Gly	Tyr	His	Lys	Asp	Leu
1160						1165					1170			
Gln	Thr	Arg	Ala	Thr	Phe	Met	Glu	Val	Leu	Thr	Lys	Ile	Leu	Gln
1175						1180					1185			
Gln	Gly	Thr	Glu	Phe	Asp	Thr	Leu	Ala	Glu	Thr	Val	Leu	Ala	Asp
1190						1195					1200			
Arg	Phe	Glu	Arg	Leu	Val	Glu	Leu	Val	Thr	Met	Met	Gly	Asp	Gln
1205						1210					1215			
Gly	Glu	Leu	Pro	Ile	Ala	Met	Ala	Leu	Ala	Asn	Val	Val	Pro	Cys
1220						1225					1230			
Ser	Gln	Trp	Asp	Glu	Leu	Ala	Arg	Val	Leu	Val	Thr	Leu	Phe	Asp
1235						1240					1245			
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1250						1255					1260			
Glu	Val	Glu	Leu	Ala	Asp	Ser	Met	Gln	Thr	Leu	Phe	Arg	Gly	Asn
1265						1270					1275			
Ser	Leu	Ala	Ser	Lys	Ile	Met	Thr	Phe	Cys	Phe	Lys	Val	Tyr	Gly
1280						1285					1290			
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1295						1300					1305			
Ile	Thr	Ser	Ser	Asp	Trp	Gln	His	Val	Ser	Phe	Glu	Val	Asp	Pro
1310						1315					1320			
Thr	Arg	Leu	Glu	Pro	Ser	Glu	Ser	Leu	Glu	Glu	Asn	Gln	Arg	Asn
1325						1330					1335			
Leu	Leu	Gln	Met	Thr	Glu	Lys	Phe	Phe	His	Ala	Ile	Ile	Ser	Ser
1340						1345					1350			
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1355						1360					1365			
Tyr	Gln	Ala	Thr	Cys	His	Ser	Leu	Leu	Asn	Lys	Ala	Thr	Val	Lys
1370						1375					1380			
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1400						1405					1410			
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1415						1420					1425			
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1460						1465					1470			
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1475						1480					1485			
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1490						1495					1500			
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1535	1540	1545
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Leu Thr Ser Ser Lys Phe Glu Glu Phe Met Thr Arg His Gln Val		
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His Glu Lys Glu Glu Phe Lys Ala Leu Lys Thr Leu Ser Ile Phe		
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Tyr Gln Ala Gly Thr Ser Lys Ala Gly Asn Pro Ile Phe Tyr Tyr		
1595	1600	1605
Val Ala Arg Arg Phe Lys Thr Gly Gln Ile Asn Gly Asp Leu Leu		
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Ile Tyr His Val Leu Leu Thr Leu Lys Pro Tyr Tyr Ala Lys Pro		
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Tyr Glu Ile Val Val Asp Leu Thr His Thr Gly Pro Ser Asn Arg		
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Phe Lys Thr Asp Phe Leu Ser Lys Trp Phe Val Val Phe Pro Gly		
1655	1660	1665
Phe Ala Tyr Asp Asn Val Ser Ala Val Tyr Ile Tyr Asn Cys Asn		
1670	1675	1680
Ser Trp Val Arg Glu Tyr Thr Lys Tyr His Glu Arg Leu Leu Thr		
1685	1690	1695
Gly Leu Lys Gly Ser Lys Arg Leu Val Phe Ile Asp Cys Pro Gly		
1700	1705	1710
Lys Leu Ala Glu His Ile Glu His Glu Gln Gln Lys Leu Pro Ala		
1715	1720	1725
Ala Thr Leu Ala Leu Glu Glu Asp Leu Lys Val Phe His Asn Ala		
1730	1735	1740
Leu Lys Leu Ala His Lys Asp Thr Lys Val Ser Ile Lys Val Gly		
1745	1750	1755
Ser Thr Ala Val Gln Val Thr Ser Ala Glu Arg Thr Lys Val Leu		
1760	1765	1770
Gly Gln Ser Val Phe Leu Asn Asp Ile Tyr Tyr Ala Ser Glu Ile		
1775	1780	1785
Glu Glu Ile Cys Leu Val Asp Glu Asn Gln Phe Thr Leu Thr Ile		
1790	1795	1800
Ala Asn Gln Gly Thr Pro Leu Thr Phe Met His Gln Glu Cys Glu		
1805	1810	1815
Ala Ile Val Gln Ser Ile Ile His Ile Arg Thr Arg Trp Glu Leu		
1820	1825	1830
Ser Gln Pro Asp Ser Ile Pro Gln His Thr Lys Ile Arg Pro Lys		
1835	1840	1845
Asp Val Pro Gly Thr Leu Leu Asn Ile Ala Leu Leu Asn Leu Gly		
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Ser Ser Asp Pro Ser Leu Arg Ser Ala Ala Tyr Asn Leu Leu Cys		
1865	1870	1875
Ala Leu Thr Cys Thr Phe Asn Leu Lys Ile Glu Gly Gln Leu Leu		
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Glu Thr Ser Gly Leu Cys Ile Pro Ala Asn Asn Thr Leu Phe Ile		
1895	1900	1905

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Ile Glu	Leu Lys His Leu Cys	Leu Glu Tyr Met	Thr	Pro Trp Leu	
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Ser Asn	Leu Val Arg Phe Cys	Lys His Asn Asp	Asp	Ala Lys Arg	
1955		1960		1965	
Gln Arg	Val Thr Ala Ile Leu	Asp Lys Leu Ile	Thr	Met Thr Ile	
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Asn Glu	Lys Gln Met Tyr Pro	Ser Ile Gln Ala	Lys	Ile Trp Gly	
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Ser Leu	Gly Gln Ile Thr Asp	Leu Leu Asp Val	Val	Leu Asp Ser	
2000		2005		2010	
Phe Ile	Lys Thr Ser Ala Thr	Gly Gly Leu Gly	Ser	Ile Lys Ala	
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Glu Val	Met Ala Asp Thr Ala	Val Ala Leu Ala	Ser	Gly Asn Val	
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Lys Leu	Val Ser Ser Lys Val	Ile Gly Arg Met	Cys	Lys Ile Ile	
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Met Trp	Asp Asp Ile Ala Ile	Leu Ala Arg Tyr	Met	Leu Met Leu	
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Ser Phe	Asn Asn Ser Leu Asp	Val Ala Ala His	Leu	Pro Tyr Leu	
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Phe His	Val Val Thr Phe Leu	Val Ala Thr Gly	Pro	Leu Ser Leu	
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Cys Thr	Cys Ser Gln Leu His	Phe Ser Glu Glu Thr	Lys	Gln Val	
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Leu Arg	Leu Ser Leu Thr Glu	Phe Ser Leu Pro	Lys	Phe Tyr Leu	
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Gln Tyr	Asn Pro Ser Leu Gln	Pro Arg Ala Leu	Val	Val Phe Gly	
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2330						2335					2340			
Thr	Leu	Asp	Ser	Leu	Arg	Ile	Phe	Asn	Asp	Lys	Ser	Pro	Glu	Glu
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2570						2575					2580			
Ile	Ser	Ser	Ser	Gln	Gln	His	Pro	His	Leu	Arg	Lys	Val	Ser	Val
2585						2590					2595			
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2630						2635					2640			
Ala	Glu	Ala	Ser	Val	Val	Phe	Pro	Lys	Val	Phe	Pro	Val	Val	His
2645						2650					2655			
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2660						2665					2670			
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2675						2680					2685			
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2690	2695	2700
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2720	2725	2730
Phe Leu Asp Ala Leu Ile Asp Thr Tyr Leu Pro Gly Ile Asp Glu		
2735	2740	2745
Glu Thr Ser Glu Glu Ser Leu Leu Thr Pro Thr Ser Pro Tyr Pro		
2750	2755	2760
Pro Ala Leu Gln Ser Gln Leu Ser Ile Thr Ala Asn Leu Asn Leu		
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Ser Asn Ser Met Thr Ser Leu Ala Thr Ser Gln His Ser Pro Gly		
2780	2785	2790
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2795	2800	2805
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Val

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<212> TYPE: PRT

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Lys Val Ser Thr Glu His Asn Lys Glu Cys Leu Ile Asn Ile Ser Lys
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Met	Met	Leu	Asn	Leu	Val	Arg	Tyr	Val	Arg	Val	Leu	Gly	Asn	Met	Val

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His	Ala	Ile	Gln	Ile	Lys	Thr	Lys	Leu	Cys	Gln	Leu	Val	Glu	Val
1010						1015					1020			
Met	Met	Ala	Arg	Arg	Asp	Asp	Leu	Ser	Phe	Cys	Gln	Glu	Met	Lys
1025						1030					1035			
Phe	Arg	Asn	Lys	Met	Val	Glu	Tyr	Leu	Thr	Asp	Trp	Val	Met	Gly
1040						1045					1050			
Thr	Ser	Asn	Gln	Ala	Ala	Asp	Asp	Asp	Ile	Lys	Cys	Leu	Thr	Arg
1055						1060					1065			
Asp	Leu	Asp	Gln	Ala	Ser	Met	Glu	Ala	Val	Val	Ser	Leu	Leu	Ala
1070						1075					1080			
Gly	Leu	Pro	Leu	Gln	Pro	Glu	Glu	Gly	Asp	Gly	Val	Glu	Leu	Met
1085						1090					1095			
Glu	Ala	Lys	Ser	Gln	Leu	Phe	Leu	Lys	Tyr	Phe	Thr	Leu	Phe	Met
1100						1105					1110			
Asn	Leu	Leu	Asn	Asp	Cys	Ser	Glu	Val	Glu	Asp	Glu	Asn	Ala	Gln
1115						1120					1125			
Thr	Gly	Gly	Arg	Lys	Arg	Gly	Met	Ser	Arg	Arg	Leu	Ala	Ser	Leu
1130						1135					1140			
Arg	His	Cys	Thr	Val	Leu	Ala	Met	Ser	Asn	Leu	Leu	Asn	Ala	Asn
1145						1150					1155			
Val	Asp	Ser	Gly	Leu	Met	His	Ser	Ile	Gly	Leu	Gly	Tyr	His	Lys
1160						1165					1170			
Asp	Leu	Gln	Thr	Arg	Ala	Thr	Phe	Met	Glu	Val	Leu	Thr	Lys	Ile
1175						1180					1185			
Leu	Gln	Gln	Gly	Thr	Glu	Phe	Asp	Thr	Leu	Ala	Glu	Thr	Val	Leu
1190						1195					1200			
Ala	Asp	Arg	Phe	Glu	Arg	Leu	Val	Glu	Leu	Val	Thr	Met	Met	Gly
1205						1210					1215			
Asp	Gln	Gly	Glu	Leu	Pro	Ile	Ala	Met	Ala	Leu	Ala	Asn	Val	Val
1220						1225					1230			
Pro	Cys	Ser	Gln	Trp	Asp	Glu	Leu	Ala	Arg	Val	Leu	Val	Thr	Leu
1235						1240					1245			
Phe	Asp	Ser	Arg	His	Leu	Leu	Tyr	Gln	Leu	Leu	Trp	Asn	Met	Phe
1250						1255					1260			
Ser	Lys	Glu	Val	Glu	Leu	Ala	Asp	Ser	Met	Gln	Thr	Leu	Phe	Arg
1265						1270					1275			
Gly	Asn	Ser	Leu	Ala	Ser	Lys	Ile	Met	Thr	Phe	Cys	Phe	Lys	Val
1280						1285					1290			
Tyr	Gly	Ala	Thr	Tyr	Leu	Gln	Lys	Leu	Leu	Asp	Pro	Leu	Leu	Arg
1295						1300					1305			
Val	Ile	Ile	Thr	Ser	Ser	Asp	Trp	Gln	His	Val	Ser	Phe	Glu	Val
1310						1315					1320			
Asp	Pro	Thr	Arg	Leu	Glu	Pro	Ser	Glu	Ser	Leu	Glu	Glu	Asn	Gln
1325						1330					1335			
Arg	Asn	Leu	Leu	Gln	Met	Thr	Glu	Lys	Phe	Phe	His	Ala	Ile	Ile
1340						1345					1350			
Ser	Ser	Ser	Ser	Glu	Phe	Pro	Ser	Gln	Leu	Arg	Ser	Val	Cys	His
1355						1360					1365			
Cys	Leu	Tyr	Gln	Ala	Thr	Cys	His	Ser	Leu	Leu	Asn	Lys	Ala	Thr
1370						1375					1380			
Val	Lys	Glu	Arg	Lys	Glu	Asn	Lys	Lys	Ser	Val	Val	Ser	Gln	Arg
1385						1390					1395			
Phe	Pro	Gln	Asn	Ser	Ile	Gly	Ala	Val	Gly	Ser	Ala	Met	Phe	Leu

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1400	1405	1410
Arg Phe Ile Asn Pro Ala Ile Val Ser Pro Tyr Glu Ala Gly Ile		
1415	1420	1425
Leu Asp Lys Lys Pro Pro Pro Arg Ile Glu Arg Gly Leu Lys Leu		
1430	1435	1440
Met Ser Lys Val Leu Gln Ser Ile Ala Asn His Val Leu Phe Thr		
1445	1450	1455
Lys Glu Glu His Met Arg Pro Phe Asn Asp Phe Val Lys Ser Asn		
1460	1465	1470
Phe Asp Leu Ala Arg Arg Phe Phe Leu Asp Ile Ala Ser Asp Cys		
1475	1480	1485
Pro Thr Ser Asp Ala Val Asn His Ser Leu Ser Phe Ile Ser Asp		
1490	1495	1500
Gly Asn Val Leu Ala Leu His Arg Leu Leu Trp Asn Asn Gln Glu		
1505	1510	1515
Lys Ile Gly Gln Tyr Leu Ser Ser Asn Arg Asp His Lys Ala Val		
1520	1525	1530
Gly Arg Arg Pro Phe Asp Lys Met Ala Thr Leu Leu Ala Tyr Leu		
1535	1540	1545
Gly Pro Pro Glu His Lys Pro Val Ala Asp Thr His Trp Ser Ser		
1550	1555	1560
Leu Asn Leu Thr Ser Ser Lys Phe Glu Glu Phe Met Thr Arg His		
1565	1570	1575
Gln Val His Glu Lys Glu Glu Phe Lys Ala Leu Lys Thr Leu Ser		
1580	1585	1590
Ile Phe Tyr Gln Ala Gly Thr Ser Lys Ala Gly Asn Pro Ile Phe		
1595	1600	1605
Tyr Tyr Val Ala Arg Arg Phe Lys Thr Gly Gln Ile Asn Gly Asp		
1610	1615	1620
Leu Leu Ile Tyr His Val Leu Leu Thr Leu Lys Pro Tyr Tyr Ala		
1625	1630	1635
Lys Pro Tyr Glu Ile Val Val Asp Leu Thr His Thr Gly Pro Ser		
1640	1645	1650
Asn Arg Phe Lys Thr Asp Phe Leu Ser Lys Trp Phe Val Val Phe		
1655	1660	1665
Pro Gly Phe Ala Tyr Asp Asn Val Ser Ala Val Tyr Ile Tyr Asn		
1670	1675	1680
Cys Asn Ser Trp Val Arg Glu Tyr Thr Lys Tyr His Glu Arg Leu		
1685	1690	1695
Leu Thr Gly Leu Lys Gly Ser Lys Arg Leu Ile Phe Ile Asp Cys		
1700	1705	1710
Pro Gly Lys Leu Ala Glu His Ile Glu His Glu Gln Gln Lys Leu		
1715	1720	1725
Pro Ala Ala Thr Leu Ala Leu Glu Glu Asp Leu Lys Val Phe His		
1730	1735	1740
Asn Ala Leu Lys Leu Ala His Lys Asp Thr Lys Val Ser Ile Lys		
1745	1750	1755
Val Gly Ser Thr Ala Val Gln Val Thr Ser Ala Glu Arg Thr Lys		
1760	1765	1770
Val Leu Gly Gln Ser Val Phe Leu Asn Asp Ile Tyr Tyr Ala Ser		
1775	1780	1785
Glu Ile Glu Glu Ile Cys Leu Val Asp Glu Asn Gln Phe Thr Leu		
1790	1795	1800

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Thr Ile	Ala Asn Gln Gly	Thr	Pro Leu Thr Phe	Met	His Gln Glu
1805		1810		1815	
Cys Glu	Ala Ile Val Gln Ser	Ile Ile His Ile	Arg	Thr Arg Trp	
1820		1825		1830	
Glu Leu	Ser Gln Pro Asp Ser	Ile Pro Gln His	Thr	Lys Ile Arg	
1835		1840		1845	
Pro Lys	Asp Val Pro Gly Thr	Leu Leu Asn Ile	Ala	Leu Leu Asn	
1850		1855		1860	
Leu Gly	Ser Ser Asp Pro Ser	Leu Arg Ser Ala	Ala	Tyr Asn Leu	
1865		1870		1875	
Leu Cys	Ala Leu Thr Cys Thr	Phe Asn Leu Lys	Ile	Glu Gly Gln	
1880		1885		1890	
Leu Leu	Glu Thr Ser Gly Leu	Cys Ile Pro Ala	Asn	Asn Thr Leu	
1895		1900		1905	
Phe Ile	Val Ser Ile Ser Lys	Thr Leu Ala Ala	Asn	Glu Pro His	
1910		1915		1920	
Leu Thr	Leu Glu Phe Leu Glu	Glu Cys Ile Ser	Gly	Phe Ser Lys	
1925		1930		1935	
Ser Ser	Ile Glu Leu Lys His	Leu Cys Leu Glu	Tyr	Met Thr Pro	
1940		1945		1950	
Trp Leu	Ser Asn Leu Val Arg	Phe Cys Lys His	Asn	Asp Asp Ala	
1955		1960		1965	
Lys Arg	Gln Arg Val Thr Ala	Ile Leu Asp Lys	Leu	Ile Thr Met	
1970		1975		1980	
Thr Ile	Asn Glu Lys Gln Met	Tyr Pro Ser Ile	Gln	Ala Lys Ile	
1985		1990		1995	
Trp Gly	Ser Leu Gly Gln Ile	Thr Asp Leu Leu	Asp	Val Val Leu	
2000		2005		2010	
Asp Ser	Phe Ile Lys Thr Ser	Ala Thr Gly Gly	Leu	Gly Ser Ile	
2015		2020		2025	
Lys Ala	Glu Val Met Ala Asp	Thr Ala Val Ala	Leu	Ala Ser Gly	
2030		2035		2040	
Asn Val	Lys Leu Val Ser Ser	Lys Val Ile Gly	Arg	Met Cys Lys	
2045		2050		2055	
Ile Ile	Asp Lys Thr Cys Leu	Ser Pro Thr Pro	Thr	Leu Glu Gln	
2060		2065		2070	
His Leu	Met Trp Asp Asp Ile	Ala Ile Leu Ala	Arg	Tyr Met Leu	
2075		2080		2085	
Met Leu	Ser Phe Asn Asn Ser	Leu Asp Val Ala	Ala	His Leu Pro	
2090		2095		2100	
Tyr Leu	Phe His Val Val Thr	Phe Leu Val Ala	Thr	Gly Pro Leu	
2105		2110		2115	
Ser Leu	Arg Ala Ser Thr His	Gly Leu Leu Ile	Asn	Ile Ile His	
2120		2125		2130	
Ser Leu	Cys Thr Cys Ser Gln	Leu His Phe Ser	Glu	Glu Thr Lys	
2135		2140		2145	
Gln Val	Leu Arg Leu Ser Leu	Thr Glu Phe Ser	Leu	Pro Lys Phe	
2150		2155		2160	
Tyr Leu	Leu Phe Gly Ile Ser	Lys Val Lys Ser	Ala	Ala Val Ile	
2165		2170		2175	
Ala Phe	Arg Ser Ser Tyr Arg	Asp Arg Ser Phe	Ser	Pro Gly Ser	
2180		2185		2190	

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Tyr	Glu	Arg	Glu	Thr	Phe	Ala	Leu	Thr	Ser	Leu	Glu	Thr	Val	Thr
2195						2200					2205			
Glu	Ala	Leu	Leu	Glu	Ile	Met	Glu	Ala	Cys	Met	Arg	Asp	Ile	Pro
2210						2215					2220			
Thr	Cys	Lys	Trp	Leu	Asp	Gln	Trp	Thr	Glu	Leu	Ala	Gln	Arg	Phe
2225						2230					2235			
Ala	Phe	Gln	Tyr	Asn	Pro	Ser	Leu	Gln	Pro	Arg	Ala	Leu	Val	Val
2240						2245					2250			
Phe	Gly	Cys	Ile	Ser	Lys	Arg	Val	Ser	His	Gly	Gln	Ile	Lys	Gln
2255						2260					2265			
Ile	Ile	Arg	Ile	Leu	Ser	Lys	Ala	Leu	Glu	Ser	Cys	Leu	Lys	Gly
2270						2275					2280			
Pro	Asp	Thr	Tyr	Asn	Ser	Gln	Val	Leu	Ile	Glu	Ser	Thr	Val	Ile
2285						2290					2295			
Ala	Leu	Thr	Lys	Leu	Gln	Pro	Leu	Leu	Asn	Lys	Asp	Ser	Pro	Leu
2300						2305					2310			
His	Lys	Ala	Leu	Phe	Trp	Val	Ala	Val	Ala	Val	Leu	Gln	Leu	Asp
2315						2320					2325			
Glu	Val	Asn	Leu	Tyr	Ser	Ala	Gly	Thr	Ala	Leu	Leu	Glu	Gln	Asn
2330						2335					2340			
Leu	His	Thr	Leu	Asp	Ser	Leu	Arg	Ile	Phe	Asn	Asp	Lys	Ser	Pro
2345						2350					2355			
Glu	Glu	Val	Phe	Met	Ala	Ile	Arg	Asn	Pro	Leu	Glu	Trp	His	Cys
2360						2365					2370			
Lys	Gln	Met	Asp	His	Phe	Val	Gly	Leu	Asn	Phe	Asn	Ser	Asn	Phe
2375						2380					2385			
Asn	Phe	Ala	Leu	Val	Gly	His	Leu	Leu	Lys	Gly	Tyr	Arg	His	Pro
2390						2395					2400			
Ser	Pro	Ala	Ile	Val	Ala	Arg	Thr	Val	Arg	Ile	Leu	His	Thr	Leu
2405						2410					2415			
Leu	Thr	Leu	Val	Asn	Lys	His	Arg	Asn	Cys	Asp	Lys	Phe	Glu	Val
2420						2425					2430			
Asn	Thr	Gln	Ser	Val	Ala	Tyr	Leu	Ala	Ala	Leu	Leu	Thr	Val	Ser
2435						2440					2445			
Glu	Glu	Val	Arg	Ser	Arg	Cys	Ser	Leu	Lys	His	Arg	Lys	Ser	Leu
2450						2455					2460			
Leu	Leu	Thr	Asp	Ile	Ser	Met	Glu	Asn	Val	Pro	Met	Asp	Thr	Tyr
2465						2470					2475			
Pro	Ile	His	His	Gly	Asp	Pro	Ser	Tyr	Arg	Thr	Leu	Lys	Glu	Thr
2480						2485					2490			
Gln	Pro	Trp	Ser	Ser	Pro	Lys	Gly	Ser	Glu	Gly	Tyr	Leu	Ala	Ala
2495						2500					2505			
Thr	Tyr	Pro	Ala	Val	Gly	Gln	Thr	Ser	Pro	Arg	Ala	Arg	Lys	Ser
2510						2515					2520			
Met	Ser	Leu	Asp	Met	Gly	Gln	Pro	Ser	Gln	Ala	Asn	Thr	Lys	Lys
2525						2530					2535			
Leu	Leu	Gly	Thr	Arg	Lys	Ser	Phe	Asp	His	Leu	Ile	Ser	Asp	Thr
2540						2545					2550			
Lys	Ala	Pro	Lys	Arg	Gln	Glu	Met	Glu	Ser	Gly	Ile	Thr	Thr	Pro
2555						2560					2565			
Pro	Lys	Met	Arg	Arg	Val	Ala	Glu	Thr	Asp	Tyr	Glu	Met	Glu	Thr
2570						2575					2580			
Gln	Arg	Ile	Pro	Ser	Ser	Gln	Gln	His	Pro	His	Leu	Arg	Lys	Val

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2585	2590	2595
Ser Val Ser Glu Ser Asn Val	Leu Leu Asp Glu Glu	Val Leu Thr
2600	2605	2610
Asp Pro Lys Ile Gln Ala Leu	Leu Leu Thr Val Leu	Ala Thr Leu
2615	2620	2625
Val Lys Tyr Thr Thr Asp Glu	Phe Asp Gln Arg Ile	Leu Tyr Glu
2630	2635	2640
Tyr Leu Ala Glu Ala Ser Val	Val Phe Pro Lys Val	Phe Pro Val
2645	2650	2655
Val His Asn Leu Leu Asp Ser	Lys Ile Asn Thr Leu	Leu Ser Leu
2660	2665	2670
Cys Gln Asp Pro Asn Leu Leu	Asn Pro Ile His Gly	Ile Val Gln
2675	2680	2685
Ser Val Val Tyr His Glu Glu	Ser Pro Pro Gln Tyr	Gln Thr Ser
2690	2695	2700
Tyr Leu Gln Ser Phe Gly Phe	Asn Gly Leu Trp Arg	Phe Ala Gly
2705	2710	2715
Pro Phe Ser Lys Gln Thr Gln	Ile Pro Asp Tyr Ala	Glu Leu Ile
2720	2725	2730
Val Lys Phe Leu Asp Ala Leu	Ile Asp Thr Tyr Leu	Pro Gly Ile
2735	2740	2745
Asp Glu Glu Thr Ser Glu Glu	Ser Leu Leu Thr Pro	Thr Ser Pro
2750	2755	2760
Tyr Pro Pro Ala Leu Gln Ser	Gln Leu Ser Ile Thr	Ala Asn Leu
2765	2770	2775
Asn Leu Ser Asn Ser Met Thr	Ser Leu Ala Thr Ser	Gln His Ser
2780	2785	2790
Pro Gly Leu Asp Lys Glu Asn	Val Glu Leu Ser Pro	Thr Ala Gly
2795	2800	2805
His Cys Asn Ser Gly Arg Thr	Arg His Gly Ser Ala	Ser Gln Val
2810	2815	2820
Gln Lys Gln Arg Ser Ala Gly	Ser Phe Lys Arg Asn	Ser Ile Lys
2825	2830	2835
Lys Ile Val		
2840		

What is claimed is:

1. A method for detecting neurofibromin expression pattern in a subject, the method comprising: obtaining a sample from a subject, the sample selected from the group consisting of a white blood cell sample, a skin fibroblast sample, a non-cancerous cell, and combinations thereof; contacting the sample with a first antibody that specifically binds neurofibromin to obtain an expression level of neurofibromin in the subject; and detecting dopamine expression level in the sample from the subject by contacting the sample with a second antibody that specifically binds dopamine.

2. The method of claim 1 further comprising normalizing the expression level of neurofibromin to an expression level of α -tubulin detected in the sample.

3. The method of claim 1 further comprising detecting phosphorylation of dopamine and cAMP regulated neuronal phosphoprotein (DARPP-32) in the sample from the subject by contacting the sample with a third antibody that specifically binds phosphorylated DARPP-32.

4. The method of claim 3 further comprising normalizing the level of phosphorylated DARPP-32 with an expression level of non-phosphorylated DARPP-32 detected in the sample.

5. The method of claim 1 further comprising normalizing the level of dopamine with total protein level detected in the sample.

6. The method of claim 1 further comprising isolating and lysing the cells of the sample.

7. The method of claim 1, wherein the method comprises a Western Blot analysis.

8. The method of claim 1, wherein the subject is a vertebrate.

9. The method of claim 8, wherein the subject is selected from the group consisting of a human and a rodent.

10. The method of claim 9, wherein the subject is a human having or suspected of having Neurofibromatosis Type I.

11. The method of claim 10, wherein the subject has or is suspected of having one of a cognitive impairment and a behavioral disorder.

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12. The method of claim 11, wherein the behavioral disorder is selected from the group consisting of attention deficit/hyperactivity disorder (ADHD), autism, and combinations thereof.

13. The method of claim 11, wherein the subject has a reduced amount of at least one of neurofibromin, dopamine and DARPP-32 as compared to a subject that does not have Neurofibromatosis Type I.

14. The method of claim 1 further comprising detecting at least one of neurofibromin, dopamine, and DARPP-32 in an additional tissue obtained from the subject.

15. The method of claim 14 wherein the additional tissue is selected from the group consisting of brain, hippocampi, and a combination thereof.

16. A method of quantifying a level of neurofibromin, phosphorylated DARPP-32, and dopamine in a subject, the method comprising

- (a) obtaining a sample from the subject, wherein the sample is selected from the group consisting of a white blood cell sample, a skin fibroblast sample, a brain sample, a hippocampus sample, and combinations thereof;
- (b) isolating cells from the sample;
- (c) lysing the cells isolated from the sample;

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(d) extracting protein content from the cells; and

(e) detecting neurofibromin, phosphorylated DARPP-32, and dopamine in the extracted protein content by contacting the extracted protein content with an antibody that specifically binds neurofibromin, an antibody that specifically binds phosphorylated DARPP-32, and an antibody that specifically binds dopamine; and

(f) quantifying the level of at least one of neurofibromin, phosphorylated DARPP-32, and dopamine.

17. The method of claim 16 further comprising normalizing the level of at least one of neurofibromin with α -tubulin and phosphorylated DARPP-32 with non-phosphorylated DARPP-32 in the sample.

18. The method of claim 16 further comprising analyzing the level of at least one of neurofibromin, phosphorylated DARPP-32, and dopamine in the sample and a reference expression level of at least one of neurofibromin, phosphorylated DARPP-32, and dopamine.

19. The method of claim 16, wherein the subject has or is suspected of having at least one of neurofibromatosis type 1, a cognitive impairment, attention deficit/hyperactivity disorder (ADHD), autism, and combinations thereof.

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