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(54) **Title**: DIAGNOSTIC AND PROGNOSTIC AND THERAPEUTIC METHODS

(57) **Abstract**: An assay for a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder (hereinafter "the conditions"), comprising assessing the level or activity of an analyte selected from ATP13A2 polypeptide, *ATP13A2* nucleic acid, Mn^{2+}, and a complex comprising ATP13A2 polypeptide and Mn^{2+}, wherein the level or activity of the analyte indicates that the test subject has or is susceptible to the conditions. A method of treatment or prevention employing the assessment assays or ATP13A2 polypeptide or *ATP13A2* nucleic acid. ATP13A2 polypeptide or *ATP13A2* nucleic acid for use in the treatment or prevention of the conditions wherein the ATP13A2 polypeptide or *ATP13A2* nucleic acid increases the level of mitochondrial Mn^{2+} in subjects exhibiting a low level of mitochondrial Mn^{2+}. An isolated cell or organism comprising same, wherein the activity of ATP13A2 polypeptide is modified, such as reduced or inhibited (ablated). An assay for agents that complement the phenotype an ATP13A2 deficient cell or non-human organism comprising such cells, the assay comprising contacting the cell or organism with a putative agent and assessing the ability of the agent to increase the level of mitochondrial Mn^{2+} in the cell. An assay for agents that modulate Mn^{2+} levels in a cell or organism, the assay comprising contacting the cell or organism with a putative agent and assessing the ability of the agent to increase the level or activity of ATP13A2 in the cell or organism.

DIAGNOSTIC AND PROGNOSTIC AND THERAPEUTIC METHODS

FIELD

The present invention relates to the field of diagnosis, prognosis and treatment of behavioural (CNS) conditions characterised by anxiety such as obsessive-compulsive disorder (OCD). The invention relates generally to the role of ATPases and manganese homeostasis in maintaining mammalian health. In one embodiment, the present invention provides medical assessment systems based upon analyte profiling, screening assays and animal models of perturbed manganese distribution.

BACKGROUND

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

The reference in this specification to any prior publication or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

ABC transporters are ubiquitous membrane proteins that facilitate unidirectional substrate translocation across lipid bilayers. One such ABC transporter gene the *ATP13A2* gene encodes a P-type ATPase, that is reported to be expressed widely but predominantly in the brain. Mutations in the gene have been linked by Ramirez *et al., Nat Genet 38*: 1184-1191, 2006 to hereditary parkinsonism with dementia who located wild type protein in lysosomes (Ramirez *et al.,* 2006 (*supra*) incorporated herein by reference in its entirety). Others have found no link between this gene and the disease and any role of ATP13A2 in parkinsonism remains poorly understood.

Manganese has been associated with toxic effects in the brain and has been considered as

an etiological factor of Parkinson's disease.

The mechanism of Mn^{2+} transport and the mechanism of accumulation in the brain and central nervous system of mammals is unknown.

There is a need in the art for methods of diagnosis, prognosis and treatment of conditions or states associated with liver, brain, blood and mitochondrial dysfunction. There is a need in the art to identify molecules and mechanisms regulating manganese ion distribution in the body in order to model these processes and identify agents that can be used to treat or prevent disorders or states associated with perturbed manganese levels. There is a need for assays that identify subjects with or susceptible to developing OCD-like conditions.

SUMMARY

In one embodiment, the present invention provides an analyte assessment approach to identify test subjects who have or who are susceptible to a behavioural disorder characterised by anxiety and/or repetitive compulsive behaviours. Also, the assessment approach identifies or is suitable to testing subjects who have a lipid and/or a lipid associated liver disorder and links this condition in a test subject with the presence or risk of onset of the behavioural condition characterised by anxiety, where appropriate. Thus, for example, presymptomatic diagnosis of a behavioural disorder associated with anxiety such as obsessive compulsive disorder (OCD) based upon an early identification of a lipid or lipid-associated liver condition will facilitate early treatment and prevention, including the use of existing therapies. The identification of affected or susceptible subjects should facilitate the delivery or critical intervention or treatment strategies. The assessment tools are also instructive as to the effectiveness of treatment, identifying types of subjects that may respond well to specific medications, and making them useful in pharmacotranslational studies and in the clinical management of patients.

Accordingly, the present invention provides a diagnostic or prognostic assay for a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, the assay comprising assessing the level or activity of an analyte in a biological fluid or tissue sample in or from a test subject, the analyte selected from one or more of ATP13A2 polypeptide, *ATP13A2* nucleic acid, Mn^{2+} , and a complex comprising ATP13A2 polypeptide and Mn^{2+}, wherein the level or activity of the analyte relative to a control indicates that the test subject has or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder.

Comparison of the individual levels or activities of the analytes with a control can be carried out in different ways.

In one illustrative embodiment, the assay comprises comparing the level of Mn^{2+} in the test subject to the level of Mn^{2+} in at least one control subject selected from a subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder and a normal subject, wherein a similarity in the level of Mn^{2+} between the test subject and the normal control subject identifies the test subject as normal or non-susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, and wherein a similarity in the level of Mn^{2+} between the test subject and the control subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder identifies the test subject as having or being susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid-associated liver disorder or being susceptible thereto.

In some embodiments, the level or activity of analyte is assessed in blood, serum or plasma.

In other embodiments, the level or activity of analyte is assessed in a liver sample.

In further embodiments, the level or activity of analyte is assessed in a brain sample.

In a still further embodiment, the level or activity of analyte is assessed in mitochondria.

In one particular embodiment, the analyte is Mn^{2+}.

In another embodiment the analyte is an *ATP13A2* nucleic acid, such as a genomic sequence or RNA.

In another embodiment, the analyte is an ATP13A2 polypeptide.

In another illustrative embodiment, the control represents the level or activity of the analyte in a normal healthy subject and (i) a decreased level or activity of ATP13A2 or *ATP13A2* relative to the level or activity in a normal healthy control subject and/or (ii) an

increased level of Mn^{2+} in the blood of the test subject relative to the level of Mn^{2+} in the blood of a normal healthy control subject and/or a decreased level of Mn^{2+} in a second tissue in or from the test subject relative to the level of Mn^{2+} in the same tissue from a normal healthy control subject, such as brain, mitochondria, and/or liver, indicates that the test subject has or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid-associated liver disorder.

In another embodiment, the control represents the level of Mn^{2+} in a normal healthy subject and an increased level of Mn^{2+} in the blood of the test subject relative to the level in a normal healthy subject and/or a decreased level of Mn^{2+} in a second sample in or from the test subject relative to the corresponding level in the same tissue in or from a normal healthy control subject, such as brain, mitochondria, and/or liver, indicates that the test subject has or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid-associated liver disorder.

In some embodiments, the second sample is in or from one or more of at least brain, mitochondria and liver.

In other embodiments, the lipid and/or lipid-associated liver disorder is one or more of fatty liver, hyperlipidemia and steatosis. Hyperlipidemia may comprise elevated levels of triglycerides, HDL, LDL, and/or total cholesterol.

In some embodiments, the assessment assays are practised *ex vivo*.

In some embodiments, the assays comprise assessing the level or activity of Mn^{2+} and ATP13A2 in the test subject to the level of Mn^{2+} in at least one control subject selected from a subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder and a normal subject, wherein a similarity in the level or activity of Mn^{2+} and ATP13A2 between the test subject and the normal subject identifies the test subject as normal or non-susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver

disorder, and wherein a similarity in the level or activity of Mn^{2+} and ATP13A2 between the test subject and the control subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder identifies the test subject as having or being susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid-associated liver disorder or being susceptible thereto.

In some embodiments, the assays comprise assessing the level or activity of *ATP13A2* in the test subject to the level of *ATP13A2* in at least one control subject selected from a subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder and a normal subject, wherein a similarity in the level or activity of *ATP13A2* between the test subject and the normal subject identifies the subject as normal or non-susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, and wherein a similarity in the level or activity of *ATP13A2* between the test subject and the subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder identifies the test subject as having or being susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid-associated liver disorder or being susceptible thereto.

The assessment of the level or activity of the analyte may be carried out in different ways.

In some embodiments, the assays comprise pre-selecting the test subject as having or suspected of having hyperlipidemia and/or lipid-associated liver disorder.

In some embodiments, the diet of the subject is assessed to determine if they are on a high-fat diet.

In another aspect, the present invention provides compositions comprising ATP13A2 polypeptide or *ATP13A2* nucleic acid for use in the treatment or prevention of a disorder characterised by anxiety, such as OCD, and/or a lipid-associated liver disorder in a subject wherein the ATP13A2 polypeptide or *ATP13A2* nucleic acid increases the level of

mitochondrial Mn^{2+} in subjects typically exhibiting a low level of mitochondrial Mn^{2+} compared to the level in a normal healthy control subject.

Liver directed gene therapy and hepatocyte transplantation methods are known in the art.

In a similar embodiment, the present invention provides a method of treatment or prophylaxis of a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid associated liver disorder, the method comprising administering ATP13A2 polypeptide or a functional analog thereof or an *ATP13A2* nucleic acid to a subject in need thereof for a time and under conditions sufficient for the treatment or prophylaxis of the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid associated liver disorder.

In another embodiment, the present invention provides a method of treatment or prophylaxis of a subject comprising assessing a test subject with respect to a behavioural disorder characterised by anxiety, such as OCD, by assessing the level or activity of an analyte in a biological fluid or tissue sample in or from a test subject, the analyte selected from one or more of ATP13A2 polypeptide, *ATP13A2* nucleic acid, Mn^{2+} , and a complex comprising ATP13A2 polypeptide and Mn^{2+}, wherein the level or activity of the analyte relative to a control indicates that the test subject has or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, and exposing the subject to therapeutic or prophylactic or behavioural intervention on the basis that the test subject tests positive to having or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder.

The present invention also provides for the use of ATP13A2 polypeptide or ATP13A2 nucleic acid, or the herein disclosed analyte assessment assays or agent screening assays in the manufacture of a medicament for the treatment or prevention of a behavioural condition characterised by anxiety, such as OCD, and/or a lipid and/or lipid-associated liver disorder.

In some embodiments, the methods comprise assessing the test subject as disclosed herein before and after treatment.

In another aspect, the present invention provides an isolated cell or non-human organism comprising such cells, wherein the activity of ATP13A2 polypeptide is modified, such as reduced or inhibited (included ablated), compared to a non-modified organism of the same species optionally for use as an ATP13A2 deficient animal or cellular model of a behavioural disorder, such as OCD, and/or a lipid and/or lipid-associated liver disorder.

In some embodiments, the cell or non-human organism is or is from a mouse or zebrafish.

In some embodiments the cell or non-human animal is modified using art recognised strategies to be substantially incapable of producing ATP13A2 or produces ATP13A2 having substantially no activity. Illustrative strategies include gene knockouts, co-suppression, gene silencing or induction of iRNA approaches.

In another aspect, the present invention provides an assay for agents that complement the phenotype an ATP13A2 deficient cell or non-human organism comprising such cells, the assay comprising contacting the cell or organism with a putative agent and assessing the ability of the agent to increase the level of mitochondrial Mn^{2+} in the cell.

In another embodiment, the invention provides an assay for agents that modulate Mn^{2+} levels in a cell or organism, the assay comprising contacting the cell or organism with a putative agent and assessing the ability of the agent to increase the level or activity of ATP13A2 in the cell or organism. In some embodiments, the cell or organs is substantially incapable of producing ATP13A2 or produces ATP13A2 having substantially no activity.

The above summary is not and should not be seen in any way as an exhaustive recitation of all embodiments of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Some figures contain colour representations or entities. Coloured versions of the figures are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

Figure 1 is a diagrammatic representation showing genetic targeting of the *ATP13A2* gene in mice. **a,** Gene targeting strategy to disrupt the *ATP13A2* gene. Primers for genotyping are indicated as b and c. **b,** Genotyping of ATP13A2 mice for the deletion of exons 2 and 3 of the ATP13A2 gene. **c,** RT-PCR measurement of ATP13A2 mRNA in wild type (WT) and ATP13A2 knock out (KO) mouse tissues.

Figure 2 is a graphical representation of data showing that ATP13A2 deficiency induces increased body weight, hyperlipidemia and hepatomegaly in young adult male mice. All data are mean±SEM. **a,** Incidence of mouse excessive groom behaviour. Mice of both sexes at the age of 6-7 and 12-13 months were observed for excessive groom behaviours in two groups of $ATP13A2^{+/+}$ and $ATP13A2^{-/-}$. Data are mean±SEM (n=38, $^{**}p<0.05$). **b,** Serum levels of lipids and glucose in 24-week-old ATP13A2 WT (blue bars) and KO (red bars) male mice. (n=5; $^{*}p<0.05$; $^{**}p<0.01$). **c,** Effect of ATP13A2 deletion on the serum levels of Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} of 24-week-old male mice. Data are mean±SEM (n=4; $^{*}p<0.05$). **d, e,** Average tissue/body weight ratios of ATP13A2 WT (light grey bars) and KO (dark grey bars) mice (n=4; $^{*}p<0.05$; $^{**}p<0.01$).

Figure 3 is a photographic representation of data showing manganese deficiency and lipid accumulation in the liver of ATP13A2 KO mice. **a,** Hematoxylin and eosin (H & E) staining of liver sections from the ATP13A2 WT (+/+) and KO (-/-) mice as indicated, at 10x (left) and 40x (right) magnifications. White arrows indicate fat droplets in hepatocytes and black arrows indicate inflammatory cells. CV=central vein. PV=portal vein. **b,** Oil Red O staining for lipid accumulation in the liver at 10x (left) and 40x (right) magnifications. **c,** Biochemical quantification of serum triglyceride and cholesterol to show increased lipid content in the livers of ATP13A2 KO mice. Cholesterol was measured in duplicate liver

samples of 16 week old male ATP13A2 WT (+/+) and KO (-/-) mice. Data are mean±SEM (n=4; *p<0.05). **d,** Quantification of the lipid staining in liver and epididymal fat tissue sections. Oil Red O staining was quantitated by microscopic imaging analysis (Olympus X51 and software AIS). Data are mean±SEM (n=12 micrographs; **p<0.001). **e,** Lipid accumulation in the epididymal fat pads of mice. Epididymal fat tissues were stained with H & E (left panels) and Oil Red O (right panels). **f,** Effect of ATP13A2 deletion on the Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} in the livers of 6-month old male mice. Approximately 10 mg liver from ATP13A2 WT (blue bars) and KO (red bars) mice were subject to Graphite Furnace Atomic Absorption Spectrometry. Data are mean±SEM (n=10).

Figure 4 is a graphical representation of data showing protein and trace metal analyses in the ATP13A2 KO livers. **a,** Measurement of Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} in the livers of neonatal WT and ATP13A2 KO mice. Data are mean±SEM (n=5).. **b,** Average levels of gene expression (a) in the livers of WT and ATP13A2 KO male mice at 24 weeks as determined by phosphor imaging of a Western blot. Data are mean±SEM (n=4; *p<0.05).

Figure 5 is a photographic representation of data showing that deletion of the *ATP13A2* gene induces cerebral neuron losses in mice. **a, b,** Cell counting analysis of the neurons (s) and microglial cells (b) in the cerebral visual cortex of ATP13A2 WT and KO mice of 24 weeks of age. Cell numbers were counted using AIS software based on cell morphology under the microscope (Olympus X51). **c,** Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} levels in the brains of WT (blue bars) and KO (red bars) ATP13A2 mice. Data are mean±SEM (n=5).

Figure 6 is a graphical representation of data showing the effect of high-fat diet on the levels of manganese in the liver and brain, hepatic steatosis and neuron losses in ATP13A2 WT and KO mice. Male mice aged ~16 week were fed with the high-fat diet for 8 weeks. **a, b,** Effect of high-fat diet on lipid accumulation in the livers of WT (a) and (b) ATP13A2 KO mice. The sections were stained with Oil Red O at magnifications of 10x (left panels) and 40x (right panels). **c,** Effect of high fat-diet on liver manganese levels. Data are mean±SEM (n=5; *p<0.05). **e, f,** Brain cortical regions WT (e) and ATP13A2 KO mice (f) were stained with Oil Red O after 8 weeks on the high-fat diet. Micrographs are at 10x

(left panel) and 40x (right panel) magnifications. **g,** Quantification of the nerve cell losses in the brain cortex and the Substantia nigra regions. Cell numbers were counted using AIS software based on cell morphology under the microscope (Olympus X51). Nissl- stained neurons in various regions were counted in Multiple areas per slide of 3-4 sections from each brain. Data are mean±SEM (n=6; *p<0.05; **p<0.001).

Figure 7 is a photographic representation of data showing Nissl staining of the various brain regions of WT and ATP13A2 KO mice fed the high-fat diet. Coronary sections from 1 mm caudal to the optic chiasm were stained with cresyl violet. Representative micrographs of different brain regions are shown at magnification of 10x or 40x.

Figure 8 is a photographic representation of immunofluorescence staining of ATP13A2 in cells in culture. **a,** Specific detection of endogenous and recombinant exogenous ATP13A2. HeLa cells were transfected with pEGFP empty plasmid, pEGFP-ATP13A2 (WT) or pEGFP-ATP13A2 shRNA for 24 hours. Cells on cover slips were incubated with mouse monoclonal antibodies against ATP13A2 follow and viewed by fluorescence microscopy. **b,** Co-localization of endogenous ATP13A2 with LAMP1 in lysosomes. **c,** Co-localization of recombinant ATP13A2 with MitoTracker in mitochondria. Images at 60x magnification.

Figure 9 is a photographic representation of data showing ATP13A2 localization, and under-, and over-expression studies in cultured cells. **a,** Co-localization of endogenous ATP13A2 with the mitochondria marker MitoTracker in cultured COS-7 cells. Confocal images were taken at 60x magnification. The panel is at 240x magnification. **b,** Confocal microscopic Z sectioning of ATP13A2 co-localization with MitoTracker. **c,** Quantitative detection of ATP13A2 gene expression in HeLa cells transfected with control plasmid, pEGFP-ATP13A2 or pEGFP-ATP13A2 shRNA. Data are mean ± SEM (n=3; **p<0.01). **d,** Knockdown of the *ATP13A2* gene induces manganese retention in the mitochondria of cultured cells. HeLa cells transfected with GFP alone, a GFP-ATP13A2 WT gene expression plasmid or a GFP-ATP13A2 shRNA expression plasmid for 48 hours were

lyzed and the mitochondria purified for measurement in triplicate of Cu^{2+}, Fe^{2+} and Mn^{2+}. Data are mean±SEM (n=3; *p<0.05).

Figure 10 is a graphical representation of data showing ATP13A2 KO mice appear to be prone to fear and stress. Using non-invasive tests on a vertical grid, ATP13A2 KO mice showed a significant delay in direction turning and walking down the grid in comparison with the age- and sex-matched control mice. The figure shows that ATP13A2 KO mice show time delays in turning directions and walking down a vertical grid. (n=30, *p<0.05)

Figure 11 is a graphical representation of data showing that ATP13A2 KO mice fear jumping down from a 30 cm height of horizontal grid with time delays in comparison with age- and sex-matched wild type controls. (n=24, **p<0.01). On a vertical grid test, ATP13A2 KO mice appeared frightened to come down with a significant time delay when it was compared to the age- and sex-matched control mice.

Figure 12 is a photographic representation of data indicating lacking of nest building behaviour and therefore of anxiety in ATP13A2 deficient mice.

Figure 13 is a photographic representation of data indicating lacking of nest building behaviour and therefore of anxiety in ATP13A2 deficient mice.

Figure 14 is a graphical representation of data showing excessive grooming and anxiety-like behaviours. Data are expressed as mean±SEM. (*p<0.05; **p<0.01). **a,** Incidence of excessive grooming. Mice were examined for grooming in each family aged 6-7 and 12-13 months in $ATP13A2^{+/+}$ and $ATP13A2^{-/-}$ mice of both sexes. **b, c,** ATP13A2 mice showed more time spent in self-grooming (**b**) and more grooming bouts (**c**) at all time examined. **d,** Excessive grooming led to severe skin lesions.

Figure 15 is a graphical representation of data showing anxiety-related behaviour of $ATP13A2^{-/-}$ and WT mice. **a-c,** Dark-bright field observation. $ATP13A2^{-/-}$ mice in the dark-light choice test showed increased time in the bright field (**a, b**) and decreased

frequency of moving between the light and dark compartments (**c**) over a 30-min period (n = 4). **d,** Nest-building test: 0, no nest; 1, partially built nest and 2, complete nest. **e,** Time spent in a horizontal grid test. Data are expressed as mean±SEM (n=24). **f,** Time recorded in a vertical grid test for turning and walking. (n=30). **g,** Rearing test: the numbers of rears were recorded for age-matched male and female ATP13A2 WT and KO mice during 5 min trial. **h,** Data are expressed as mean±SEM (n=8).

BRIEF DESCRIPTION OF THE TABLES

Table 1 provides a description of the SEQ ID NOs provided herein.

Table 2 provides an amino acid sub-classification.

Table 3 provides exemplary amino acid substitutions.

Table 4 provides a list of non-natural amino acids contemplated in the present invention.

DETAILED DESCRIPTION

Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. Thus, use of the term "comprising" and the like indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

The use of numerical values in the various ranges specified in this specification, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges were both preceded by the word "about". In this manner, slight variations above and below the stated ranges can be used to achieve substantially the same results as values within the ranges. Also, the disclosure of these ranges is intended as a continuous range including every value between the minimum and maximum values.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

Unless defined otherwise, all technical and scientific terms used herein have the same

meaning as commonly understood by those of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practise or testing of the present invention, preferred methods and materials are described.

As used herein the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a mutation" includes a single mutation, as well as two or more mutations; reference to "a control" includes one control, as well as two or more control; and so forth.

Each embodiment in this specification is to be applied *mutatis mutandis* to every other embodiment unless expressly stated otherwise.

Genes and other genetic material (e.g. mRNA, nucleic acid constructs etc) are represented herein in italics while their proteinaceous expression products are represented in non-italicised form. Thus, for example ATP13A2 polypeptide is the expression product of ATP13A2 nucleic acid (genetic) sequences. ATP13A2 includes mRNA, gDNA, cDNA amongst other forms.

Understanding how manganese (Mn^{2+}) is transported in the body is a necessary basis for understanding the role of Mn^{2+} in maintaining or modulating biological systems.

In accordance with the present invention, ATP13A2 has been identified as a Mn^{2+} transporter. In some embodiments, ATP13A2 is identified as a mitochondrial Mn^{2+} transporter. Low levels of Mn^{2+} in the mitochondria of the brain of ATP13A2 deficient mice were associated with significant neural loss and increased microglial activity in the cerebral cortex. Lesions induced by ATP13A2 deficiency were augmented and expanded in mice that were fed a high-fat diet. Inhibition of ATP13A2 gene expression in normal adult mice led to a significant decrease in the levels of mitochondrial Mn^{2+}.

Furthermore, as illustrated in the Examples, ATP13A2 deficient mice, although normal at

weaning, exhibited elevated levels of Mn^{2+} in the blood and depressed levels in the liver. Significantly, low levels of Mn^{2+} in the liver were associated with fatty liver, steatosis and hyperlipidemia.

In one embodiment, ATP13A2 transports Mn^{2+} and has a profound effect on the ability of the liver to store Mn^{2+}, and ATP13A2 deficiency leads to increased levels of Mn^{2+} in the circulation.

In another embodiment, ATP13A2 transports Mn^{2+} and ATP13A2 deficiency reduces the ability of the brain to store or regulate the distribution of Mn^{2+}.

In another embodiment, ATP13A2 deficiency leads to increased levels of Mn^{2+} in the circulation and decreased levels of Mn^{2+} in the liver and central nervous system including the brain.

In one broad embodiment, the level or activity of Mn^{2+} is proposed as a diagnostic or prognostic marker for one or more of neurodegeneration, a mental or behavioural disorder, steatosis, fatty liver and hyperlipidemia, or in the manufacture of a medicament for the treatment or prevention of one or more of these conditions.

In another broad embodiment, ATP13A2 in proteinaceous or genetic form is proposed for use in the diagnosis or prognosis of one or more of neurodegeneration, a mental or behavioural disorder, steatosis, fatty liver and hyperlipidemia or in the manufacture of a medicament for the treatment or prevention of a disorder in a mammalian subject characterised by one or more or two or more or three or more of neurodegeneration, a mental or behavioural disorder, steatosis, fatty liver and hyperlipidemia.

Any subject who could benefit from the present methods or compositions is encompassed and these are referred to as test subjects. The term "subject" includes, without limitation, humans and non-human primates, livestock animals, companion animals, laboratory test animals, captive wild animals, reptiles and amphibians, fish, birds and any other organism.

The most preferred subject of the present invention is a human subject. A subject, regardless of whether it is a human or non-human organism may be referred to as a patient, individual, subject, animal, host or recipient.

Behavioural (central nervous system (CNS)) and mental disorders may occur in the absence of detectable neurodegeneration. As disclosed herein, ATP13A2 deficient mice display a mental or behavioural disorder characterized by an anxiety disorder including obsessive compulsive disorder (OCD), or a paroxysmal dyskinesia such as paroxysmal kinesigenic choreoathetosis (PKC) otherwise known as paroxysmal kinesigenic dyskinesia (PKD). The OCD-like phenotype includes increased anxiety and repetitive compulsive behaviours with an element of self-injury. Accordingly, in some embodiments, the behavioural disorder is selected from an anxiety disorder, such as obsessive compulsive disorder or a paroxysmal dyskinesia such as PKC or PKD. In other embodiments, the mental or behavioural disorder is characterised by signs that include neural loss and/or increased microglial cell activity.

While ATP13A2 has previously be linked to Parkinson's disease which involves neurodegeneration, the present invention relates to the link between Mn^{2+} and ATP13A2 as a carrier for Mn^{2+} and the effect of perturbed Mn^{2+} distribution on liver and blood and brain function. Thus, for example, a subject identified as hyperlipidemic could be tested in accordance with the present invention to determine the likelihood of developing further conditions in the group of conditions herein associated with a perturbed (dysfunctional) Mn^{2+} distribution.

Accordingly, the present invention provides a diagnostic or prognostic assay for a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, the assay comprising assessing the level or activity of an analyte in a biological fluid or tissue sample in or from a test subject, the analyte selected from one or more of ATP13A2 polypeptide, *ATP13A2* nucleic acid, Mn^{2+} , and a complex comprising ATP13A2 polypeptide and Mn^{2+}, wherein the level or activity of the analyte relative to a control indicates that the test subject has or is susceptible to developing a

behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder.

The term "susceptible" or "susceptibility", as described herein, refer to the proneness of an individual subject towards the development of a certain state (e.g., OCD), or towards being less able to resist a particular state than the average individual. The term encompasses both increased susceptibility and decreased susceptibility.

In one illustrative embodiment, the assay comprises comparing the level of Mn^{2+} in the test subject to the level of Mn^{2+} in at least one control subject selected from a subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder and a normal subject, wherein a similarity in the level of Mn^{2+} between the test subject and the normal subject identifies the test subject as normal or non-susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, and wherein a similarity in the level of Mn^{2+} between the test subject and the subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder identifies the test subject as having or being susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid-associated liver disorder or being susceptible thereto.

In some embodiments, the level or activity of the analyte in a susceptible or affected subjects is at least 101%, 102%, 103%, 104%, 105%, 106%, 107% 108%, 109%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% (i.e. an increased or higher level), or no more than about 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.01%, 0.001% or 0.0001% (i.e. a decreased or lower level) of the level or activity of the same analyte in a non-susceptible or normal subject.

Suitably, the test subject is identified as being effected or susceptible to developing a behavioural condition characterised by anxiety, such as OCD, when the level or activity of

the analyte in the test subject varies from the level or activity of the same analyte in a susceptible or affected control subject or in a susceptible or affected control population of subjects by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%.

Alternatively, the test subject is identified as being non-susceptible or normal with respect to having or developing a behavioural condition characterised by anxiety, such as OCD, when the level or activity of an analyte in the subject varies from the level or activity of the same analyte in a normal healthy control subject or population of subjects by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%.

In some embodiments, the analyte is assessed in a biological fluid or tissue in or from a subject. Reference herein to a "sample" or "biological sample" includes any biological tissue or fluid sample in or obtained from a subject. Examples of suitable samples or biological fluids or tissues include those obtained from cells, or a biological fluid such as blood, serum, plasma, bile, saliva, urine, sweat, tears, tissue biopsy, synovial, amniotic, peritoneal, cerebrospinal fluid, aqueous or vitreous humor, or any bodily secretion. Samples may also be obtained from tissues or organs including preferably biopsy sample, or cells from culture. DNA or protein may be extracted or isolated from the sample prior to testing. Samples from cells include mitochondrial, lysosomal or endosomal preparations.

The level or activity of ATP13A2 or ATP13A2 may be determined by any art recognised method. In particular, the enzymatic activity or Mn^{2+} binding of the polypeptide can be assessed. In other embodiments, ATP13A2 is tested for mutations associated with ATP13A2 dysfunction or its level determined by quantitative amplification reaction-based methods such as RT-PCR.

In some embodiments, elevated levels of Mn^{2+} in the blood and low levels in the liver (the normal storage organ for manganese in healthy subjects) compared to the levels in normal healthy subjects indicates that the subject has or is at risk of developing hyperlipidemia and fatty liver. Furthermore, elevated levels in the blood indicate that the brain will also

experience high levels of Mn^{2+}, especially in subject on a high-fat diet, but low mitochondrial Mn^{2+}, indicating that the subject has or is at risk of developing a mental or behavioural disorder.

In some embodiments, early detection of manganese deficiency in the liver facilitates early medical and/or other behavioural or dietary changes and intervention to increase levels of Mn^{2+} in the liver useful in preventing or delaying onset or progression of fatty liver, hyperlipidemia, brain neural loss, microglial activity, and related physiological, psychological and behavioural sequelae.

Manganese (Mn^{2+}) may be detected instrumentally or *via* any suitable method known in the art. For instance, manganese levels may be measured by spectrometry such as atomic absorption spectrometry, or inductively coupled plasma mass spectrometry (ICS-MS) or by flow injection analysis based upon the ability of Mn^{2+} to interact with one of a range of binding partners.

In some embodiments, "a control" includes the level or activity in a normal healthy subject group or other suitable reference. In some embodiments, the relative level of Mn^{2+} is determined in one or more samples (from different tissues) from a subject such that the level or ratio in/between different tissues may be determined. In some embodiments, the blood and liver or blood and brain Mn^{2+} levels in a single subject are used to characterise an elevated or decreased level of Mn^{2+}.

In some embodiments, the level of analyte is assessed by comparing the level of analyte in a test subject to the respective levels of the same analyte in a control subject. Reference to a "control subject" includes a single control subject and a population or cohort of control subjects.

The control level (reference level or concentration) may be expressed as a mean or mode level or a range from a cohort of control subjects or a mean together with a standard

deviation to determine threshold levels. Levels or concentrations of Mn^{2+} are determined from fluid or tissue and expressed as nmol/g.

The term "level" or "levels" also encompasses ratios of levels of analyte in different samples, and odds ratios of levels or ratios of odds ratios. Analyte levels in cohorts of subjects may be represented as mean levels and standard deviations as known to those of skill in the art. The term "level" includes an increase in a level or a ratio of levels and a decrease in a level or a ratio levels.

Reference to a "control" broadly includes data that the skilled person would use to facilitate the accurate interpretation of technical data. In an illustrative example, the level analyte (s) from a test subject are compared to the respective reference level or levels of the same analyte(s) in one or more cohorts (populations/groups) of control or reference subjects whose anxiety behavioural disease status or risk is known or established.
In some embodiments, the control may be the level or activity or ratio of levels or activities of an analyte from the test subject taken at an earlier time point. Thus, a temporal change in analyte levels can be used to identify susceptibility or provide a correlation as to the state of the disorder.

In some embodiments, a control subject is a group of control subjects. The level or activity of analyte in a control subject group may be a mean value or a preselected level, threshold or range of levels that define, characterize or distinguish a particular group. Thresholds may be selected that provide an acceptable ability to predict diagnostic or prognostic risk, treatment success, etc. In illustrative examples, receiver operating characteristic (ROC) curves are calculated by plotting the value of one or more variables *versus* its relative frequency in two populations (called arbitrarily "OCD" and "normal" or "hyperlipidemic" and "fatty liver" groups for example). The area under the curve provides the C-statistic which is a measure of the probability the measurement will allow correct identification of a condition or risk. For any particular analyte(s) or class(es), a distribution of level(s) for subjects in two control populations will likely overlap. Under such conditions, a test level may not absolutely distinguish between populations with 100% accuracy, and the area of

overlap indicates where the test cannot distinguish between groups. Accordingly, in some embodiments, a threshold or range is selected, within which the test is considered to be "indicative" i.e., able to discriminate between disease status and without which the test is considered to be "non-indicative" i.e., unable to discriminate. Various further controls will be routinely applied by the skilled artisan.

In some embodiments, Mn^{2+} levels in control groups are used to generate a profile of analyte levels reflecting difference between levels in two control populations. The data may be represented as an overall signature score or the profile may be represented as a barcode or other graphical representation to facilitate analysis or diagnosis. The analyte levels from a test subject may be represented in the same way and the similarity with the signature scope or level of "fit" to a signature barcode or other graphical representation may be determined.

The herein disclosed conditions, or analogous conditions provoked by surgery, injury, genetic or idiopathic causes can now be diagnosed or prognosed by monitoring subjects for modification in the level or activity of ATP13A2 or specific mutations or epigenetic modifications, aberrations (such a methylation events) in *ATP13A2.* In some embodiments, genetic testing is proposed in conjunction with or as a consequence of the results of Mn^{2+} testing.

One particular mutation results in a non-conservative substitution in ATP13A2 such as in an Mn^{2+} binding site, phosphorylation site, ATP binding site or hydrolase site.

One form of assessing the activity of ATP13A2 is to assess the gene or part of the gene for mutations that confer or are likely to confer ATP13A2-deficiency. A wide range of mutation detection screening methods are available as would be known to those skilled in the art. Any method which allows an accurate comparison between a test and control nucleic acid sequence may be employed. Scanning methods include sequencing, denaturing gradient gel electrophoresis (DGGE), single-stranded conformational polymorphism (SSCP and rSSCP, REF-SSCP), chemical cleavage methods such as CCM,

ECM, DHPLC and MALDI-TOF MS and DNA chip technology. Specific methods to screen for pre-determined mutations include allele specific oligonucleotides (ASO), allele specific amplification, competitive oligonucleotide priming, oligonucleotide ligation assay, base-specific primer extension, dot blot assays and RFLP-PCR. The strengths and weaknesses of these and further approaches are reviewed in Sambrook, *Chapter 13*, *Molecular Cloning*, 2001.

The diagnostic and prognostic methods of the present invention detect or assess an aberration in the wild type *ATP13A2* gene or locus to determine if *ATP13A2* will be produced or if it will be over-produced or under-produced or if its function is affected. The term "aberration" in the *ATP13A2* gene or locus encompasses all forms of mutations including deletions, insertions, point mutations and substitutions in the coding and non-coding regions of *ATP13A2*. It also includes changes in methylation patterns of *ATP13A2* or of an allele of *ATP13A2*. Deletions may be of the entire gene or only a portion of the gene. Point mutations may result in stop codons, frameshift mutations or amino acid substitutions. Somatic mutations are those which occur only in certain tissues, e.g. in the tumor tissue and are not inherited in the germline. Germline mutations can be found in any of a body's tissues and are inherited. An *ATP13A2* allele which is not deleted (e.g. that found on the sister chromosome to a chromosome carrying a *ATP13A2* deletion) can be screened for other mutations such as insertions, small deletions, point mutations and changes in methylation pattern. Illustrative ATP13A2 amino acid and nucleic acid sequences are known in the art.

Useful diagnostic techniques to detect aberrations in the *ATP13A2* gene include but are not limited to fluorescent *in situ* hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern blot analysis, single-stranded conformational analysis (SSCA), Rnase protection assay, allele-specific oligonucleotide (ASO hybridization), dot blot analysis and PCR-SSCP (see below). Also useful is DNA microchip technology.

Predisposition to the herein disclosed conditions associated with liver, blood and/or mental or behavioural defects such as OCD-like behaviour can be ascertained by testing any

sample of a human or other mammal for mutations in a *ATP13A2* gene. This can be determined by testing DNA from any sample of a subject's body. In addition, pre-natal diagnosis can be accomplished by testing fetal cells, placental cells or amniotic fluid for mutations of the *ATP13A2* gene. Alteration of a wild type allele whether, for example, by point mutation or by deletion or by methylation, can be detected by any number of means.

There are several methods that can be used to detect DNA sequence variation. Direct DNA sequencing, either manual sequencing or automated fluorescent sequencing, can detect sequence variation. Another approach is the single-stranded conformation polymorphism assay (SSCP) (Orita *et al.*, *Proc. Nat. Acad. Sci. USA, 86:*2776-2770, 1989). This method can be optimized to detect most DNA sequence variation. The increased throughput possible with SSCP makes it an attractive, viable alternative to direct sequencing for mutation detection on a research basis. The fragments which have shifted mobility on SSCP gels are then sequenced to determine the exact nature of the DNA sequence variation. Other approaches based on the detection of mismatches between the two complementary DNA strands include clamped denaturing gel electrophoresis (CDGE) (Sheffield *et al.*, *Am. J. Hum. Genet., 49:*699-706, 1991), heteroduplex analysis (HA) (White *et al.*, *Genomics, 12:*301-306, 1992) and chemical mismatch cleavage (CMC) (Grompe *et al., Proc Natl Acad Sci U S A. 86*(15): 5888-5892, 1989). Other methods which might detect mutations in regulatory regions or which might comprise large deletions, duplications or insertions include the protein truncation assay or the asymmetric assay. A review of methods of detecting DNA sequence variation can be found in Grompe *et al.*, 1989 (*supra*). Once a mutation is known, an allele specific detection approach such as allele specific oligonucleotide (ASO) hybridization can be utilized to rapidly screen large numbers of other samples for that same mutation. Such a technique can utilize probes which are labeled with gold nanoparticles to yield a visual color result (Elghanian *et al.*, *Science 277:* 1078-1081, 1997).

Other tests for confirming the presence or absence of a wild type or mutant *ATP13A2* allele include single-stranded conformation analysis (SSCA) (Orita *et al.*, 1989 (*supra*)); denaturing gradient gel electrophoresis (DGGE) (Wartell *et al.*, *Nucl. Acids Res., 18:*2699-

2705, 1990; Sheffield *et al.*, *Proc. Natl. Acad. Sci. USA, 86:*232-236, 1989); RNase protection assays (Finkelstein *et al.*, *Genomics, 7:*167-172, 1990; Kinszler *et al.*, *Science, 251:*1366-1370, 1991); denaturing HPLC; allele-specific oligonucleotide (ASO hybridization) (Conner *et al.*, *Proc. Natl. Acad. Sci. USA, 80:*278-282, 1983); the use of proteins which recognize nucleotide mismatches such as the *E. coli* mutS protein (Modrich, *Ann. Rev. Genet., 25:*229-253, 1991) and allele-specific PCR (Ruano *et al., Nucl. Acids. Res., 17:*8392, 1989). For allele-specific PCR, primers are used which hybridize at their 3' ends to a particular *ATP13A2* mutation or to junctions of DNA caused by a deletion of *ATP13A2*. If the particular *ATP13A2* mutation is not present, an amplification product is not observed. Amplification Refractory Mutation System (ARMS) can also be used, as disclosed in European Patent Publication No. 0 332 435 and in Newtown *et al.* (*Nucl. Acids. Res., 17:*2503-2516, 1989). Insertions and deletions of genes can also be detected by cloning, sequencing and amplification.

Microchip technology is also applicable to the present invention. In this technique, thousands of distinct oligonucleotide or cDNA probes are built up in an array on a silicon chip or other solid support such as polymer films and glass slides. Nucleic acid to be analysed is labelled with a reporter molecule (e.g. fluorescent label) and hybridized to the probes on the chip. It is also possible to study nucleic acid-protein interactions using these nucleic acid microchips. Using this technique, one can determine the presence of mutations or sequence the nucleic acid being analysed or one can measure expression levels of a gene of interest or multiple genes of interest such as genes encoding products in a biochemical pathway. The technique is described in a range of publications including Hacia *et al,. Nature Genetics, 14:* 441-447, 1996; Shoemaker *et al., Nature Genetics, 14:* 450-456, 1996; Chee *et al., Science, 274:* 610-614, 1996; Lockhart *et al., Nature Biotechnology, 14:*1675-1680, 1996; DiRisi *et al., Nature Genetics, 14:* 457-460, 1996; and Lipshutz *et al. Biotechniques, 19:* 442-447, 1995.

Alteration of wild type *ATP13A2* genes can also be detected by screening for alteration of wild type ATP13A2 proteins. For example, monoclonal antibodies immunoreactive with ATP13A2 can be used to screen a sample. Lack of cognate antigen would indicate an

ATP13A2 mutation. Antibodies specific for products of mutant alleles such as those that fail to bind Mn^{2+} or have defective catalytic or hydrolase or other binding activity could also be used to detect mutant ATP13A2 gene product. Such immunological assays can be done in any convenient format known in the art. These include Western blots, immunohistochemical assays and ELISA assays. Other assays include standard binding assays known in the art.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production is derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation (i.e. comprising ATP13A2) or can be done by techniques which are well known to those who are skilled in the art. (See, for example, Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol. II, ed. by Schwartz, 1981; Kohler *et al.*, *Nature, 256:*495-499, 1975; Kohler *et al., European Journal of Immunology. 6:*511-519, 1976).

The activity of ATP13A2 may be monitored using DNA or protein binding assays, reporter assays or direct or indirect assays of ATP13A2 activity including the use of ATPase assays antibodies or other proteinaceous or genetic agents in a number of assays which are well known to those of skill in the art. Antibodies, for example, may be used to detect ATP13A2 by Western Blotting, cytometric histochemical or ELISA procedures. As discussed herein below, such agents may also distinguish between active and inactive forms of the ATP13A2 or between mutant and normal forms of *ATP13A2*. In accordance with some embodiments, mutant forms of ATP13A2 are forms of *ATP13A2* (found in a population of subjects) which are associated with aberrant or Mn^{2+} binding or enzymatic activity or liver or brain dysfunction or predisposition thereto. In some embodiments, normal forms of ATP13A2 are forms of ATP13A2 which are not associated with the subject herein disclosed conditions. Mutant forms of *ATP13A2* may also be conveniently be detected using nucleic acid based assays well know in the art and as described herein. Low levels of active polypeptide may be produced as a result of mutations in *ATP13A2*

leading to altered expression levels, altered transcript stability or altered functional activity. Thus, ATP13A2 activity may be monitored indirectly by monitoring RNA production and/or stability or the levels of regulatory molecules such as enhancers and repressors. The activity of variant forms of ATP13A2 may be monitored using an *in vivo* or non-human or mammalian or teleost bioassays.

The level or activity of ATP13A2 or *ATP13A2* may be determined by any art recognised method. In particular, the enzymatic or Mn^{2+} binding activity of the polypeptide can be assessed. In other embodiments, *ATP13A2* is tested for mutations associated with ATP13A2 dysfunction or its level determined by quantitative amplification reaction-based methods such as RT-PCR.

In further embodiments, the diagnosis or prognosis is practised using a kit comprising instructions for use. In some embodiments, the instructions may include dietary advice, such as to maintain a low fat diet. In some embodiments, the kit comprises an antibody or antigen-binding fragment of an antibody specific to ATP13A2.

In another embodiment, the present invention provides an isolated cell or non-human organism comprising such cells, wherein the activity of ATP13A2 is reduced or inhibited compared to a non-modified organism of the same species.

In some embodiment, the organism is a recognised animal model organism such as a mouse or zebrafish.

In another aspect the present invention provides modified animals or cells for use *inter alia* in the development or testing of agents as described herein.

The genetically modified animals described herein and cells therefrom provide a model or sensitized system in which to study the affects of a range of agents.

The term "genetically modified" refers to changes at the genome level and refers herein to a cell or animal that contains within its genome a specific gene which has been altered. Alternations may be single base changes such as a point mutation or may comprise deletion of the entire gene such as by homologous recombination. Genetic modifications include alterations to regulatory regions, insertions of further copies of endogenous or heterologous genes, insertions or substitutions with heterologous genes or genetic regions etc. Alterations include, therefore, single of multiple nucleic acid insertions, deletions, substitutions or combinations thereof.

Cells and vertebrates which carry a mutant *ATP13A2* allele or where one or both alleles are modified can be used *inter alia* as model systems to study the effects of ATP13A2 or Mn^{2+} supplementation and/or to test for agents which have potential as therapeutic or teratogenic agents when ATP13A2 functional activity is impaired or as a marker for manganese deficiency. Animals for testing therapeutic agents can be selected after mutagenesis, knock-down, or introduction of over expression molecules of whole animals or after treatment of germline cells or zygotes. Such treatments include insertion of mutant *ATP13A2* alleles (including those carrying loxP flanking sequences), usually from a second animal of the same species, as well as insertion of disrupted homologous genes. Alternatively, the endogenous *ATP13A2* gene of the animals may be modified by insertion or deletion mutation or other genetic alterations using conventional techniques. These animal models provide an extremely important testing vehicle for potential therapeutic products. The cells may be isolated from individuals with *ATP13A2* mutations, either somatic or germline. Alternatively, the cell line can be engineered to carry the mutation in the *ATP13A2* allele, as described above, or by gene modification using zinc finger nucleases (see Meng *et al.*, *Nat. Biotechnology, 26(6)*: 650-701, 2008; Doyon *et al.*, *Nat. Biotech. 26*: 702-708, 2008). After a test substance is applied to the cells, the phenotype of the cell is determined. Any trait of the cells can be assessed.

Thus a genetically modified animal or cell includes animals or cells from a transgenic animal, a "knock in" or knock out" animal, conditional variants or other mutants or cells or animals susceptible to co-suppression, gene silencing or induction of RNAi.

Conveniently, targeting constructs are initially used to generate the modified genetic sequences in the cell or organism. Ta rgeting constructs generally but not exclusively modify a target sequence by homologous recombination. Alternatively, a modified genetic sequence may be introduced using artificial chromosomes. Targeting or other constructs including reporter constructs for screening potential ATP13A2 modulators are produced and introduced into target cells using methods well known in the art which are described in molecular biology laboratory manuals such as, for example, in Sambrook, 2001 (*supra*); Ausubel (Ed) *Current Protocols in Molecular Biology*, 5th Edition, John Wiley & Sons, Inc, NY, 2002. Targeting constructs may be introduced into cells by any method such as electroporation, viral mediated transfer or microinjection. Selection markers are generally employed to initially identify cells which have successfully incorporated the targeting construct.

One method for modifying expression of *ATP13A2* is the GAL4-UAS system described for example by Fischer *et al., Nature, 332*: 853-856, 1998, as reviewed by Scheer *et al., Mechanism of Development, 80*: 153-158, 1999, this technique is based on two different kinds of transgenic strains, called activator and effector lines. In an activator line the gene for the yeast transcriptional activator GAL4 is placed under the control of a specific promoter, while in the effector line the gene of interest is fused to the DNA-binding motif of GAL4. The effector gene will be transcriptionally silent unless animals carrying it are crossed to those of an activator line. In the progeny of this cross, expression of the effector gene will reflect the pattern of expression of GAL4 in the activator, which is ultimately dependent on the promoter that has been used to control it. This, of course, allows controlled ectopic expression of the effector gene. The use of activators with different expressivities, which arise due to positional effects acting on the activator construct, allows the experimenter to exploit a relatively wide range of levels of effector gene expression.

The human ATP13A2 GenBank Accession No is AY4617/2.1. A mutation K509E in this gene abolished ATP13A2 ATPase activity and expression of the mutant reduced Mn^{2+} levels in brain mitochondria.

The homologous mouse ATP13A2 gene is published at NM_029097, the mRNA/cDNA sequence occurs at GenBank Accession No. BC042661 (35936p mRNA), NM_029097.

Representative examples of the nucleic acid and amino acid sequences of ATP13A2 molecules provided in publically available databases include homologous proteins from human, mouse and zebrafish which are generally more than 60% identical. Accordingly, the terms ATP13A2 or *ATP13A2* in the claims encompass all homologs and isoforms in any animal species including human homologs and isoforms and homologs of veterinary interest. Preferably, a homolog of ATP13A2 or *ATP13A2* has at least 60% identity to publish human, mouse or zebrafish amino acid sequences at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99% identity or their encoding sequences. Percentage identity is a well known concept in the art and can be calculated using, for example but without limitation, the BLAST software available from NCBI (Altschul *et al., J. Mol. Biol., 215*: 403-410, 1990; Gish and States, *Nature Genet., 3*: 266-272, 1993; Madden *et al., Meth. Enzymol., 266*: 131-141, 1996; Altschul *et al., Nucleic Acids Res., 25*: 3389-3402, 1997; Zhang and Madden, *Genome Res., 7*: 649-656, 1997).

Alternatively, the Cre-LoxP system can be used to provide appropriate conditional ATP13A2 levels (see Sternberg and Hamilton, *J. Mol. Biol., 150*: 467-486., 1981; Lakso *et al., Proc Natl Acad Sci U S A., 89*(14): 6232-6, 1992; Langenau *et al., Proc Natl Acad Sci U S A., 102*(17): 6068-73, 2005) by providing targeted activation (or inactivation).

Genetically modified organisms are generated using techniques well known in the art such as described in Hogan *et al., Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbour Laboratory Press, CSH NY, 1986 especially Sections E, F and G; Mansour *et al., Nature 336*: 348-352, 1988. Stem cells including embryonic stem cells (ES cells) are introduced into the embryo of a recipient organism at the blastocyst stage of development. There they are capable of integration into the inner cell mass where they develop and contribute to the germ line of the recipient organism. ES cells are

conveniently obtained from pre-implantation embryos maintained *in vitro*. Once correct targeting has been verified, modified cells are injected into the blastocyst or morula or other suitable developmental stage, to generate a chimeric organism. Alternatively, modified cells are allowed to aggregate with dissociated embryonic cells to form aggregation chimera. The chimeric organism is then implanted into a suitable female foster organism and the embryo allowed to develop to term. Chimeric progeny are bred to obtain offspring in which the genome of each cell contains the nucleotide sequences conferred by the targeting construct. Genetically modified organism may comprise a heterozygous modification or alternatively both alleles may be affected.

ATP13A2 overexpression may be achieved by transgenesis in which *ATP13A2* overexpression is driven by a later promoter or by an inducible promoter (e.g. the tet-on or tet-off system, see Hillen and Berens, *Annu. Rev. Microbiol., 48*: 345-369, 1994; Gossen and Bujadt, *Proc. Nati. Acad. Sci. USA, 89*: 5547-5551, 1992; Huang *et al., Dev Dyn, 233*(4): 1294-1303, 2005), allowing screening of drug effects at later timepoints following endogenous or exogenous activation of the promoter driving *ATP13A2* overexpression. Again, an expected readout for normal overexpression of *ATP13A2* may be increased mitochondrial Mn^{2+}.

The terms "genetic material", "genetic forms", "nucleic acids", "nucleotide" and "polynucleotide" include RNA, cDNA, genomic DNA, synthetic forms and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog (such as the morpholine ring), internucleotide modifications such as uncharged linkages (e.g. methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g. phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g. polypeptides), intercalators (e.g. acridine, psoralen, etc.), chelators, alkylators and modified linkages (e.g. α-anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence *via*

hydrogen binding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

The present invention provides a method of treatment or prophylaxis of a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid associated liver disorder, the method comprising administering a composition comprising ATP13A2 polypeptide or a functional analog or *ATP13A2* nucleic acid to a subject in need thereof for a time and under conditions sufficient for the treatment or prophylaxis of the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid associated liver disorder.

The term "composition" and terms such as "agent", "medicament", "active" and "drug" are used interchangeably herein to refer to a chemical compound or cellular composition which induces a desired pharmacological and/or physiological effect. The terms encompass pharmaceutically acceptable and pharmacologically active ingredients including but not limited to salts, esters, amides, pro-drugs, active metabolites, analogs and the like. The term includes genetic and proteinaceous molecules or analogs thereof as well as cellular compositions as previously mentioned. The instant compounds and compositions are suitable for the manufacture of a medicament for the treatment and/or prevention of herein described conditions/disorders. In relation to cellular compositions, the present invention extends to cellular compositions including genetically modified cells such as liver cells (hepatocytes) which are capable of regenerating or augmenting tissues and/or organs of an animal subject *in situ* or *in vivo.* Stem cells or stem cell-like cells are preferably multipotent or pluripotent. Other cellular compositions comprise vectors such as viral vectors for delivery of nucleic acid constructs capable of producing ATP13A2 in a cell of a subject as described later herein.

Composition include those comprising or encoding all or an active part of ATP13A2 or an active variant thereof. Polypeptide variants may include a small number of conservative substitutions as illustrated in Tables 2 and 3 and as well known in the art. Such variants

comprise at least 95% amino acid sequence identity to a published sequence of a wild-type or naturally occurring variant of ATP13A2.

"Percentage similarity" between a particular sequence and a recited amino acid or nucleotide sequence includes at least about 95% or above such as at least about 96%, 97%, 98%, 99% or greater. Percentage identities between 60% and 100% are also contemplated such as 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%.

The term "mammalian" as used herein includes humans, primates, livestock animals, laboratory test animals, companion animals and wild captive animals, preferably, the mammal is a human or a laboratory test animal. Even more preferably, the mammal is a human.

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease or condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

Analogs contemplated herein include the use of non-proteogenic and non-naturally occurring amino acids which may be prepared by side-chain modification or total synthesis. Examples of side-chain modifications contemplated by the present invention are those well known in the pharmaceutical art. A list of unnatural amino acids contemplated is included in Table 4.

Similarly, polynucleotide sequences contemplated for use in the present invention are substantially similar and have at least 95% or at least 99% sequence identity to active wild-type ATP13A2 coding sequences but may comprise routine modifications as known in the art to enhance their expression levels, half-life or other pharmacological attributes by expected amounts.

In accordance with another aspect of the present invention, one or more samples from a subject may be tested using the herein disclosed methods to determine whether gene or cell therapy with an agent comprising or encoding *ATP13A2* or a functional analog thereof is indicated. The provision of wild type or enhanced ATP13A2 function to a cell which carries a mutant or altered form of *ATP13A2* should in this situation complement the deficiency and result in an improvement in the subject. Alternatively, cells capable of providing normal or enhanced ATP13A2 activity are provided. The *ATP13A2* allele may be introduced into a cell in a vector such that the gene remains extrachromosomally. Alternatively, artificial chromosomes may be used. Typically, the vector may combine with the host genome and be expressed therefrom.

Gene therapy would be carried out according to generally accepted methods, for example, as described by Friedman, Ed., *Therapy for Genetic Disease*, Oxford University Press, pp. 105-121, 1991 or Culver, *Gene Therapy: A Primer for Physicians*, 2nd Ed., 1996. Suitable vectors are known, such as disclosed in U.S. Patent No. 5,252,479, International Patent Publication No. WO 93/07282 and U.S. Patent No. 5,691,198. Gene transfer systems known in the art may be useful in the practice of the gene therapy methods of the present invention. These include viral and non-viral transfer methods as well known in the art.

Liver directed gene therapy and hepatocyte transplantation methods are known in the art. Hepatocytes injected into the hepatic cords are detectable in the liver within days. Hepatocyte transplantation has resulted in partial correction of metabolic disorders in LDL-receptor deficient Watanabe Heritable Hyperlipidemic rabbits, and Long-Evans Cinnamon rats. Genes may be delivered to the liver by systemic administration, infusion into the portal vein, hepatic artery or bile duct, or direct injection into the liver.

Non-viral gene transfer methods are also known in the art such as chemical techniques including calcium phosphate co-precipitation, mechanical techniques, for example, microinjection, membrane fusion-mediated transfer *via* liposomes and direct DNA uptake and receptor-mediated DNA transfer. Viral-mediated gene transfer can be combined with direct *in vivo* gene transfer using liposome delivery.

In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization and degradation of the endosome before the coupled DNA is damaged. For other techniques for the delivery of adenovirus based vectors, see U.S. Patent No. 5,691,198. Liposome/DNA complexes are also capable of mediating direct *in vivo* gene transfer.

Expression vectors in the context of gene therapy are meant to include those constructs containing sequences sufficient to express a polynucleotide that has been cloned therein. In viral expression vectors, the construct contains viral sequences sufficient to support packaging of the construct. If the polynucleotide encodes a polypeptide having the relevant functional activity of ATP13A2, expression will produce a polypeptide having the functional activity of ATP13A2. If the polynucleotide encodes a sense or antisense polynucleotide or a ribozyme or DNAzyme, expression will produce the sense or antisense polynucleotide or ribozyme or DNAzyme. Thus, in this context, expression does not

require that a protein product be synthesized. In addition to the polynucleotide cloned into the expression vector, the vector also contains a promoter functional in eukaryotic cells. The cloned polynucleotide sequence is under control of this promoter. Suitable eukaryotic promoters are routinely determined.

Receptor-mediated gene transfer may be achieved by conjugation of DNA to a protein ligand *via* polylysine. Ligands are chosen on the basis of the presence of the corresponding ligand receptors on the cell surface of the target cell/tissue type. These ligand-DNA conjugates can be injected directly into the blood if desired and are directed to the target tissue where receptor binding and internalization of the DNA-protein complex occurs. To overcome the problem of intracellular destruction of DNA, co-infection with adenovirus can be included to disrupt endosome function.

Accordingly, in some embodiments, patients who carry an aberrant *ATP13A2* allele are treated with a gene delivery vehicle such that some or all of their cells receive at least one additional copy of a functional normal ATP13A2 allele. Preferably, only specific cells such as liver or brain cells are targeted.

Alternatively, peptides or mimetics or other functional analogs which have ATP13A2 activity can be supplied to cells which carry aberrant *ATP13A2* alleles. Protein can be produced by expression of the cDNA sequence in bacteria or other suitable host cells known in the art, for example, using known expression vectors. In addition, synthetic chemistry techniques can be employed to synthesize the instant active molecules. Active molecules can be introduced into cells by microinjection or by use of liposomes, for example. Alternatively, some active molecules may be taken up by cells, actively or by diffusion. In some embodiments, supply of molecules with ATP13A2 activity leads to enhanced liver and mitochondrial function.

In some embodiments, the present invention employs recombinant nucleic acids including a recombinant construct comprising all or part of *ATP13A2*. The recombinant construct may be capable of replicating autonomously in a host cell. Alternatively, the recombinant

construct may become integrated into the chromosomal DNA of the host cell. Such a recombinant polynucleotide comprises a polynucleotide of genomic, cDNA, semi-synthetic or synthetic origin which, by virtue of its origin or manipulation: (i) is not associated with all or a portion of a polynucleotide with which it is associated in nature; (ii) is linked to a polynucleotide other than that to which it is linked in nature; or (iii) does not occur in nature. Where nucleic acids according to the invention include RNA, reference to the sequence shown should be construed as reference to the RNA equivalent with U substituted for T. Such constructs are useful to elevate ATP13A2 levels or to down-regulate *ATP13A2* levels such as *via* antisense means or RNAi-mediated gene silencing. As will be well known to those of skill in the art, such constructs are also useful in generating animal models carrying modified alleles of *ATP13A2* and, as pharmaceutical compositions for modulating the activity of ATP13A2 in a subject *in vivo*.

Genetically modified cells or non-human organisms may be provided in the form of cells or embryos for transplantation. Cells and embryos are preferably maintained in a frozen state and may optionally be distributed or sold with instructions for use.

In a further aspect, the present invention provides a genetically modified cell, or non-human animal comprising such cells, wherein a *ATP13A2* gene is modified and the cell or animal produces a substantially enhanced level or activity of ATP13A2 polypeptide, or substantially reduced level or activity of ATP13A2 polypeptide compared to a non-modified animal of the same species, or is substantially incapable of producing ATP13A2 polypeptides.

The genetically modified cells and non-human animals may be a non-human primate, livestock animal, companion animal, laboratory test animal, captive wild animal, reptile, amphibian, fish, bird or other organism. Preferably the genetically modified non-human animal is a mouse.

In one aspect, the modified cell or non-human animal is genetically modified and produces a substantially reduced level of ATP13A2 or is substantially incapable of producing

ATP13A2 or produces ATP13A2 having substantially reduced or no activity. Preferably the *ATP13A2* gene is modified. Modification may be in one or both alleles and may optionally be within a regulatory region of the gene. In another embodiment, the genetic modification resulting in a cell or animal capable of exhibiting a modified level or activity of ATP13A2 comprises genetic modification outside the *ATP13A2* gene to cause expression of genetic or proteinaceous molecules which effectively modulate the activity of ATP13A2 or *ATP13A2.* In a preferred aspect, the modified cell or non-human animal is genetically modified and substantially overproduces ATP13A2 having normal or altered activity relative to an unmodified cell or animal of the same species.

In yet another aspect, the invention provides a method of screening for or testing an agent capable of complementing a phenotype shown by a cell or non-human animal comprising a modified *ATP13A2* nucleic acid or ATP13A2 polypeptide and exhibiting a substantially modified level or activity of ATP13A2 polypeptide. Prefer ably, the cell or animal is contacted with the agent and its effect on the activity of ATP13A2 or its binding targets determined. In one aspect the method comprises screening for mutants which exhibit a complementing phenotype and then mapping and identifying the modifying gene. In another aspect the method comprises screening for agents which enhance the level or activity of ATP13A2 in a normal or modified cell. In some embodiments, small-molecule libraries are screened for agents which directly or indirectly modulate ATP13A2 polypeptide activity.

One method is described by Stegmaier *et al.*, *PLOS Medicine, 4*(4): 702-714, 2007. Here, expression profiles diagnostic of an ATP13A2-on activity and an ATP13A2-off activity are chosen, and the ability of small-molecules to produce either the ATP13A2-on or the ATP13A2-off profile is determined. Antisense knockdown strategies for selecting the ATP13A2-off activity are routine in the art and include ShRNAs or morpholinos directed against the ATP13A2 transcript. Specifically, chemical screening can be performed as described in Lieschke and Curry, *Nat. Rev. Genetics, 8*(5): 353-367, 2007. Small groups of zebrafish embryos or larvae are arrayed in multi-well microtitre plates and standard concentrations of small molecules are robotically pipetted into the raising media in

individual wells. Throughput is increased if suppression can be assessed directly in the larvae using fluorescent read-outs, or if it can be made quantitative in some way, particularly if the scoring process is suited to automation. Scoring can also be coupled with an immunological or gene-expression assay to monitor cell-cycle progression. The active compounds that are identified can undergo a secondary process of validation, dose and toxicity assessment, and can be extended by exploration of analogs generated by combinatorial chemistry, before proceeding to testing in other animal models. For examples of chemical screening in zebrafish are described in Schlueter & Peterson, *Circulation, 120*(3): 255-63, 2009; Mukhopadhyay *et al., Current Opinion in Hematology, 15*(3): 221-227, 2008.

In further embodiment, the subject invention provides a use of a cell or non-human animal comprising a modified *ATP13A2* or ATP13A2 and exhibiting a substantially enhanced level or activity of ATP13A2 in screening for or testing agents for use in the treatment or prophylaxis of a condition, states or disorders as described herein.

A substantially modified level or activity of ATP13A2 is conveniently assessed in terms of a percent reduction relative to normal cells or animals or pre-treatment/pre-administration. A substantial increase includes one which results in, for example, increased mitochondria Mn^{2+} levels in a subject or cell. Alternatively, a reduced level of gene expression of transcription targets or a reporter thereof is detected. Preferably, the modification is at least 20% enhanced or reduced compared to normal cells, more preferably about 25%, still more preferably at least about 30% reduction, more preferably at least about 40% enhanced or reduced ATP13A2 level or activity. The reduction may of course be complete loss of ATP13A2 activity in a cell or animal. A "modified" level or activity includes enhanced levels of ATP13A2 activity relative to pre-treatment levels and may equate to or exceed the level or activity of ATP13A2 detectable in controls. Overexpression includes a forced expression in all tissue or more particularly specific tissue or regions. No particular level of expression is prescribed. The terms refer to expression that is not essentially normally developmentally regulated.

In another aspect, the present invention provides, a method of screening for agents that complement the phenotype of an ATP13A2 deficient animal model. Typically, putative useful agents are screened in the subject animal or cell models, *inter alia,* for their ability to increase Mn^{2+} storage in the liver, and decrease levels of Mn^{2+} in the circulation, or to increase mitochondrial Mn^{2+}. In some embodiments, agents are tested for their ability to prevent or slow progressive neural loss in the brain or to stimulate neural regeneration. In other embodiments, agents are tested for their ability improve one or more symptoms or signs of a mental or behavioural disorder as disclosed herein, such as excessive grooming behaviours, anxiety, dyskinesia.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Generation of ATP13A2 KO mice

A targeting vector was constructed using the plasmid FLSniper (Ozgene, WA, Australia). DNA fragments were generated by PCR from mouse 129Sv/J genomic DNA. The targeting sequence contains LoxP - Exon 2 - Exon 3 - FRT- Neo Cassette - FRT - LoxP flanked by homologous arms (see Figure 1A). The targeting vector was inserted by electroporation into W9.5 embryonic stem (ES) cell line (passage 28) derived from a sub-strain of 1291/Sv-p+Tyr+KitlSl-J/+ (Szabo and Mann, *Development 120*: 1651-1660, 1994). After having undergone homologous recombination, the targeted W9.5 ES cells with two loxP sites were isolated using standard procedures and injected into C57BL/6 blasts. The resulting chimaeras of Donor (W9.5) and Host (C57BL/6) ES cells were bred to C57BL/6 mice to obtain germ-line transmission, and the agoutis carrying genetic modification were further mated to C57BL/6 mice to generate a heterozygous targeted line (wt/floxP) where exons 2 and 3 and the pkg-neo cassettes are flanked by loxP sites, in which the pkg-neo cassette is flanked by FRT sites. The resulting wt/floxp germ line was bred to a cre deleter strain (C57BL/6-OzCRE) from which the Cre cassette had been knocked into the ROSA-26 locus (Ozgene, WA, Australia). The breeding generated a heterozygous knockout line (wt/KO/cre) from which exons 2 and 3, and pkg-neo cassettes were deleted. ATP13A2 KO mice were obtained by intercrossing ATP13A2 heterozygous (+/-) mice and the genotype of the KO line was verified by Southern Blotting (not shown), genotyping and gene expression analysis (Figure 1b, 1c).

Genotyping and animal experiments

Mice were bred, genotyped and maintained with 12-hr light and dark cycles (Jones *et al., Proc Natl Acad Sci USA 97*: 12735-12740, 2000). KO offspring were genotyped by PCR amplification of genomic DNA isolated from the mouse tail tip (Figure 1c). The primers for ATP13A2 KO genotype were 5'-ATGCCAGTAGTAGCAAGACAGGTG-3' (SEQ ID NO: 1) (b in Figure 1a) and 5'-CAGTCTTATCTATGTGGCTTTGGTG-3' (SEQ ID NO: 2) (c in Figure 1a). The following conditions were used: Incubation at 94°C for 3 min, then 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by 1 cycle of

72°C for 10 min. Male mice of 8-24 weeks were used, unless otherwise specified. They were fed standard rodent chow or a high-fat diet containing 60% fat-derived calories. Body weight was measured weekly for a period of 28 weeks. The food intake of mice was monitored using singly housed mice. For the diet-induced effect, mice at ~24 weeks of age were fed a high-fat diet for 8 weeks, based on Research Diets D12451 with some modifications. Tissues and organs were dissected and collected by embedding and freezing in O.C.T. compound and snap freezing for protein and quantitative RT-PCR (qRT-PCR).

Measurement of copper, zinc, iron and manganese, and serum biochemistry

Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} were measured on a sample of tissue using Graphite Furnace Atomic Absorption Spectrometry. Random samples of liver and brain (from 1 mm caudal to the optic chiasm) were acid digested and analyzed against aqueous standards (Pamphlett *et al., Neurotoxicology 22*: 401-410, 2001). The blood levels of lipids, glucose, and hormones were measured at the Pathology and Biochemistry Laboratories, Sydney South West Area Health Service.

Histology and immunofluorescence microscopy

Histological observation was carried out on frozen tissue sections (5 μm thick or as indicated) stained with hematoxylin and eosin (HE), Oil Red O or periodic acid-Schiff (PAS) using standard protocols. Brain coronal cryosections were 10 μm thick, taken from the optic chiasm to 2 mm caudal to the optic chiasm. Frozen brain sections, or cultured cells grown on glass cover slips as indicated were rinsed in PBS and subjected to immunofluorescence staining (Yang *et al., J Biol Chem 276*: 4251-4260, 2001). Briefly, cells were prefixed with 4% (v/v) paraformaldehyde in PBS (pH 7.4) for 15 min, the free aldehyde groups quenched in 50 mM NH_4Cl in PBS, and the cells permeabilised with 0.1% (v/v) Triton X-100 for 5 min at room temperature. After washing and blocking for 30 min with 1% (w/v) bovine serum albumin (BSA), cells were incubated for 1 h at room temperature with the primary antibodies diluted in 1% BSA in PBS, then washed and incubated with fluorescein isothiocyanate-conjugated second antibody for 1 h at room temperature. After a further wash in PBS, the cover slips were mounted onto glass slides with 2.6% (v/v) DABCO (1,4-diazabicyclo-(2,2,2)octane; Sigma) in 90% (v/v) glycerol in

PBS (pH 8.6). Slides were visualized using a Nikon Eclipse TE2000 Plan Fluor 100× oil emersion objective, DS-5MC CCD camera and NIS-Elements F 2.20 software (Nikon). Confocal microscopy was conducted using a laser confocal imaging system (Nikon D-eclipse equipped with NIS-elements AR30 software). The confocal images were captured by a 60x/1.4 Nikon oil lens (Nikon ECLIPSE 90i).

TUNEL apoptosis detection kit (Millipore) was used for fluorescence DNA fragmentation staining according to the manufacturer's protocol. Briefly, brain sections were fixed with 1% paraformaldehyde in PBS overnight at 4°C, followed by 5 min permeabilization with 2:1 ethanol:acetic acid at -20°C. After equilibration, terminal deoxynucleotidyl transferase (TdT) was applied at 37°C for an hour. The samples were blocked and incubated with anti-digoxigenin FITC-conjugated antibody for 30 min at room temperature. The sections were counterstained with 1:200 of anti-NeuN (Millpore) at 4°C overnight before mounted with cover-slips.

Cell culture, gene expression, and cellular fractionation

The human HeLa cervical and HepG2 hepatic cell lines were obtained from American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium. Cell culture media were supplemented with 10% (w/w) heat-inactivated fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded in 24-well plastic plates, 6-10 cm dishes or eight-chamber glass slides (Nune, Napervile, CT) and transfected according to a standard procedure (Yang *et al.*, 2001 (*supra*)). All expression plasmids, WT and shRNA were verified before use by DNA sequencing. All plasmids used in transfection assays were prepared with the endotoxin-free plasmid Maxi-kit (Qiagen) and resuspended in endotoxin-free 0.1 x Tris/EDTA buffer to a concentration of 1 µg/µl. MCF-7 cells (2×10^5) were placed in 1 ml of medium in 24-well tissue culture plates and incubated overnight. Gene expression vectors were transfected with Fugene 6 (Roche). After 24 hours, the cells were harvested, washed in PBS, and lysed in cell lysis buffer (Promega). Subcellular fractionation was performed as described (Yang *et al.*, 2001 (*supra*)). Briefly, mouse tissue or cultured cells as indicated were homogenized in hyperosmotic buffer (440

mM mannitol, 60 mM sucrose, 40 mM HEPES). Nuclei were removed by centrifugation at 800 x g for 10 min and the supernatants were centrifuged at 15,000 x g for 10 min to collect the mitochondrial pellet. Proteins in each fraction were analyzed by immunoblotting and immunoprecipitation.

Reverse transcription and quantitative PCR

Total RNA was extracted using a High Pure RNA Tissue kit (Roche). Equal amounts of RNA were reverse transcribed with oligo(dT) using the ThermoScript system (Invitrogen) according to the manufacturer's instructions. RT-PCR and Real Time PCR were used to measure gene expression of TERT, c-Myc and p53. Expression of each gene was normalized to the housekeeping gene GAPDH. Conditions for amplification were optimized to ensure that reactions were within the logarithmic linear range. PCR products were analyzed on 1.5% (w/v) agarose gels stained with ethidium bromide. Products were quantitated by phosphor imaging and the expression of each gene normalized to GAPDH expression. Primers used for ATP13A2 RT-PCR were 5'-TCAATCGATCCCCTCAGCTCCTCAG-3' (SEQ ID NO: 3) and 5'-AGGCAGATGGAGATGGCTGAGATGA -3' (SEQ ID NO: 4), to yield a 734-bp DNA product, or 5'-GGTCAAGTTGTCCATGCGGGTGTG-3' (SEQ ID NO: 5) and 5'-AGAGGCACCCGGTTTCGGTAGAGGA-3' (SEQ ID NO: 6), for a 450-bp product. Primers used for GAPDH RT-PCR were 5'-CATGACAACTTTGGCATTGTGG-3' (SEQ ID NO: 7) and 5'-CAGATCCACAACGGATACATTGGG-3' (SEQ ID NO: 8). Products were quantitated by phosphor imaging and the expression of each gene normalized to GAPDH expression.

Western blotting

Extracted tissue proteins were quantitated using a Bio-Rad protein assay. Proteins were separated by 10% (w/v) SDS-PAGE and transferred onto nitrocellulose membrane by electroblotting. The nitrocellulose membranes were blocked at 4°C overnight in 10% (w/v) nonfat milk, 0.2% (v/v) FCS, 0.05% (w/v) Tween 20 in 1x PBS and incubated at room temperature for 1 h with antibodies as indicated, then with the appropriate horseradish peroxidase-conjugated secondary antibody (DAKO Australia) diluted 1:1000. Antibodies

against MTP (N-17), CD68 (H-255), SOD-2 (N-20) and transferrin (M-70) were from Santa Cruz Biotechnology Inc. A SuperSignal West PICO (Calbiochem) chemiluminescent substrate kit was used to detect and visualize protein antigens after exposure to BioMax autoradiographic film (Kodak). Alternatively X-ray film or Odyssey was used for autoradiography. The autoradiograph films were scanned and the bands quantified on a Phosphor-Imager (Fujifilm FLA-2000, Berthold).

Behaviour analyses

Grooming was observed in adult mice aged 4-14 months using a video camera for four hours in four-hour intervals. The time spent grooming, including all sequences of face-wiping, scratching/rubbing of head and ears, and full body grooming was analyzed for individual mice. The dark-light choice test (DLC) was carried out using a dark box placed inside the open field chambers to divide the chamber into a bright compartment and a dark compartment. Mice were placed in the dark compartment, and the time and ambulatory exploration were recorded in both areas for 30 min essentially as described (Zhou *et al., Proc Natl Acad Sci U S A 107*: 4401-4406, 2010). The rearing behaviour test was videoed of the animal's engagement in exploratory behaviour in a clear plexiglass cylinder (height 30 cm, diameter 20 cm) for 5 min. Rearing was determined as the contact of both forelimbs with the wall of the cylinder above shoulder level (Cannon *et al., Neurobiol Dis 34*: 279-290, 2009). The nest-building test was carried out in a single open-space with nesting material placed for 15 h as previously described (Sager *et al., Behav Brain Res 208*: 444-449, 2010). The vertical and horizontal grid tests were conducted according to the methods described (Kim *et al., Brain Res 1306*: 176-183, 2010).

Statistical Analysis

Data were analyzed using student t-tests and ANOVA Post Hoc tests. A probability (P) value of less than 0.05 was considered statistically significant.

EXAMPLE 2

Effect of disrupting the ATP13A2 gene

To investigate the physiological function of ATP13A2 in the trafficking of trace metal in mammals, the *ATP13A2* gene was disrupted by creating a mouse line in which exons 2 and 3 of the *ATP13A2* gene locus were floxed by loxP sites (Figure 1a). Heterozygous mice were generated by crossing the Zlox mice with Cre deleter transgenic mice. The *ATP13A2* lox/lox (knockout, KO) mice were obtained by intercross of *ATP13A2* lox/- mice. Genotyping and gene expression analysis confirmed the genetic disruption and gene expression deficiency of TP13A2 (Figure 1b, c). By the age of 4-6 months, ATP13A2 KO mice developed excessive losses of hairs, first noticed as a patch of hairless skin on the body back and neck. During maintenance of these mice, it was further observed that individually housed ATP13A2 homozygous mutants displayed large bald patches on their backs, necks, lateral body surfaces and faces (Figure 2a). The over-grooming phenotype occurred in both sexes although much more frequent in females than males of age and was continued to more than one year old in ATP13A2 -/- mice compared to the aged-matched wild type littermates (Figure 2a). The extent of hair loss varied in mice from displayed bald spots to the more severely bald skins as the loss of eyebrows, the loss of whiskers, bald face, bald head, bald neck back and bald body back. Examination of ATP13A2 -/- mice revealed significant amounts of body hair trapped between the gums and teeth, and present in their stomachs, suggesting that the ATP13A2 deficient mice actively removed their hair.

ATP13A2 KO mice appeared to be prone to fear and stress. Using noninvasive tests on a vertical grid, ATP13A2 KO mice showed a significant delay in direction turning and walking down the grid in comparison with the age- and sex-matched control mice (Figure 11). Nest building test showed that ATP13A2 KO mice displayed anxiety in building nests in open fields compared to control littermates. Consistent with fear and anxiety, ATP13A2 KO mice appeared frighted to come down from the horizontal test with significant time delays (Figure 12). Together, these excessive pathological grooming and anxiety

behaviours indicate a striking similarity of the ATP13A2 mice to the mental disorder obsessive compulsive disorder (OCD).

Another significant difference between ATP13A2 KO mice and their WT littermates was the slightly larger size of young adult ATP13A2 KO males. The average body weight of 8-16 week-old male ATP13A2 KO mice was 10-20% greater than that of the age-matched controls, whereas female ATP13A2 KO mice were the same size as WT females (not shown). The caloric intake of KO and WT mice was the same. The serum lipid levels were significantly higher in ATP13A2 male KO mice than WT counterparts (total cholesterol 4 mM vs. 3 mM, $p<0.05$, n=5; TG 5 mM vs. 3 mM, $p<0.01$, n=5; HDL-cholesterol 4 mM vs. 3 mM, $p<0.05$, n=5) (Figure 2b). To determine the substrate for ATP13A2, serum levels of calcium (Ca^{2+}), magnesium (Mg^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}), iron (Fe^{2+}) and Mn^{2+} were measured in WT and ATP13A2 KO mice. The serum levels of Mn^{2+} were markedly increased from ~50 nmol/g to ~97 nmol/g (Figure 2c, $p<0.05$, n=4), whereas serum levels of Ca^{2+}, Mg^{2+}, Zn^{2+} , Fe^{2+} and Cu^{2+} were unchanged in ATP13A2 KO mice (Figure 2c). These data demonstrate that the ATP13A2-deficient mice are hyperlipidemic and high manganese in blood circulation.

Histopathological examination showed that apart from liver at 24 weeks, the abdominal viscera and the brain of ATP13A2 KO male and female mice appeared normal. The livers of ATP13A2 KO mice were significantly larger than those of WT animals (Figure 2d, e). The average liver weight in the ATP13A2 KO male mice was approximately 125% that of WT animals (Figure 2d). Under the microscope, hematoxylin and eosin (HE) staining of hepatocytes revealed substantial numbers of unstained fat droplets of diverse sizes (Figure 3a). Staining for lipids with Oil Red O confirmed marked lipid accumulation in the macrovesicular and microvesicular forms in ATP13A2 KO livers (Figure 3b, c). Affected hepatocytes were around central and portal veins throughout the livers (Figure 3a, b). Hepatic triglyceride levels were significantly higher in ATP13A2 KO mice compared with WT (Figure 3c). Consistent with the increase in body weight, the weight of epididymal fat pads from KO mice was also significantly higher than in WT mice and they contained larger lipid droplets (Figure 3d, e). There was no significant difference in glycogen and

glycolipid deposition in the livers of ATP13A2 KO and WT mice. Thus, ATP13A2 deficiency caused disorders of lipid metabolism that resulted specifically in fatty liver in association with hyperlipidemia.

PAS staining for glycogen and glycolipid deposition in the livers of WT and ATP13A2 KO mice.

To determine if ATP13A2 deficiency alters hepatic levels of the trace metals, Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} in the liver tissues of WT and ATP13A2 KO mice were measured. There was no significant difference in the levels of the four ions between newborn KO and WT animals (Figure 4a). By 24 weeks, however, Mn^{2+} levels differed markedly (Figure 3f). Hepatic Mn^{2+} levels in the 24-week-old ATP13A2 KO mice were 28 ±2 nmol/g compared to 41±4 nmol/g ($p<0.01$) in age-matched WT mice (Figure 3f). Analysis of hepatic gene expression showed a moderate increase Mn^{2+}-dependent superoxide dismutase (SOD-2), decrease of triglyceride transfer protein (MTP), and comparable transferrin, CD68 (a macrophage marker related to lysosomal glycoproteins) and actin (Figure 4c). There was a significant change of SOD-2 activity in the liver (Figure 4c). These data indicate that ATP13A2 is required for hepatic Mn^{2+} homeostasis in addition to blood circulation, such that ATP13A2 deficiency causes the redistribution of Mn^{2+} from liver to the circulation.

EXAMPLE 3

ATP13A2 deficiency and loss of cerebrocortical neurons

There was no discernible difference in the gross morphology and microscopic architecture of the brains of ATP13A2-deficient and WT mice. More specifically, the dentate gyrus and hippocampus (Figure 5e, f) were similar. However, the cerebral cortex of ATP13A2 KO mice contained fewer neurons and more microglial cells than matching sections from WT mice (Figure 51a, b). The number of ameboid microglial cells was much greater in the ATP13A2 KO cerebral cortex than that of controls.

Coronary sections from the region 1 mm caudal to the optic chiasm of WT and ATP13A2 mice KO were incubated with anti-NeuN specific antibodies for immunofluorescence staining. DNA was stained blue by DAPI. Micrographs of NeuN-positive neurons in the auditory (Au) regions. Immunoflorescence staining NeuN-positive neurons in the CA1 region of the hippocampus in the dentate gyrus (DG) regions and in the auditory region of brain cortex, by the Image-G program indicate that the cerebral cortex of ATP13A2 KO mice contained about 30% fewer NeuN-positive neurons than WT (577±94 vs. 846±77, $p<0.05$), but that the number of neurons in the Substantia nigra (SN) was the same. Data are NeuN-positive cell counts presented as mean±SEM (n=3; *$p<0.05$).

Thus, lack of ATP13A2 caused neuronal losses and increased microglial cell scavenger activity in the cerebral cortex. Comparison of the levels of Cu^{2+}, Zn^{2+}, and Fe^{2+}, between the brains of ATP13A2 KO and WT mice, showed no significant differences (Figure 5k). However, significant reduction in Mn^{2+} levels was noted (Mn^{2+} was 20±2.4 nmol/g in WT brains and 16.2±1.1 nmol/g in the ATP13A2 KO brains, n=8, $p>0.05$), demonstrating an impairment of Mn^{2+} metabolism in the brains of the ATP13A2 KO mice at 24 weeks.

EXAMPLE 4

High-fat diet intensifies ATP13A2 deficiency-induced lesions of liver and brain

To determine if the Mn^{2+} deficit in the ATP13A2 KO liver was secondary to fatty liver, the formation of fatty livers was induced in WT mice by feeding them a high-fat diet and measuring the effect on the levels of liver and brain Mn^{2+} and lipids. Wild type and ATP13A2 KO male mice aged about 16 weeks were fed a high-fat diet for 8 weeks, and the accumulation of lipids in the liver confirmed by Oil Red O.

More hepatic lipid accumulated in the KO mice (Figure 6a, b). Hepatic Mn^{2+} levels of WT mice fed the high-fat diet appeared to be variably increased with no statistical significance compared with that on normal chow (64±14 vs. 41±4 nmol/g, $p>0.05$, respectively). These data suggest that Mn^{2+} deficiency is not secondary to lipid accumulation in the liver. In the ATP13A2 KO mice fed the high-fat diet, the hepatic Mn^{2+} level was significantly lower

than that of the WT fed the high-fat diet (64±14 vs. 24±5 nmol/g, $p<0.05$) (Figure 6c). There was no difference in the hepatic Mn^{2+} level of ATP13A2 KO mice fed chow or the high-fat diet (levels of 24±5 vs. 28±2 nmol/g, $p>0.05$), suggesting that ATP13A2 deficiency plays a key role in Mn^{2+} uptake by the liver.

After 8 weeks of the high-fat diet. Although no significant lipid accumulation was observed in the brain (Figure 6e, f), neuronal loss was apparent in a number of regions of the brain cortex (Figure 6g, Figure 7), SN, hippocampus and DG (Figure 7). Significant neuronal loss was also observed in the CA1, CA2 and CA3 regions of the hippocampus (Figure 7, Figure 8g). Thus, these data demonstrate that the loss of functional ATP13A2 predisposes mice to cellular degeneration in the brain and liver, and that a high-fat diet precipitates and aggravates the injuries incurred by the disorder in Mn^{2+} metabolism and perturbed Mn^{2+} distribution.

EXAMPLE 5

ATP13A2 is localized and imports Mn^{2+} in mitochondria

To further investigate the mechanisms of ATP13A2 transport of Mn^{2+} at the cellular and molecular level, monoclonal antibodies to ATP13A2 were generated. Green fluorescence protein (GFP) tagged ATP13A2 WT, mutant with a single amino acid mutation in the ATP-binding site and short-hairpin (sh) RNA (Figure 8a, b).

Confocal immunofluorescence microscopy showed endogenous ATP13A2 to be present in the perinuclear and juxtanuclear regions radiating to periphery regions of COS-7 cells (Figure 9a and Figure 8b). With specific antibodies and recombinant GFP-ATP13A2 (Figure 8a, b), endogenous (Figure 8c) and recombinant (not shown) ATP13A2 were co-localized with lysosome marker Lyso-tracker, consistent with a lysosome location demonstrated previously (Ramirez *et al.*, 2006 (*supra*)). However, further investigation revealed that endogenous (Figure 9a, b) and recombinant (not shown) ATP13A2 were significantly co-localized with the mitochondrial marker MitoTracker in both COS-7 cells

(Figure 9a, b) and human cervical cancer HeLa cells (not shown), demonstrating that ATP13A2 operates in the mitochondria of mammalian cells.

To examine the role of ATP13A2 in the transport of Mn^{2+} in mitochondria, brain mitochondria were isolated from ATP13A2 KO and WT mice and subjected to trace metal measurement. Remarkably, although the Mn^{2+} levels were significantly increased in ATP13A2 deficient brain, in the mitochondria of brain tissues, Mn^{2+} was dramatically decreased (Figure 9c). The mitochondrial Mn^{2+} in ATP13A2 KO brain was reduced to the levels of about a third of that in the matching ATP13A2 WT brain mitochondria (Figure 9d).

To control potentially an indirect effect of the ATP13A2 gene disruption in mice, cultured HeLa cells were used to inhibit endogenous ATP13A2 by gene silencing and dominant negative gene expression of the ATP13A2 mutant (GFP-ATP13A2ΔK509E) and to increase ATP13A2 by expressing recombinant GFP ATP13A2 over a short time course of 40 hours. Transient transfection of HeLa cells with GFP-ATP13A2 or GFP-ATP13A2 shRNA resulted in a 150% increase or a 60-70% decrease, respectively, in *ATP13A2* expression (Figure 10c). Over-expression of ATP13A2 was associated with a significant increase (~25%) in mitochondrial Mn^{2+}, whereas silencing of ATP13A2 gene expression was associated with a significant decrease (~40%) of mitochondrial Mn^{2+} (Figure 9d). Consistently, over-expression of GFP-ATP13A2ΔK509E mutant resulted in a decrease of Mn^{2+} in mitochondria, with the Mn^{2+} levels in the mitochondria of GFP-ATP13A2ΔK509E transfected cells being half of that of vector only controls (Figure 9d). As a specificity control, no significant effect of altering ATP13A2 was observed on the levels of Cu^{2+} and Zn^{2+} (Figure 9d). These data demonstrate that ATP13A2 is required for the admission of Mn^{2+} into mitochondria in mouse brain and HeLa cells, and lacking ATP13A2 causes Mn^{2+} deficiency in mitochondria even in the presence of high levels of Mn^{2+} in mouse brain.

EXAMPLE 6

Discussion

The present study has shown that ATP13A2 is indispensible in Mn^{2+} homeostasis in mice, and that the lack of ATP13A2 leads to lower brain and hepatic Mn^{2+} levels and raised levels in serum, resulting in steatosis, hyperlipidemia, loss of brain neurons, increased microglial activity and a mental or behavioural disorder. While the liver is the major site of Mn^{2+} storage, the mechanism of Mn^{2+} uptake by the liver is not known. The presently disclosed data that Mn^{2+} was normal in the newborn liver of ATP13A2 KO mice and that the high-fat diet increased hepatic levels of Mn^{2+} in WT mice suggest that Mn^{2+} deficiency in liver of adult ATP13A2 KO mice is due to disrupted Mn^{2+} absorption from the diet. The deficit of Mn^{2+} in the liver of ATP13A2 KO mice is not a consequence of lipid accumulation, as demonstrated in the WT mouse fed the high-fat diet to induce lipid accumulation. The findings disclosed herein that lipid accumulation is confined to the Mn^{2+}-deficient liver supports the idea that fatty liver and hyperlipidemia occur as a result of the Mn^{2+} deficit rather than the lack of ATP13A2. Thus, it is demonstrated herein that ATP13A2 has an obligatory role in hepatic Mn^{2+} trafficking and that hepatic Mn^{2+} deficiency leads to fatty liver and hyperlipidemia. Deficiency of ATP13A2 (Figure 3f) causes perturbed Mn^{2+} distribution including in one embodiment decreased Mn^{2+} levels in brain and increased levels in the blood/serum.

In contrast to the phenotype in ATP13A2 KO mice of low Mn^{2+} and high lipids in the liver in association with hyperlipidemia, the brain phenotype is one of low Mn^{2+} and no lipid accumulation. The mechanism of Mn^{2+} homeostasis in the CNS is not known (Gitler *et al., Nat Genet 41*: 308-315, 2009; Uchino *et al., Neuroradiology 49*: 715-720, 2007; Culotta *et al., Eukaryot Cell 4*: 1159-1165, 2005; Jason *et al., Molecular Microbiology 72*: 12-25, 2009; Aschner and Dorman, *Toxicol Rev 25*: 147-154, 2006) however, the finding of markedly decreased Mn^{2+} in the brains of ATP13A2 KO mice demonstrates that ATP13A2 plays an obligatory role in Mn^{2+} transport in brain. Thus, in some embodiments, the present invention links ATP13A2 to Mn^{2+} deficiency in the brain and neuronal cell death occurring in the development of neural degenerative diseases (Mena *et al., N Engl J Med*

282: 5-10, 1970; Cotzias *et al., Science 176*: 410-412, 1972). The findings of concurrent decreases in Mn^{2+} concentration and neuronal loss in the ATP13A2-deficient brain demonstrate a causative role of ATP13A2 deficiency in the required effects of Mn^{2+} and mental, behaviour or neurodegenerative pathogenesis. In other embodiments of the present invention, it is shown that brain neuronal losses are increased in mice fed a high-fat diet indicating that ATP13A2 mutations found in patients (Ramirez *et al.,* 2006 (*supra*); Di Fonzo *et al., Neurology 68*: 1557-1562, 2007; Ning *et al., Neurology 70*: 1491-1493, 2008; Lin *et al., Neurology 71*: 1727-1732, 2008) might also confer a genetic susceptibility to neuron losses. The embodiment herein that a high-fat diet contributes to the genetic defect-induced neurodegeneration and mental or behavioural disorder could explain the presence of *ATP13A2* gene mutations in human populations with no clinical phenotype (Sutherland *et al., Mov Disord 24*: 833-838, 2009; Rakovic *et al., Mov Disord 24*: 429-433 2009).

As ATP13A2 is found herein in mitochondria and ATP13A2 deficiency results in mitochondrial Mn^{2+} deficiency it is proposed that that Mn^{2+} has an anti-oxidative role against ROS production and oxidative damage to macromolecules such as those produced by microglial cells. The demonstration herein of ATP13A2 being a Mn^{2+} transporter in mitochondria provides a new target in regulating Mn^{2+} and mitochondria function. The demonstration herein of ATP13A2 deficiency-induced Mn^{2+} shortage in liver and brain and excess in blood, underlying the pathogenesis of fatty liver, hyperlipidemia and a mental or behavioural disorder in mice provides novel methods for diagnosing, prognosing and treating these metabolic disorders in humans.

EXAMPLE 7

New model of paroxysmal kinesogenic dyskinesia (PKD) or paroxysmal kinesogenic choreoathetosis (PKC)

Mice, 129Sv/C57Black6 ATP13A2-/- male mice, were crossed with SJL/J ATP13A2 +/+ mice, to produce 129Sv/C57Black6/SJL/J ATP13A2 -/-. The SJL/J strain is susceptible to behaviour and movement disorders. The mice are viable and showed apparently normal reproduction and development of the pups. However, upon weaning, mice developed

abnormal movement and behaviour disorders. They were characterized by episodes of fast running around in circles which was triggered by sudden movements or startle. Frequency of attacks was between multiple times in a day. Severe mouse undertook spinning for several sections to several minutes in both directions. Mice were conscious between attacks without much difference from wild type controls except hyper-activity.

EXAMPLE 8

Anxiety tests

ATP13AO deficient mice were prone to anxiety. Using a nest building test ATP13A2 KO mice showed a significant loss of nest building activity relative to wild-type controls (See Figure 12 and Figure 13).

EXAMPLE 9

Further behavioural analysis of ATP13A2 homozygous knockout mice

The excessive grooming phenotype occurred with greater frequency in female than male mice (Figure 14a). The incidence in male mice was 17-47% in the ATP13A2 KO compared to 0% of WT, and in females, 34-68% in the KO compared to 12-23% in WT mice. Video recording of habituated mice showed that ATP13A2 KO mice spent significantly more time engaged in repetitive grooming behaviours than their WT littermates. The total time the mice engaged in self-grooming was about 3-fold higher in ATP13A2 KO mice than WT (Figure 14b) and the number of bouts of self-grooming was almost 5-fold greater (Figure 14c). Over grooming led to hair loss from the face, eyebrows, whiskers, cheek, head and chest. The extent of hair loss ranged from small to large patches of bald skin (Figure 14d). Severe skin lesions from self-mutilation were observed occasionally in association with repeated grooming bouts on the backs of ATP13A2 mice (Figure 14e). No lesions were observed in WT or heterozygous mice, whether housed alone or in the same cage as ATP13A2 KO mice.

In addition to compulsive behaviours, ATP13A2 KO mice had a significantly higher level of anxiety. In a 30-minute dark-bright field observation, ATP13A2 KO mice were less settled, and spent significantly more time in the bright field, than the WT mice (Figure 15a-b). ATP13A2 KO mice moved to and from the dark field less frequently than WT mice (Figure 15c). In contrast, ATP13A2 KO mice showed no significant difference in nest building than WT littermates (Figure 15d). Consistent with an increased state of fear and anxiety, ATP13A2 KO mice were slower to jump than WT mice during a horizontal grid test (Figure 15e). In a vertical grid test, ATP13A2 KO mice were relatively averse to turning and walking down from the grid (Figure 15f). There was a significant increase in rearing behaviours in ATP13A2 KO mice, both males and females, compared to WT mice (Figure 15g). These data together suggest that the phenotype of ATP13A2-deficient mice is one of high-level anxiety and compulsive repetitive behaviours.

Table 1: ***Summary of sequence identifiers***

SEQUENCE ID NO:	Description
1	PCR primer ATP13A2 KO
2	PCR primer ATP13A2 KO
3	RT-PCR primers for ATP13A2
4	RT-PCR primers for ATP13A2
5	RT-PCR primers for ATP13A2
6	RT-PCR primers for ATP13A2
7	RT-PCR primers for GAPDH
8	RT-PCR primers for GAPDH

Table 2: ***Amino acid sub-classification***

Sub-classes	Amino acids
Acidic	Aspartic acid, Glutamic acid
Basic	Noncyclic: Arginine, Lysine; Cyclic: Histidine
Charged	Aspartic acid, Glutamic acid, Arginine, Lysine, Histidine
Small	Glycine, Serine, Alanine, Threonine, Proline
Polar/neutral	Asparagine, Histidine, Glutamine, Cysteine, Serine, Threonine
Polar/large	*Asparagine, Glutamine*
Hydrophobic	*Tyrosine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan*
Aromatic	*Tryptophan, Tyrosine, Phenylalanine*
Residues that influence chain orientation	*Glycine and Proline*

Table 3: ***Exemplary and Preferred Amino Acid Substitutions***

Original residue	Exemplary substitutions	Preferred substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, His, Lys, Arg	Gln
Asp	Glu	Glu
Cys	Ser	Ser
Gln	Asn, His, Lys,	Asn
Glu	Asp, Lys	Asp
Gly	Pro	Pro
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleu	Leu
Leu	Norleu, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, Gln, Asn	Arg
Met	Leu, Ile, Phe	Leu
Phe	Leu, Val, Ile, Ala	Leu
Pro	Gly	Gly
Ser	Thr	Thr
Thr	Ser	Ser
Trp	Tyr	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Leu, Met, Phe, Ala, Norleu	Leu

Table 4: ***Codes for non-conventional amino acids***

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-Nmethylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut

	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
5	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
10	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
15	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
20	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
25	γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
	L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L-α-methylalanine	Mala
	L-α-methylarginine	Marg	L-α-methylasparagine	Masn
30	L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
	L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
	L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
	L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
	L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
35	L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
	L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
	L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
	L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
	L-α-methylserine	Mser	L-α-methylthreonine	Mthr
40	L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
	L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
	N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
	1-carboxy-1-(2,2-diphenyl-	Nmbc		
45	ethylamino)cyclopropane			

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CLAIMS:

1. A diagnostic or prognostic assay for a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, the assay comprising assessing the level or activity of an analyte in a biological fluid or tissue sample in or from a test subject, the analyte selected from one or more of ATP13A2 polypeptide, *ATP13A2* nucleic acid, and Mn^{2+}, wherein the level or activity of the analyte relative to a control indicates that the test subject has or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder.

2. The assay of claim 1, comprising comparing the level of Mn^{2+} in the test subject to the level of Mn^{2+} in at least one control subject selected from a subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder and a normal subject, wherein a similarity in the level of Mn^{2+} between the test subject and the normal control subject identifies the test subject as normal or non-susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, and wherein a similarity in the level of Mn^{2+} between the test subject and the control subject with the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder identifies the test subject as having or being susceptible to a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder.

3. The assay of claim 1 or 2 wherein the level or activity of analyte is assessed in blood, serum or plasma.

4. The assay of claim 1, 2 or 3 wherein the level or activity of analyte is assessed in a liver sample.

5. The assay of claim 1, 2, 3 or 4 wherein the level or activity of analyte is assessed in a brain sample.

6. The assay of any one of claims 1 to 5 wherein the level or activity of analyte is assessed in mitochondria.

7. The assay of claim 1 wherein the control represents the level or activity of the analyte in a normal healthy subject and wherein (i) a decreased level or activity of ATP13A2 or *ATP13A2* relative to the level or activity in a normal healthy control subject and/or (ii) an increased level of Mn^{2+} in the blood of the test subject relative to the level of Mn^{2+} in the blood of a normal healthy control subject and/or a decreased level of Mn^{2+} in a second tissue in or from the test subject relative to the level of Mn^{2+} in the same tissue from a normal healthy control subject, such as brain, mitochondria, and/or liver, indicates that the subject has or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid-associated liver disorder.

8. The assay of claim 2 wherein the control represents the level of Mn^{2+} in a normal healthy subject and wherein an increased level of Mn^{2+} in the blood of the test subject relative to the level in a normal healthy subject and/or a decreased level of Mn^{2+} in a second sample in or from the test subject relative to the level in the same tissue in or from a normal healthy control subject, such as brain, mitochondria, and/or liver, indicates that the test subject has or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid-associated liver disorder.

9. The assay of claim 7 or 8 wherein the second sample is in or from one or more of brain, mitochondria and liver.

10. The assay of any one of claims 1 to 9 wherein the lipid and/or lipid-associated liver disorder is one or more of fatty liver, hyperlipidemia and steatosis.

11. The assay of any one of claims 1 to 10 practised *ex vivo*.

12. The assay of claim 2 comprising assessing the level or activity of ATP13A2 polypeptide or *ATP13A2* nucleic acid.

13. The assay of any one of claims 1 to 12 including selecting the test subject as having or suspected of having a lipid and/or lipid-associated liver disorder.

14. ATP13A2 polypeptide or *ATP13A2* nucleic acid for use in the treatment or prevention of a disorder characterised by anxiety, such as OCD, and/or a lipid-associated liver disorder in a subject wherein the ATP13A2 polypeptide or *ATP13A2* nucleic acid increases the level of mitochondrial Mn^{2+} in subjects exhibiting a low level of mitochondrial Mn^{2+} compared to the level in a normal healthy control subject.

15. A method of treatment or prophylaxis of a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid associated liver disorder, the method comprising administering a composition comprising ATP13A2 polypeptide or a functional analog thereof or *ATP13A2* nucleic acid to a subject in need thereof for a time and under conditions sufficient for the treatment or prophylaxis of the behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or lipid associated liver disorder.

16. A method of treatment or prophylaxis of a subject comprising assessing a test subject with respect to a behavioural disorder characterised by anxiety, such as OCD, by assessing the level or activity of an analyte in a biological fluid or tissue sample in or from a test subject, the analyte selected from one or more of ATP13A2 polypeptide, *ATP13A2* nucleic acid, Mn^{2+} , and a complex comprising ATP13A2 polypeptide and Mn^{2+}, wherein the level or activity of the analyte relative to a control indicates that the test subject has or is susceptible to developing a

behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder, and exposing the subject to therapeutic or prophylactic or behavioural intervention on the basis that the test subject tests positive to having or is susceptible to developing a behavioural disorder characterised by anxiety, such as OCD, and/or a lipid and/or a lipid-associated liver disorder.

17. An isolated cell or non-human organism comprising such cells, wherein the activity of ATP13A2 polypeptide is modified, such as reduced or inhibited, compared to a non-modified organism of the same species optionally for use as an ATP13A2 deficient animal or cellular model of a behavioural disorder, such as OCD, PKD or PKC, and/or a lipid and/or lipid-associated liver disorder.

18. The cell or organism of claim 17 wherein the cell and organism are of mammalian or zebrafish origin.

19. An assay for agents that complement the phenotype an ATP13A2 deficient cell or non-human organism comprising such cells, the assay comprising contacting the cell or organism with a putative agent and assessing the ability of the agent to increase the level of mitochondrial Mn^{2+} in the cell.

20. An assay for agents that modulate Mn^{2+} levels in a cell or organism, the assay comprising contacting the cell or organism with a putative agent and assessing the ability of the agent to increase the level or activity of ATP13A2 in the cell or organism.

a

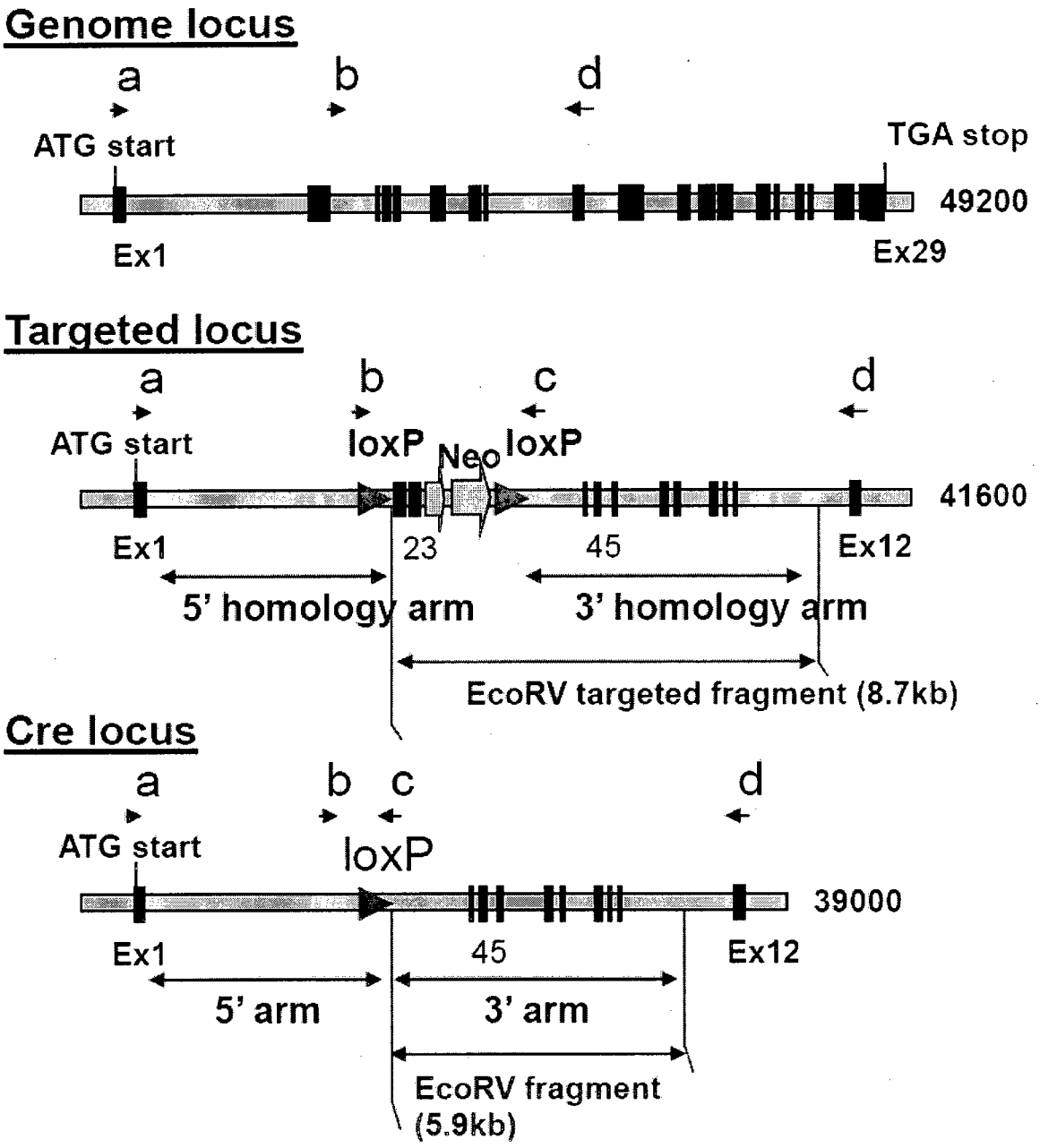

Genome locus
a
b
d
ATG start
TGA stop
49200
Ex1
Ex29
Targeted locus
a
b
c
d
ATG start
loxP
Neo
loxP
41600
Ex1
23
45
Ex12
5' homology arm
3' homology arm
EcoRV targeted fragment (8.7kb)
Cre locus
a
b
c
d
ATG start
loxP
39000
Ex1
45
Ex12
5' arm
3' arm
EcoRV fragment
(5.9kb)

FIGURE 1

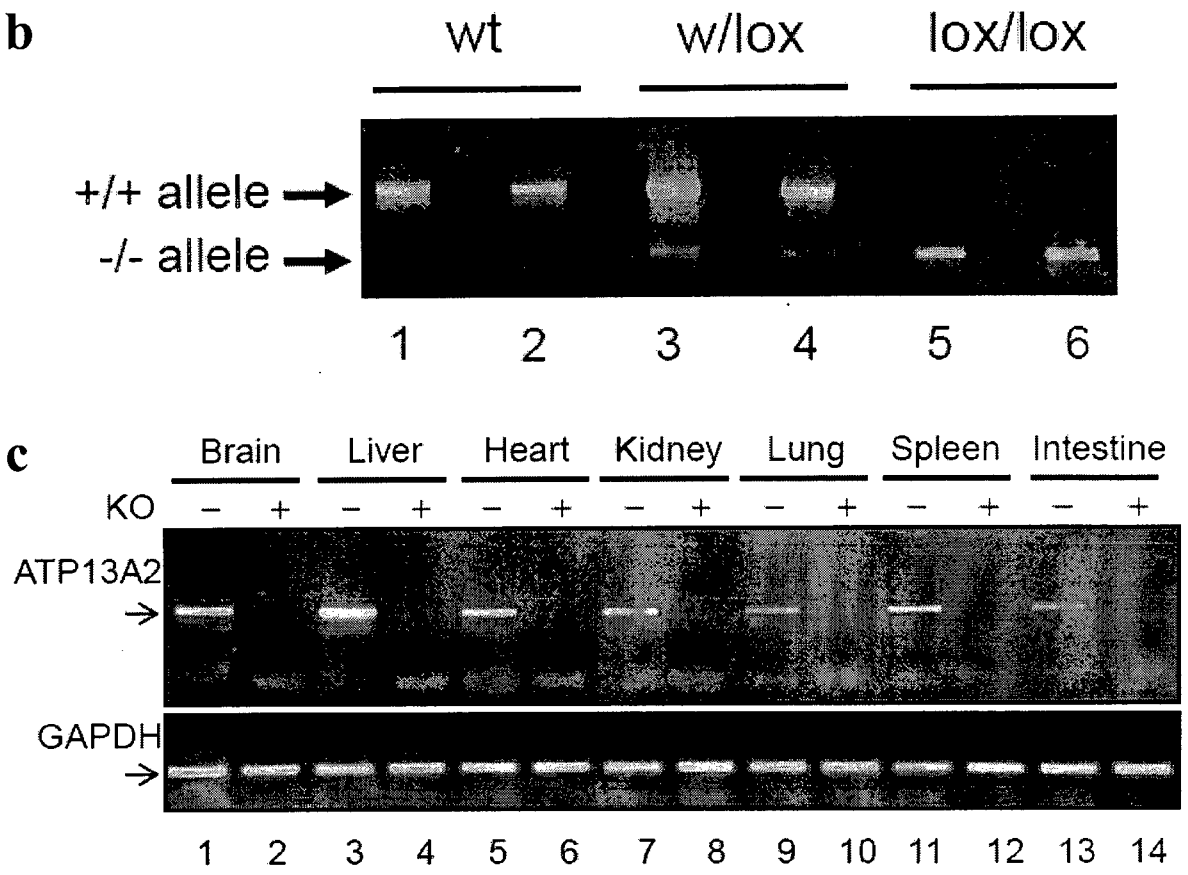


FIGURE 1 (CONTINUED)

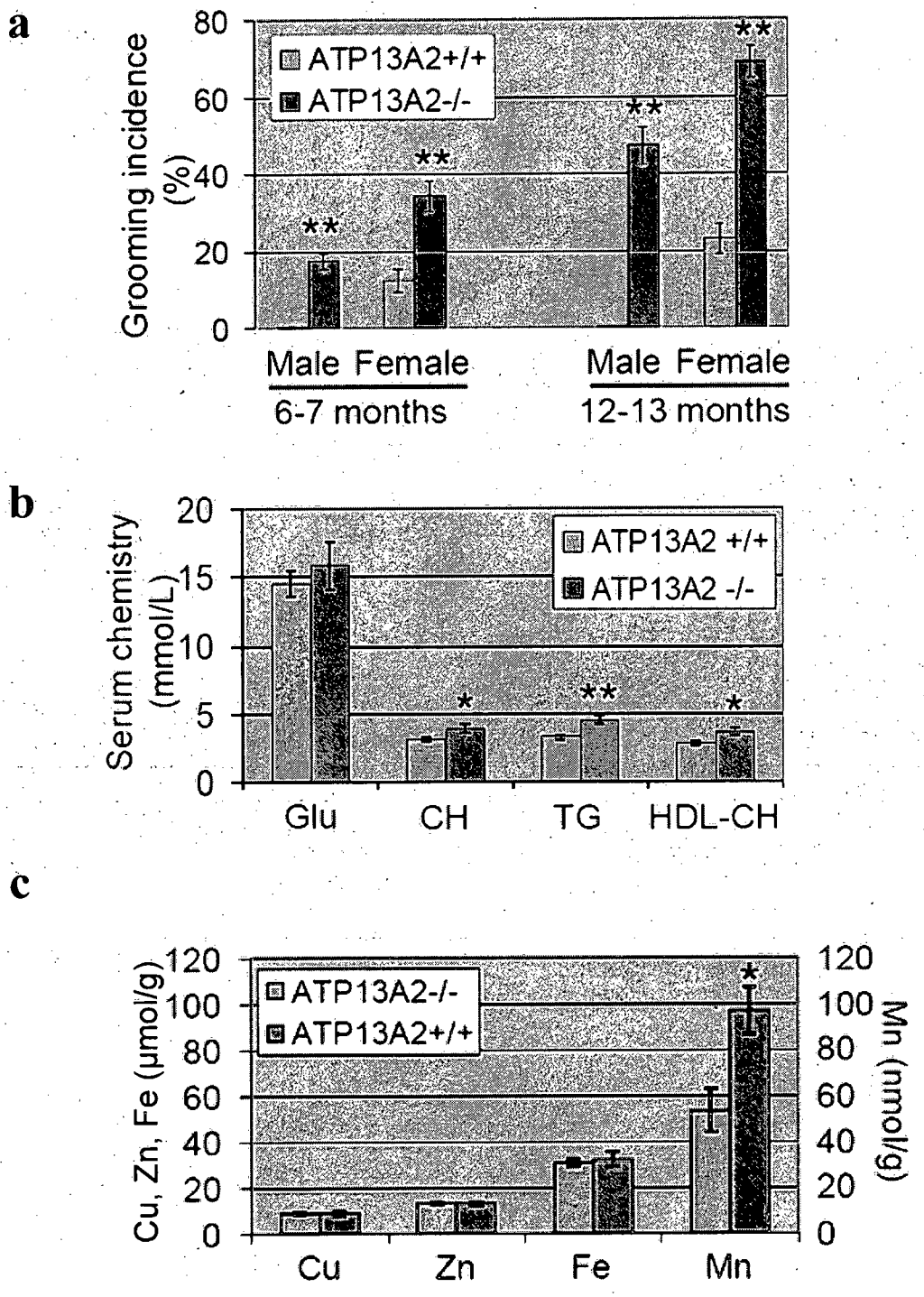
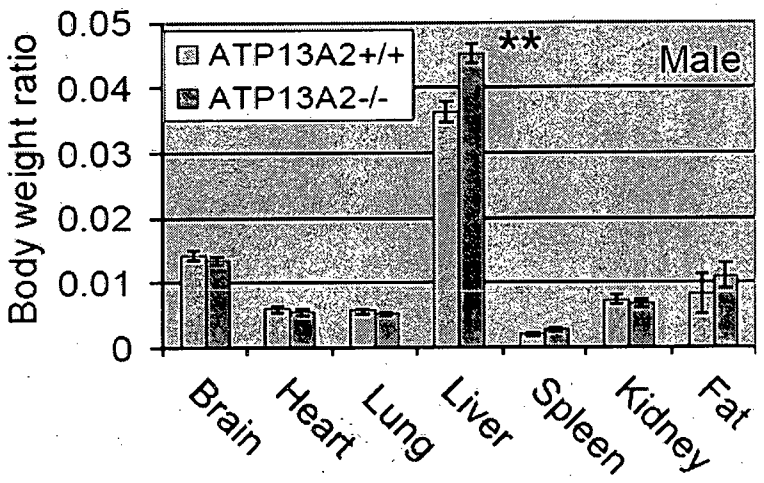

a
Grooming incidence (%)
ATP13A2+/+
ATP13A2-/-
Male Female
6-7 months
Male Female
12-13 months
b
Serum chemistry (mmol/L)
ATP13A2 +/+
ATP13A2 -/-
Glu
CH
TG
HDL-CH
c
Cu, Zn, Fe (µmol/g)
Mn (nmol/g)
ATP13A2-/-
ATP13A2+/+
Cu
Zn
Fe
Mn

FIGURE 2

d



e

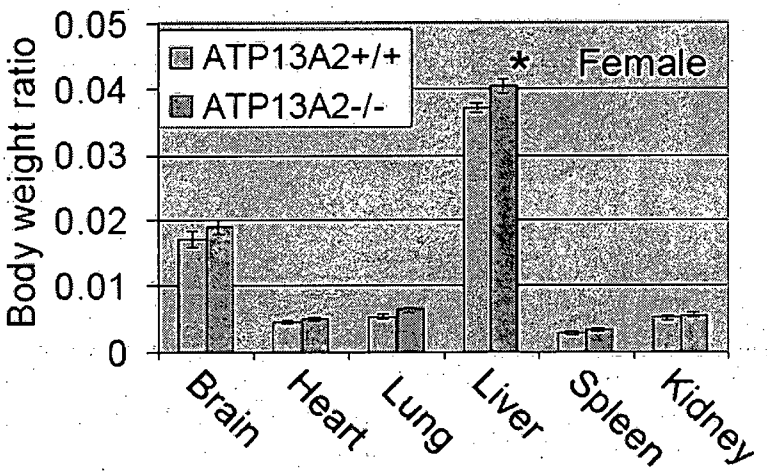


FIGURE 2 (CONTINUED)

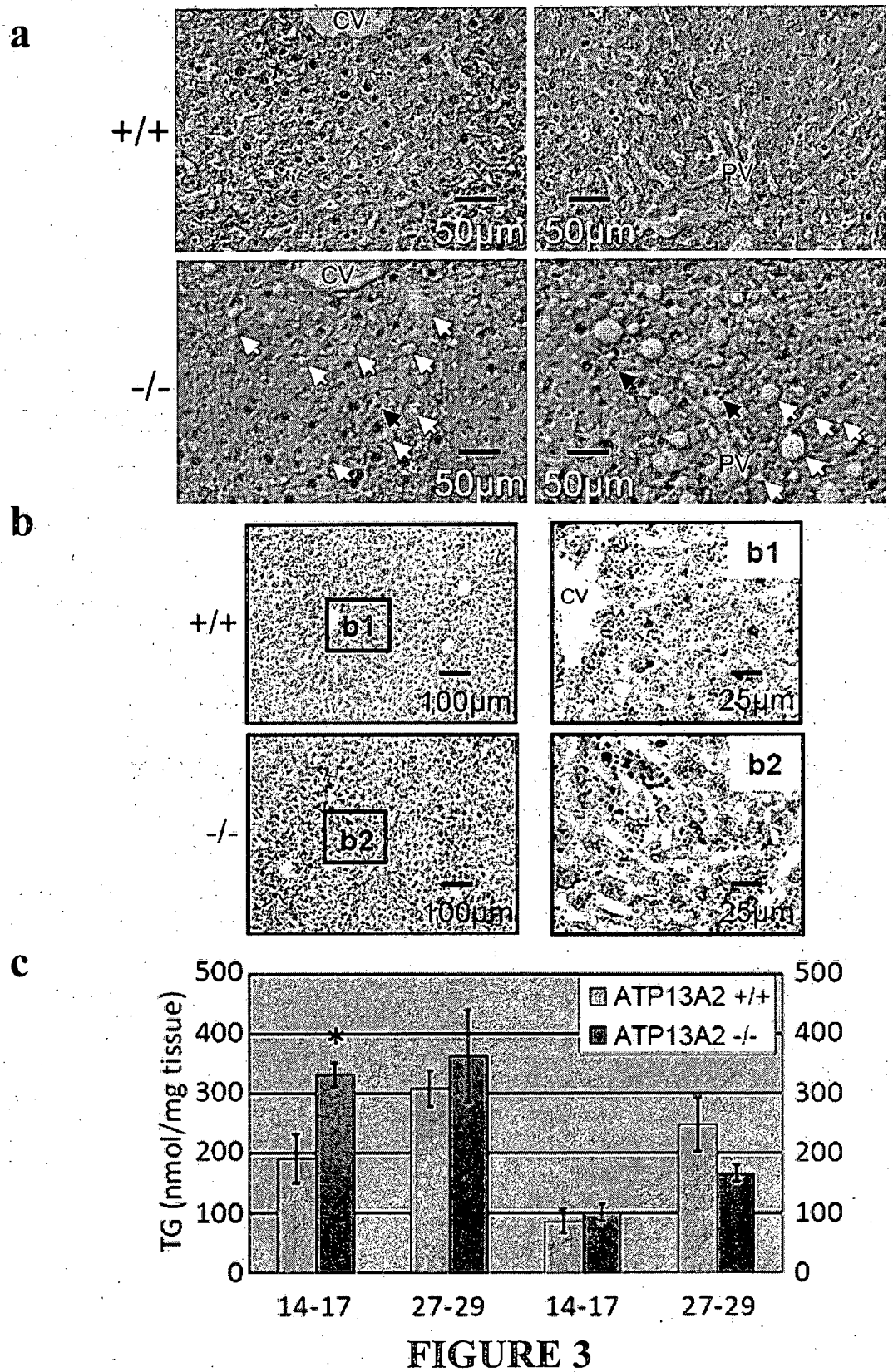

a
+/+
-/-
CV
PV
50µm
b
b1
b2
100µm
25µm
c
TG (nmol/mg tissue)
ATP13A2 +/+
ATP13A2 -/-
0
100
200
300
400
500
14-17
27-29
14-17
27-29

FIGURE 3

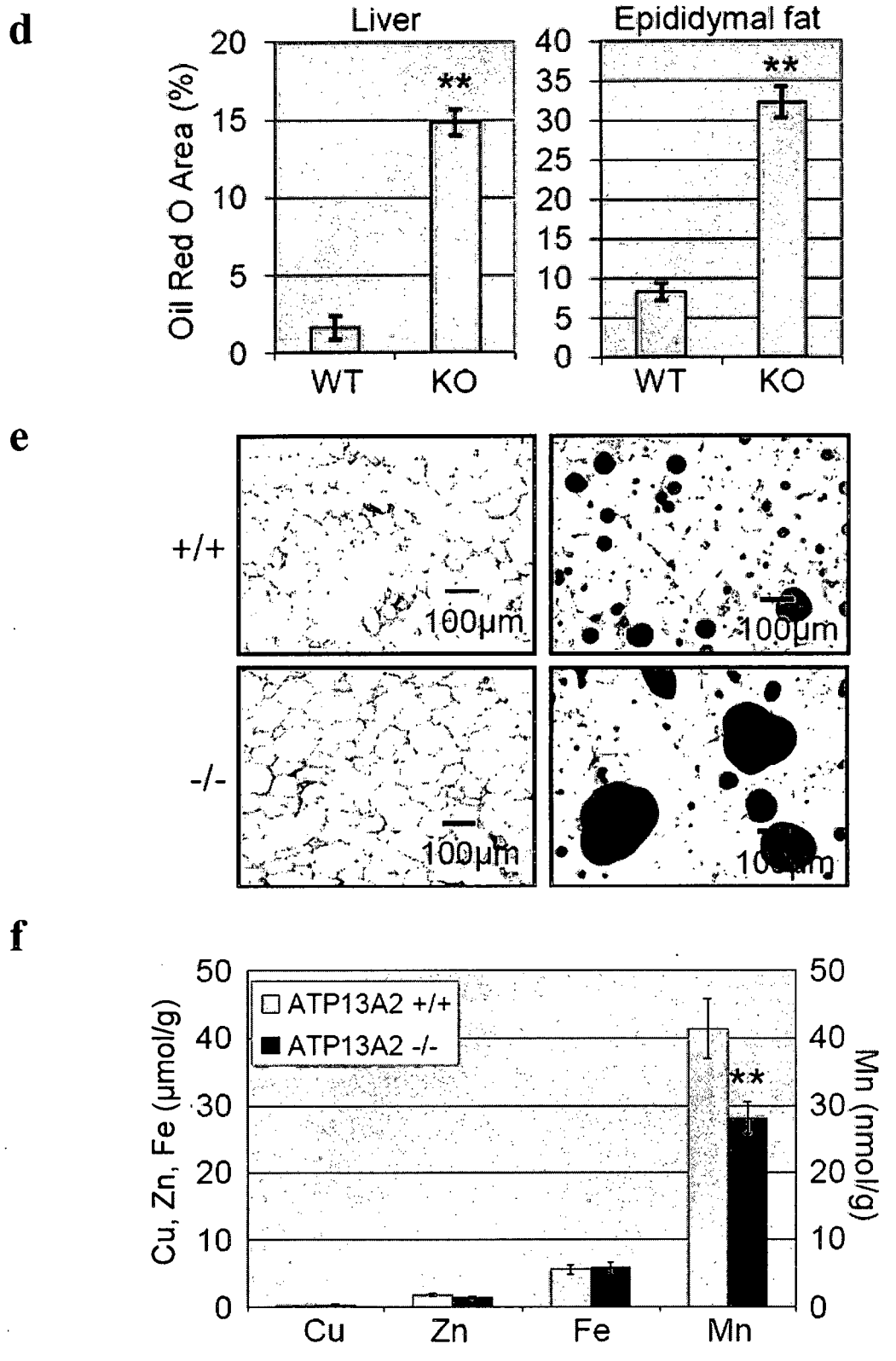


FIGURE 3 (CONTINUED)

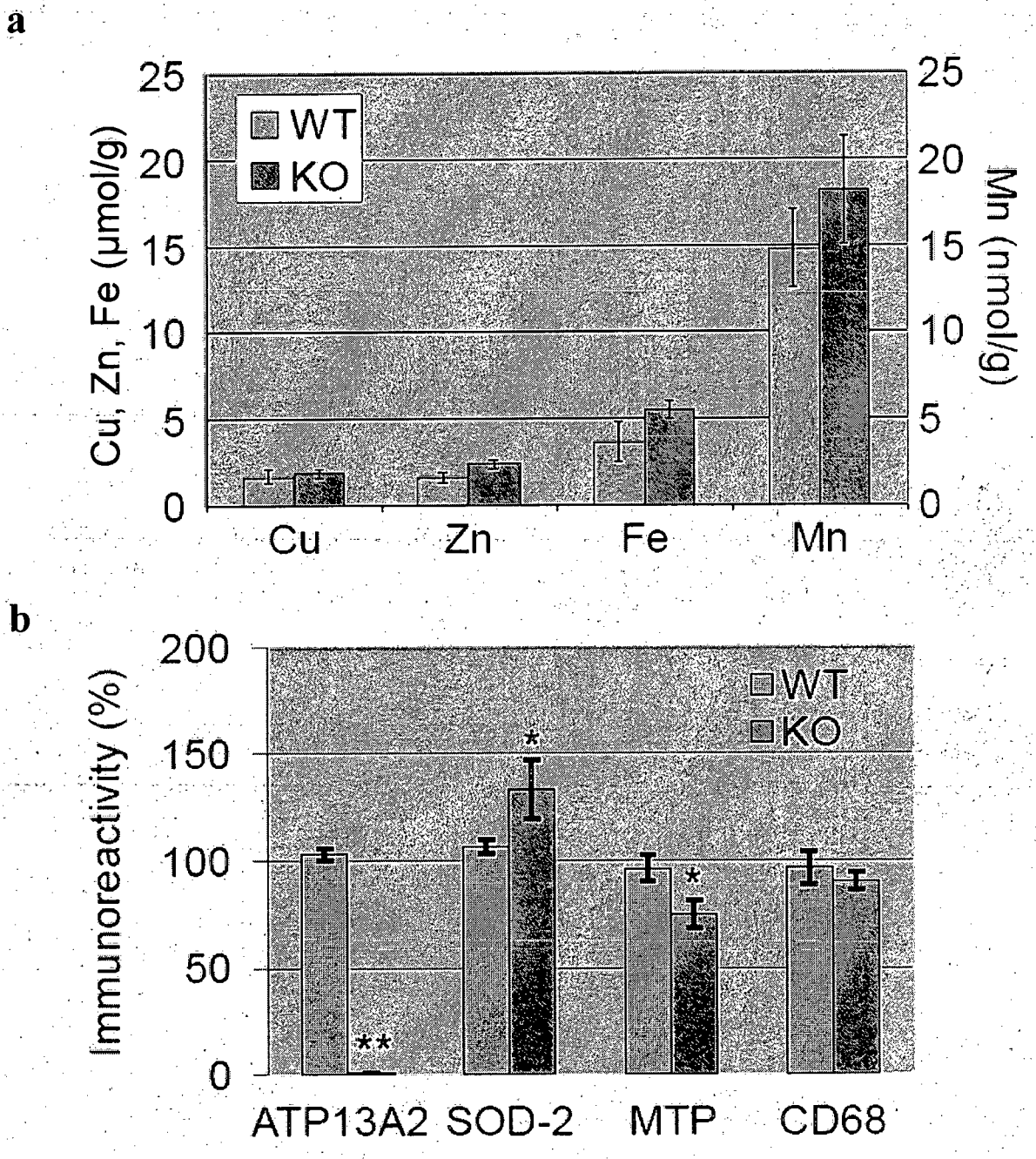

a
25
20
15
10
5
0
Cu, Zn, Fe (µmol/g)
WT
KO
Mn (nmol/g)
Cu
Zn
Fe
Mn
b
200
150
100
50
0
Immunoreactivity (%)
WT
KO
**
*
*
ATP13A2
SOD-2
MTP
CD68

FIGURE 4

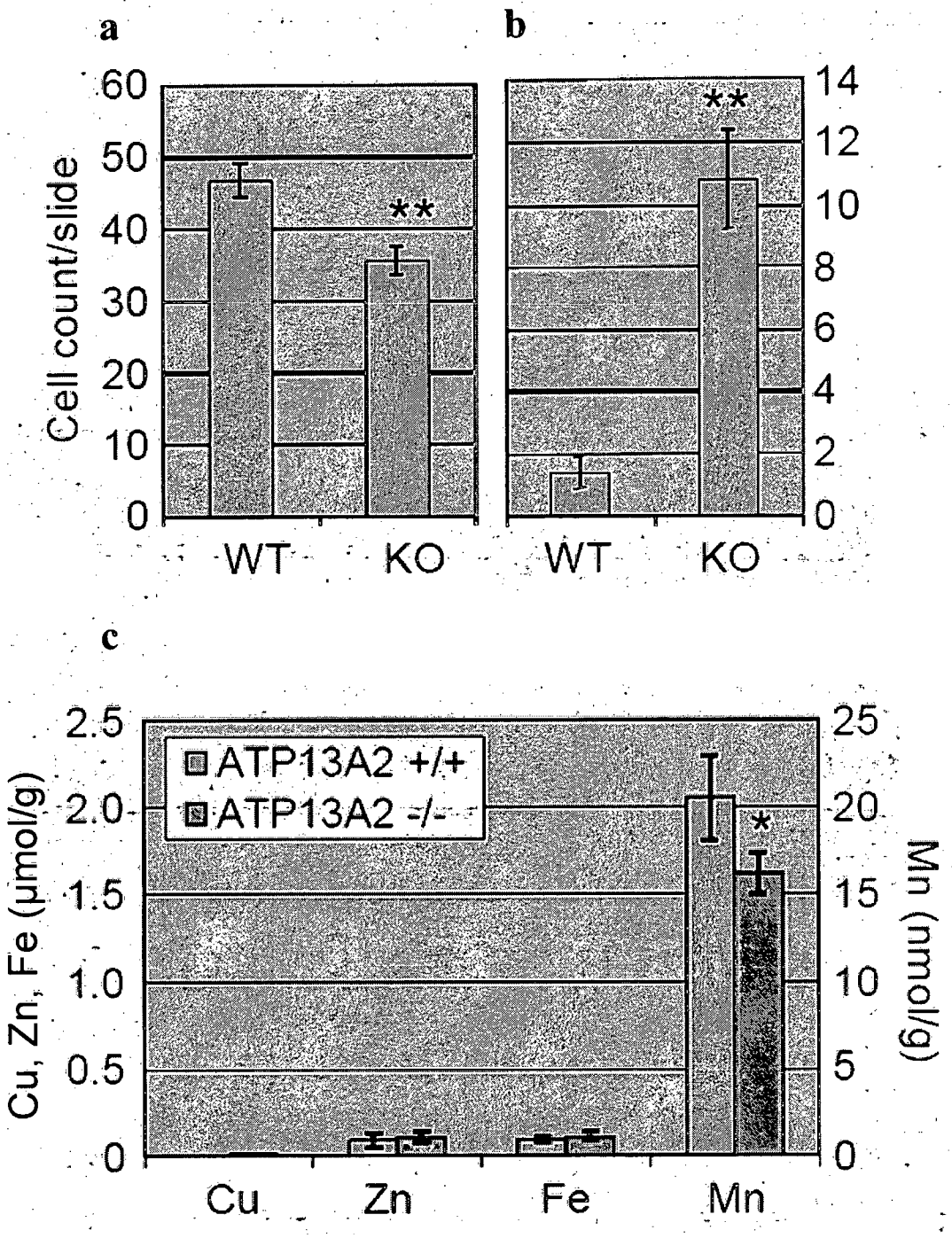

a
b
c
Cell count/slide
60
50
40
30
20
10
0
**
WT
KO
14
12
10
8
6
4
2
0
Cu, Zn, Fe (µmol/g)
2.5
2.0
1.5
1.0
0.5
ATP13A2 +/+
ATP13A2 -/-
Cu
Zn
Fe
Mn
25
20
15
10
5
Mn (nmol/g)
*

FIGURE 5

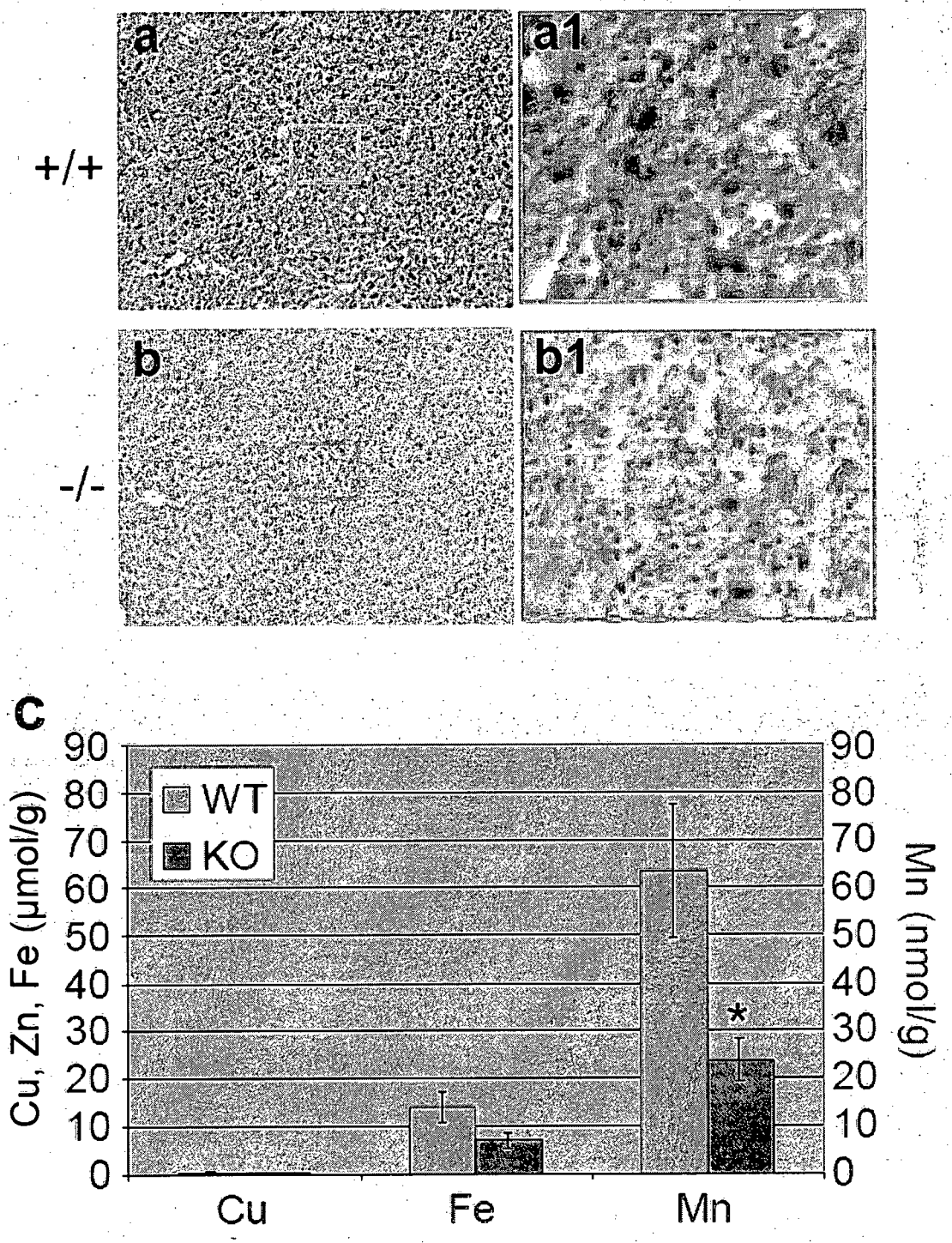

a
a1
+/+
b
b1
-/-
c
WT
KO
Cu, Zn, Fe (µmol/g)
Mn (nmol/g)
0
10
20
30
40
50
60
70
80
90
Cu
Fe
Mn
*

FIGURE 6

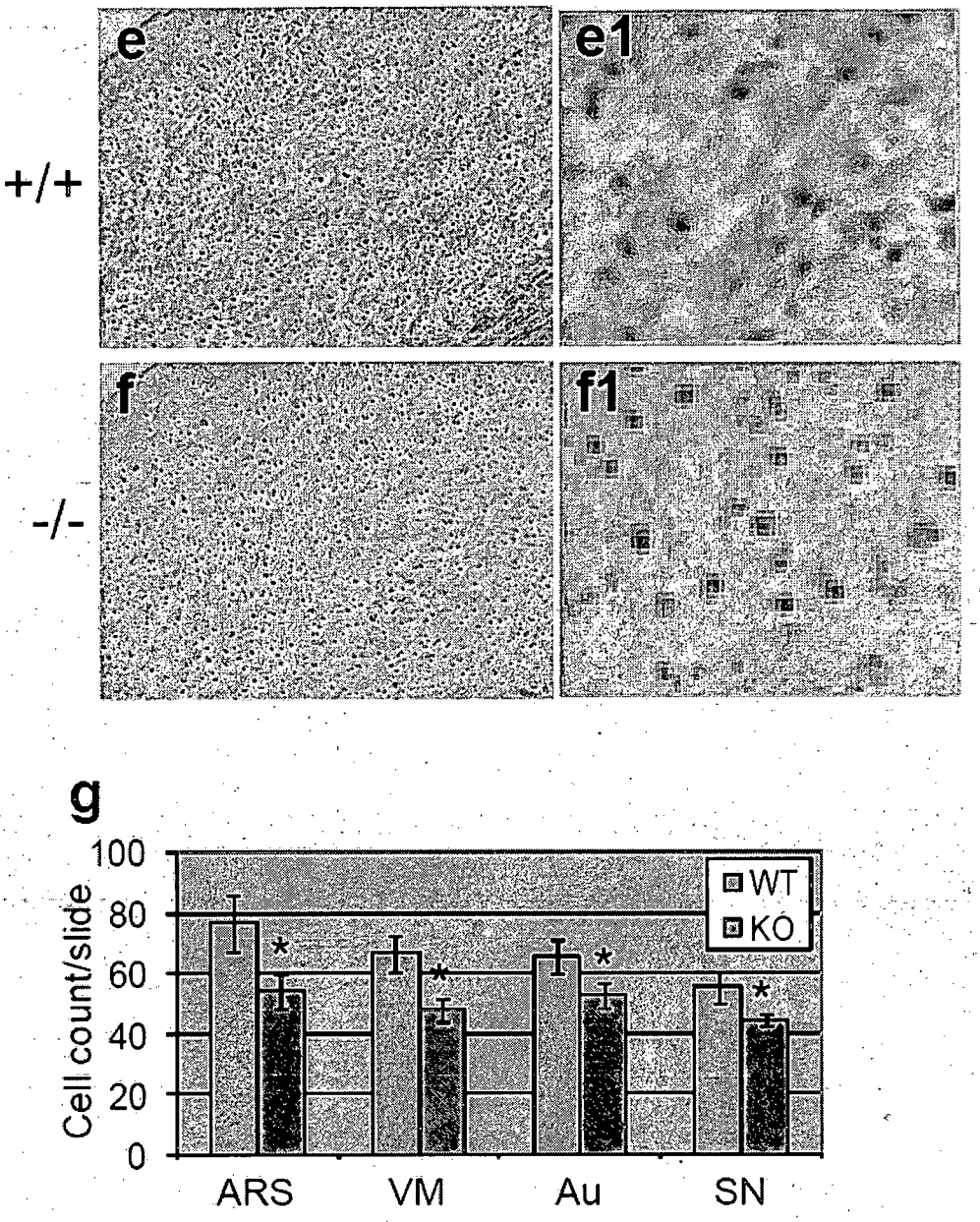


FIGURE 6 (CONTINUED)

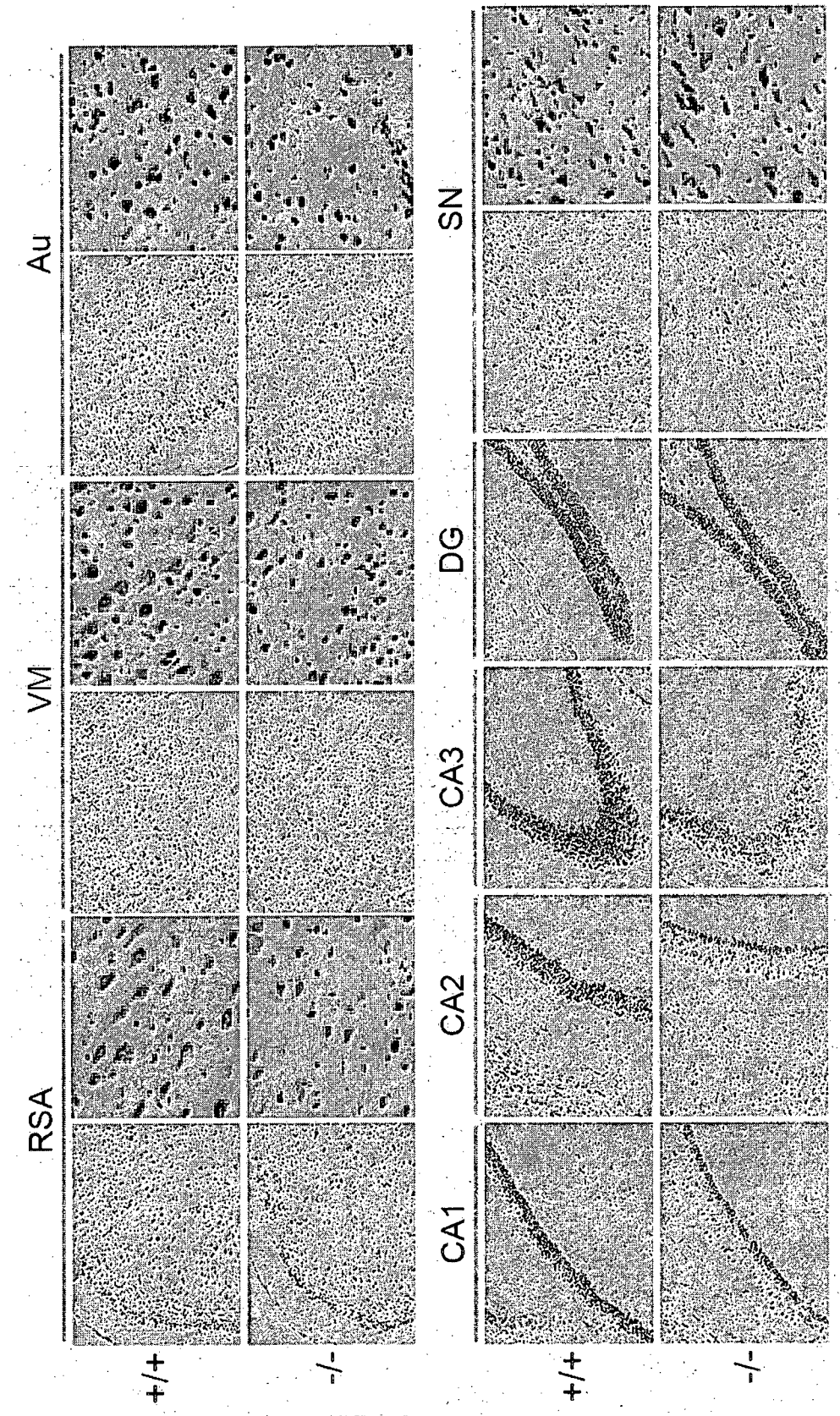


FIGURE 7

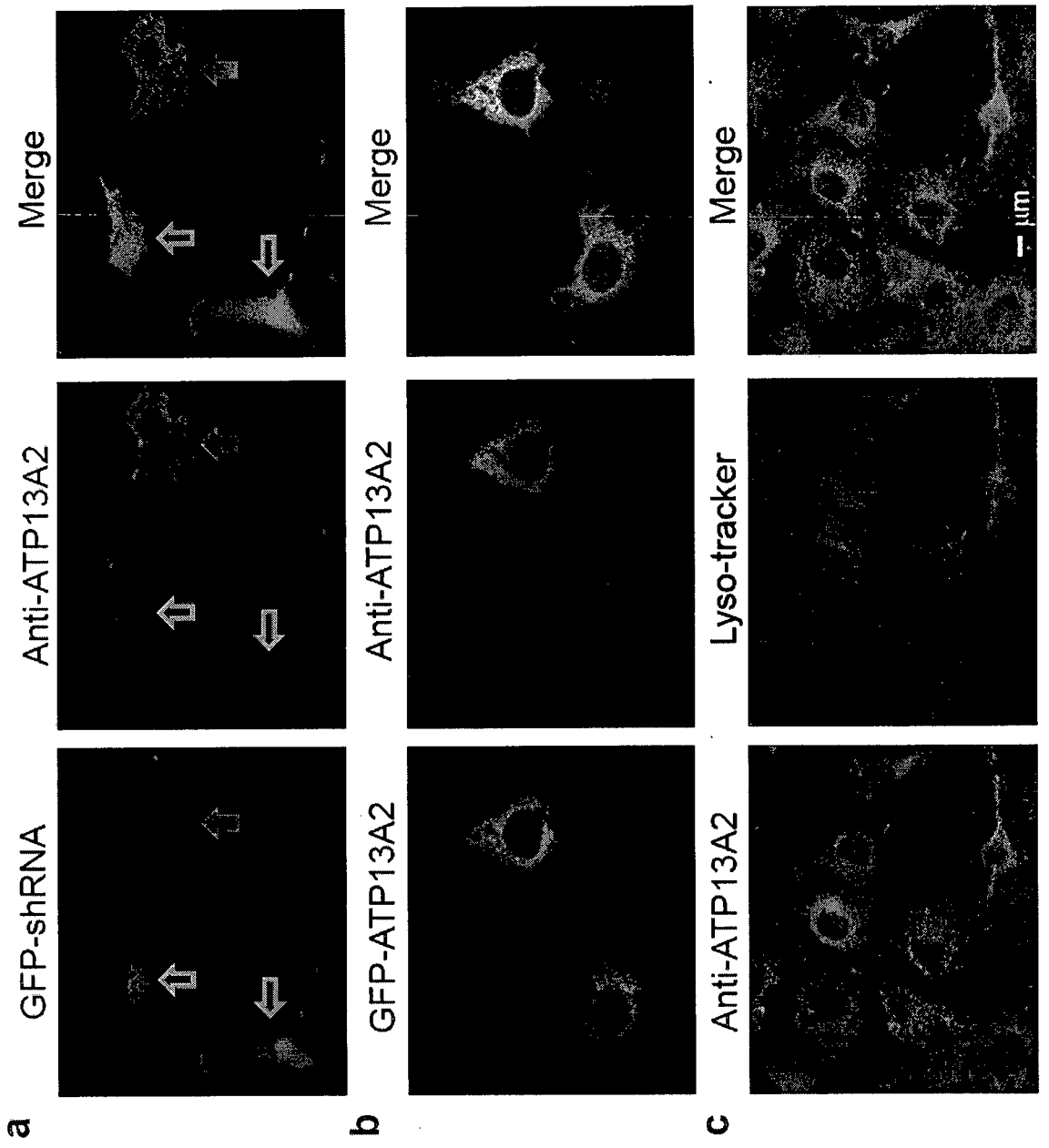

a
GFP-shRNA
Anti-ATP13A2
Merge
b
GFP-ATP13A2
Anti-ATP13A2
Merge
c
Anti-ATP13A2
Lyso-tracker
Merge
1 μm

FIGURE 8

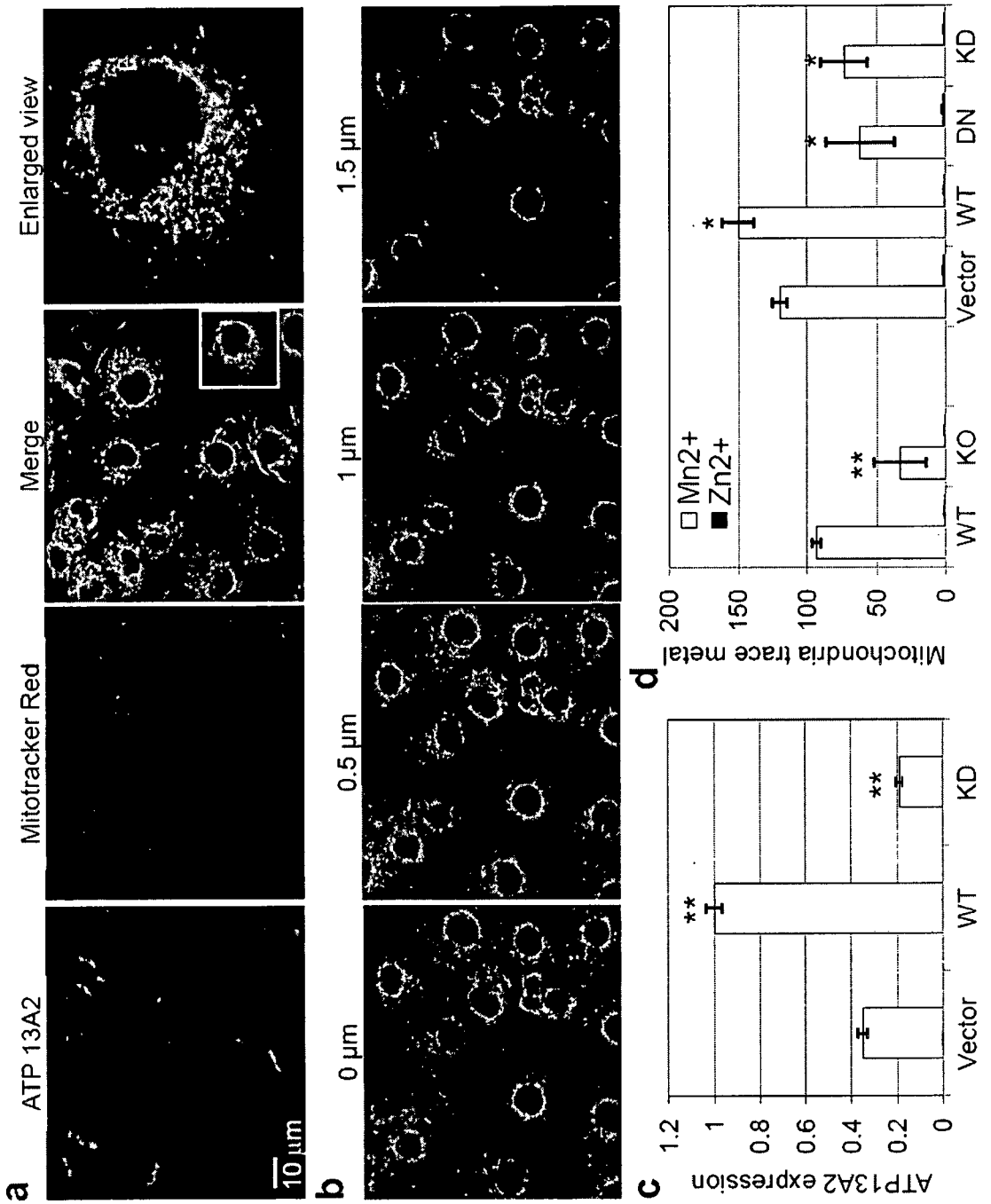

a
ATP 13A2
Mitotracker Red
Merge
Enlarged view
10 µm
b
0 µm
0.5 µm
1 µm
1.5 µm
c
ATP13A2 expression
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Vector
WT
KD
d
Mitochondria trace metal
200
150
100
50
0
□Mn2+
■Zn2+
**
*
*
*
WT
KO
Vector
WT
DN
KD

FIGURE 9

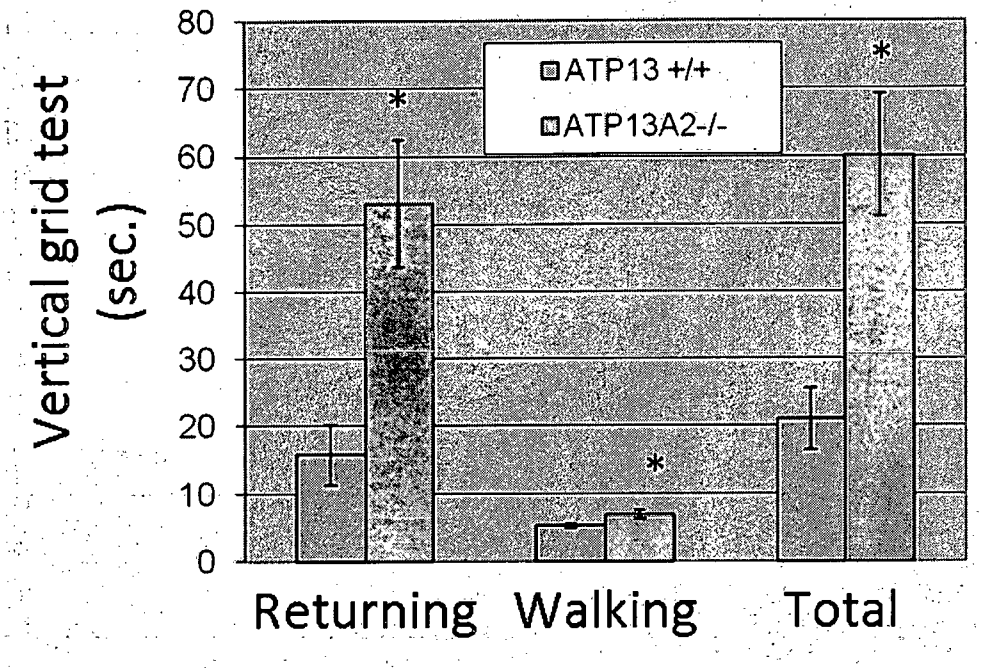


FIGURE 10

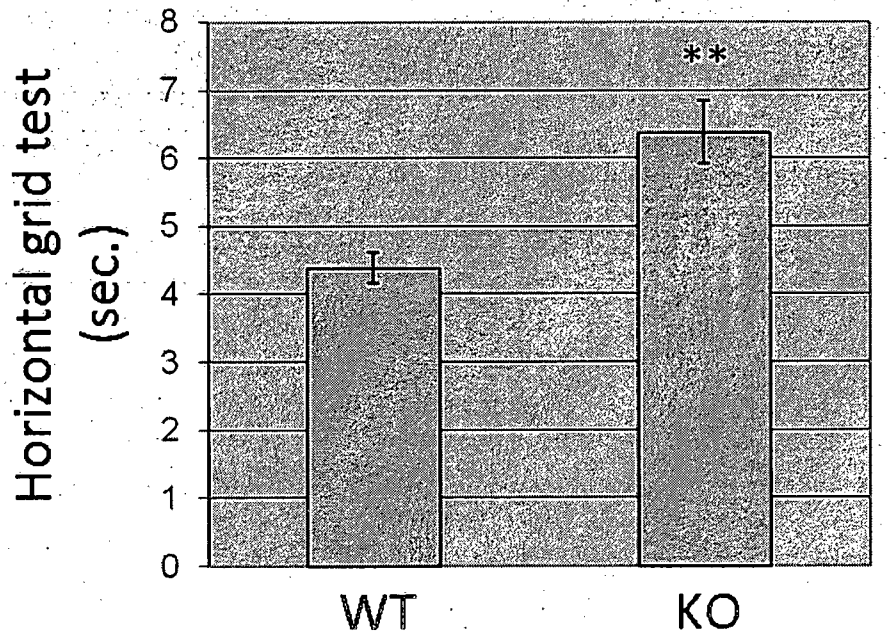
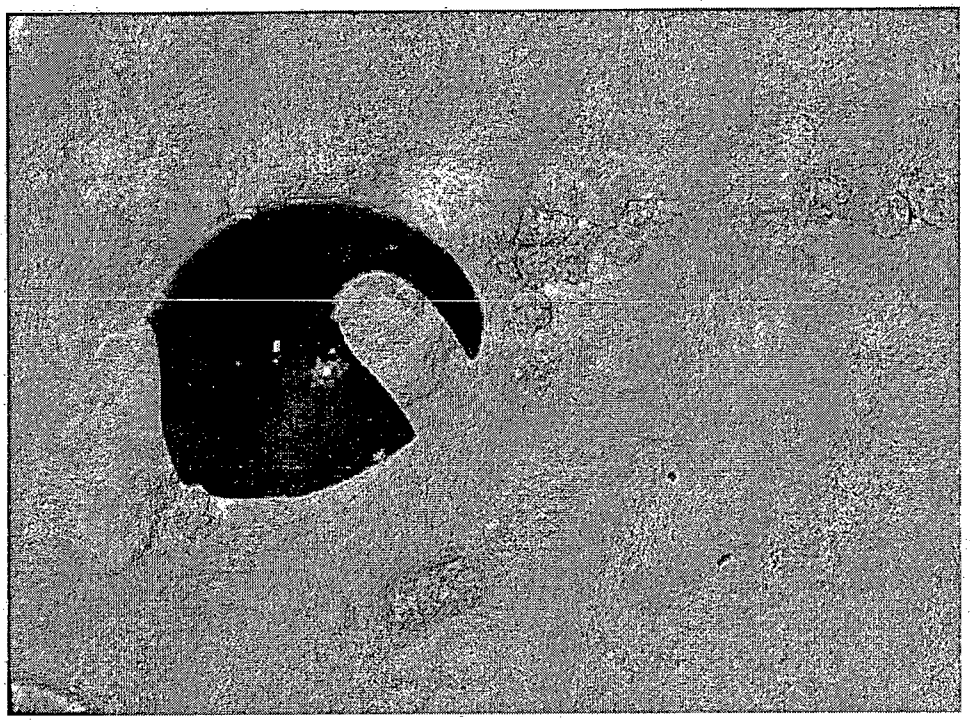


FIGURE 11

WT mice nest building



ATP13A2 KO mice nest building

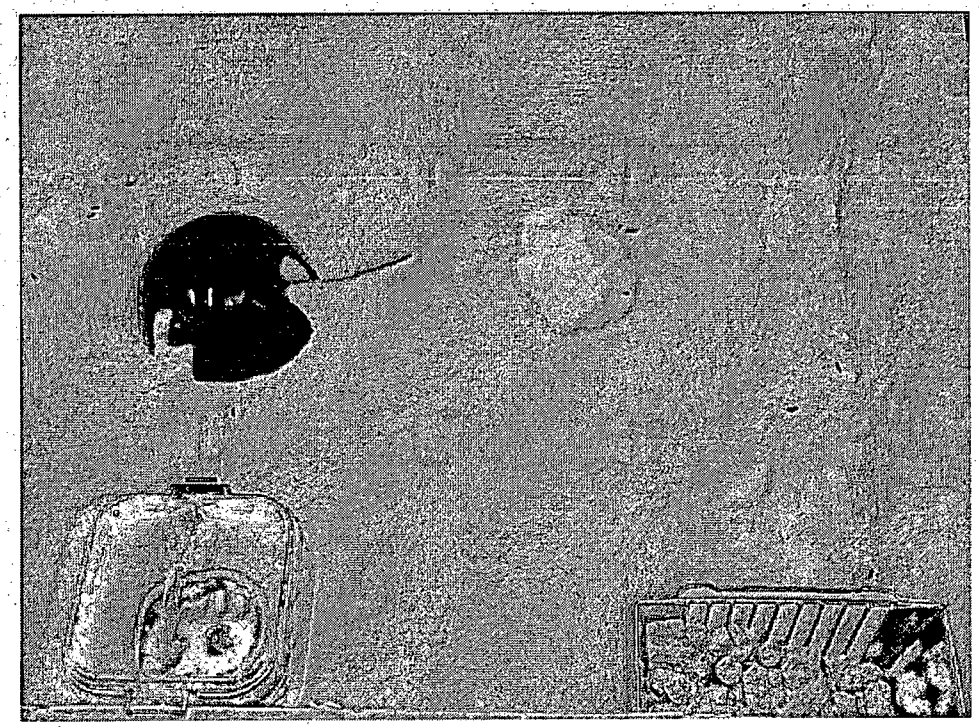
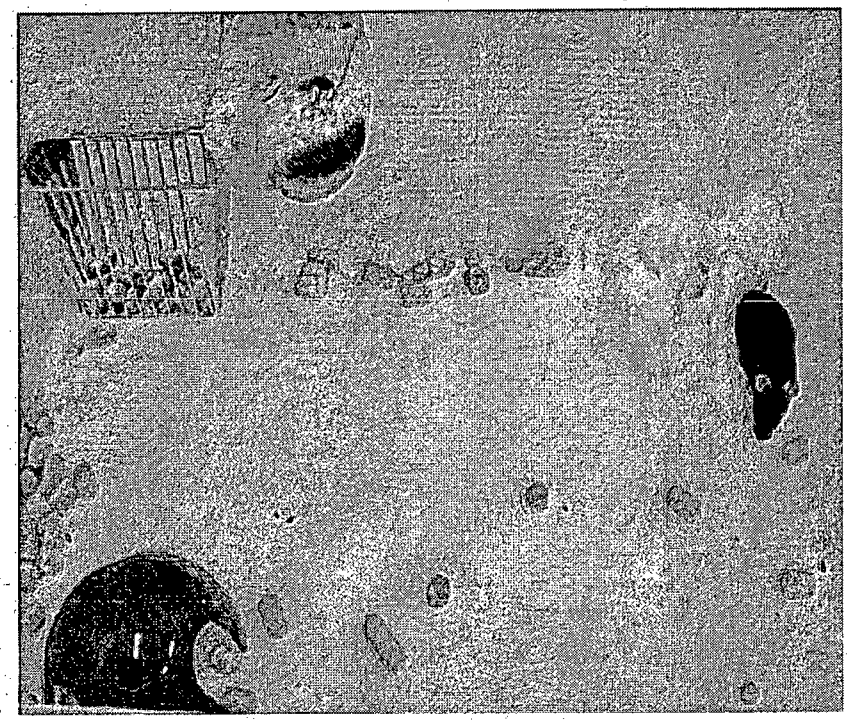


FIGURE 12

WT mice nest building



ATP13A2 KO mice nest building

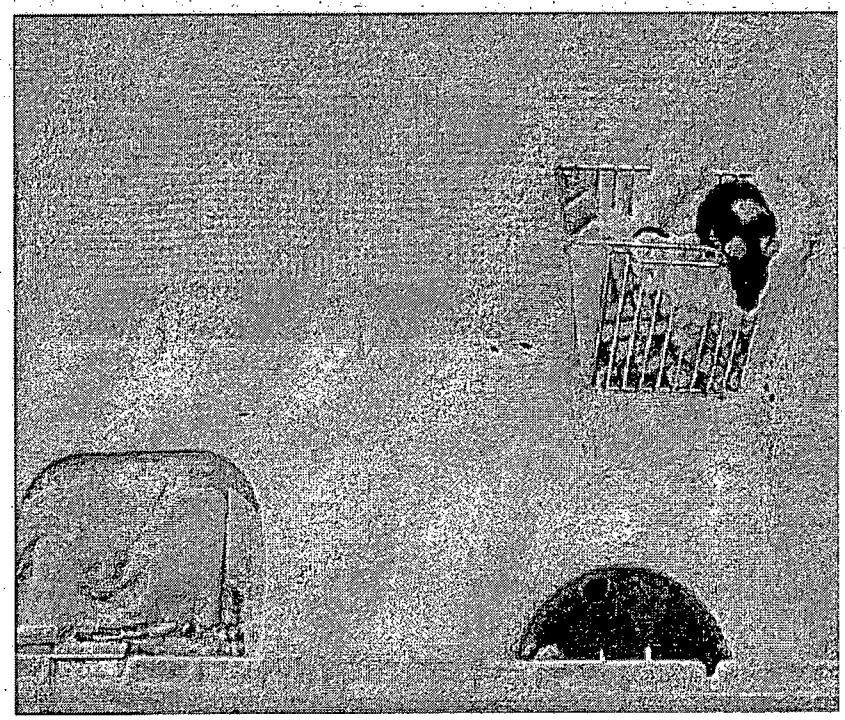


FIGURE 13

a



b

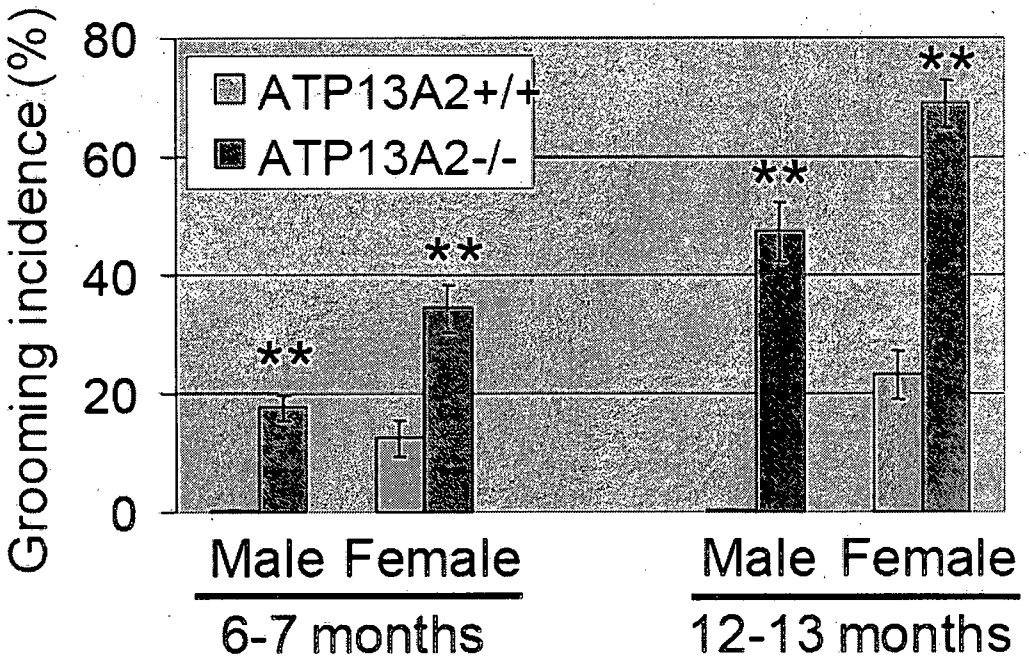
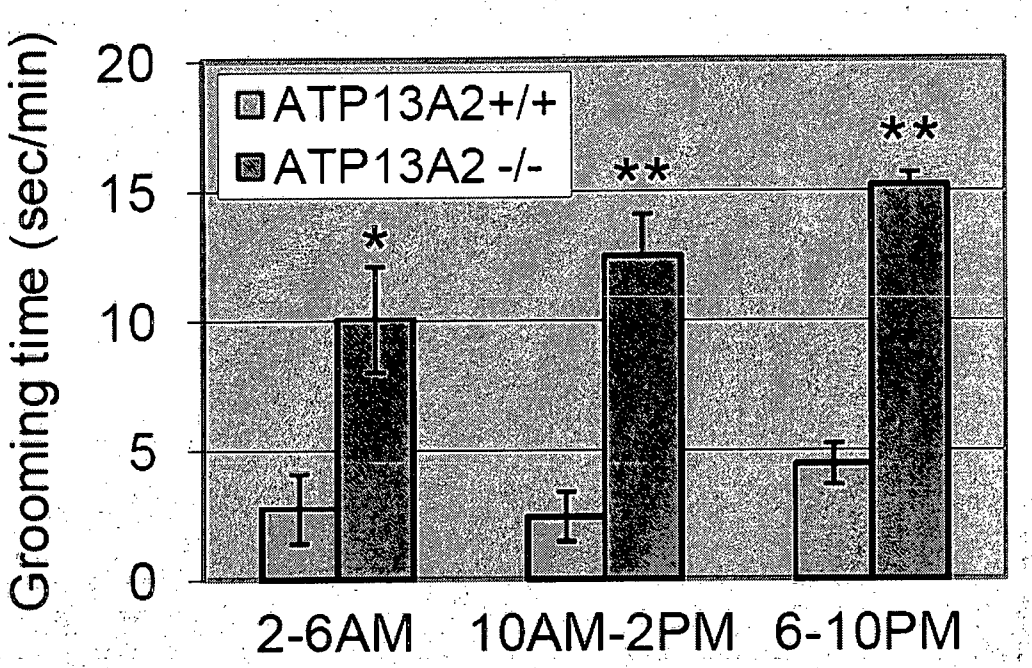

Grooming incidence (%)
80
60
40
20
0
ATP13A2+/+
ATP13A2-/-
**
**
**
**
Male Female
6-7 months
Male Female
12-13 months

FIGURE 14

c



d

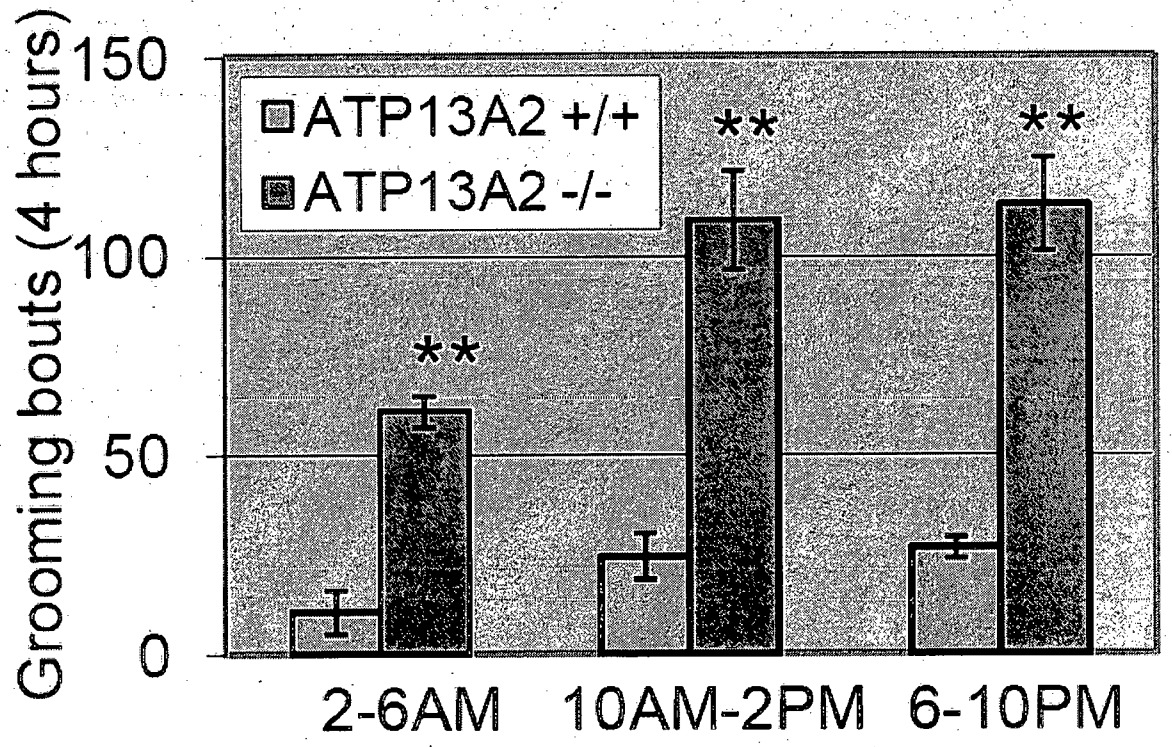
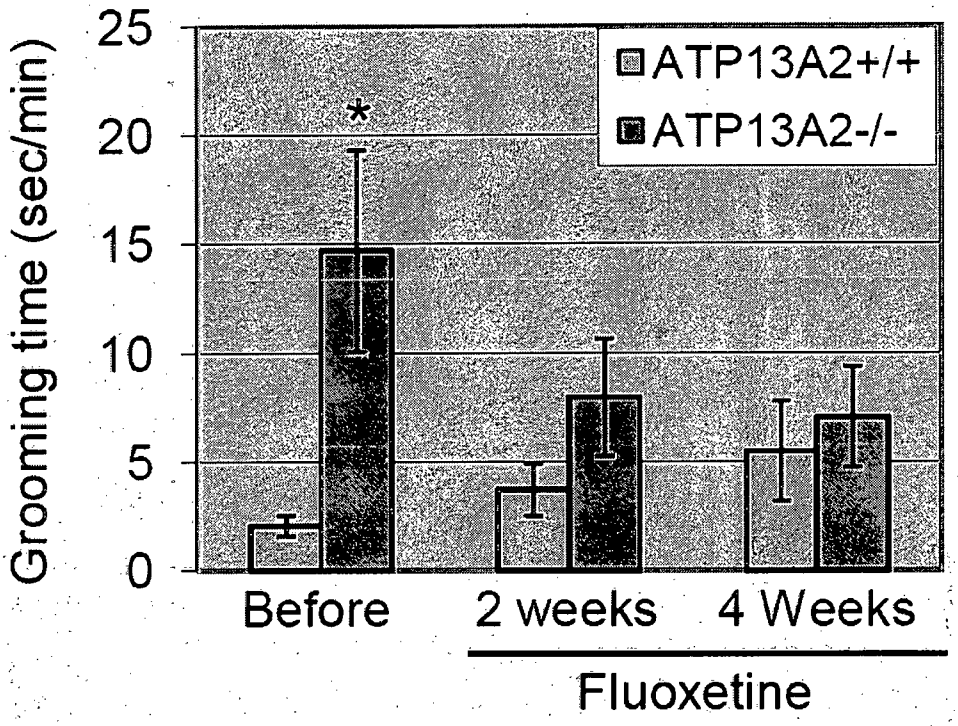


FIGURE 14 (CONTINUED)

e



f

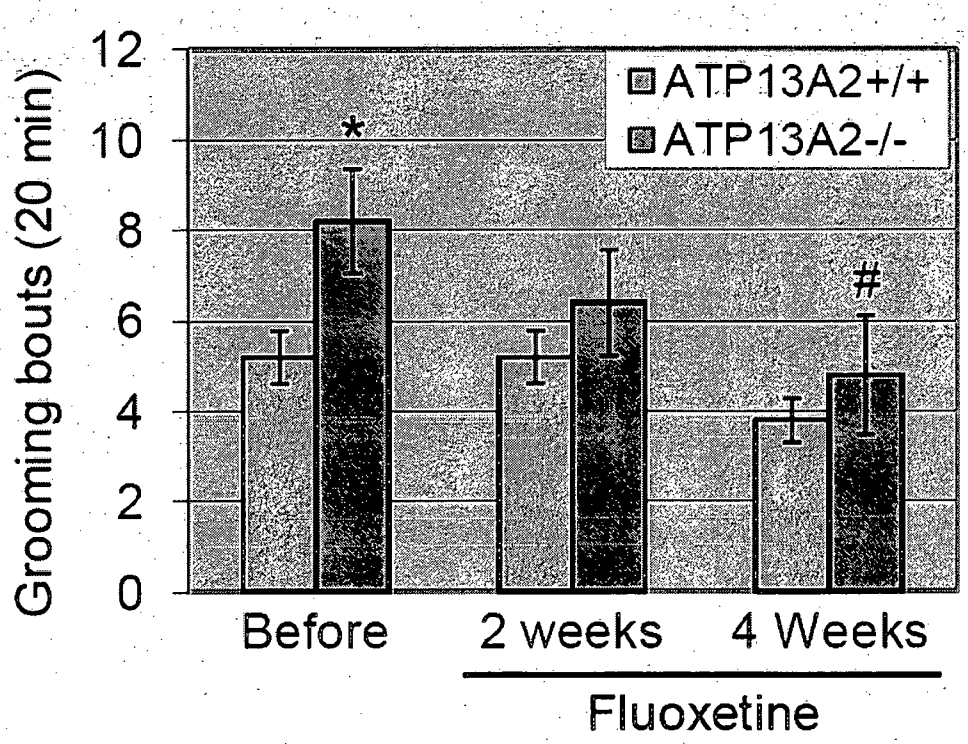


FIGURE 14 (CONTINUED)

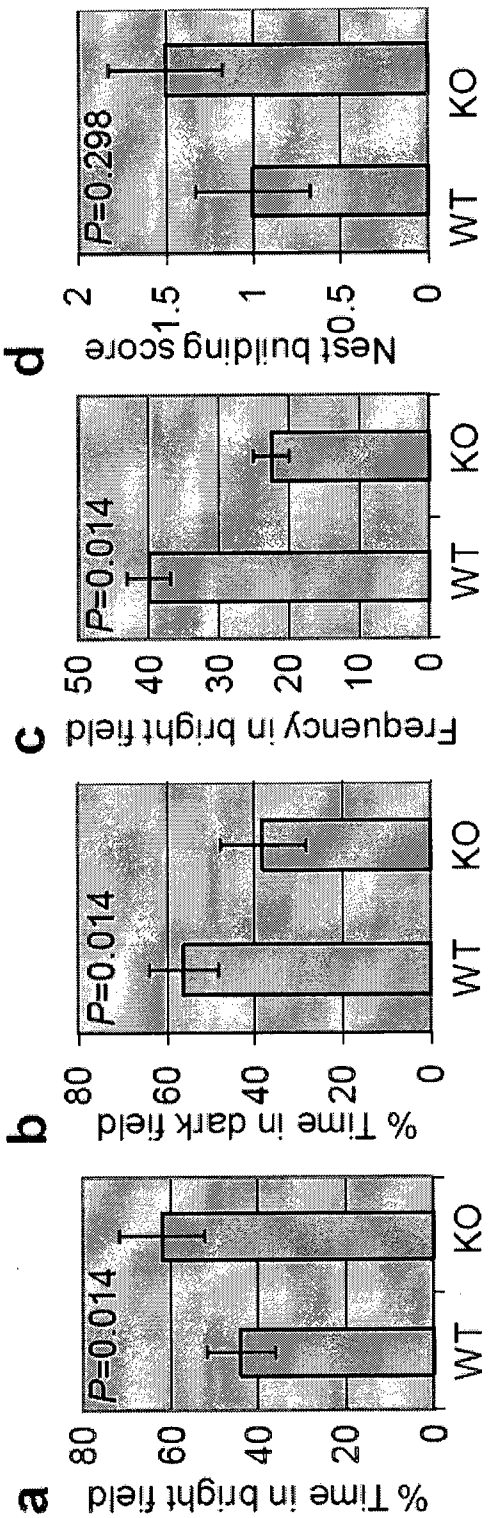

a
% Time in bright field
80
60
40
20
0
P=0.014
WT
KO
b
% Time in dark field
80
60
40
20
0
P=0.014
WT
KO
c
Frequency in bright field
50
40
30
20
10
0
P=0.014
WT
KO
d
Nest building score
2
1.5
1
0.5
0
P=0.298
WT
KO

FIGURE 15

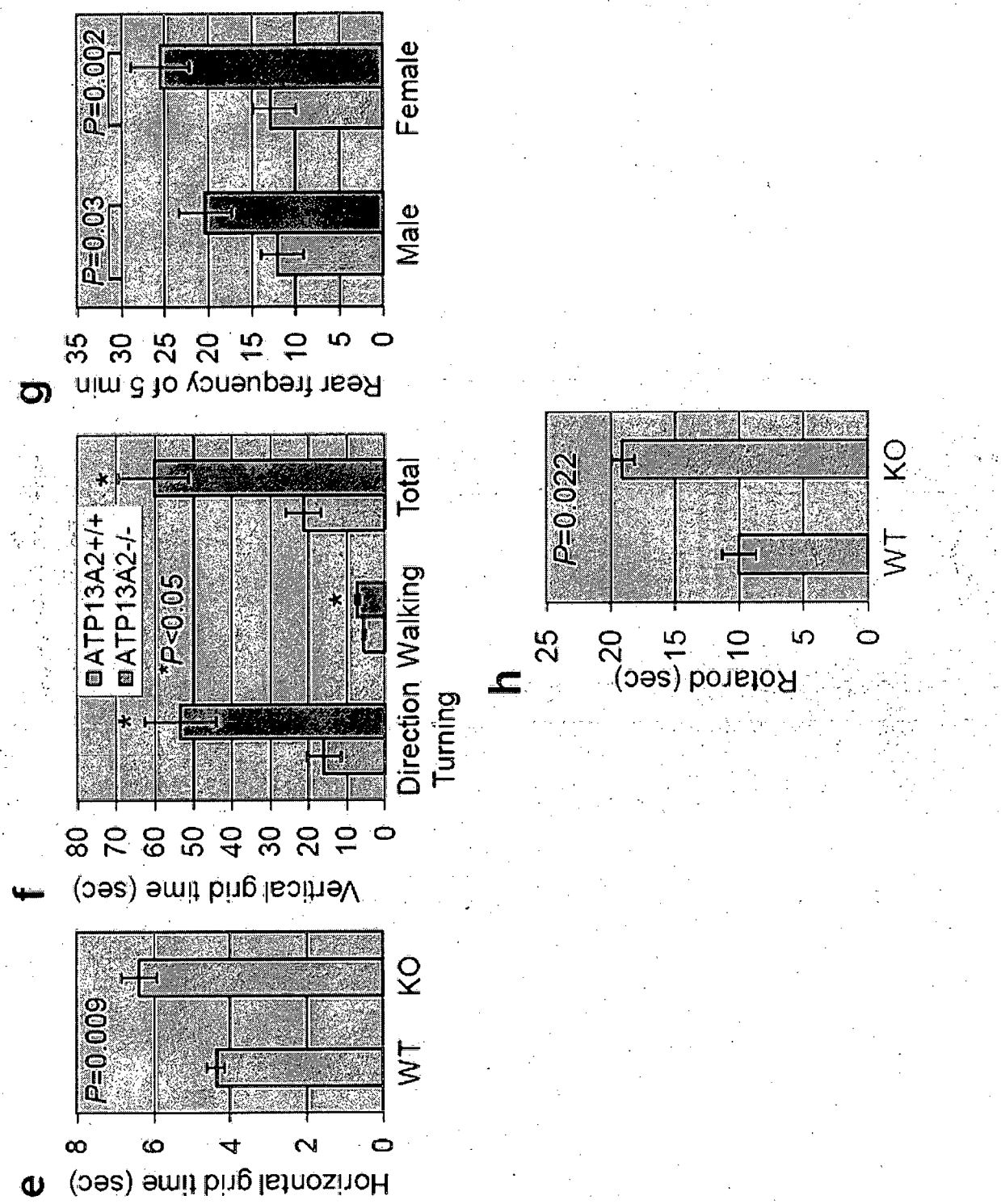


FIGURE 15 (CONTINUED)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2011/000513

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12Q 1/68 (2006.01) ***C12N 15/06*** (2006.01) ***G01N 33/48*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI, EPODOC, MEDLINE, CAPLUS, BIOSIS. Keywords ATP13A2, manganese, OCD, liver disorder and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GITLER, A.D. *et al.*, 'Alpha-synuclein is part of a diverse and highly conserved interaction network that includes PARK9 and manganese toxicity', Nature Genetics 2009, vol. 41, no. 3, pages 308-315 The whole document, but particularly abstract; p312, paragraph 5 – p313, paragraph 3	17, 18
X	BOWLER, R.M. *et al.*, 'Neuropsychiatric effects of manganese on mood', NeuroToxicology, 1999, vol. 20, pages 367-378 The whole document, but particularly abstract; tables 1 and 2; page 372, column 2, paragraph 2; page 376, column 1, paragraph 2	1-3, 7, 8, 11
A	WO 2006/091964 A2 (THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ALABAMA) 31 August 2006	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 2011	21 JUN 2011
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **Julie Christie** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2463

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TUSCHL, K. *et al.*, 'Hepatic cirrhosis, dystonia, polycythaemia and hyper manganesaemia – a new metabolic disorder', J. Inherit. Metab. Dis., 2008, vol. 31, pages 151-163	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2011/000513

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
WO 2006091964	AU	2006216442	AU	2006318447	CA	2599182
	CA	2624361	CN	101142482	CN	101365451
	EP	1856527	EP	1951262	KR	20070110104
	KR	20080067628	RU	2007135320	US	2007203079
	US	2007204352	WO	2007062186		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX