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Evidence Revealing Deregulation of The KLF11-Mao A Pathway in Association with Chronic Stress and Depressive Disorders

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The biochemical pathways underlying major depressive disorder (MDD) and chronic stress are not well understood. However, it has been reported that monoamine oxidase A (MAO A, a major neurotransmitter-degrading enzyme) is significantly increased in the brains of human subjects affected with MDD and rats exposed to chronic social defeat (CSD) stress, which is used to model depression. In the current study, we compared the protein levels of a MAO A-transcriptional activator, Kruppel-like factor 11 (KLF11 , also recognized as transforming growth factor-beta-inducible early gene 2) between the brains of 18 human subjects with MDD and 18 control subjects. We found that, indeed, the expression of KLF11 is increased by 36% ($p < 0.02$) in the postmortem prefrontal cortex of human subjects with MDD compared with controls. We also observed a positive correlation between KLF11 levels and those of its target gene, MAO A, both in association with MDD. KLF11 protein expression was also increased by 44% ($p < 0.02$) in the frontal cortex of KLF11 wild-type mice ($Klf11^{+/+}$) *vs* $Klf11^{-/-}$ when both exposed to CSD stress. In contrast, locomotor activities, central box duration and sucrose preference were significantly reduced in the stressed $Klf11^{+/+}$ mice, suggesting that $Klf11^{+/+}$ mice are more severely affected by the stress model compared with $Klf11^{-/-}$ mice. These results serve to assign an important role of KLF11 in upregulating MAO A in MDD and chronic social stress, suggesting that inhibition of the pathways regulated by this transcription factor may aid in the therapeutics of neuropsychiatric illnesses. Thus, the new knowledge derived from the current study extends our understanding of transcriptional mechanisms that are operational in the pathophysiology of common human diseases and thus bears significant biomedical relevance.
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INTRODUCTION

Major depressive disorder (MDD) is a highly prevalent psychiatric illness and is considered as one of the most burdensome diseases in the world (World Health Organization, 2002), which has become a leading cause of death and disability in middle-to-high income nations (World Health Organization, 2008). The average lifetime prevalence of MDD is 17% for all Americans (Krishnan, 2010), affecting a variety of people ranging from adolescents to senior citizens with different employment statuses, education levels, marital statuses, and race/ethnicity across the United States (Kessler *et al*, 2003). The Sequenced Treatment Alternatives to Relieve Depression study has revealed that the disorder is highly resistant to treatment, requiring new therapeutic approaches (Gaynes *et al*, 2008). Thus, many laboratories, including ours, are devoting significant efforts to identify novel relevant pathophysiological mechanisms underlying the development and progression of MDD, which can be targeted for therapeutic purposes.

The current study seeks to shed light on the potential contribution of the Monoamine oxidase A-Kruppel-like factor 11 (MAO A-KLF11) pathway to MDD using a design that includes analyses of the brain tissue from affected human subjects and a relevant animal model. MAO A, an enzyme that degrades monoamine neurotransmitters such as serotonin, norepinephrine, and dopamine (Bach *et al*, 1988), has a well-documented implication in major depressive disorder (Du *et al*, 2004; Lung *et al*, 2011; Meyer *et al*, 2006, 2009). Significant increase in the levels of MAO A is found in association with the pathogenesis of

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MDD (Meyer *et al*, 2006, 2009; Sherif *et al*, 1991). As neurotransmitters are oxidized by MAO, reactive oxygen species, such as hydrogen peroxide, are produced causing oxidative stress that impairs neuronal homeostasis (Maurel *et al*, 2003). Stress-induced MAO A expression is mediated by transcription factors, in particular, KLF11 (also identified as transforming growth factor-beta-inducible early gene 2 (TIEG2) (Grunewald *et al*, 2012).

KLF11 regulates the transcription of neuronal genes by binding to distinct sequences within their promoter region (Cook *et al*, 1998; Tachibana *et al*, 1997), triggering Pol II-mediated transcriptional initiation. Through this key biochemical mechanism KLF11 regulates multiple cellular processes including cell growth, differentiation, apoptosis, endocrine disorders, and malignancy (Buck *et al*, 2006; Cook *et al*, 1998; Fernandez-Zapico *et al*, 2003; Tachibana *et al*, 1997). Relevant to the current study, we have previously shown that KLF11 (Fernandez-Zapico *et al*, 2003; Zhang *et al*, 2001) is a robust transcriptional activator for MAO B (Lu *et al*, 2008; Ou *et al*, 2004), increasing the transcription of this gene via distinct GC-rich sites, which are located adjacent to its transcription start sites (Ou *et al*, 2004). Similarly, the MAO A promoter also contains Sp1-binding sites which have been recently reported to be a target of KLF11, increasing the expression of this gene (Grunewald *et al*, 2012). Unfortunately, however, in spite of this valuable biochemical information, the role that this protein plays in MDD remains to be defined.

Consequently, the current study was designed with the goal of helping to fill this important gap of knowledge in the pathogenesis of this common neuropsychiatric disorder. Our experiments find that: (1) KLF11 protein levels are elevated in human individuals with MDD; (2) Changes in KLF11 expression correlate with changes in MAO A, which has been shown to be elevated in human MDD; and (3) chronic social defeat (CSD) stress, in genetically engineered *Klf11* mice, which is used as a model for MDD, increases both KLF11 and MAO A expression. Notably, our results support a pathophysiological role of the KLF11-MAO A pathway in the pathogenesis of MDD, a fact that should be taken into consideration for the future design of psychotherapeutic strategies for the treatment of this disorder.

MATERIALS AND METHODS

Human Subjects and Tissue Collection

The study was performed in concurrence with the declaration of Helsinki (Stockmeier *et al*, 2009) and Institutional Review Board policies at University Hospitals of Cleveland and the University of Mississippi Medical Center. Prefrontal cortex samples (Brodmann area 8/9; right hemisphere) were collected upon autopsy at the Cuyahoga County Coroner's Office (Cleveland, Ohio). The next-of-kin for the subjects provided written consent (Miguel-Hidalgo *et al*, 2006; Ou *et al*, 2010; Stockmeier *et al*, 2009). A trained interviewer administered the Structured Clinical Interview for DSM-IV Psychiatric Disorders to knowledgeable informants to determine current and lifetime Axis I psychopathology (First *et al*, 1996). The validity of diagnoses resulting from retrospective interviews concurs with diagnoses based on reviewing the subject's medical records (Deep-Soboslay *et al*, 2005; Dejong and Overholser, 2009; First *et al*, 1996; Johnson *et al*, 2011; Kelly and Mann, 1996).

Two groups were formed: 18 subjects that met the DSM-IV criteria for MDD (American Psychiatric Association (Table 1 and Supplementary Data Table 1), (Johnson *et al*, 2011), and psychiatrically normal control subjects (18) were matched intently to MDD subjects (Table 1 and Supplementary Data Table 1) (Johnson *et al*, 2011).

MDD Subjects with Corresponding Control Subjects

Eighteen (18) psychiatrically normal control subjects. The average age (years) for this control group was 49.6 ± 3.4, 11 subjects were male and 8 subjects were smokers (Table 1 and Supplementary Data Table 1A). Eight subjects were African American and 10 subjects were Caucasian. The average postmortem interval (PMI) prior to the collection of brain specimens was 19.6 ± 1.89 (hours). The average pH of the brain tissue for this group was 6.58 ± 0.08. The average freezer storage time at the time of this investigation was 11.67 ± 3.40 (years). Assessment of postmortem blood and urine for the normal control

Table 1 Demographics, Sample Conditions, and Clinical Characteristics Data of Subjects with Major Depressive Disorder and Corresponding Psychiatrically Normal Control Subjects

	Control ($n = 18$) mean ± SEM	MDD ($n = 18$) mean ± SEM	Statistic[a]	*P*-value
Age of onset (years)	NA	46.4 ± 4.7		
Deaths due to suicide	0	12 (66.7%)		
Age (years)	49.6 ± 3.4	54.6 ± 4.7	$t_{34} = 0.87$	0.3908
Gender (%male)	61.1%	66.7%	$\chi^2_1 = 0.12$	0.7286
Race (%AA)	44.4%	27.8%	$\chi^2_1 = 1.08$	0.2979
PMI (h)	19.8 ± 2.0	20.7 ± 1.6	$t_{34} = 0.34$	0.7340
Tissue pH	6.60 ± 0.08	6.56 ± 0.07	$t_{34} = 0.35$	0.7285
Storage time (years)	13.3 ± 0.8	13.5 ± 1.2	$t_{17} = 0.15$	0.8782
Smokers (%yes)	9 (50.0)	7(38.9)	$\chi^2_1 = 0.45$	0.5023
KLF11/actin	1.40 ± 0.15	2.17 ± 0.25	$t_{34} = 2.62$	**0.0131**

Abbreviations: MDD, major depressive disorder; *n*, number of subjects; NA, not applicable; PMI (h), postmortem interval in hours; SEM, standard error of the mean; y, years.
Categorical data compared with χ^2-test with 1 degree of freedom. General outcomes: there were no significant differences between cohorts for demographic variables (age, gender, race, PMI, pH, storage time, and smoking history). The detailed analysis for KLF11 (TIEG2): in the univariate analysis using Student's *t*-test and KLF11/actin ratio as the outcome, the mean value for subjects with MDD was significantly greater than control subjects ($t_{34} = 2.62$, *p*-value = 0.0131). KLF11 levels were not significantly correlated with age, PMI, tissue pH, or storage time, nor did gender, race, or smoking status significantly affect KLF11 levels. There was no significant correlation between KLF11 levels and either age of onset, duration of illness, or number of depressive episodes (data not included). Among subjects with MDD, there is no significant difference in KLF11 levels between those dying by suicide ($n = 12$, 2.15 ± 0.31) and those not dying by suicide ($n = 6$, 2.21 ± 0.50)($t_{16} = 0.09$, *p*-value = 0.9256).
[a]Continuous data compared with t-statistic with 34 degrees of freedom.
Bold entries indicate that statistically significant elevation in KLF11 protein levels in the prefrontal cortex of MDD samples compared to controls was not accompanied by any change in demographic data between the groups.

subjects did not reveal the presence of psychoactive drugs. These control subjects were matched as closely as possible to the 18 subjects with MDD taking into account age, sex, race, PMI, pH, toxicology, smoking status, and freezer storage time (Supplementary Data Tables 2).

Eighteen (18) untreated subjects with MDD. The average age for subjects with MDD is 54.6 ± 4.7 and the average age of onset for MDD in subjects was 46.41 ± 4.7. Twelve subjects were male (Table 1 and Supplementary Data Table 1B). Sixty-seven percent (12/18) of the MDD subjects were victims of suicide (data not shown). All MDD subjects had postmortem toxicology screenings that were negative for the presence of antidepressant drug therapy. Fifteen MDD subjects (15/18) had not taken antidepressant drugs in the last 3 years before death. The remaining three MDD subjects were prescribed antidepressants within the last 30 days before death but were considered noncompliant as these medications were not detected in the postmortem toxicology screen (Johnson *et al*, 2011). Toxicology reports revealed positive blood alcohol content in three MDD subjects at the time of their deaths; nevertheless, these subjects were not diagnosed with alcohol use disorders, including alcohol dependence.

Additional information is disclosed for subjects with MDD (Supplementary Data Table 1C). The MDD exhibited by these subjects included classifications such as single/recurrent episodes with/without psychotic and/or melancholic features. Among the 18 MDD subjects, six subjects had recurrent major depressive episodes and 12 had MDD with a single depressive episode throughout their lifetime. In addition, eight of the MDD subjects were actively in a major depressive episode at the time of death and five MDD subjects were likely in a major depressive episode at death based on correlating onset, duration, and time of death; the remaining five MDD subjects were either not in a depressive episode at the time of death or relevant information was unavailable. The duration of illness (depression) for these subjects ranged from 1 month to 34 years. The duration of individual episodes ranged from 3 weeks to 15 years. The average number of depressive episodes was 1.4 (Supplementary Data Table 1C) (Johnson *et al*, 2011).

Western Blot Analysis

Protein lysates were obtained from the brain tissue samples of each subject (human) following homogenization in a 0.5 ml solution containing 1 mM EDTA, 10 mM Tris-HCL, and fresh protease inhibitor (Sigma), and centrifuged at 4 °C (550 *g*) for 10 min. Forty micrograms (40 μg) of total protein were separated by 10.5% SDS-polyacrylamide gel electrophoresis. After transfer, membranes were incubated with mouse anti-TIEG2 antibody (1:500; BD Transduction Laboratory; 611402) or mouse anti-MAO A antibody (clone G-10, 1: 250; Santa Cruz Biotechnology) overnight at 4 °C and anti-mouse secondary antibody at room temperature for 2 h.

An equal number of samples from each group (control and MDD human subjects) were immunoblotted on separate membranes as duplicates. Protein bands were visualized by the ChemiDoc XRS + Imaging System (Bio-Rad). Expression of *β*-actin was also quantified from stripped membranes (immunoblotted for the determination of KLF11, or MAO A, respectively) to establish loading controls. The band intensities for KLF11 or MAO A were calculated and normalized to the band intensities of *β*-actin using Quantity One analysis software. Using increasing protein concentrations from a human control subject, a standard curve was established for KLF11, which exhibited a linear relationship relative to the total protein concentration as shown in Supplementary Data Figure 1 (Johnson *et al*, 2011; Ou *et al*, 2010).

Statistical Analysis of Human Subjects

Student's *t*-test for two group comparison was utilized in the evaluation of statistical significance. The data were reported as mean ± SEM, and a value of $p<0.05$ was considered statistically significant. The potential influence of age, sex, race, smoking habit, PMI, tissue pH, tissue storage time on KLF11, duration of illness, and number of depressive episodes were examined by linear regression (Table 1).

Animal Subjects

The *Klf11* homozygous knockout (*Klf11*$^{-/-}$) model was generated at the University of Washington (Seattle, WA)

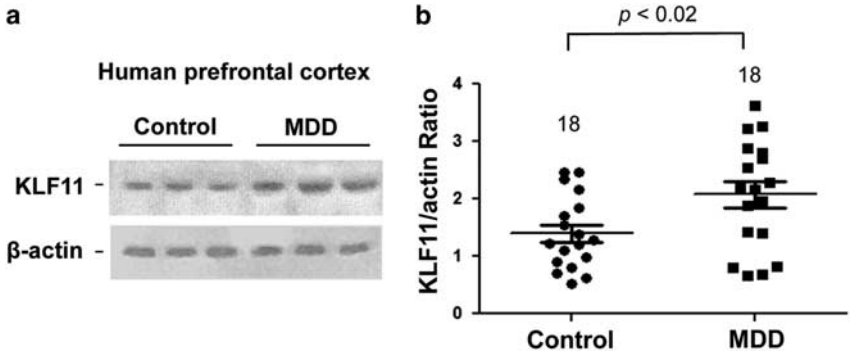

Figure 1 Western blot analysis of KLF11 (TIEG2) protein levels in the human postmortem prefrontal cortex of 18 subjects with major depressive disorder (MDD) *vs* 18 non-affected, psychiatrically normal control subjects. (a) A representative immunoblot showing three healthy controls and three MDD subjects is shown. *β*-actin was used as the loading control. (b) Quantitative analysis. Each KLF11 band was evaluated by its relative intensity and normalized to the density of *β*-actin. Graphs of the average optical density of KLF11/actin for the individual subjects (solid circles or squares) and mean values (horizontal lines) are shown.

following homologous recombination techniques to inactivate the endogenous *Klf11* gene in embryonic stem cells, generating chimeras, and isolating colony founders carrying the knockout gene (Bonnefond *et al*, 2011). These animals were originally generated in a mixed background and subsequently transferred to the Mayo Animal Facilities (Rochester, MN) where it was crossed back into a pure C57BL/6 background for >20 generations to produce the inbred strain used in this study. *Klf11* wild-type ($Klf11^{+/+}$) and knockout mice ($Klf11^{-/-}$)(C57BL6) were obtain from Mayo Clinic. CD1 breeder mice were obtained from Harlan Laboratories. Mice were housed at a constant temperature with a 12 h light/dark cycle with *ad libitum* food and water. In all of the experiments, male $Klf11^{-/-}$ animals were compared with age-matched male $Klf11^{+/+}$ littermates. All procedures were in accordance with the University of Mississippi Medical Center IACUC (protocol # 1316).

CSD Stress

Mice (2–3 months old) were subjected to social defeat stress for 10 consecutive days. Each mouse was introduced into the home cage of an unfamiliar resident for 5 min; during this time the mice were physically defeated. Unfamiliar resident mice were CD1 breeders; these mice are well-known for their consistently short-attack latencies. Once submissive posturing was exhibited, mice were kept in the same cages with the unfamiliar resident for 24 h separated by perforated plexiglass partitions in the resident's home cage allowing for sensory contact. Each day, experimental mice were exposed to new resident mice (Berton, 2006).

Behavioral Evaluation

Open field. One day after the last session of social defeat stress, mice were placed in a $40 \times 40 \times 40$ cm box with opaque walls and allowed to roam freely for 30 min during which their activities were recorded with a digital camera and analyzed with the Noldus Ethovision 8.5 Software. Total distance travelled, immobility time, and duration in the central box were analyzed to indicate the depressive behaviors (Prut and Belzung, 2003; Rygula *et al*, 2005)

Sucrose preference. Sucrose preference was performed during the dark phase. The mice were trained to task by three presentations of sweetened *vs* tap water during the course of 1 week. Following 4 h water restriction, mice were given 1 h access to two bottles: sucrose (1% during training initiation for 3 days before chronic social defeat; mice were then transitioned to 2% sucrose for testing during 10-day's CSD on day 1, day 5, and finally on day 10) and tap water placed side by side. Bottle position was reversed with each presentation (Peng *et al*, 2012; Rygula *et al*, 2005). Fluid consumption was measured by subtracting the drinking bottle weight following presentation from the initial filled bottle weight. Baseline preference was determined 1 h prior to the onset of stress (day 1) and the final preference tests were conducted on day 10 of CSD stress.

Quantitative Real-time RT-PCR

Using mice brain tissue, mRNA was extracted with TRIzol reagent (Invitrogen) and reverse transcription was conducted with the SuperScript III first-strand synthesis system (Invitrogen). Resultant cDNA was quantified with the iCycler MyiQ real-time PCR detection system (Bio-Rad) using mice KLF11 and MAO A primers and the 18 S ribosomal RNA primer as an internal control (Grunewald *et al*, 2012).

MAO A Catalytic Activity Assay

Mice brain tissue was homogenized in 50 mM sodium phosphate buffer. For each sample, 100 µl of total protein was incubated with 100 µM [^{14}C]5-hydroxytryptamine (in sodium phosphate buffer) at 37 °C for 20 min. One hundred microliter of 6 N HCl was added to discontinue the reaction. Subsequently, reaction products were extracted with a benzene/ethyl acetate mixture and radioactivity was determined by liquid scintillation spectroscopy (Grunewald *et al*, 2012).

Triple-label Immunofluorescence

Triple immune-labelling was performed to reveal the cell-type-specific expression of MAO A, with specific cell phenotype markers NeuN (for neuronal) and GFAP (for astrocytes) in human prefrontal cortex. Sections were blocked with 5% goat serum and incubated with rabbit anti-MAO A (H-70, 1:100, Santa Cruz, CA) antibody with mouse anti-NeuN monoclonal (1:500; Millipore, Billerica, MA) and chicken anti-GFAP polyclonal (1:1000; Abcam, Cambridge, England). Following primary antibody incubation, sections were visualized by incubation with FITC-conjugated Goat anti-Rabbit IgG (1:1000, Vector Laboratories, Burlingame, CA), Cy3-conjugated AffiniPure Goat anti-Chicken (1:1000, Jackson ImmunoResearch, West Grove, PA), and Cy5-conjugated Goat anti-mouse IgG. Last, all sections were rinsed with PBS and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA).

Statistical Analysis of Animal Subjects

Statistical significance was evaluated using student *t*-test for two group comparison or analysis of variance followed by Bonferroni adjusted tests when comparing more than two groups. A value of $p<0.05$ was considered significant.

RESULTS

Upregulation of the KLF11-MAO A Pathway in the Prefrontal Cortex is a Biochemical Feature of MDD in Humans

KLF11 is known to inhibit cell growth (Buck *et al*, 2006; Cook *et al*, 1998), and increase oxidative stress (Neve *et al*, 2005) and apoptosis (Tachibana *et al*, 1997; Wang *et al*, 2007); which are mechanisms known to contribute to the development and progression of MDD. We hypothesized that KLF11 may be elevated in postmortem brain tissue from subjects with MDD because of the reduced prefrontal cortex volume, signs of cell death, and oxidative damage that have been

reported in the brains of depressive patients (Andreazza *et al*, 2010; Duman and Monteggia, 2006; Dwivedi *et al*, 2009), including patients with MDD (Duman and Monteggia, 2006; Dwivedi *et al*, 2009; Gawryluk *et al*, 2011; Kang *et al*, 2007). In addition, the significantly increased MAO A levels have been observed in the brains of human MDD subjects compared with healthy control subjects (Du *et al*, 2004; Johnson *et al*, 2011; Lung *et al*, 2011; Meyer *et al*, 2006, 2009). However, whether the expression of KLF11 is altered in this disease remains to be defined.

Consequently, we measured the levels of the KLF11 protein in both the postmortem prefrontal cortex of 18 subjects with MDD and 18 psychiatrically normal control subjects matched closely for age, sex, smoking habits, and other conditions (Johnson *et al*, 2011). The results of these experiments show that KLF11 levels were increased by 36% ($p<0.02$) in subjects with MDD compared with control subjects (Figure 1a and b) as determined by western blot analysis. In additon, using linear regression analyses, we evaluated the potential influence of age, sex, race, smoking habit, PMI, tissue pH, tissue storage time on KLF11, duration of illness, number of depressive episodes, and suicide. Interestingly, we find that KLF11 levels do not correlate with any demographic features of MDD (Table 1). Thus, KLF11 is a prevalent change of MDD regardless of the disease stage and/or patient characteristics.

We sought to determine the relationship between the levels of KLF11 and MAO A in MDD subjects using western blot analyses. Figure 2a shows that the levels of the MAO A protein are increased (28% increase, $p<0.02$) in the prefrontal cortex of subjects with MDD. Interestingly, statistical analysis shows a significant positive correlation between the protein expression levels of KLF11 and MAO A ($p<0.03$, Figure 2b).

We have recently demonstrated that KLF11 is specifically expressed in both neurons and astrocytes in prefrontal cortex of postmortem brain (Udemgba *et al*, 2014). In this study, we identified that the majority of MAO A immunoreactivity is localized in astrocytes with a weaker signal in neurons in the prefrontal cortex of controls and those with MDD (Figure 2c). This result is consistent with others showing that MAO A is expressed in astrocytes and neurons (Konradi *et al*, 1987; Riederer *et al*, 1987; Saura *et al*, 1992).

Evidence for the Upregulation of the KLF11-MAO A Pathway in the Brains of a relevant murine model of MDD

The expression of mRNA of KLF11 and MAO A in the prefrontal cortex of postmortem brains of subjects with MDD would provide important insights for MDD-related regulation on this pathway at transcriptional level. Owing to

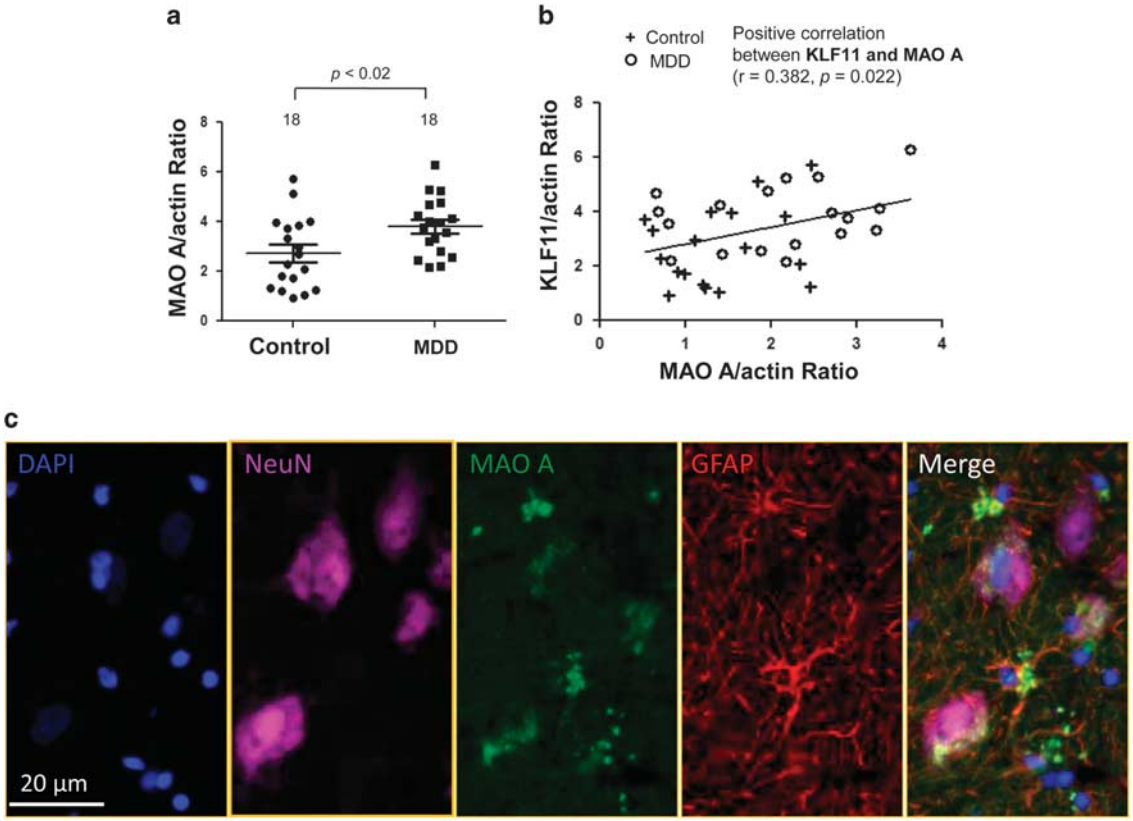

Figure 2 Western blot analysis of MAO A in the human postmortem prefrontal cortex of 18 subjects with major depressive disorder (MDD) *vs* 18 non-affected control subjects. (a) Quantitative analysis. Each MAO A band was evaluated by its relative intensity and normalized to the density of β-actin. Graphs of the average optical density of MAO A/actin for the individual subjects (solid circles or squares) and mean values (horizontal lines) are shown. (b) Graphic representation demonstrating the positive correlation between KLF11 and MAO A levels in psychiatrically normal control subjects (black circles) and subjects with MDD. (c) Representative images showing cell phenotype-specific localization of MAO A in neurons (NeuN positive) and astrocytes (GFAP positive) in the prefrontal cortex of the postmortem human brain.

the limitation to obtain high-quality mRNA from the long term-freezer storage postmortem samples, we analyzed the stress-regulated KLF11-MAO A pathway using the mRNA and protein from the prefrontal cortex of mice following exposure to CSD stress (Figure 3). We found that KLF11 mRNA levels are increased by approximately fourfold ($p<0.01$) in the frontal cortex of mice following CSD stress compared with control mice (Figure 3a, $n=7$ mice per group). Congruently, the levels of KLF11 protein were more than doubled of the basal control values ($p<0.02$) in the frontal cortex of stressed mice (Figure 3b). In parallel, both MAO A mRNA and enzymatic activity were increased in the frontal cortex of stressed mice (Figure 4, lane 2 *vs* 1 in A and B). Together, these results reveal that the regulation of the normal levels of both KLF11 and its target, MAO A are impaired in diseased human brain in a manner that is recapitulated by a murine model for this disorder.

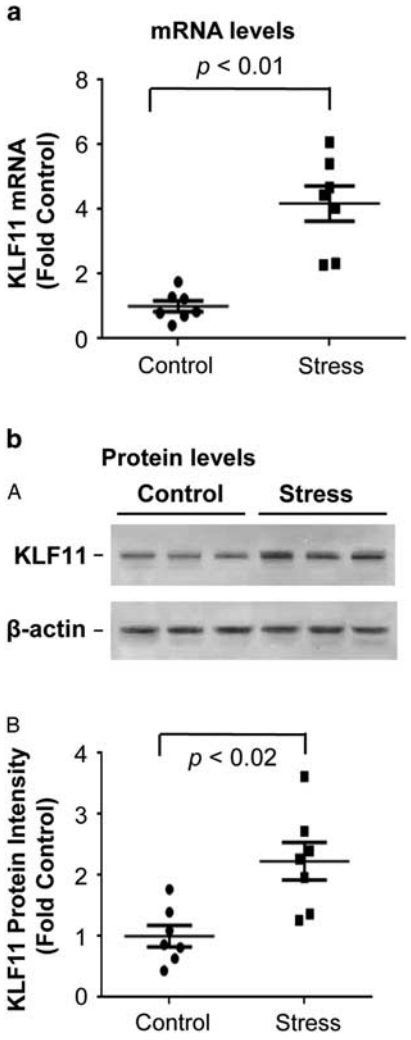

Figure 3 Analysis of KLF11 in the frontal cortex of seven mice following exposure to chronic social defeat (CSD) stress *vs* seven control mice. (a) Quantitative analysis. Real-time RT-PCR results of KLF11 mRNA are shown for mice exposed to CSD stress. (b) Quantitative analysis. (A) A representative immunoblot of KLF11 and β-actin is shown for three control mice and three stressed mice is shown. (B) Each KLF11 band was assessed based on its relative intensity and normalized to β-actin.

Genetic Inactivation of KLF11 results in reduced MAO A Expression After CSD Stress

To better elucidate the role of KLF11 in stress-induced MAO A expression, we assessed the levels and enzymatic activity of the MAO A protein in the frontal cortex of seven KLF11-wild-type *Klf11* $^{+/+}$ and seven *Klf11* $^{-/-}$ mice following CSD stress (Figure 4). Figure 4a illustrates that, MAO A mRNA levels were increased (by 3.7-fold, $p<0.01$, lane 2 *vs* 1) in *Klf11* $^{+/+}$ mice after exposure to CSD. In addition, we observe that *Klf11* $^{-/-}$ mice exhibited reduced MAO A mRNA levels compared with wild-type mice following CSD (by 3.4-fold, $p<0.01$, lane 4 *vs* 2). Likewise, MAO A catalytic activity was significantly increased in *Klf11* $^{+/+}$ mice upon exposed to chronic social stress compared with stress-free *Klf11* $^{+/+}$ mice by 24% ($p<0.05$, Figure 4b, lane 2 *vs* 1). However, MAO A catalytic activity was not significantly increased (only by 12%) in *Klf11* $^{-/-}$ mice after CSD compared with control Klf11 $^{-/-}$ mice (Figure 4b, lanes 4 *vs* 3). Last, CSD significantly reduced MAO A catalytic activity in *Klf11* $^{-/-}$ mice as compared with *Klf11* $^{+/+}$ by 36% ($p<0.02$, Figure 4b, lane 4 *vs* 2). Therefore, these mechanistic experiments demonstrate that the inactivation of KLF11 leads to an impairment in its target gene, MAO A.

Mice Carrying a Genetic Inactivation of KLF11 Exhibit Significantly Less Depressive-like Behavior Following Chronic Stress Exposure

Experimental mice live in social groups and small cages. In the open-field test, mice are separated from their social group and in an anxiety-provoking condition. Therefore, the CSD-stressed mice decrease their locomotor activities and exploratory behavior (Prut and Belzung, 2003; Rygula *et al*, 2005). In addition, stress-induced reduction of sucrose preference is another common characteristic that assesses anhedonia in animal models of chronic stress (Peng *et al*, 2012; Rygula *et al*, 2005; Tang *et al*, 2013). To further establish whether KLF11 behaves as a gene modifier for the manifestation of stress and depression, we determined the effect of CSD stress on locomotor activities, central box exploration, and sucrose preference in both *Klf11* $^{-/-}$ and *Klf11* $^{+/+}$ mice.

These experiments show that *Klf11* $^{+/+}$ mice display a significant increase in immobility (Figure 5a, $p<0.01$, $n=7$ mice per group), decreased total distance traveled (Figure 5b, $p<0.02$), and reduced time in the central box (Figures 5c, $p<0.001$) in the open-field test. The *Klf11* $^{+/+}$ mice also showed a significantly reduced sucrose preference upon completion of the CSD period (Figures 5d, $p<0.0001$). In contrast, *Klf11* $^{-/-}$ mice displayed no change in depressive-like behavior following CSD stress compared with control. There was also a slight reduction in sucrose preference in control *Klf11* $^{-/-}$ mice compared with control *Klf11* $^{+/+}$ mice, nonetheless, this difference was not statistically significant. Thus, as predicted by our hypothesis, disruption of the key MAO A regulator KLF11 confers resilience to depression-like symptoms in an animal model relevant to the study of MDD.

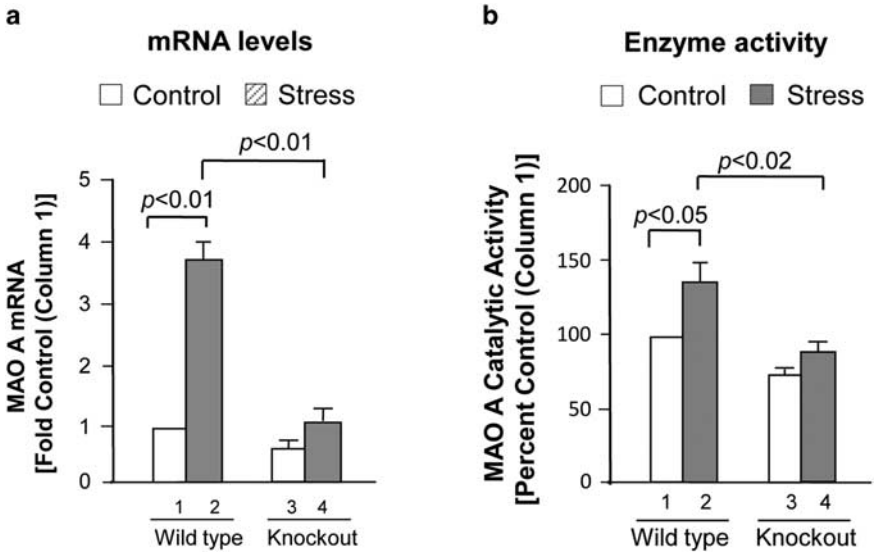

Figure 4 Analysis of MAO A in the frontal cortex of KLF11-wild-type and knockout mice following exposure to chronic social defeat (CSD) stress. (a) Quantitative analysis. Real-time RT-PCR results assessing MAO A mRNA are shown. (b) Quantitative analysis. Catalytic activity levels are illustrated for both control and stressed mice.

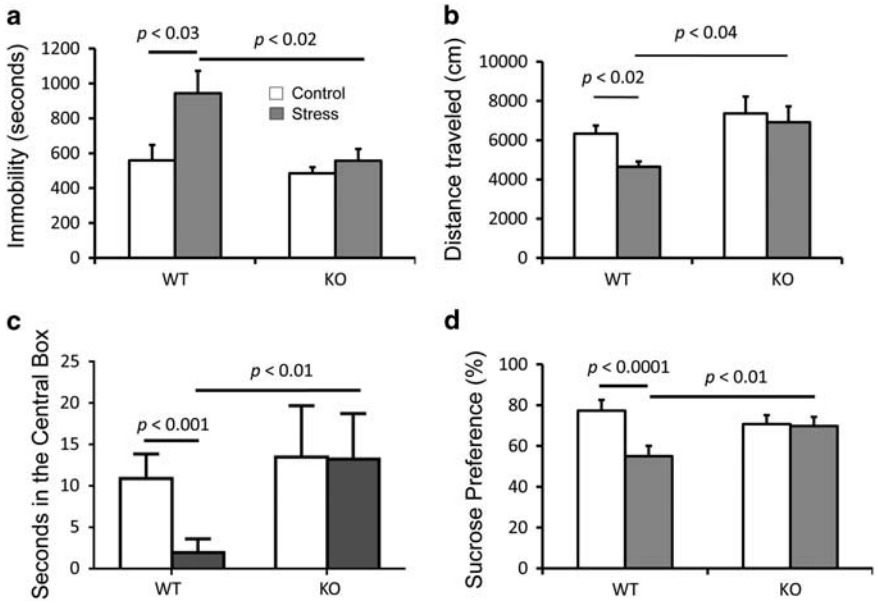

Figure 5 Evaluation of the depressive behaviors. (a) Immobility, (b) total distance traveled, and (c) time in central box in open-field tests and (d) sucrose preference of KLF11-wild-type and knockout mice following exposure to chronic social defeat (CSD) stress. Open-field tests were performed 1 day after the sucrose preference tests that were performed on day 10 of the chronic social defeat procedure and compared with baseline.

DISCUSSION

Abnormal monoamine oxidase expression is associated with several common psychiatric disorders and neurodegenerative diseases (Cases *et al*, 1995; Du *et al*, 2002, 2004; Meyer *et al*, 2006, 2009; Ou *et al*, 2010; Sacher *et al*, 2010; Shih *et al*, 1999; Youdim *et al*, 2006). Therefore, understanding the molecular mechanisms that regulate MAO A expression and enzymatic function is crucial for advancing the clinical treatment of depressive disorders. This study supports the role of the transcription factor, KLF11, in the manifestation of MDD by gathering data directly from human postmortem brain of subjects diagnosed with MDD and the brains of mice exposed to CSD.

Previous evidence has characterized KLF11 as a transcriptional regulator of MAO A via the binding of this protein to GC-rich, Sp1-like binding sites located on the promoter of this gene (Grunewald *et al*, 2012). Various studies have implicated alterations in MAO A and depressive disorders (Du *et al*, 2002, 2004; Fan *et al*, 2010; Sacher *et al*, 2010). More specifically, positron emission tomography (PET) and carbon 11-labeled harmine measurements in the brain from living patients with MDD show that MAO A is significantly elevated in major

depressive episodes (Meyer *et al*, 2006, 2009). The results reported in the current study illustrates that KLF11 protein levels are also significantly increased in the brains of postmortem human subjects with MDD, implicating an upregulation of KLF11 in the pathogenesis of MDD. Moreover, the increased KLF11 seen in MDD positively correlates with MAO A expression, further supporting our previous findings that KLF11 is a transcriptional activator for MAO A.

It has been reported that cigarette smoke reduces brain MAO A activity (Bacher *et al*, 2011; Berlin *et al*, 1995; Fowler *et al*, 1996). In the current study, we have not observed a significant reduction of MAO A protein expression in smokers either in the MDD cohort or the controls with non-smokers (Supplementary Figure 2C, D). The reason for this discrepancy may be because in the current work, we measured the content of MAO A protein in homogenates of frontal cortex from postmortem brain. While those who found reduction of MAO A activity in heavy smokers analyzed the binding of MAO A inhibitors, such as the [11C]clorgyline (Fowler *et al*, 1996) or [11C]harmine (Bacher *et al*, 2011). Cigarettes contain harmines, and harmine has a Ki of $\sim$50 nM for MAO-A (Bacher *et al*, 2011). Therefore, there is some occupancy of MAO-A by Harmine in the brains of smokers. When the level of brain MAO A was measured using [11C]clorgyline or [11C]harmine binding with PET, it was likely reduced owing to occupancy by harmine from cigarette smoke. Therefore, our results may suggest that cigarette smoking may not alter MAO A content itself, but inhibitors in cigarette smoke reduce the amount of free MAO A available to bind to PET ligands *in vivo*. In addition, it is reported that, in withdrawal, heavy cigarette-smoking subjects ($\geqslant$25 cigarettes/day) did not show a reduction of MAO-A binding as compared with healthy subjects (Bacher *et al*, 2011). This implies that in withdrawal, more free MAO A is available for binding to the PET ligand. In our study, the average amount of cigarettes smoked in the control group (1.5 packs/day, $n=8$) and in the MDD cohort (1.38 packs/day, $n=6$, one of the smoker has no record for the amount of cigarettes smoked) are similar. Therefore, we have not observed a significant effect of cigarettes smoked on MAO A protein expression after rigorous analysis.

This study also demonstrates that the levels of both the KLF11 mRNA and protein are upregulated in the frontal cortex of mice following CSD stress, an accepted animal model for depression (Czeh *et al*, 2007; Rygula *et al*, 2005; Vialou *et al*, 2010). Moreover, MAO A levels are decreased in KLF11 knockout mice after exposure to CSD stress, further supporting the role of this transcription factor as an activator for MAO A. Last, KLF11-wild-type mice had reduced locomotor activities, sucrose preference, increased anxiety (less time in the central box of an open-field) compared with KLF11-knockout mice following the CSD stress, indicating that KLF11-knockout mice exhibit less depressive-like behavior. Collectively, these findings document that the KLF11-MAO A pathway is altered at the level of both the neurotransmitter metabolism enzyme and its regulator, which bears implications for better understanding pathophysiological mechanisms underlying MDD. In addition, it is likely that compounds to modulate the levels of expression and function of the KLF11-MAO-mediated pathway may increase neuroprotection, neuroplasticity, and synaptic activities. Maximizing the therapeutic effects upon these targets is also essential toward achieving comprehensive management of stress-induced, frequently treatment-resistant, psychiatric illnesses, and addictions (Barr *et al*, 2004; Beasley *et al*, 2005; Dwivedi *et al*, 2006; Frazer, 1997; Mitchell *et al*, 2012; Sanacora, 2008; Sawada *et al*, 2005; Silberman *et al*, 2009; Wallace *et al*, 2007). Thus, the new knowledge generated by the current study has mechanistic relevance and also provides the rationale for targeting this pathway to develop potential novel therapeutics approaches in the treatment of MDD.

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