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ORIGINAL ARTICLE

Decreased NOX2 expression in the brain of patients with bipolar disorder: association with valproic acid prescription and substance abuse

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Neuroinflammation and increased oxidative stress are believed to contribute to the development of psychiatric diseases. Animal studies have implicated NADPH oxidases (NOX) as relevant sources of reactive oxygen species in the brain. We have analyzed the expression of NOX isoforms in post-mortem brain samples from patients with psychiatric disorders (schizophrenia, bipolar disorder) and non-psychiatric subjects. Two collections from the Stanley Medical Research Institute were studied: the Array Collection (RNA, 35 individuals per group), and a neuropathology consortium collection (paraffin-embedded sections, 15 individuals per group). Quantitative PCR analysis revealed expression of NOX2 and NOX4 in prefrontal cortex. No impact of psychiatric disease on NOX4 levels was detected. Remarkably, the expression of NOX2 was specifically decreased in prefrontal and cingulate cortices of bipolar patients, as compared with controls and schizophrenic patients. NOX2 expression was not statistically associated with demographic parameters and post-mortem interval, but correlated with brain pH. Immunostaining demonstrated that NOX2 was predominantly expressed in microglia, which was corroborated by a decrease in the microglial markers CD68 and CD11b in the cingulate cortex of bipolar disorder patients. The analysis of potentially confounding parameters showed association of valproic acid prescription and heavy substance abuse with lower levels of NOX2. Taken together, we did not observe changes of NOX2 in schizophrenic patients, but a marked decrease of microglial markers and NOX2 in the brain of bipolar patients. This might be an underlying feature of bipolar disorder and/or a consequence of valproic acid treatment and substance abuse.

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INTRODUCTION

Psychiatric diseases, including depression, bipolar disorder (BD) and schizophrenia (SZ), are a major challenge to public health. Mechanisms underlying their pathogenesis are still poorly understood, but evidence points toward a combination of genetic and environmental factors. Patients with mental disorders are given symptomatic treatments, such as antipsychotics (for example, clozapine) for schizophrenics, mood stabilizers (lithium, valproic acid (VPA)) for bipolar patients. Many of these drugs have multiple modes of action, including anti-oxidative[1] and anti-inflammatory activities.[2]

Although BD and SZ are distinct diagnoses, neuropathological differences and biochemical quantitative biomarkers distinguishing the two disorders are virtually non-existing. SZ and BD symptoms are overlapping and patients are often given similar treatments. At the neuropathological level, most evidence argues for a contribution of neuroinflammation, dysfunction in glutamate neurotransmission, altered microglial function and oxidative stress. Changes in reactive oxygen species (ROS) generation are commonly observed in neuronal disorders and psychiatric diseases make no exception. Oxidative modifications are documented in SZ or BD patients. This includes higher levels of 4-hydroxynonenal (peroxidized lipids),[3] oxidized guanine in post-mortem brain samples[4,5] as well as increased thiobarbituric acid reactive substances (byproducts of lipid peroxidation)[6] and decreased levels of intracellular antioxidants in the blood.[7] Also, plasma levels of the oxidized form of cysteine were found to be elevated in schizophrenic[8,9] and bipolar patients. Similarly, several animal models of psychosis recapitulate observations made in humans and present increased oxidation in both central nervous system (CNS) parenchyma and the blood (reviewed in ref. 10). It therefore has been speculated, that the increased oxidative stress in the CNS is due to an increased ROS generation (see below) and/or to a decrease in antioxidant capacity and in enzymes mediating ROS degradation. For example, genetic associations between polymorphisms in enzymes involved in the glutathione synthesis and SZ have been described,[11,12] which might account for low glutathione levels found in SZ.[13] Sources of ROS in the CNS are multiple, and their relative contribution to oxidative stress is a subject of intense investigations. NADPH oxidases (NOX), in particular, the phagocyte NADPH oxidase NOX2 might be important.[14] A role for NOX2 in psychiatric disorders was first proposed in a study from Behrens and collaborators, which use a drug-induced model of psychosis (administration of the NMDA antagonist ketamine to mice). The authors proposed that increased ROS generation by neuronal NOX2 is causative in psychogenesis and induced the loss of parvalbumin-positive neurons, a key pathological hallmark of the schizophrenic brain.

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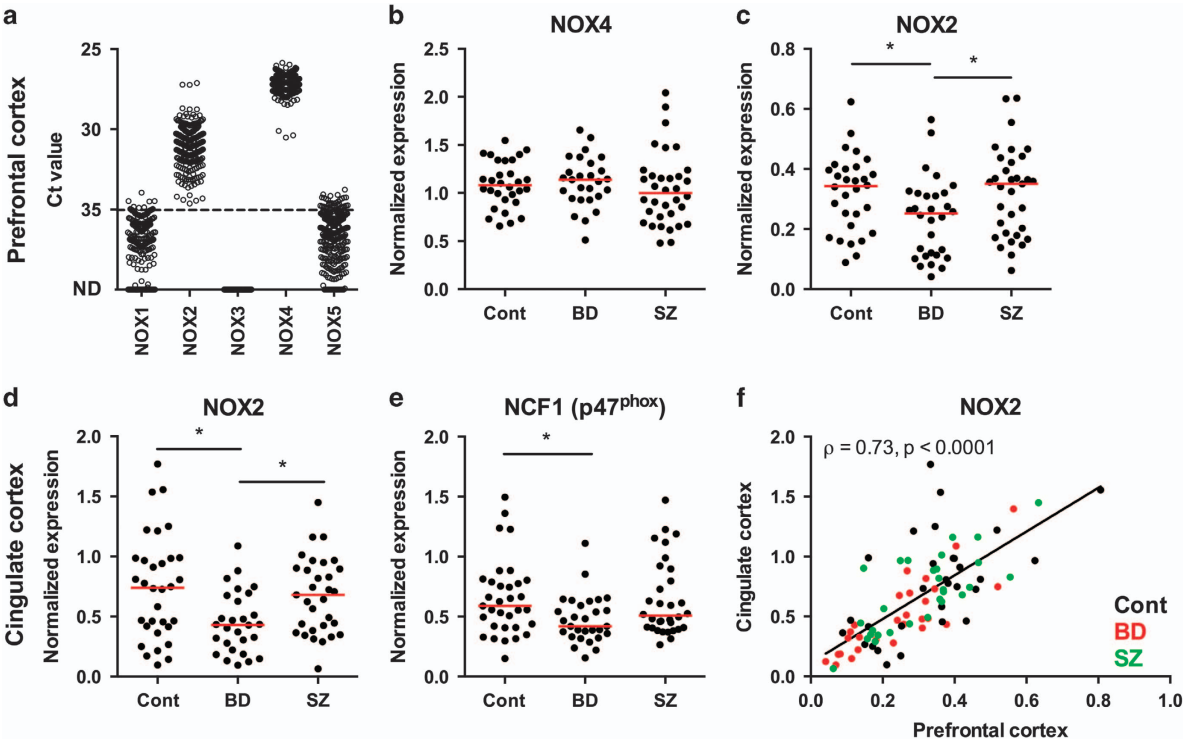

Figure 1. Expression of NADPH oxidases (NOX) mRNA in prefrontal and cingulate cortices of psychiatric patients. (**a**) Ct values from the qPCR analysis of the expression of NOX1–NOX5 are shown. ND, not detected. Only values above a threshold of Ct = 35 (dotted line) were used for further analysis. The expression of (**b**) NOX4 was unchanged, whereas NOX2 (**c**) was decreased in prefrontal cortex (PFC) of patients with bipolar disorder. The expression of (**d**) NOX2 and (**e**) p47phox were decreased in cingulate cortex of bipolar patients. (**f**) Significant correlation was observed for NOX2 expression in PFC and in cingulate cortex, Spearman ρ and *P*-value are shown on the graph. Data is presented as median. *ANOVA Kruskal–Wallis test followed by Dunn's multiple comparison test. Cont, control group (black); BD, bipolar disorder (red); SZ, schizophrenia (green).

Interestingly, ketamine, has received much attention as a promising drug for the treatment of major depression,[15] raising the possibility that some fundamental differences in redox systems regulate SZ and depressive psychiatric disorders.

Although animal models of psychosis may shed light on several redox-dependent processes, translation of these findings to human is required. Clinical trials using the reducing agent *N*-acetylcysteine have shown encouraging results on some specific symptoms of SZ and BD (reviewed in ref. 16). However, redox mechanisms in the human brain and their relationship to psychiatric diseases remain insufficiently understood. Clinical studies using material collected from psychiatric patients should help filling the gap between human and mouse studies, and address the role of NOX in psychiatric diseases. In this study, we investigated the expression of NOX in the brain of patients diagnosed with BD or SZ.

MATERIALS AND METHODS

Post-mortem brain samples

For the studies of gene expression, we used mRNA from cingulate cortex and prefrontal cortex (PFC) from the Array Collection provided by Stanley Medical Research Institute. Selection of samples, informed consent and criteria of exclusion are documented in http://www.stanleyresearch.org/brain-research. The collection consists of 105 brains from patients diagnosed with SZ, BD and unaffected controls (35 cases in each group). Demographic information is provided in Supplementary Table S1.

For immunostaining, we used fixed paraffin 10 µm thick sections from anterior cingulate cortex from the Neuropathology Consortium collection provided by Stanley Medical Research Institute. This collection consists of 60 brains from patients diagnosed with SZ, BD, depression and unaffected controls (15 each). In this study, we focused on three groups (SZ, bipolar and controls) matched by age, sex, race, post-mortem interval, pH and side of brain. Diagnosis and socio-demographic distribution of patients considered in this study are shown in Supplementary Table S2. The study was approved by the Stanley Medical Research Institute.

Real-time quantitative polymerase chain reaction

Total RNA (500 ng) was used for cDNA synthesis using the superscript II kit according to the manufacturer's instructions (Invitrogen, Basel, Switzerland). Real-time PCR was performed using SYBR green assay on a 7900HT SDS systems from ABI at the Genomics Platform, National Center of Competence in Research Frontiers in Genetics, Geneva. The efficiency of each primer was verified with serial dilutions of cDNA. Relative expression levels were calculated by normalization to geometric mean of the three house-keeping genes (GAPDH, β-2-microglobulin and EEF1A1) as described previously.[17] The highest normalized relative quantity was arbitrarily designated as a value of 1. Fold-changes were calculated from the quotient of means of these normalized quantities and reported as ± s.e.m. Sequences of all primers used in this study are provided in Supplementary Table S3. Threshold cycle (Ct) values for NOX isoforms corresponding to the intersection between an amplification curve and a threshold line set at 0.20 by the software are provided in Figure 1a. Specificity of NOX2 and NOX4 primers was verified by subcloning the generated amplicons using the *TOPO* TA Cloning Kit (Thermo Fischer Scientific, Reinach, Switzerland) followed by sequencing at Microsynth (Balgach, Switzerland).

Immunohistochemistry and imaging

Brain sections were deparaffinized through graded alcohols, subjected to heat-induced epitope retrieval for 15 min in 0.01 mol l^{-1} citrate buffer (pH 6), and incubated overnight at 4 °C in PBS-0.3% Triton-X100 with the monoclonal anti-human NOX2 antibody Mo48[18] (1:250 LSBio LS-C85347, Seattle, WA, USA). Sections were then incubated for 1 h at room temperature with specific biotinylated secondary antibody (1:100, Vector Laboratories, Peterborough, UK) and, after several washes in PBS, for 1 h in horseradish peroxidase-avidin/biotin complex solution (1:100, Vector

Laboratories) and detected with 3,3-diaminobenzidinetetrahydrochloride hydrate (DAB, Sigma-Aldrich, St Louis, MO, USA) and H_2O_2. For PFC, the images were acquired using a Digital Image Hub (Leica Biosystems, Wetzlar, Germany) and number of brown/white objects was quantified using in-house —developed software analyzed as previously described.[19]

Statistical analysis

Spearman's rank correlations were calculated for the relationship between NOX2 expression and all available clinical data to determine whether any other factors were contributing to the variation seen in the expression. The difference between two groups was studied with the Mann–Whitney *U* test; groups of more than two were studied using the nonparametric Kruskal–Wallis method with the application of a Dunn's correction for multiple comparisons. Data is presented as median on all figures. To examine for effects of continuous confounding measures, we performed Spearman's correlations on measures that emerged as significant with the nonparametric analyses of variance. There were nine continuous confounding measures available for this analysis (post-mortem interval, age, estimated lifetime exposure to neuroleptics, age of illness onset, illness duration, brain pH, brain weight, and freezer or fixation storage time). There were also three non-continuous confounding measures (hemisphere, gender, and any current or past history of substance abuse) available for analysis. These were used as grouping measures for Mann–Whitney *U* tests for any abnormal measure. The level of significance was defined as $P < 0.05$. All statistical analyses were performed using Sigma Stat (version 3.0, San Jose, CA, USA), GraphPad Prism (version 4.0, La Jolla, CA, USA) and SPSS (version 10, Armonk, NY, USA).

Blindness of the study

Researchers performing qPCR of NOX isoforms and NOX2 immunostaining analysis were blinded to patient diagnosis as well as to socio-demographic distributions. Patients were initially identified by a code. Blindness of the study was broken after returning results to the Stanley Medical Research Institute. Further qPCR using the NOX2 subunit $p47^{phox}$ (NCF1) and the microglia markers CD11b, Iba1 and CD68 were subsequently performed.

RESULTS

Expression of NOX mRNA in prefrontal and cingulate cortices of psychiatric patients

To evaluate the expression of NOX NADPH oxidases in brain regions associated with psychiatric disorders we obtained RNA samples from PFC and cingulate cortex from the Stanley Array Collection. The expression of mRNA for five NOX isoforms (NOX1–NOX5) in PFC was measured by qPCR. We did not analyze the expression of two other members of NOX family: DUOX1 and DUOX2 as previous qPCR and RNAseq data suggest that they are not expressed in the brain tissue.[20,21] We found medium to high levels of NOX2 and NOX4 mRNA, and very low levels of NOX1 and NOX5, whereas NOX3 was below the detection level (Figure 1a). We confirmed the identity of NOX2 and NOX4 by sequencing the qPCR products (data not shown).

The Stanley Array Collection is a collection of mRNA samples from patients with BD, SZ and healthy individuals without psychiatric diagnosis. We analyzed the expression of NOX2 and NOX4 in disease groups. No changes of NOX4 mRNA levels were found in the three analyzed groups (Figure 1b). In contrast to what has been suggested from mouse studies,[22,23] we did not observe significant changes of NOX2 expression in the SZ group. However, we found a significant decrease of NOX2 mRNA levels in bipolar patients ($n = 28$; median = 0.4316; interquartile range (IQR) = 0.4271), which distinguished this group both from controls ($n = 32$; median = 0.7413; IQR = 0.5687) and from schizophrenic patients ($n = 31$; median = 0.6802; IQR = 0.5181) (Figure 1c). Interestingly, this decrease was not limited only to PFC. Levels of NOX2 and its subunit $p47^{phox}$ were also decreased in cingulate cortex (Figures 1e and f). Overall, a strong correlation was found for NOX2 mRNA expression between the two brain regions (Figure 1d).

Association of NOX expression with demographic, social and technical factors

The analysis of demographic factors (age, sex, brain weight and hemisphere), technical factors related to sample preparation (post-mortem interval, refrigerator period (from estimated time of death to refrigeration of body) and of disease-related factors (age of onset, time in the hospital) showed no statistically significant correlation neither with NOX2 expression in PFC and cingulate cortex (Supplementary Figure 1) nor with NOX4 expression in PFC (Supplementary Figure 2). The expression of NOX2 significantly correlated with brain pH (Supplementary Figures 1i and s). It was previously shown that the brain pH in bipolar patients from the Stanley Array Collection was lower than in the control group.[24] This observation can have multiple interpretations including post-mortem sample preservation or changes of brain pH inherent to disease. In our data set, the brain pH was also lower in bipolar patient as well as in SZ patients as compared with the controls (data not shown). It has been suggested that decreased pH is associated with lower RNA quality.[24] However, it is unlikely that the decrease in NOX2 expression in bipolar patients is due to the lower pH for the following reasons: (i) the expression of NOX4 was not affected (Supplementary Figures 2i and j); (ii) no association between NOX2 and post-mortem interval (Supplementary Figures 1e and m) nor with refrigerator interval (Supplementary Figures 1f and n) were found; (iii) NOX2 mRNA levels were not decreased in SZ patients and (iv) NOX2 levels were significantly lower even after elimination from the analysis of samples with pH lower than 6.4 in PFC (Supplementary Figures 1j and t). Furthermore, no statistical impact of VPA prescription or substance abuse on pH was identified (data not shown).

Out of the social factors (smoking, alcohol consumption and substance abuse) and of the disease-related factors (psychotic features, suicide status and duration of illness) only substance abuse was significantly associated with decreased expression of NOX2 both in PFC (Figure 2b: no/low users; $n = 58$; median = 0.739; IQR = 0.5534 vs users; $n = 32$; median = 04372; IQR = 0.3971) and in cingulate cortex (Figure 2h: no/low users; $n = 58$; median = 0.3429; IQR = 0.2239 vs users; $n = 32$; median = 0.2485; IQR = 0.2024).

Localization of NOX2 in microglia and correlation with microglial markers

To address the question of NOX2 localization in the brain of psychiatric patients, we stained paraffin-embedded sections of PFC with a monoclonal anti-human NOX2 antibody.

The Mo48 antibody is currently the only validated antibody specific for human NOX2 in immunohistochemistry applications. Its epitope is known[25] and Mo48 is routinely used (also with the 7D5 monoclonal antibody) to characterize patients with NOX2 loss of function.[26] Immunohistochemical analysis detected NOX2 staining mainly in microglia (Figures 3a–f). We quantified microglia separately from gray and white matter because of intrinsic differences in microglial density in these regions.[27] No difference in object count was detected between different disease groups in gray (Figure 3g) and in white matter (Figure 3h) in spite of a trend for a decrease in the white matter of the PFC.

Recently RNASeq analysis of specific cell populations of the mouse and human CNS (neurons, astrocytes, oligodendrocytes, microglia/macrophages and endothelial cells) has been made publicly available.[20] We therefore analyzed the expression of NOX2 (aka *CYBB*) in these samples (Figure 3i). By far, the largest expression of NOX2 was found in microglia/macrophages. There was also some degree of NOX2 expression in the oligodendrocyte fraction; however, according to the authors of the publication, there was an ~5% contamination of microglia/macrophages population within the oligodendrocyte fraction. Finally, the amount of NOX2 in astrocytes, endothelial cells and neurons

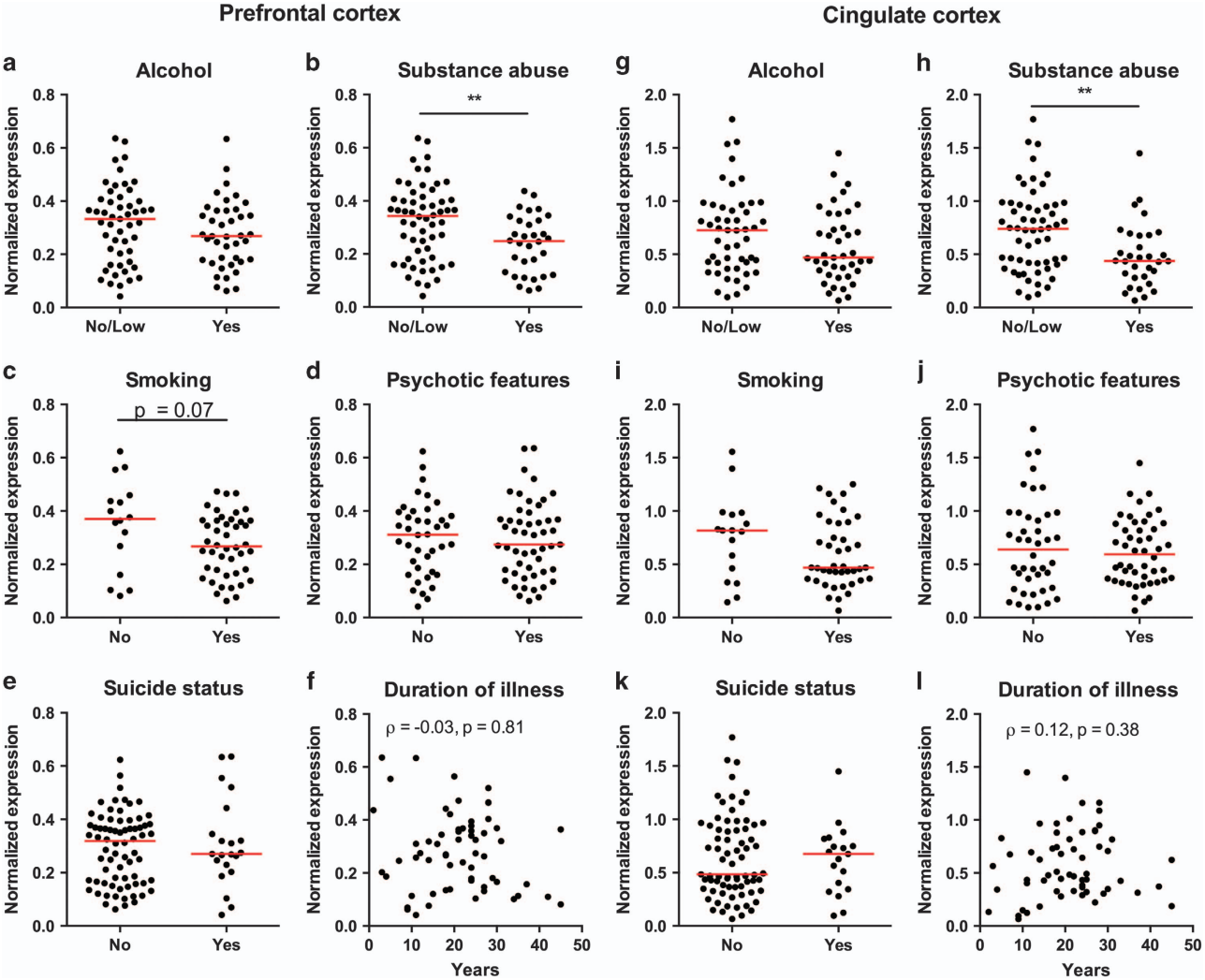

Figure 2. Association of NOX2 expression with social and disease-relevant factors. NOX2 mRNA expression in prefrontal cortex (PFC) and in cingulate cortex was not significantly associated with alcohol consumption (**a**, **g**) and smoking (**c**, **i**). NOX2 expression was significantly lower in the patients with substance abuse (**b**, **h**). No association with the disease-relevant factors such as psychotic features (**d**, **j**), suicide status (**e**, **k**) and duration of illness (**f**, **l**) were found. $^{**}P < 0.01$, Mann–Whitney *U* test.

was negligible. This corroborates our immunohistochemical data suggesting that the large majority of NOX2 in the studied human brain samples are localized to microglia/macrophages.

If NOX2 is indeed located in microglia, one would predict a correlation between the expression of NOX2 and microglia markers. We therefore performed additional qPCR with mRNA samples from the cingulate cortex to evaluate the expression of microglial markers. We found a significant correlation between the expression of NOX2 and microglial markers: CD11b, Spearman $\rho = 0.87$, $P < 0.0001$ (Figure 3j); CD68, Spearman $\rho = 0.85$, $P < 0.0001$ (Supplementary Figure 3a); Iba1, Spearman $\rho = 0.34$, $P < 0.0008$ (Supplementary Figure 3b). The analysis of microglial markers in patient groups showed that the expression of CD11b (Figure 3k) and CD68 (Figure 3l) was significantly decreased in BD group as compared with control. There were no significant differences for IBA-1 (Figure 3m). This is in line with the weaker correlation between NOX2 and Iba1 mRNA expression ($\rho = 0.34$; see above and Supplementary Figure 3b). This difference might be due to different activation stages of microglia in these samples and thereby may illustrate the relative specificity of microglial markers in identifying surveillant versus activated microglia.

Association of NOX expression with prescribed medications and substance abuse

Almost all patients in our study were prescribed multiple medications including antipsychotics, mood stabilizers and antidepressants. Most patients, have a history of smoking, alcohol consumption and substance abuse, in particular, those with BDs. These substances might impact microglia homeostasis.[28] By investigating the association of NOX expression levels with the documented prescribed medications, we found that the decrease of NOX2 levels in PFC and in cingulate cortex of BD patients was significantly associated with the prescription of VPA (Figures 4a and c). Such association was not found for NOX4 (PFC, Figure 4b). There was also a decrease in microglia markers associated with VPA prescription (CD11b (Figure 4d), CD68 (Figure 4e), but not with Iba1 (Figure 4g). The prescription of another frequently used mood stabilizer, lithium, was not associated with the decrease in NOX2 levels nor with microglial markers. Note that no information is available regarding the fact that patients would actually use VPA. No association between VPA prescription and a particular BD subtype was observed (data not shown).

As VPA is often prescribed to both BD and SZ patients, we compared these two patient groups. NOX2 expression was lower in BD patients receiving VPA than in schizophrenic patients to

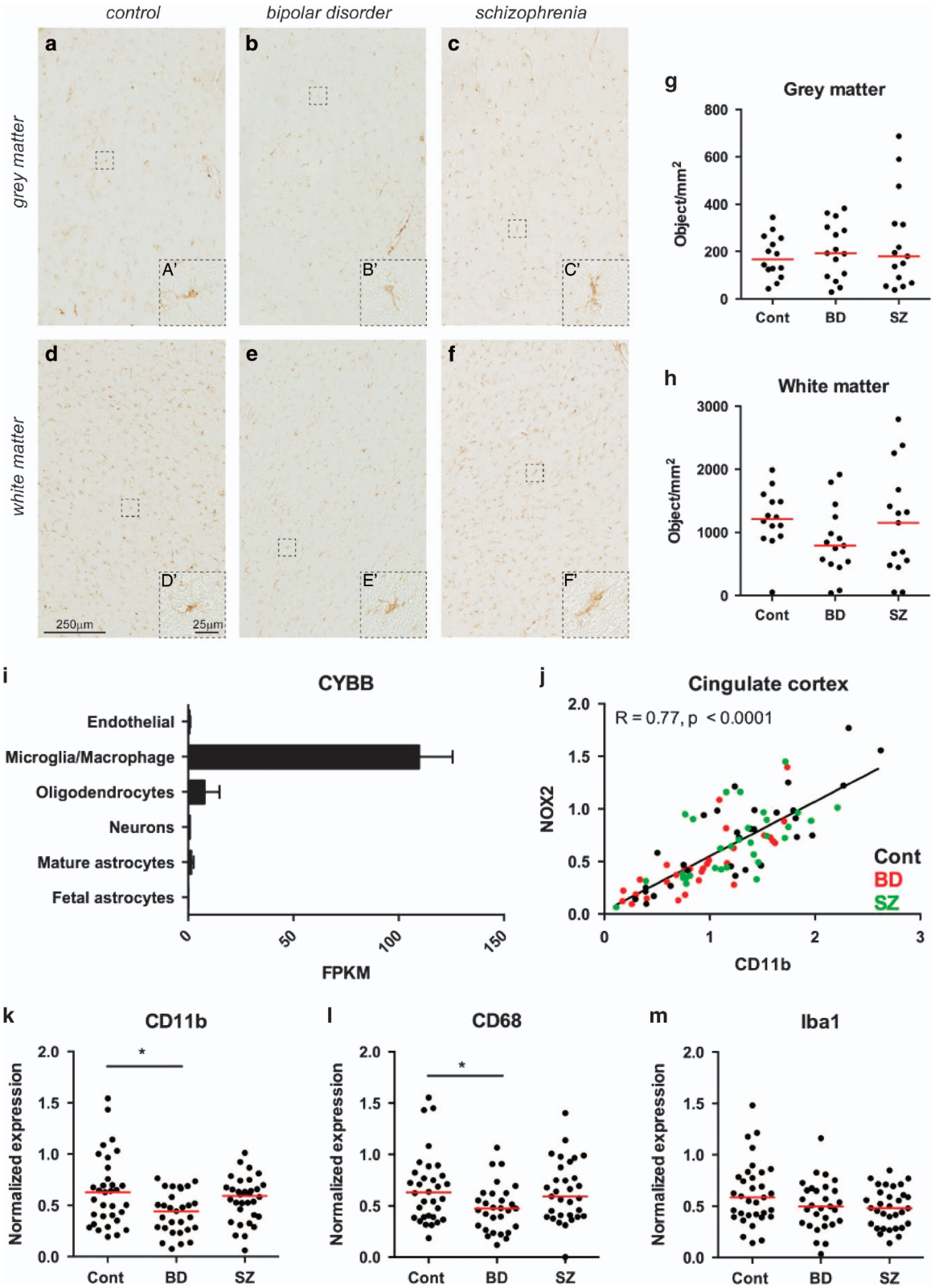

Figure 3. NOX2 localization in microglia and correlation with microglial markers. (**a**) Representative images of NOX2 staining in the indicated patient groups are shown for gray matter (**a**–**c**) and for white matter (**d**–**f**). Inset depicts typical microglial morphology in these samples. No significant differences in the number of objects were detected in gray matter (**g**) nor in white matter (**h**). (**i**) RNAseq data showing NOX2 expression in central nervous system (CNS) cell populations in healthy human brain.[20] (**j**) Significant correlation with CD11b expression was found for NOX2 in cingulate cortex. Spearman ρ and *P*-values are shown on the graph. The expression of microglial markers CD11b (**k**), CD68 (**l**) but not Iba1 (**m**) is decreased in the cingulate cortex of BD patients. *ANOVA Kruskal–Wallis test followed by Dunn's multiple comparison test.

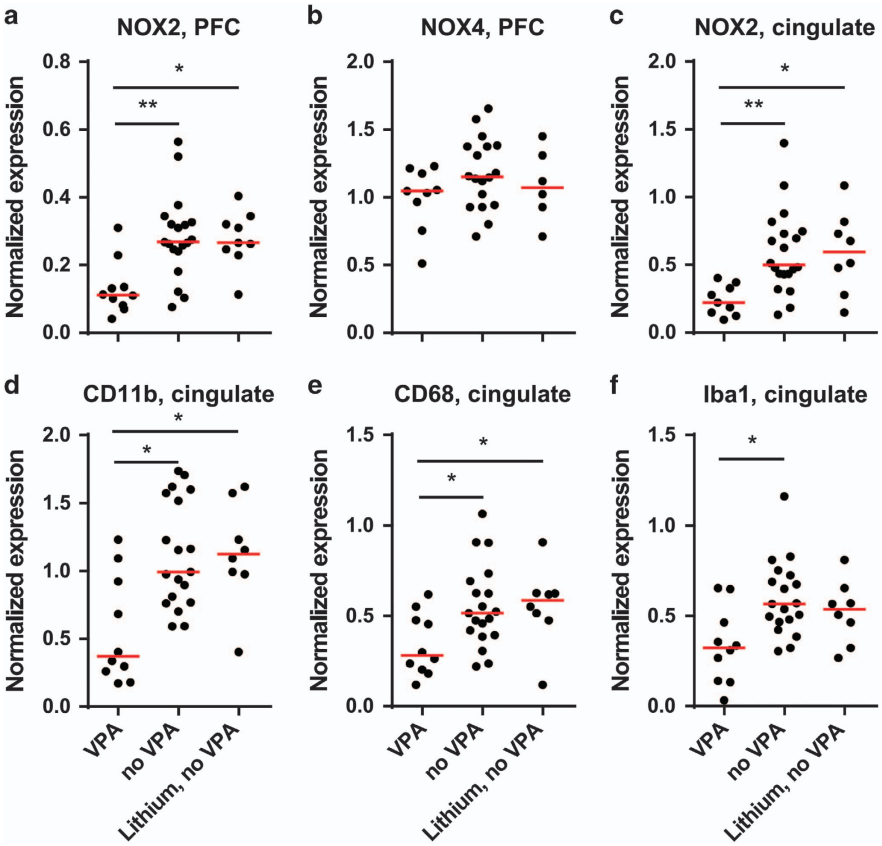

Figure 4. Association between mood stabilizers and NOX2 expression in patients with BD. Valproic acid (VPA) but not lithium prescription is associated with decreased levels of NOX2 (**a**) but not NOX4 (**b**) in prefrontal cortex (PFC); and with decreased levels of NOX2 (**c**) and microglial markers CD11b (**d**), CD86 (**e**) and Iba1 (**f**) in cingulate cortex. The axis legend applies for the graphs of both rows. *, **ANOVA Kruskal–Wallis test followed by Dunn's multiple comparison test.

whom the drug has been prescribed (Figures 5a and c). There was no statistically significant difference between schizophrenic patients receiving or not receiving VPA (albeit there was a trend). These data were corroborated by expression levels of the activated microglia marker CD11b (Figure 5d). In contrast, VPA has no impact on NOX4 levels in our analysis (Figure 5b). We also performed a similar analysis among subpopulations of patients with documented substance abuse. NOX2 expression was significantly decreased only between BD patients with substance use and abstinent SZ in PFC and cingulate cortex, although there was a trend for a decrease of NOX2 and CD11b in BD users. No difference was detected for NOX4. The groups were probably too small to generate more significant data. In addition, the substance use is a vague terminology and most likely represents very diverse drug-related behaviors and various types of recreational drug with fundamentally different mode of action.

DISCUSSION

In this study we demonstrate—as opposed to what had been predicted based on animal experimentation—that there is no increase of NOX2 expression in schizophrenic patients. Unexpectedly, however, we found that NOX2 expression was decreased in patients with BD. This decrease was essentially seen in patients that were either prescribed VPA or in patients with a history of substance abuse. NOX2 expression was mostly found in microglia suggesting a decreased activity of brain inflammatory cells in a subset of patients with BD.

We evaluated the expression of the ROS-generating NADPH oxidases NOX1-5 in human brain samples from the Stanley Collection (schizophrenic, BDs and control). The overall pattern of expression was as follows: we found high mRNA expression levels of the NOX2 and NOX4, whereas NOX1 and NOX5 were at the limit of detection and NOX3 below detection levels. Although the expression of NOX2 in the human brain has been convincingly documented before, the question of which cell type within the CNS expresses NOX2 is still a matter of debate. Expression in microglia has been previously shown,[19,21] but NOX2 expression has been suggested in mature neurons,[29] as well as adult neural stem cells.[30] The interpretation of these—at least in part contradictory—results is complex: (i) in many instances, antibodies with a poorly documented specificity have been used (discussed in ref. 14); (ii) the cell type that expresses NOX2 might depend on the brain region and developmental state; and (iii) disease-specific expression of NOX2 might also exist. Our findings in prefrontal and cingulate cortices of psychiatric patients and control individuals argue in favor of a specific expression of NOX2 in microglia: (i) brain immunostaining with the specific and fully validated NOX2 antibody mo48[ref. 18] detected cells with an obvious microglial morphology, and (ii) NOX2 expression correlated with macrophage/microglial markers CD11b, CD68 and Iba1. Along these lines, data extracted from an RNAseq study[31] indicates strong microglial enrichment of NOX2 and its subunits $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$, whereas NOX2 was virtually absent in neurons. Data from mouse experiments suggest that NOX2 is also expressed in adult neural stem cells of the subventricular zone and the dentate gyrus.[30,32] However, this has not been investigated in humans, and, in addition, neural stem cells are most likely absent in the regions (cingulate and prefrontal cortices) investigated in our study. We did not address the cellular

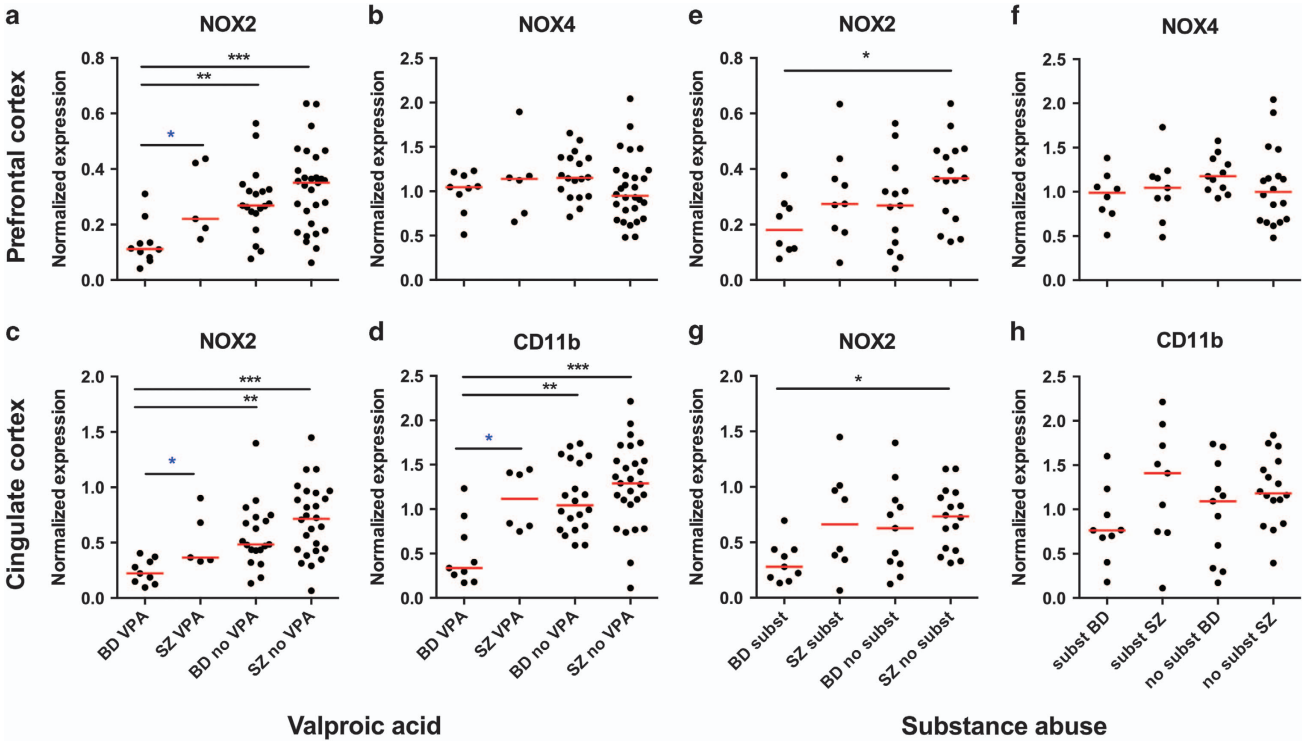

Figure 5. Comparison of valproic acid (VPA) prescription and substance abuse in bipolar disorder (BD) and schizophrenia (SZ) patients. NOX2 expression is lower in BD patients prescribed with VPA compared with other BD patients and SZ patients with or without VPA prescription in both prefrontal cortex (PFC) (**a**) and cingulate cortex (**c**). NOX4 is not changed (**b**) and CD11b shows the same pattern as NOX2 in cingulate cortex (**d**). Similar analysis for substance abuse shows no difference in NOX2 and NOX4 expression in PFC (**e**, **f**) and only a difference in NOX2 expression in the cingulate cortex for BP patients with substance abuse compared with abstinent SZ patients (**g**). No differences in CD11b expression was detected between subgroups. The axis legend applies for the graphs of both rows. *, **ANOVA Kruskal–Wallis test followed by Dunn's multiple comparison test.

CNS localization of NOX4 by immunohistochemistry, but recent RNAseq data indicate a strong NOX4 enrichment in human brain endothelial cells,[33] whereas microarray data indicate NOX4 expression in human pericytes.[34]

A major goal of our study was to understand whether NOX2 expression was altered in psychiatric disease. SZ and BD are distinct and yet overlapping clinical entities. The etiologies of the two psychiatric diseases remain poorly understood. Several lines of evidence argue for a role of inflammation[35] and oxidative dysregulation.[36] Oxidative modifications in the brain of schizophrenic patients have been widely reported[10] making NOX2 an attractive candidate to be involved in the disease process. On the basis of animal models of psychosis,[37] a role of increased NOX2 activity and expression in the pathophysiology of schizophrenia has been suggested. Other NOX isoforms have hardly been studied in this context. To our knowledge, our study represents the first systematic analysis of NOX enzymes in samples from patients with psychiatric disease. The NOX1, NOX3 and NOX5 isoforms showed very low expression levels, and for this reason we did not perform in-depth analysis. NOX4 expression levels were high, but we did not observe any changes between patient groups, socio-demographic factors, and technical parameters. However, marked variations in NOX2 expression levels were detected. Our initial working hypothesis, that there might be an increased level of NOX2 in schizophrenic patients was not confirmed. Indeed, NOX2 levels were virtually identical in schizophrenic patients and in control. Unexpectedly, however, we found that BD patients—as compared to controls and schizophrenic patients—had significantly lower levels of NOX2 in both brain regions tested. The most widely used microglia marker Iba1, is not changed in BD patients. However, the activation-dependent microglia markers CD11b and CD68 follow a similar pattern as NOX2, suggesting that microglia activation rather than microglia number is affected. This robust decrease of NOX2 and other microglial activation markers in BD post-mortem material is a novel and unexpected finding. Indeed, most publications support a role of exaggerated microglia activity and increased pro-inflammatory cytokines in psychiatric diseases. Our study challenges this theory. Importantly, our results indicate that there are differences in microglial response in BD and SZ.

Our results raise the possibility that decreased NOX2 expression is linked to BD. At this point, however, it is difficult to distinguish whether this is an intrinsic feature of the disease or whether this is due to external factors. At the first glance, our data appears to favor the latter explanation: decreased NOX2 expression is limited to a subgroup of BD patients, namely patients that have been prescribed VPA, and patients with a history of heavy substance abuse. Thus, one working hypothesis might be that VPA and recreational drugs suppress NOX2 expression. VPA is drug with a complex mode of actions, including inhibitions of histone deacetylases.[38] It is used since the 1960s for the treatment of several CNS disorders, including epilepsy and BDs. High concentrations of VPA have been shown to be inhibitory to human microglia[39] or even toxic to mouse microglia[40] and/or rat peripheral leukocytes.[41] Thus, the decreased NOX2 expression and microglial activation could be due to VPA treatment. Although this possibility is real, we are not aware of a common biochemical pathway that links the use of VPA and recreational drugs. No information on the compliance towards VPA and the type of substances abused by the patients is available for the Stanley Collection. Also, a decrease in NOX2 is observed only in BD, but not in SZ patients with VPA prescription. For this reason, an alternative explanation should also be considered. It is conceivable that patients that are prescribed VPA and patients prone to

substance abuse belong to a pathophysiologically distinct subgroup of BD patients. Indeed, VPA is often prescribed to BD patients that are not responsive or intolerant to lithium.[42] Thus, a more generalized microglia pathology might contribute to the pathophysiology of this subgroup of BD patients. Primary alterations in microglia function have been implicated in autism[43] and obsessive compulsive behavior.[44] Assuming that decreased microglia activation in BD is not a drug-induced epiphenomenon, how could it contribute to disease progression? Microglia has a key role in brain homeostasis by regulating neurogenesis both during development and in adults by removing superfluous synapses (synaptic pruning).[45,46] Thus, the decreased microglia activity in BD patients might alter synaptic pruning. Alternatively, one might consider a paracrine pathway where NOX2-derived ROS regulate redox-sensitive transcription factors and signaling in neural cells.

Altogether, our results demonstrate low microglial NOX2 in a subgroup of patients with BD, whereas NOX2 expression and microglial activation were unchanged in schizophrenia. The decrease in NOX2 expression might either be secondary to VPA and recreational drugs, or represent a pathophysiologically relevant feature of a subgroup of BD patients. In the future, it will be important to develop techniques of *in vivo* redox imaging in the human brain, which will allow distinguishing between these possibilities.

CONFLICT OF INTEREST

VJ and KHK hold shares in Genkyotex SA, a company developing NOX inhibitors. The remaining authors declare no conflict of interests.

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Supplementary Information accompanies the paper on the *Translational Psychiatry* website (http://www.nature.com/tp)