Altered Expression of Regulators of the Cortical Chloride Transporters NKCC1 and KCC2 in Schizophrenia

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Context: Disturbances in markers of cortical γ-aminobutyric acid neurotransmission are a common finding in schizophrenia. The nature of γ-aminobutyric acid neurotransmission (hyperpolarizing or depolarizing) depends on the local intracellular chloride concentration. In the central nervous system, the intracellular chloride level is determined by the activity of 2 cation-chloride transporters, NKCC1 and KCC2. The activities of these transporters are in turn regulated by a network of serine-threonine kinases that includes OXSR1, STK39, and the WNK kinases WNK1, WNK3, and WNK4.

Objective: To compare the levels of NKCC1, KCC2, OXSR1, STK39, WNK1, WNK3, and WNK4 transcripts in prefrontal cortex area 9 between subjects with schizophrenia and healthy comparison subjects.

Design: Real-time quantitative polymerase chain reaction technique was used to measure transcript levels in the prefrontal cortex.

Setting: Human brain specimens were obtained from autopsies conducted at the Allegheny County Medical Examiner’s Office, Pittsburgh, Pennsylvania.

Participants: Postmortem brain specimens from 42 subjects with schizophrenia and 42 matched healthy comparison subjects. Brain specimens from 18 macaque monkeys exposed to haloperidol, olanzapine, or sham long-term.

Main Outcome Measures: Relative expression levels for NKCC1, KCC2, OXSR1, STK39, WNK1, WNK3, and WNK4 transcripts compared with the mean expression level of 3 housekeeping transcripts.

Results: OXSR1 and WNK3 transcripts were substantially overexpressed in subjects with schizophrenia relative to comparison subjects. In contrast, NKCC1, KCC2, STK39, WNK1, and WNK4 transcript levels did not differ between subject groups. OXSR1 and WNK3 transcript expression levels were not changed in antipsychotic-exposed monkeys and were not affected by potential confounding factors in the subjects with schizophrenia.

Conclusion: In schizophrenia, increased expression levels, and possibly increased kinase activities, of OXSR1 and WNK3 may shift the balance of chloride transport by NKCC1 and KCC2 and alter the nature of γ-aminobutyric acid neurotransmission in the prefrontal cortex.


A core feature of schizophrenia is the impairment of certain cognitive functions, such as working memory, that are dependent on the circuitry of the dorsolateral prefrontal cortex (DLPFC). These cognitive deficits in schizophrenia are associated with altered neural network synchrony, that may be attributable, at least in part, to functional abnormalities in γ-aminobutyric acid (GABA) neurotransmission in the DLPFC.

Disturbed GABA neurotransmission in schizophrenia might arise from alterations in 1 or more of the following factors: (1) the strength of GABA neurotransmission, which depends on the amount of GABA available at the synapse; (2) the kinetics of GABA neurotransmission, which is determined, in part, by the subunit composition of GABA_{A} receptors; and (3) the nature of GABA neurotransmission, which can be hyperpolarizing, depolarizing, or shunting depending on the flow of chloride ions when GABA_{A} receptors are activated. Presynaptic and postsynaptic markers of the strength and kinetics of GABA neurotransmission are known to be altered in the DLPFC in subjects with schizophrenia, but measures of the nature of GABA neurotransmission have not been evaluated.

The flow of chloride ions through GABA_{A} receptors depends on intracellular...
lular levels of chloride, which are regulated by the relative activities of the sodium-potassium-chloride cotransporter 1 (NKCC1; SLC12A2), which mediates chloride uptake, and the potassium-chloride cotransporter 2 (KCC2; SLC12A5), which mediates chloride extrusion from the cell. In the central nervous system, NKCC1 is found in both neurons and glial cells, but KCC2 is strictly neuronal.9-11 The activities of both transporters are sensitive to intracellular chloride levels, and their activation depends on their phosphorylation (NKCC1) or dephosphorylation (KCC2) status.12-15 Oxidative stress response kinase (OXSR1; also known as OSR1) and Ste 20–related, proline-alanine–rich kinase (STK39; also known as SPAK) are highly expressed in the brain16-19 and bind to NKCC1 where they phosphorylate residues present in the N-terminal domain, resulting in an increase of NKCC1 activity.17,20–23 In addition, NKCC1 can be phosphorylated by the coexpression of STK39 and WNK4 (with no K [lysine] protein kinase 4) kinases in vitro.24 Conversely, STK39-mediated phosphorylation has a dominant negative effect on KCC2 function.25 Another WNK kinase, WNK1, activates both STK39 and OXSR1 via phosphorylation.25,26-27 Finally, a fifth kinase, WNK3, both activates NKCC1 and inhibits KCC2, regardless of cellular toxicity.28,29 Consequently, alterations in the relative expression levels of NKCC1 and KCC2, or of their regulatory kinases, in the DLPFC of subjects with schizophrenia could, by shifting intracellular chloride levels, alter the nature of GABA transmission and thereby contribute to impaired neural network synchrony and cognitive dysfunction in affected individuals.

To examine this possibility, we quantified messenger RNA (mRNA) expression levels of NKCC1 and KCC2 and their associated regulatory kinases STK39, OXSR1, WNK1, WNK3, and WNK4 in the DLPFC from subjects with schizophrenia and matched healthy comparison subjects. In subjects with schizophrenia, OXSR1 and WNK3 were always processed together. The subject groups (Table) did not significantly differ in mean age, PMI, brain pH, RNA integrity number (RIN), or tissue storage time at −80°C (all t < 1.67; all F > .10) or in race (χ² = 1.587; P = .21). All procedures were approved by the University of Pittsburgh’s Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

### TISSUE PREPARATION

The right hemisphere of each brain was blocked coronally, immediately frozen, and stored at −80°C, as described previously.31 Area 9 of the DLPFC was identified cytoarchitectonically from Nissl-stained coronal sections spanning the rostrocaudal axis of the superior frontal sulcus. The cortical gray matter was dissected from cryostat sections (40 µm) in a manner that ensured limited white matter contamination and excellent RNA preservation, as described previously.32 Total RNA was isolated from tissue homogenates using the TRizol protocol from Invitrogen (Invitrogen Corporation, Carlsbad, California) and further purified using the RNeasy kit (QIAGEN, Valencia, California). RNA integrity was assessed by measuring the RIN using the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, California).

### QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was converted to complementary DNA using the High-Capacity cDNA Archive Kit from Applied Biosystems (Foster City, California). For each reaction, we used 50 ng of total RNA from each subject. Priming was performed with random hexamers, according to the manufacturer’s recommendations. The efficiency for each primer set (eTable 2) was between 92% and 100%, and the amplified product resulted in a specific single product in dissociation curve analysis. Given the high level of homology between certain domains of OXSR1 and STK39, the quantitative real-time polymerase chain reaction (qPCR) primer sets for these 2 transcripts were designed within a unique sequence in the 3’ untranslated region of each mRNA.

Samples from each matched pair of subjects with schizophrenia and comparison subjects were always assayed on the
same plate. For each sample, amplified product differences for each transcript were measured with 4 replicates using SYBR Green chemistry-based detection.31–33

β-Actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase were used as endogenous reference genes. These 3 transcripts were selected based on their previously demonstrated stable expression across both subjects with schizophrenia and healthy comparison subjects.32–33 The qPCR reactions were carried out in an ABI Prism 7000 thermal cycler (Applied Biosystems) using the ABI Prism 7000 SDS software with the automatic baseline and threshold detection options selected. These data were exported to Microsoft Excel (Microsoft, Redmond, Washington) and delta cycle thresholds (dCTs) were calculated for each sample by using the geometric mean of the 3 endogenous reference genes as the normalization factor (ie, cycle threshold [CT] for each transcript in a sample minus the geometric mean of β-actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase CTs for the same sample).34 The expression level for each transcript was then calculated as the expression ratio value (where expression ratio = 2^(-ΔΔCT)), and all results are reported as the expression ratio.

Two isoforms of the WNK3 gene are expressed in the brain: isoform 1, which is brain specific, and isoform 2, which is ubiquitoutous. Isoform 1 differs from isoform 2 only by the presence of an additional stretch of 47 amino acids in the protein.35 Given the differential expression pattern of both isoforms, we designed 1 primer set detecting WNK3 isoform 1 (primer set WNK3-1) and 1 primer set detecting both isoforms 1 and 2 (primer set WNK3-1&2). WNK3-1 was used on the 42 pairs of subjects, and WNK3-1&2 was used only on an arbitrary subset of 12 pairs of subjects with schizophrenia and healthy comparison subjects (eTable 1A, pairs with * ) that had been used in a previous real-time qPCR study.32

**HIERARCHICAL CLUSTERING**

Two-way cluster analyses were performed using delta-delta CTs (subject with schizophrenia dCT minus matched healthy comparison subject dCT) across all 42 subject pairs for all transcripts using Pearson mean analysis in Genes@Work (IBM, Armonk, New York).36

**ANTIPSYCHOTIC-TREATED MONKEYS**

The effect of long-term exposure to antipsychotic medication on the levels of transcripts showing altered expression in schizophrenia was examined using macaque monkeys exposed to haloperidol, olanzapine, and placebo, as described before37,38 (eAppendix). All housing and experimental procedures were conducted in accordance with National Institutes of Health guidelines and with approval of the University of Pittsburgh Institutional Animal Care and Use Committee.

**IMMUNOCYTOCHEMICAL EXPERIMENTS**

To assess the distribution and relative levels of OXSR1 protein, we conducted immunocytochemical experiments using immersion-fixed cryostat sections (40 µm) of DLPFC area 9 from 6 white male human subjects (17-62 years of age; 3 subjects with a short PMI [5-8.2 hours] and 3 subjects with a long PMI [14.5-37 hours]). Tissue sections were processed for OXSR1 immunoreactivity using a 1:200 dilution of a goat anti-OXSR1 (SC49473; Santa Cruz Biotechnology, Santa Cruz, California) and the Vectastain ABC kit (Vector Laboratories, Burlingame, California), according to a previously described protocol.39 The OXSR1 antibody was raised against the C-terminal portion (amino acids 370-420) of the human OXSR1 protein. Antibi

d specificity was demonstrated by the absence of labeling in the thymus40 and by the absence of specific labeling when the primary antibody was omitted.

**WESTERN BLOTTING AND SDS GEL ELECTROPHORESIS**

Western blots were conducted using brain tissue lysate prepared as previously described41 (eAppendix). The specificity of the anti-OXSR1 antibody was confirmed by detection of a band of identical molecular weight (58 kDa) compared with cloned human OXSR1 expressed in vitro and by lack of cross-reactivity with a cell lysate of in vitro–expressed STK39 (OriGene, Rockville, Maryland) (data not shown).

To assess the effect of PMI on OXSR1 immunoreactivity, 4 adjacent coronal tissue blocks (2-3 mm thick) were prepared from the DLPFC of the same adult macaque monkey, stored in room-temperature artificial cerebrospinal fluid for 0, 6, 12, or 24 hours, and then flash frozen.42 The OXSR1 protein content was reported as percentage relative to the 0-hour PMI.

**LASER MICROROBODISSECTION ANALYSES**

To assess the laminar and cellular patterns of the expression differences in OXSR1 and WNK3-1&2, we used laser microdissection techniques to obtain mRNA samples from layer 3 tissue and from individually dissected layer 3 pyramidal cells from a subset of 10 subject pairs (eTable 1A and B, pairs with *). Using the results from qPCR experiments conducted on the entire area 9 gray matter for all 42 subject pairs, we selected the 10 pairs with mean expression differences between subjects with schizophrenia and control subjects that were closest to the mean of the entire group.

Cryostat sections (12 µm) were cut and thaw-mounted onto glass polyethylene naphthalate membrane slides (Leica Microsystems, Bannockburn, Illinois) that had been previously UV-treated at 254 nm for 30 minutes, dried, and stored at -80°C. On the day of the microdissection, slides were immersed in an ethanol–acetic acid fixation solution, stained with thionin, dehydrated through 100% ethanol, and air dried. Using a Leica microdissection system (LMD 6500), 2 independent samples of layer 3 pyramidal neurons (eFigure 3A) per subject from 2 different slides (×40 objective; power, 15; aperture, 9; speed, 12; balance, 14; and offset, 120) or 3 strips of tissue from layer 3, as previously described43 (×5 objective; power, 43; aperture, 18; speed, 13; balance, 25; and offset, 60), were obtained. An average of 50 single pyramidal cells or 4.1 mm² of layer 3 tissue were collected per sample in 0.5-ml microtube caps (Ambion/Applied Biosystems) and lysed by vortexing for 30 seconds in 200 µL of RLT Buffer Plus (QIAGEN). The RNA was extracted and PCR analyses were conducted, and cell type specificity and absence of glial contamination of the single pyramidal cell samples were confirmed, as described in the eAppendix. We elected to measure both isoforms of WNK3 using the WNK3-1&2 primer set because of the lower expression of WNK3-1, although the expression difference between schizophrenia and comparison samples using WNK3-1&2 was only half of the expression difference obtained using WNK3-1.

**STATISTICAL ANALYSES**

The effect of diagnosis and the influence of potential confounding variables on the transcript expression ratios were assessed using 2 analyses of covariance (ANCOVA) models. In the first model, transcript expression ratio was used as the dependent variable; diagnostic group, as the main effect; and subject pair, as a blocking factor. The RIN, pH, and freezer

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storage time were entered as covariates because they may affect RNA integrity. Subject pairing may be considered an attempt to control for experimental variance by the parallel processing of tissue samples from each subject pair and not a true statistical pair design. Therefore, to validate the first model, a second unpaired ANCOVA model was performed, using diagnostic group as the main effect and with age, sex, PMI, RIN, pH, and freezer storage time as covariates. Because the 42 pairs of subjects were used in 2 successive qPCR runs of 24 and 18 pair cohorts (eTable 1A) and 18 (eTable 1B) that involved different lots of reagents, qPCR run was also entered as a covariate in both models. Tissue storage time did not have a significant effect in either model for any transcript and was excluded in the reported analyses. The reported P values for each model were corrected for multiple comparisons (n=21) using the Bonferroni procedure.

The influences of potential confounding variables on the expression ratio values in subjects with schizophrenia were assessed with ANCOVA models using each confounding variable as the main effect and sex, age, PMI, pH, RIN, and run as covariates. Pearson correlation was used to assess the relationships of the expression ratios for all pairs of transcripts; reported P values were also corrected for multiple comparisons (n=21) using the Bonferroni procedure.

A 1-way analysis of variance model with expression ratios as the dependent variable and treatment group as the main effect was used to compare transcript expression levels in the haloperidol-, olanzapine-, and placebo-exposed monkeys.

**RESULTS**

**TRANSCRIPT LEVELS FOR CHLORIDE TRANSPORTERS AND RELATED TRANSCRIPTS**

After corrections for multiple comparisons, neither the mean level of NKCC1 mRNA (Figure 1A) nor KCC2 mRNA (Figure 1B) was significantly altered in subjects with schizophrenia (paired ANCOVA, both F1,38<3.8 and P>.43; unpaired ANCOVA, both F1,73<4.1 and P>.33). For the chloride transporter–related kinases, group differences in gene expression levels were not significant for STK39, WNK1, or WNK4 (paired, all F1,38<5.1 and all P>.20; unpaired, all F1,73<4.11 and all P>.32). In contrast, the mean expression level of OXSR1 mRNA (Figure 1C) was significantly (paired,
Because of the low expression level of the brain-specific WNK3-1 mRNA (primer set WNK3-1) in the DLPFC, we designed a new set of primers binding to both isoforms 1 and 2 of WNK3 in the human brain (primer set WNK3-1&2). This primer set produced lower CT values than the WNK3-1 primer set in the same subjects (n=12 pairs), reflecting the higher level of expression for both WNK3 isoforms combined than for isoform 1 alone. Consistent with the findings for isoform 1 alone, the combined expression of both WNK3 isoforms was also higher (+27.0%; paired, F1,8=3.97 and P=.08; unpaired, F1,10=7.1 and P=.01) in the subjects with schizophrenia, and the expression levels from both primer sets were highly correlated (r=0.561; P<.01) across all subjects.

ANALYSIS OF POTENTIAL ANTIPSYCHOTIC MEDICATION EFFECTS

The expression ratios for OXSR1, WNK3-1, and WNK3-1&2 did not differ (all F1,13<0.92 and all P>.42) across the monkeys with long-term exposure to placebo, haloperidol, or olanzapine (Figure 2).

ANALYSIS OF POTENTIAL CONFOUNDING FACTORS

The mean expression ratio in the subjects with schizophrenia did not differ for OXSR1 (Figure 3A) (all F<1.941 and all P≥.17, uncorrected) or WNK3-1 (Figure 3B) (all F≤3.99 and all P>.05, uncorrected) as a function of sex; diagnosis of schizoaffective disorder; suicide; antidepressant medication, benzodiazepines or sodium valproate, or antipsychotic medication use at the time of death; or diagnosis of substance abuse/dependence at time of death.

HIERARCHICAL CLUSTERING AND CORRELATIONS

A 2-way cluster analysis performed on delta-delta CT values across the 42 pairs of matched subjects for all 7 transcripts with Pearson mean distance analysis resulted in the separation of the transcript expression changes in 2 different clusters (eFigure 1). OXSR1 clustered in a pathway with WNK1, WNK4, NKCC1, and STK39, whereas WNK3-1 and KCC2 clustered in a distinct pathway.

Pearson correlations were performed using the dCT values obtained for each transcript for each of the 84 subjects (Figure 4 and eTable 3); the reported P values were calculated using Bonferroni correction for multiple comparisons. Expression levels of OXSR1 were significantly positively correlated with those for WNK3-1 (r=0.744; P<10^-7) and WNK1 (r=0.478; P<10^-4), and WNK3-1 expression was significantly correlated with WNK1 (r=0.346; P=.03). In contrast, WNK3-1 expression was negatively correlated with KCC2 (r=-0.356; P=.02). NKCC1 expression was positively correlated with WNK1 (r=0.583; P<10^-7), WNK4 (r=0.425; P=.001), and STK39 (r=0.371; P=.01).

IMMUNOLOCALIZATION OF OXSR1

In human DLPFC from subjects with PMIs less than 6 hours, immunoreactivity for OXSR1 was highest in layers 3 and 5, intermediate in layers 2 and 6, and low in layers 1 and 4 (Figure 5A and B). OXSR1 immunoreactivity was mainly located in the perinuclear cytoplasmic compartment of neurons, most of which had the morphology of pyramidal cells. In contrast, OXSR1 labeling was severely reduced with PMIs longer than 12 hours (Figure 5C). This finding was confirmed by Western blotting in samples of monkey DLPFC with artificially induced PMIs. At PMIs of 12 and 24 hours, levels of OXSR1 protein were only 60% and 40%, respectively, of that detected at 0 hours (eFigure 2). Thus, a quantitative study of OXSR1 protein levels in subjects with schizophrenia and control subjects could not be conducted.

LAMINAR AND CELLULAR ASSESSMENTS OF OXSR1 AND WNK3-1&2 mRNA EXPRESSION

To determine if the greater expression of OXSR1 and WNK3-1&2 transcripts in the subjects with schizophrenia arose from the same cortical layer and neurons, we used laser microdissection to obtain samples of tissue re-
stricted to layer 3 and samples of 50 pyramidal cells individually cut from layer 3. In the layer 3 tissue samples, the expression levels of OXSR1 and WNK3-1&2 mRNA were significantly correlated ($r=0.88; P<.001$) across all 20 subjects, and the within-pair expression differences for OXSR1 and WNK3-1&2 mRNA levels were also significantly correlated ($r=0.92; P<.001$). Consistent with these findings, in the samples of layer 3 pyramidal cells, the expression levels of OXSR1 and WNK3-1&2 mRNA were significantly correlated ($r=0.56; P=.001$) across all 20 subjects, confirming that both transcripts are expressed in layer 3 pyramidal cells and suggesting that their expression is coregulated. In addition, both transcripts showed greater expression in the subjects with schizophrenia (mean percentage increases: OXSR1, +12.0%; WNK3-1&2, +12.4%), although because of the smaller

Figure 3. Mean (bar) and individual (circle) expression ratio (ER) values for OXSR1 (A) and WNK3 isoform 1 (WNK3-1) (B) are shown for the subjects with schizophrenia grouped by potential confounding factors. Neither sex, diagnosis of schizoaffective disorder, suicide, antidepressant medication use at the time of death, use of benzodiazepines or sodium valproate at the time of death, antipsychotic medication use at the time of death, nor diagnosis of substance abuse or dependence at the time of death significantly affected OXSR1 (A) or WNK3-1 (B) transcript expression. Numbers in bars indicate the number of subjects with schizophrenia in each category.
sample size (n=10 pairs) these findings did not achieve statistical significance (t=−1.007; P=.15 and t=−0.682; P=.25, respectively). Together, these findings suggest an upregulation of both transcripts in layer 3 pyramidal cells in the illness.

**COMMENT**

In the DLPFC of subjects with schizophrenia, mRNA expression levels for the chloride transporters NKCC1 and KCC2 were not significantly altered. Although it is possible that our measures of mRNA in total gray matter obscured schizophrenia-associated expression differences in neuronal subpopulation(s) in a different layer, we previously found that neither NKCC1 nor KCC2 mRNA showed a preferential laminar pattern of expression in control human subjects. In contrast, the mRNA expression levels of 2 regulatory kinases, OXSR1 and WNK3, were markedly and consistently higher in the DLPFC of subjects with schizophrenia. Although an increase in protein kinase activity may not always follow an increase in the corresponding mRNA transcript, previous reports have shown that increasing WNK3 mRNA levels or silencing OXSR1 expression is associated with alterations in NKCC1 transport activity. Thus, our results suggest that the increased expression of OXSR1 and WNK3 are likely to lead to shifts in the activity of the chloride transporters that could substantially alter the nature of cortical GABA neurotransmission in schizophrenia. Indeed, the finding that both transcripts are upregulated in the same layer 3 pyramidal neurons suggests that altered GABA signaling may be particularly prominent in these cells. The findings for OXSR1 and WNK3 are striking for several other reasons. First, the higher levels of these transcripts in subjects with schizophrenia contrast with the more commonly observed lower levels of GABA-related transcripts in the illness. Second, the elevated transcript levels in schizophrenia indicate that the findings are not attributable to poorer RNA quality in these subjects, consistent with the excellent brain pH and RIN measures in all subjects used in this study and the absence of group differences in these variables. Third, relative to the matched healthy comparison subjects, almost all of the 42 subjects with schizophrenia had higher levels of both OXSR1 and WNK3 mRNA. The apparent consistency of these findings suggests that they are more likely to be related to a conserved downstream aspect of the disease process (eg, impaired GABA neurotransmission) than to reflect the etiological complexity of schizophrenia. Fourth, consistent with the findings that these transcripts are not altered in monkeys exposed to antipsy-
Chotic medications long-term, the conservation of these alterations across almost all subjects examined strongly suggests that they are not attributable to other factors (e.g., substance use, suicide, mood symptoms) that were present in some of the subjects with schizophrenia, an interpretation confirmed by the direct assessment of these factors (Figure 3).

A conserved OXSR1 binding motif present on NKCC1 has also been detected on WNK1 and WNK4 and the activation of NKCC1 by OXSR1 is regulated through upstream interactions with WNK1 and WNK4. Consistent with these observations, we found strong correlations across all subjects between OXSR1 and WNK1 ($r = 0.478; P < .01$) and between WNK1 and WNK4 ($r = 0.516; P < .001$) mRNA levels. The expression differences between subjects with schizophrenia and control subjects for WNK1 and WNK4 kinases clustered together, consistent with the idea that WNK1 and WNK4 regulate each other's activity. Nevertheless, neither WNK1 nor WNK4 transcript expression level was significantly altered in subjects with schizophrenia, despite suggestions of overexpression (+7%; $P = .36$ and +12.6%; $P = .17$, respectively), indicating that the overexpression of the OXSR1 transcript could be primarily responsible for an increase in NKCC1 transporter activity.

WNK3 is a potent activator of NKCC1 and they are colocalized in neurons. Furthermore, WNK3 possesses an OXSR1/STK39 consensus binding motif, suggesting that an increase in WNK3 expression in schizophrenia could lead to increased NKCC1 transporter activity through OXSR1. Consistent with this hypothesis, we found very strong correlations ($r = 0.744; P < 10^{-7}$) between OXSR1 and WNK3 mRNA levels across all subjects as well as within-pair expression differences in layer 3 samples ($r = 0.92; P < .001$), suggesting that their effects on chloride transporter activity are synergistic.

WNK3 can inhibit KCC2 activity regardless of cellular tonicity, suggesting that the elevated WNK3 ex-
Thus, when GABAA receptors are activated, chloride influx is reduced (or perhaps reversed) and the nature of GABA neurotransmission is altered.

In normal adult neurons, intracellular chloride concentration is low because of low levels of NKCC1 and high levels of KCC2. The binding of GABA to GABA<sub>A</sub> receptors triggers chloride entry (chloride flow is represented by green arrows) and hyperpolarization. In schizophrenia, increased OXSR1 and WNK3-1 kinase levels lead to increased phosphorylation (black P) and consequently increased NKCC1 activity and decreased KCC2 activity, producing a greater intracellular chloride concentration. Thus, when GABA<sub>A</sub> receptors are activated, chloride influx is reduced (or perhaps reversed) and the nature of GABA neurotransmission is altered.

Because kinase-dead WNK3 activates KCC2, KCC2 inhibition triggered by the increase in WNK3 could be indirect, through the potential inhibition of a yet to be identified phosphatase or the activation of another kinase. Coimmunoprecipitation studies have shown that WNK3 can interact with WNK4 via their carboxy termini and it has been suggested that WNK4 could act as a regulator of WNK3 activity and therefore KCC2/NKCC1 activity. In our data set, WNK4 gene expression did display an upregulated trend (+12.6%; P = .17) but its expression level in all 84 subjects was correlated with NKCC1 and not with WNK3. This suggests that NKCC1 and KCC2 regulation by WNK3 may happen through different regulatory pathways. Finally, the strong Pearson correlation between OXSR1 and WNK3 (0.744; P < .01) suggests that the regulation of both NKCC1 and KCC2 activities could be linked through the interaction of these 2 kinases.

**SYNERGISTIC EFFECTS OF ALTERED KINASE LEVELS ON CHLORIDE TRANSPORT IN SCHIZOPHRENIA**

The combined and coordinated greater expression of OXSR1 and WNK3 in schizophrenia would be expected to increase both NKCC1 and KCC2 phosphorylation levels. Because NKCC1 and KCC2 are phosphorylated and dephosphorylated, respectively, our findings suggest that schizophrenia is associated with both increased NKCC1 activity and decreased KCC2 activity. Interestingly, the latter finding may be consistent with preliminary reports of decreased levels of KCC2 mRNA in the hippocampus of patients with schizophrenia. This combined shift in transporter activities would be expected to increase intracellular chloride levels, reducing or reversing the net inward flow of chloride ions when GABA<sub>A</sub> receptors are activated, and thus altering the nature of synaptic GABA neurotransmission.

Early in development, the ratio of NKCC1 to KCC2 is high, resulting in high intracellular chloride content and a depolarizing excitatory response on GABA<sub>A</sub> receptor activation. Later, as the expression of NKCC1 declines and KCC2 increases, the level of intracellular chloride decreases and GABA<sub>A</sub> receptor activation triggers chloride influx, hyperpolarization, and inhibition. Interestingly, an increase in OXSR1 and WNK3 kinase activity could promote a change of transporter activities (ie, increased NKCC1 to KCC2 ratio) and a shift in the nature of GABA neurotransmission to be more similar to that observed earlier in development. Specifically, our findings suggest that schizophrenia is associated with a tendency for GABA neurotransmission to be less strongly inhibitory in the DLPFC, at least in the neurons that have elevated levels of OXSR1 and WNK3 expression.

A provisional model summarizing these potential interactions between NKCC1 and KCC2 chloride transporters and OXSR1 and WNK3 kinases is shown in **Figure 6**. In normal adult neurons (Figure 6A), NKCC1 levels are low and KCC2 levels are high resulting in a low level of intracellular chloride. GABA binding to GABA<sub>A</sub> receptors triggers an inward flow of chloride leading to hyperpolarization. In schizophrenia (Figure 6B), greater levels of OXSR1 and WNK3 result in greater phosphorylation of both chloride transporters, and a consequent increase in NKCC1 and decrease in KCC2 activities, leading to a higher level of intracellular chloride. This change in intracellular chloride concentration has the potential to reduce the hyperpolarizing effect triggered by GABA binding and therefore to substantially change the nature of GABA neurotransmission.

**Figure 6.** Putative interaction model between NKCC1 and KCC2 and OXSR1 and WNK3 isoform 1 (WNK3-1) kinases. In both panels, the orange bar represents the cell membrane, with the extracellular domain above and the intracellular domain below the bar. The size and orientation of the green arrows indicate the magnitude and direction of chloride ion flow mediated by NKCC1, KCC2, and γ-aminobutyric acid<sub>A</sub> (GABA<sub>A</sub>) receptor chloride channels. A, In normal adult neurons, intracellular chloride concentration is low because of low levels of NKCC1 and high levels of KCC2. The binding of GABA to GABA<sub>A</sub> receptors triggers chloride entry (chloride flow is represented by green arrows) and hyperpolarization. B, In schizophrenia, increased OXSR1 and WNK3-1 kinase levels lead to increased phosphorylation (black P) and consequently increased NKCC1 activity and decreased KCC2 activity, producing a greater intracellular chloride concentration. Thus, when GABA<sub>A</sub> receptors are activated, chloride influx is reduced (or perhaps reversed) and the nature of GABA neurotransmission is altered.
The affected cortical neurons are likely to include pyramidal cells, given the immunocytochemical evidence that OXSR1 is preferentially localized in pyramidal neurons in layers 3 and 5 and the correlated expression of OXSR1 and WNK3-1&2 mRNA in layer 3 pyramidal neurons. Effective synchronization of networks of pyramidal neurons at the gamma band frequencies associated with working memory requires strongly hyperpolarizing inputs to a network of pyramidal cells so that the postsynaptic pyramidal neurons then escape from inhibition simultaneously and fire subsequent action potentials in unison, producing synchronized activity. Thus, a shift to increased NKCC1 and decreased KCC2 activity in pyramidal neurons in schizophrenia could alter the reversal potential of the GABA<sub>a</sub> receptor current to be closer to the resting membrane potential, reducing the strong inhibition required for gamma oscillations, and thus contributing to the alterations in the prefrontal gamma oscillations associated with impaired working memory in schizophrenia.

OTHER POTENTIAL EFFECTS OF ALTERED OXSR1 AND WNK3 EXPRESSION IN SCHIZOPHRENIA

The increased expression of OXSR1 and WNK3 could also contribute to other circuitry disturbances in the DLPFC. For example, vesicular glutamate transport appears to be highly dependent on the chloride content outside of synaptic vesicles. Thus, the change in intracellular chloride concentration predicted by our findings could also alter vesicular glutamate transporter activity and contribute to alterations in glutamate transmission and synaptic plasticity reported in schizophrenia.

Recent work also indicates that KCC2, through its interaction with the dendritic cytoskeleton, regulates spine maturation. Thus, a decrease in KCC2 activity, secondary to increased OXSR1 and WNK3 expression, could contribute to impaired spine maturation and thus to lower dendritic spine density in the DLPFC. Consistent with this interpretation, we observed higher OXSR1 and WNK3 expression in layer 3 pyramidal cells where the spine deficit in schizophrenia is most pronounced.

In summary, we report substantial increases in the expression of the regulatory kinases OXSR1 and WNK3 in the DLPFC that appear to be highly conserved across subjects with schizophrenia. This combination is likely to alter the relative activities of the chloride transporters NKCC1 and KCC2, producing a higher than normal intracellular chloride concentration, such that activation of GABA<sub>a</sub> receptors results in less chloride influx and a smaller increase in hyperpolarization, or depending on the chloride reversal potential, could even lead to an outflow of chloride ions, and some degree of membrane depolarization of the postsynaptic cell.

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Author Contributions: Dr Lewis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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