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Chemokine Receptors and Cortical Interneuron Dysfunction in Schizophrenia

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Abstract

Alterations in inhibitory (GABA) neurons, including deficiencies in the GABA synthesizing enzyme GAD67, in the prefrontal cortex in schizophrenia are pronounced in the subpopulations of neurons that contain the calcium-binding protein parvalbumin or the neuropeptide somatostatin. The presence of similar illness-related deficits in the transcription factor Lhx6, which regulates prenatal development of parvalbumin and somatostatin neurons, suggests that cortical GABA neuron dysfunction may be related to disturbances *in utero*. Since the chemokine receptors CXCR4 and CXCR7 guide the migration of cortical parvalbumin and somatostatin neurons from their birthplace in the medial ganglionic eminence to their final destination in the neocortex, we sought to determine whether altered CXCR4 and/or CXCR7 mRNA levels were associated with disturbances in GABA-related markers in schizophrenia. Quantitative PCR was used to quantify CXCR4 and CXCR7 mRNA levels in the prefrontal cortex of 62 schizophrenia and 62 healthy comparison subjects that were previously characterized for markers of parvalbumin and somatostatin neurons and in antipsychotic-exposed monkeys. We found elevated mRNA levels for CXCR7 (+29%; $p < .0001$) and CXCR4 (+14%, $p = .052$) in schizophrenia subjects but not in antipsychotic-exposed monkeys. CXCR7 mRNA levels were inversely correlated with mRNA levels for GAD67, parvalbumin, somatostatin, and Lhx6 in schizophrenia but not in healthy subjects. These findings suggest that higher mRNA levels for CXCR7, and possibly CXCR4, may represent a compensatory mechanism to sustain the migration and correct positioning of cortical

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Contributors

Dr. Volk oversaw all aspects of the design and implementation of the study and was the primary author of the manuscript. Ms. Chitrapu and Ms. Edelson contributed to the design of the study, collected the data, and assisted with the interpretation of the data. Dr. Lewis contributed to the design of the study and the preparation of the manuscript. All authors contributed to and have approved the final manuscript.

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Disclosures

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parvalbumin and somatostatin neurons in the face of other insults that disrupt the prenatal development of cortical GABA neurons in schizophrenia.

Keywords

GABA; parvalbumin; somatostatin; GAD67; prenatal development; postmortem

1. Introduction

Alterations in inhibitory (GABA) neurons in the prefrontal cortex in schizophrenia, including lower transcript levels for the GABA synthesizing enzyme glutamate decarboxylase (GAD67) (Akbarian et al. 1995; Volk et al. 2000; Guidotti et al. 2000; Straub et al. 2007; Duncan et al. 2010; Curley et al. 2011; Kimoto et al. 2014; Volk et al. 2014), are among the most consistently reported postmortem findings in schizophrenia. Specific subpopulations of GABA neurons, in particular those that contain the calcium-binding protein parvalbumin or the neuropeptide somatostatin, have been reported to be severely affected in the illness (Hashimoto et al. 2003; Morris et al. 2008; Mellios et al. 2009; Fung et al. 2010; Volk et al. 2012; Glausier et al. 2014). Recent evidence suggests that the disease process that leads to dysfunction of cortical parvalbumin and somatostatin neurons may have a prenatal origin. For example, approximately 50% of parvalbumin neurons have been reported to fail to complete their phenotypic specification into fully functioning GABA neurons (Hashimoto et al. 2003), while disrupted migration of cortical somatostatin neurons has also been reported (Yang et al. 2011). Furthermore, we recently reported disease-related deficits in the transcription factor Lhx6 (Volk et al. 2012; Volk et al. 2014), which is important for the prenatal development of cortical parvalbumin and somatostatin neurons as they tangentially migrate out of the medial ganglionic eminence and toward the cerebral cortex (Liodis et al. 2007; Zhao et al. 2008; Neves et al. 2013; Vogt et al. 2014). However, subsequent animal models revealed that a partial loss of Lhx6 similar to that seen in schizophrenia was not sufficient in isolation to reproduce the pattern of disturbances in cortical GABA neurons seen in schizophrenia (Neves et al. 2013; Volk et al. 2014). These findings indicate the need to explore whether additional factors that regulate the prenatal ontogeny of cortical GABA neurons may also be altered in schizophrenia and perhaps may interact with deficits in Lhx6 to produce GABA-related disturbances similar to those seen in schizophrenia.

Interestingly, the chemotactic cytokine (i.e. chemokine) receptors CXCR4 and CXCR7, which bind the ligand CXCL12 (also known as Stromal cell-derived factor-1; SDF-1) (Balabanian et al. 2005), are expressed by migrating cortical interneurons, including future cortical parvalbumin and somatostatin neurons (Stumm et al. 2003; Stumm et al. 2007; Li et al. 2008; Lopez-Bendito et al. 2008; Tanaka et al. 2010; Sanchez-Alcaniz et al. 2011; Wang et al. 2011). CXCR4 and CXCR7 play critical roles in sustaining the tangential migration of cortical parvalbumin and somatostatin neurons from their birth in the medial ganglionic eminence to their final destination in the neocortex, including the timing of their radial migration through the cortical plate (Li et al. 2008; Tanaka et al. 2010; Sanchez-Alcaniz et al. 2011; Wang et al. 2011; Vogt et al. 2014). Furthermore, Lhx6 can affect the expression

of CXCR7 by binding to an intronic enhancer element of CXCR7, while transduction of CXCR7 can, in turn, partially compensate for the deleterious effects of a loss of Lhx6 on cortical GABA neuron migration (Vogt et al. 2014). These findings raise the question of whether alterations in CXCR4 and/or CXCR7 in the prefrontal cortex in schizophrenia may act in concert with, or may instead compensate for, deficits in Lhx6 to affect prenatal ontogeny of cortical parvalbumin and somatostatin neurons in schizophrenia.

2. Materials and methods

2.1. Human subjects

Brain specimens were obtained during routine autopsies conducted at the Allegheny County Office of the Medical Examiner (Pittsburgh, Pennsylvania) after consent was obtained from next-of-kin. An independent committee of experienced research clinicians made consensus DSMIV (American Psychiatric Association 1994) diagnoses for each subject using structured interviews with family members and review of medical records, and the absence of a psychiatric diagnosis was confirmed in healthy comparison subjects using the same approach (Volk et al. 2011). To control for experimental variance, subjects with schizophrenia or schizoaffective disorder (n=62) were matched individually to one healthy comparison subject for sex and as closely as possible for age (Supplemental Table S1). Tissue samples from subjects in a pair were processed together throughout all stages of the study. The mean age, postmortem interval, RNA integrity number (RIN), and tissue freezer storage time did not differ between subject groups ($t_{(122)} = 0.45$, $p = 0.65$) (Table 1). Mean ($\pm$ standard deviation) brain pH was different between the schizophrenia (6.6 ± 0.3) and healthy subject groups (6.7 ± 0.2 ; $t_{(122)} = 2.6$, $p = 0.01$), but the difference was quite small and of uncertain significance. 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.

2.2. Quantitative PCR

Frozen tissue blocks containing the middle portion of the right superior frontal sulcus were confirmed to contain prefrontal cortex area 9 using Nissl-stained, cryostat tissue sections for each subject (Volk et al. 2000). The gray-white matter boundary of prefrontal cortex area 9 in a tissue block from each subject was carefully scored with a scalpel blade where the gray matter had uniform thickness and the gray-white matter boundary was easily delineated. The scored gray matter region of the tissue block was then digitally photographed, and the number of tissue sections (40 μ m) required to collect $\sim 30 \text{ mm}^3$ of gray matter was determined for each subject. The calculated number of required tissue sections for each subject was then cut by cryostat, and gray matter was separately collected into a tube containing TRIzol reagent in a manner that ensured minimal white matter contamination and excellent RNA preservation (Volk et al. 2013). Standardized dilutions of total RNA for each subject were used to synthesize cDNA. All primer pairs (Supplemental Table S2) demonstrated high amplification efficiency ($>98\%$) across a wide range of cDNA dilutions and specific single products in dissociation curve analysis, and control studies in which the cDNA template was not included in the quantitative PCR reaction resulted in a complete lack of amplification.. Quantitative PCR was performed using the comparative cycle

threshold (CT) method with Power SYBR Green dye and the ViiA-7 Real-Time PCR System (Applied Biosystems), as previously described (Volk et al. 2014). Three reference genes (beta actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were used to normalize target mRNA levels (Hashimoto et al. 2008). The difference in CT (dCT) for each target transcript was calculated by subtracting the geometric mean CT for the three reference genes from the CT of the target transcript (mean of four replicate measures). Because dCT represents the log₂-transformed expression ratio of each target transcript to the reference genes, the relative level of the target transcript for each subject is reported as 2^{-dCT} (Vandesompele et al. 2002; Volk et al. 2010). The mean coefficient of variance ($\pm$ SD) of the replicate measures for CXCR4 was 0.048 ($\pm$ 0.031) and for CXCR7 was 0.053 ($\pm$ 0.036). Furthermore, the mean relative expression level for each reference gene relative to the two other reference genes did not differ between schizophrenia and healthy comparison subjects, respectively (beta actin: 1.36 ± 0.25 versus 1.35 ± 0.17 ; cyclophilin: 0.41 ± 0.05 versus 0.43 ± 0.04 ; GAPDH: 1.85 ± 0.24 versus 1.78 ± 0.15).

2.3. Antipsychotic-exposed monkeys

Young adult, male, long-tailed monkeys (*Macaca fascicularis*) received oral doses of haloperidol, olanzapine or placebo (n=6 monkeys per group) twice daily for 17–27 months, as previously described (Dorph-Petersen et al. 2005). RNA was isolated from prefrontal cortical area 9, and qPCR was conducted for the same three reference genes and CXCR4 and CXCR7 (Supplemental Table S2) with all monkeys from a triad processed together on the same plate. All animal studies followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.4. Statistical analysis

The ANCOVA model we report includes mRNA level as the dependent variable, diagnostic group as the main effect, and age, postmortem interval, brain pH, RIN, and freezer storage time as covariates. Because each schizophrenia subject was individually matched to a healthy subject to account for the parallel processing of tissue samples from a pair and to balance diagnostic groups for sex and age, a second ANCOVA model with subject pair as a blocking factor and including postmortem interval, brain pH, RIN, and freezer storage time was also used, and both models produced similar results. Subsequent analyses of differences in mRNA levels between schizophrenia subjects grouped by predictors and indicators of disease severity, psychotropic medications, and smoking were conducted using the unpaired ANCOVA models. For the antipsychotic-exposed monkey study, an ANOVA model with mRNA level as the dependent variable, treatment group as the main effect, and triad as a blocking factor was employed.

3. Results

3.1. Quantification of CXCR4 and CXCR7 mRNA levels in the prefrontal cortex in schizophrenia

Mean ($\pm$ standard deviation) mRNA levels in the prefrontal cortex of schizophrenia subjects were statistically significantly higher for CXCR7 (+29%; 0.0052 ± 0.0016 ; $F_{(1,117)}=24.9$,

$p < .0001$) and nearly significantly higher for CXCR4 (+14%; 0.0042 ± 0.0028 ; $F_{(1,117)} = 3.9$, $p = .052$) relative to healthy comparison subjects (0.0040 ± 0.0009 and 0.0037 ± 0.0010 , respectively; Figure 1). Furthermore, we found no relationship between use of antipsychotic, antidepressant, or benzodiazepine medications, smoking, or substance abuse or dependence at time of death and CXCR7 (all $F = 2.5$, $p = 0.12$) or CXCR4 (all $F = 3.4$, $p = 0.07$) mRNA levels in schizophrenia subjects. In addition, among schizophrenia subjects, CXCR7 mRNA levels did not differ as a function of factors that predict a more severe course of illness (male sex, a diagnosis of schizophrenia rather than schizoaffective disorder, first-degree relative with schizophrenia, early age at illness onset [≥ 18 years of age]) or measures of illness severity (suicide, no history of marriage, low socioeconomic status (as measured by the Hollingshead Index of Social Position), or living dependently at the time of death) (all $F = 2.1$, $p = .15$). CXCR4 mRNA levels also did not differ as a function of these same factors that predict or measure illness severity (all $F = 3.7$, $p = .06$) with one exception: schizophrenia subjects with a history of marriage had 17% lower mRNA levels than schizophrenia subjects without a history of marriage ($F_{(1,55)} = 4.6$, $p = 0.036$).

3.2. Relationship between mRNA levels for chemokine receptors and GABA neuron-related markers in the prefrontal cortex of schizophrenia subjects

Interestingly, CXCR7 mRNA levels were inversely correlated with mRNA levels for parvalbumin ($r = -.46$, $p = .0002$), GAD67 ($r = -.46$, $p = .0002$), Lhx6 ($r = -.30$, $p = .018$), and somatostatin ($r = -.46$, $p = .0001$) in schizophrenia subjects ($n = 62$) but not in healthy comparison subjects ($n = 62$; for all, $r = .19$, $p = .14$; Figure 2). In contrast, CXCR4 mRNA levels did not correlate with mRNA levels for parvalbumin, GAD67, or Lhx6 in schizophrenia (all $r = .08$, $p = .56$) or in healthy comparison subjects (all $r = .16$, $p = .21$; Figure 2). CXCR4 mRNA levels also did not correlate with somatostatin mRNA levels in schizophrenia subjects ($r = -.16$, $p = .20$), but were inversely correlated with somatostatin mRNA levels in healthy subjects ($r = -.33$, $p = .008$).

3.3. CXCR7 and CXCR4 mRNA levels in the prefrontal cortex of antipsychotic-exposed monkeys

We also found that CXCR7 ($F_{(2,10)} = 1.5$, $p = .28$) and CXCR4 ($F_{(2,10)} = 0.2$, $p = .82$) mRNA levels did not differ in the prefrontal cortex of monkeys chronically exposed to haloperidol, olanzapine, or placebo (Figure 3).

4. Discussion

CXCR4 and CXCR7 play important roles in the prenatal migration of cortical parvalbumin and somatostatin neurons (Stumm et al. 2003; Stumm et al. 2007; Li et al. 2008; Lopez-Bendito et al. 2008; Tanaka et al. 2010; Sanchez-Alcaniz et al. 2011; Wang et al. 2011; Vogt et al. 2014). Consequently, in this study, we sought to determine whether alterations in CXCR4 and CXCR7 mRNAs in the prefrontal cortex in schizophrenia may be related to disturbances in GABA neuron-related markers in schizophrenia. We found higher mRNA levels for CXCR7 and CXCR4 in the schizophrenia subjects; however, only the elevation in CXCR7 mRNA levels achieved statistical significance. Furthermore, evidence from the schizophrenia subjects and antipsychotic-exposed monkeys suggests that the elevated

CXCR7 and CXCR4 mRNA levels were not attributable to exposure to psychotropic medication, substance abuse or dependence, or smoking. Finally, the presence of inverse correlations between mRNA levels for CXCR7 with mRNA levels for multiple GABA neuron-related markers in schizophrenia subjects suggest that higher mRNA levels for CXCR7 may represent a compensatory mechanism to sustain the migration and ensure the correct positioning of cortical parvalbumin and somatostatin neurons in the face of other insults to the prenatal ontogeny of cortical GABA neurons.

Several different mechanisms may potentially underlie the over-expression of CXCR7 and possibly CXCR4 in the prefrontal cortex in schizophrenia. First, several transcription factors have been reported to regulate levels of CXCR7. For example, the transcription factors Dlx1, Dlx2, Zfhx1b (also known as Zeb2 and Sip1), and Lhx6 are expressed early in gestation in the medial ganglionic eminence and regulate different aspects of the migration, specification, and maturation of cortical parvalbumin and somatostatin neurons (Anderson et al. 1997; Cobos et al. 2005; Liodis et al. 2007; Zhao et al. 2008; McKinsey et al. 2013; Neves et al. 2013; van, V et al. 2013; Vogt et al. 2014). Animal models of a loss of expression of Dlx1/Dlx2, Zfhx1b, or Lhx6 each result in markedly lower levels of CXCR7 in the medial ganglionic eminence and in migrating interneurons (Zhao et al. 2008; McKinsey et al. 2013; Long et al. 2009). Furthermore, in schizophrenia, cortical mRNA levels for Dlx1 and Lhx6 have been previously been reported to be lower (Joshi et al. 2012; Volk et al. 2012; Volk et al. 2014). However, in the current cohort of schizophrenia subjects, Lhx6 mRNA levels are inversely correlated with CXCR7 mRNA levels. These data suggest that the more subtle losses of Dlx1 and Lhx6 may be insufficient to have a direct effect on CXCR7 mRNA levels and that CXCR4 and CXCR7 mRNA levels are instead up-regulated in schizophrenia through another, yet to be identified, mechanism. Second, CXCR4 levels have been reported to be dependent upon the expression of CXCR7 (Sanchez-Alcaniz et al. 2011), suggesting that higher levels of CXCR7 in schizophrenia may in part contribute to an upregulation of CXCR4 in the illness. Finally, while the status of CXCL12, the ligand that binds to CXCR4 and CXCR7, in the prefrontal cortex in schizophrenia is presently unknown, such knowledge would be a useful next step in investigating the pathophysiological mechanisms underlying our findings of higher mRNA levels of CXCR7 and CXCR4 in schizophrenia.

Several lines of evidence suggest that higher levels of CXCR7 may have a compensatory effect that at least partially mitigates disturbances in the prenatal development of cortical interneurons. For example, transduction of CXCR7 in Lhx6 mutant cells in the medial ganglionic eminence in a mouse model partially rescued the lamination pattern of cortical Lhx6-expressing neurons, but did not rescue levels of parvalbumin and somatostatin (Vogt et al. 2014). Strikingly, these compensatory effects of overexpression of CXCR7 mirror alterations in the prefrontal cortex in schizophrenia where the lamination pattern of parvalbumin neurons is preserved (Woo et al. 1997; Hashimoto et al. 2003) while mRNA levels for parvalbumin and somatostatin remain lower (Hashimoto et al. 2003; Morris et al. 2008; Mellios et al. 2009; Fung et al. 2010; Volk et al. 2012). Thus, evidence from schizophrenia subjects and animal models suggests that overexpression of CXCR7 may help preserve the lamination pattern of parvalbumin neurons in schizophrenia but is not sufficient to retain the phenotype of parvalbumin and somatostatin neurons. However, additional

studies of the effects of overexpression of CXCR7 on cortical parvalbumin and somatostatin neuron migration are needed to further investigate the potential compensatory role that higher CXCR7 levels may play in schizophrenia. Furthermore, CXCR4 and CXCR7 have been reported to preferentially regulate parvalbumin neurons with minimal effects on somatostatin neurons (Meechan et al. 2012; Vogt et al. 2014) (but also see (Tanaka et al. 2010)). Reductions of CXCR4 have been reported to alter the migration and laminar distribution of parvalbumin neurons while not affecting the distribution of somatostatin neurons (Meechan et al. 2012). Similarly, CXCR7^{-/-} transplants into the medial ganglionic eminence of normal embryos resulted in lower levels of parvalbumin, but not somatostatin (Vogt et al. 2014). In schizophrenia, fewer somatostatin neurons have been reported to be detectable in the gray matter of the prefrontal cortex (Morris et al. 2008), while more somatostatin neurons have been reported in the white matter of the prefrontal cortex in the illness (Yang et al. 2011). This pattern of findings has been interpreted to reflect an abnormality in the migration of cortical somatostatin neurons in schizophrenia (Volk and Lewis 2013). Thus, the overexpression of CXCR4 and CXCR7 may have a compensatory effect to preserve the migration and correct cortical positioning of parvalbumin neurons, but not that of somatostatin neurons, in response to other insults that disrupt the prenatal ontogeny of these neurons in schizophrenia. However, future studies investigating abnormalities in the multiple other molecular markers that regulate cortical parvalbumin and somatostatin neuron development are required to determine the extent to which a disrupted prenatal developmental process could contribute to cortical parvalbumin and somatostatin neuron pathology in schizophrenia (Volk and Lewis 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Quantitative PCR determination of relative mRNA levels for chemokine receptors in the prefrontal cortex in schizophrenia

Transcript levels for CXCR7 (left panel) and CXCR4 (right panel) in schizophrenia subjects relative to matched healthy comparison subjects in a pair are indicated by open circles. Data points to the left of the unity line indicate higher mRNA levels in the schizophrenia subject relative to the healthy comparison subject and vice versa. CXCR7 mRNA levels were higher in the schizophrenia subject relative to the healthy comparison subject in 47 of the 62 subject pairs, while CXCR4 mRNA levels were higher in the schizophrenia subject relative to the healthy comparison subject in only 34 of the 62 subject pairs.

Figure 2. Relationships between mRNA levels of chemokine receptors and GABA neuron-related markers in schizophrenia

Quantitative PCR analysis revealed that CXCR7 mRNA levels (left graphs) were inversely correlated with mRNA levels for parvalbumin, GAD67, Lhx6, and somatostatin in schizophrenia subjects (grey circles; black linear regression lines, $n=62$ subjects), but not in healthy subjects (open circles; dashed linear regression lines, $n=62$ subjects). In contrast, CXCR4 mRNA levels (right graphs) were not correlated with mRNA levels for parvalbumin, GAD67, Lhx6, and somatostatin in schizophrenia or in healthy subjects with the exception of a significant inverse correlation between CXCR4 and somatostatin in healthy subjects.

Figure 3. Chemokine receptor mRNA levels in prefrontal area 9 of antipsychotic-exposed monkeys

Quantitative PCR analysis revealed no statistically significant differences in CXCR7 or CXCR4 mRNA expression levels in monkeys chronically exposed to either olanzapine (light gray triangles) or haloperidol (dark gray triangles) compared to placebo (open triangles). Mean values are shown as horizontal black bars.

Table 1

Summary of demographic and postmortem characteristics of human subjects

Parameter	Healthy Comparison	Schizophrenia
N	62	62
Sex	47M / 15F	47M / 15F
Race	52W / 10B	46W / 16B
Age (years)	48.7 ± 13.8	47.7 ± 12.7
Postmortem Interval (hours)	18.8 ± 5.5	19.2 ± 8.5
Freezer Storage Time (months)	131.8 ± 56.2	128.1 ± 60.7
Brain pH	6.7 ± 0.2	6.6 ± 0.3
RNA Integrity Number	8.2 ± 0.6	8.1 ± 0.6

For brain pH, $t_{(122)}=2.6$, $p=0.01$. For all others, $t_{(122)} \leq 0.45$, $p \geq 0.65$. Values are group means ± standard deviation.