

Cortical Opioid Markers in Schizophrenia and across Postnatal Development

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Certain cognitive deficits in schizophrenia have been linked to dysfunction of prefrontal cortical (PFC) γ -aminobutyric acid (GABA) neurons and appear neurodevelopmental in nature. Since opioids suppress GABA neuron activity, we conducted the first study to determine 1) whether the μ opioid receptor (MOR), δ opioid receptor (DOR), and opioid ligand proenkephalin are altered in the PFC of a large cohort of schizophrenia subjects and 2) the postnatal developmental trajectory in monkey PFC of opioid markers that are altered in schizophrenia. We used quantitative polymerase chain reaction to measure mRNA levels from 42 schizophrenia and 42 matched healthy comparison subjects; 18 monkeys chronically exposed to haloperidol, olanzapine, or placebo; and 49 monkeys aged 1 week–11.5 years. We found higher levels for MOR mRNA (+27%) in schizophrenia but no differences in DOR or proenkephalin mRNAs. Elevated MOR mRNA levels in schizophrenia did not appear to be explained by substance abuse, psychotropic medications, or illness chronicity. Finally, MOR mRNA levels declined through early postnatal development, stabilized shortly before adolescence and increased across adulthood in monkey PFC. In schizophrenia, higher MOR mRNA levels may contribute to suppressed PFC GABA neuron activity and might be attributable to alterations in the postnatal developmental trajectory of MOR signaling.

Keywords: delta opioid receptor, GABA, mu opioid receptor, prefrontal cortex, proenkephalin

Introduction

Cognitive impairments in schizophrenia are disabling and difficult to treat and have been linked to disturbances in the inhibitory (γ -aminobutyric acid [GABA]) circuitry of the prefrontal cortex (PFC), including the parvalbumin (PV)- and somatostatin (SST)-containing GABA neuron subpopulations (Hashimoto et al. 2003; Morris et al. 2008; Mellios et al. 2009; Fung et al. 2010; Curley et al. 2011). For example, in schizophrenia, PV and SST neurons appear to have a reduced capacity to synthesize GABA due to lower levels of a GABA synthesizing enzyme (GAD67) (Hashimoto et al. 2003; Morris et al. 2008; Curley et al. 2011) and may also receive reduced excitatory drive from pyramidal neurons (Lisman et al. 2008; Bitanihirwe et al. 2009). Since PV neurons regulate the synchronous firing of neurons at gamma frequency (Sohal et al. 2009), PV neuron dysfunction has been proposed (Gonzalez-Burgos et al. 2010) to contribute to the reduced power of PFC gamma oscillations reported in schizophrenia subjects performing cognitive tasks (Cho et al. 2006; Minzenberg et al. 2010). These data suggest that the development of novel treatments for cognitive impairments in schizophrenia may be advanced by understanding the

pathophysiological mechanisms that contribute to disturbed PFC GABA neurotransmission in the disorder.

Interestingly, the endogenous opioid system, which has been relatively unexamined in schizophrenia, modulates GABA neuron function in a manner that could directly impact GABA-related disturbances in the disorder. For example, activation of the μ opioid receptor (MOR), which is a G protein-coupled receptor, suppresses vesicular GABA release from axon terminals (Capogna et al. 1993; Lupica 1995) and activates G protein-coupled inwardly rectifying potassium channels which hyperpolarize the cell body and reduce responsiveness to excitatory input (Wimpey and Chavkin 1991; Glickfeld et al. 2008). Furthermore, activation of MOR has been reported to disrupt gamma oscillations (Whittington et al. 1998; Gulyas et al. 2010). Interestingly, MOR are primarily found in the axon terminals and somatodendritic region of virtually all PV and SST neurons but not in pyramidal neurons or glial cells (Drake and Milner 1999, 2002; Stumm et al. 2004). These lines of evidence suggest that if markers of cortical opioid signaling are higher (or lower) in the illness, they may potentially contribute to (or compensate for) deficits in GABA neuron activity, GABA release, and gamma oscillations in the PFC in schizophrenia.

Determining how altered MOR signaling could contribute to the cause or compensation of GABA neuron dysfunction in schizophrenia may be further informed by knowledge of the normal development of this system. For example, the development of certain cognitive functions, including working memory, across childhood and early adolescence exhibits a persistent “lag” in children who are later diagnosed with schizophrenia (Reichenberg et al. 2010), suggesting that cortical circuitry may not develop into a fully adult state in affected individuals. Furthermore, determinants of PFC GABA signaling that are disturbed in schizophrenia, including markers of PV neuron function (Lewis et al. 2001; Hashimoto et al. 2003), also undergo marked changes across childhood and early adolescence (Erickson and Lewis 2002; Cruz et al. 2003; Beneyto and Lewis 2011; Fung et al. 2010). These lines of evidence raise speculation that the development of important regulators of PFC GABA signaling, such as MOR, may not occur normally in schizophrenia.

Because the expression levels for opioid signaling proteins are relatively low in human PFC, prior microarray studies might have lacked the sensitivity to detect disease-related alterations in these transcripts. Consequently, we used quantitative reverse transcription polymerase chain reaction (qPCR) to quantify mRNA levels for MOR and other opioid markers (i.e., the δ opioid receptor, DOR, and the opioid ligand propeptide proenkephalin) in the PFC of a large cohort of schizophrenia and healthy comparison subjects. We also sought to determine whether any schizophrenia-related findings were associated with exposure to substances of abuse, antipsychotic

medications, or predictors or measures of disease severity. Finally, we assessed the postnatal developmental trajectory of MOR in the PFC of monkeys. Our findings suggest that increased MOR expression in schizophrenia may contribute to suppressed GABA neuron function and may reflect impaired development of PFC neural circuitry.

Materials and Methods

Human Subjects

Brain specimens ($n = 84$) were obtained during routine autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA) after consent was obtained from next-of-kin. An independent committee of experienced research clinicians made consensus DSM-IV (American Psychiatric Association 1994) diagnoses for each subject using structured interviews with family members and review of medical records (Volk et al. 2010), and the absence of a psychiatric diagnosis was confirmed in healthy comparison subjects. To control for experimental variance, subjects with schizophrenia or schizoaffective disorder ($n = 42$) were matched individually to one healthy comparison subject for sex and as closely as possible for age (Supplementary Table S1), and samples from subjects in a pair were processed together throughout all stages of the study. The mean age, postmortem interval, brain pH, RNA integrity number (RIN) (Agilent Bioanalyzer, Waldbronn, Germany), and tissue storage time did not differ between subject groups (Table 1). 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.

Preparation of Human Brain Tissue

Frozen tissue blocks containing the anterior-posterior level corresponding to the middle portion of the right superior frontal sulcus for each subject were confirmed to contain PFC area 9 using Nissl-stained, cryostat tissue sections ($40 \mu\text{m}$) (Volk et al. 2000). The gray-white matter boundary of the tissue block was then 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 required to collect $\sim 30 \text{ mm}^3$ of gray matter was determined for each subject (typically between 3 and 12 sections per subject depending upon the amount of gray matter available from the tissue block). The calculated number of required tissue sections for each subject was then cut by cryostat ($40 \mu\text{m}$), 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 (Hashimoto et al. 2008).

Quantitative PCR

cDNA was synthesized from standardized dilutions of total RNA ($10 \text{ ng}/\mu\text{L}$) for each subject. All primer pairs (Supplementary Table S2) demonstrated high amplification efficiency ($>96\%$) across a wide range

of cDNA dilutions and specific single products in dissociation curve analysis. Quantitative PCR was performed using the comparative cycle threshold (CT) measurement method with Power SYBR Green dye and the StepOnePlus Real-Time PCR System (Applied Biosystems). cDNA samples from both subjects in the same pair were processed together on a single quantitative PCR plate, and a different plate was utilized for each of the 42 subject pairs. Based on their stable relative expression level between schizophrenia and comparison subjects (Hashimoto et al. 2008), 3 reference genes (beta actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were used to normalize the target gene expression levels. The difference in CT for each target transcript was calculated by subtracting the geometric mean CT for the 3 reference genes from the CT of the target transcript. Because this difference in CT (dCT) represents the log₂-transformed expression ratio of each target transcript to the geometric mean of the 3 reference genes, the relative expression level of the target transcript is determined as $2^{-\text{dCT}}$ (Vandesompele et al. 2002; Hashimoto et al. 2008; Volk et al. 2010). Four replicate measures were performed for each transcript for each subject with a detection threshold for each gene applied consistently for all subjects, and the mean coefficient of variance (\pm standard deviation) of the replicate measures were MOR 0.044 (± 0.026), DOR 0.041 (± 0.023), and proenkephalin 0.061 (± 0.031).

Antipsychotic-Exposed Monkeys

Experimentally naïve, young adult, male, long-tailed macaque monkeys (*Macaca fascicularis*) received oral doses of haloperidol, olanzapine, or placebo ($n = 6$ monkeys per group) twice daily for 17–27 months (Dorph-Petersen et al. 2005). One monkey from each of the 3 medication groups was euthanized together on the same day, and the 6 triads were euthanized on separate days. RNA was isolated from PFC area 9, and qPCR was conducted for the same 3 reference genes and MOR (Supplementary Table S2) with all monkeys from a triad processed together on the same plate. Primers were designed using monkey-specific cDNA sequences, and primer amplification efficiencies were $>96\%$. 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.

Developmental Monkeys

Forty-nine female rhesus macaque monkeys (*Macaca mulatta*) ranging in age from 1 week to 11.5 years were used (Supplementary Table S3) (Hashimoto et al. 2009; Eggan et al. 2010). Twenty-one monkeys were perfused transcerebrally with artificial cerebrospinal fluid (Gonzalez-Burgos et al. 2008), and in 5 animals, a small tissue block from the left principal sulcus was surgically excised for electrophysiology studies 2–4 weeks prior to perfusion. All remaining animals were unperfused and experimentally naïve. Monkeys were assigned to age groups (Supplementary Table S3) that were established by previous studies (Hashimoto et al. 2009; Eggan et al. 2010). RNA was isolated from the frontal pole (PFC area 10) due to the availability of existing tissue. Relative mRNA levels were quantified by qPCR as described above, and subjects of different developmental ages were assigned to qPCR runs using a randomized block design. Although GAPDH mRNA levels appeared not to be stable through early development, beta actin and cyclophilin mRNA levels were constant across development ($F_{7,41} = 1.75$, $P = 0.123$; Supplementary Fig. S1), and these 2 transcripts were used to normalize MOR mRNA levels.

Statistical Analysis

For the schizophrenia study, 2 analysis of covariance (ANCOVA) models were used (Volk et al. 2010). The first model included mRNA level as the dependent variable, diagnostic group as the main effect, storage time, brain pH, and RIN as covariates, and subject pair as a blocking factor. Subject pairing may be considered an attempt to account for the parallel processing of tissue samples from a pair and to balance diagnostic groups for sex and age and not a true statistical paired design. Therefore, we also utilized a second model without subject pair as a blocking factor that included age, sex, postmortem interval, storage time, brain pH, and RIN as covariates. The Bonferroni correction for multiple comparisons was

Table 1

Summary of demographic and postmortem characteristics of human subjects

Parameter	Healthy comparison	Schizophrenia
N	42	42
Sex	31 M/11 F	31 M/11 F
Race	34 W/8 B	29 W/13 B
Age (years)	48 \pm 13	47 \pm 13
Postmortem interval (hours)	17.8 \pm 5.9	18.1 \pm 8.7
Freezer storage time (months)	106 \pm 43	106 \pm 46
Brain pH	6.8 \pm 0.2	6.6 \pm 0.4
RIN	8.3 \pm 0.6	8.2 \pm 0.7

Note: M, male; F, female; W, white; B, black. For all, $t_{82} < 2.0$, $P > 0.05$. Values are group means \pm standard deviation.

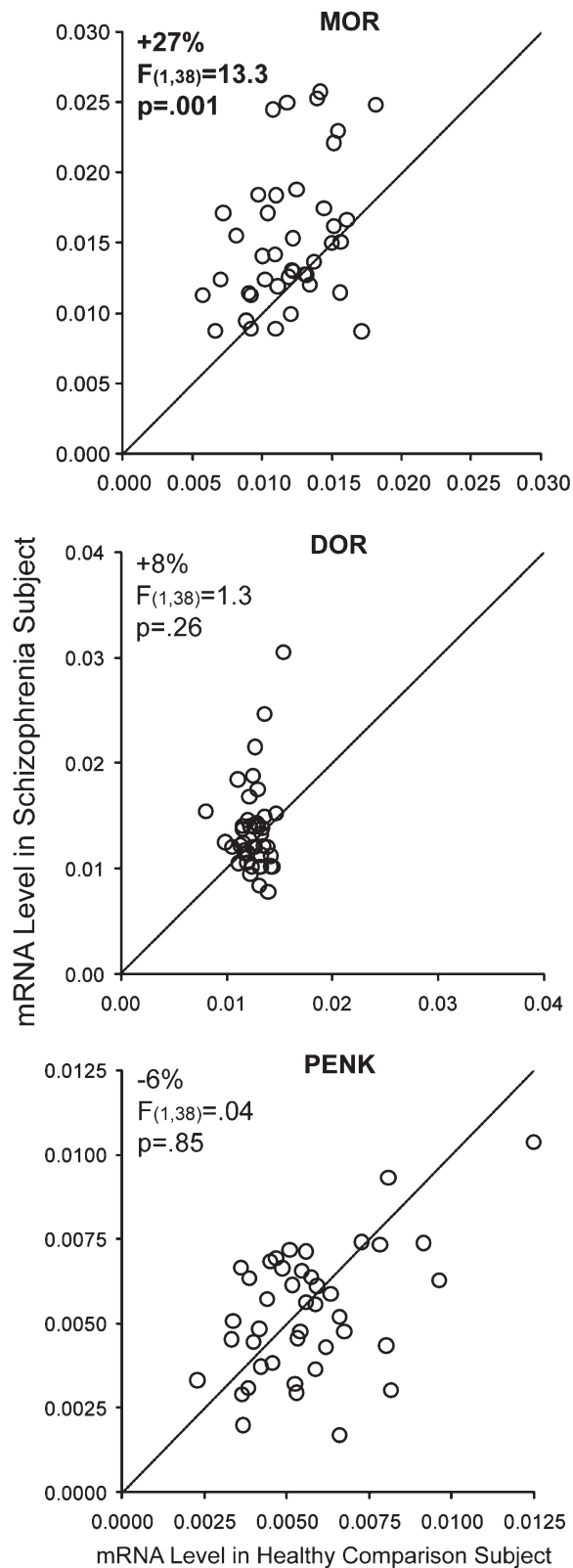


Figure 1. mRNA expression levels of opioid markers in schizophrenia. mRNA expression levels for schizophrenia subjects relative to matched healthy comparison subjects in a pair are indicated by open circles. Data points to the left of the unity line indicate higher levels of mRNA expression in the schizophrenia subject relative to the healthy comparison subject and vice versa. Statistically significant differences ($\alpha = 0.016$) in mRNA expression levels in schizophrenia subjects were found for MOR but not DOR or proenkephalin (PENK).

employed to set the threshold for statistical significance to $\alpha = 0.016$. Schizophrenia subjects were then divided into subgroups based upon the following factors: presence of a comorbid diagnosis of substance abuse or dependence at time of death (ATOD); history of cannabis use; nicotine use ATOD; use of antipsychotic, antidepressant, or benzodiazepine medications ATOD; sex; schizophrenia or schizoaffective disorder diagnosis; family history of a first degree relative with schizophrenia; age at onset of schizophrenia; age ATOD; suicide or other cause of death; history of marriage; and socioeconomic status as indicated by Hollingshead Two Factor Index of Social Position. MOR mRNA levels for schizophrenia subjects within each of these subgroups were then compared with their matched comparison subjects using one-tailed paired t tests with $\alpha = 0.05$ as higher MOR mRNA levels had already been demonstrated in the primary analysis of the schizophrenia group.

For the antipsychotic-exposed monkey study, an ANCOVA model with MOR mRNA level as the dependent variable, treatment group as the main effect, and triad as a blocking factor was employed. For the developmental monkey series, an ANOVA model with MOR mRNA level as the dependent variable and age group as main effect was employed, and least significant difference was employed as a post hoc test with $\alpha = 0.05$.

Results

Tissue Transcript Levels of Opioid Markers in Schizophrenia

We found elevated MOR mRNA levels (+27%; paired: $F_{1,38} = 13.3$, $P = 0.001$; unpaired: $F_{1,76} = 21.3$, $P < 0.001$) in the PFC of schizophrenia subjects relative to healthy comparison subjects (Fig. 1), and MOR mRNA levels were higher in the schizophrenia subject in 31 of 42 subject pairs. Use of a second primer set designed against a different MOR mRNA region (Supplementary Table S2) revealed similarly higher MOR mRNA levels in schizophrenia subjects (+25%; paired: $F_{1,38} = 11.4$, $P = 0.002$; unpaired: $F_{1,76} = 18.1$, $P < 0.001$), and MOR mRNA levels from the 2 primer sets were highly correlated across all subjects ($r = 0.98$, $P < 0.001$; Supplementary Fig. S2). In contrast, we found no differences in mRNA levels for DOR (+8%; paired: $F_{1,38} = 1.3$, $P = 0.26$; unpaired: $F_{1,76} = 1.8$, $P = 0.19$) or proenkephalin (-6%; paired: $F_{1,38} = 0.04$, $P = 0.85$; unpaired: $F_{1,76} = 0.33$, $P = 0.57$) in schizophrenia (Fig. 1; Supplementary Fig. S2).

Multiple mRNA splice variants of MOR that alter intracellular signaling capacity have been identified in some human cells (Pan et al. 2003, 2005; Pan 2005; Shabalina et al. 2009; Xu et al. 2009). The primer sets for MOR in this study were designed against the transmembrane region (exons 2-3) that is common across MOR splice variants (Pan et al. 2003, 2005; Pan 2005; Shabalina et al. 2009; Xu et al. 2009). Therefore, we investigated whether higher PFC MOR mRNA levels in schizophrenia were largely attributable to overexpression of specific splice variants. We tested multiple primer sets against human splice variant-specific exons reported in the NCBI database (exons 3b, 4, 5, 11, 13, O, X, Y) in the PFC from 3 healthy human subjects. Primer sets targeting exon 4 demonstrated excellent efficiency (>96%) and resulted in readily detectable mRNA levels. In contrast, primer sets targeting other exons revealed very low (exons 3b, 5, 13, O, X, Y) or undetectable (exon 11) mRNA levels (data not shown). Exon 4 expression likely reflects mRNA levels for the MOR-1 splice variant because the other exon 4-containing splice variants also possess either exon 11 (Xu et al. 2009) or 13 (Shabalina et al. 2009) whose mRNA levels were either very low or not detectable by qPCR in human PFC. Using the exon 4-specific primer set, we found that exon 4-containing MOR mRNA levels were elevated in

schizophrenia similar to that observed with the original primer set (Fig. 2). These data suggest that higher PFC MOR mRNA levels in schizophrenia are predominantly attributable to higher mRNA levels of the MOR-1 splice variant.

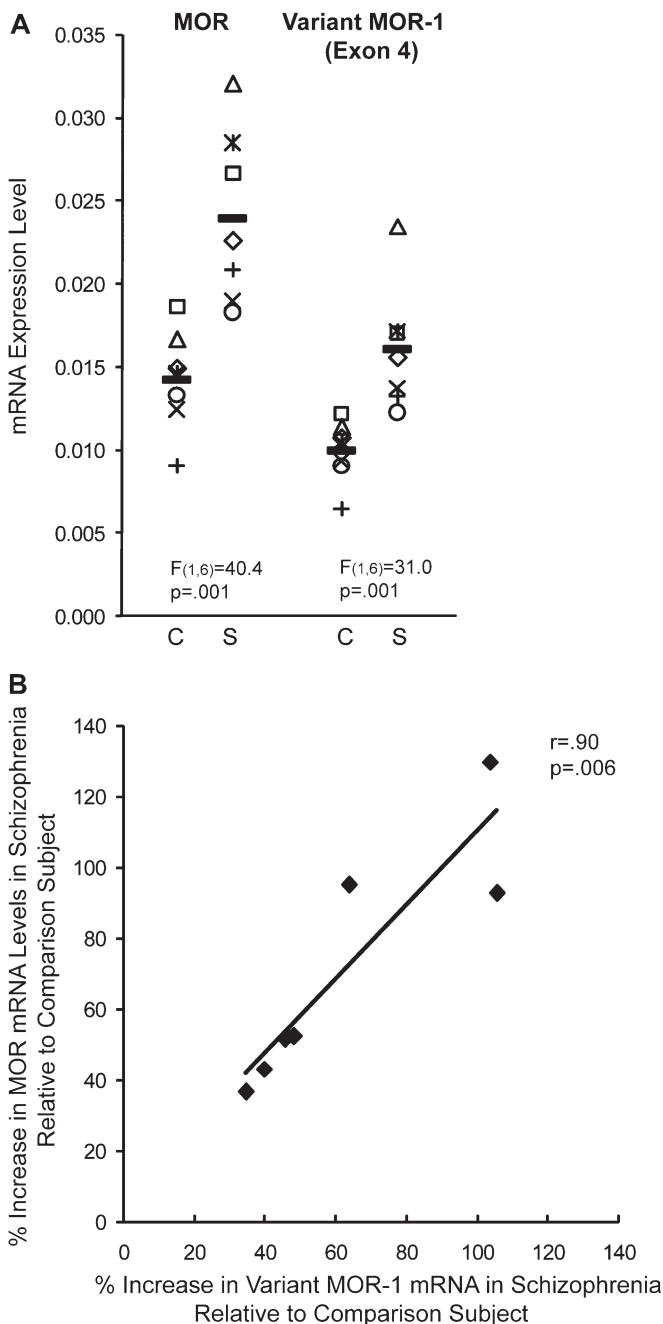


Figure 2. Expression levels of exon 4-containing MOR mRNA in schizophrenia subjects. mRNA expression levels were determined in 7 schizophrenia subjects relative to comparison subjects using primer sets targeting the transmembrane region common to MOR mRNA splice variants (MOR primer set 1; Supplementary Table S2) and exon 4 of MOR mRNA (Supplementary Table S2), which appears to selectively target the MOR-1 splice variant in human PFC. (A) An ANCOVA with mRNA level as the dependent variable, diagnostic group as the main effect, and subject pair as a blocking factor revealed significant elevations in exon 4-containing MOR mRNA levels in schizophrenia subjects. Data points that share a symbol indicate subjects in the same pair. Horizontal black lines indicate the mean mRNA expression level for the diagnostic group. (B) Differences in MOR mRNA expression levels and splice variant MOR-1 mRNA expression levels between schizophrenia and matched healthy comparison subjects were highly correlated.

Effects of Substance Abuse and Psychotropic Medications on MOR mRNA Levels

We also evaluated whether higher MOR mRNA levels were present in schizophrenia subjects with or without a history of substance abuse and psychotropic medications ATOD. We found that MOR mRNA levels were similarly and significantly higher in all subsets of schizophrenia subjects relative to matched comparison subjects regardless of the presence of a comorbid diagnosis of substance abuse or dependence ATOD; history of cannabis use; nicotine use ATOD; or use of antipsychotic, antidepressant, or benzodiazepine medications ATOD (Fig. 3; Supplementary Table S4). Furthermore, within-subject pair differences in MOR mRNA levels did not differ between subsets of schizophrenia subjects for any of these variables (for all, $t < 1.8$, $P > 0.08$). Only one schizophrenia subject in our cohort had a diagnosis of opioid abuse or dependence (subject 581; Supplementary Table S1). Medical records indicate that opioid medications were prescribed ATOD for 2 comparison subjects (818-morphine and 988-hydrocodone) and one schizophrenia subject (781-fentanyl and meperidine), and toxicology results indicated the presence of opioids in one comparison subject (551-morphine in urine) and in 2 schizophrenia subjects (781-meperidine in serum and 581-propoxyphene in urine). Exclusion of the pairs containing these 5 subjects still resulted in higher MOR mRNA levels (+23%) in the schizophrenia subjects ($F_{1,33} = 7.4$, $P = 0.011$).

Only 6 schizophrenia subjects in our study were off antipsychotic medications ATOD. Therefore, we evaluated whether PFC MOR mRNA levels are affected by antipsychotic medications in monkeys chronically exposed to haloperidol, olanzapine, or placebo. We found that MOR mRNA levels were similar ($F_{2,10} = 0.27$, $P = 0.77$) in haloperidol-exposed (0.0143 ± 0.003) and olanzapine-exposed monkeys (0.0152 ± 0.002) compared with placebo-exposed monkeys (0.0154 ± 0.004 ; Supplementary Fig. S3).

Aging and MOR mRNA

MOR protein levels in the frontal cortex have been reported to increase with age in adult humans (Gross-Isseroff et al. 1990; Gabilondo et al. 1995), and we also found a prominent effect of aging on MOR mRNA expression in human PFC (Supplementary Fig. S4). In healthy subjects, MOR mRNA levels steadily rose with age ($r = 0.89$, $P < 0.001$), and MOR mRNA expression was highest in the oldest healthy subject (82 years old; 0.0181) and lowest in the youngest healthy subject (19 years old; 0.0058)—an increase of greater than 300% through the adult aging process. In schizophrenia subjects, a similar, though somewhat weaker, correlation of MOR mRNA levels and age was also found ($r = 0.44$, $P = 0.003$). Diagnostic group differences in MOR mRNA levels were largest in the youngest age group (<40 year old) and smallest in the oldest age group (≥ 60 year old), and MOR mRNA levels in the oldest schizophrenia group were not statistically different from matched comparison subjects (Supplementary Fig. S4 and Table S4).

Predictors and Measures of Disease Severity and MOR mRNA Levels in Schizophrenia

We also evaluated whether higher MOR mRNA levels were associated with a more severe illness course in schizophrenia. Information available from psychological autopsy included 1)

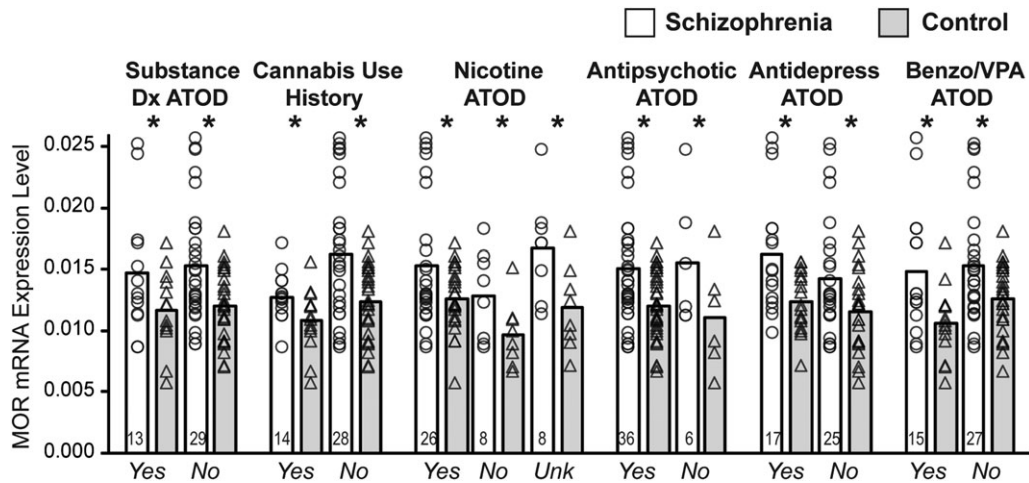


Figure 3. Effects of substance abuse and psychotropic medications on MOR mRNA expression levels in schizophrenia. Schizophrenia subjects and matched comparison subjects were divided in subsets of subjects based on the presence in the schizophrenia subject of substance abuse or dependence ATOD; history of cannabis use; nicotine use ATOD; and antipsychotic, antidepressant, or benzodiazepine and valproic acid medications ATOD. MOR mRNA levels for schizophrenia subjects within each of these subgroups were then compared with MOR mRNA levels in their matched comparison subjects. Open columns and gray columns denote the average MOR mRNA expression level in the schizophrenia and corresponding comparison subjects, respectively. Open circles and open triangles denote MOR mRNA expression levels in individual schizophrenia and healthy comparison subjects, respectively. Number of subjects in each subgroup are provided in the open column. * denotes $P < 0.05$. Abbreviations: Dx, diagnosis; ATOD, at time of death; Benzo, benzodiazepine; VPA, valproic acid.

predictive factors of a greater illness severity such as male sex, schizophrenia rather than schizoaffective disorder diagnosis, family history of schizophrenia, and earlier age of onset; and 2) more direct measures of disease severity and functional impairment, such as suicide as cause of death, no history of marriage, lower socioeconomic status, and inability to live independently (Sadock BJ and Sadock VA 2003). Relative to matched comparison subjects, MOR mRNA levels were similarly higher in schizophrenia subjects with/without a first degree relative with schizophrenia; onset of illness before or after age 18; suicide or other cause of death; with/without history of marriage; and high or low socioeconomic status as indicated by the Hollingshead Two Factor Index of Social Position (Supplementary Table S4). However, MOR mRNA levels were significantly higher than comparison subjects in males and subjects with “pure” schizophrenia but showed only trend increases in females and subjects with schizoaffective disorder (Supplementary Table S4). No effect of a diagnosis by sex interaction on MOR mRNA expression was found ($F_{1,80} = 0.57, P = 0.45$). Interestingly, relative to matched comparison subjects, although MOR mRNA levels were significantly higher in schizophrenia subjects not living independently ATOD (+34%; $t_{30} = 5.2, P < 0.001$), MOR mRNA levels did not differ in schizophrenia subjects living independently ATOD (+7%; $t_{10} = 0.57, P = 0.29$).

Relationship between MOR and GAD67 mRNA Levels in the PFC in Schizophrenia

MOR activation suppresses GABA signaling (Glickfeld et al. 2008), and lower GAD67 mRNA levels are a highly replicated finding in the PFC in schizophrenia (Akbarian et al. 1995; Volk et al. 2000; Duncan et al. 2010), including in the current cohort of 42 schizophrenia subjects (Curley et al. 2011). Consequently, we sought to determine whether a relationship may exist between higher MOR mRNA and lower GAD67 mRNA levels in schizophrenia subjects as quantified by qPCR (Curley et al. 2011). We found a significant negative correlation between within-subject pair differences in MOR mRNA and

GAD67 mRNA levels ($r = -0.41, P = 0.007, n = 42$ subject pairs) (Supplementary Fig. S5). Furthermore, a significant negative correlation was found between relative MOR mRNA and GAD67 mRNA expression levels in the schizophrenia subjects ($r = -0.39, P = 0.011, n = 42$ subjects) but not in the control subjects ($r = 0.17, P = 0.29; n = 42$ subjects) (Supplementary Fig. S5), suggesting convergent presynaptic mechanisms for downregulating GABA signaling in schizophrenia.

Postnatal Development of MOR mRNA Levels in Monkey PFC

We also examined the developmental trajectory of MOR mRNA levels from the neonatal period to adulthood in the PFC of female macaque monkeys, which recapitulates the cytoarchitecture and protracted development of human PFC. Statistical analysis revealed a significant effect of age group on MOR mRNA levels in monkey PFC ($F_{7,41} = 12.0, P < 0.001$). Post hoc comparisons revealed a significant 71% decrease in MOR mRNA levels from the 1 week old to 8–9 month old age groups (Fig. 4). MOR mRNA levels were relatively stable from pre- and early adolescence to late adolescence (8–48 months). Finally, similar to the aging process in adult human PFC, MOR mRNA levels were significantly higher (+72%) in adult compared with late adolescent monkeys.

Discussion

In a large cohort of subjects, we found higher MOR mRNA levels in the PFC of schizophrenia subjects relative to healthy comparison subjects, and this finding was validated using multiple primer sets, including a primer set targeting the predominant splice variant, MOR-1. Higher MOR mRNA levels appear to be a remarkably consistent feature of the illness and are present in schizophrenia subjects regardless of the presence or absence of substance abuse (including exposure to prescribed opioids) or psychotropic medications. In addition, higher MOR mRNA levels in schizophrenia subjects were largely independent of markers of illness severity,

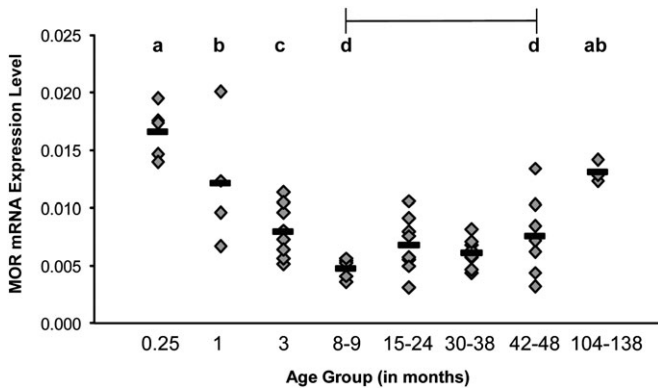


Figure 4. MOR mRNA expression levels across postnatal development in the PFC of female monkeys. MOR mRNA expression levels in PFC area 10 from 49 individual monkeys are plotted in age-defined groups. Mean values are shown as horizontal black bars. Statistical analysis revealed a significant ($F_{7,41} = 12.0, P < 0.001$) effect of age group on MOR mRNA expression levels. Age groups not sharing the same letters (a-d) are significantly different ($P < 0.05$) in MOR mRNA expression levels. Note the prominent decline in MOR mRNA expression levels from 1 week to 8-9 months (-71%), following by a period of relatively stable MOR mRNA expression levels across adolescence (8-48 months) and subsequent age-related increase in MOR mRNA expression levels (+72%) from late adolescence (42-48 months) to middle adulthood (104-138 months).

suggesting that this finding is a part of the disease process rather than a consequence of illness chronicity. Furthermore, alterations in the PFC opioid system in schizophrenia appear to be specific for MOR since we found no changes in mRNA levels for DOR and proenkephalin, and a prior report found no differences for the κ opioid receptor and the opioid ligand prodynorphin (Peckys and Hurd 2001). These data suggest that higher MOR mRNA levels reflect a selective alteration in one component of opioid signaling that may contribute to altered PFC function in schizophrenia.

In schizophrenia, higher MOR mRNA levels are likely to be accompanied by a corresponding increase in protein levels due to transcriptional regulation of MOR activity (Law et al. 2004). Consistent with this interpretation, both mRNA (present study) and protein (Gross-Isseroff et al. 1990; Gabilondo et al. 1995) levels of MOR increase with age in human PFC. Increased MOR activity is likely to have a suppressive effect on PFC GABA neuron function in schizophrenia (Fig. 5). For example, MOR activation has been reported to 1) reduce GABA neurotransmitter release from axon terminals (Capogna et al. 1993; Lupica 1995), 2) hyperpolarize interneuron cell bodies which reduces neuronal excitability (Wimpey and Chavkin 1991; Glickfeld et al. 2008), and 3) reduce the power of gamma oscillations (Whittington et al. 1998; Gulyas et al. 2010). Unfortunately, the cell-type-specific localization of MOR in human and monkey PFC has not been studied, and we have found MOR transcript levels to be generally too low to permit definitive dual-label in situ hybridization studies of MOR mRNA expression in subtypes of neurons in human and monkey PFC. However, studies in rat brain have localized MOR to the axon terminals and somatodendritic region of most PV and SST neurons but not in pyramidal neurons or glial cells (Drake and Milner 1999, 2002; Stumm et al. 2004). Thus, higher MOR activation may serve as another factor contributing to dysfunction of PFC GABA neurons in schizophrenia.

These data also raise consideration of whether MOR may represent a possible novel treatment target for cognitive deficits in schizophrenia. For example, a MOR antagonist may

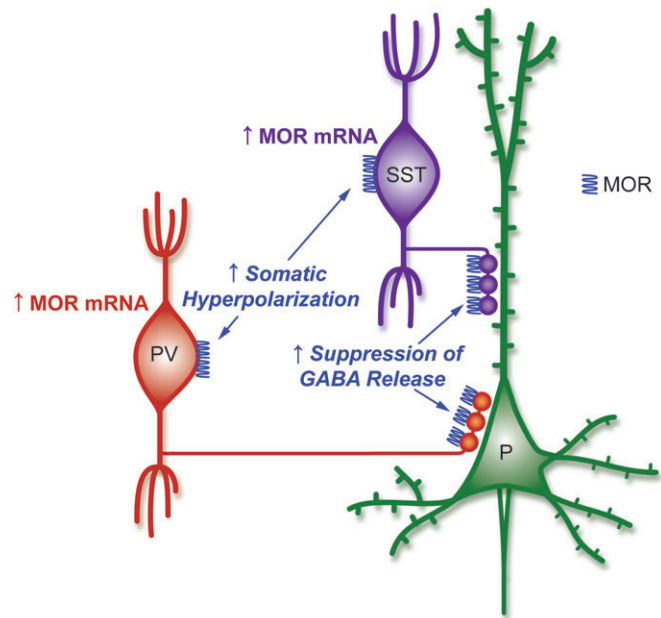


Figure 5. Schematic of alterations in MOR in schizophrenia and the potential downstream impact on GABA neurotransmission. Studies in rat brain have found that MOR are primarily found in the axon terminals and somatodendritic region of virtually all parvalbumin (PV)- and most somatostatin (SST)-containing neurons but not in pyramidal neurons (P) or glial cells (Drake and Milner 1999, 2002; Stumm et al. 2004). MOR activation suppresses GABA release from axon terminals and hyperpolarizes the cell body that reduces responsiveness to excitatory input (Lupica 1995; Glickfeld et al. 2008). In schizophrenia, higher MOR mRNA levels, if accompanied by a corresponding increase in protein levels, may both suppress GABA release from PV and SST axon terminals and hyperpolarize their cell bodies, reducing their ability to fire in response to excitatory inputs.

reduce somatic hyperpolarization of GABA neurons, enhancing their ability to fire in response to otherwise deficient excitatory inputs (Lisman et al. 2008; Bitanirhirwe et al. 2009). In addition, a MOR antagonist may enhance vesicular release of GABA from inhibitory axon terminals, which may help compensate for deficient GABA synthesis in the disorder (Akbarian et al. 1995; Guidotti et al. 2000; Volk et al. 2000; Lewis et al. 2005; Duncan et al. 2010; Curley et al. 2011). Interestingly, earlier studies involving administration of a MOR antagonist to patients with schizophrenia found some improvements in, or at least a lack of exacerbation of, positive and negative symptoms (Davis et al. 1977; Emrich et al. 1977; Gunne et al. 1977; Janowsky et al. 1977; Kurland et al. 1977; Volavka et al. 1977; Watson et al. 1978; Rapaport et al. 1993; Marchesi et al. 1995; Petrakis et al. 2004; Batki et al. 2007). These studies were in part limited by small sample size, brief duration of treatment (often including one time intravenous administration of the short acting MOR antagonist naloxone), and/or inclusion of mostly subjects with alcohol dependence. However, no studies to our knowledge have yet investigated the potential beneficial effects of a MOR antagonist on cognitive functioning in schizophrenia.

Some studies have suggested that cognitive impairments in schizophrenia may reflect disturbances in the development of PFC circuitry. For example, working memory function normally improves through adolescence (Diamond 2002; Luna et al. 2004) and is associated with increasing involvement of PFC circuitry (Alexander and Goldman 1978; Alexander 1982; Crone et al. 2006). Furthermore, in schizophrenia, working memory impairments are present before the onset of psychosis (Reichenberg et al. 2010). In addition, some PFC circuitry

markers that undergo marked developmental changes prior to adolescence are also disturbed in schizophrenia. For example, mRNA levels for PV, the GABA synthesizing enzyme GAD67, and the $\alpha 1$ subunit found in the majority of GABA-A receptors all rise across development in the normal PFC but are reduced in the PFC of schizophrenia subjects (Akbarian et al. 1995; Volk et al. 2000; Huang et al. 2007; Hashimoto et al. 2009; Beneyto and Lewis 2011; Duncan et al. 2010; Fung et al. 2010). Interestingly, exactly the opposite pattern was observed in our study where we found a marked decline in MOR mRNA levels from the neonatal period to adolescence in the PFC of female monkeys (tissue from male monkeys at different developmental stages was not available for this study) and higher MOR mRNA levels in the PFC in schizophrenia. These data invite speculation that higher MOR mRNA levels may in part reflect incomplete maturation of PFC neural circuitry in schizophrenia (Beneyto and Lewis 2011). If so, then MOR antagonists might represent a rational preemptive intervention for high-risk individuals in the premonitory or prodromal phases of the illness. However, given that MOR mRNA levels continue to rise with aging across adulthood in human PFC, higher MOR mRNA levels in schizophrenia could alternatively reflect a premature aging process in the disorder.

Importantly, exploration of MOR as a potential treatment target in schizophrenia also requires a clearer understanding of the pathogenetic origin of higher PFC MOR mRNA levels in schizophrenia. While alterations in cortical GABA neurons may represent a primary defect in the disorder, perhaps due to impaired maturation as discussed previously, alternate explanatory hypotheses merit consideration. For example, alterations in MOR mRNA levels and other GABA markers may also represent a compensatory, homeostatic response to deficits in glutamatergic neurotransmission in schizophrenia. Some studies have reported reduced glutamatergic input to PV neurons in schizophrenia (Lisman et al. 2008; Bitanirwe et al. 2009), and animal models have demonstrated that reduced excitatory drive to PV neurons during adolescence can result in lower GAD67 levels (Belforte et al. 2010), suggesting that deficient excitatory neurotransmission may lead to a compensatory downregulation of inhibition in schizophrenia. If higher MOR mRNA levels are present in GABA neurons in schizophrenia (as suggested by the correlation of MOR and GAD67 mRNA levels and by the anatomical localization of MOR in rat brain [Drake and Milner 2002; Stumm et al. 2004]), then increased MOR expression and lower GABA synthesis may represent convergent approaches to suppress GABA signaling in an attempt to normalize PFC excitatory-inhibitory balance in schizophrenia. Further testing of this hypothesis may reveal whether, and when in the course of the illness, pharmacological manipulations of MOR signaling may be useful for treating cognitive dysfunction in schizophrenia.

Supplementary Material

Supplementary material can be found at: <http://www.cercor.oxfordjournals.org/>

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