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Lamina- and cell-specific alterations in cortical somatostatin receptor 2 mRNA expression in schizophrenia

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ABSTRACT

Disturbed cortical γ-aminobutyric acid (GABA) neurotransmission in schizophrenia is evident from lamina- and cell type- specific alterations in presynaptic markers. In the dorsolateral prefrontal cortex (DLPFC), these alterations include lower transcript expression of glutamic acid decarboxylase (GAD67) and somatostatin (SST), a neuropeptide expressed in the Martinotti subpopulation of GABA neurons whose axons innervate the distal apical dendrites of pyramidal neurons. However, whether the alterations in SST-containing interneurons are associated with changes in post-synaptic receptors for SST has not been examined. Thus, we used *in situ* hybridization to quantify the mRNA expression levels of SST receptors subtype 1 (SSTR1) and subtype 2 (SSTR2) in DLPFC area 9 from 23 matched pairs of subjects with schizophrenia and normal comparison subjects. We also assessed the effects of potential confounding variables within the human subjects and in brain specimens from macaque monkeys with long term exposure to antipsychotic drugs. SSTR1 mRNA levels did not differ between subject groups. In contrast, mean cortical SSTR2 mRNA levels were significantly 19% lower in the subjects with schizophrenia. Laminar and cellular level analyses revealed that lower SSTR2 mRNA levels were localized to pyramidal cells in cortical layers 5–6. Expression of SSTR2 mRNA did not differ between monkeys exposed chronically to high doses of haloperidol or olanzapine and control animals, or between subjects with schizophrenia on or off antipsychotic medications at the time of death. However, levels of SSTR2 mRNA were significantly 37.6% lower in monkeys exposed chronically to low dose haloperidol, suggesting that the lower levels of SSTR2 mRNA selectively in pyramidal neurons in DLPFC layers 5–6 in schizophrenia should be interpreted with caution. In concert with prior findings of lower SST mRNA expression in the same subjects, the results of this study suggest the convergence of pre- and post-synaptic mechanisms to reduce inhibitory inputs to pyramidal neurons in the infragranular layers of the DLPFC.

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1. Introduction

Dysfunction of the dorsolateral prefrontal cortex (DLPFC) in schizophrenia is associated with abnormalities in cortical γ-aminobutyric acid (GABA) neurotransmission. These abnormalities include lower levels of the mRNA for the key enzyme regulating GABA synthesis, the 67 kDa isoform of glutamic acid decarboxylase (GAD_{67}). Several lines of evidence suggest that these alterations are not present in the ~50% of GABA neurons that express calretinin (Hashimoto et al., 2008; Sakai et al., 2008; Woo et al., 1998), but are prominent in a subset (~25–35%) of interneurons that express either the calcium-binding protein parvalbumin (Hashimoto et al., 2008) or the neuropeptide somatostatin (Morris et al., 2008). For example, a reduction in the expression of SST mRNA in DLPFC, initially described by microarray analysis (Hashimoto et al., 2008), has been confirmed by real-time qPCR and *in situ* hybridization (Hashimoto et al., 2008; Morris et al., 2008). However, whether the alterations in presynaptic markers of inhibitory neurotransmission in SST-containing interneurons are associated with alterations in post-synaptic receptors for SST has not been yet examined.

The effects of SST are mediated by five known G-protein coupled receptor subtypes (SSTR1-5) (Moller et al., 2003); stimulation of these receptors appears to have an inhibitory effect on neuronal excitability (Baraban and Tallent, 2004; Vezzani and Hoyer, 1999). SSTR1 and SSTR2 subtypes are the most abundant subtypes in the cerebral cortex (Videau et al., 2003), and although both are present in pyramidal and nonpyramidal neurons (Hervieu and Emson,

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1998a,b; Schindler et al., 1997), these receptor subtypes appear exhibit a high degree of specialization with regard to their cellular expression (Kumar, 2005) and subcellular targeting (Schulz et al., 2000). For example, SSTR2 immunolabeling is present mainly in pyramidal neurons, suggesting a major postsynaptic role in these neurons, whereas SSTR1 shows comparable expression in both pyramidal cells and interneurons (Kumar, 2005). In addition, SSTR1 immunoreactivity is found on varicose axons, suggesting a presynaptic role for this receptor (Schulz et al., 2000).

Consequently, in this study we used *in situ* hybridization to examine the lamina- and cell-specific expression levels of SSTR1 and SSTR2 transcripts in the DLPFC from schizophrenia and matched normal comparison subjects in which we had previously measured SST mRNA expression using an identical approach (Morris et al., 2008).

2. Material and methods

2.1. Human subjects

Brain specimens from 46 subjects were obtained after consent from the next-of-kin during autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA). 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.

In order to control for experimental variance and to reduce biological variance between groups, each subject in the schizophrenia group ($n = 23$), which included 8 subjects diagnosed with schizoaffective disorder, was matched for sex and, as closely as possible, for age, with one normal comparison subject (for demographic details, see Table 1). Subject groups did not differ in mean age, postmortem interval (PMI), RNA integrity number (RIN), brain pH, or tissue storage time at −80 °C (Table 1).

2.2. Tissue preparation

For each brain specimen, coronal blocks from the right frontal cortex were frozen and stored at −80 °C. Serial cryostat sections (20 μm) containing the superior frontal gyrus were thaw-mounted onto glass slides and stored at −80 °C until processed. Adjacent sections were collected into tubes containing Trizol (Invitrogen Corp, Carlsbad, CA) in order to obtain RNA for RIN measures using the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's protocol as previously described (Hashimoto et al., 2008). The location of DLPFC area 9 was identified by cytoarchitectonic criteria in Nissl-stained sections as previously described (Glantz et al., 2000; Volk et al., 2001, 2000b). Three sets of adjacent sections per subject, separated at rostro-caudal intervals of approximately 300 μm, were matched within subject pairs and used to assess SSTR1 and SSTR2 mRNA expression.

2.3. In situ hybridization

Templates for the synthesis of the antisense and sense riboprobes for human SSTR1 and SSTR2 mRNAs were first generated by polymerase chain reaction (PCR). The specific primers amplified 484 and 247 base pair fragments of human SSTR1 and SSTR2, respectively. These fragments corresponded to bases 886–1370 of the human SSTR1 (GenBank NM_001049) and 1018–1264 of the human SSTR2 (GenBank NM_001050) genes. Nucleotide sequencing confirmed 100% homology of the amplified fragment to the previously reported sequences. The fragments were then subcloned into a plasmid (pSTBlue-1, Novagen, Madison, WI). The antisense and sense riboprobes were transcribed in the presence of ^{35}S-CTP (Amersham Biosciences, Piscataway, NJ) using T7 and SP6 RNA polymerase, respectively. DNase I was used to digest the DNA template. The riboprobes were purified using RNeasy mini-spin columns (Qiagen, Valencia, CA).

Table 1

Parameter	Comparison	Schizophrenia[a]
Sex	17 M, 6 F	17 M, 6 F
Race	18 W, 5 B	15 W, 8 B
Age (years)	48.0 (15.5)	47.9 (14.1)
PMI (hours)	18.0 (5.5)	17.8 (9.3)
Brain pH	6.9 (0.2)	6.8 (0.3)
RIN	8.7 (0.4)	8.4 (0.7)
Storage time (months at −80 °C)	113.6 (23.5)	117.8 (23.5)

Note: Abbreviations: PMI, postmortem interval; RIN, RNA integrity number. Values are mean (±SD).

[a] This group includes 8 individuals with diagnosed with schizoaffective disorder.

One section from each subject pair was processed side-by-side in three separate runs for each gene of interest. Prior to the hybridization reaction, tissue sections were fixed with 4% paraformaldehyde in PBS solution, acetylated with 0.25% acetic anhydrate in 0.1 M triethanolamine/0.9% NaCl for 10 min, dehydrated with a graded alcohol series, and then defatted in chloroform for 10 min. The sections were then hybridized with ^{35}S-labeled riboprobes (1.0×10^6cpm/μl) in hybridization buffer at 56 °C for 16 h. The hybridization buffer contained 50% formamide, 0.75 M NaCl, 20 mM 1,4-piperazine diethane sulfonic acid, pH 6.8, 10 mM EDTA, 10% dextran sulfate, 5× Denhardt's solution (0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml BSA), 50 mM dithiothreitol, 0.2% SDS, and 100 μg/ml yeast tRNA. Following the hybridization reaction, sections were washed in a solution of 0.3 M NaCl, 20 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 50% formamide at 63 °C, treated with RNase A (20 μg/ml) at 37 °C, washed in 0.1 X SSC (1.5 mM NaCl, 150 μM sodium citrate) at 67 °C, dehydrated through a graded ethanol series, and air dried. Sections from both subjects in a pair were exposed on the same BioMax MR film (Kodak, Rochester, NY) for 7 days, and then coated with undiluted NTB2 emulsion (Kodak) for SSTR2 and NTB2 emulsion diluted 2:1 with water for SSTR1. Utilizing DLPFC sections from normal comparison subjects, different emulsion exposure times were systematically evaluated in order to achieve an optimal signal to noise ratio. The emulsion was exposed for 77 and 71 days at a constant temperature of 4 °C for SSTR1 and SSTR2, respectively. The slides were developed with D-19 (Kodak) and counterstained with Cresyl-violet.

2.4. Quantification of mRNA expression levels

Each section was randomly coded, so that subject number and diagnosis were unknown to a single rater. Trans-illuminated autoradiographic film images were captured by a video camera under precisely controlled conditions, digitized, and quantified using a Microcomputer Imaging Device (MCID) system (Imaging Research Inc., London, ON, Canada). Quantification was performed without knowledge of subject diagnosis by random coding of the sections. Images of Nissl-stained sections were also captured and superimposed onto the autoradiographic images to draw contours of the full thickness of the cortex exclusively in the zones where the cortex was cut perpendicular to the pial surface. Optical density (OD) was measured within the contours and expressed as nCi/g of tissue by reference to radioactive ^{14}C standards (ARC Inc., St Louis, MO, USA) exposed on the same autoradiographic film. The mean (SD) total area of gray matter sampled in each subject was 360 (178) and 344 (117) mm^2 for SSTR1, and 358 (185) mm^2 and 304 (91) mm^2 for SSTR2, in comparison subjects and subjects with schizophrenia, respectively.

2.5. Laminar analysis of mRNA expression

SSTR1 and SSTR2 mRNA expression as a function of cortical layer was determined in a series of cortical traverses (1–2 mm in width) extending from the pial surface to the white matter. Three cortical traverses were sampled for each section (9 traverses per subject). Each traverse was divided into 50 equal bins parallel to the pial surface and the OD was determined for each bin. These bins were then combined into six zones that approximated the laminar boundaries in the DLPFC based on previous studies (Akbarian et al., 1995; Pierri et al., 1999). These zones (i.e., bins 1–5, 6–10, 11–25, 26–30, 31–40, and 41–50) corresponded to cortical layers 1–6, respectively. The mean OD was calculated for each zone. Background measures in each section were sampled from layer 1, where no specific expression of mRNA for either receptor subtype was observed. All sampled areas for both total gray and laminar analyses were corrected by subtracting the corresponding background measure from the same slide.

2.6. Cellular analysis of mRNA expression

Evaluation of mRNA expression at the cellular level was performed for SSTR2 transcript in a subset of 13 subject pairs where the total cortical expression level was decreased by >15% in the subject with schizophrenia relative to the matched comparison subject. Silver grain accumulation on emulsion-dipped, Nissl-counterstained sections was conducted as previously described (Beneyto and Meador-Woodruff, 2006, 2008; Hashimoto et al., 2003; Morris et al., 2008). Briefly, using the MCID imaging software and a Zeiss microscope with a motorized stage, four 1 mm-wide cortical traverses extending from the pial surface to the white matter were placed on each tissue section in locations where area 9 was cut perpendicular to the cortical surface. In each of the cortical traverses, four sampling frames (120×170 μm) were systemically and randomly placed in deep layer 5-superficial layer 6 (defined as 70%–90% of the distance from the pial surface to the white matter border), corresponding to the laminar distribution of the major change in mRNA expression observed for SSTR2. The edges of the frames were equidistant from the border of each traverse and the edge of the next sampling frame.

Because RNase-A treatment during the in situ hybridization procedure degrades Nissl-stainable substances within the cytoplasm, it is not possible to draw contours around neuronal soma. Thus, the number of grains/cell was counted in each frame by placing circles over nuclei of cells in a bright field image. As previously described, grain clusters confined within a 22 μm diameter circle were considered to be interneurons and those within a 30 μm diameter circle were considered to be

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