Association of An Orexin 1 Receptor 408^{Val} Variant with Polydipsia–Hyponatremia in Schizophrenic Subjects

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Background: *Primary polydipsia is a common complication in patients with chronic psychoses, particularly schizophrenia. Disease pathogenesis is poorly understood, but one contributory factor is thought to be dopamine dysregulation caused by prolonged treatment with neuroleptics. Both angiotensin-converting enzyme (ACE) and orexin (hypocretin) signaling can modulate drinking behavior through interactions with the dopaminergic system.*

Methods: *We performed association studies on the insertion/deletion (I/D) sequence polymorphism of ACE and single nucleotide polymorphisms within the prepro-orexin (HCRT), orexin receptor 1 (HCRTR1), and orexin receptor 2 (HCRTR2) genes. Genotypes were determined by polymerase chain reaction amplification, followed by either electrophoretic separation or direct sequencing.*

Results: *The ACE I/D polymorphism showed no association with polydipsic schizophrenia. Screening of the orexin signaling system detected a 408 isoleucine to valine mutation in HCRTR1 that showed significant genotypic association with polydipsic–hyponatremic schizophrenia (*p = *.012). The accumulation of this mutation was most pronounced in polydipsic versus nonpolydipsic schizophrenia (*p = *.0002 and* p = *.008, for the respective genotypic and allelic associations). The calcium mobilization properties and the protein localization of mutant HCRTR1 seem to be unaltered.*

Conclusion: *Our preliminary data suggest that mutation carriers might have an increased susceptibility to polydipsia through an undetermined mechanism.*

Key Words: Schizophrenia, drinking behavior, neuroleptics, hypocretin, angiotensin-converting enzyme, dopamine system

Polydipsia, or the excessive intake of fluids, is commonly observed in patients with psychoses, particularly those with chronic schizophrenia (de Leon et al 1994; Vieweg et al 1985). Simple polydipsia often goes unrecognized until patients develop the more severe condition, water intoxification, which manifests both neurological and psychiatric symptoms (Goldman 1991). This disorder is comparable to neuroleptic-induced tardive dyskinesia and malignant syndrome in the management of psychiatric illnesses and is responsible for nearly 20% of premature deaths among schizophrenic subjects (Vieweg et al 1985).

Little is known about causative genetic factors, but the existence of genetically polydipsic mice suggests that the condition might have a genetic basis (Silverstein and Sokoloff 1958). Other studies have suggested alcohol dependence, male gender, and smoking as co-associating factors (Ahmed et al 2001; Shinkai et al 2003). Polydipsia is thought to be a side effect of chronic neuroleptic treatment, generating a hypersensitivity to dopamine that alters hypothalamic function. The observation that clozapine, an atypical antipsychotic with limited dopamine D2 receptor activity, alleviates the symptoms of polydipsia, lends weight to this theory (Canuso and Goldman 1999). Additionally, the behavioral pattern of increased drinking episodes and clustering of these episodes without an increase in the volume ingested in each session suggests that polydipsia might represent a stereotypy, a compulsive and repetitive behavior often observed in rodents after dopaminergic stimulation (Shutty and Song 1997); however, there is no genetic evidence linking dopamine-related genes with polydipsia.

Verghese et al (1993) suggested that normal drinking behavior is modulated by the interaction of dopamine D2 receptors with angiotensin. This precursor molecule from the renin–angiotensin system is converted in two stages to angiotensin II (ATII), a powerful dipsogen (Simpson and Routtenberg 1978). The rate-limiting enzyme in this catalysis is the angiotensin-converting enzyme (ACE), and inhibitors of this enzyme have been shown to reduce water intake in both rats and humans (Greendyke et al 1998; Rowland et al 1994). Association studies based on an *ACE* insertion/deletion (I/D) polymorphism that controls ATII levels, however, are inconclusive. The first study described a modest association with simple polydipsia, and the second failed to confirm this finding (Ouyang et al 2001; Shinkai et al 2003).

Orexins (also known as hypocretins) play a role in animal feeding and drinking behavior. Kunii et al (1999) reported that central administration of orexin A in rats stimulated an acute increased water intake, with a longer-lasting effect than ATII. Similar studies in rats have noted an increase in both feeding and drinking behavior, as well as hyperlocomotion and stereotypy, after orexin administration (Dube et al 1999; Hagan et al 1999; Sakurai 1999; Sakurai et al 1998). Orexin neurons are specifically localized in the lateral hypothalamic area and the medial part of the zona incerta, both anatomical regions thought to be involved in the regulation of drinking and feeding behaviors (Gonzalez-

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Lima et al 1993; Sakurai et al 1998). Orexin neurons also send dense excitatory projections to all monoaminergic cell groups in the midbrain, including dopamine neurons (Nakamura et al 2000). This physiological role for orexin as a mediator of drinking behavior and its interaction with the dopaminergic system prompted us to examine the possible involvement of the orexin system in polydipsic schizophrenic subjects who were previously treated with dopamine blockers. We also analyzed the *ACE* I/D polymorphism to clarify the role of this variant in polydipsia.

Methods and Materials

Subjects

We selected 168 chronic schizophrenic subjects without apparent polydipsic behavior and with normal sodium serum levels who were institutionalized for at least 2 years before recruitment. Sodium levels were routinely measured every 1–3 months, depending on the hospital. The diagnosis of schizophrenia was made by consultation according to DSM-IV criteria and required the consensus of at least two experienced psychiatrists. Diagnostic reliability was confirmed among participating psychiatrists. All available medical records and information from relatives and hospital staff were also considered. The schizophrenic subjects included 83 men (aged 48.9 ± 11.7 years [mean ± SD]; duration of illness 24.8 ± 12.9 years) and 85 women (aged 46.6 ± 12.0 years; duration of illness 23.2 ± 13.1 years). Polydipsic subjects were selected from schizophrenic inpatients who had been hospitalized for at least 2 years before the study and showed hyponatremia ($Na^+ \leq 130$ mEq/L) at least once during the previous 2 years, in addition to observed excessive repetition of normal drinking behavior. No patients suffered from systemic diseases, nor were they treated with agents that could affect water–ion balance or disturb sodium homeostasis. Additionally, none of the schizophrenic subjects had received clozapine (this drug is not approved in Japan) or olanzapine (at the time of this study, olanzapine was not available in Japan), drugs that could potentially improve polydipsia (Canuso and Goldman 1999; Littrell et al 1997). Our polydipsia–hyponatremia group comprised 59 men (aged 48.8 ± 11.6 years; duration of illness 25.2 ± 11.4 years) and 23 women (aged 48.0 ± 10.6 years; duration of illness 24.9 ± 9.3 years). Control subjects were recruited from among hospital staff and volunteers who showed no evidence of psychoses during brief interviews with psychiatrists; this group comprised 90 men (aged 47.1 ± 8.5 years) and 90 women (aged 46.8 ± 10.3 years). All subjects were from central Japan. The study was approved by the Ethics Committee of RIKEN, and all participants provided written informed consent.

Mutation Analysis and Genotyping

For the *ACE* I/D polymorphism rs1799752, genomic fragments were amplified by polymerase chain reaction (PCR) with the forward primer 5′-AGACCACTCCCATCCTTTCT-3′ and reverse primer 5′-GGCCATCACATTCGTCAGAT-3′ (Genbank accession no. X62855) (Rigat et al 1990). Products were electrophoresed on a 2% agarose gel to detect the I allele of 470 base pairs (bp) and D allele at 182 bp. The first-round products were re-amplified with the *ACE* forward primer and a primer located within the inserted sequence (5′-AGAGACGGGGTTTCACCGTTTTAG-3′) and separated on 2% agarose, to accurately distinguish between the I/D and the D/D genotypes. The locations and sequences of primers used in the mutation screening of *HCRT* (Genbank accession no. AF118885*), HRCTR1* (AF202078-202084), and *HRCTR2* (AF202085-202091) are shown in Supplement 1. Details of amplification conditions are available upon request. Briefly, primer pairs covering 823 bp upstream of exon 1, exons 1 and 2, and 197 bp downstream of exon 2 for *HCRT* and primers for *HCRTR1* and *HCRTR2* covering all exons and flanking intronic sequences were used to amplify genomic fragments from subjects. The genotypes were determined by sequencing both deoxyribonucleic acid (DNA) strands with the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, California) and the ABI PRISM 3700 Genetic Analyzer (Applied Biosystems).

Statistical and Haplotype Structure Analyses

All empirical significance levels for genotypic and allelic distributions were assessed by the Fisher exact test. Hardy-Weinberg equilibrium was evaluated with Arlequin software (http://lgb.unige.ch/arlequin/) (Schneider and Excofier 2000). Genetic Power Calculator (Purcell et al 2003) (http://statgen.iop.kcl.ac.uk/gpc/) was used to compute statistical power. To examine linkage disequilibrium structures of *HCRTR1* in the Japanese population, we analyzed data on Japanese samples from the HapMap project (http://www.hapmap.org/). Haplotype block structures in the region were constructed with the Haploview program (Barrett et al 2005) (http://www.broad.mit.edu/mpg/haploview/).

Prediction of Phosphorylation Sites

The phosphorylation site prediction was calculated with the NetPhos 2 server (http://www.cbs.dtu.dk/services/NetPhos/).

Prediction of Messenger Ribonucleic Acid Folding

GENETYX-MAC version 12 software (GENETYX, Tokyo, Japan) was used to analyze the wild-type and polymorphic *HCRTR1* messenger ribonucleic acid (mRNA) sequences.

Preparation of Normal and Mutant *HCRTR1* Constructs

Five prime and 3′ *HCRTR1* sequences tagged with an *Eco*RI site and *Not*I site, respectively, were used to amplify complementary DNA from the SK-N-SH neuroblastoma cell line, known to be heterozygous for the 1222A>G mutation (forward 5′-ATGGAGGCCCGAATTCTGTAGAGCCTAGGAATGCCCCT-3′ and reverse 5′-CGGATAATGCGGCCGCAGGAAGTGACTTATCCAGAGT-3′) (Wieland et al 2002). Products were double digested with *Eco*RI and *Not*I and cloned into appropriately digested pIRESneo2 (BD Biosciences, Clontech, Palo Alto, California). Clones were generated under standard conditions and sequenced to check fidelity and to select constructs that contained position 1222A (408Ile) or 1222G (408Val).

Intracellular Ca^{2+} Measurement

COS7 cells were transiently transfected with either HCRTR1 408Ile or HCRTR1 408Val expressing constructs with the Fugene6 transfection kit (Roche, Indianapolis, Indiana). Cultures were grown for 24 hours, trypsinized, washed three times in *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES)-buffered saline and loaded with fura-2/AM (Molecular Probes, Eugene, Oregon) for 1 hour at room temperature. Cells were then washed again in HEPES-buffered saline, filtered and loaded into 96-well plates (30,000 cells per well). Agonist HCRT1 (0.1 nmol/L–10 μmol/L) was added to the cells, and the changes in intracellular Ca^{2+}-dependent fluorescence were recorded with a fluorescence imaging plate reader (FDSS6000; Hamamatsu Photonics, Shizuoka, Japan). Transfection efficiency was corrected for using the bombesin-like receptor construct and ligand, and dye loading was corrected for using uridine triphosphate (UTP)