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Regulation of a Novel α N-Catenin Splice Variant in Schizophrenic Smokers

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Abstract

The α N-catenin (*CTNNA2*) gene represents a promising candidate gene for schizophrenia based upon previous genetic linkage, expression, and mouse knockout studies. *CTNNA2* is differentially regulated by smoking in schizophrenic patients. In this report, the genomic structure of a primate-specific α N-catenin splice variant (α N-catenin III) is described. A comparison of α N-catenin III mRNA expression across postmortem hippocampi from schizophrenic and non-mentally ill smokers and non-smokers revealed a significant decrease in expression among patient non-smokers compared to all other groups. The recent evolutionary divergence of this gene, as well as the differences in gene expression in postmortem brain of schizophrenic non-smokers, supports the role of α N-catenin III as a novel disease susceptibility gene.

Keywords

schizophrenia; nicotine addiction; *CTNNA2*; evolution; primate

INTRODUCTION

Decades of research have demonstrated a significant genetic component in the development of schizophrenia [Harrison and Weinberger, 2005]. While progress has recently been made in identifying disease genes, candidates have not yet been identified at several loci achieving genome-wide significance in recent meta-analyses [Badner and Gershon, 2002; Lewis, 2003], including a region at 2p11–22. α N-catenin (*CTNNA2*), the gene closest to a peak linkage marker on chromosome 2p [DeLisi et al., 2002], may represent a promising candidate gene for schizophrenia.

Alpha-catenins are cytoskeletal proteins that mediate cell–cell and cell–matrix interactions [Takeichi, 1991]. They may also be involved in clustering of nicotinic acetylcholine receptors in the membrane [Zhang et al., 2007]. Three subtypes of α -catenins have been identified to date: α E-catenin (*CTNNA1*), expressed mainly by non-neural tissues [Nagafuchi et al., 1991], α N-catenin (*CTNNA2*), expressed across the central nervous system [Hirano and Takeichi, 1994; Uchida et al., 1994], and α T-catenin (*CTNNA3*), predominantly expressed in testes and

cardiac tissues [Janssens et al., 2001]. α N-catenin can be further subdivided into isoforms I and II, with the former predominating in expression during adulthood [Uchida et al., 1994].

The neuronal expression pattern and function of *CTNNA2* are consistent with a gene that could contribute to the disturbances in cognitive function characteristic of schizophrenia. *CTNNA2* is enriched in primate brain regions that exhibit a high degree of cortical folding and lamination [Smith et al., 2005]. The expression of this gene is critical for maintaining the stability of dendritic spines in rodent hippocampal neurons in culture. In the absence of *CTNNA2*, spines display abnormally rapid protrusion and retraction of filopodia, while the over-expression of this gene is associated with reduced spine turn-over [Abe et al., 2004]. *CTNNA2* also plays a role in regulating hippocampal development and sensorimotor gating. Gene deletions in mice are associated with hippocampal pyramidal cell disorganization and deficits in prepulse inhibition of the startle response [Park et al., 2002], two abnormalities that are observed in schizophrenic patients [Braff et al., 1978; Grillon et al., 1992; Hamm et al., 2001; Harrison, 2004].

Individuals with schizophrenia are most often smokers. The prevalence of smoking in schizophrenia is greater than 80%, compared to approximately 22% in the general population [Dalack et al., 1998; Leonard and Giordano, 2002; de Leon et al., 2002]. We have recently shown that *CTNNA2* is differentially regulated by smoking in schizophrenic patients compared to controls [Mexal et al., 2005]. Gene expression in human postmortem hippocampus from schizophrenic and control smokers and non-smokers was compared by micro-array analysis. There were 77 genes that were differentially regulated by smoking in schizophrenia. The patterns were generally the same; expression was either increased or decreased in schizophrenic non-smokers compared to controls and was brought to control levels or normalized by smoking. *CTNNA2* mRNA and protein expression were reduced in patient non-smokers compared to non-mentally ill subjects. In schizophrenic smokers, *CTNNA2* was expressed at control levels [Mexal et al., 2005]. Because cigarette smoking has been hypothesized as a form of self-medication among schizophrenics [Adler et al., 1993; Olincy et al., 1998; Leonard et al., 2001], *CTNNA2* and other genes that are differentially regulated by smoking in patient and control groups, may constitute risk factors underlying schizophrenia and the development of nicotine addiction in this patient population [Mexal et al., 2005].

In the current report we describe the genomic structure and human expression pattern of a primate-specific α N-catenin splice variant (α N-catenin III, GenBank ID: BX537769) and its regulation by smoking in schizophrenia. The nucleotide and amino acid sequence of this transcript was compared across species, and α N-catenin III mRNA expression was analyzed in postmortem hippocampal tissue from schizophrenic and non-mentally ill smokers and non-smokers.

MATERIALS AND METHODS

Human Postmortem Brain Collection

The Schizophrenia Research Center Brain Bank at the VA Medical Center in Denver, Colorado, provided postmortem brain tissue. Human brain was collected at autopsy from local services following family consent. At autopsy, the brain was weighed and examined for gross pathology. It was then divided sagittally and one hemisphere, selected randomly, was preserved in formalin for neuropathological analysis at the macroscopic and microscopic level. Microscopic evaluations included standard Bielchowsky silver stain on multiple cerebral areas to rule out abnormal neuropathology, such as plaques and tangles, associated with Alzheimer's and other conditions. Patients with positive neuritic findings or ambiguous neuropathology reports were excluded from the current study. The hemisphere that was not subjected to neuro-pathological analysis was sliced coronally into 1 cm slices, from which multiple regions were dissected in

1 g blocks, frozen in dry ice snow, and packaged for storage at -80°C . Hippocampal tissue was always derived from coronal section 5. Each sample included CA4, CA3, CA2, CA1, and subiculum. Brain hemisphere, left or right, was randomly collected for quantitative RT-PCR experiments.

Pre- and postmortem parameters were recorded for each subject from an extensive review of hospital, autopsy, and neuropathology reports, as well as structured interviews with physicians and family, as described in detail previously [Mexal et al., 2005, 2006]. These included diagnosis, age, sex, cause of death, postmortem interval, pH, freezer storage time, neuroleptic medication, and smoking history. Based upon this information, a DSM-IV diagnosis was confirmed by two independent psychiatrists (RGR and RF). Additionally, an agonal state score was assigned following the Hardy four-point rating scale [Hardy et al., 1985]. Briefly, the four agonal state categories included (1) violent and fast death, (2) fast death of natural causes, (3) intermediate death, and (4) slow death. Subjects utilized for the quantitative RT-PCR studies in each experimental group were matched as best possible utilizing these pre- and postmortem criteria (Table I).

Western Blot

Protein isolation and western blotting were performed as described [Mexal et al., 2005]. Briefly, frozen tissue was homogenized in NP-40 lysis buffer, consisting of 150 mM NaCl, 1% Amersham Nonidet P40, 50 mM Tris (pH 8.0), and deionized water to reach a volume of 1 L. Protease inhibitors (200 mg/ml aprotinin, 100 mg/ml pepstatin A, 50 mg/ml leupeptin, 50 mg/ml phenylmethylsulfonyl fluoride [PMSF]) were added to the freshly made NP-40 buffer. Samples were centrifuged for 5 min at 10,000 rpm at 4°C . A 1:10 dilution was prepared by combining 40 μl of the supernatant and 360 μl of the NP-40 buffer. Protein quantification was determined by the BCA protein assay kit (Pierce, Rockford, IL).

Protein extracts were resolved by gel electrophoresis, transferred to Hybond-P Polyvinylidene Fluoride (PVDF) transfer membranes (Amersham, Piscataway, NJ), and blocked for 1 hr in BLOTTO (5% powdered milk diluted with Tris-buffered saline with 0.5% Tween-20 [TBST]) at room temperature. Membranes were then incubated overnight at 4°C in BLOTTO including the appropriate primary antibody: goat anti-GAPDH (SC-20357; Santa Cruz Biotechnology, Santa Cruz, CA), 1:1,000 dilution, or goat anti- αN -catenin (SC-1498, Santa Cruz Biotechnology), 1: 500 dilution. Blots were rinsed in BLOTTO and incubated for 1 hr at room temperature in the donkey-anti-goat (Santa Cruz Biotechnology) peroxidase-conjugated secondary antibody. Membranes were processed using the ECL detection system (Amersham), and exposed to chemiluminescence film (Amersham). Films were digitized using a scanner (Epson, Long Beach, CA) and the resulting images were analyzed using UN-SCAN-IT gel digitizing software, version 5.1 (Silk Scientific, Inc., Orem, UT).

DNA Sequencing

DNA was isolated from human postmortem brain utilizing a standard method [Sambrook and Russell, 2001]. To sequence the novel *CTNNA2* splice variant, PCR was carried out using 200nM of forward and reverse primers (Table II), 1 μl of 500ng/ μl single stranded cDNA, as well as buffer from the GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA), which consisted of 0.2 mM dNTPs, 1 $\times$ buffer, 1.5mM MgCl₂, 0.25 units/ml AmpliGold Taq, and DEPC water to reach 25 μl volume reaction. The samples were amplified in a Perkin Elmer GeneAmp PCR 9600 System using the following conditions: 93°C for 3 min, 30 cycles of 93°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, followed by a 1 min incubation at 72°C and a 4°C hold at the end. The amplification of a single 139 base pair product (Table II) was confirmed by running an aliquot of the sample on a 6% acrylamide gel. The PCR product was also sequenced by dye-terminator cycle sequencing using ddNTPs

labeled with rhodamine-based dyes (ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems). A separate sequencing reaction was carried out with either the forward or the reverse primer (Table II). Each primer (3–5 pmol) was mixed with 1.0 µl of the PCR product generated above, 2.5 × terminator ready reaction mix, and 1.3 M betaine (Sigma–Aldrich Corp, St Louis, MO), in a total volume of 20 µl. Reaction mixtures were incubated in a GeneAmp PCR 9600 System (Perkin-Elmer) with the following PCR program of 25 cycles at 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min, with a final incubation at 4°C. Following the re-amplification, the contents of the tubes were briefly spun down in a micro-centrifuge and spin column purified to remove excess dye-labeled terminators and buffers (Performa DTR Gel Filtration Cartridges, Edge Biosystems, Gaithersburg, MA). Purified samples were loaded onto a polyacrylamide gel on the ABI 377 sequencer and sequencing data was collected and analyzed with the ABI 377 software.

Quantitative RT-PCR Procedure (QRT-PCR)

RNA was isolated using the Trizol Reagent manufacturer's protocol (Life Technologies, Gaithersburg, MD) and as described elsewhere [Mexal et al., 2005, 2006]. RNA was purified by utilizing the RNeasy mini kit (Qiagen, Valencia, CA). The integrity of total RNA was assessed at several steps. The ratio of 18S–28S ribosomal RNA was determined by gel electrophoresis. Housekeeping gene (GAPDH and α -actin) 3' to 5' hybridization ratios on the Affymetrix Test3 arrays (Affymetrix, Santa Clara, CA) were available for 27/28 of the subjects evaluated in the QRT-PCR analysis. GAPDH mRNA levels as determined by QRT-PCR were available for all 28 subjects.

Methods for cDNA synthesis, quantitative RT-PCR amplification and data collection were reported previously [Mexal et al., 2005, 2006]. Briefly, single strand cDNA was synthesized from total RNA using random hexamer primers and the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). To limit amplification from genomic DNA, primer sets for each gene were designed to cross intron/exon boundaries. Primers were designed for α N-catenin III and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table II) using the Oligo Analysis and Plotting Tools (<http://www.operon.com/oligos/toolkit.php?>). PCR amplification of cDNA was performed using 500 nM of primers and the Quantitect SYBR Green PCR Kit (Qiagen). PCR cycling conditions were 50°C for 2 min, 95°C for 15min, and 40 cycles of 94°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec. Formation of PCR products was monitored using an iCycler detection system (BioRad, Hercules, CA). All sample assays were performed in triplicate. For each subject, values for the gene of interest were normalized to the GAPDH mRNA levels. For each experiment, a tube without the addition of reverse transcriptase or without the addition of cDNA was included to assess amplification from genomic DNA and non-specific product formation, respectively. A melt curve analysis was also conducted to examine the uniformity of product formation, primer–dimer formation, and amplification of non-specific products.

Bioinformatics Analysis

Sequence similarity across species was evaluated using NCBI BLAST (<http://http://www.ncbi.nlm.nih.gov/blast/>). Genomic structures of α N-catenin isoforms across species were analyzed with Ensembl Genome Browser, version 9.30a.1 (<http://www.ensembl.org>). Alignment of nucleotide and amino acid sequences was performed with ClustalW program (www.ebi.ac.uk/clustalw/). Potential promoter sites were identified with the Promoter 2.0 prediction server (www.cbs.dtu.dk/services/promoter). Predicted peptide sequences were translated from nucleotide sequence using the ExpASY translate tool (<http://www.expasy.ch/tools/dna.html>). DNA sequences were screened for interspersed repeats, such as Alu repeats, utilizing RepeatMasker Program 3.0.8 (<http://www.repeatmasker.org>).

Statistical Analysis

All statistical analyses were performed using SPSS version 12 (SPSS, Chicago, IL). Measurements of RNA quality and pre-and postmortem continuous variables, including age, brain pH, freezer storage time, and postmortem interval, were evaluated by two-way ANOVA to identify any significant differences across the four groups included in the expression study (control non-smoker, control smoker, schizophrenic non-smoker, schizophrenic smoker). To further evaluate the potential confounding effects of these parameters, a bivariate correlation was also carried out between the variables and α N-catenin III mRNA expression. To determine whether gender or agonal state was significantly different across the mental illness and control groups a Fisher's exact test was performed. Relative expression values from the QRT-PCR study were $\log_{10}$ transformed and differences in mean gene expression were determined in planned comparisons based upon expression findings for other α N-catenin III transcripts by Student's *t*-test ($P < 0.05$, Excel).

RESULTS

Genetic Locus of *CTNNA2* and the Identification of a Novel Splice Variant

The α N-catenin gene (*CTNNA2*) lies approximately 0.11 Mb downstream from marker D2S139 on chromosome 2p (Fig. 1). D2S139 previously gave a peak lod score of 2.99 in a schizophrenia linkage study [DeLisi et al., 2002]. The genetic locus of *CTNNA2* is closer to D2S139 than any other gene on 2p (Fig. 1).

Ensembl lists three major α N-catenin transcripts (Fig. 2A), including a hypothetical differentially spliced α N-catenin cDNA identified in two separate human genome projects [Venter, 2001; Wambutt et al., 2003]. The expression of this transcript and its predicted 65 kDa protein has not yet been characterized. An α N-catenin isoform including all 19 exons has not been identified to date in NCBI and Ensembl, and was not detected by QRT-PCR or Western blot analysis in this report (data not shown). AlphaN-catenin was previously found to be differentially expressed in the postmortem hippocampus of schizophrenic non-smokers compared to patient smokers and non-mentally ill subjects [Mexal et al., 2005]. In this report, there was no significant difference in expression of α N-catenin by diagnosis [Mexal et al., 2005].

A 65 kDa peptide was detected in human hippocampus using an antibody targeting the carboxy terminus of the human α N-catenin peptide (Fig. 2B). This is the predicted size of the hypothetical protein for an α N-catenin splice variant (Gene-Bank GenPept ID: CAD97832). The 65 kDa protein was more abundantly expressed than the larger protein detected at approximately 100 kDa. The larger band most likely represents the 102 kDa α N-catenin I protein, as this peptide is more predominantly expressed in the adult brain compared to the 108 kDa α N-catenin II protein [Uchida et al., 1994]. However, because these peptides differ by only 48 amino acid residues, α N-catenin isoforms I and II may be difficult to distinguish with our standard Western blot technique. Unlike the larger α N-catenin I protein(s), the 65 kDa protein was absent in both mouse and rat brains (Fig. 2B). Neither protein was expressed in human fibroblast homogenates (data not shown). Although the specificity of the antibody cannot be guaranteed, BlastP analysis of the antibody's epitope sequence revealed 100% sequence identity (19/19) with the hypothetical protein (GenBank GenPept ID: CAD97832) coded for by the differentially spliced α N-catenin cDNA.

Genomic Structure of α N-catenin III and Sequence Comparison Across Species

To compare the genomic structure of these transcripts in silico, the exonic sequences were obtained from Ensembl and aligned using ClustalW. This comparison revealed that the splice variant is missing exons 1–6 (Fig. 2A). The exclusion of exons 1–6 removes the first 352 amino

acids of the full-length peptide, truncating 22% of the vinculin protein domain and 100% of the β -catenin binding domain [Taniguchi et al., 2005]. The actin cytoskeleton-binding domain is intact.

In addition to the exclusion of several exons, the transcript coding for the α N-catenin III splice variant contains a novel 5', 276 base pair insertion that is absent in isoforms I and II (Fig. 2A). The predicted peptide sequence of this exon failed to show sequence identity with any known conserved protein domains. The novel exon is located within intron 6 (79,831 base pairs from the start of exon 7, not shown) and is apparently spliced in utilizing a non-canonical TG 3'-splice site (Fig. 3A). Screening of the sequence for the novel exon in intron 6 with RepeatMasker failed to identify interspersed repeats. A bioinformatics examination of the 1,000 base pair sequence upstream of the novel exon revealed no putative promoter region.

BlastN analysis of the nucleotide sequence for the human novel exon across the genomes of several species, including mouse, rat, chicken, chimpanzee and rhesus monkey, failed to identify orthologs in non-primates. The chimpanzee novel exon is similar to the human exon at both the nucleotide (99.6%) and protein (100%) levels (Fig. 3A,B). The rhesus monkey novel exon is also similar to the human exon at the nucleotide level (94.9%), while the protein is missing the first 18 out of 31 amino acids according to Ensembl (Fig. 3A,B). The use of an alternate translational start site in the monkey compared to human and chimpanzee peptides may be due to a nucleotide difference at position +196 of the novel exon. If the upstream ATG were used, this G/A polymorphism would introduce a premature stop codon in the monkey α N-catenin III peptide, truncating the peptide after three residues. The exon/intron sequence is conserved across species at the 3' splice site, while the 5' splice site consists of a TG in chimpanzee and human and a TC in rhesus monkey (Fig. 3A). Compared with the entire human protein sequence, the sequence identity shared by the chimpanzee and rhesus monkey peptides is 99.1% and 96.9%, respectively (Fig. 3B).

The genomic structure of α N-catenin III was validated by PCR amplification. Hippocampal cDNA was amplified using primers spanning the novel exon to exon 7, generating a single PCR product (Fig. 4). Sequence analysis of this 139 base pair product confirmed that the novel exon was spliced in preceeding exon 7 (data not shown).

Comparison of α N-catenin III mRNA Expression in Postmortem Hippocampus of Control and Schizophrenic Smokers and Non-Smokers

SYBR green quantitative RT-PCR (QRT-PCR) was utilized to evaluate α N-catenin III mRNA expression in postmortem hippocampus of non-mentally ill and schizophrenic smokers and non-smokers. Overall, 28 postmortem human brains were evaluated in this study (Table I). Postmortem tissues from 27 of these subjects had been analyzed in a previous microarray study [Mexal et al., 2006]. Measurements of RNA quality, including α -actin and GAPDH 3'/5' test chip ratios, were not significantly different across the groups (GAPDH: $F=0.61$, $P=0.61$; α -actin: $F=1.20$, $P=0.32$). Additionally, GAPDH mRNA levels determined by QRT-PCR for all 28 subjects were also similar across groups ($F=0.10$, $P=0.96$). Age was significantly higher among patient smokers compared to non-smokers and tissue storage time was significantly lower in control non-smokers relative to smokers (Table I). These parameters, as well as other pre- and postmortem variables, were therefore considered in the expression analysis.

Consistent with our Western blot results, QRT-PCR analysis demonstrated that the α N-catenin III transcript was not expressed in the non-neuronal tissues evaluated, or in mouse whole brain (Fig. 5A). Post-mortem hippocampal expression of α N-catenin III was compared across control and schizophrenic smokers and non-smokers. Expression was not significantly different between smokers and non-smokers ($F_{1,24}=0.67$, $P=0.67$) or patients and controls ($F_{1,24}=0.94$, $P=0.51$). There was, however, a significant interaction of smoking $\times$ schizo-schizophrenia

($F_{1,24}=4.53$, $P=0.44$) on α N-catenin III mRNA expression. Post-hoc analysis revealed that expression was reduced in schizophrenic non-smokers relative to control nonsmokers ($P=0.01$) and schizophrenic smokers ($P=0.03$) (Fig. 5B). Overall, our findings did not appear to be related to age, brain pH, PMI, or storage time, as none of these variables were significantly correlated with α N-catenin III mRNA expression (Table III). We did not find a significant correlation in patients, controls, or in the combined groups, between mRNA expression and packs of cigarettes smoked daily (Table IV). The altered expression of α N-catenin III between schizophrenic non-smokers relative to patient smokers and control smokers also appeared to be independent of gender and alcohol use, as these parameters were not significantly different across these groups ($P=0.56$ to $P=1.00$, Fisher's Exact Tests). While the current sample size restricted a statistical evaluation of the effects of antipsychotic drug use on α N-catenin III mRNA expression, schizophrenic non-smokers, regardless of medication status, consistently displayed lower expression levels compared to control non-smokers (Fig. 5B).

DISCUSSION

This study reports the characterization of a novel α N-catenin splice variant, α N-catenin III, in human postmortem hippocampus. The α N-catenin III protein is more abundantly expressed than the other α N-catenin isoforms, suggesting that this splice variant may have a functional significance. While the role of this isoform remains unclear, α N-catenin III expression was affected by the interaction of smoking and schizophrenia, consistent with our previously published micro-array study [Mexal et al., 2005]. α N-catenin III may therefore contribute to the neurobiology of schizophrenia and/or tobacco dependence in the disorder.

The truncated domain in α N-catenin III could alter the protein-protein interactions, and hence the function, of this protein, compared to α N-catenins I and II. Isoform III is missing a putative β -catenin interaction region [Taniguchi et al., 2005]. However, a deletion of the NH₂ terminus expressed in oocytes was found to have no dominant negative effect and resulted in normal development, suggesting that the actin binding domain in the COOH terminus may be more important for α -catenin function [Sehgal et al., 1997]. Extensive alternative splicing at both the carboxy and N-terminus appears to be a general feature of the catenin gene family. Because α N-catenin III is absent in non-primates, it could be critical for the more complex human neuronal development and cognitive functioning that is generally disrupted in schizophrenia. Promising candidate genes for schizophrenia, including *G72*, *DISC1*, and *CHRNA7*, also display recent evolutionary divergence [Gault et al., 1998; Chumakov, 2002; Fortna et al., 2004; Bord et al., 2006]. Because schizophrenia is associated with disturbances in higher cognitive functions, it has been hypothesized that such recently diverged genes, including *CTNNA2*, represent significant factors in the inheritance of the disorder [Enard et al., 2002; Clark et al., 2003; Penny, 2004; Bord et al., 2006].

Although it is clear that the *CTNNA2* sequence has recently diverged, the evolutionary origin of the novel exon of α N-catenin III remains unknown. Based upon in silico analysis, this exon is derived from intron 6 in the *CTNNA2* gene. While one possible mode by which this exon may have arisen involves the insertion of a primate-specific short interspersed element (SINE), or other repeat element into the intronic sequence [Singer et al., 2004], we did not identify any interspersed repeats in the novel exon sequence.

The characterization of a primate-specific α N-catenin splice variant in this report, and converging evidence from previous genetic linkage and functional studies, suggest a function for α N-catenin in the pathophysiology of schizophrenia. *CTNNA2* is the most prominent gene in the schizophrenia linkage region on 2p. Mice null for this gene exhibit properties found in schizophrenic subjects, and *CTNNA2* functions in cognitive processes [Park et al., 2002]. Because α N-catenin III is abundantly expressed in human postmortem brain and is

differentially regulated by smoking in patients, this novel splice variant may play a role in the disorder. For example the novel exon could be an alternative β -catenin interaction site. Future experiments, including immunoprecipitation studies, will be conducted to confirm that the 65 kDa peptide identified in this report is indeed a novel *CTNNA2* splice variant. However, based upon the 100% epitope sequence identity and species specificity, it is likely that this peptide does represent a new *CTNNA2* isoform. Future studies will also investigate α N-catenin III mRNA findings in an extended cohort, including additional drug-naïve patients, allowing a more thorough evaluation of the effects of various pre- and postmortem parameters.

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Fig. 1. Proximity of the CTNNA2 locus to marker D2S139. Linkage to chromosome 2p was previously reported in a schizophrenia study, with marker D2S139 giving the peak lod score [DeLisi et al., 2002]. CTNNA2 is the closest gene to this marker, lying 0.11Mb downstream. The location of D2S139 on the Gene Map is shaded and also indicated with the square symbol ■.

Fig. 2.

Genomic structure of the major α N-catenin transcripts and protein expression of a novel α N-catenin splice variant. **A:** α N-catenin isoforms I and II are missing the novel exon, which is included only in isoform III. The sequence of this exon is 99.9% identical between humans and chimpanzees, but does not display any sequence similarities with non-primates. Both isoforms I and III are missing exon 17. The location of the N-catenin III quantitative RT-PCR and sequencing primers are shown in gray and black, respectively, below this transcript. The GenBank number for each transcript is listed next to each gene. The genomic structure is not drawn to scale. **B:** A 65 kDa protein was identified in human postmortem hippocampus, along with the larger α N-catenin peptide(s). The lower molecular weight protein was absent from rat and mouse brain. For each lane, 25 μ g of total protein was loaded.

Fig. 3.

Species comparison of the α N-catenin III isoform. **A:** Alignment of the chimpanzee, monkey, and human nucleotide sequences for the α N-catenin III novel exon. The 5' and 3' splice sites are indicated in bold and putative ATG transcription start sites are underlined and in bold. Human and chimpanzee share 99.6% sequence identity, while the human and monkey share 94.9% sequence identity. **B:** Amino acid sequences of the chimpanzee, monkey, and human peptides. The coding region for the novel exon is indicated by the gray box. Human and chimpanzee share 99.1% sequence identity, while the human and monkey share 96.9% sequence identity.

Fig. 4.

Confirmation of α N-catenin III genomic structure. Primers spanning the novel exon and exon 7 amplified a single PCR product from human brain cDNA. This 139 base pair product was consistent with the α N-catenin III genomic structure, which is missing exons 1–6 and has the novel exon spliced in at the N-terminus of the transcript. DNA sequencing of this PCR product confirmed that the novel exon is spliced in next to exon 7 (not shown).

Fig. 5. QRT-PCR analysis of α N-catenin III mRNA expression. **A:** Consistent with the protein expression, mRNA levels of this transcript were not detected in mouse brain and non-neuronal human tissues. WB, whole brain; FIBRO, fibroblast; IM LYMPH, immortalized lymphoblasts; 1° LYMPH, primary lymphocytes; HC, hippocampus; NC, negative control; CNS, central nervous system. **B:** The α N-catenin III isoform displayed significantly reduced expression in schizophrenic non-smokers relative to controls and significantly elevated expression in patient smokers compared to patient non-smokers. Medication status is shown among schizophrenics. Drug-naïve is represented by the filled diamond, atypical antipsychotics by a filled square ■, typical antipsychotics by an open triangle △, and atypical and typical antipsychotics by a filled circle ●. Mean relative mRNA amounts ($\pm$ SEM) for each subject group are shown. * $P < 0.05$.

Demographics of Subjects Evaluated in the QRT-PCR Analysis

Cohort	Age	Gender		Race		Tobacco use (PPD)		Alcohol use		Medication			Agonal score ^a		PMI (hr)	Brain pH	Tissue storage (months)	Hem ^b	
		M	F	C	H	yes	no	none	typ	atyp	typ and atyp	1 or 2	3 or 4	L				R	
CT NS (n=7)	57.4±3.3	6	1	6	1	n/a	7	0	7	0	0	0	6	1	13.4±3.1	6.52±0.13	6.8±1.8 ^d	3	3
CT SM (n=6)	57.3±5.0	4	2	6	0	1.1±0.28	3	3	6	0	0	0	5	1	10.7±3.6	6.44±0.11	32.2±13.0 ^d	5	0
SZ NS (n=6)	66.0±6.9 ^c	4	2	6	0	n/a	6	0	2	3	1	0	5	1	17.8±3.8	6.43±0.05	24.2±8.2	1	2
SZ SM (n=9)	47.1±4.6 ^c	7	2	9	0	1.5±0.32	8	1	0	5	3	1	9	0	18.3±2.4	6.59±0.07	16.5±2.9	7	1

C, Caucasian; H, Hispanic; F, female; M, male; NS, non-smoker; SM, smoker; PPD, packs per day of cigarettes; Hem, hemisphere; L, left; R, right; atyp, atypical antipsychotic; typ, typical antipsychotic; N/A, not applicable.

^a Agonal score was assigned following the guidelines established by Hardy et al. [1985].

^b Hemisphere information was available for 20/28 subjects.

^c Age was significantly higher in patient non-smokers relative to smokers ($P<0.05$).

^d Tissue storage time was significantly lower in the control non-smokers compared to smokers ($P<0.05$).

TABLE II

Quantitative RT-PCR and Sequencing Primers

Gene name (symbol)	GenBank number	Forward primer	Reverse primer	Product	Product size (bp)
Quantitative RT-PCR primers					
α Na-catenin III	BX537769	5'-GTCAGTAGGC AAAAGTCTGTG-3'	5'-GTCATCTTATCAATCGCAATGTTG-3'	Novel exon 7	121
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AB062273	5'-GGTATCGTGGAAGGACTC-3'	5'-GGATGATGTTCTGGAGAGC-3'	Exon 7-exon 8	118
Sequencing primers					
α N-catenin III	BX537769	5'-CAGATGGATGGATGGAGAAG-3'	5'-CAATCGCAATGTTGAGAGG-3'	Novel exon 7	139

TABLE IIICorrelation Between α N-Catenin III mRNA Expression and Various Parameters

Parameter	Correlation coefficient	<i>P</i> -value
Age	−0.252	0.195
Brain pH	0.164	0.405
Brain freezer Storage time	−0.172	0.382
PMI	−0.065	0.741

TABLE IVCorrelation Between α N-Catenin III mRNA Expression and Packs of Cigarettes Smoked per day

Parameter	Correlation coefficient	<i>P</i> -value
Control smokers	−0.398	0.435
Schizophrenic smokers	0.414	0.308
All smokers	0.282	0.290