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Fine Mapping of Calcineurin (*PPP3CA*) Gene Reveals Novel Alternative Splicing Patterns, Association of 5'UTR Trinucleotide Repeat With Addiction Vulnerability, and Differential Isoform Expression in Alzheimer's Disease

MATTHEW J. CHIOCCO¹, XUGUANG ZHU¹, DONNA WALTHER¹, OLGA PLETNIKOVA², JUAN C. TRONCOSO², GEORGE R. UHL¹, and QING-RONG LIU³

¹Molecular Neurobiology Branch, NIH-IRP, NIDA, DHHS, Baltimore, Maryland, USA

²Department of Pathology, Neuropathology Division, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

³Behavior Neuroscience Research Branch, NIH-IRP, NIDA, DHHS, Baltimore, Maryland, USA

Abstract

Fine mapping of calcineurin (*PPP3CA*) gene identified single nucleotide polymorphisms (SNPs) and simple sequence repeat polymorphisms that are associated with addiction vulnerability. A trinucleotide repeat marker, located in the 5' untranslated region (5'UTR) of the *PPP3CA* mRNA, exhibited significantly different genotype and allele frequencies between abusers and controls in the NIDA African-American sample. The polymorphism showed allelic-specific expression in mRNA extracted from postmortem brain specimens. Novel alternatively spliced isoforms of *PPP3CA* were identified and their expressions were found altered in brain regions of postmortem Alzheimer's disease patients. These data underscore the importance of calcineurin gene in the molecular mechanism of addiction and Alzheimer's diseases.

Keywords

phosphatase; SNP association; gene regulation; alternative splicing

Introduction

Genome wide association using denser and denser single nucleotide polymorphism (SNP) sets has allowed identification of a number of genes that display SNPs whose allelic frequencies distinguish individuals with substance dependence from control individuals (Johnson et al., 2006; Liu et al., 2005a,b; Uhl, Liu, Walther, Hess, & Naiman, 2001). rs1395475, an SNP whose allele frequencies distinguished dependent from control individuals, lies at the locus for the *PPP3CA* gene (Liu et al., 2005a,b) that encodes the alpha catalytic subunit of a Ca²⁺/calmodulin(CaM)-dependent serine/threonine phosphatase, also called calcineurin or *PP2B*.

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Address correspondence to Qing-Rong Liu, Behavior Neuroscience Research Branch, NIH-IRP, NIDA, DHHS, 251 Bayview Blvd, Suite 200, Baltimore, MD 21224; qliu@intra.nida.nih.gov..

Declaration of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

PPP3CA/calcineurin acts as a calcium-activated heteromeric complex that contains calcium, calmodulin, a catalytic subunit and a regulatory subunit (Klee, Crouch, & Krinks, 1979). Other *PPP3CA* gene family members include the additional catalytic subunits, *PPP3CB* and *PPP3CC* and the regulatory subunits, *PPP3R1* and *PPP3R2*.

Calcineurin functions include (a) regulation of presynaptic phosphorylation of dynamin and the PP1 inhibitor DARPP-32, (b) postsynaptic phosphoregulation via actions from its anchored localization to scaffolding proteins in postsynaptic densities, and (c) regulation of transcriptional modulators that include the transcription factor NFAT (Yakel, 1997). Calcineurin plays roles in multiple brain circuits important for reward, memory, and aging in ways thought to link dopaminergic and glutamatergic signals (Gerber et al., 2003). Altered calcineurin expression or function can alter rewarding effects of abused substances (Biala, Betancur, Mansuy, & Giros, 2005; Gerdjikov and Beninger, 2005), alter long-term potentiation (LTP; Winder, Mansuy, Osman, Moallem, & Kandel, 1998) and change memory functions (Malleret et al., 2001; Mansuy, Mayford, Jacob, Kandel, & Bach, 1998). Evidence that directly implicates calcineurin in the memory-associated affliction, Alzheimer's disease (AD), include observations that overall calcineurin expression levels and calcineurin activities are reduced in AD brains (Gong, Liu, Grundke-Iqbal, & Iqbal, 2006). *PPP3CA* activity is reduced and its aggregation is increased in the aging brain (Agbas, Zaidi, & Michaelis, 2005).

Although *PPP3CA* is thus a strong candidate to play central roles in addiction and memory-associated disorders, the details of *PPP3CA* associations with these disorders, the detailed structure of the *PPP3CA* gene and its variants have not all been elucidated. We have thus sought to follow up on initial evidence for association of *PPP3CA* markers with substance dependence. We assemble, for the first time, a complete profile of the *PPP3CA* gene structure, delineating novel splice variants and polymorphisms. We define isoform-selective differences in *PPP3CA* expression in mRNAs extracted from AD brains. These observations point to selective roles for *PPP3CA* and its variants in the pathogenesis of addiction, AD and, perhaps, other disorders that involve reward and/or memory-like features.

Materials and Methods

Substance Abuser and Control Research Subjects

Genomic DNAs came from NIH IRP (NIDA) research volunteers of self-reported European and African-American descent whose drug use and dependence were characterized as described (Liu et al., 2005a,b; Uhl et al., 2001). A total of 389 unrelated European-American "abusers," averaged 35 years of age, scored 3+ on a total drug use scale of drug use of addictive substances, and demonstrated DSMIII-R or DSMIV dependence on at least one illegal abused substance. Two hundred forty "control" European-Americans with average age of 32 years reported no significant lifetime histories of use of any addictive substance and 0–1+ DUS (drug use scale) total drug use scores. DNAs from "abusers" of self-reported African-American descent numbered two hundred forty in total, while one hundred "control" African-Americans were analyzed for the current study.

Human Brain Tissue

Brain tissue from 57 pathologically confirmed AD patients and 20 non-AD "control" individuals were obtained postmortem from the Department of Pathology, Johns Hopkins Medical Institutions. For each individual sample, portions of medial temporal gyrus (Brodmann area 21) and medial frontal gyrus (Brodmann area 46) were subjected to extraction of RNA using RNazolB (Tel-test, Friendswood, TX), as previously described (Liu et al., 2006). cDNA was prepared using SuperScript First strand cDNA synthesis

(Invitrogen, Carlsbad, CA) for reverse transcription-PCR reaction. cDNA samples were used to analyze allelic and alternative splicing isoform expression (described in the following section). Additional cDNA was synthesized from total RNA from amygdala, caudate, putamen, cerebellum, cortex, hippocampus, hypothalamus, substantia nigra, heart, intestine, kidney, leukocyte, liver, lung, muscle, spleen, and testis purchased from BD Bioscience Clontech (Palo Alto, CA).

Simple Sequence Repeat Genotyping

Simple sequence length polymorphisms were identified throughout the *PPP3CA* gene by alignment of genomic with expressed sequence tag (EST) and mRNA sequences available from NCBI (www.ncbi.nlm.nih.gov) using Sequencher software (Version 3.0, Gene Code Co., Ann Arbor, MI).

SSLPs were genotyped using PCR amplifications in which one primer of the pair was labeled with carboxylfluorescein (6-FAM) at the 5'-terminal end of the primer (Table S1). For the *PPP3CA*_Prmt and *PPP3CA*_Int2 primer pairs, genomic DNA samples (10 ng/ μ l) were added to the reaction using 1 $\times$ Gold buffer, 1.5 mM MgCl₂, 0.8 mM dNTPs, 20 μ M of each oligonucleotide primer, and 0.13 U of *AmpliTaq* Gold polymerase. Each 5 μ l reaction used a procedure of 94°C for 5 min, 35 cycles of 94°C for 0.5 min, 55°C for 0.5 min, 72°C for 0.75 min, followed by 72°C for 7 min. The *PPP3CA*_EX1 primer pair required use of *Herculase* Hotstart DNA polymerase (Stratagene, La Jolla, CA) and 5% dimethylsulfoxide (DMSO) to amplify this GC-rich region. For the 5 μ l reaction, genomic DNA samples were amplified using a procedure of 95°C for 10 min, 45 cycles of 94°C for 0.5 min, 62°C for 0.5 min, 72°C for 0.75 min, followed by 72°C for 10 min. The PCR products were denatured with Hi-Di Formamide (Applied Biosystems, Foster City, CA) at 95° for 5 min. The fragments were electrophoresed using a DNA Sequencer (ABI 3100, Applied Biosystems) with a fluorescent ladder ROX350 (Applied Biosystems) as an internal size marker and analyzed with GENOTYPER software (Version 3.7).

SNP Genotyping With Taqman Assays. SNPs rs2851060, rs713455, and rs3730251 were genotyped using Allelic Discrimination software (SDS v2.1) and Taqman assays for ABI Prism 7900HT Sequence Detection System (Applied Biosystems). To distinguish between each SNP allele, one Taqman minor groove binding (MGB) probe was labeled with VIC reporter dye and another labeled with FAM reporter dye included with SNP sequence-specific forward and reverse primers. Reactions were performed in a 384-well format in a total reaction volume of 5 μ l containing 6.0 ng of genomic DNA and 1 $\times$ TaqMan Universal PCR Master Mix without AmpErase UNG (Applied Biosystems) using 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 0.25 min, 60°C for 1 min. Fluorescent signals between 500–660 nm were analyzed for each plate and genotypes were scored using automated software.

SNP Genotyping Using Multiplex PCR and SNaPshot Assays

PCR primers (Table S2) were generated using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplicons were designed to display sizes of 80 bp–400 bp. Five microliters PCR reactions were performed using 3 ng–10 ng of genomic DNA, 2.5 mM MgCl₂, 0.1 mM dNTP, 0.025 U AmpliTaq Gold DNA polymerase, and 1 pmol of eight mixed primers. Initial denaturation at 95° for 10 min was followed by 40 cycles of 95° for 30 s, 55° for 15 s, and 72° for 30 s using GeneAmp PCR Biosystem 9700 in 384-well reaction plates. PCR conditions were verified by direct DNA sequencing using mixed PCR products after treatment with exonuclease (USB), 0.5 units per sample, and shrimp alkaline phosphatase (SAP, USB), 0.50 U/sample for 30 min at 37°, and 5 min at 95°. Following multiplex DNA amplification,

unincorporated PCR primers and dNTPs were degraded by adding 0.5 units per sample exonuclease and 0.50 U/sample SAP, incubating for 30 min at 37° and incubating for a further 5 min at 95°. After purification, 1.5 µl of cleaned PCR product was mixed with 5.5 µl of water, 2.0 µl of SNaPshot ready Reaction Mix and 1.0 µl of mixed SNaPshot extension primers (2 pmol/µl). Twenty-five thermocycles of 96° for 10 s, 50° for 5 s, 60° for 30 s were followed by treatment of postextension products 0.50 U SAP/sample, incubation for 30 min at 37° and for 5 min at 95°. One microliter of final product was denatured with 8.5 µl Hi-Di Formamide (ABI) and 0.5 µl Liz-120 Size Standards (ABI) at 95° for 5 min. Fragments were then electrophoresed using a 3100 DNA Analyzer (ABI) and analyzed using ABI GeneScan version 3.7 software. Genotypes were scored manually, using size standards for peak verification.

Allele-Specific Expression

cDNA samples from human brain region tissues and genomic samples, both from individuals who were heterozygous for the *PPP3CA* 5'UTR tri-nucleotide repeat (GCC), were used for allele-specific expression assays. Genomic DNA and cDNA samples from medial temporal gyrus (n = 13) and/or medial frontal gyrus (n = 14) were added to 384-well plates containing 1× reaction buffer (Stratagene), 0.8 mM dNTPs (Stratagene), CYBR-1 nucleic acid green stain dye (BioWhittaker Molecular Applications), 1× Rox reference dye (Invitrogen), 5% DMSO, 20 uM each of *PPP3CA_EX1F4* and *PPP3CA_EX1R5*, and Herculase enzyme (Stratagene). Reactions used an ABI Prism 7900HT Sequence Detection System with 95°C denaturation for 10 min followed by 45 cycles of 94°C for 0.5 min, 62°C for 0.5 min, 72°C for 0.75 min, and then terminal 10 min 72°C incubation. Dissociation, involved a hold at 50°C for 5 min, followed by one cycle of 95°C for 0.25 min, 60°C for 0.25 min, and 95°C for 0.25 min, and was used to degrade short fragment primer dimers. The linear range for each sample was identified using SDS v2.1 software. Using the determined linear range, each sample was analyzed in duplicate by PCR using the FAM-labeled primer for *PPP3CA_EX1*. Products were resolved using an ABI 3100 sequence analyzer, as described. Peak area measurements were averaged. Relative peak areas for each allele were compared to produce an assessment of relative peak areas for each heterozygous sample for brain cDNAs and heterozygote genomic DNA standards.

Isoform-Specific Expression

PPP3CA isoforms were identified by aligning genomic sequence with human *PPP3CA* ESTs using Sequencher software (Version 3.0, Gene Code Co.). As described previously (Liu et al., 2006), exon-specific primers and fluorescent FAM-labeled MGB probes were designed (Table S3) across two alternatively spliced exons using Primer Express (Applied Biosystems) to avoid nonspecific amplification of genomic DNA. The cDNA templates were amplified using ABI Prism 7900HT Sequence Detection System (Applied Biosystems) with a cycling program of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 0.25 min and 60°C for 1 min. Predeveloped human glyceraldehyde-3-phosphate dehydrogenase (GAPD) (Applied Biosystems) provided a control. Quantification used methods in user bulletin #2 (ABI Prism 7700 Sequence Detection System) and graphs used Prism 3.0 (GraphPad Software, Inc. San Diego, CA).

Genetic Data and Statistical Analyses

GOLD software (<http://www.sph.umich.edu/csg/abecasis/GOLD>) allowed graphical display of LD between SNP and microsatellite markers (Abecasis and Cookson, 2000). PHASE version 2.1 (<http://www.stat.washington.edu/stephens/software.html>) provided haplotype estimates (Stephens and Donnelly, 2003; Stephens, Smith, & Donnelly, 2001). Chi-square tests (Microsoft Excel, MS Office 2003) identified genotype or allele frequency association

for *PPP3CA* microsatellite and SNP markers. Statistical analyses for *PPP3CA* brain region expression differences used Prism 3.0 software (GraphPad, Inc., San Diego, CA).

Results

rs1395475 Genotypes in Individually Genotyped Substance Dependence versus Control Samples

We sought verification of the microarray-based genome scan data that used pooled DNA samples (Liu et al., 2005a,b) to identify association of rs1395475 [Figure 1(A)], by genotyping this SNP in 379 individual African-American abuser and control samples (a subset of the individuals genotyped in pools in Liu et al., 2005a,b). These results produced significant allele frequency differences in this marker, which lies in *PPP3CA* intron 3 ($\chi^2 = 6.07, p = .014$).

Genomic Assembly and Identification of Novel *PPP3CA* Variants and Splicing Isoforms

To identify potential functional variants that might contribute to associations with the phenotype, we reassembled >300 kb sequences at the *PPP3CA* locus. We searched these sequences for simple sequence repeats and SNPs, relying on alignments of genomic BAC and EST sequences from NCBI and Celera. We identified several simple sequence length polymorphism/microsatellite markers. A trinucleotide repeat lies in *PPP3CA*'s 5'untranslated region (UTR) (ss68756203). We observed six and seven GCC allelic variants, containing between 2 and 10 repeat, in European and African-American samples, respectively. A 16 nucleotide tandem repeat (ss68756205) with eight nucleotide insertion lies in intron 1. A pentanucleotide repeat (ss68756204) lies in intron 3. The SNP now termed rs2850328 lies in 5' flanking putative promoter sequences [Figure 1(A)]. Several of these markers were used in genotyping assays (see above and below) to identify allele frequency differences between disease and control samples.

By aligning 15 exons of *PPP3CA* gene with ESTs (dbEST: <http://www.ncbi.nlm.nih.gov/dbEST/>) and sequences of the RT-PCR products generated here, we were able to identify novel, alternatively spliced *PPP3CA* isoforms that can be all translated in frame. Exon 1 can be individually spliced to four different downstream exons [Figure 1(A)], generating isoforms that we term CNEX1-2, 1-3, 1-4, and 1-10 [Figure 1(B)]. Different translation initiation sites for CNEX1-2 and 1-3 create different N-terminal sequences of 6 and 19 amino acids; these are followed by the translation products of exon 3 and other exons [Figure 2(A)]. CNEX1-4 and 1-10 delete 67 and 366 amino acids, respectively. These "deletions" include part or whole the phosphatase catalytic domain [Figure 2(B)], respectively. CNEX9-11 and 13-15 skip the single exons 10 and 14, respectively. Such skipped exons delete the domains responsible for binding subunit B as well as amino acids that lie N-terminal to calcineurin's autoinhibitory domain [Figure 2(B)]. CNEX3-12 skips exon 4-11. The resultant 274 amino acid deletion removes both phosphatase catalytic and subunit B binding domains [Figure 2(B)]. More than two dozens of calcineurin isoforms can thus be formed by these alternative-splicing events. CNEX1-3, 1-4, 13-14, and 13-15 isoforms have been previously described (Guerini and Klee, 1989; Kincaid et al., 1990; Reuter, Mi, Sehrsam, Ludolph, & Volkel, 2001). CNEX1-2, 1-10, 3-12, and 9-11 isoforms are reported here for the first time.

Genotyping Additional *PPP3CA* Variants in Substance Dependent and Control Samples

To seek additional information from these *PPP3CA* variants in substance abuse vulnerability, we genotyped additional simple sequence repeat and SNP markers in abuser/control populations of European and/or African-American descent (Tables 1 and 2, respectively). In African-American samples, there was a nominally significant genotype

frequency difference for the 5' 16 nucleotide repeat intron 1 VNTR, ss68756205 ($p = .024$) [Figure 1(A) and Table 2].

These data received support from genotypes for the trinucleotide repeat in the *PPP3CA* 5'UTR. The most frequent alleles for the 5'UTR trinucleotide repeat are distinguished by 8, 9, or 10 GGC repeats, forming the alleles that we denote as "B," "C," and "D," respectively (Figure 3). In African-American samples, in which these three alleles display similar frequencies (B 0.35, C 0.20, D 0.42), there were significant, ca 10% "C" allele frequency differences between substance abuser and control samples ($p = .03$ after Bonferroni correction; Figure 3). D alleles were correspondingly more frequent in controls than in abusers, though the p -value was only 0.09 after Bonferroni correction. Corresponding odds ratios and confidence intervals were: OR = 1.75; CI = 1.16–2.63, for the C, and OR = 0.69; CI = 0.49–0.99, for the D alleles.

For the other markers (Tables 1 and 2), allele or genotypic frequency differences did not reach statistical significance. Distributions of allele and genotype frequencies for the promoter SNP rs2850328 deviate from Hardy-Weinberg equilibriums, especially in the African-American substance dependent samples (Table 2; χ^2 p -value = 9.95×10^{-4}).

Allele-Specific Expression Based on *PPP3CA* 5' Utr Trinucleotide Repeat Alleles

Based on the data noted, haplotypes marked by variants that tag mRNA and lie at the 5' end of the *PPP3CA* gene are thus candidates to play roles in some of the association findings noted here. To seek allele-specific expression based on 5' *PPP3CA* haplotypes, we compared effects of haplotypes marked by "B" versus "D" alleles in mRNAs extracted from postmortem brain samples, using genomic DNA from heterozygotes as a control. Using the linear range for amplification of cDNA and genomic DNA for each sample, we were able to determine the relative trinucleotide repeat expression by quantifying peak area ratios (i.e., B allele peak area to D allele peak area) produced using GENESCAN. In mRNA extracted from 13 medial temporal gyrus samples, we observed significant differences in the B/D allele ratio in comparison to control values observed in studies of genomic DNA (Figure 4) ($p = .0154$). Such data support the idea that the tri-nucleotide repeats identify 5' *PPP3CA* haplotypes that can alter *PPP3CA* expression in ways that are preserved in postmortem cerebral cortical samples. Conceivably, the repeat sequences itself might even contribute to such differential expression.

Tissue-Specific Expression of *PPP3CA* Alternatively Spliced Isoforms

To supplement fragmentary prior descriptions of the human tissue-specific expression of *PPP3CA* isoforms, we studied expression patterns in eight brain regions and nine peripheral tissues using relatively quantitative Taqman assays. MGB probes were designed according to junction sequences of two alternatively spliced exons [Figure 1(B) and S3] to avoid detecting genomic DNA. We selected CNEX1-3 expression in amygdala as "100%" reference, and compare isoform expression data from other brain regions and peripheral tissues to this value (Table 3). The most prominent isoforms, CNEX1-3 and 13-14, are expressed at the highest levels in caudate and putamen (200%–400% of amygdala levels), high levels in cortex, amygdala, cerebellum, and hippocampus (100%–200%), and lower levels in hypothalamus and substantia nigra (5%–20%). CNEX1-3 and 13-15 were expressed at intermediate levels in leukocytes, muscle, spleen, and testis (7%–36%). CNEX13-14 contains a 10 amino acid insertion [Figure 2(B), 427-ATVEAIEADE-436] as compared to CNEX13-15. CNEX13-14 is more concentrated in brain, whereas CNEX13-15 is more concentrated in peripheral tissues (Table 3). CNEX1-2, CNEX1-4, and CNEX9-11 with truncated N-terminus, partial deletion of phosphatase domain, and complete deletion of subunit b binding domain, respectively, are expressed at low levels in brain regions and

peripheral tissues (<2%). CNEX3-12 and 1-10, which have completely deleted phosphatase domains, were expressed at very low levels (<0.2%) and only detected in brain (Table 3).

We compared *PPP3CA* expression in brain samples from 57 pathologically verified AD patients and 15 non-AD control using real time PCR/Taqman assays. We focused on *PPP3CA* isoforms with intact functional domains, namely CNEX1-3, 1-4, 13-14, and 13-15. There was significant reduction of levels of CNEX1-3, 1-4, and 13-14 isoforms in medial temporal gyrus. Reduction in CNEX1-4 and CNEX13-14 expression also reached significance in mRNA from medial frontal gyrus samples (Figure 5). Expression of the CNEX13-15 isoform, by contrast, was not significantly reduced in medial temporal gyrus and was actually significantly increased in AD medial frontal gyri (Figure 5, Mann-Whitney $p = .032$).

Discussion

In this report, we describe a more complete image of the structure of the *PPP3CA* gene. This picture can be linked to convergent data from association, genomic, and expression studies that support roles for *PPP3CA*, also known as the calcineurin catalytic subunit A, in addiction and in AD.

The *PPP3CA* gene encodes a number of alternative calcineurin isoforms, some previously described (Guerini and Klee, 1989; Kincaid et al., 1990; Reuter et al., 2001) and some described here for the first time, which are likely to acquire alternative functions as a result of deleting functional amino acid domains due to differential splicing of the calcineurin primary transcript. Many of these splice variants delete portions or all of the catalytic domain [Figures 2(A) and (B)] and/or regulatory subunit b binding domain. Removal of exon 14 (e.g., in CNEX13-15) removes a 10 amino acid domain whose function is currently unknown [Figure 2(B)]. Interestingly, each *PPP3CA* isoform appears to retain its calmodulin-binding domain and thus retain the potential for calcium/calmodulin regulation. Expression studies demonstrate that CNEX1-3, 13-14, and 13-15 represent the major *PPP3CA* isoforms in brain and peripheral tissues. Other isoforms appear to be expressed at less than 1% of the levels of expression of these major isoforms. CNEX1-10 and 3-12 isoforms are expressed in brain only, where they are likely to participate in regulation of brain-specific calcineurin isoform activity. However, isoforms that are expressed at low levels in whole tissue samples might be selectively expressed in small cell subpopulations where they might play more substantial roles.

The 5' portions of the *PPP3CA* gene harbor haplotypes that display nominally significant association with polysubstance abuse vulnerability in an African-American poly-substance abuse versus control comparison. The evidence is consistent with modest, polygenic contributions from variants at the *PPP3CA* locus in addiction. Nevertheless, roles for variants in levels of calcineurin expression in reward- and memory-related phenotypes are well documented in animal model studies (Biala et al., 2005; Gerdjikov and Beninger, 2005; Mansuy et al., 1998). Data in the present report supports prior observations of altered *PPP3CA* expression in postmortem samples from AD brains. The present haplotype-specific expression data, based on 5' UTR markers, provides a direct link between association data and prior data that describes behavioral effects of altered *PPP3CA* expression and/or calcineurin activities.

The alternative spliced *PPP3CA* variants include or delete exons in this region of that might exert significant differences in *PPP3CA* function with unique patterns of brain and/or peripheral tissue expression, in manners that suggest specific regulation. CNEX13-15 appears to be selectively preserved in mRNAs from postmortem AD brains in ways that

support the specificity of effects on other *PPP3CA* transcripts and might even suggest that this CNEX13-15 isoform might be expressed by different cell types.

Calcineurin upregulation has been postulated in AD brain due to its involvement in calcium homeostasis (Liu et al., 2005a,b) and inflammatory responses AD (Norris et al., 2005), whereas calcineurin downregulation has been postulated based on oxidative stress (Celsi et al., 2007), pathological cell losses and/or other pathological cellular damage (Brion, Couck, & Conreur, 1995). Alterations in phosphoregulation do provide major pathways by which cytoskeleton proteins, especially *tau*, (Lian, Ladner, Magnuson, & Lee, 2001) contribute to the neurofibrillary pathology characteristic of AD. Our observations that majority of *PPP3CA* isoform expression is reduced in AD postmortem brains thus supports much, though not all, prior work. Evidence for the specificity of this observation also comes from the study of expression of specific isoforms. Identification of the isoform that excludes exon 14 and is expressed at levels that are higher in AD than in control samples is of special interest, despite the fact that the function of the amino acids deleted in this isoform has not been well documented. Conceivably, the alternative splicing factors that produce CNEX13-14 and 13-15 might be altered in AD brain. Alternatively, it may be that this relative preservation could indicate that the CNEX13-14 and 13-15 isoforms might be differentially expressed by cell types (e.g., microglia) whose abundance can be increased in AD brains. Understanding the functions of the individual *PPP3CA* isoforms and understanding the mechanisms whereby the differential splicing is regulated might each provide unique insights into specific AD pathogenetic features.

Messenger RNAs from brain samples can provide evidence for effects of agonal state, effects of acute gene regulation, consequences of illness, death, developmental underrepresentation, developmental overrepresentation, or trait differences, among other features, in the relative levels of expression. Studies that document features of the clinical course and brain handling that could influence mRNA levels and work that uses the “haplotype” (or allele) specific expression approaches employed in the present study provide some of the best current means to control for these issues. Studies of relative expression levels of specific splice variants of the same primary transcript also provide significant control for several of these issues. Nevertheless, replication of these findings in independent samples of brains from different sources will also help solidify the observations made in postmortem RNA.

Despite these and other limitations, the convergence between the molecular biologic and association data presented here, when added to prior information about the behavioral consequences of altering calcineurin and/or *PPP3CA* activities, provides a collective strength. Taken together, these findings support careful examination of effects of polygenic *PPP3CA* variations that might include effects of 5' haplotypes on levels of expression and 3' haplotypes on relative expression of different splicing variants on the effects of this interesting gene in brain systems and disorders of memory and, perhaps, memory-related phenomena in addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Calcineurin (*PPP3CA*) marker map. Physical representation of polymorphisms and splicing isoforms throughout the 330 kb calcineurin A subunit gene on chromosome 4. (A) Physical map of the *PPP3CA* genomic locus with lines depicting various alternative splicing sites. Also shown are novel polymorphisms identified in genetic analysis: rs2850328, 5'UTR trinucleotide repeat (ss#68756203), variable nucleotide repeat (ss#68756205), penta-nucleotide repeat (ss#68756204), as well as the SNP originally identified in "10K SNP chip" association genome scan, rs1395475 (Liu et al., 2005a,b). (B) Alternative splicing schematic depicting possible isoforms for the 15 exon transcript. Dashes illustrate the location primer and probe to detect each transcript by RT-PCR. Asterisks indicate novel isoforms identified.

A

Ex2/Ex3 phosphatase catalytic domain
 CNEX1-2 MKDTATAVPPPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLEESV 47
 Ex1/Ex3
 CNEX1-3 MSEPKAIDPKLSTTDRVVKAVPPPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLEESV 60

Ex3/Ex4
 CNEX1-2 ALRIITEGASILRQEKNLDDIDAPVTVCGDHGGQFFDLMKLFEVGGSPANTRYLFLGDYV 107
 CNEX1-3 ALRIITEGASILRQEKNLDDIDAPVTVCGDHGGQFFDLMKLFEVGGSPANTRYLFLGDYV 120

Ex4/Ex5 Ex5/Ex6
 CNEX1-2 DRGYFSIECVLYLWALKILYPKTLFLLRGHNECRHLTEYFTFKQECKIKYSERVYDACMD 167
 CNEX1-3 DRGYFSIECVLYLWALKILYPKTLFLLRGHNECRHLTEYFTFKQECKIKYSERVYDACMD 180

Ex6/Ex7
 CNEX1-2 AFDCLPLAALMNQQFLCVHGGLSPEINTLDDIRKLDRFKEPPAYGPMCDILWSDPLEDFG 227
 CNEX1-3 AFDCLPLAALMNQQFLCVHGGLSPEINTLDDIRKLDRFKEPPAYGPMCDILWSDPLEDFG 240

Ex7/Ex8 Ex8/Ex9
 CNEX1-2 NEKTQEHFTHNTVRGCSYFYSPAVCEFLQHNNLLSILRAHEAQDAGYRMYRKSQTTGFP 287
 CNEX1-3 NEKTQEHFTHNTVRGCSYFYSPAVCEFLQHNNLLSILRAHEAQDAGYRMYRKSQTTGFP 300

Ex9/Ex10 subunit B binding Ex10/
 CNEX1-2 SLITIFSAPNYLDVYNNKAAVLKYENNVNIRQFNCSPPHYWLPNFMVDVFTWSLFPVGEK 347
 CNEX1-3 SLITIFSAPNYLDVYNNKAAVLKYENNVNIRQFNCSPPHYWLPNFMVDVFTWSLFPVGEK 360

/Ex11 domain Ex11/Ex12 calmodulin domain Ex12/Ex13
 CNEX1-2 VTEMLVNVNLNICSDELGSEEDGFDGATAAARKEVIRNKIRAIKGMARVFSVLRRESESV 407
 CNEX1-3 VTEMLVNVNLNICSDELGSEEDGFDGATAAARKEVIRNKIRAIKGMARVFSVLRRESESV 420

Ex13/ Ex14 /Ex15 autoinhibitory
 CNEX1-2 LTLKGLTPTGMLPSGVLGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEEAKGLDRIN 467
 CNEX1-3 LTLKGLTPTGMLPSGVLGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEEAKGLDRIN 480

domain
 CNEX1-2 ERMPPRRDAMPDANLNSINKALTSETNGTDSNGSNSNNIQ 508
 CNEX1-3 ERMPPRRDAMPDANLNSINKALTSETNGTDSNGSNSNNIQ 521

B

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Ex1/          Ex1/Ex3          phosphatase catalytic domain
CNEX13-14 MSEPKAIDPKLSTTDRVVKAVPPPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLEESV 60
CNEX13-15 MSEPKAIDPKLSTTDRVVKAVPPPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLEESV 60
CNEX9-11 MSEPKAIDPKLSTTDRVVKAVPPPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLEESV 60
CNEX1-4 MSEPKAIDPKLSTTDRVVK----- 19
CNEX3-12 MSEPKAIDPKLSTTDRVVKAVPPPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLEESV 60
CNEX1-10 MSEPKAIDPKLSTTDRVVK----- 19

          Ex3/Ex4
CNEX13-14 ALRIITGASILRQEKNLDDAPVTVCDDIHQFFDLMLKFEVGGSPANTRYLFLGDVY 120
CNEX13-15 ALRIITGASILRQEKNLDDAPVTVCDDIHQFFDLMLKFEVGGSPANTRYLFLGDVY 120
CNEX9-11 ALRIITGASILRQEKNLDDAPVTVCDDIHQFFDLMLKFEVGGSPANTRYLFLGDVY 120
CNEX1-4 -----VCDDIHQFFDLMLKFEVGGSPANTRYLFLGDVY 53
CNEX3-12 ALRIITGASILRQEKNLDDAPVT----- 86
CNEX1-10 ----- 19

          Ex4/Ex5          Ex5/Ex6
CNEX13-14 DRGYFSEICVLYLWALKILYPTLFLLRGNHECRHLTEYTFPKQCKIKYSERYVDACMD 180
CNEX13-15 DRGYFSEICVLYLWALKILYPTLFLLRGNHECRHLTEYTFPKQCKIKYSERYVDACMD 180
CNEX9-11 DRGYFSEICVLYLWALKILYPTLFLLRGNHECRHLTEYTFPKQCKIKYSERYVDACMD 180
CNEX1-4 DRGYFSEICVLYLWALKILYPTLFLLRGNHECRHLTEYTFPKQCKIKYSERYVDACMD 113
CNEX3-12 ----- 86
CNEX1-10 ----- 19

          Ex6/Ex7
CNEX13-14 AFDCLPLAALMNQQLCVHGGLSPEINTLDDIRKDRFKEPPAYGPMCDILMSDFLEDGF 240
CNEX13-15 AFDCLPLAALMNQQLCVHGGLSPEINTLDDIRKDRFKEPPAYGPMCDILMSDFLEDGF 240
CNEX9-11 AFDCLPLAALMNQQLCVHGGLSPEINTLDDIRKDRFKEPPAYGPMCDILMSDFLEDGF 240
CNEX1-4 AFDCLPLAALMNQQLCVHGGLSPEINTLDDIRKDRFKEPPAYGPMCDILMSDFLEDGF 173
CNEX3-12 ----- 86
CNEX1-10 ----- 19

          Ex7/Ex8          Ex8/Ex9
CNEX13-14 NEKTQEHPTHNTVRGCSYFYSYPAVCEFLQHNNLLSILRAHEAQDAGYRMYRSQTTF 300
CNEX13-15 NEKTQEHPTHNTVRGCSYFYSYPAVCEFLQHNNLLSILRAHEAQDAGYRMYRSQTTF 300
CNEX9-11 NEKTQEHPTHNTVRGCSYFYSYPAVCEFLQHNNLLSILRAHEAQDAGYRMYRSQTTF 300
CNEX1-4 NEKTQEHPTHNTVRGCSYFYSYPAVCEFLQHNNLLSILRAHEAQDAGYRMYRSQTTF 233
CNEX3-12 ----- 86
CNEX1-10 ----- 19

          Ex9/Ex10          subunit B binding Ex10/
CNEX13-14 SLITIFSAPNYLDVNNKAAVLKYENNVMNIQPNCSHPHYWLPNFMVDVTFWSLPPVGEK 360
CNEX13-15 SLITIFSAPNYLDVNNKAAVLKYENNVMNIQPNCSHPHYWLPNFMVDVTFWSLPPVGEK 360
CNEX9-11 SLITIFSAPNYLDVNNK----- 318
CNEX1-4 SLITIFSAPNYLDVNNKAAVLKYENNVMNIQPNCSHPHYWLPNFMVDVTFWSLPPVGEK 293
CNEX3-12 -----AALVKYENNVMNIQPNCSHPHYWLPNFMVDVTFWSLPPVGEK 128
CNEX1-10 ----- 19

/Ex11 domain          Ex11/Ex12 calmodulin domain          Ex12/Ex13
CNEX13-14 VTEMLNVNLNCSDDLGSSEDDGPDGATAAARKEVIRNKIRAIQGMARVFSVLRSEESV 420
CNEX13-15 VTEMLNVNLNCSDDLGSSEDDGPDGATAAARKEVIRNKIRAIQGMARVFSVLRSEESV 420
CNEX9-11 VTEMLNVNLNCSDDLGSSEDDGPDGATAAARKEVIRNKIRAIQGMARVFSVLRSEESV 378
CNEX1-4 VTEMLNVNLNCSDDLGSSEDDGPDGATAAARKEVIRNKIRAIQGMARVFSVLRSEESV 353
CNEX3-12 VTEMLNVNLNCSDDLGSSEDDGPDGATAAARKEVIRNKIRAIQGMARVFSVLRSEESV 188
CNEX1-10 -----GATAAARKEVIRNKIRAIQGMARVFSVLRSEESV 54

          Ex13/          Ex14          /Ex15          autoinhibitory
CNEX13-14 LTLKGLTPTGMLPSGVLGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEAKGLDRIN 480
CNEX13-15 LTLKGLTPTGMLPSGVLGGKQTLQ-----AIKGFSPQHKITSFEAKGLDRIN 470
CNEX9-11 LTLKGLTPTGMLPSGVLGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEAKGLDRIN 428
CNEX1-4 LTLKGLTPTGMLPSGVLGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEAKGLDRIN 413
CNEX3-12 LTLKGLTPTGMLPSGVLGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEAKGLDRIN 248
CNEX1-10 LTLKGLTPTGMLPSGVLGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEAKGLDRIN 114
domain
CNEX13-14 ERMPFPRDAMPDANLNSINKALTSETNGTDSNGSNSNTIQ 521
CNEX13-15 ERMPFPRDAMPDANLNSINKALTSETNGTDSNGSNSNTIQ 511
CNEX9-11 ERMPFPRDAMPDANLNSINKALTSETNGTDSNGSNSNTIQ 469
CNEX1-4 ERMPFPRDAMPDANLNSINKALTSETNGTDSNGSNSNTIQ 454
CNEX3-12 ERMPFPRDAMPDANLNSINKALTSETNGTDSNYGIEINTQ 289
CNEX1-10 ERMPFPRDAMPDANLNSINKALTSETNGTDSNGSNSNTIQ 155

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Figure 2.

Amino acid sequences of calcineurin (*PPP3CA*) isoforms. (A) CNEX1–2 and 1–3 isoform amino acid sequences. (B) CNEX13–14, 13–15, 1–4, 3–12, and 1–10 isoform amino acid alignment. The isoform names are listed on the left-hand side of the amino acid sequences and exon junctions and functional domains are marked above amino acid sequences. Protein domains: red refers to phosphatase catalytic domain; green refers to calcineurin B subunit binding domain; blue refers to calmodulin binding domain; and purple refers to calcineurin autoinhibitory domain.

Figure 3.

5'UTR repeat allele and genotype frequencies in NIDA populations. (A, B): NIDA European–American (“Abusers” $n = 389$; “Controls” $n = 240$). (C, D): NIDA African–American (“Abusers” $n = 240$; “Control” $n = 100$). C * refers to significant allele frequency difference between drug abuser population and controls, p value = .031 (Bonferroni correction). D * refers to significant genotype frequency difference between control and drug abuser populations, p value = .093 (Bonferroni correction).

Figure 4.

Allele-specific expression of calcineurin 5'UTR tri-nucleotide repeat alleles. Black, medial temporal gyrus cDNA (n = 13); Hatched, medial frontal gyrus cDNA (n = 14); Gray, genomic DNA. Values represent the average ratio of the area for B allele (8 repeats) relative to the area for the D allele (10 repeats). Medial temporal gyrus peak area ratio is significantly higher than genomic DNA peak area ratio, p value = .0154. Inset: Genotyping profile depicting peaks for B and D alleles.

Figure 5.

PPP3CA isoform expression analysis in Alzheimer's disease (AD). Expression profile for four major isoforms compared between control samples (n = 15) and AD samples (n = 57) in medial temporal gyrus (A) and medial frontal gyrus (B). After normalizing to GAPDH, the expression level for each isoform was determined relative to control tissue samples. * refers to significant values by nonparametric Mann–Whitney test (p value < .05).

Table 1

NIDA Caucasian genotyping results

SNP/Marker	n	Allele frequencies (%)		p value	Genotype frequencies (%)			p Value	HWE
rs2850328		A	G		AA	AG	GG		
Control	157	66.9	33.1	0.962	43.3	47.1	9.55	0.968	0.758
Abuser	270	67.0	33.0		44.1	45.9	10.0		
SS68756205 (intron1_VNTR)		Allele 1 (1 rep.)	Allele2 (2 rep.)		1 1	1 2	2 2		
Control Abuser	195	75.9	24.1	0.660	59.0	33.8	7.18	0.893	0.614
Abuser	323	77.1	22.9		60.4	33.4	6.19		
rs713455		C	G		CC	CG	GG		
Control	210	72.9	27.1	0.958	52.9	40.0	7.14	0.956	0.901
Abuser	350	73.0	27.0		52.6	40.9	6.57		
SS68756204 (intron3_CTTT)		Allele 1 (1 rep.)	Allele2 (2 rep.)		1 1	1 2	2 2		
Control	232	60.3	39.7	0.782	38.4	44.0	17.7	0.880	0.561
Abuser	372	59.5	40.5		36.6	46.0	17.5		
rs2851060		C	T		CC	CT	TT		
Control	219	23.1	76.9	0.068	6.39	33.3	60.3	0.082	0.999
Abuser	360	18.6	81.4		2.78	31.7	65.6		
rs7840		A	T		AA	AT	TT		
Control	240	79.6	20.4	0.944	63.5	32.0	4.41		
Abuser	389	79.7	20.3		64.2	31.2	4.66	0.977	0.923

Table 2

NIDA African–American genotyping results

SNP/Marker	n	Allele frequencies (%)	p Value	Genotype frequencies (%)	p Value	HWE
rs2850328		A		AA	AG	GG
Control	62	20.2	0.313	11.3	17.7	71.0
Abuser	237	24.5		12.7	23.6	63.7
SS68756205 (intron1_VNTR)		Allele1 (1 rep.)		1 1	12	22
Control	95	52.6	0.100	25.3	55.8	18.9
Abuser	234	59.6		39.7	40.6	19.7
rs713455		C		CC	CG	GG
Control	95	64.7	0.466	42.1	45.3	12.6
Abuser	234	61.7		37.9	47.7	14.5
SS68756204 (intron3_CTTT)		Allele1 (1 rep.)		1 1	1 2	2 2
Control	97	41.8	0.104	17.5	48.5	34.0
Abuser	243	35.0		14.1	41.9	44.0
rs2851060		C		CC	CT	TT
Control	97	11.3	0.135	2.06	18.6	79.4
Abuser	237	15.8		1.69	28.3	70.0
rs7840		A		AA	AT	TT
Control	100	89.2	0.752	80.0	18.5	1.54
Abuser	240	90.2		83.3	13.7	2.99

Table 3Relative expression of *PPP3CA* isoforms (expressed in percentage relative to Amygdala CNEX1-3 isoform)

Tissues	CNEX1-3 (%)	CNEX1-2 (%)	CNEX14 (%)	CNEX13-14 (%)	CNEX13-15 (%)	CNEX9-11 (%)	CNEX1-10 (%)	CNEX3-12 (%)
Amygdala	100	4.19×10^{-3}	0.624	101	7.76	0.574	1.74×10^{-2}	2.01×10^{-2}
Caudate	262	5.01×10^{-3}	1.47	404	28.3	0.969	0.118	0.200
Putamen	169	4.67×10^{-3}	0.873	207	6.67	0.502	6.89×10^{-2}	5.46×10^{-2}
Cerebellum	111	2.77×10^{-2}	0.381	103	22.8	0.906	1.33×10^{-2}	1.00×10^{-2}
Cortex	137	7.38×10^{-3}	0.722	166	9.09	1.01	4.81×10^{-2}	3.96×10^{-2}
Hippocampus	114	1.08×10^{-3}	0.593	108	14.5	0.536	ND	3.65×10^{-2}
Hypothalamus	5.05	7.09×10^{-4}	3.19×10^{-2}	11.0	3.36	9.97×10^{-2}	3.89×10^{-3}	ND
Substantia								
Nigra	19.9	4.71×10^{-3}	6.44×10^{-2}	11.9	5.07	0.237	ND	ND
Heart	2.49	6.18×10^{-4}	1.89×10^{-3}	1.40	0.994	1.78×10^{-2}	ND	ND
Intestine	4.29	4.50×10^{-3}	3.80×10^{-3}	0.174	2.40	0.216	ND	ND
Kidney	3.54	1.96×10^{-3}	5.66×10^{-3}	1.37×10^{-2}	1.53	9.99×10^{-2}	ND	ND
Leukocytes	22.0	3.16×10^{-2}	1.89×10^{-2}	0.493	8.01	1.80	ND	ND
Liver	2.30	ND	9.73×10^{-3}	1.16×10^{-2}	1.21	ND	ND	ND
Lung	1.04	1.25×10^{-3}	5.35×10^{-4}	0.897	6.56×10^{-2}	2.21×10^{-2}	ND	ND
Muscle	32.8	2.87×10^{-2}	8.30×10^{-2}	1.43	16.0	0.390	ND	ND
Spleen	36.1	2.35×10^{-2}	0.141	1.59	15.4	0.504	ND	ND
Testis	19.0	1.73×10^{-2}	7.14×10^{-2}	3.25	7.31	0.156	ND	ND