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Abstract

To identify novel risk genes and better understand the molecular pathway underlying Alzheimer’s disease (AD), whole-exome sequencing was performed in 215 early-onset AD (EOAD) patients and 255 unrelated healthy controls of Han Chinese ethnicity. Subsequent validation, computational annotation and in vitro functional studies were performed to evaluate the role of candidate variants in EOAD. We identified two rare missense variants in the phosphodiesterase 11A (PDE11A) gene in individuals with EOAD. Both variants are located in evolutionarily highly conserved amino acids, are predicted to alter the protein conformation and are classified as pathogenic. Furthermore, we found significantly decreased protein levels of PDE11A in brain samples of AD patients. Expression of PDE11A variants and knockdown experiments with specific short hairpin RNA (shRNA) for PDE11A both resulted in an increase of AD-associated Tau hyperphosphorylation at multiple epitopes in vitro. PDE11A variants or PDE11A shRNA also caused increased cyclic adenosine monophosphate (cAMP) levels, protein kinase A (PKA) activation and cAMP response element-binding protein phosphorylation. In addition, pretreatment with a PKA inhibitor (H89) suppressed PDE11A variant-induced Tau phosphorylation formation. This study offers insight into the involvement of Tau phosphorylation via the cAMP/PKA pathway in EOAD pathogenesis and provides a potential new target for intervention.

Introduction

Alzheimer's disease (AD) is a leading cause of dementia, affecting between 23 and 35 million people worldwide (1). AD is one of the major contributors to disability and causes increases in the burdens of patients and the families of patients, as well as of the health care systems (2). The neuropathologic hallmarks of AD brains are extracellular accumulation of diffuse and neuritic amyloid plaques, composed of amyloid-β (Aβ) peptide, and the intraneuronal accumulation of neurofibrillary tangles. The presence of these hallmarks is strongly correlated with clinical dementia in AD. The amyloid-β (Aβ) hypothesis postulates an abnormal processing of amyloid precursor protein (APP) is the initiating factor in the disease process. The discovery of the APP gene in 1986 (3) and subsequent studies have revealed a complex interaction of genetics, biology and environment that contributes to AD risk. Increasing evidence has indicated that both genetic and environmental factors play important roles in the pathogenesis of AD. Although several susceptibility genes have been identified (4), there is still no effective intervention to delay the progression of AD.

A growing body of evidence suggests that tau hyperphosphorylation is a critical event in the disease process of AD (5). Abnormal phosphorylation of tau protein results in the formation of paired helical filaments (PHFs), which are the major component of neurofibrillary tangles and are considered the disease-specific hallmark of AD (6). The cAMP/PKA signaling pathway has been implicated in the regulation of tau phosphorylation (7). Phosphorylation of tau at multiple sites by PKA is involved in the regulation of tau function and is considered an early event in the development of AD (8). The cAMP/PKA signaling pathway is involved in the regulation of tau phosphorylation and is considered an early event in the development of AD (8). In neurons, cAMP/PKA signaling can regulate tau phosphorylation through multiple mechanisms, such as increasing the expression level of tau, altering the distribution of tau, and affecting the activity of PKA (9). Furthermore, cAMP/PKA signaling activates PKA, which can directly phosphorylate tau at multiple sites (8,10). The cAMP/PKA signaling pathway is involved in the regulation of tau phosphorylation and is considered an early event in the development of AD (8). In neurons, cAMP/PKA signaling can regulate tau phosphorylation through multiple mechanisms, such as increasing the expression level of tau, altering the distribution of tau, and affecting the activity of PKA (9). The cAMP/PKA signaling pathway is involved in the regulation of tau phosphorylation and is considered an early event in the development of AD (8). In neurons, cAMP/PKA signaling can regulate tau phosphorylation through multiple mechanisms, such as increasing the expression level of tau, altering the distribution of tau, and affecting the activity of PKA (9). The cAMP/PKA signaling pathway is involved in the regulation of tau phosphorylation and is considered an early event in the development of AD (8). In neurons, cAMP/PKA signaling can regulate tau phosphorylation through multiple mechanisms, such as increasing the expression level of tau, altering the distribution of tau, and affecting the activity of PKA (9). The cAMP/PKA signaling pathway is involved in the regulation of tau phosphorylation and is considered an early event in the development of AD (8). In neurons, cAMP/PKA signaling can regulate tau phosphorylation through multiple mechanisms, such as increasing the expression level of tau, altering the distribution of tau, and affecti...
tangles (NFTs) composed of hyperphosphorylated protein Tau (p-Tau). Based on age at onset (AAO), AD is classified as either early onset (AAO < 65 years) or late onset (AAO ≥ 65 years) (3). Of all AD patients, around 10% are diagnosed with early onset AD (EOAD) (4). Late-onset AD (LOAD) is a complex disorder with a heterogeneous etiology and a heritability of 58–79% (3,5).

Genome-wide association studies (GWAS) have identified over 50 risk loci associated with AD (5). However, most GWAS loci are non-coding common variants of uncertain function. Indeed, GWAS hits explain a relatively small proportion of the phenotype (6). However, rare but functionally important variants may be explained by rare variants in amyloid precursor protein (APP) (7–9). High-penetrant variants are non-coding common variants of uncertain function. Indeed, GWAS hits explain a relatively small proportion of the phenotype (6). However, rare but functionally important variants may be explained by rare variants in amyloid precursor protein (APP) (7–9). High-penetrant variants in amyloid precursor protein (APP) (3), presenilins 1 (PSEN1) and presenilins 2 (PSEN2) are the main genetic risk factors underlying EOAD, but are only found in ~11% of all EOAD patients (6). Exome sequencing has recently identified rare missense variants in TIM22, ABCA7, SORL1, granulin precursor (GRM) and other genes that increase the risk for developing AD (7–9). However, a large number of EOAD patients remain genetically unexplained. EOAD patients are more likely to carry pathogenic variants (5) and suitable for identifying novel AD risk genes. The number of patients with dementia in China accounts for ~25% of the entire population with dementia worldwide (1). However, few studies on risk genes in EOAD cohorts have been carried out in China.

In this study, exome sequencing was performed in 215 EOAD patients and 255 unrelated healthy controls of Han Chinese ethnicity to identify novel AD risk genes. We identified two rare non-synonymous variants (p.Arg202His and p.Leu756Gln) in phosphodiesterase 11A (PDE11A) in individuals with EOAD. Functional analyses in vitro revealed that both variants enhanced Tau phosphorylation. We also investigated the mechanisms through which PDE11A may be relevant in AD.

Results

Identification of PDE11A variants

We sequenced and analyzed the whole exome of 470 individuals (the pipeline is shown in Fig 1). The cohort consisted of 215 EOAD cases and 255 unrelated control samples (Table 1). About 80,000 variants per sample passed our quality control filters. For investigation of novel genes, patients carrying known EOAD risk genes (TREM2, VPS35, SORL1, MARK4, RUFY and TCRG1) or genes associated with LOAD (ABCA7, ADAM17, IGHG3, PLD3, UNC5C,BIN1, CD2AP, CLU, CR1, EPHA1, MS4A4A, PLEC2, ABI3, AKAP9 and ZNF655) were excluded (number of alleles = 13 (Supplementary Material, Table S1)). We then selected only variants rare in the population (<0.01% minor allele frequency (MAF)) and coding variants, lowering the count to 317. Next, we excluded synonymous variants and used in silico analysis to restrict our findings to those predicted as damaging for the protein, revealing 32 variants (Supplementary Material, Table S2). To further narrow the search for variants of interest, variant pathogenicity was evaluated using sorting intolerant from tolerant (SIFT), Polyphen-2, MutationTaster, Mendelian Clinically Applicable Pathogenicity (M-CAP), Combined Annotation Dependent Depletion (CADD), Likelihood Ratio Test (LRT), Protein Variation Effect Analyzer (PROVEAN), deleterious annotation of genetic variants using neural networks (DANN), Variant Effect Scoring Tool 3 (VEST3), fathmm-MKL, Genomic Evolutionary Rate Profiling (GERP), SiPhy, phastCons and phylop tools. The variants that all the tools predicted pathogenic were from ADRA2B, AHS2, PDE11A, MYO5A, COL6A6 and ETFDH (Supplementary Material, Table S3). Then, we used data from Online Mendelian Inheritance in Man (OMIM), Mouse Genome Informatics (MGI), Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), The American College of Medical Genetics and Genomics (ACMG) and UKBiobank PheWeb to perform a systems-level analysis of the six genes.

Among them, PDE11A met all criteria. We identified two variants in the PDE11A gene (NM_016953.4: rs752822096: c.605G>A; p.Arg202His and NM_016953.4: rs201572288: c.2267 T>A: p.Leu756Gln). One p.Arg202His heterozygote and one p.Leu756Gln heterozygote was found in EOAD patients, but none in normal controls. Confirmation by Sanger sequencing is shown in Figure 2A. PDE11A is a gene located on an autosome. A significant association was shown (P-value = 1.0 × 10−13) between PDE11A and AD using the UKBiobank PheWeb tool (https://pheweb.org/UKB-Neale/gene/PDE11A). Based on genetic databases, these two variants are rare in the East Asian population (p.Arg202His, gnomAD exomes_EAS: allele frequency = 0.0000; p.Leu756Gln, gnomAD exomes_EAS: allele frequency = 0.0043). The two variants are also rare in other populations. The p.Arg202His and p.Leu756Gln variants are predicted by 11 bioinformatics tools, including Polyphen2 HDIV, Polyphen2 HVAR, SIFT, LRT, PROVEAN, MutationTaster, DANN, VEST3, fathmm-MKL, CADD and M-CAP, to be damaging to the protein and by three algorithms (GERP, phastCons, phyloP) to be conserved (Table 2). The p.Arg202His and p.Leu756Gln variants are likely pathogenic and of uncertain significance, respectively, according to the American College of Medical Genetics and Genomics guidelines (10). Subsequent Sanger sequencing analysis was performed in an expanded cohort of individuals (N = 5024: 535 EOAD, 4489 controls). We identified two p.Arg202His heterozygotes in EOAD patients but none in normal controls, and six p.Leu756Gln heterozygotes in EOAD patients and 16 heterozygotes in normal controls. Meta-analysis for the two variants with two-stage sequencing data revealed a significant association with AD (p.Arg202His, P = 0.04; p.Leu756Gln, P = 0.01).

PDE11A variants and clinical features

The PDE11A p.Arg202His variant was detected in a 54-year-old female patient who had visited our hospital complaining of progressive memory decline over the past 4 years. She presented with amnesia as well as executive function and orientation deficits. She scored 9/30 on the Mini-Mental State Examination (MMSE) and 8/30 on the Montreal Cognitive Assessment, which was below the recommended cutoff values of 22 and 24, respectively. She had a Clinical Dementia Rating (CDR) score of 3. He denied a family history of dementia.

Figure 1. Workflow of the current study. Exome sequencing was performed in 215 Chinese individuals with EOAD and 255 controls. Subsequent direct sequencing was performed in an independent cohort to validate the selected rare variants. Then in vitro functional studies were performed to evaluate the role of candidate variants in EOAD and the underlying mechanisms.

Table 1. Demographics

<table>
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<tr>
<th>Characteristic</th>
<th>WES cohort</th>
<th>Direct sequencing cohort</th>
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<tr>
<td></td>
<td>Controls (n = 255)</td>
<td>Cases (n = 215)</td>
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<tr>
<td>Male, n</td>
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<td>101</td>
</tr>
<tr>
<td>Female, n</td>
<td>125</td>
<td>114</td>
</tr>
<tr>
<td>Age-at-study, mean (SD), years</td>
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<tr>
<td>Age at onset, mean (SD), years</td>
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<td>48.5 (3.8)</td>
</tr>
<tr>
<td>Education (years)</td>
<td>11 (6.3)</td>
<td>11 (4.6)</td>
</tr>
<tr>
<td>MMSE score, mean (SD)</td>
<td>27.8 (1.7)</td>
<td>12.1 (2.5)</td>
</tr>
<tr>
<td>CDR score, mean (SD)</td>
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<td>2.7 (0.8)</td>
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<tr>
<td>No. of APOE ε4 alleles, No. (%)</td>
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<td>198 (77.6)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>57 (22.4)</td>
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APOE, apolipoprotein E; CDR, Clinical Dementia Rating Scale; MMSE, Mini-Mental State Examination.

Functional annotation of rare PDE11A variants

PDE11A p.Arg202His is uniquely present in the PDE11A4 isoform. The PDE11A protein sequence in which the two rare variants are located contains highly conserved amino acids across different species (Fig. 2B), and their GERP scores are 4.34 and 5.57, respectively, implicating important protein biological functions by the variants. PDE11A p.Arg202His and p.Leu756Gln are predicted by Poly-Phen2 and SIFT to be damaging or possibly damaging (Table 1). As depicted in the schematic diagram of full-length PDE11A in Figure 2C, the two variants are located near or in functional domains, including the cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE), adenylyl cyclase and FhlA (GAF) and catalytic domains, suggesting a potential functional impact of these variants on the PDE11A protein.
Figure 2. Functional annotation of PDE11A variants. (A) Sanger sequencing confirmation of PDE11A variants. (B) Protein homologs in different species were aligned. The p.Arg202His and p.Leu756Gln are conserved in all PDE11A orthologs. The variants are highlighted in the red boxes. (C) Predicted domain maps of PDE11A protein with the identified missense variants marked: G: GAF domain (amino acids 217-380 and amino acids 402-568), P: HD/PDEase domain (amino acids 663-839), C: 3’-5’-cyclic nucleotide phosphodiesterase, catalytic domain (amino acids 588-912). The p.Arg202His variant is located near the GAF domain, and p.Leu756Gln variant in the 3’-5’-cyclic nucleotide phosphodiesterase, catalytic domain. (D) Predicted three-dimensional structure of wild-type (WT) and mutant PDE11A protein. (E) Structure comparison between wild-type PDE11A and R202H variant. Critical hydrogen bonds with surrounding amino acids were predicted to be eliminated. Dashed lines indicate hydrogen bonds. (F) Structure comparison between wild-type PDE11A and L756Q variant. Q756 mutant was predicted to induce more hydrogen bonds and then affect helix structure.

Global conformations of the p.Arg202His and p.Leu756Gln variants changed significantly from wild-type human PDE11A in three-dimensional (3D) homology models (Fig. 2D–F). Specifically, the 3D model predicts that p.Arg202His abolishes critical hydrogen bonds with surrounding amino acids; in contrast, p.Leu756Gln leads to a new hydrogen-bonded network, which affects the helical structure. Taken together, the model predicts that both p.Arg202His and p.Leu756Gln variants identified in patients with AD may impair PDE11A function.

PDE11A expression in AD brain tissues

The PDE11A gene is expressed in several regions of the mouse hippocampus, including the CA1, the subiculum and the amygdalohippocampal area (11). Moreover, Pde11a-knockout mice exhibit enlarged lateral ventricles and abnormal social investigation (11). PDE11A is also reported to be involved in the regulation of cGMP-mediated signaling. These results suggest that PDE11A has an important role in the brain, with a possible role in central nervous system disorders.

We assessed PDE11A expression in a publicly available single-nuclei RNA sequencing (RNA-seq) study from AD cases and control brain samples (12) and found the PDE11A gene to be expressed in almost all types of cells, including neurons, astrocytes and microglia (Fig. 3A and B).

To characterize the involvement of PDE11A in AD, we analyzed the PDE11A protein in fresh frozen postmortem brain tissues from cognitively normal healthy controls (n = 6) and patients with AD (n = 6). All participants were matched for age and sex. This analysis revealed significantly decreased
levels of PDE11A in those with AD relative to healthy controls (Fig. 3C and D).

**Effects of PDE11A variants on Aβ homeostasis**

To further confirm the pathogenesis of PDE11A variants, we performed in vitro studies to test the effect on Aβ homeostasis. Lower levels of the PDE11A protein was observed in AD patients. Therefore, we used PDE11A short hairpin RNA (shRNA) to knockdown (KD) PDE11A levels in cell models. A markedly lower PDE11A messenger RNA (mRNA) and protein levels were obtained in SH-SY5Y cells (Supplementary Material, Fig. S1A–C).

SH-SYSY cells were transduced with APP695 lentiviruses and PDE11A lentiviruses (PDE11A wide type (WT), p.Arg202His, p.Leu756Gln, scramble or shRNA). PDE11A variants did not significantly change PDE11A levels. And it showed no significant differences in secreted Aβ40 or Aβ42 levels or the Aβ42/Aβ40 ratio (Supplementary Material, Fig. S2A–C). PDE11A variants or KD did not change the levels of APP or Beta-Secretase 1 (BACE-1) (Supplementary Material, Fig. S3A–C). These results suggest that PDE11A may not affect Aβ homeostasis.

**PDE11A variants affect Tau phosphorylation**

To understand the influence of PDE11A variants on Tau phosphorylation, PDE11A lentiviruses (WT PDE11A, PDE11A p.Arg202His, PDE11A p.Leu756Gln, shRNA or scramble) were used to transduce SH-SYSY. Because of the relative low levels of endogenous phosphorylation Tau in cells, human Microtubule Associated Protein Tau (MAPT) lentiviruses were transduced with PDE11A lentiviruses simultaneously. Effects on Tau phosphorylation levels in transduced cells were assessed by immunoblotting. A significant reduction in Tau phosphorylation was detected at multiple sites, including T181, S404, S202/T205, S416, S214 and S396, in WT PDE11A-expressing cells compared with cells infected with mock lentivirus (Fig. 4). Moreover, both variants notably increased Tau phosphorylation at multiple sites compared with the WT (Fig. 4A and B). PDE11A shRNA treatment reduced PDE11A mRNA and protein levels by 60% and significantly increased Tau phosphorylation at multiple sites compared with scramble shRNA treatment (Fig. 4A–C). These results suggest that both variants could be loss-of-function.

The protein level of glycogen synthase kinase-3β (GSK-3β), a major kinase involved in Tau phosphorylation, and its
Figure 4. The PDE11A variants led to significantly high Tau phosphorylation levels. The cells were co-infected with MAPT and PDE11A lentivirus (PDE11A WT, mutants, scramble or shRNA). A total of 72 h after infection, cell lysates were used to detect levels of p-Tau(T181), p-Tau(S404), p-Tau(S202), p-Tau(S416), p-Tau(S214), p-Tau(S396), p-Tau(AT8) and Tau. (A) Representative western blots illustrate the expressions of phosphorylated Tau. (B and C) The histograms show the quantification of phosphorylated Tau levels detected by immunoblot relative to control levels. The data are represented as the mean ± SEM, based on three unrelated measurements. ∗P < 0.05, ∗∗P < 0.01 by one-way ANOVA and Dunnett test or Student’s t-test.

inactive form p-Ser9-GSK3β was not significantly altered in any of the groups (Supplementary Material, Fig. S4A–C). These data suggest that PDE11A variants likely affect Tau phosphorylation independent of GSK-3β signaling.

PDE11A variants exhibit alterations in cAMP/protein kinase A (PKA) signaling

The cAMP/PKA/cAMP response element-binding protein (CREB) signaling plays an important role in AD. However, the link with PDE11A is unknown. To further clarify the underlying mechanisms, we tested the effects of PDE11A on cAMP/PKA/CREB signaling. Transduction of WT PDE11A (with MAPT) in cells decreased cAMP levels compared with the mock group (with MAPT). The p.Arg202His and p.Leu756Gln variants increased cAMP levels relative to WT PDE11A (Fig. 5A and B). These results suggest that the p.Arg202His and p.Leu756Gln variants reduced the ability of PDE11A to degrade cAMP. Total PKA and p(Thr197)-PKA levels, as well as the ratio of phosphorylated CREB (p-CREB) to CREB, were also increased in variants PDE11A-expressing cells compared with WT PDE11A-expressing cells (Fig. 5C and D). Similar results were obtained in PDE11A shRNA-treated cells compared with scramble shRNA-treated cells (Fig. 5C and E).

In addition, pretreating the cells with the PKA inhibitor (H89) reduced PDE11A p.Arg202His and p.Leu756Gln variant-induced Tau phosphorylation (Fig. 6A-C). H89 pretreatment also decrease phosphorylated PKA (p-PKA) and p-CREB/CREB levels (Supplementary Material, Fig. S5A–E). Furthermore, these results were recapitulated using PDE11A shRNA. Therefore, these data demonstrate that the PDE11A variants affect the cAMP/PKA pathway, which is associated with increased Tau phosphorylation through a loss-of-function mechanism.

Discussion

In the present study, we report PDE11A as a novel candidate risk gene for EOAD. Our results showed that PDE11A variants did not affect amyloid production but cause dysregulation of cAMP/PKA signaling and increase Tau phosphorylation. These findings potentially improve our understanding of the molecular mechanisms of EOAD and reveal new AD target pathways.

We selected 215 cases of very EOAD (with age of onset before 55 years) to detect new AD risk genes. EOAD has a higher heritability than LOAD and absence of known causal or risk AD gene variants, sequencing EOAD increases the chances of discovering new AD genes. Using a systematic analysis of exome sequencing data, we identified PDE11A as a novel candidate risk gene for EOAD. Combined with the subsequent direct sequencing of the two variants in 4962 individuals, it revealed the two variants were significantly enriched in AD. The human PDE11A gene is located at 2q31.2 and consists of 23 exons (13). Four transcript isoforms designated PDE11A1–4, which result from different transcription initiation sites and alternative splicing, encode different PDE11A isoforms with unique N-terminal domains. Germline mutations, expression changes and functional alterations in PDE11A have been linked to brain function, tumorigenesis and inflammation (11,14–16). Moreover, PDE11A negatively regulates the efficacy of lithium in treating bipolar disorder (17). Expression of PDE11A4 mRNA in the brain tends to occur in CA1
Figure 5. The PDE11A variants affect cAMP/PKA signaling. ELISA analysis showed cAMP levels in cells after infection with WT PDE11A (with MAPT), decreased as expected; they increased following infection with PDE11A p.Arg202His, PDE11A p.Leu756Gln (A) or shRNA (with MAPT) (B). (C) Western blot of PKA signaling. The histograms show the quantification of p-PKA, PKA, p-CREB/CREB levels detected by immunoblot relative to control levels in cells infected with PDE11A mutants (D) or shRNA (with MAPT) (E). The data are represented as the mean ± SEM. *P < 0.05, **P < 0.01 by one-way ANOVA and Dunnett test or Student's t-test.

neurons and in the subiculum in ventral hippocampal (VHIPP) formation. Atrophy of VHIPP has been observed in patients with AD (18), and the deficits in the VHIPP appear to correlate with the severity of clinical impairment in patients with AD (19).

In addition, PDE11A4 can directly influence synaptic plasticity (20). PDE11A4 also plays a role in systems consolidation, which has relevance for cognitive deficits (21). PDEs have been linked to mental processes of emotions, learning and memory, with
Table 2. Pathogenicity prediction of PDE11A variants

| Variant        | Polyphen2 | PhyloPhy | SIFT | Mutation Taster | LRT | PolyPhen2 | HDIV | PolyPhen2 | HDIV | PolyPhen2 | HDIV | PolyPhen2 | HDIV | PolyPhen2 | HDIV |
|----------------|-----------|----------|------|----------------|-----|------------|------|------------|------|------------|------|------------|------|------------|------|------------|------|
| c.605A         | 0.998     | 6.12     | 0.995 | 0.995          | 0.999 | 6.12       | 0.995 | 0.995      | 0.999 | 6.12       | 0.995 | 0.995      | 0.999 | 6.12       | 0.995 | 0.995      |
| p.Arg202His    | 0.098     | 6.06     | 0.998 | 0.998          | 0.998 | 6.06       | 0.998 | 0.998      | 0.998 | 6.06       | 0.998 | 0.998      | 0.998 | 6.06       | 0.998 | 0.998      |
| p.Leu756Gln    | 0.098     | 6.12     | 0.995 | 0.995          | 0.995 | 6.12       | 0.995 | 0.995      | 0.995 | 6.12       | 0.995 | 0.995      | 0.995 | 6.12       | 0.995 | 0.995      |

Table 2. Pathogenicity prediction of PDE11A variants

implications in mood and cognitive disorders, including Huntington disease, Parkinson’s disease and AD (22–25). This evidence suggests that PDE11A plays a role in brain function and may also be involved in AD.

In our study, we identified two rare non-synonymous variants p.Arg202His and p.Leu756Gln in PDE11A in EOAD patients. The p.Arg202His variant is only present in the PDE11A4 isoform, whereas the p.Leu756Gln variant is present in all PDE11A isoforms. PDE11A isoforms are expressed in a tissue-specific manner (26). In a previous study, Kelly (27) found that PDE11A4 is highly expressed in hippocampal areas but not in any of the other tissues examined. PDE11A4 is the only PDE with restricted expression in hippocampal areas, which are affected in AD. Both variants identified in this study affect PDE11A4 isoforms through a loss-of-function mechanism. Both variants are highly conserved evolutionarily and located near or in an important PDE11A4 domain. In addition, both mutations disrupt hydrogen bonding and dramatically alter protein conformation. Furthermore, both variants are predicted to be damaging by 11 in silico tools and are considered conserved by all four algorithms applied. Taken together, these findings suggest that important biological functions can be attributed to these variants. Moreover, using bulk RNA-seq data derived from 1536 individuals (28), we found that the PDE11A gene is significantly associated with neuronal proportion in cortical tissues. Neuronal loss in the cerebral cortex is one of the main characteristic pathological changes of AD. We also detected significantly decreased protein levels of PDE11A in brain tissues from AD patients. It indicates that these variants may affect PDE11A function in the brain and facilitate disease onset.

We conducted cellular and biochemical studies and found that the PDE11A variants increase Tau phosphorylation at multiple sites. PDE11A shRNA also induces significant Tau hyperphosphorylation, whereas overexpression of PDE11A markedly decreases Tau phosphorylation. These findings suggest a putative role for PDE11A and variants in Tau phosphorylation. In contrast, the conditioned medium of cells transduced with lentivirus carrying the p.Arg202His or p.Leu756Gln variant or PDE11A shRNA showed no significant differences in Aβ42 or Aβ40 levels or Aβ42/ Aβ40 ratio, indicating that the variants may not affect Aβ peptides homeostasis. GSK-3β signaling affects Tau hyperphosphorylation. However, PDE11A variants or shRNA did not affect GSK-3β levels, indicating that the GSK-3β pathway is unaffected by the PDE11A variants. It is likely that other signaling pathways contribute to Tau hyperphosphorylation. The underlying mechanisms connecting PDE11A and Tau needs to be further evaluated.

Our data demonstrate that PDE11A affects Tau phosphorylation through the cAMP/PKA pathway. PDEs hydrolyze cAMP and are key enzymes regulating intracellular cAMP levels. cAMP is known as a crucial second messenger that participates in transducing signals in many types of cells. cAMP activates PKA, which phosphorylates and activates CREB. Activated CREB binds to cAMP-response elements (CREs) in target genes to regulate their expression. cAMP/PKA signaling pathway dysregulation has been reported in AD patients, and the pathway plays a crucial role in AD pathogenesis (29). Therefore, we reasoned that PDE11A variants might affect AD-relevant phenotypes through cAMP/PKA signaling. Our results showed higher cAMP levels in lysates from cells transduced with PDE11A p.Arg202His, p.Leu756Gln or shRNA compared with those expressing WT PDE11A or scramble shRNA. We also found that PDE11A p.Arg202His or p.Leu756Gln variant led to elevated PKA, pPKA and p-CREB levels. Increases in cAMP and PKA levels have
been observed in the cerebrospinal fluid (CSF) or cortex in AD patients (30,31). Expression of PKA and p-CREB is decreased in AD patients and in vitro models (32,33), suggesting that PKA and p-CREB levels may vary on different tissues at different stages of disease progression.

PKA phosphorylates Tau, and it is important for the formation of NFTs (34,35). Martinez et al. (30) showed that cAMP levels correlate with levels of Tau protein in the CSF. In the present study, H89 (a specific PKA inhibitor) pretreatment suppressed PDE11A mutation-associated p-Tau formation, which further confirmed the role of the cAMP/PKA/CREB signaling pathway in PDE11A-mediated regulation of Tau phosphorylation. Carlyle et al. (36) reported an age-related increase in cAMP-dependent PKA-mediated phosphorylation of Tau as a risk factor for aged cortex degeneration. PKA activation induces Tau hyperphosphorylation and spatial memory deficits, which are reversed by the PKA inhibitor H89 inhibits (39). Ikezu et al. (37) identified two rare variants in AKAP9 in African American AD patients. AKAP9 variants had no effect on Ap, but significantly increased Tau phosphorylation in cells treated with a PDE4 inhibitor (38). PKA enhances Tau phosphorylation by other Tau kinases (39). Thus, the effect of PKA on AD-associated Tau phosphorylation may be either direct or indirect, and the exact mechanism has yet to be elucidated.

There are some limitations in our study. First, an independent replication of the association of PDE11A variants with EOAD in other Han Chinese population and other different populations would have been supportive of our genetic findings. Second, hopefully future studies into the pathogenesis of EOAD will be able to use our findings regarding PDE11A. Third, some other candidate risk genes for AD were missing using our selection strategies. Further analysis and functional study on other risk genes need to be investigated in future study. Fourth, frozen postmortem AD brain samples were not from EOAD patients. When the adequate EOAD postmortem brains are available, the PDE11A protein levels should be detected in these samples.

Conclusions

In summary, we report for the first time two novel rare PDE11A variants in individuals with EOAD. These PDE11A variants enhance both cAMP/PKA signaling and Tau phosphorylation. PDE11A could be a novel candidate predisposing genetic factor for AD.

Materials and Methods

Study design and subjects

Data from 215 individuals with EOAD from Xuanwu Hospital were evaluated in this observational study and compared with 4489 healthy controls recruited at the Xuanwu Hospital from 2015 to 2020 and used for Sanger sequencing. Signed informed consent was provided by all the patients and control subjects. The study protocol was approved and monitored by the ethics committee of Xuanwu Hospital.

Relative PDE11A protein levels were quantified using immunoblot in samples from fresh frozen postmortem parietal lobe tissue of six AD patients and six cognitively healthy controls, recruited at Washington University in St. Louis Charles F. and Joanne Knight Alzheimer’s Disease Research Center Brain Bank and approved by the institutional review board of Washington University. Written informed consent for brain autopsy was obtained from all participants or their legal representatives.

Exome sequencing and data analysis

Genomic DNA was obtained from the peripheral blood of all participants. Briefly, the exome was enriched using Agilent SureSelect Human All Exon V5 Kit (Agilent Technologies, Santa Clara, CA, USA) and analyzed using Illumina HiSeq 2500 (150 bp paired-end, Illumina, San Diego, CA, USA). After conducting quality control, high-quality paired-end reads were mapped to the human genome build GRCh37 using Burrows-Wheeler Aligner software. Verita Trekker was employed to identify variants. Enliven® and Annotate Variation (ANNOVAR) were utilized to perform annotation for Variant Call Format, and variants were selected according to an autosomal dominant model. Non-synonymous exonic or close-to splice-site variants with minor allele frequency <0.01% were prioritized for analysis. Variant pathogenicity was evaluated using SIFT, Polyphen-2, MutationTaster, M-CAP, DANN, VEST3, fathmm-MKL, GERP, SiftPhy, phastCons and phyloP tools. Candidate variants were prioritized based on OMIM, MGI, GO, KEGG, ACGM and the published literature.

Annotation of PDE11A variants

InterPro was used to predict domain maps of the PDE11A protein. DNAMAN software (Lynnon Corporation, Quebec, Canada) was applied for multiple sequence alignment. A homology model of the wild-type PDE11A structure was built in MODELLER software using the crystal structure of the PDE template (PDB: 3IBJ). Based on the homology modeling structure of wild-type PDE11A, Arg202His and Leu756Gln-mutated proteins were generated with Pymol software. AMBER software was used for molecular dynamics simulations for wild-type PDE11A and the Arg202His and Leu756Gln variants. To investigate in which major cell type the PDE11A gene is expressed, we queried the gene in a publicly available single-cell/nuclei RNA-seq dataset of AD cohorts of human brain tissues (http://adsn.ddnetbio.com/) (12).

Generation of PDE11A variants

Human wild-type PDE11A complementary DNA (cDNA) (NM_016953) was cloned into the pcDNA3.1-enhanced green fluorescent protein (EGFP) vector. Site-directed PDE11A mutagenesis (p.Arg202His and p.Leu756Gln) was performed using a KOD-Plus-Mutagenesis kit (Stratagene, La Jolla, CA, USA). For shRNA, three optimal targeting human PDE11A and one scrambled control were designed (Supplementary Material, Table S4). Lentiviral
particles were generated by co-transfecting the transfer vector (psh2-u6-egfp-puro containing shRNA or scramble, pCDH-CMV-MCS-EF1-GFP-T2A-Puro containing WT PDE11A, PDE11A p.Arg202His or PDE11A p.Leu756Gln) and the packaging vectors into HEK293T cells.

Cell culture
SH-SY5Y cells were maintained in 60 mm culture dishes with Eagle’s Minimal Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (1% penicillin–streptomycin; Invitrogen). Cells (10^5/cm²) were transduced with lentiviruses.

Real-time quantitative polymerase chain reaction (PCR)
Total RNA was purified from freshly harvested cells using an RNAeasy Mini Kit (Thermo Fisher Scientific) and TRizol (Thermo Fisher Scientific). Reverse transcription was carried out using a SuperScript First-Strand Synthesis Kit (Thermo Fisher Scientific), and total mRNA was measured by real-time quantitative PCR. Diluted cDNA templates, upstream and downstream primers, and SYBR Green I Master mix (Thermo Fisher Scientific) were mixed in a 20 μl volume, and reactions were performed using the StepOne Plus real-time PCR platform. Human Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (forward, 5′-ACACCTCAAGATCATCAGCAAT-3′; reverse, 5′-GATGCGATGGACTGTGCTAT-3′) was used as the internal reference gene, and relative expression levels of the PDE11A gene were calculated using the 2^ΔΔCt method. PDE11A quantitative PCR (q-PCR) primers were as follows: forward, 5′-CTGGAGTTGATGATGCTCTG-3′; reverse, 5′-CAGTGTCTTTTTTGTTGTAGCTCT-3′.

Western blotting
Briefly, cells were lysed in radio immunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktails and phosphatase inhibitors. The cell lysate was pelleted by centrifugation, and the protein concentration was measured by the Bicinchoninic Acid (BCA) assay. Samples were separated by 10% Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electroblotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking in 5% non-fat dry milk, the membrane was probed with appropriate primary antibodies (listed in Supplementary Material, Table S5) overnight at 4°C. The next day, the membrane was washed with Tris-buffered saline containing Tween-20 and incubated with horseradish peroxidase-linked secondary antibodies (1:5000; Santa Cruz Biotechnology) for 1 h at room temperature. After washing, the proteins were visualized by chemiluminescence. Western blots were normalized using the housekeeping protein β-actin.

Enzyme-linked immunosorbent assay (ELISA)
The concentrations of secreted Aβ40 and Aβ42 in conditioned medium were quantified using commercial ELISA kits (JBL International, Hamburg, Germany). Levels of cAMP in cell lysates were determined using a commercially available assay kit (R&D Systems, Minneapolis, MN, USA), according to the protocol provided by the manufacturer. Briefly, after washing cells with phosphate-buffered saline, the cells were resuspended in cell lysis buffer. A total of 300 μl of cell lysates were used to perform the assay in duplicate. The optical density of each well was determined with a microplate reader which is set to 450 nm. The concentrations of cAMP were calculated corresponding to the mean absorbance from the standard curve.

Statistical analysis
Quantification data were obtained from three independent repeats. Two-tailed unpaired Student’s t-test or one-way analysis of variance (ANOVA) was performed using GraphPad Prism, version 8.00 (San Diego, CA, USA). Dunnett test was performed for multiple comparisons after one-way ANOVA. To determine if the candidate risk gene is enriched in AD, a Fisher’s exact test on the variant count data was performed. Meta-analysis of the two-stage sequencing data was done using the Review Manager S.3 (The Cochrane Collaboration, Oxford, UK). A P-value <0.05 was used to indicate a statistically significant difference.

Supplementary Material
Supplementary Material is available at HMG online.

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