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CHRNA5 links chandelier cells to protection against amyloid pathology in human aging and Alzheimer's Disease

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Abstract

Changes in high-affinity nicotinic acetylcholine receptors are intricately connected to neuropathology in Alzheimer's Disease (AD). Protective and cognitive-enhancing roles for the nicotinic α5 subunit have been identified, but this gene has not been closely examined in the context of human aging and dementia. Therefore, we investigate the nicotinic α5 gene *CHRNA5* and the impact of relevant single nucleotide polymorphisms (SNPs) in prefrontal cortex from 922 individuals with matched genotypic and *post-mortem* RNA sequencing in the Religious Orders Study and Memory and Aging Project (ROS/MAP). We find that a genotype robustly linked to expression of *CHRNA5* (rs1979905A2) predicts significantly reduced cortical β-amyloid load. Yet, co-expression analysis shows a clear dissociation between expression of *CHRNA5* and other cholinergic genes, suggesting a distinct cellular expression profile for the human nicotinic α5 subunit. Consistent with this prediction, single nucleus RNA sequencing from 22 individuals reveals disproportionately-elevated *CHRNA5* expression in chandelier cells. These interneurons are enriched in amyloid-binding proteins and also play a vital role in excitatory/inhibitory (E/I) balance. Cell-type proportion analysis from 549 individuals demonstrates chandelier cells have increased amyloid vulnerability in individuals homozygous for the missense *CHRNA5* SNP (rs16969968A2) that impairs function/trafficking of nicotinic α5-containing receptors. These findings suggest that *CHRNA5* and its nicotinic α5 subunit exert a neuroprotective role in aging and Alzheimer's disease potentially centered on chandelier interneurons.

Keywords: Alzheimer's disease, prefrontal cortex, acetylcholine, nicotinic receptors, chandelier cells, interneurons, amyloid, attention

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Introduction

The cholinergic system plays a critical role in the pathology of Alzheimer's disease (AD) (1), a neurodegenerative disease marked by the accumulation of β-amyloid peptide (β-amyloid) and neurofibrillary tangles of phosphorylated tau in the brain (2). In AD, there are well-characterized disturbances in the excitation/inhibition (E/I) balance in cerebral cortex (3,4) arising from the disruption of inhibitory signalling. The shift toward higher excitation in the cortex is associated with cognitive impairment in AD.

The cholinergic system is an important regulator of E/I balance in the prefrontal cortex (PFC) (5,6) and is central to one of the first mechanistic explanations of cognitive deficits in AD; the so-called *cholinergic hypothesis* (7). In AD, there is a decrease of cortical nicotinic acetylcholine receptor binding (8,9), and β-amyloid binding to nicotinic receptors has been postulated as a potential mediator of AD pathology (10,11) possibly via blockade of these receptors (12,13). By contrast, stimulation of neuronal nicotinic receptors has been found to improve neuron survival in AD pathology (14), promote neurogenesis, and improve cognition (15,16). Promoting nicotinic signalling using acetylcholinesterase inhibitors is one of the mainstay AD treatments (17).

High-affinity nicotinic acetylcholine receptors are hetero-pentamer cation channels most commonly composed of α4 and β2 subunits (α4β2) (18). Deep layer PFC pyramidal cells express nicotinic receptors also containing the auxiliary α5 subunit (18,19). Nicotinic α5 subunits do not contribute to the acetylcholine binding site and cannot form functional receptors on their own (19), requiring the binding sites provided by partner subunits α4 and β2, and forming the α4β2α5 nicotinic receptor. The α5 subunit alters the kinetics of nicotinic receptors (20) and increases their permeability to calcium ions (21,22). Importantly, β-amyloid binds less readily to α4β2α5 than α4β2 nicotinic receptors (12), which raises the question of a possible protective role of the α5 subunit in AD pathology.

The nicotinic α5 subunit has previously been linked to cognitive performance, with loss or disruption of this subunit impairing performance in attentional tasks in rodents (23,24). In humans, single nucleotide polymorphisms (SNPs) affecting the expression and function/trafficking of the *CHRNA5* gene, which codes for the α5 subunit, have been linked to attentional and cognitive deficits (25,26). These SNPs are also linked to smoking (27), a major AD risk factor (28). However, the role of *CHRNA5* in aging and AD is unknown.

To address this critical gap, we built a multi-step model of the connections between SNPs affecting the expression and function of *CHRNA5*, and age-related cognitive and neuropathological phenotypes using detailed clinical and post-mortem data from the Religious Order Study and Memory and Aging Project (ROS/MAP) (29). Next, we leveraged single-nucleus RNAseq to determine the cell-type expression pattern of *CHRNA5* in the PFC. We then used a gene ontology analysis of cortical patch-seq data (30) to elucidate the functional makeup of the cell type with the highest *CHRNA5* levels in the PFC, the chandelier cells. Finally, we probed an estimated cell type proportion dataset from the PFC (31) to assess the interaction effect of *CHRNA5* SNPs and Alzheimer's disease pathology on this *CHRNA5*-enriched cell type. The results of our study suggest a novel role for *CHRNA5* in maintaining the E/I balance in the forebrain and as a potential new target for therapies aiming to promote neuronal survival in AD.

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Methods

Study cohort

We accessed data from 2004 deceased individuals from the ROS/MAP cohort study (29), of whom 1732 were autopsied. Both studies enrolled individuals without known dementia. ROS enrols elderly nuns, priests, and members of clergy, whereas MAP enrols individuals from community facilities and individual homes. Both studies were approved by an Institutional Review Board of Rush University Medical Centre. Participants gave informed consent for annual clinical evaluation, completed a repository consent allowing their resources to be shared, and signed an Anatomic Gift Act for brain donation at the time of death. Most individuals assessed were female (68%). The average age at study entry was 80.5 ± 0.16 years and the average age at death was 89.2 ± 0.2 years. All data was retrieved from the Synapse AMP-AD Knowledge Portal (Synapse ID: syn2580853).

Selection of candidate single nucleotide polymorphisms (SNPs)

Nicotinic α5 subunits (encoded by *CHRNA5*) do not contribute to the acetylcholine binding site and cannot form functional receptors on their own (19). In prefrontal cortex, the nicotinic α5 subunits participate in pentameric receptors with two binding sites contributed by partner subunits: α4 (encoded by *CHRNA4*) and β2 (encoded by *CHRNB2*) (18). Together, these subunits form α4β2α5 nicotinic receptors. While the current focus is *CHRNA5*, we also probed the impact of polymorphisms relevant to its receptor partners *CHRNA4* or *CHRNB2* for perspective. The specific polymorphisms were selected based on their reported effects on expression, coding, or clinical response. (*CHRNA5*:(27,32,33); CHRNB2: (34,35),; *CHRNA4*: (36).

Genotype data preparation and imputation, quality control, generation of bulk gene expression residuals

Details on the ROS/MAP cohort genotyping and handling of the post-mortem samples have been previously published (37) and are described briefly together with the quality control approaches and generation of the gene expression residuals in **Supplemental Methods**.

Neuropathology and cognitive scores

A detailed description of the neuropathology and cognitive variables in ROSMAP is included in **Supplemental Methods** and on the RADC Research Resource Sharing Hub.

Single-nucleus RNA sequencing data processing

The single-nucleus gene counts and metadata available from Cain et al. 2020 (31) on synapse (ID: syn16780177) were converted into a Seurat object (38) in R Studio for further processing. Potential doublets were removed by filtering out cells with over 2500 detected features, and potential dead or dying cells were removed by excluding cells expressing over 5% mitochondrial genes. Cell type annotations were indicated in the data as described in the metadata downloaded from the Cain et al. 2020 Synapse repository (ID: syn16780177). The snRNAseq data was log-normalized and matched with genotype data (n = 22 individuals). *CHRNA5* expression was averaged per cell type per individual and *CHRNA5* levels in different cell types were then compared between cell types by one-way ANOVA with Tuckey's post-hoc t-test. To prevent bias for rare cell types in calculating average *CHRNA5* expression per cell type per individual, cell types with fewer than 100 individual cells represented in the original data

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(not aggregated) were removed (the layer 5 FEZF2 ET cell type was excluded from analysis as only 93 cells of this type were present in the genotype-matched snRNAseq dataset).

Gene ontology of chandelier cell genes

A set of genes which are upregulated in PVALB+ chandelier cells versus PVALB+ chandelier cells was previously generated by Bakken and colleagues (30). From this list we first excluded all genes not specifically upregulated in humans (final n = 222) To determine the ontology of this gene set we used the Gene Ontology Resource (geneontology.org), searching specifically for molecular function.

Estimates of relative cell type proportions from bulk DLPFC RNAseq

Estimates of cell-type proportions from bulk DLPFC RNAseq data from 640 ROS/MAP participants was performed and described by Cain et al. 2020 (31). In brief: The authors developed a custom regression-based consensus model, CelMod, to extract cell cluster specific genes from the snRNAseq dataset from 24 ROS/MAP participants, and then used these genes to estimate the proportions of different cell subtypes in the bulk DLPFC RNAseq dataset from 640 ROS/MAP individuals. The deconvolved cell-type proportion data from the DLPFC was available on request from the research group (Cain et al. 2020 personal communication). We matched the cell type proportion data with genotype, bulk DLPFC RNAseq, and neuropathology (brain levels of β-amyloid and tau) data (final n = 549 individuals). At the time of analysis full cell-type annotations (like that in the snRNAseq data) were only available for the different classes of inhibitory neuron proportions. The estimated proportions of a GABAergic neuron subtype defined by its expression of the marker gene combination PVALB+/LHX6+/THSD7A+, were determined (Cain et al. 2020 personal communication) to represent chandelier cell proportions in DLPFC. Observed differences in the estimated proportions of this cell subtype represent a difference in the proportion of this cell subtype in the broad cell class (GABAergic neurons). Further information on the cell type proportions is provided in **Supplemental Methods**.

Statistical approaches

A detailed description of the statistical analyses is provided in **Supplemental Methods**.

Results

Expression of α4β2α5 receptor component genes is affected by single nucleotide polymorphisms

To identify effects in aging and dementia of gene variants previously shown in younger adults to influence *CHRNA5* expression (33) and α5 coding (21,39), we examined brain expression quantitative trait loci (eQTL). The variants were in weak-moderate linkage disequilibrium in our European ancestry sample (r^2=0.34), in agreement with previous work (33). We also found that dosage of the A allele of the missense SNP rs16969968 (minor allele frequency (MAF) = 0.33) in the coding region of *CHRNA5* (**Fig 1B**) was associated with lower *CHRNA5* expression (t = -13.93 $p = 3.98*10^{-40}$), consistent with existing data (40). A different SNP haplotype in the regulatory region upstream of *CHRNA5* (**Fig 1B**), denoted here by the A allele of the tag SNP rs1979905 (MAF = 0.43), was associated with higher *CHRNA5* expression (t= 27.87, $p = 5.94*10^{-124}$) (**Fig. 1C**). Furthermore, analyses of the coding-SNP rs16969968 and the regulatory-SNP rs1979905 together (**Fig. 1D**) showed that *CHRNA5* expression is predominantly regulated by the regulatory-SNP rs1979905 rather than the coding-SNP, rs16969968, as all rs1979905 A allele non-carriers showed similar levels of *CHRNA5* mRNA regardless of the rs16969968 A allele (Nested one-way ANOVA: F(2) = 229.6; Šidák's post-hoc test for multiple comparisons: rs1979905 A1 vs. A0 $p = 8*10^{-6}$, A2 vs. A1 p = 0.004, A2 vs A0 $p = 4*10^{-6}$). This was also demonstrated using a conditional eQTL model, where the effect

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of rs16969968 A allele on *CHRNA5* expression was lost when co-varying for rs1979905 A allele (rs16969968A: t = -1.068, p = 0.2815; rs1979905A: t = 21.931, $p = 6.140*10^{-86}$). No association between *CHRNA5* expression and disease state was detected (**Supplemental Fig. S1**). No trans-eQTL effects were detected (**Fig. 1E**) between either of these SNPs and the expression of required partner nicotinic subunit genes, *CHRNA4* and *CHRNB2*.

To assess the α4 and β2 nicotinic subunits required for the formation of α5-containing α4β2α5 receptors, we extended our eQTL analyses to SNPs in *CHRNA4* and *CHRNB2*, focusing on those associated with altered gene expression or clinical effects(35,36,41). Without exception, eQTL SNP effects for these genes were weaker than those of rs16969968 and rs1979905 for *CHRNA5*: the T allele of *CHRNB2* intronic variant rs2072660 (MAF = 0.23) was associated with lower *CHRNB2* expression (t = -5, $p = 7.05*10^{-7}$), and a similar association with *CHRNB2* expression was seen with the T allele of the *CHRNB2* non-coding variant rs4292956 (MAF = 0.07) (t = -5.244, $p = 2.02*10^{-7}$) (**Fig. 1E**). For *CHRNA4,* the G allele of missense variant rs1044396 (MAF = 0.45) in the coding region of *CHRNA4* was associated with higher *CHRNA4* expression (t = 3.67, $p = 0.0003$). We also used the Gene Query function of the xQTLServe online tool (DLPFC of 534 ROS/MAP participants) to identify the T allele of the intronic variant rs45497800 as associated with decreased *CHRNA4* expression (t = -6.92, $p = 1.32 * 10^{-11}$). We then replicated this association in our larger cohort of 924 ROS/MAP individuals (MAF = 0.07) (t = -4.57, $p = 5.54*10^{-6}$) (**Fig. 1E**). No associations were found between these SNPs and the expression of other high-affinity nicotinic receptor subunit genes (**Fig. 1E**).

CHRNA5 polymorphisms are not associated with smoking status in this largely non-smoking population

The percentage of participants identified as never, previous, and current smokers (**Supplemental Methods**) was 68.2, 29.4, and 2.4 respectively. To investigate previously-reported (25,42) associations between genotype for the *CHRNA5* SNPs and smoking status at baseline (current/former/never smoked), we used a Chi-squared test and found no relationship (rs1979905_A : $\chi2(4) = 1.575$, p = 0.813; rs16969968_A: $\chi2(4) = 1.317$, p = 0.858) in this largely non-smoking population. Smoking status was not used in further analysis, unless specifically indicated.

A *CHRNA5* polymorphism is negatively associated with brain β-amyloid levels

To address interrelationships among nicotinic subunit expression, nicotinic SNPs, and neuropathological and cognitive phenotypes, we used clinical and post-mortem data in a multi-step model, the inclusion and exclusion criteria for this model can be found in **Supplemental Fig. S2**. As illustrated in **Fig. 1F**, both β-amyloid and tau pathology were negatively associated with global cognitive performance proximal to death (tau: t = -18.87 $p = 5.77 *10^{-70}$; amyloid: t = -7.88, $p = -6.99*10^{-15}$) and positively associated with each other (t = 12.59, $p = 2.14*10^{-34}$). Of the SNPs examined, only the SNP increasing *CHRNA5* expression had a significant association with AD neuropathology, with the A allele of the regulatory-SNP rs1979905 negatively associated with β-amyloid load (t = -2.79, $p = 0.005$). This association remained significant after false discovery rate (FDR) correction for multiple comparisons ($p_{FDR} = 0.021$)(43), *CHRNA5* expression was associated negatively with β-amyloid load prior to FDR correction (t = -2.23, $p = 0.026$). By contrast, the expression of the major nicotinic subunit genes *CHRNA4* and *CHRNB2* showed significant positive associations with the last global cognition score, which remained significant after FDR correction (*CHRNA4*: t = 2.98, $p_{FDR} = 0.013$; *CHRNB2*: t = 3.43, $p_{FDR} = 0.003$). Conversely, the rs2072660 T allele, associated with lower *CHRNB2* expression, was negatively associated with the last global cognition score (t = -1.98, $p = 0.047$). A similar negative association with the last global cognition score (t = -2.29, $p = 0.021$) was found for the T allele of the *CHRNB2* SNP rs4292956.

CHRNA5 expression does not tightly correlate with other components of the cholinergic system

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To further investigate the interrelationships among *CHRNA5* and the major nicotinic subunits as well as other components of the cholinergic system, we performed a series of expression correlation analyses. Most of the major components of the cholinergic system which were detected in bulk DLPFC data of the ROS/MAP individuals (*CHRNA2*, *CHRNA4*, *CHRNA7*, *CHRNB2*, *CHRM3*, *CHRM1* and *ACHE*) showed significant positive correlation with each other (**Fig. 1G**). By contrast, *CHRNA5* stood out as showing no positive correlation with any of the other major cholinergic genes and only weak negative correlations with the expression of *CHRNB2* and *CHRM1* (**Table 1**). Considering that the expression of *CHRNA5*, *CHRNB2,* and *CHRNA4* are required for the assembly of the high-affinity α4β2α5 nicotinic receptor, it was surprising to see that *CHRNA5* expression was not positively correlated with either *CHRNA4* or *CHRNB2* (**Fig. 1G** and **Table 1**). Therefore, we next investigated whether this lack of correlation may arise from differences in the cell-type specific expression of *CHRNA5* compared to the major nicotinic receptor subunits, *CHRNA4* and *CHRNB2,* which are more broadly expressed.

CHRNA5 shows stronger expression in chandelier interneurons than most other cell classes

To assess the cell-type specificity of *CHRNA5* expression in the ROS/MAP cohort, we calculated the average *CHRNA5* expression per cell type per individual using the genotype-matched single-nucleus RNAseq data available from the DLPFC in a subset of 22 ROS/MAP participants (31)(2 individuals lacked genotyping data). In this small dataset, *CHRNA5* was expressed at a low level across a number of different excitatory, inhibitory, and nonneuronal cell types (**Fig. 2A**), with significantly higher expression in inhibitory PVALB+ chandelier cells (as identified by Cain et al. 2020). Chandelier cells had significantly-higher expression of *CHRNA5* compared to most other cell types (One-way ANOVA: $F(21) = 3.439$, $p = 6.1*10^{-7}$; Tukey's post-hoc t-test Chandelier cells vs. 18 out of 19 other cell types: $p < 0.05$) (**Fig. 2A**). By contrast, chandelier cell expression of *CHRNA4* and *CHRNB2* were at a similar level in chandelier cells to their expression levels in many other cell types (**Fig. 2B**).

To confirm the novel finding that chandelier cells show stronger *CHRNA5* expression compared to other classes of neurons, we probed publicly available cell-type specific gene expression databases of human brain tissue. Using the Allen Institute SMART-seq single-cell transcriptomics data from multiple cortical areas https://celltypes.brain-map.org/rnaseq/human_ctx_smart-seq we found *CHRNA5* expression to be highest in a PVALB+/SCUBE3+ inhibitory cell type (0.06 trimmed mean *CHRNA5* expression) likely representing chandelier cells (44), and in a co-clustering PVALB+/MFI+ cell type (0.06 trimmed mean *CHRNA5* expression). Highest expression of *CHRNA5* in chandelier cells compared to all other cell types is also replicated in the Seattle Alzheimer's Disease Brain Cell Atlas (https://knowledge.brain-map.org/data/5IU4U8BP711TR6KZ843/2CD0HDC5PS6A58T0P6E/compare?cellType=WholeTaxonomy&geneOption=CHRNA5&metadata=Cognitive Status&comparison=dotplot.) In the Human Protein Atlas database (brain single cell tissue) https://www.proteinatlas.org/ENSG00000169684-CHRNA5/single+cell+type/brain, *CHRNA5* showed highest expression in a PVALB+ inhibitory cell type (c-41) which also showed highest expression of *SCUBE3* (Inhibitory neurons c-41, 15.1 normalized *CHRNA5* transcripts per million), likely also representing chandelier cells.

To investigate the cell type-specificity of the *CHRNA5* eQTL effects of the regulatory-SNP rs1979905 in the single nucleus data from ROS/MAP, we stratified the averaged *CHRNA5* expression by genotype for the rs1979905 A allele. We found that higher allelic dosage of the rs1979905 A allele was associated with greater *CHRNA5* expression (**Fig. 2C**), and that this pattern was most pronounced in subtypes of layer 5 (L5 RORB IT: $t = 2.460$, $p = 0.0249$) and layer 6 (L6 IT THEMIS: $t = 2.402$, $p = 0.028$) excitatory neurons (**Fig. 2D**). In the stronger *CHRNA5*-expressing PVALB+ chandelier cells, however, the association did not reach significance. Other neuronal cell types appeared to diverge completely from the typical eQTL

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pattern of rs1979905 (**Fig. 2C,D**). This analysis suggests that only a subset of cell-types contribute to the stepwise expression pattern observed in the prefrontal cortex by rs1979905 genotype.

Cell type specific data from ROS/MAP and the Allen database supports the hypothesis that *CHRNA5* possesses a distinctive expression pattern with enrichment in chandelier cell interneurons, compared to its more abundant and widely-expressed subunit partners.

Chandelier cells are significantly enriched for genes interacting with β-amyloid

To investigate the potential contribution of chandelier cells to β-amyloid processing, especially one that might be driven by nicotinic α5-containing receptors, we assessed the molecular function of genes which differentiate the chandelier cells from a different class of PVALB+ interneurons (basket cells). For this analysis we took advantage of a list of 222 such genes previously generated by Bakken and colleagues (30), who investigated the cellular identities of chandelier neurons in the cortex across species, including humans. Molecular functional pathway analysis revealed this list to be significantly enriched for genes defined as "amyloid-beta binding" (fold enrichment = 7.79, *p*(FDR) = 0.01) (**Fig. 3A**), including *SORL1,* an endocytic receptor that directs the amyloid precursor protein away from the amyloidogenic pathway (45–47) and *EPHA4,* a receptor tyrosine kinase involved in amyloid regulation (48). Since α5-containing nicotinic receptors are highly permeable to calcium ions (21,22), we note that chandelier neurons are also significantly enriched for genes with a "calcium ion binding" molecular function (fold enrichment = 3.77, *p*(FDR) = $4.12*10^{-6}$) (**Fig. 3B)**, including genes potentially protective against amyloid pathology such as *MME and SPOCK1*. Two genes are at the intersection of these functional pathways in chandelier cells, *PRNP and CLSTN2*. The former has been implicated in nicotinic receptor-mediated regulation of amyloid (49–52) and the latter is essential for normal cortical levels of GABAergic neurotransmission (53) These findings underscore the importance of investigating the relationship between the functional status of nicotinic α5 subunits and chandelier neuron vulnerability to AD neuropathology.

A genotype-specific reduction in proportion of chandelier cells with increasing brain β-amyloid levels

To determine whether impaired function/trafficking α5-containing nicotinic receptors might promote chandelier neuron vulnerability to neurodegeneration, we examined the interaction of rs16969968 genotype and AD neuropathology on estimated proportions of chandelier neurons in the bulk RNAseq dataset. This investigation was based on cell type proportion estimates for chandelier cells and several other interneuron subclasses, from sets of single-cell-informed marker genes, in a subset of 640 ROS/MAP participants. Overall, β-amyloid levels were negatively associated with the proportion of chandelier cells (t = -4, $p = 7.26*10^{-5}$). However, the missense rs16969968 A allele homozygotes showed significantly lower chandelier cell proportions with increasing β-amyloid load, compared to rs16969968 A allele non-carriers (interaction term t = -2.86, *p* = 0.004)(**Fig. 4A,B,C**). In a secondary analysis, there is a suggestion of an opposite relationship of rs1979905 A allele and β-amyloid levels with chandelier cells but this does not reach statistical significance (interaction term t = 1.79, *p* = 0.074)(**Fig. 4D,E,F**). The observed relationships between chandelier cell proportions, *CHRNA5* genotypes and amyloid were not altered by the inclusion of smoking status as a covariate in the analysis. Chandelier cell proportions did not correlate with tau pathology (t = -0.4, *p* = 0.687), nor was there an interaction of *CHRNA5* genotype and tau pathology with chandelier cell proportions (data not shown).

To assess whether the interaction between β-amyloid levels and *CHRNA5* SNPs was driven by the effects of these SNPs on *CHRNA5* expression, we assessed the effect of the interaction of *CHRNA5* levels and β-amyloid load on chandelier cell proportion but found no significant effect (interaction term, *t* = 0.5, *p* = 0.619). This suggests that the genotype-specific association between amyloid load and chandelier cell

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proportion is more likely driven by changes in nicotinic α5 protein structure and/or trafficking (54) as a consequence of having two copies of the missense SNP in *CHRNA5*, rather than by the altered *CHRNA5* expression associated with the rs1979905 SNP genotype.

The schematic in **Fig. 5** illustrates a working model of the impact of the rs16969968 A allele homozygosity for chandelier cell vulnerability, as well as example mechanisms enriched in chandelier cells and known to alter β-amyloid processing.

Discussion

We examined human prefrontal cortical *CHRNA5* expression in aging, probing the connections between SNPs affecting the expression and function/trafficking of the nicotinic α5 subunit gene and AD-related neuropathology. The aging prefrontal cortex demonstrates strong eQTL effects of the common regulatory-SNP rs1979905, and we show that the A allele of rs1979905 is associated with lower levels of brain β-amyloid. Single-nucleus RNAseq data revealed that chandelier cells have the greatest abundance of *CHRNA5* in human prefrontal cortex. These neurons are significantly enriched in amyloid-binding proteins, including some that may be activated via nicotinic receptors. Lastly, we examined the impact of AD neuropathology on the proportions of chandelier neurons and determined that the α5-altering common coding-SNP rs16969968 renders this interneuron population vulnerable to β-amyloid levels. Our findings, summarized in the working model in **Fig 5**, suggest a potential cell-type specific neuroprotective role for C*HRNA5* to reduce β-amyloid levels and toxicity.

Inhibitory signalling is disrupted in Alzheimer's disease

The disruption of the excitatory/inhibitory balance in the cortex is a hallmark of AD pathology and is associated with cognitive AD symptoms (3,4). Previous studies have shown disruption of cortical inhibitory signalling in AD stems from the alteration of the activity of inhibitory neurons and inhibitory cell loss (55–57). The susceptibility of inhibitory neurons to AD pathology is not uniform, however. While studies have shown significant drops in somatostatin-positive interneurons in the cortex of AD patients (58,59), the numbers of parvalbumin-positive cortical interneurons, including the PVALB+ chandelier cells, appear comparatively more resilient to AD pathology (59,60). Our study expands on these findings, demonstrating that the preservation of chandelier cells in AD pathology depends on the genotype of SNP in a nicotinic receptor subunit. The rs16969968 SNP is a missense mutation found to functionally alter the α5-containing nicotinic receptors in cell systems and *in vivo*, through altered channel biosynthesis, trafficking, properties and/or modulation (21,39,54,61,62). Recent optophysiological work suggests that the nicotinic α5 subunit accelerates the endogenous cholinergic response in prefrontal cortex and protects it against desensitization (20), but little is known about endogenous cholinergic modulation of chandelier neurons.

Chandelier cells are vulnerable to β-amyloid pathology

Chandelier cells are a specialized subtype of PVALB+ interneurons. They differ anatomically from the PVALB+ basket cells by their large number of vertically oriented axonal cartridges, which specifically innervate the axon initial segments of pyramidal neurons (63,64). New evidence suggests chandelier cells regulate excitation dynamics of neuronal networks (65). Impairment of these neurons has been implicated in diseases involving pathological excitation in the cortex, such as epilepsy (66,67) and AD (3,4,60). Our RNASeq findings are in agreement with previous work showing that the inhibitory output of

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chandelier cells is sensitive to β-amyloid pathology (68) but unaffected by tau pathology (69). Chandelier cell axons near β-amyloid plaques have been found to show deformations, and pyramidal neurons proximal to plaques show loss of inhibitory input onto their axon initial segments (68). Our findings suggest that in people homozygous for A allele of the missense *CHRNA5* SNP rs16969968 (11% of the ROSMAP participants), this vulnerability of chandelier cells to β-amyloid pathology may be exacerbated, possibly leading to cell death.

Potential mechanisms for a neuroprotective effect of α5-containing nicotinic receptors

Our results suggest that polymorphisms affecting *CHRNA5* expression and function, may alter both the total β-amyloid levels in the brain, and alter the susceptibility of specific *CHRNA5*-expressing cell types, such as the chandelier cells, to β-amyloid-mediated toxicity. One possible explanation for these observations may be the lowered binding of β-amyloid to the α4β2α5 nicotinic receptors (13) expressed by these cells. This protection against β-amyloid binding and inhibition of the nicotinic response (12) could promote resilience of nicotinic signalling in the chandelier cells, potentially leading to improved cell survival (14) in AD pathology. Furthermore, since α4β2α5 nicotinic receptors support higher conductance of calcium ions into the cell (21,22), another putative neuroprotective mechanism of the α5 subunit may be through driving possible calcium-dependent neuroprotective pathways in the neurons which express the α4β2α5 nicotinic receptors (70,71). Such calcium-regulated pathways may include *MME*, *SORL1*, *SPOCK1* or *PRNP*, which are specifically enriched in PVALB+ chandelier cells compared to PVALB+ non-chandelier cells (30), and which have been previously suggested to alter β-amyloid production and clearance (46,49,50,72).

Caveats and opportunities for additional investigation

While the ROS/MAP database offered an opportunity to assess the impact of *CHRNA5* expression and *CHRNA5*-related SNPs on AD pathology in a large sample, some caveats exist. As *CHRNA5* expression has previously been shown to be important for animal performance in demanding attentional tasks (23,24), one of the critical limitations of our study is that a robust attention assessment of the ROS/MAP individuals was not part of the study design, potentially explaining the lack of any association between *CHRNA5* expression or polymorphisms and a cognitive readout. While the ROS/MAP dataset presented an opportunity to study the effects of rs16969968 on a background of an unusually-low smoking prevalence (73), future work would benefit from more robust assessment of smoking history.

Although most prevalent in the prefrontal cortex, the α4β2α5 receptor is not the only type of α5-containing nicotinic receptor. Another type of interest is the α3β4α5 receptor, which is expressed primarily in the habenula (22), and intriguingly has all its subunits within the same locus (74). Unfortunately, expression data for the relevant *CHRNA3* and *CHRNB4* genes were not included in the prefrontal bulk RNAseq dataset, complicating investigation of hypotheses pertaining to the *CHRNA3, CHRNA5*, *CHRNB4* locus. β-amyloid pathology affects other types of nicotinic receptors besides the α4β2* subtype, including the abundant and widely-expressed low-affinity homomeric α7 receptor (10). However, since the activity and β-amyloid-sensitivity of the neuronal α4β2* receptor can be further modified by the inclusion of the auxiliary subunit α5 (13), we focus on the high-affinity nicotinic receptor as a potentially rewarding target of study in the context of altered nicotinic signalling in AD.

A limitation of the single-nucleus RNA sequencing data (31) was its relatively low number of individuals, limiting the robustness of comparing the effects of rs1979905 on *CHRNA5* expression across the different cell types, and preventing a similar examination of cell-type specific effects of rs16969968

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on *CHRNA5* expression in the ROSMAP dataset. This limitation should be considered when interpreting the findings of our study. Furthermore, the low numbers of individuals in the single-nucleus cohort also prevented a comparison between our findings from the estimated cell-type proportions in the bulk RNAseq data and the actual proportions of different cell types present in the single-nucleus data. Future studies involving more subjects with single-nucleus RNAseq data could extend our findings on the interaction association of the rs16969968 genotype and β-amyloid with chandelier cell proportions.

Since cortical cell-type proportions were estimated using patterns of marker gene expression (31), the decreased chandelier cell proportions may instead reflect reductions of chandelier cell cartridges in AD (60). While the disruption of cortical E/I balance would remain similar, a different interpretation of our data would be that the rs16969968 A allele homozygous genotype exacerbates chandelier cartridge loss and resulting in lower expression of chandelier-cell-specific marker genes in individuals with elevated β-amyloid.

Finally, while SNP exploration provides novel insight into the relationship between *CHRNA5* and neuropathology in aging, this work is limited by being correlational. Moreover, gene expression does not necessarily denote protein levels in AD brains (75) and thus differences in nicotinic receptor gene expression may not fully predict receptor levels or binding (76). Many questions remain about the mechanisms by which nicotinic receptors in chandelier cells regulate amyloid processing, as well as the consequences for this cell population when these mechanisms are disrupted. Work in model systems and larger snRNAseq datasets will be necessary to test specific hypotheses raised in this work.

Summary and implications

A growing body of work suggests that cortical excitability is perturbed early in Alzheimer's disease through impairment of inhibitory interneurons (55,77). *CHRNA5* is positioned to modulate the overall excitability of the prefrontal cortex in two ways: through the excitation of a population of deep layer cortical pyramidal neurons (20,78,79) that send projections throughout prefrontal cortex and, as ascertained from our findings in this study, through the excitation of specific subsets of cortical interneurons, the chandelier cells. Our findings suggest that *CHRNA5* is involved in Alzheimer's disease neuropathology. The A allele of the *CHRNA5* regulatory-SNP rs1979905 is associated with higher expression of *CHRNA5* and with reduced β-amyloid load in the brain. In parallel, the A allele of the missense SNP, rs16969968, is associated with fewer chandelier cells in individuals with high β-amyloid levels, suggesting that differences in the protein structure of *CHRNA5* contributes cellular resiliency to β-amyloid pathology. This combination suggests neuroprotective roles of *CHRNA5* in β-amyloid pathology and makes *CHRNA5* a target for therapies aiming to improve neuron survival in Alzheimer's disease.

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Figure legends

Figure 1. SNPs affecting expression of α4β2* nicotinic receptor subunit genes highlight a link between *CHRNA5* and amyloid pathology. *A,* Schematics illustrating different subunit compositions of prefrontal α4β2* nicotinic receptors, with and without the α5 subunit. ***B,*** Localization of the rs16969968 and rs1979905 SNPs in relation to the *CHRNA5* locus. ***C,*** The A allele of the missense SNP rs16969968 (left) in the coding region of *CHRNA5* is associated with lowered *CHRNA5* expression, while the A allele of the rs1979905 SNP (middle) upstream of the *CHRNA5* gene is associated with enhanced *CHRNA5* expression. Data from the DLPFC of ROS/MAP individuals. ***D,*** *CHRNA5* expression appears to be controlled by the zygosity of the rs1979905 A allele (colors) instead of the rs16969968 A allele (x-axis). Data shown as *CHRNA5* expression for each subject with means indicated. ***E,*** eQTL effects of SNPs in nicotinic subunit genes on their respective gene expression. Shown as β-coefficient with significance (p) in brackets. **F,** Network plot depicting the relationships between SNPs (black), gene expression (red), neuropathology (blue), and last global cognition score (green). Solid and dashed lines indicate whether association was significant after correction for FDR or not, respectively. **G,** Network plot depicting the correlations present between the expression of select cholinergic genes in the DLPFC. Colour of lines indicates direction of correlation (negative or positive) while the thickness indicates correlation strength. All correlations shown are significant after adjustment for FDR.

Figure 2. *CHRNA5* expression is elevated in chandelier cells and is affected by genotype for the rs1979905 A allele. *A,* Expression of *CHRNA5* averaged per cell type per individual, original gene count values were normalized for each cell by total expression. F-test significance of ANOVA shown on graph, with red asterisk denoting post-hoc tests demonstrating *CHRNA5* expression is stronger in chandelier cells compared to all but one other cell type. Mean expression of *CHRNA5* in chandelier cells displayed (red line). ***B,*** Expression of *CHRNA4* (left) and *CHRNB2* (right) across different cell types in the ROS/MAP DLPFC snRNAseq dataset. Mean expression of *CHRNA4* or *CHRNB2* in chandelier cells displayed (red line). Data shown as mean + SEM of the data averaged per cell-type per individual. ***C,*** Expression of *CHRNA5* across cell types in the PFC in individuals split by genotype for the rs1979905 A allele, expression averaged per cell type per individual. Number of individuals per rs1979905 A allele genotype: 0, n = 5; 1, n = 13; 2, n = 4. ***D,*** Effect of genotype for rs1979905 A allele on expression of *CHRNA5* in selected cell types, data shown as *CHRNA5* expression averaged per cell type per individual. A pattern of increasing *CHRNA5* expression with increasing rs1979905 A zygosity is present in some cell types. Significance shown for linear regression models for L5 and L6 excitatory neurons. Displayed as mean + SEM. Number of cells per subtype indicated (n_{cell}).

Figure 3. Chandelier cells are significantly enriched for genes interacting with amyloid. *A,* Ontology (molecular function) of gene set upregulated in cortical PVALB+ chandelier cells vs. PVALB+ non-chandelier cells. Only molecular functions with significant fold enrichment after FDR are displayed. "Calcium ion-binding" and "amyloid-beta binding" functions are highlighted. ***B,*** Venn diagram displaying genes from ***A*** with either a "calcium ion-binding" or a "amyloid-beta binding" molecular function, and their overlap.

Figure 4. Association of chandelier cell proportions with β-amyloid load is dependent on the rs16969968 A allele genotype. Cell type proportion data for interneuron populations is available for almost a third of the deceased ROS/MAP subjects, allowing the assessment of the interaction among chandelier cell proportion, brain amyloid load, and the *CHRNA5* SNP haplotype. ***A,B*** Stratifying by rs16969968 A allele reveals a significant interaction effect between rs16969968 A allele and amyloid load

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on chandelier cell proportions with rs16969968 A allele homozygotes showing a more negative association between brain amyloid load and chandelier cell proportions compared to rs16969968 A allele non-carriers (interaction term t = -2.86, $p = 0.004$). Scatter plots show 95% confidence intervals of linear model predictions, β-coefficients and p values of individual linear regression models are displayed. ***C,*** Overlay of the linear models from A,B, showing 95% confidence intervals. ***D,E,*** Stratifying by rs1979905 A allele shows a suggestion of an opposite interaction between rs1979905 A allele and amyloid load on chandelier cell proportions (interaction term t = 1.769, $p = 0.078$). Scatter plots show 95% confidence intervals of linear model predictions, β-coefficients and p values of individual linear regression models are displayed. ***F***, Overlay of the linear models from D,E, showing 95% confidence intervals.

Figure 5. A working model of the potential role of chandelier cells in β-amyloid processing, and of the impact of *CHRNA5* genotype on chandelier cell (ChC) resilience and vulnerability. Top: Chandelier cells are significantly enriched for multiple genes involved in β-amyloid processing and degradation including for example neprilysin (NEP), a potentially *CHRNA5*-regulated degrader of β-amyloid, and SORL1, a vital component of the APP-recycling pathway. Bottom: In coding-SNP rs16969968 non-carriers, the α4β2α5 nicotinic receptor is resistant to inhibition by β-amyloid, preserving nicotinic signalling even at high β-amyloid levels that inhibit the α4β2 receptors. In coding-SNP rs16969968 homozygous individuals, the disruption of the α5 subunit may reduce its representation in the receptors or block its protective function against β-amyloid, leading to disrupted nicotinic signalling at higher β-amyloid levels, possibly triggering a cytotoxic response in the chandelier cells.

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Table 1. Expression correlation of selected cholinergic genes. Results of Pearson's correlation analysis of the expression of selected cholinergic genes using the bulk tissue RNAseq data from the DLPFC of ROS/MAP subjects.

Gene 1	Gene 2	r	p	FDR
CHRNA4	*ACHE*	0.556	<0.001	<0.001
	CHRM1	0.542	<0.001	<0.001
	CHRM3	0.242	<0.001	0.002
	CHRNA2	0.307	<0.001	<0.001
	CHRNA7	0.131	0.039	0.464
	CHRNB2	0.457	<0.001	<0.001
CHRNA5	*ACHE*	0.063	0.324	1.000
	CHRM1	-0.309	<0.001	<0.001
	CHRM3	-0.095	0.134	1.000
	CHRNA2	-0.045	0.477	1.000
	CHRNA4	-0.130	0.041	0.464
	CHRNA7	-0.061	0.342	1.000
	CHRNB2	-0.248	<0.001	0.001
CHRNB2	*ACHE*	0.472	<0.001	<0.001
	CHRM1	0.846	<0.001	<0.001
	CHRM3	0.645	<0.001	<0.001
	CHRNA2	0.283	<0.001	<0.001
	CHRNA4	0.457	<0.001	<0.001
	CHRNA7	0.359	<0.001	<0.001

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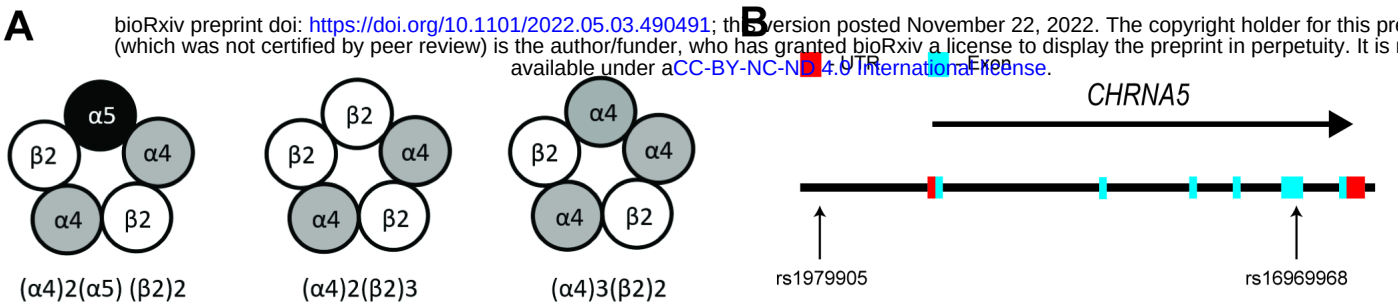

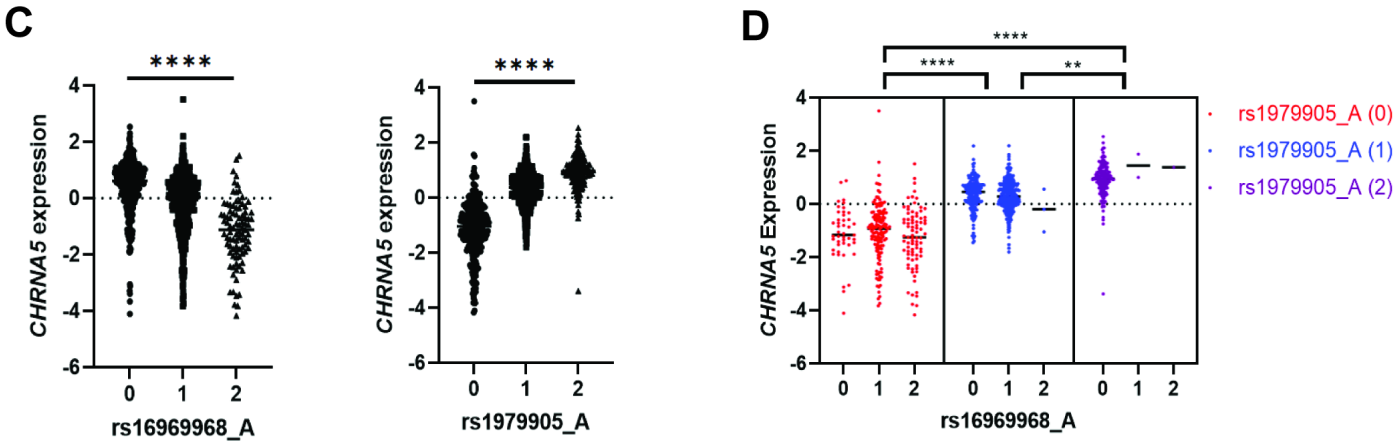

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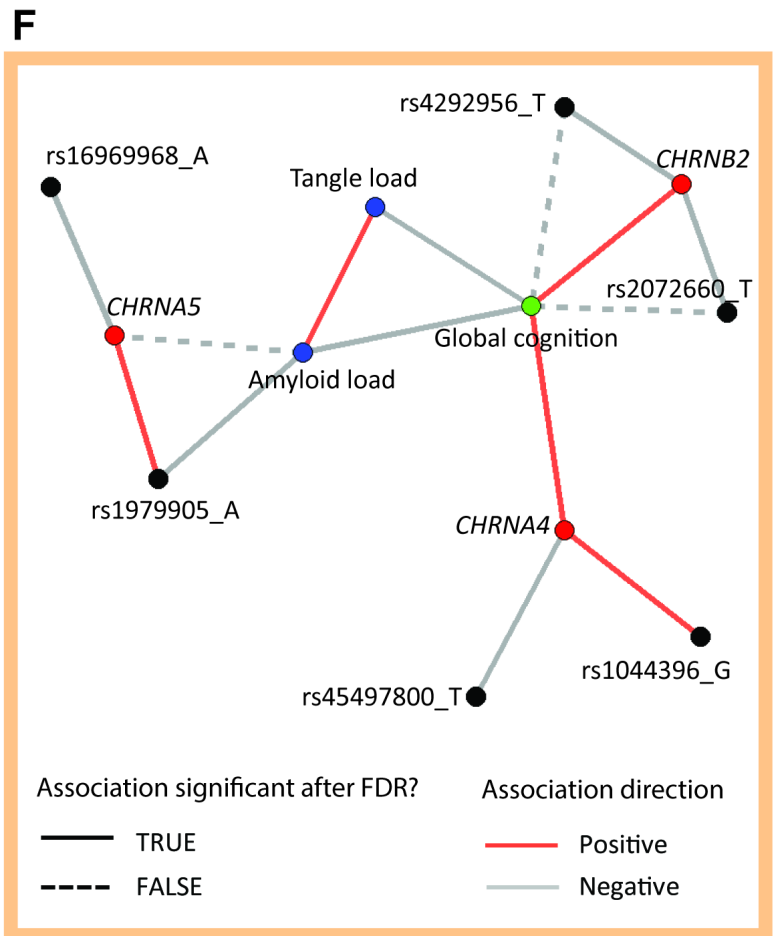
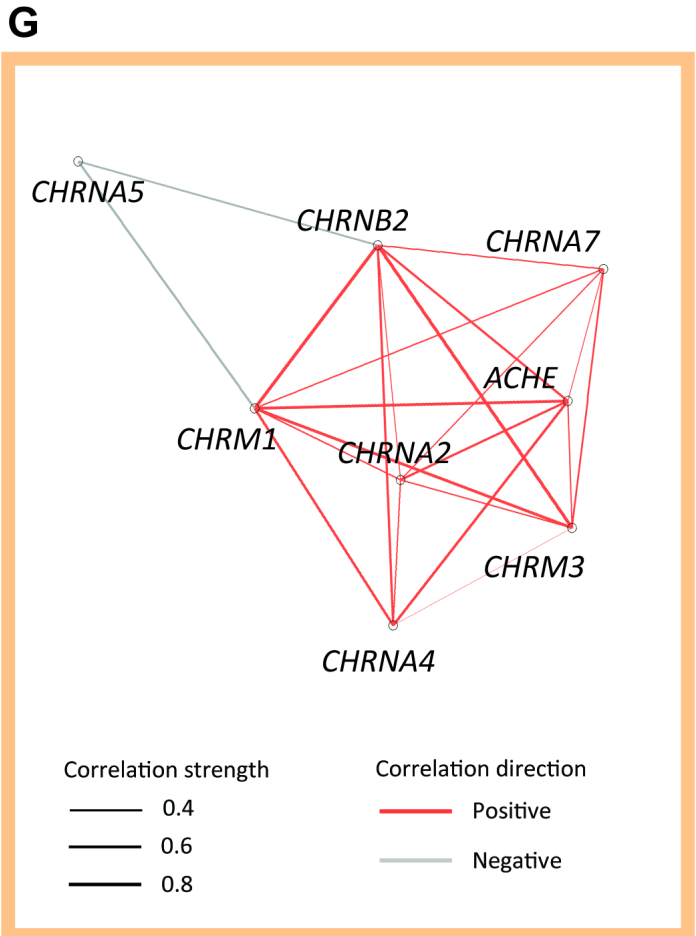
SNP/Gene	*CHRNA5*	*CHRNA4*	*CHRNB2*
rs1979905_A	1.083 ($5.94*10^{-124}$)	-	-
rs16969968_A	-0.713 ($3.98*10^{-40}$)	-	-
rs1044396_G	-	0.075 (0.0003)	-
rs4549700_T	-	-0.181 ($1.32 * 10^{-11}$)	-
rs4292956_T	-	-	-0.209 ($2.02*10^{-7}$)
rs2072660_T	-	-	-0.128 ($7.05*10^{-7}$)





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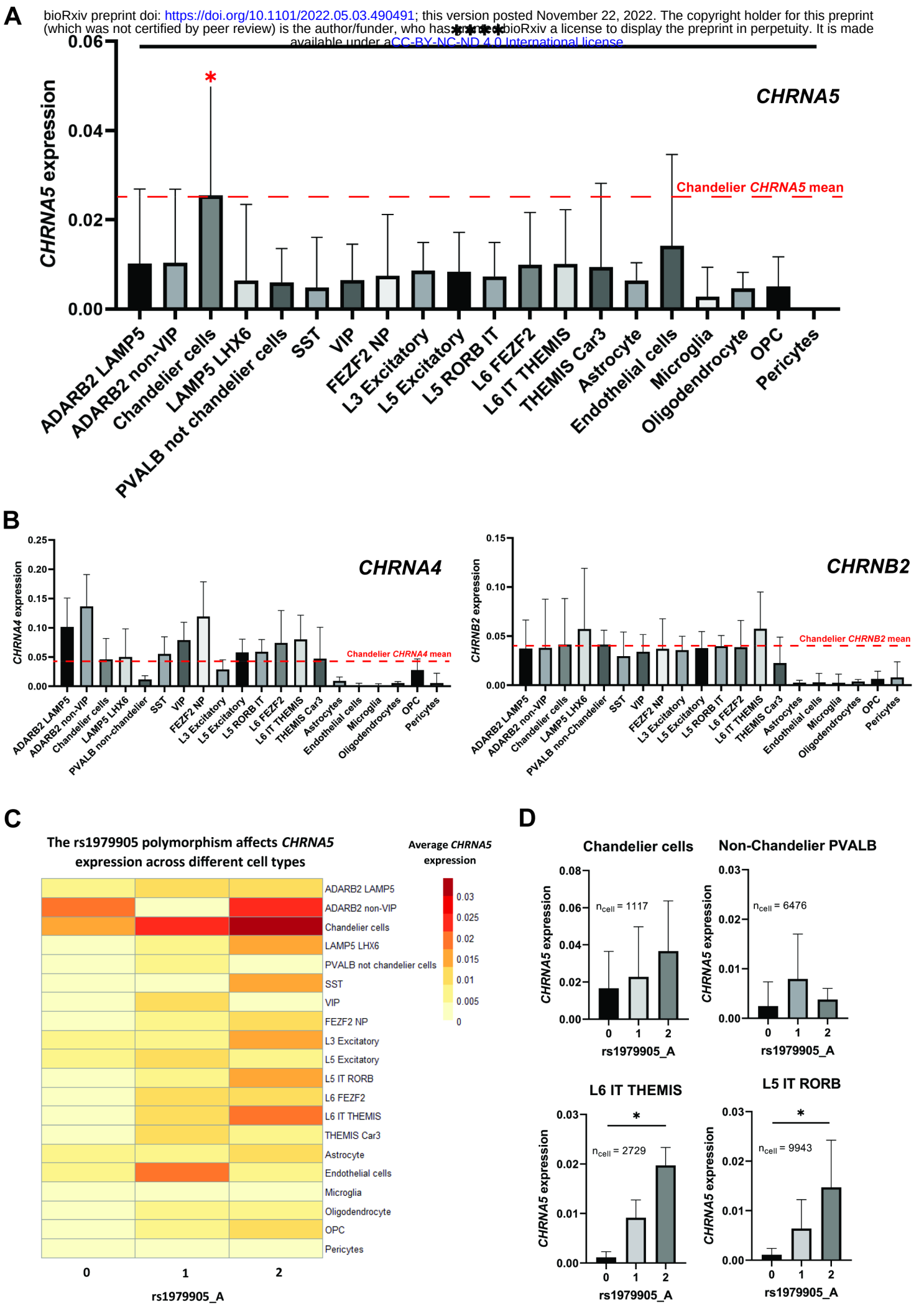

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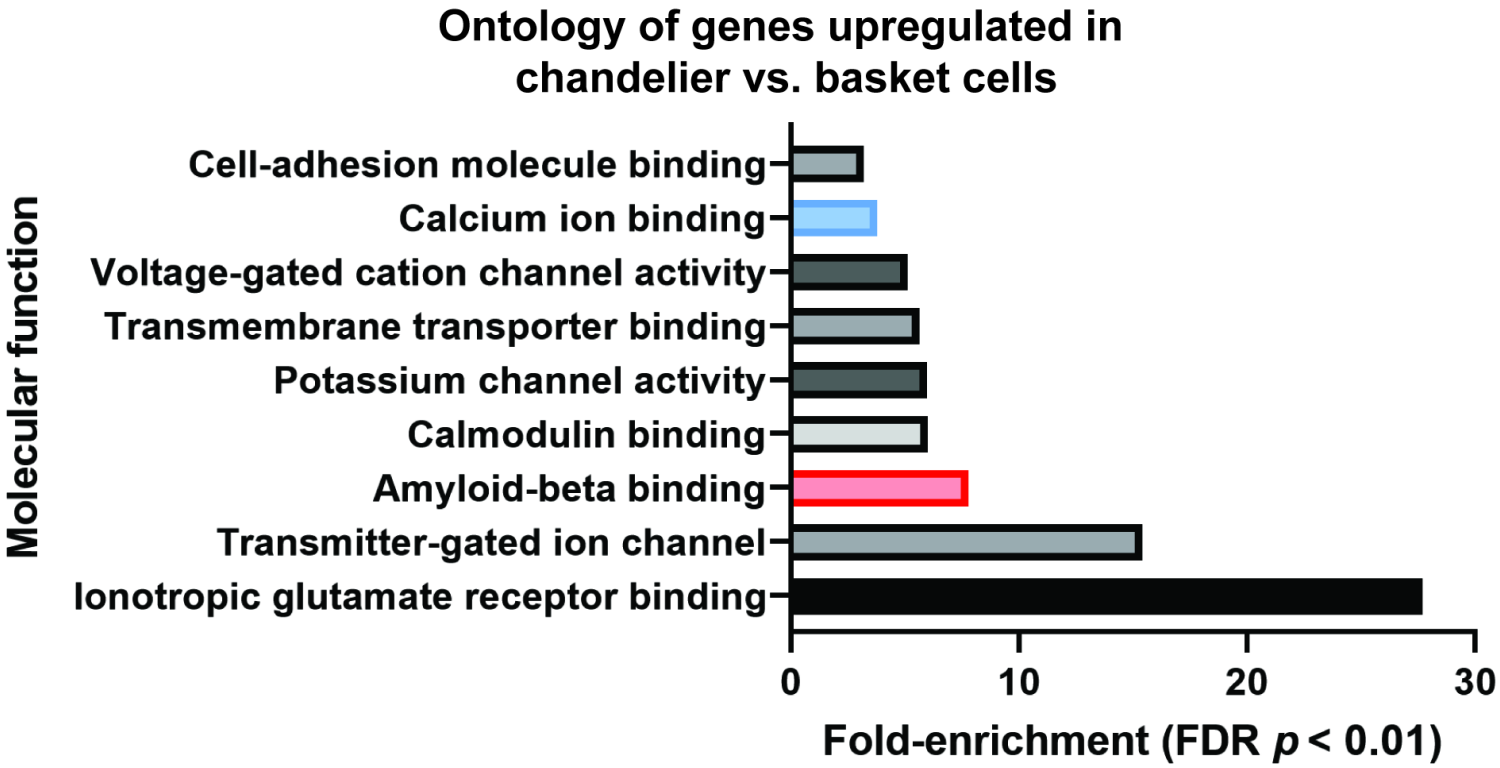

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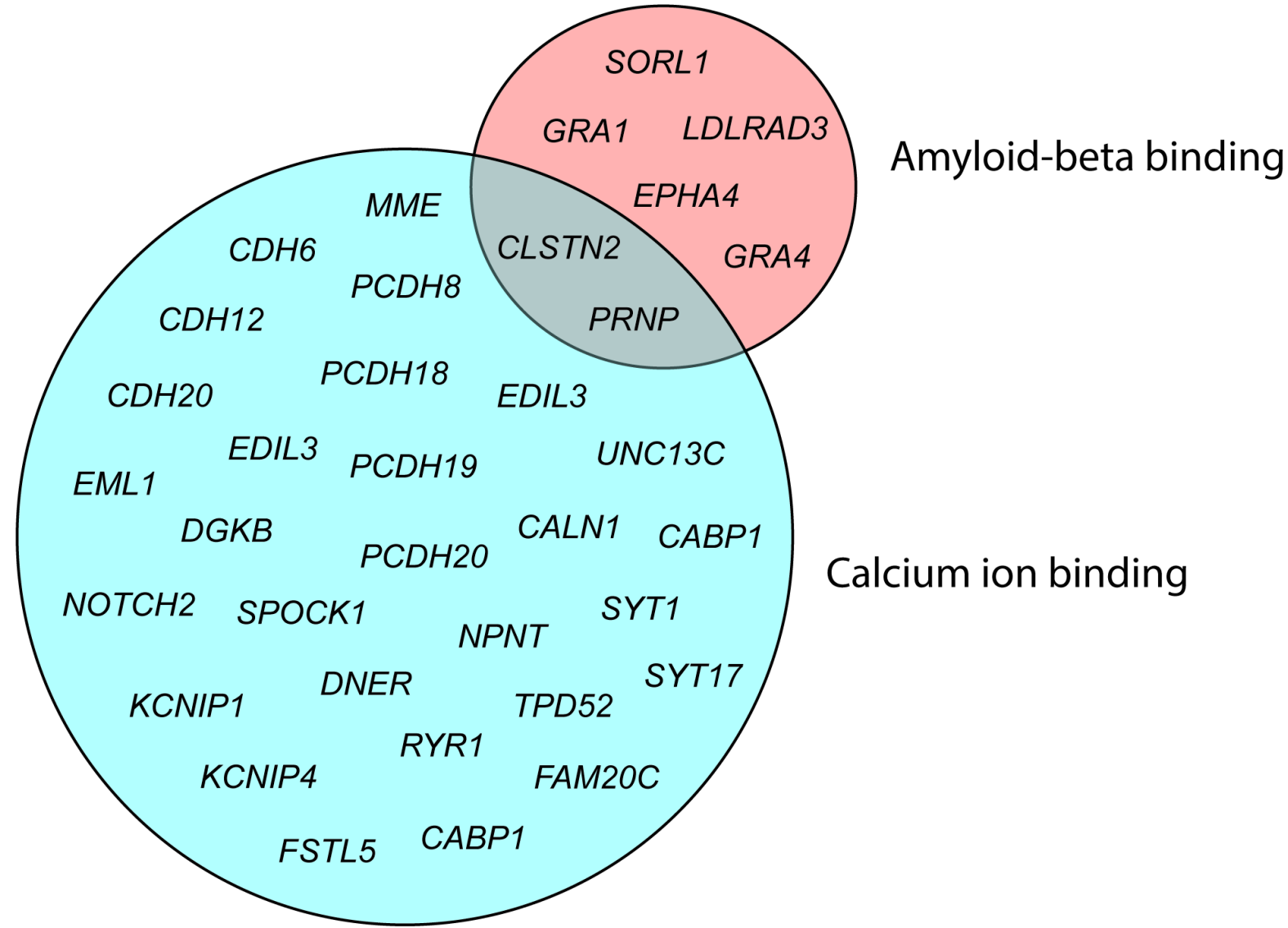

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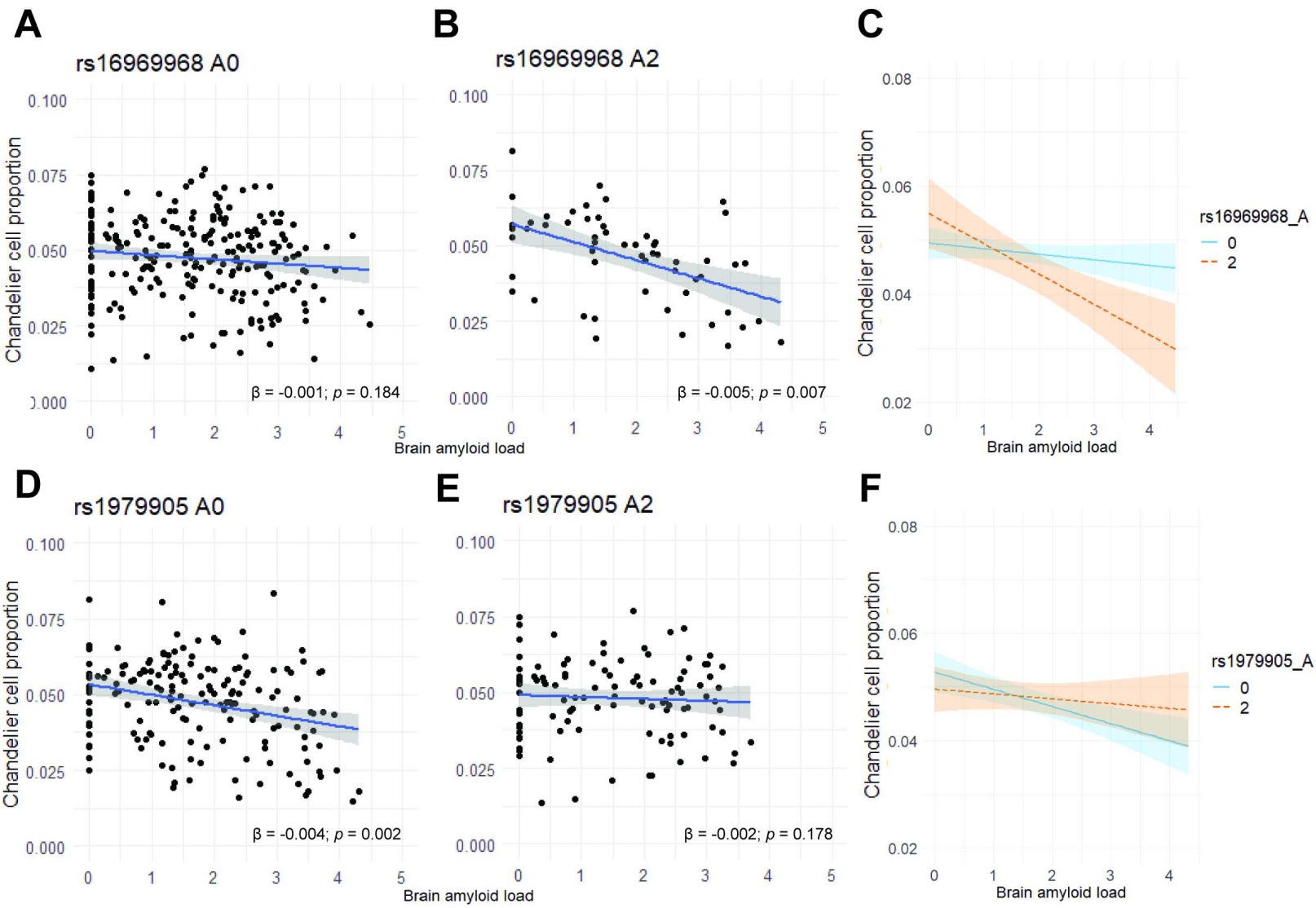

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