TACR1 Genotypes Predict fMRI Response to Alcohol Cues and Level of Alcohol Dependence

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Background: The tachykinin receptor 1 (TACR1) gene is a promising candidate gene in the search for the genetic basis of alcohol dependence (AD); TACR1 antagonists improve symptomology not only in preclinical models of AD but also in a clinical sample of detoxified alcoholics (George et al., *Science* 319:1536, 2008). The purpose of the current study was to determine whether TACR1 single nucleotide polymorphisms (SNPs) were associated with (i) blood oxygen level dependent (BOLD) activation in response to gustatory alcohol cues in a sample of heavy drinkers and (ii) Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR) AD symptom count in a large, publicly available data set—the Study of Addictions: Genetics and Environment Genome Wide Association study (SAGE GWAS) (Bierut et al., 2010).

Methods: First, we examined relationships between TACR1 genotypes and neural responses during a craving task in 326 individuals with alcohol use disorders. Next, correlational analyses between 69 TACR1 SNPs and DSM-IV-TR AD symptoms were performed on the SAGE data set.

Results: rs3771863, rs3755459, and rs1106855 predicted BOLD activation in response to alcohol cues in those same reward and reinforcement brain areas, especially in the medial prefrontal cortex, striatum, and insula. rs3771863 also predicted AD symptom count in the SAGE data set and BOLD activation in the mesocorticolimbic pathway response to alcohol cues.

Conclusions: Each of the 5 SNPs in the TACR1 gene that was significantly related to AD severity in the SAGE data set and/or the BOLD response to the craving task is near the 3′ or 5′ areas of the gene and may therefore be near mutations with potential functional significance. In particular, the potential functional significance of rs1106855 should be explored because of its location within a stop codon.

Key Words: Alcoholism, TACR1, fMRI, SAGE.

CURRENT PHARMACOLOGICAL AND psychosocial treatments for alcohol dependence (AD) are only modestly effective. For example, naltrexone, 12-step facilitation, motivational enhancement approaches, and cognitive behavioral therapies have led to 12-month abstinence rates between 17 and 35% (Miller et al., 2001). These results speak to the large variability of biological and psychosocial mechanisms involved in the etiology and course of AD. Many of the most important mechanisms in the development of AD are heritable susceptibility factors, which contribute 50 to 60% of the disease risk (Dick and Bierut, 2006). However, molecular genetic studies to date have only explained 2 to 3% of the genetic variance in diagnosis (Dick and Bierut, 2006), perhaps due to the complexity and heterogeneity of the AD phenotype.

Previously, Seneviratne and colleagues (2009) reported a significant association between a single nucleotide polymorphism (SNP) of the tachykinin receptor 1 (TACR1) gene (rs6715729) and 2 haplotypes (formed by combinations of rs6715729–rs735668–rs6741029) to susceptibility for AD in Caucasians (odds ratio for first haplotype 1.89 [95% CI = 1.16, 3.11] and 11.31 [95% CI = 3.62, 32.35] for the second haplotype). The gene resides on the 2p11 region of chromosome 2, is approximately 115 kB in length, and contains 5 exons. It is transcribed into 4 variants (2 found in the human brain) via alternative promoter usage, differential slicing, or both (Seneviratne et al., 2009). A peptide known as substance P (SP) is the endogenous ligand that binds to the TACR1 receptor, a G protein-coupled receptor found in both the central and peripheral nervous systems. SP is released in response to pain or stress (Sinha et al., 2011). In the human brain, TACR1 receptors are the predominantly expressed subtype of neurokinin receptors (Rigby et al., 2005).

In preclinical studies performed by George and colleagues (2008), mice genetically deficient in TACR1 receptors showed a marked lack of voluntary alcohol consumption and an increased sensitivity to the sedative effects of alcohol relative to wild-type mice, an effect that was mimicked in wild-type mice by administering a TACR1 antagonist (Thorsell et al., 2010). Additionally, intracerebral infusion of SP into the amygdala reduces alcohol consumption in both

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wild-type and "anxious" rats (June et al., 2009). Importantly, the effect of TACR1 genotypes on alcohol consumption has been shown to be via a direct regulation of alcohol consumption, rather than a developmental effect on TACR1 function or structure (Thorsell et al., 2010).

Gilman and Hommer (2008) demonstrated beneficial effects of a TACR1 antagonist in humans via a functional magnetic resonance imaging (fMRI) study of affective responses. Placebo-treated alcoholics showed robust responses to negative affective images, while alcoholics who received a TACR1 antagonist (LY 686017) demonstrated less activation in the insula and medial temporal gyrus in response to negative images. Furthermore, alcoholics who received the TACR1 receptor antagonist showed greater activation to positive images, suggesting a shift in the emotional valence of positive and negative stimuli (Gilman and Hommer, 2008). This suggests potential clinical utility of TACR1 antagonists, because greater activation to positive stimuli is predictive of lower alcohol consumption in the first 6 months after detoxification (Heinz et al., 2007).

George and colleagues (2008) went on to demonstrate the clinical efficacy of a TACR1 antagonist for AD. In a randomized controlled experimental study, recently detoxified alcoholic in-patients were treated with a TACR1 receptor antagonist, LY 686017 ($n = 25$), or placebo ($n = 25$). The TACR1 receptor antagonist suppressed spontaneous alcohol cravings, improved overall well-being, blunted cravings induced by the Trier Social Stress Test (Kirschbaum et al., 1993), and attenuated concomitant cortisol responses.

Given the promising preclinical and clinical effects of TACR1 antagonism as a pharmacological treatment for AD, the current research aimed to better understand which variants within the candidate gene TACR1 influence susceptibility to AD as well as neural responses to alcohol cues. First, it was hypothesized that TACR1 SNPs would be significantly associated with AD symptom count in an independent sample: the Study of Addictions: Genetics and Environment Genome Wide Association study (SAGE GWAS) (Bierut et al., 2010). Second, it was also hypothesized that the same SNPs that show an association with AD in the SAGE GWAS would also show an association with neural responses to alcohol cues in regions such as the striatum, anterior cingulate, orbitofrontal cortex, and insula in a different sample of heavy drinkers.

MATERIALS AND METHODS

fMRI Participants

Three hundred and twenty-six heavy drinking individuals (100 females, 31%) were recruited from a large metropolitan area in the southwest region of the United States (for details of recruitment and sample characteristics, see Claus et al., 2011). Briefly, the sample consisted of a racially diverse group, with 48.1% identifying themselves as White, 27.8% Latino, 4.7%, Native American, 0.6% Asian, 0.6% Native Hawaiian/Pacific Islander, 1.9% Black, and 16.3% mixed. The average age of participants was 31.86 (SD = 9.72). The average number of drinks per drinking day in the last month was 7.35 (SD = 4.16), the mean AD Score was 13.47 (SD = 8.17), and the mean Alcohol Use Disorders Identification Test score was 19 (SD = 8).

Taste Task

We utilized a taste cue task previously reported to elicit blood oxygen level dependent (BOLD) response in mesocorticolimbic areas (Filbey et al., 2008). All taste stimuli were delivered to the participants, while they were in the MR machine via Teflon tubing using a computer-controlled delivery system. The alcohol stimuli used were each subject's preferred alcoholic beverage, whereas the control stimulus was kept constant across subjects. The control stimulus (litchi juice) provided an appetitive control for the activation of the mesocorticolimbic circuitry. During each of two 9-minute echo-planar imaging runs, there were 12 pseudorandomized alcohol and control trials (6 of each). Each trial consisted of a 24-second taste delivery period, followed by a washout period to allow the liquid taste to dissipate before the next trial. The word "TASTE" was visually presented throughout the taste period. The washout period consisted of a 16-second rest period during which the word "REST" appeared on the screen; no gustatory stimulus was delivered during the rest period.

Image Acquisition

All MRI data were collected on a 3T Siemens Trio (Erlangen, Germany) whole body scanner. Prior to the acquisition of anatomical scans, localizer scans were acquired. An echo-planar gradient-echo pulse sequence (time to repeat [TR] = 2,000 ms, time to echo [TE] = 29, flip angle = 75°) was acquired with an 8-channel head coil, and images were acquired parallel to the ventral surface of a participant's orbitofrontal cortex to reduce signal dropout and distortion in this region. Each volume acquired consisted of 33 axial slices (64 × 64 matrix, 3.75 × 3.75 mm^2, 3.5 mm thickness, 1 mm gap). An amount of 275 echo-planar imaging volumes were collected in each run of the task; participants completed two 9:00 minute runs each. In addition to fMRI, we also collected a high-resolution T1-weighted MP-RAGE image for registration purposes (TR = 2,530 ms, TE = 1.64 ms, flip angle = 7°, 192 sagittal slices, 256 × 256 matrix, slice thickness 1 mm, no gap).

Image Analysis

Nonbrain tissue was removed from high-resolution anatomical images (T1-weighted) using FSL's Brain Extraction Tool (BET) (Smith, 2002). The first 7 volumes of each functional run were discarded to allow the magnet to reach steady state. MCFLIRT (FMRIB) was used to motion correct images within a run; each image within the run was aligned to the first volume within the run. Images were then deskulled using BET, spatially smoothed with an 8-mm full-width half-max Gaussian kernel, temporally filtered using a high-pass filter of 100 seconds, and grand mean intensity normalized; all of these steps were performed using FMRIB Expert Analysis Tool (FEAT). Statistical analyses were performed using the general linear model as implemented in FEAT. Customized square waveforms representing the onset times of each condition of interest (alcohol taste, alcohol rest, alcohol urge, control taste, control rest, and control urge) and the duration of stimulus presentation were convolved with a double-gamma hemodynamic response function. Time series analyses were conducted using FMRIB Improved Linear Model (FILM) with local autocorrelation estimation. Contrast maps were created by contrasting alcohol taste versus control taste conditions, that is, activation in response to alcohol with activation in response to the control stimulus subtracted. Contrast maps were then registered to the participant's high-resolution anatomical

image and the MNI 152 brain template using FMRIB Linear Image Registration Tool (FLIRT). Individual runs were then combined within participants using a fixed effects model, which determines estimates of each participant's contrast map as well as variance map. These second-level analyses were then used in a third-level analysis using FMRIB Local Analysis of Mixed Effects (FLAME) stage 1 only. Before computing group level statistics, all second-level images were registered to the MNI template.

DNA Collection

DNA was extracted from saliva consistent with previously published methods (Filbey et al., 2010). The 69 TACR1 SNPs assayed in SAGE were genotyped using the Illumina 1M Duo array. To be included, each SNP had to have a call rate >0.9, minor allele frequency >5%, and could not deviate from Hardy–Weinberg equilibrium.

SNP Association Analyses

The association test for each SNP was a regression of the allelic variation on a measure of the spatial extent of significant BOLD response in each mask. The first step was to examine the association between the group variable (in this case an SNP) and the alcohol greater than control difference in BOLD activation at each voxel within the whole brain. Each voxel was tested for an association with each SNP, and only those voxels that exceed a statistical threshold ($z > 2.32$) were highlighted in a spatial map. Cluster size was defined as the largest number of spatially contiguous voxels, which exceed the statistical threshold within the whole brain. To control for the number of voxels tested in the first step, the analysis software evaluated cluster size in relation to the number of voxels tested and calculated a probability distribution for how likely it would be to find a contiguous cluster of a specific size by chance (5% of the time for $p < 0.05$), given the number of voxels tested. To control for false positives, we used a false discovery rate (FDR) approach for all associations between SNPs and whole brain response. For each SNP, we found all clusters of activation that exceed the first pass voxel–level threshold ($z > 2.3$) and the p-values associated with each cluster (range 0 to 1). Using this approach, 3,498 clusters of 1 or more voxels and associated p-values were found across all SNPs in the positive and negative direction. p-Values were then adjusted using FDR (Benjamini and Hochberg, 1995).

TACR1 Association with a Clinical Phenotype

The health research sample of the SAGE data set was downloaded from the National Center for Biotechnology Information database of genotypes and phenotypes (NCBI dbGap study accession phs000092 v1.p1) to test the association between TACR1 SNPs and a clinical phenotype. We included only nonrelated Caucasian individuals (n = 2,605) to match our mostly Caucasian and Hispanic fMRI sample, as the rest of the SAGE sample was mostly African American and only 3% Hispanic. This sample was genotyped on the Illumina Human 1M beadchip. For the purpose of the current analyses, we selected the 69 TACR1 SNPs represented on this array. In the SAGE sample, genotypes were coded log-additively (0, 1, 2 copies of the minor allele). Further descriptions of sample demographics and quality controls for the genotyping are described elsewhere (Bierut et al., 2010). Covariates for sex and age were also included in the analysis. All subjects provided written informed consent for genetic studies and agreed to have their DNA and clinical data available through the National Institutes of Health repositories. All data for this study were de-identified. Effect sizes were calculated based on FDR-corrected p-values and sample size.

RESULTS

SNP Associations with BOLD Response to Alcohol Cue Task

The pattern of BOLD activity in response to the alcohol taste cue showed activation in the alcohol minus litchi contrast mainly in the mesocorticolimbic pathways where TACR1 receptors are highly expressed. The alcohol greater than control effect was significant in the anterior and posterior cingulate cortex, in addition to the dorsal striatum, insula, amygdala thalamus, and brainstem (Claus et al., 2011). BOLD activation in response to gustatory alcohol cues was predicted by rs3755459, rs3771863, and rs1106855 (Fig. 1). The minor allele (T) of rs3771863, which was predictive of greater AD symptom count in the SAGE sample, was associated with greater activation to alcohol cues in the lateral orbitofrontal cortex, the pallidum, and the putamen. The dominant allele (A) of rs3755459 was associated with greater activation in the caudate, cingulate, insula, pallidum, and putamen. For rs1106855, the minor allele (G) was associated with greater activation in the caudate, cingulate, and putamen. The effect sizes for each of these SNPs were small to medium, ranging from 0.103 to 0.30 (Table 1). Analyses were performed on the Caucasian ethnic groups individually, and the results were not significantly different from the overall group results (data not reported here).

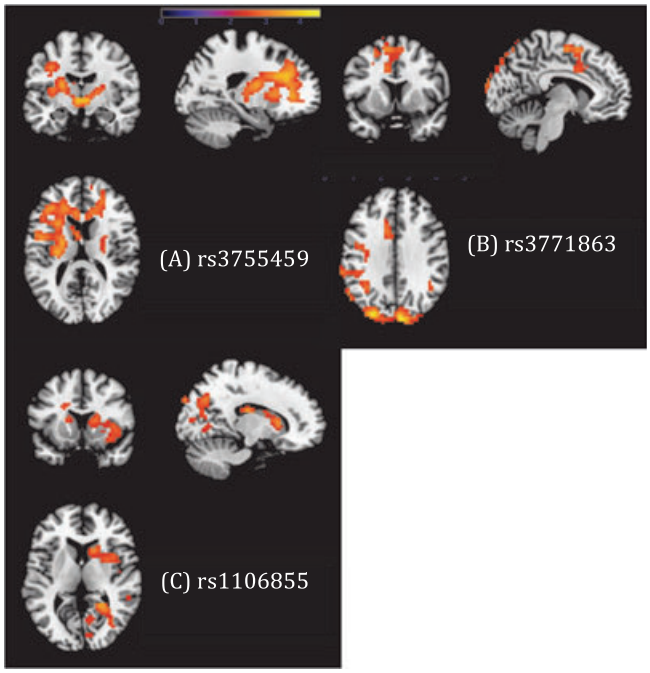

Fig. 1. (**A**) The major allele of rs3755459 predicted greater activation to gustatory alcohol cues in the caudate, putamen, and dorsolateral prefrontal cortex. (**B**) The minor allele of rs3771863 predicted greater activation to gustatory alcohol cues in the medial prefrontal cortex and cingulate. (**C**) The minor allele rs1106855 predicted greater activation to gustatory cues in the cingulate, insula, and lentiform nucleus.

SNP Associations in the SAGE GWAS

Three TACR1 SNPs predicted AD symptom count in the SAGE sample (rs10490308, rs11688000, and rs3771863) at a statistically significant level before the correction for multiple tests (p = 0.0076, 0.0343, 0.0415, respectively, df = 2,603) (Table 2). In this sample, the minor allele (T) of rs3771863 was associated with higher AD symptom count than the major allele (C). Only the association between rs10490308 and AD symptom count passes an FDR correction (FDR = 0.05, p threshold = 0.008625). The effect sizes as measured by Cohen's d were very small, ranging from 0.078 to 0.104 (Table 2).

Table 1. Cluster Sizes with FDR-Corrected p-Levels and Cohen's d for Blood Oxygen Level Dependent Response to Alcohol Cues as a Function of TACR1 Genotype

SNP	Whole brain largest cluster size, contiguous voxels	FDR-corrected p-value	Cohen's d
rs3755459	4,189	7.98e−9	0.1082
rs3771863	3,348	2.5e−7	0.3076
rs1106855	2,138	1.39e−4	0.1031

SNP, single nucleotide polymorphism; FDR, false discovery rate.

Table 2. SNPs with Highest Association with Alcohol Use Disorders Symptom Count Based on the SAGE GWAS Data Set

SNP	Position	Allele A	Allele B	FDR-corrected p-value	Cohen's d
rs10490308	75335883	**A**	C	**0.007608812**	**0.1041**
rs11688000	75146665	**A**	G	**0.0342919987**	**0.081**
rs3771863	75273222	C	**T**	**0.041533356**	**0.0781**

Allele names are assigned in alphabetical order and for the forward strand for each SNP. Significant associations are presented in bold type.

SAGE GWAS, Study of Addictions: Genetics and Environment Genome Wide Association study; SNP, single nucleotide polymorphism; FDR, false discovery rate.

DISCUSSION

The goal of this research was to find genetic variations in the TACR1 gene that predicted not only the clinical phenotype of AD, but also an intermediate neurobiological phenotype, BOLD response during alcohol cue presentation. Each of the SNPs associated with AD symptom count in the SAGE sample (rs10490308, rs11688000, and rs3771863) and the 3 SNPs associated with BOLD response to gustatory alcohol cues (rs3771863, rs3755459, rs1106855) are in regions of the TACR1 gene with high potential for functional significance (Fig. 2). rs3771863 and rs10490308 are in the 5′ untranslated region and thus could be in linkage disequilibrium (LD) with an SNP that influences regulatory element that would increase or decrease production of TACR1 receptors. rs11688000, rs1106855, and rs3755459 are in the 3′ untranslated region and could be in LD with an SNP that might alter the stability of the messenger ribonucleic acid as it travels from the nucleus to ribosomes.

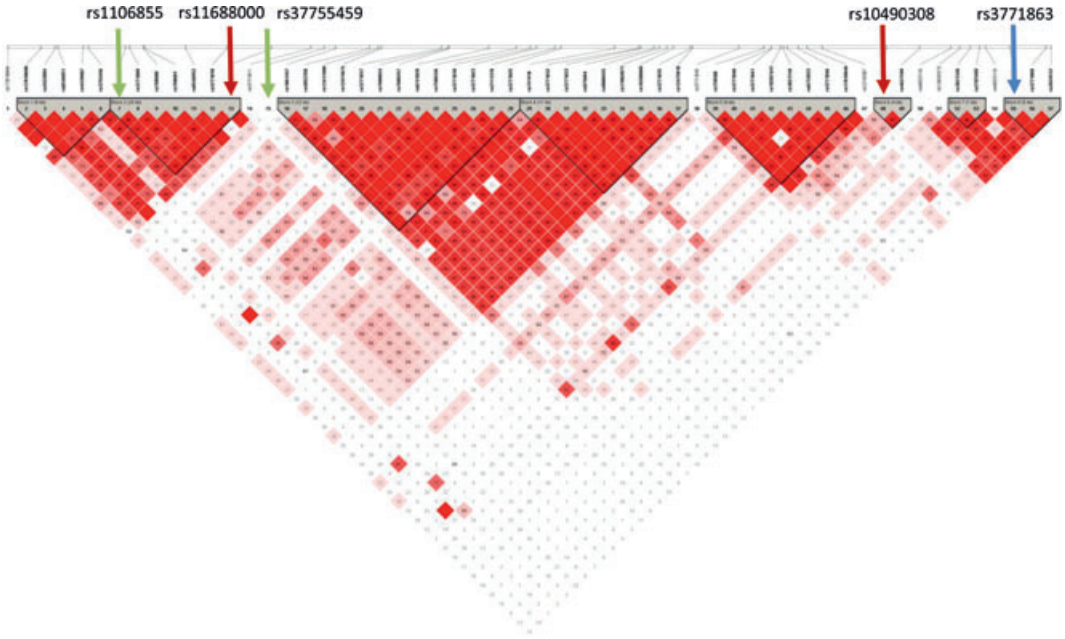

Fig. 2. Linkage disequilibrium plot from Haploview 4.1 for EA subjects based on Hapmap (phase II) samples of individuals of European Ancestry for the single nucleotide polymorphisms (SNPs) evaluated in this study. Pairwise SNP |D'| values (×100) of linkage are shown along with 2 haplotype blocks identified using the 4-gamete rule. Darkened blocks indicate SNP pairs without evidence of extensive recombination (i.e., 4-gamete rule for haplotype block characterization with at least one 2-SNP haplotype having a frequency <0). The second and fourth arrows (left to right) indicate SNPs significantly associated with alcohol dependence symptom count in the SAGE sample. The first and third arrows indicate SNPs significantly associated with the blood oxygen level dependent response to gustatory alcohol cues. The fifth arrow indicates the SNP that was significantly associated with the SAGE sample and the imaging sample.

The 3 SNPs that were significantly related to BOLD response to alcohol cues were associated with activity in the putamen and caudate, areas involved in learning and memory (Mattfeld et al., 2011). There were also significant associations between areas involving emotional perception and processing, such as the cingulate (Etkin et al., 2010), and areas involved in impulse regulation and interoceptive monitoring, including the orbitofrontal cortex and insula (Naqvi and Bechara, 2010; Zeeb et al., 2010). Given that the natural ligand for the receptor coded by TACR1, SP, is released in response to stress, TACR1 may play a role in the learning of using alcohol consumption as a coping mechanism or in relapse to alcoholic drinking under stressful conditions via its interaction with serotonergic, dopaminergic, and noradrenergic systems in the brain (Schank et al., 2011).

One SNP, rs3771863, was predictive of both BOLD activation in response to alcohol cues and AD symptom count in the SAGE GWAS (at a nominal level). However, it is unclear whether or not this SNP is a potential functional determinant of TACR1 receptor structure or activity because it is within an intron in the gene. The functions of introns remain largely unknown, although some have been implicated in differential RNA slicing (Mattick, 2004). Alternatively, it might be in high LD with a functional SNP in the 5′ untranslated or promoter region, which is more likely to alter TACR1 receptor number, structure, and function. For example, the signal from this end of the gene is also reflected in the statistically significant association between rs10490308 and AD symptom count in the SAGE GWAS.

Another SNP with greater potential functional significance, rs1106855, was associated with BOLD activation in response to alcohol cues. This SNP is in an intron, but is located within a stop codon. Therefore, it could affect the amount of mRNA produced by a cell and thereby alter the number of receptors or the physical confirmation of the protein and its associated binding properties. These findings must be interpreted within the limitations of this study. First, the role of any 1 SNP or gene in the determination of the complex phenotype of AD is likely to be one of many contributing to interconnected neurobiological pathways. Personalized medicine based on single gene function is therefore limited, and a cellular systems approach, combining the study of many genes or many thousands of SNPs that underlie neural activity, might be a more fruitful alternative.

A limitation of this study was the use of Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR) AD symptom count as the clinical phenotype in the SAGE GWAS. The DSM-IV-TR AD diagnosis has limited utility because 2 people can be diagnosed with AD without sharing even 1 symptom. Thus, as a phenotype, it is not uniform. However, the association with BOLD activation in response to alcohol cues identified more potential SNPs of interest, suggesting that BOLD responses may be a more sensitive phenotype for identifying intermediate effects of SNPs on risk for dependence. In fact, the effect size for rs3771863 is much larger in our sample than the SAGE sample, despite our much smaller sample size. As an intermediate phenotype, BOLD activation is theoretically closer to the gene's neurobiological activity and thus was also theoretically able to detect smaller, but potentially important effects of SNPs on the corresponding clinical phenotype, AD. This underscores the importance of intermediate phenotypes, such as BOLD activation, in the parsing of AD variation to aid in the development of novel medications.

In sum, SNPs were identified in areas of the TACR1 gene that are most likely to be functional. Further study of the role of the TACR1 receptor and its natural ligand of SP in the development and maintenance of AD might prove to be useful in the search for pharmacological treatments for AD.

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