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# Allelic Variation of Calsyntenin 2 (*CLSTN2*) Modulates the Impact of Developmental Tobacco Smoke Exposure on Mnemonic Processing in Adolescents

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## Abstract

**Background—**Exposure to nicotine in tobacco smoke during development has been linked to subsequent deficits in attention and memory. The present study tested for evidence that genetic variation may contribute to individual differences in vulnerability to the effects of developmental exposure to tobacco smoke on memory and medial temporal lobe function in adolescents.

**Methods—**Verbal and visuospatial memory were assessed and functional magnetic resonance imaging (fMRI) data were acquired in 101 adolescents systematically characterized for prenatal and adolescent exposure to tobacco smoke, while they performed an encoding and recognition memory task. The impact of allelic variation at loci within *CLSTN2* (encoding synaptic protein calsyntenin 2) and *KIBRA*, shown previously to modulate early and delayed recall of words, on the dependent measures was examined.

**Results—***KIBRA* genotype did not exert significant main or interacting effects with prenatal or adolescent exposure to tobacco smoke on verbal or visuospatial memory. Previous observations of a beneficial effect of the *CLSTN2* C allele on verbal recall were replicated. Adolescent exposure to tobacco smoke reversed this beneficial effect and was associated with increased activation of parahippocampal gyrus during early and delayed recognition in *CLTSN2* C allele carriers. While the *CLSTN2* C allele conferred enhanced functional connectivity between brain regions subserving accurate verbal recognition, adolescent exposure to tobacco smoke reversed this effect.

**Conclusions—**These findings extend previous work demonstrating that calsyntenins play an essential role in learning and indicate that this role is modulated both by *CLSTN2* genotype and, during adolescent development, by exposure to tobacco smoke.

### Keywords

Adolescent; calsyntenin 2; nicotine; parahippocampal gyrus; prenatal; verbal memory

Nicotine in tobacco smoke binds to nicotinic acetylcholine receptors (nAChRs), which, when stimulated by endogenous acetylcholine, play a key role in regulating all phases of brain development (1–3). Rodent studies have shown that stimulation of nAChRs by



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nicotine during gestation and/or adolescence disrupts neurodevelopment, possibly by disrupting the regulatory actions of acetylcholine (1,4,5). In humans, prenatal exposure to maternal smoking elevates risk for deficits in auditory and visual attention and verbal memory in offspring (6,7). Adolescent exposure to nicotine via tobacco smoking has been linked to chronic impairments in verbal working memory that worsen during nicotine withdrawal (8,9). Nicotine withdrawal exacerbated working memory deficits in adolescent smokers are associated with reduced efficiency of brain regions that support mnemonic processing, including hippocampus and parahippocampal gyrus (8,10).

Genetic variation has been shown to contribute to individual differences in the acute effects of nicotine on the accuracy and efficiency of mnemonic processing in adults (11). Given evidence that developmental nicotine exposure can disrupt mnemonic processing and the function of medial temporal lobe structures critical to mnemonic function 8–10,12,13), we tested for evidence that genetic variation may mediate individual differences in the vulnerability to the effects of developmental exposure to tobacco smoke on memory and medial temporal lobe function in adolescents. The impact of allelic variation at loci within two genes (*CLSTN2* and *KIBRA*) previously implicated in human memory and hippocampal function (14–16) was examined in a sample of adolescents who underwent functional magnetic resonance imaging (fMRI) while they performed a task permitting assessment of the neural circuitry supporting encoding, rehearsal, and early and delayed recognition. As effective mnemonic processing requires functional integration between medial temporal lobe and anterior cortical structures (17–20), the impact of genotype and developmental exposure on functional connectivity of the medial temporal lobe during task-related activation was also examined.

*KIBRA* encodes a neuronal protein that may be involved with synaptic plasticity (21). Polymorphic variation at a locus within *KIBRA* (Single Nucleotide Polymorphism database [dbSNP] number rs17070145) has been shown to modulate verbal memory (early and delayed recall of words) and hippocampal function during memory retrieval (14–16). *CLSTN2* encodes the synaptic protein calsyntenin 2 (also called alcadein gamma). Polymorphic variation at a locus within *CLSTN2* (dbSNP number rs6439886) has been shown to modulate early and delayed recall of words in a large young Swiss sample but not in an older racially heterogenous U.S. sample (14). *CLSTN2* is expressed exclusively in brain, with high levels occurring in cortical gamma-aminobutyric acid (GABA)ergic interneurons and in medial temporal lobe regions (22). Recent work has demonstrated that calsyntenins are essential for learning, that metabolism of calsyntenins and amyloid precursor protein (APP) is coordinated in neurons, and that coordination of this metabolism may be regulated by nAChRs (23–27). Given evidence that nAChRs may play a role in the regulation of calsyntenin metabolism and the lack of evidence supporting a role for nAChRs in the function of *KIBRA*, we anticipated that the impact of developmental nicotine exposure on mnemonic processing would be modulated by allelic variation within *CLSTN2* but not by allelic variation within *KIBRA.*

## Methods and Materials

### Participants

Participants included 34 adolescent daily tobacco smokers with prenatal exposure to active maternal smoking, 21 smokers with no prenatal exposure to maternal smoking, 14 nonsmokers (defined as having a lifetime history of smoking no more than two cigarettes and expired air carbon monoxide [CO] < 8 ppm at screening and assessment) with prenatal exposure to maternal smoking, and 32 nonsmokers with no prenatal exposure to maternal smoking. Status as a current tobacco smoker was verified by expired air CO > 8 ppm or urine cotinine concentration > 200 ng/mL at screening, determined using Accutest NicAlert

Test Strips (Jant Pharmacal Corporation, Encino, California). Subjects were 13 to 18 years of age and were free of medical and psychiatric illness and substance abuse or dependence disorders, other than nicotine dependence, as determined by physical exam and structured clinical interview (28,29). At initial screening, general intelligence, reading achievement, symptoms of depression and inattention, and prenatal exposure to maternal smoking and to environmental tobacco smoke were assessed as previously described (30)(Table 1).

Parental consent was obtained for subjects 17 years of age and younger. This study was approved by the Yale University School of Medicine Human Investigation Committee. Subjects provided written assent or, for 18-year-olds, consent for study participation.

## Procedure

Test sessions were conducted in the late afternoon for all subjects and consisted of behavioral testing followed by fMRI scanning. To minimize potentially confounding effects of acute nicotine withdrawal on cognitive function (9,31), smokers were permitted to smoke their own brand of cigarettes during a break midway through behavioral testing and immediately prior to scanning. During assessment, prior to scanning, blood or, in 38 subjects who declined phlebotomy, saliva was obtained for genotyping and to measure nicotine and cotinine, the primary metabolite of nicotine, using gas chromatography.

## Assessment of Verbal and Visuospatial Memory

Verbal memory was assessed using the Hopkins Verbal Learning Test-Revised (HVLT-R) (32), which begins with three trials in which a list of 12 words is read by the examiner. At the end of each trial, the subject is asked to recall as many words from the list as possible. The three immediate recall trials are followed by a delayed recall trial 25 minutes later. Scores derived include total recall (the sum of the number of words correctly recalled during the three immediate recall trials), delayed recall (the number of words recalled after the 25-minute delay), and percent retention (the number of words recalled after the delay relative to the number of words recalled after the higher of trials two or three).

Visuospatial memory was assessed using the Medical College of Georgia (MCG) Complex Figure Test, a visuospatial learning and memory test in which subjects are initially presented with a complex geometric figure and instructed to draw an exact copy of the figure on a clean sheet of paper. Subjects are subsequently asked to draw the figure from memory after a 3-minute and a 30-minute delay interval. Scores, reflecting the number of components drawn correctly, include copy, immediate recall, and delayed recall (33).

## Assessment of Brain Function

Each imaging run consisted of four cycles, the components of which included rest, encode, rehearse, and forced choice recognition, each of which were of 20 seconds duration. Verbal and nonverbal cycles alternated during each imaging run. The structure of the verbal cycles is schematically depicted in Figure 1. Verbal stimuli consisted of 276 monosyllabic English words that were three to six letters in length. During the verbal encode phase, subjects saw six words sequentially for 3.333 seconds each, followed by a 20-second rehearsal period. During the early recognition period, subjects saw three familiar words and three new words, with the sequence of old and new randomized across recognition blocks and runs. Subjects indicated whether each word was old (seen during the encode phase) or new with a button press. Nonverbal cycles were identical in design to the verbal cycles except that stimuli were characters from South Asian languages. Each run was of 5 minutes 40 seconds duration.

After six runs, a high-resolution anatomic scan was obtained, followed by two runs in which delayed recognition was assessed. Each delayed recognition run consisted of five cycles that

included a 20-second rest block followed by a 20-second verbal recognition and a 20-second nonverbal recognition block. Each delayed recognition block consisted of three stimuli presented during the encoding blocks, but not the recognition blocks, during the first six runs and three new stimuli. Subjects indicated whether each stimulus was old or new with a button press. Only stimuli presented during encoding blocks within the first five runs were included as targets in the delayed recall runs. Average duration between the end of the fifth run and the beginning of the delayed recall runs was 15.5 ± 1.2 minutes.

Subjects were scanned using a 3.0 Tesla Siemens Trio magnetic resonance imaging (MRI) system (Erlangen, Germany). Axial oblique T1-weighted anatomic images were acquired parallel to the anterior commissure-posterior commissure (AC-PC) line using a Fast Low Angle Shot (FLASH) sequence (25 contiguous slices, slice thickness = 5 mm, echo time [TE] = 2.47 msec, repetition time [TR] = 300 msec, flip angle [FA] = 60°, matrix = 256 × 256 pixels, field of view [FOV] = 20 $cm^2$). Epibold functional images were acquired in the same relative slice locations using a single-shot, gradient-echo pulse sequence (TE = 30 msrc, TR = 2000 msec, FA = 80°, matrix = 64 × 64 pixels, FOV = 20 $cm^2$, 1 average). One hundred seventy-one images were acquired per slice per run. The high-resolution anatomic scan was acquired using a sagittal magnetization-prepared rapid acquisition with gradient-echo (MPRAGE) pulse sequence (TE = 3.66 msec, TR = 2530 msec, FA = 7°, FOV = 256 × 256 pixels, slice thickness = 1 mm, 176 slices, 1 average).

A total of 97 subjects provided HVLT-R data, 96 subjects provided MCG Complex Figure Test data, and 93 subjects provided fMRI data. Four subjects provided fMRI data but no HVLT-R or MCG Complex Figure Test data. Eighty-nine subjects provided data on all measures.

## Genotyping

DNA was extracted from whole blood or saliva using standard methods. Single nucleotide polymorphisms (SNPs) rs17070145 (*KIBRA*) and rs6439886 (*CLSTN2*) were genotyped using a fluorogenic 5' nuclease assay method (the Taqman method [Applied Biosystems, Foster City, California]) (34). All samples were genotyped in duplicate for quality control, with no discrepancies. Genotype counts were as follows: *KIBRA*: 37 C homozygotes, 41 heterozygotes, 22 T homozygotes, 1 subject in whom genotyping failed, T allele carriers were combined into one group for statistical analyses (14); *CLSTN2*: 76 T homozygotes, 22 heterozygotes, 3 C homozygotes, C allele carriers were combined into one group for statistical analyses. Allele frequencies are provided in Supplement 1.

## Data Analysis

**Behavioral Data—**Verbal and visuospatial immediate and delayed scores were analyzed using linear regression implemented in S-Plus (Insightful Corporation, Seattle, Washington). Regression models were estimated that included variables for genotype, adolescent smoking, prenatal exposure status, and the two- and three-way interaction effects between these variables. Plasma nicotine and cotinine concentrations were estimated from salivary concentrations using previously described methods 9,).

**fMRI Data—**Image analysis was performed using locally developed software written in Matlab (MathWorks, Natick, Massachusetts). Prior to statistical analysis, functional images were sinc interpolated to correct for slice acquisition time and then were motion corrected for three translational directions and for the three possible rotations using SPM 99 (35). Images were then spatially smoothed with a 6.25 millimeter full-width at half maximum (FWHM) Gaussian kernel. Individual subject maps of activation associated with transient processes (time-locked responses evoked by the encoding and retrieval trials) and sustained

processes (average activation levels during the self-guided rehearsal blocks) were generated using regression-based methods implemented within the general linear model (GLM) (10,36). Transient effects were coded in the GLM with a reference hemodynamic response function modeled as a gamma function (tau = .9, $n = 4$, delay = 2.3; peaking at 5 seconds poststimulus) (37). The sustained effects of rehearsal were similarly coded by convolving a block-length boxcar function with this gamma function (36,37). Prior to across-subjects comparisons, fMRI data were spatially normalized to standard stereotactic space (Montreal Neurologic Institute [MNI] template) using nonlinear spatial normalization. Interacting effects of genotype and developmental exposure to tobacco smoke were assessed using voxelwise mixed-model repeated measures analysis of variance (ANOVA). A univariate voxel threshold of $p < .001$, corrected for mapwise false discovery rate (FDR) (38), was used with a further cluster threshold of 10 contiguous significant voxels. Percent signal change data were extracted from regions the voxelwise ANOVA identified as significant and were submitted to regression analysis as described above to confirm significance after controlling for potential confounding variables. Location of peak differences in activation were estimated from Talairach and Tournoux, after adjustment for differences between MNI and Talairach coordinate space using the nonlinear transformation by Brett (www.mrc-cbu.cam.ac.uk/Imaging/Common/mnispace.shtml).

**Connectivity Analysis—**Effects of genotype and developmental exposure to tobacco smoke on functional connectivity were examined using partial least squares (PLS) analysis (8,39,40). This multivariate analysis tests for interregional correlations in functional activity that show the greatest changes in strength or direction across genotype and tobacco smoke exposure conditions. Maps of correlations are computed for each group and condition between a source region and every other voxel in the brain. Partial least squares is then used to extract the primary components of the correlational pattern. Within each PLS component, loadings to brain regions are overlaid on anatomical images and indicate the set of areas that correlate with the source region. Each component also includes loadings to genotype and tobacco smoke exposure condition (i.e., how strongly each group reflects a given correlation pattern). In this analysis, a region of the right parahippocampal gyrus showing a significant *CLSTN2* genotype by adolescent smoking interaction effect on activation during delayed verbal recognition testing was used as the source region.

## Results

Demographic, clinical and cognitive characteristics of both groups are presented in Table 1.

The groups did not differ in age, years of education, birth weight, general intelligence, reading achievement, or symptoms of inattention. Parents of nonsmokers with prenatal exposure to maternal smoking completed fewer years of education than did parents of smokers and parents of nonsmokers with no prenatal exposure ($\beta = 4.0$, $t = 2.5$, $p < .02$). Smokers reported significantly more symptoms of depression at screening (Beck Depression Inventory scores: $\beta = 5.3$, $t = 3.0$, $p < .004$) and a greater history of cannabis consumption ($\beta = 296.4$, $t = 2.8$, $p < .007$). These effects of adolescent smoking and prenatal exposure were not significantly modified by genotype. There was a significant prenatal exposure by *CLSTN2* rs6439886 genotype interaction effect on race ($\beta = 1.2$, $t = 2.8$, $p < .007$). All Hispanic subjects were *CLSTN2* T homozygotes and were equally distributed across prenatal exposure groups. *CLSTN2* C allele carriers with prenatal exposure to maternal smoking included 11 European American (EA) and 2 African American (AA) subjects, while *CLSTN2* C allele carriers with no prenatal exposure included 5 EA, 6 AA, and 1 Asian American subject. There was a significant *CLSTN2* genotype by adolescent smoking by prenatal exposure interaction effect on gender ($\beta = 1.4$, $t = 2.2$, $p < .04$); all exposure groups included more female subjects than male subjects except for C allele smokers with

no prenatal exposure and nonsmokers with prenatal exposure, both of whom included more male subjects than female subjects. The *CLSTN2* genotype by adolescent smoking by prenatal exposure effect on history of alcohol consumption was also significant ($\beta = 9.3$, $t = 2.1$, $p < .05$); rate of alcohol consumption was greatest among smokers with no prenatal exposure who were carriers of the *CLSTN2* C allele, intermediate among the remaining smokers, and lowest among nonsmokers across prenatal exposure and genotype groups. Among smokers, effects of prenatal exposure to maternal smoking and *CLSTN2* or *KIBRA* genotype on age at onset of smoking, years of daily smoking, number of cigarettes smoked per day, and symptoms of nicotine dependence (measured using the Fagerstrom Test for Nicotine Dependence [FTND] [41]) were not significant. Adolescents with prenatal exposure to active maternal smoking also had more prenatal exposure to alcohol ($\beta = .3$, $t = 2.2$, $p < .05$). This effect was not significantly modified by smoking status or genotype. Rates of prenatal exposure to cannabis or cocaine did not significantly differ across the groups. Among smokers, effects of genotype and prenatal exposure on estimated plasma concentrations of nicotine and cotinine at the time of assessment were not significant.

Potential confounding effects of group differences in race, gender, alcohol use, parental education, baseline symptoms of depression, prenatal exposure to maternal alcohol consumption, and cannabis use were controlled for by including these variables in the regression models used to analyze the behavioral and brain imaging data. As our prior work in a larger sample has shown that adolescent smoking is associated with lower estimated general intelligence 6,), IQ was also included in the regression models.

## Effects of Genotype on the Impact of Developmental Exposure to Tobacco Smoke on Accuracy of Memory Performance

*KIBRA* genotype did not exert significant main or interacting effects with prenatal or adolescent exposure to tobacco smoke on accuracy of verbal or visuospatial immediate or delayed recall. Similarly, *CLSTN2* genotype did not exert significant main or interacting effects on accuracy of visuospatial immediate or delayed recall or verbal immediate recall. However, delayed verbal recall and percent retention was significantly more accurate in *CLSTN2* C allele carriers (main effect of genotype on delayed verbal recall: $\beta = 2.2$, $t = 3.1$, $p = .003$; on verbal percent retention: $\beta = 12.7$, $t = 2.3$, $p = .02$). This benefit of the *CLSTN2* C allele on verbal delayed recall was occluded by adolescent smoking (genotype by adolescent smoking interaction effect on delayed verbal recall: $\beta = -4.9$, $t = -2.3$, $p = .03$; Figure 2). Effects of *CLSTN2* genotype on verbal memory were not significantly modified by prenatal exposure to maternal tobacco smoking.

## Impact of *CLSTN2* Genotype and Adolescent Smoking on Medial Temporal Lobe Function During Verbal Encoding and Recognition

Given the observed significant interaction between *CLSTN2* genotype and adolescent smoking on accuracy of verbal recall, the lack of effects of *KIBRA* genotype on verbal or visuospatial memory, and the known importance of the medial temporal lobe to mnemonic function (12), fMRI data were examined for evidence of interacting effects of *CLSTN2* genotype and adolescent smoking on medial temporal lobe function during verbal mnemonic processing. Effects of genotype and adolescent smoking on accuracy of task performance during scanning were not significant (*p* values > .2).

*CLSTN2* genotype by adolescent smoking interaction effects on medial temporal lobe activation during encoding and rehearsal were not significant. Significant *CLSTN2* genotype by adolescent smoking interaction effects were observed on activation of bilateral parahippocampal gyrus during early recognition of verbal stimuli presented during encoding (Talairach coordinates: right parahippocampal gyrus: x = 26, y = −32, z = −12, volume =

528 $mm^3$, Brodmann area [BA] 35/36; left parahippocampal gyrus: x = −30, y = −32, z = −12, volume = 80 $mm^3$, BA 35/36). Percent signal change data extracted from these regions indicated that activation of bilateral parahippocampal gyrus during early recognition was greater in C allele carriers who were smokers than in T homozygotes or nonsmokers carrying the C allele (Figure 3). Genotype by adolescent smoking interaction effects observed on activation of right fusiform gyrus (Talairach coordinates: x = 35, y = −65, z = −10, BA 19) and right medial frontal gyrus (Talairach coordinates: x = 0, y = 30, z = −15, BA 11) during early recognition testing were not significant after controlling for potential confounding variables. A similar significant *CLSTN2* genotype by adolescent smoking interaction effect was observed on activation of right parahippocampal gyrus during delayed recognition of verbal stimuli presented during encoding (Talairach coordinates: x = 28, y = − 32, z = −12, volume = 1096 $mm^3$, BA 35/36). Percent signal change data extracted from this region indicated that activation of right parahippocampal gyrus during delayed recognition was greater in C allele carriers who were smokers than in T homozygotes or nonsmokers carrying the C allele (Figure 4). The genotype by adolescent smoking interaction effect on activation of left superior frontal gyrus (Talairach coordinates: x = −26, y = 52, z = −15, BA 11) during verbal delayed recognition testing stemmed from increased activation of this region in nonsmokers carrying the *CLSTN2* C allele, decreased activation in smokers carrying the *CLSTN2* C allele, and minimal activation of this region in *CLSTN2* T homozygotes.

### Impact of *CLSTN2* Genotype and Adolescent Smoking on Functional Connectivity

Given the significant interaction between *CLSTN2* genotype and adolescent smoking on accuracy of delayed verbal recall during HVLT-R testing, we examined functional connectivity using data acquired during delayed verbal recognition testing. The right parahippocampal gyrus region (Talairach coordinates: x = 28, y = −32, z = −12, volume = 1096 $mm^3$) showing a significant *CLSTN2* genotype by adolescent smoking interaction effect on activation during delayed verbal recognition testing was used as a seed region.

This analysis produced two components that accounted for the majority of the variance in the imaging data. The first component, accounting for 69.5% of the variance, reflected a modest main effect of *CLSTN2* genotype, whereby activation of the right parahippocampal gyrus seed region was more strongly positively correlated with activation of adjacent tissue in C allele carriers than in T homozygotes regardless of smoking status (factor loadings: smokers: T homozygotes = .34, C allele carriers = .59; nonsmokers: T homozygotes = .35, C allele carriers = .64; Supplement 2). The second component, accounting for 18.1% of the variance, strongly differentiated adolescent smokers and nonsmokers by *CLSTN2* genotype (factor loadings: smokers: T homozygotes = .16, C allele carriers = −.80; nonsmokers: T homozygotes = .13, C allele carriers = .57; Figure 5, left panel). Connectivity maps for this component are shown on the right side of Figure 5, where red/yellow indicates regions demonstrating positive functional connectivity with the seed region (encircled in green) that is consistent with the factor loadings. Positive loadings were observed at the medial frontal gyrus (x = 4, y = 53, z = −15), bilateral caudate nucleus (x = 8, y = 19, z = −2 and x = −5, y = 19, z = −2), anterior cingulate gyrus (x = 0, y = 35, z = 6; BA 24), right inferior/middle frontal gyrus (x = 38, y = 41, z = 6; BA 10/46), bilateral inferior/middle frontal gyrus (x = 50, y = 28, z = 23; BA 46 and x = −48, y = 22, z = 22; BA 46), and left posterior precuneus (x = −7, y = −59, z = 25; BA 31). During delayed verbal recognition, activation of right parahippocampal gyrus seed voxels was strongly positively correlated with activation of medial frontal gyrus, bilateral caudate nucleus, anterior cingulate gyrus, right inferior/middle frontal gyrus, bilateral inferior/middle frontal gyrus, and left posterior precuneus in nonsmokers who carried the *CLSTN2* C allele. Connectivity between these regions and right parahippocampal gyrus was less strongly positive in T homozygote smokers and

nonsmokers. Strikingly, in smokers who carried the C allele, inverse connectivity was observed between the right parahippocampal gyrus seed region and frontal cortical regions, caudate nuclei, and precuneus, indicating that as activation of right parahippocampal gyrus increased during delayed recognition in these subjects, activation of frontal cortical regions, caudate nuclei, and precuneus decreased.

## Discussion

In the present study, the previous observation of a beneficial effect of the *CLSTN2* C allele on delayed recall of words was replicated (14). We further observed that exposure to tobacco smoke during adolescent development reverses this beneficial effect of the *CLSTN2* C allele on verbal memory. *CLSTN2* encodes calsyntenin 2 (22). Calsyntenins are transmembrane proteins, the extracellular domain of which is essential for multiple types of learning in *Caenorhabditis elegans* (23). Calsyntenins form a tripartite complex with the scaffolding protein X11L and APP that suppresses intracellular metabolism of both APP and calsyntenin (24). When dissociated from this tripartite complex, calsyntenin and APP undergo proteolytic cleavage by presenilin dependent γ-secretase, leading to the extracellular secretion of β-alc and amyloid β peptide (Aβ)(42). Accumulation of Aβ is a hallmark of Alzheimer's disease, the cardinal symptom of which is loss of memory (43,44). These cleavage events also generate the intracellular domain fragments AlcICD and AICD from calsyntenin and APP, respectively (42). Recent evidence suggests that AICD interacts with the proteins FE65 and TAG1 to regulate gene transactivation and suppress neurogenesis (45). AlcICD also associates with FE65 and can inhibit the FE65-dependent gene transactivation activity of AICD (42). Given that *CLSTN2* is expressed in the medial temporal lobe and cortex (22), these observations suggest that the *CLSTN2* C allele may constitutively improve memory by enhancing the stability of the calsyntenin-X11L-APP complex, thereby reducing the generation of Aβ and AICD and enhancing neurogenesis in brain structures critical to the adequate development of neurocircuitry that supports memory.

Nicotinic acetylcholine receptor agonists have been shown to modify the secretion of Aβ in mature neurons, which may contribute to the therapeutic action of these agents in cognitive decline (27,46–49). The mechanism by which nAChR ligands modify Aβ secretion is not known. However, if activation of nAChRs modifies Aβ secretion by altering the stability of the calsyntenin-X11L-APP complex, the present observations could reflect an effect of nAChR activation by nicotine in developing neurons that eliminates the beneficial effect of the *CLSTN2* C allele by altering the stabilizing effect of the allele on the calsyntenin-X11L-APP complex. This would lead to increased generation of Aβ and AICD and consequent reductions in neurogenesis in regions subserving memory, relative to *CLSTN2* C allele carriers with no developmental exposure to nicotine. Our failure to observe significant interacting effects between *KIBRA* genotype and developmental exposure to tobacco smoke is consistent with the lack of evidence that nAChRs modulate the function of this gene.

Among *CLSTN2* C allele carriers, adolescent exposure to tobacco smoke was associated with increased activation of bilateral parahippocampal gyrus during early verbal recognition and right parahippocampal gyrus during delayed verbal recognition, relative to T homozygotes and C allele carriers with no tobacco exposure during adolescence. Converging evidence indicates that the parahippocampal gyrus and perirhinal cortex play a key role in recognition memory (50–53). Firing rates of neurons in these regions decrease in response to previously encountered stimuli, while activation of parahippocampal and perirhinal regions during recognition is inversely related to memory strength (50–53). Thus, loss of the beneficial effect of the *CLSTN2* C allele on memory in adolescent smokers may stem from maldevelopment of the parahippocampal gyrus that consequently reduces the

ability of neurons in this region to modulate activity to convey information about prior stimulus occurrence.

Connectivity analysis revealed that the benefit of the *CLSTN2* C allele on delayed recognition of words in adolescent nonsmokers is associated with enhanced positive functional connectivity between right parahippocampal gyrus, bilateral caudate nuclei, and frontal cortical regions, including anterior cingulate gyrus and bilateral inferior/middle frontal gyrus. Loss of the beneficial effect of the *CLSTN2* C allele in adolescent smokers was associated with inverse functional connectivity between these regions, while connectivity between these regions was weaker but still positive in noncarriers of the *CLSTN2* C allele. Frontal cortical regions, including inferior/middle frontal gyrus (BA 9/10/46), are recruited during successful verbal recognition, with stronger medial temporal lobe-frontal cortical functional connectivity being associated with better verbal memory performance (17,54–56). Suppression of competing verbal memories, an essential component of accurate recognition memory performance, is associated with the recruitment of anterior cingulate cortex that closely correlates with the recruitment of right inferior middle frontal cortex (57). Together, these observations suggest that, in the absence of exposure to nicotine during adolescent development, the *CLSTN2* C allele confers strengthened connectivity between brain regions critical to accurate retrieval during recognition memory. This strengthened connectivity likely supports more efficient neural processing during retrieval and thus may contribute to more accurate task performance. Inverse functional connectivity between right parahippocampal gyrus and bilateral caudate and frontal cortical regions conferred by adolescent exposure to nicotine in *CLSTN2* C allele carriers, together with the observation of increased activation of right parahippocampal gyrus during delayed verbal recognition testing, is consistent with maldevelopment of neurocircuitry supporting mnemonic processing in these subjects, possibly stemming from reversal of a stabilizing effect of the *CLSTN2* C allele on the calsyntenin-X11L-APP complex by activation of nAChRs.

The possibility that the group differences in verbal memory, brain function, and functional connectivity observed in the present study stem from factors unrelated to genotype or developmental exposure to tobacco smoke cannot be excluded. However, the fact that the observed interaction effects between developmental exposure to tobacco smoke and *CLSTN2* genotype remained significant after group matching and statistical controls for potential confounding variables argues against this possibility. While we have focused on developmental exposure to nicotine in the discussion of mechanisms that may underlie the observed findings, the possibility that developmental exposure to other components of tobacco smoke may have contributed to the observed effects cannot be excluded by these data. In the present study, immediate and delayed recall were assessed before scanning, while early and delayed recognition memory were assessed during scanning. While there are important distinctions between recall and recognition memory, both are forms of memory that rely on medial temporal lobe structures when there is a delay between the encoding and retrieval events (12,58–60), such as that employed during assessment of delayed recall using the HVLT-R and the MCG Complex Figure Test and d uring early and delayed recognition testing during scanning. Other limitations include the measurement of prenatal exposure by retrospective self-report. Work comparing prospectively and retrospectively collected data about pregnancy has supported the accuracy of pregnancy data collected by retrospective self-report (61). However, given the social stigma associated with tobacco smoking during pregnancy, underreporting of prenatal exposure may have weakened observed effects of prenatal exposure on the dependent measures. Further work is needed to address this limitation in samples prospectively characterized for prenatal exposure to maternal smoking.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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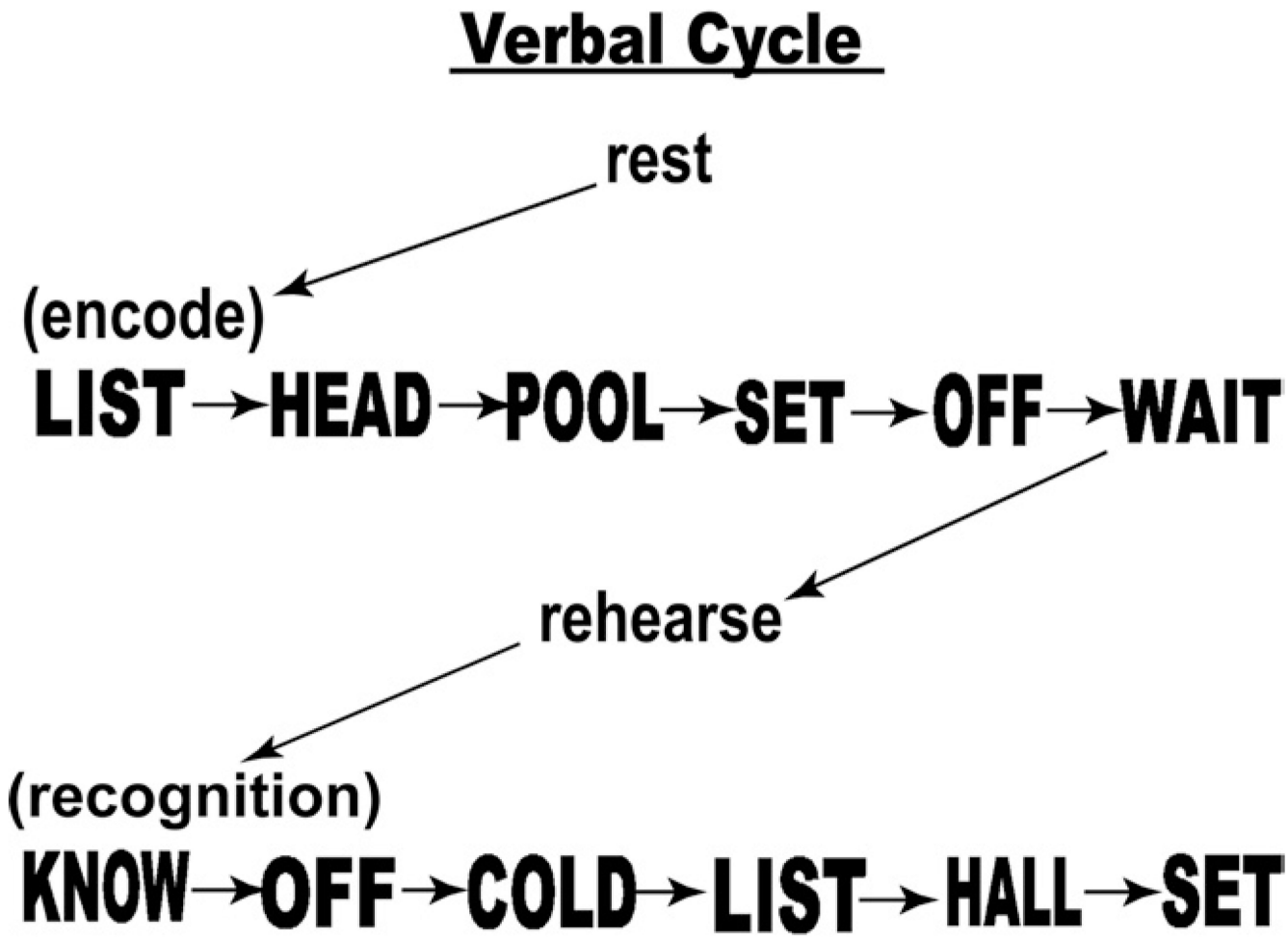


**Figure 1.**
Schematic representation of the structure of the verbal cycles during fMRI scanning. During the verbal encode phase, subjects saw six words sequentially for 3.333 seconds each, followed by a 20-second rehearsal period. During the early recognition period, subjects saw three familiar words and three new words, with the sequence of old and new randomized across recognition blocks and runs. fMRI, functional magnetic resonance imaging.

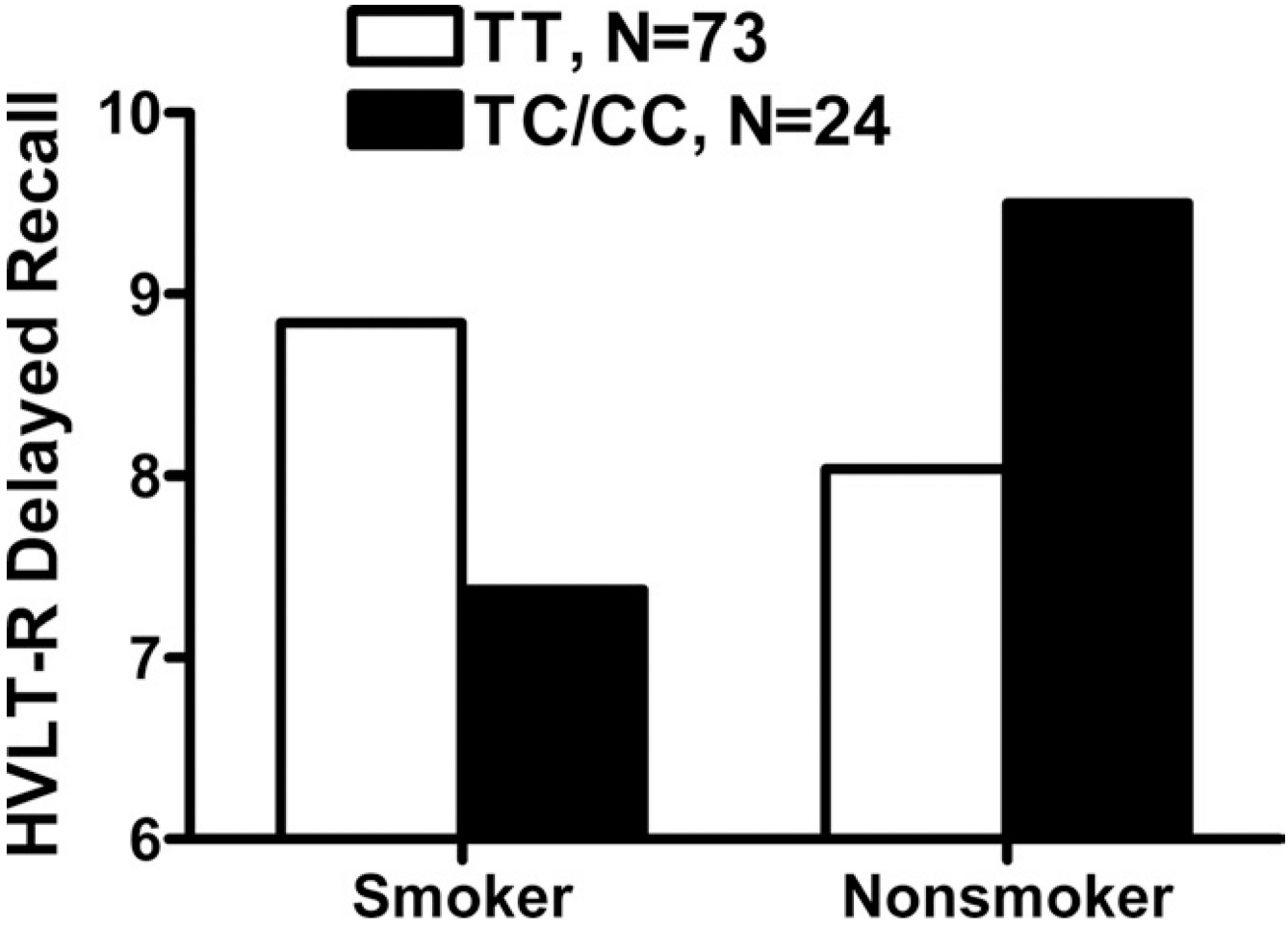


**Figure 2.**
*CLSTN2* genotype by adolescent smoking interaction effect on accuracy of delayed verbal recall (β = −4.9, $t$ = −2.3, $p$ = .03). HVLT-R, Hopkins Verbal Learning Test-Revised.

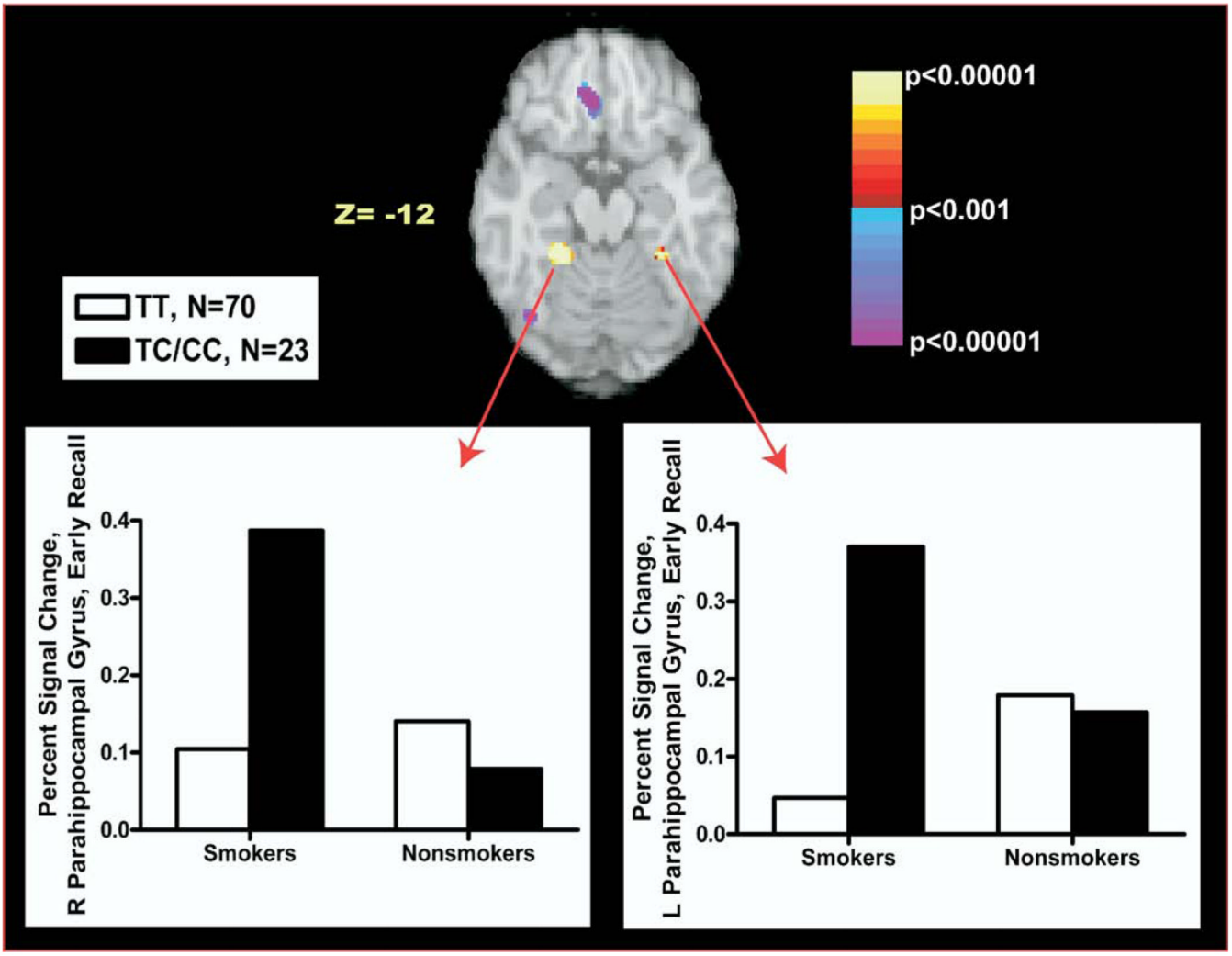


**Figure 3.**

*CLSTN2* genotype by adolescent smoking interaction effects on activation of left and right parahippocampal gyrus during early recognition of verbal stimuli. Images are displayed per radiological convention, with the right side of the brain on the left side of the figure. Voxel threshold: $p < .001$, FDR corrected; cluster threshold: 10 contiguous significant voxels. Plots are shown of average percent signal change for left and right parahippocampal gyrus voxels demonstrating significant *CLSTN2* genotype by adolescent smoking interaction effects during early verbal recognition. FDR, false discovery rate.

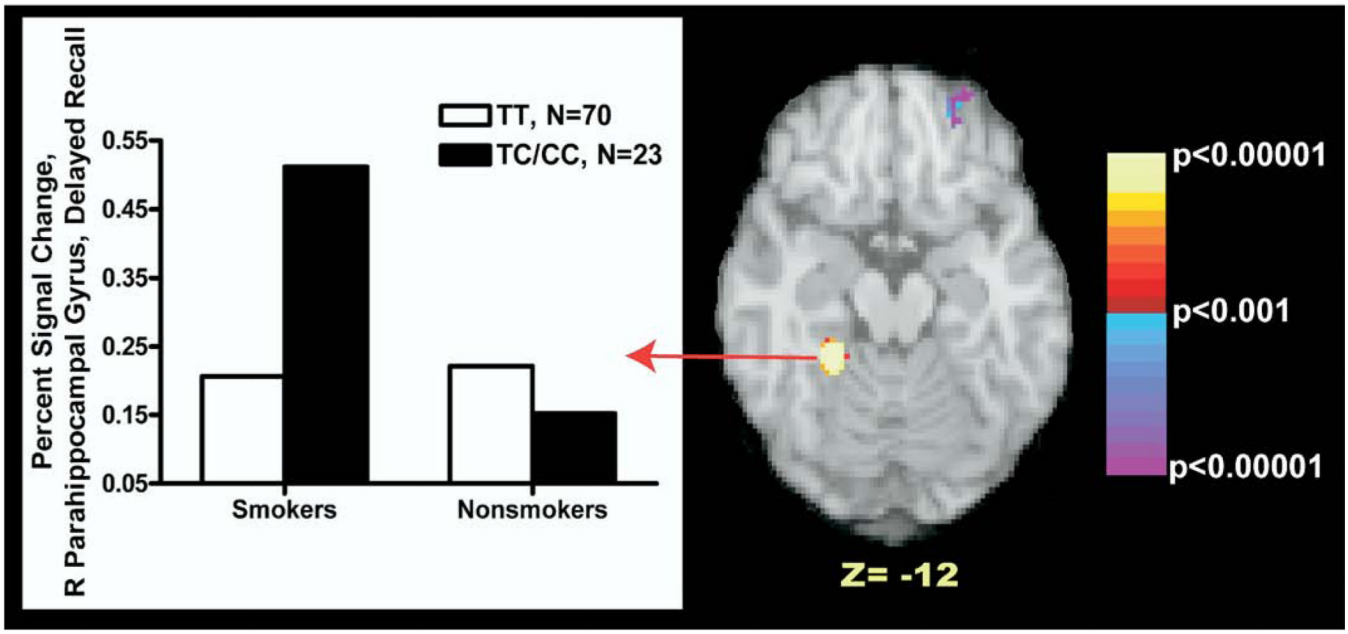


**Figure 4.**

*CLSTN2* genotype by adolescent smoking interaction effects on activation of right parahippocampal gyrus during delayed recognition of verbal stimuli. Images are displayed per radiological convention, with the right side of the brain on the left side of the figure. Voxel threshold: $p < .001$, FDR corrected; cluster threshold: 10 contiguous significant voxels. Average percent signal change of right parahippocampal gyrus voxels demonstrating significant *CLSTN2* genotype by adolescent smoking interaction effects during delayed recognition is plotted on the left side of the figure. FDR, false discovery rate.

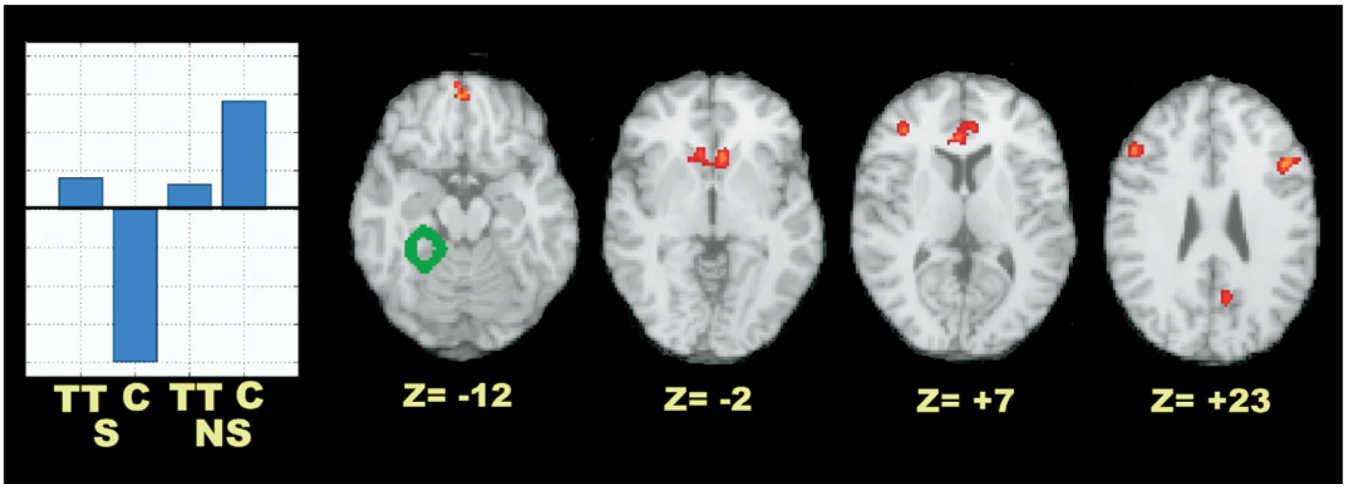


**Figure 5.**

Partial Least Squares Analysis: On the left are shown factor loadings for the second component of the PLS analysis, which accounted for 18.1% of the variance and differentiated the groups in terms of loadings to *CLSTN2* genotype and adolescent smoking (factor loadings: smokers: T homozygotes = .16, C allele carriers = −.80; nonsmokers: T homozygotes = .13, C allele carriers = .57; left panel). Connectivity maps for this component are shown on the right, where red/yellow indicates regions demonstrating positive functional connectivity with the seed region (encircled in green; right parahippocampal gyrus; Talairach coordinates: x = 28, y = −32, z = −12) that is consistent with the factor loadings. C, *CLSTN2* C allele carrier; NS, adolescent nonsmoker; PLS, Partial Least Squares; S, adolescent smoker; TT, *CLSTN2* T homozygote.

## Table 1

Demographic, Clinical and Cognitive Characteristics of 55 Adolescent Smokers and 46 Adolescent Nonsmokers

| Characteristic | Smokers, Prenatally Exposed *n* = 34 | Smokers, No Prenatal Exposure *n* = 21 | Nonsmokers, Prenatally Exposed *n*= 14 | Nonsmokers, No Prenatal Exposure *n* = 32 |
|---|---|---|---|---|
| Age (years) | 16.6 ± 1.2 | 16.9 ± .9 | 16.2 ± 1.2 | 16.4 ± 1.3 |
| Gender (M/F)[a] | 12/22 | 9/12 | 6/8 | 13/19 |
| Education (years) | 9.5 ± 1.3 | 9.8 ± 1.1 | 9.3 ± 1.3 | 9.5 ± 1.4 |
| Parented Education (years)[b] | 15.3 ± 4.7 | 14.0 ± 2.3 | 12.8 ± 1.7 | 14.6 ± 2.2 |
| Cigarettes Smoked/Day | 12.2 ± 7.4 | 10.7 ± 6.7 | — | — |
| Age at Onset of Smoking | 12.7 ± 2.2 | 13.1 ± 1.8 | — | — |
| Years of Daily Smoking | 2.8 ± 1.8 | 2.8 ± 1.6 | — | — |
| Estimated Plasma Nicotine, 7:30 PM | 14.0 ± 8.1 | 11.5 ± 5.8 | — | — |
| FTND Score | 4.5 ± 2.0 | 3.4 ± 2.1 | — | — |
| Weeks Prenatal Exposure to Tobacco | 33.4 ± 11.7 | .0 | 27.5 ± 16.2 | .0 |
| Birth Weight (kg) | 3.11 ± .52 | 3.48 ± .60 | 3.36 ± .42 | 3.30 ± .54 |
| KBIT Composite Score | 98.6 ± 7.9 | 98.7 ± 9.3 | 99.8 ± 11.2 | 101.9 ± 11.9 |
| WJR Word Attack SS | 107.1 ± 16.8 | 108.7 ± 16.5 | 112.1 ± 18.5 | 103.7 ± 15.3 |
| Beck Depression Score[c] | 8.2 ± 7.3 | 7.5 ± 6.4 | 2.6 ± 3.2 | 3.3 ± 3.9 |
| Conners Score | 23.5 ± 12.5 | 17.2 ± 8.7 | 14.6 ± 11.5 | 13.5 ± 7.7 |
| Rate of Alcohol Consumption (Drinks/ Week)[d] | 3.4 ± 5.3 | 2.5 ± 3.3 | .1 ± .2 | .2 ± .6 |
| Lifetime Episodes of Cannabis Use[e] | 266.6 ± 355.1 | 321.3 ± 407.4 | 7.5 ± 26.7 | 30.3 ± 107.7 |

Data are presented as mean ± standard deviation, unless otherwise specified.

Beck Depression Score, Beck Depression Inventory (62); Conners Score, Conners Adolescent Self Report Scale (63); F, female; FTND, Fagerstrom Test for Nicotine Dependence (41); KBIT, Kaufman Brief Intelligence Test; M, male; WJR Word Attack SS, Woodcock-Johnson Revised Test of Achievement Word Attack subtest standard scores.

[a] *CLSTN2* genotype × adolescent smoking × prenatal exposure: $\beta = 1.4$, $t = 2.2$, $p < .04$.

[b] Prenatally exposed nonsmokers < all other groups: $\beta = 4.0$, $t = 2.5$, $p < .02$.

[c] Smokers > nonsmokers: $\beta = 5.3$, $t = 3.0$, $p < .004$.

[d] *CLSTN2* genotype × adolescent smoking × prenatal exposure: $\beta = 9.3$, $t = 2.1$, $p < .05$.

[e] Smokers > nonsmokers: $\beta = 296.4$, $t = 2.8$, $p < .007$.