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ORIGINAL INVESTIGATION

Nicotinic acetylcholine receptor availability in cigarette smokers: effect of heavy caffeine or marijuana use

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Abstract
Rationale Upregulation of $\alpha_4\beta_2$* nicotinic acetylcholine receptors (nAChRs) is one of the most well-established effects of chronic cigarette smoking on the brain. Prior research by our group gave a preliminary indication that cigarette smokers with concomitant use of caffeine or marijuana have altered nAChR availability.
Objective We sought to determine if smokers with heavy caffeine or marijuana use have different levels of $\alpha_4\beta_2$* nAChRs than smokers without these drug usages.
Methods One hundred and one positron emission tomography (PET) scans, using the radiotracer 2-FA (a ligand for β_2*-containing nAChRs), were obtained from four groups of males: non-smokers without heavy caffeine or marijuana use, smokers without heavy caffeine or marijuana use, smokers with heavy caffeine use (mean four coffee cups per day), and smokers with heavy marijuana use (mean 22 days of use per month). Total distribution volume (Vt/fp) was determined for the brainstem, prefrontal cortex, and thalamus, as a measure of nAChR availability.
Results A significant between-group effect was found, resulting from the heavy caffeine and marijuana groups having the highest Vt/fp values (especially for the brainstem and prefrontal cortex), followed by smokers without such use, followed by non-smokers. Direct between-group comparisons revealed significant differences for Vt/fp values between the smoker groups with and without heavy caffeine or marijuana use.
Conclusions Smokers with heavy caffeine or marijuana use have higher $\alpha_4\beta_2$* nAChR availability than smokers without these drug usages. These findings are likely due to increased nicotine exposure but could also be due to an interaction on a cellular/molecular level.

Keywords Positron emission tomography · Tobacco dependence · Nicotinic acetylcholine receptors · Caffeine · Marijuana · Cannabis · Brain imaging · Neuroimaging · Human · Cigarette smoking

Introduction

Despite the health risks (Bartal 2001; Mokdad et al. 2004) and societal costs (Leistikow 2000; Leistikow et al. 2000; Leistikow and Miller 1998) of cigarette smoking, the prevalence of smoking in the USA remains high at ~19 % (Brown 2009; Cdc 2008; Goren et al. 2014). Roughly 44 % of cigarettes are used by smokers with substance abuse/dependence and/or mental illness (Lasser et al. 2000), and people with almost all substance abuse and mental illness diagnoses have elevated rates of cigarette smoking (Dani and Harris 2005). Cigarette smokers have elevated rates of both caffeine and marijuana use. Roughly half of smokers drink coffee and report drinking almost twice as much coffee per day as non-smokers (Research 2008). Similarly, among smokers, 57.9 % have ever used marijuana, and smokers are about 8 times more likely than non-smokers to have a marijuana use

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disorder (Agrawal et al. 2012), with cigarette smoking and marijuana use being associated even after controlling for potential confounding variables, such as depression, alcohol use, and stressful life events (Badiani et al. 2015). Given the high comorbidity of smoking and both caffeine and marijuana use, it is important to better understand biological factors that may be associated with these co-occurrences.

One of the most well-established effects of chronic cigarette smoking on the human brain is widespread upregulation of $\alpha_4\beta_2$* nicotinic acetylcholine receptors (nAChRs). Recent studies using single-photon emission computed tomography (SPECT) (Cosgrove et al. 2009; Mamede et al. 2007; Staley et al. 2006) and positron emission tomography (PET) (Brody et al. 2013; Mukhin et al. 2008; Wullner et al. 2008) have consistently demonstrated significant upregulation of these receptors in smokers compared to non-smokers. These in vivo studies were an extension of much prior research, including human postmortem brain tissue studies, demonstrating that chronic smokers have increased nAChR density compared to non-smokers and former smokers (Benwell et al. 1988; Breese et al. 1997). Additionally, many studies of laboratory animals have demonstrated upregulation of markers of nAChR density in response to chronic nicotine administration (Pauly et al. 1996; Pauly et al. 1989; Pistillo et al. 2016; Shoaib et al. 1997; Yates et al. 1995; Zhang et al. 2002).

In a previous study by our group comparing nAChR availability (as measured with PET) between smokers and non-smokers (Brody et al. 2013), we explored the effect of many variables, including caffeine and marijuana use. Both heavy caffeine and marijuana use were exclusionary, such that participants drank an average of 1.3 coffee cup equivalents per day and only 12 % of the study sample reported occasional marijuana use. PET results indicated that caffeine and marijuana use had significant relationships with $\alpha_4\beta_2$* nAChR availability in this group with low levels of usage. Based on these preliminary findings, we undertook a study of the effect of heavy caffeine or marijuana usage on $\alpha_4\beta_2$* nAChR density in cigarette smokers.

Methods

Participants and screening methods

One hundred and one otherwise healthy male adults (27 non-smokers without heavy caffeine or marijuana use, 34 smokers without heavy caffeine or marijuana use, 22 smokers with heavy caffeine use, and 18 smokers with heavy marijuana use) completed the study and had usable data. Participants were recruited and screened using the same methodology as in our prior reports (Brody et al. 2011; Brody et al. 2013; Brody et al. 2014), with the exception that this study only included Veterans. For smokers, the central inclusion criteria were current nicotine dependence and smoking 10 to 40 cigarettes per day, while for non-smokers, the central inclusion criterion was no cigarette usage within the past year. Heavy caffeine use was defined as the equivalent of ≥3 cups of coffee per day, and heavy marijuana use was defined as ≥4 uses of at least 1 marijuana cigarette per week. Exclusion criteria for all participants were as follows: use of a medication or history of a medical condition that might affect the central nervous system at the time of scanning, any history of mental illness, or any substance abuse/dependence diagnosis (by DSM-IV criteria) within the past year other than caffeine or marijuana diagnoses. Occasional use of alcohol or illicit drugs (not meeting DSM-IV criteria for a substance use disorder) was not exclusionary. There was no overlap between this study and prior research by our group.

During an initial visit, screening data were obtained to verify participant reports and characterize smoking history. Rating scales obtained were as follows: the Smoker's Profile Form (containing demographic variables, a rating of depth of inhalation, and a detailed smoking history), Fagerström Test for Nicotine Dependence (FTND) (Fagerstrom 1978; Heatherton et al. 1991), Beck Depression Inventory (BDI) (Beck et al. 1961), Hamilton Depression Rating Scale (HAM-D) (Hamilton 1967), and Hamilton Anxiety Rating Scale (HAM-A) (Hamilton 1969). An exhaled carbon monoxide (CO) level was determined using a MicroSmokerlyzer (Bedfont Scientific Ltd, Kent, UK) to verify smoking status. A breathalyzer (AlcoMatePro) test and urine toxicology screen (Test Country I-Cup Urine Toxicology Kit) were obtained at the screening visit to support the participant's report of no current alcohol abuse or other drug dependencies. This study was approved by the local institutional review board (IRB), and participants provided written informed consent.

Positron emission tomography protocol

Roughly 1 week after the initial screening session, participants underwent PET scanning following the same general procedure as in our prior reports (Brody et al. 2013; Brody et al. 2014). Participants from the smoker groups began smoking/nicotine abstinence two nights (36 h) prior to each PET session and were monitored as described previously (Brody et al. 2009; Brody et al. 2011), so that nicotine from smoking would not compete with the radiotracer for receptor binding during PET scanning. Caffeine/marijuana abstinence was initiated 12 h prior to PET scanning, so that acute ingestion/intoxication would not affect study results.

At 11 AM on the scanning day, participants arrived at the VA Greater Los Angeles Healthcare System PET Center, and smoking abstinence was verified by participant report and having an exhaled CO ≤ 4 ppm. Each participant had an intravenous line placed at 11:45 AM in a room adjacent to the PET

scanner. At 12 PM, bolus-plus-continuous-infusion of 2-[^{18}F]fluoro-3-(2(S)azetidinylmethoxy pyridine (abbreviated as 2-FA) was initiated, with 2-FA administered as an intravenous bolus in 5-ml saline over 10 s (mean doses of 145.5 ± 6.4, 147.2 ± 5.0, 144.1 ± 7.0, and 144.0 ± 6.6 MBq for the non-smokers, smokers without heavy drug use, smokers with heavy caffeine use, and smokers with heavy marijuana use, respectively). Roughly, the same amount of 2-FA (mean doses of 146.5 ± 4.4, 147.3 ± 4.7, 144.2 ± 7.5, and 143.8 ± 6.8 MBq for the four groups, respectively) was also diluted in 60-ml saline, and 51.1 ml was infused over the next 420 min (7.3 ml/h) by a computer-controlled pump (Harvard model 22, Harvard Instruments, Natick, MA). 2-FA-specific activities were similar for the study groups (8.2 ± 3.3, 7.9 ± 4.8, 7.2 ± 2.5, and 7.1 ± 2.2 Ci/micromol for the four groups, respectively). Groups did not significantly differ for injected or infused doses of 2-FA, or for specific activity (ANOVAs; Fs = 1.6, 2.2, and 0.5, respectively; nonsignificant). Thus, the amount of 2-FA administered as a bolus was equal to the amount that would be infused over 500 min (K_{bolus} = 500 min) (Kimes et al. 2008). This K_{bolus} was effective for reaching an approximate steady state in recent studies by our group and collaborators (Brody et al. 2009; Brody et al. 2011; Brody et al. 2013; Kimes et al. 2008). After initiation of the bolus-plus-continuous-infusion, participants remained seated in the room adjacent to the PET scanner for the next 4 h to allow the radiotracer to reach a relatively steady state in the brain. At 4 PM, PET scanning commenced and continued for 3 h, with a 10-min break after 90 min of scanning. Scans were acquired as series of 10-min frames.

PET scans were obtained using the Philips Gemini TruFlight (Koninklijke Philips Electronics N.V., Eindhoven, the Netherlands), a fully three-dimensional PET-CT scanner, which was operated in non-TOF mode. Reconstruction was done using Fourier rebinning and filtered back projection, and scatter and random corrections were applied. The mean spatial resolution (FWHM) for brain scanning is 5.0 mm (transverse) by 4.8 mm (axial). 2-FA was prepared using a published method (Dolle et al. 1998); this radiotracer was developed as a ligand specific for β_2*-containing nAChRs (Koren et al. 1998). A magnetic resonance imaging (MRI) scan of the brain was obtained for each participant within a week of PET scanning on a 1.5-T Magnetom Symphony System scanner (Signa; GE Medical Systems, Milwaukee, WI), in order to aid in localization of regions on the PET scans. The MRI had the following specifications: three-dimensional Fourier-transform (3DFT) spoiled-gradient-recalled acquisition with TR = 30 ms, TE = 7 ms, 30 degree angle, 2 acquisitions, 256 × 192 view matrix. The MRI scanning procedure typically lasted ~30 min. The acquired volume was reconstructed as roughly 90 contiguous 1.5-mm-thick transaxial slices.

Blood samples (5 ml) were drawn during PET scanning for determinations of free, unmetabolized 2-FA and nicotine levels in plasma. For 2-FA levels, four samples were drawn as standards prior to 2-FA administration, and nine samples were drawn at predetermined intervals during PET scanning. 2-FA levels were determined using previously published methods (Shumway et al. 2007; Sorger et al. 2007). For plasma nicotine levels, blood samples were drawn prior to and following PET scanning. These samples were centrifuged, and venous plasma nicotine concentrations were determined in Dr. Peyton Jacob's laboratory at UCSF, using a modified version of a published GC-MS method (Jacob et al. 1991). The lower limit of quantification for this method was 0.2 ng/ml. In addition to the participants described in this paper, 11 smokers completed study procedures but were excluded from the data analysis because their plasma nicotine levels were unacceptably high (>0.4 ng/ml) (determined after study participation). This issue of smokers using nicotine/tobacco during the abstinence period of a brain-imaging study has been reported in prior studies by our group and others (Brody et al. 2013; Esterlis et al. 2010; Staley et al. 2006), presumably related to difficulty in having tobacco-dependent smokers remain abstinent for a prolonged period.

PET image analysis

After decay and motion correction, each participant's PET scan was co-registered to his/her MRI using PMOD version 3.608 (http://www.pmod.com/technologies/index.html). Regions of interest (ROIs) were drawn on MRI using PMOD and transferred to the co-registered PET scans. ROIs were the left and right prefrontal cortices, brainstem, and left and right thalami, which were chosen based on prior reports indicating a range of receptor binding of 2-FA in these regions (Brody et al. 2006; Kimes et al. 2008; Mukhin et al. 2008). The brainstem and thalami were drawn as whole structures, while representative slices of the prefrontal cortices (middle frontal gyrus parallel to the body of the cingulate) were drawn. ROI placement was visually inspected for each PET frame in order to minimize effects of co-registration errors and movement; this procedure was repeated if there was a noticeable problem.

Total distribution volume (V_T/f_P) (Innis et al. 2007), which is proportional to unbound nAChR density, was calculated for each region and used for the central study analyses. V_T/f_P values were determined from the seventeen 10-min PET frames, as the ratio $C_T/(C_P \cdot f_P)$, where C_T is the total concentration of 2-FA in the ROIs, $(C_P \cdot f_P)$ is the concentration of free 2-FA in plasma, and f_P is the fraction of free (unbound) 2-FA in plasma. In addition, for scans in which participants had a measurable plasma concentration of nicotine (≥0.2 ng/ml), V_T/f_P values were corrected for plasma nicotine concentration at the time of scanning using the following equation: $V_T/f_P = (V_T/f_P)_{obs} + (V_T/f_P)_{obs} * I/IC_{50}$, where $(V_T/f_P)_{obs}$ is the observed value of total distribution volume, I is the plasma-

nicotine level at the time of scanning, and IC_{50} is the plasma nicotine concentration resulting in 50 % reduction in Vt/f_P. The IC_{50} value used here of 0.87 ng/ml was previously reported by our group (Brody et al. 2006).

Statistical analysis

To determine if the four study groups differed on demographic, rating scale, or substance-use variables, analyses of variance (ANOVAs) were performed with the variables as dependent measures and group as a between-subject factor. ANOVAs were also performed for the three groups of smokers for smoking-related variables (cigarettes per day, FTND scores, and exhaled CO levels). These analyses were performed to verify that groups differed on caffeine and marijuana use and to determine if groups had potentially confounding variables that would need to be considered when evaluating the PET data.

For evaluating group differences in $\alpha_4\beta_2$* nAChR availability, overall analyses of covariance (ANCOVAs) were performed using Vt/f_P values for each of the three ROIs (brainstem, PFC, and thalamus) as dependent measures, group as a between-subject factor, and education level as a covariate based on results of the above analysis demonstrating group differences for this variable. To clarify results of these overall tests, post hoc Student s *t* tests were performed to determine which between-group differences accounted for significant findings. Bonferroni corrections for multiple comparisons were applied to all statistical tests, with the ANCOVA results being corrected for the three regions tested and post hoc Student s *t* tests being corrected for the six group comparisons performed for each region. Results were considered significant if corrected results passed a threshold of $P < 0.05$. To maximize power, the means of left and right Vt/f_P values for prefrontal cortex and thalamus were used in statistical analyses, along with values for the whole brainstem. For descriptive purposes, percent group differences in Vt/f_P values were determined between the smoker groups and the group of non-smokers, and between smokers with and without heavy caffeine or marijuana use. Statistical tests were performed using SPSS Statistics version 23 (SPSS, Inc., Chicago, IL).

Results

Demographics, rating scale scores, and substance use

The four study groups had no significant differences in demographic or rating scale variables, except for education levels, which were lower for smokers with heavy caffeine or marijuana use than for the other study groups (ANOVA, $df = 3.97$, $P < 0.01$) (Table 1). Therefore, education level was used as a nuisance covariate in statistical analyses of the PET data below. For smoking-related variables, exhaled CO and depth of inhalation levels were higher in smokers with heavy caffeine or marijuana use than for smokers without such use (ANOVAs, $df = 2.71$, F values = 4.4 and 3.8, P values = 0.02 and 0.03, respectively), but differences between the smoker groups were not significant for cigarettes per day or FTND scores (ANOVAs, $df = 2.71$, $F = 1.9$ and 1.4, respectively, nonsignificant). As expected, study groups differed in coffee cup equivalents per day (ANOVA, $df = 3.97$, $F = 45.9$, $P < 0.0005$) and marijuana cigarettes used per week (ANOVA, $df = 3.97$, $F = 86.8$, $P < 0.0005$), but not in alcohol use.

PET findings

In analyzing ROI Vt/fp values for the four groups, the brainstem, PFC, and thalamus had significant between-group effects (ANCOVAs, $df = 3.96$, F's = 14.9, 11.5, and 4.7; $P < 0.0005$, $P < 0.0005$, and $P = 0.004$, respectively). These ANCOVA results for all three brain regions pass Bonferroni correction and indicate group differences in Vt/fp values (the measure of $\alpha_4\beta_2$* nAChR availability) for all three brain regions. Overall, PET results did not change if other variables previously found to be related to $\alpha_4\beta_2$* nAChR density (e.g., number of cigarettes per day, age, and menthol cigarette preference) were included in the model. Overall results also did not change if data uncorrected for plasma-nicotine levels at the time of scanning were used.

Using post hoc Student s *t* tests to compare the smoker groups to the non-smoker group, all of the smoker groups had higher Vt/fp values than the non-smoker group for the brainstem and PFC (Table 2; Figs. 1 and 2). For the brainstem, in comparing Vt/fp values for the smoker groups without heavy drug use, with heavy caffeine use, and with heavy marijuana use to the group of non-smokers, P values were 0.0003, 2.3×10^{-5}, and 2.9×10^{-9}, respectively. For the PFC, P values were 0.003, 9.3×10^{-5}, and 5.6×10^{-7}, respectively. All of these results pass Bonferroni correction. For the thalamus, P values were 0.60, 0.02, and 0.0007, respectively, such that only the group of smokers with heavy marijuana use had a result that passed Bonferroni correction. In quantifying group differences for descriptive purposes, Vt/fp values were higher for the three smoker groups listed above compared to the non-smoker group for the brainstem (18, 63, and 59 %, respectively), PFC (13, 51, and 42 %), and thalamus (3, 29, and 30 %).

Using post hoc Student s *t* tests to compare the smoker groups with heavy caffeine or marijuana use to the group of smokers without such use, the groups with heavy caffeine or marijuana use had higher Vt/fp values for the brainstem, PFC, and thalamus than the smoker group without such use (Table 2 and Fig. 2). For the brainstem, in comparing Vt/fp values for smoker groups

Table 1 Demographics, rating scale scores, and substance use for the four study groups

Variable	Non-smokers ($n = 27$)	Smokers without comorbidity ($n = 34$)	Smokers with heavy caffeine use ($n = 22$)	Smokers with heavy marijuana use ($n = 18$)
Age	40.5 (±13.4)	39.1 (±13.1)	46.0 (±12.9)	42.3 (±12.6)
Race (% white)	55.6	41.2	45.5	38.9
Height (inches)	70.7 (±2.9)	70.6 (±3.3)	70.7 (±2.0)	69.6 (±3.7)
Weight (pounds)	179.3 (±32.4)	184.1 (±32.3)	195.3 (±27.6)	190.8 (±37.1)
Education (highest completed grade)**	15.1 (±1.9)	14.4 (±2.2)	13.2 (±1.3)	13.9 (±1.7)
Mother's education (highest completed grade)	13.4 (±2.2)	13.6 (±2.2)	13.5 (±2.1)	13.4 (±2.4)
Cigarettes per day	N/A	18.5 (±3.9)	16.0 (±7.6)	15.8 (±6.1)
Fagerström Test for Nicotine Dependence	N/A	4.1 (±2.3)	5.0 (±2.1)	3.9 (±2.7)
Exhaled carbon monoxide (ppm)*	1.2 (±1.0)	11.6 (±6.3)	17.2 (±9.4)	15.0 (±4.2)
Depth of inhalation rating*	N/A	3.0 (±0.6)	3.3 (±0.5)	3.3 (±0.6)
Hamilton Anxiety Rating Scale	2.0 (±2.1)	1.9 (±2.7)	2.7 (±2.3)	2.8 (±2.8)
Hamilton Depression Rating Scale	1.8 (±1.9)	1.8 (±2.6)	2.4 (±2.0)	2.2 (±2.5)
Beck Depression Inventory	1.0 (±3.7)	1.9 (±2.1)	2.9 (±2.8)	2.7 (±3.3)
Caffeine use (coffee cup equivalents/ day)***	1.0 (±1.0)	0.8 (±0.7)	4.1 (±1.6)	1.3 (±1.2)
Alcohol drinks per week	1.9 (±2.6)	2.1 (±2.5)	1.3 (±2.6)	2.3 (±3.8)
Marijuana cigarettes per week**	0.1 (±0.2)	0.3 (±0.7)	0.1 (±0.2)	7.6 (±4.1)

All values are presented as means (± standard deviation) or percentages; *$P < 0.05$ between study groups, analysis of variance (ANOVA); **$P < 0.01$ between study groups, ANOVA; ***$P < 0.005$ between study groups, ANOVA. All variables were compared between the four study groups, except for smoking-related variables (cigarettes per day, Fagerström Test for Nicotine Dependence, exhaled carbon monoxide levels, and depth of inhalation levels), which were compared between the three groups of smokers. All other statistical tests for between-group effects were not significant. *ppm* parts per million

with heavy caffeine or marijuana use to the group without such use, P values were 0.0001 and 6.1×10^{-8}, respectively. For the PFC, P values were 0.0003 and 1.0×10^{-5}, respectively. All of these results pass Bonferroni correction. For the thalamus, P values were 0.013 and 0.0003, respectively, such that only the comparison of smokers with heavy marijuana use to smokers without heavy caffeine or marijuana use passed

Table 2 Total distribution volume (Vt/f_P) values for the brain regions of interest

Brain region	Vt/f_P values-non-smokers ($n = 27$)	Vt/f_P values-smokers without heavy use ($n = 34$)	Vt/f_P values-smokers with heavy caffeine use ($n = 22$)	Vt/f_P values-smokers with heavy marijuana use ($n = 18$)
Brainstem	9.6 (±2.0)	11.5 (±1.9)***	18.4 (±9.5)***,###	17.6 (±5.1)***,###
Prefrontal cortex	7.1 (±1.3)	8.0 (±1.2)**	11.9 (±5.7)***,###	10.8 (±2.9)***,###
Thalamus	15.9 (±3.5)	16.4 (±2.8)	21.4 (±10.9)*,#	21.5 (±6.6)***,###

All values are mean ± standard deviation and statistical significances are for analyses of covariance. ANCOVAs for all three brain regions were significant for between-group effects and passed Bonferonni correction for multiple comparisons. For post hoc Student s t tests, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for comparisons of the smoker groups to the non-smoker group (uncorrected). For additional post hoc Student s t tests, # $P < 0.05$ and ### $P < 0.001$ for comparisons of the smoker groups with heavy caffeine or marijuana use to the smoker group without such use (uncorrected)

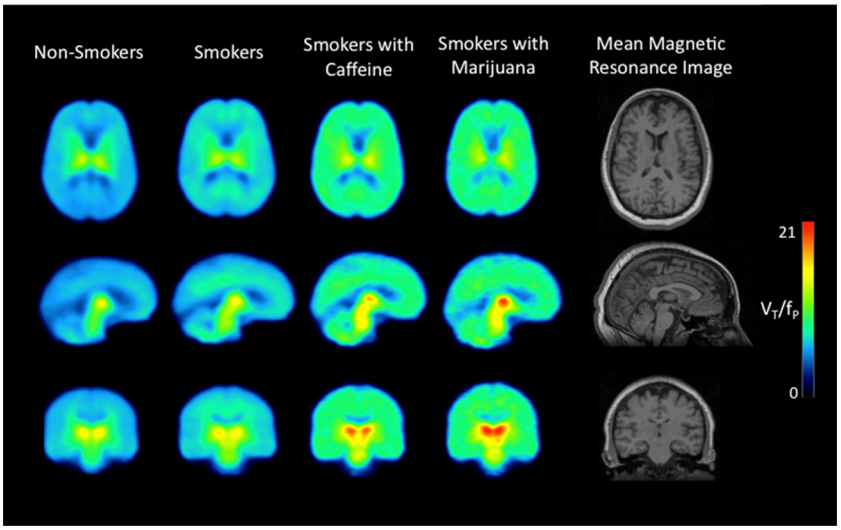

Fig. 1 Mean positron emission tomography (PET) images from the four study groups: 27 non-smokers without heavy caffeine or marijuana use, 34 smokers without heavy caffeine or marijuana use, 22 smokers with heavy caffeine use, and 18 smokers with heavy marijuana use. The figure shows higher 2-FA binding for smokers with heavy caffeine or marijuana use compared to smokers without such use or non-smokers. The images for each row are transaxial sections (*top*), saggital slices (*middle*), and coronal slices (*bottom*). PET images were spatially normalized to the group mean magnetic resonance imaging (MRI) scan (far right column). Vt/fp = total volume of distribution

Bonferroni correction. For descriptive purposes, Vt/fp values were higher for the two groups of smokers with versus without heavy caffeine or marijuana use for the brainstem (46 and 42 %, respectively), PFC (39 and 30 %), and thalamus (26 and 27 %).

For the post hoc Student s *t* tests comparing the smoker groups with heavy caffeine versus heavy marijuana use, no signficant between-group differences were found.

Discussion

The central study finding was that smokers with concomitant heavy caffeine or marijuana use have higher Vt/fp values (a marker for $\alpha_4\beta_2$* nAChR availability) in the brainstem and prefrontal cortex than smokers without such use. The study also replicated earlier work demonstrating higher Vt/fp values in the prefrontal cortex and brainstem of smokers than non-

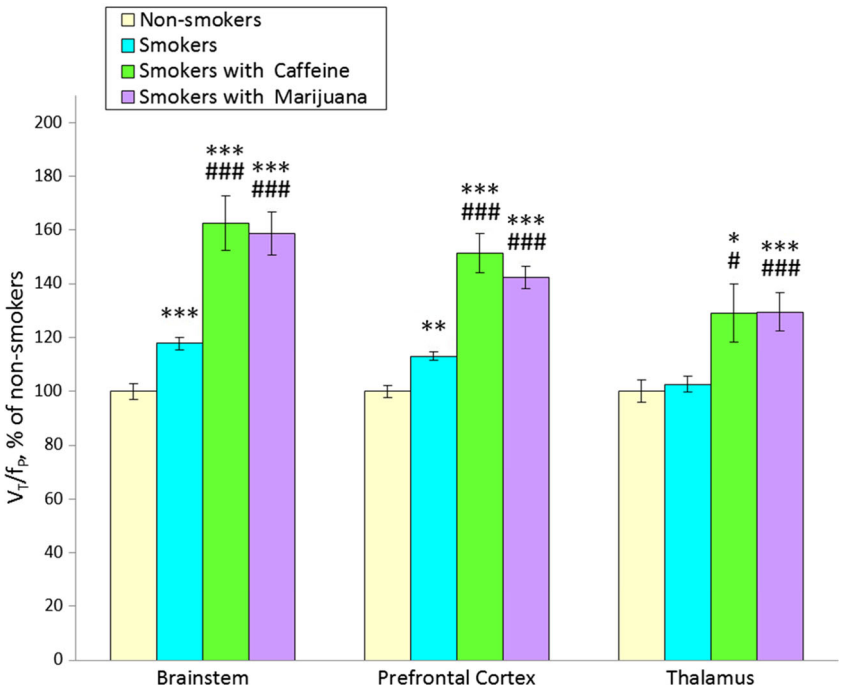

Fig. 2 Cigarette smokers with either heavy caffeine or marijuana use have higher α4β2* nAChR availabilities in the prefrontal cortex, brainstem, and thalamus than smokers without such use or non-smokers. Levels of nAChR availabilities for all three smoker groups were compared to the non-smoker group (100 %), and these levels were compared between smoker groups. For comparisons with the non-smoker group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Student s *t* tests, uncorrected). For comparisons between smokers with heavy caffeine or marijuana use versus smokers without such use, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ (Student s *t* tests, uncorrected)

smokers. Taken together, these findings indicate that smokers with concomitant heavy caffeine or marijuana use have greater nAChR upregulation than smokers without concomitant heavy use.

The most straightforward and likely explanation for the central study finding is that smokers who use caffeine or marijuana heavily have more nicotine exposure than smokers without such use. This explanation is supported by study data demonstrating that smokers with concomitant heavy caffeine or marijuana use had higher exhaled CO and greater depth of inhalation levels at baseline than smokers without such use (Table 1) (though these smokers did not report a higher number of cigarettes per day or have higher FTND scores). Other data supporting this theory include research demonstrating that caffeine (and other adenosine receptor antagonists) (Justinova et al. 2009) increase nicotine intake in laboratory animals (Liu and Jernigan 2012; Rezvani et al. 2013) and a study of smokers with heavy marijuana use who had altered lung permeability (Gil et al. 1995), which resulted in greater cigarette smoke exposure. Thus, smokers with concomitant heavy caffeine or marijuana use may have increased brain nicotine exposure due to altered smoking topography, effects of caffeine or marijuana on other aspects of nicotine absorption/intake, or both.

While the smoker groups with heavy caffeine or marijuana use did have higher exhaled CO levels and greater depth of smoking inhalation than the smoker group without concomitant use, the absence of group differences in cigarettes per day and FTND scores indicate that explanations for increased nAChR availability other than greater nicotine exposure are possible. We are not aware of studies that would fully explain direct effects of caffeine or marijuana on nAChR availability; however, recent research has begun to elucidate interactions between nicotine and caffeine (El-Mas et al. 2011; Kordosky-Herrera and Grow 2009; Singh et al. 2008; Thany et al. 2008) or marijuana (Mahgoub et al. 2013) on a cellular level, and future research could determine a mechanism by which caffeine or marijuana exposure directly affects nAChR availability.

The specific regional findings here may have functional significance, given the roles of the brainstem and PFC in the mediation of addiction. For the brainstem, many studies demonstrate that addictive drugs (including tobacco) acutely stimulate neurons originating in the brainstem leading to the ventral striatum to produce reward (see (Koob and Volkow 2010) and (Subramaniyan and Dani 2015) for reviews). For the PFC, this region is known to mediate executive functions, such as attention, working memory, and decision-making (Tanji and Hoshi 2008; Wallace and Bertrand 2013), which are associated with drug use. Extensive prior research has examined associations between smoking-related symptoms (e.g., cigarette craving and other withdrawal symptoms) and nAChR availability in the regions studied here (Brody et al. 2013; Cosgrove et al. 2009) without finding strong evidence for associations between these variables. Future research could utilize specific testing for functions of the brainstem (e.g., monetary reward tasks) or PFC (e.g., working memory tasks) to further evaluate the functional significance of increased nAChR availability in these regions in smokers (as has been done in other imaging studies examining brain function (e.g., (Ezekiel et al. 2013; Goya-Maldonado et al. 2015; Pecina et al. 2014; Wager et al. 2014)).

Study results also have clinical implications regarding the co-use of cigarettes and other drugs. Prior research examining smokers trying to quit has demonstrated that concomitant use of caffeine (Westmaas and Langsam 2005) or marijuana (Bowes et al. 2015; Ford et al. 2002) predicts less likelihood of smoking cessation. Recent research by our group (Brody et al. 2014) showed that greater nAChR availability was associated with less likelihood of smoking cessation during a quit attempt with nicotine or placebo patch administration. Taken together, our findings imply that smokers with heavy caffeine or marijuana use have greater exposure to nicotine, more upregulation of nAChRs, and more trouble quitting in smoking cessation programs than smokers without concomitant heavy drug use. Future brain imaging research in smokers with concomitant heavy drug use who undergo smoking cessation treatment could confirm this implication of the current study.

This study had several limitations. First, we did not examine non-smokers with heavy caffeine or marijuana use to determine if study findings were independent of cigarette smoking. Future research with such non-smokers could determine if caffeine and marijuana use affect nAChR density directly or if the effect on nAChR density is mediated through greater nicotine exposure in smokers with heavy caffeine or marijuana usage. Second, while we did determine exhaled CO levels, depth of inhalation, reported cigarettes per day, FTND scores, and plasma nicotine levels at the time of scanning, we did not collect blood for plasma nicotine levels at baseline during normal cigarette smoking. These levels would have been helpful in determining if the primary study results were due to increased nicotine exposure in smokers with heavy caffeine or marijuana use. And third, some smokers had small measurable plasma nicotine levels at the time of scanning (presumably due to difficulty in maintaining smoking/nicotine abstinence), which led to mathematical corrections for these levels. While overall study results did not differ with or without these corrections, an improved method of ensuring nicotine abstinence (e.g., inpatient monitoring) could have been helpful. Additionally, in the exploratory analysis from our previous study (Brody et al. 2013), lower caffeine use (in a group with a range of 0–2 coffee cup equivalents/day) was associated with greater nAChR availability. Results from this prior exploratory analysis of a group with modest caffeine use would not have passed Bonferroni correction. In contrast, the finding here of greater nAChR availability in heavy

caffeine users (range of 3–8 coffee cup equivalents/day) was highly significant (passing Bonferroni correction). Thus, the present findings indicate a robust elevation of nAChR availability in heavy caffeine using smokers.

In conclusion, smokers with concomitant heavy caffeine or marijuana use have greater $\alpha_4\beta_2$* nAChR availability than smokers without such heavy use. These findings are consistent with prior research demonstrating more severe dependence on cigarettes in caffeine and marijuana users (Rabin and George 2015; Westmaas and Langsam 2005).

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Compliance with ethical standards Participants gave written informed consent, using a form approved by the Institutional Review Board at the VA Greater Los Angeles Healthcare System.

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