

Brain nicotinic acetylcholine receptor occupancy: effect of smoking a denicotinized cigarette



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Abstract

Our group recently reported that smoking a regular cigarette (1.2–1.4 mg nicotine) resulted in 88% occupancy of brain $\alpha_4\beta_2^*$ nicotinic acetylcholine receptors (nAChRs). However, this study did not determine whether nicotine inhalation or the many other pharmacological and behavioural factors that occur during smoking resulted in this receptor occupancy. If nicotine is solely responsible for $\alpha_4\beta_2^*$ nAChR occupancy from smoking, then (as estimated from our previous data) smoking a denicotinized (0.05 mg nicotine) or a low-nicotine (0.6 mg nicotine) cigarette (commonly used for research and clinical purposes) would result in substantial 23% and 78% $\alpha_4\beta_2^*$ nAChR occupancies, respectively, and a plasma nicotine concentration of 0.87 ng/ml would result in 50% $\alpha_4\beta_2^*$ nAChR occupancy (EC₅₀). Twenty-four positron emission tomography sessions were performed on tobacco-dependent smokers, using 2-[F-18]fluoro-A-85380 (2-FA), a radiotracer that binds to $\alpha_4\beta_2^*$ nAChRs. 2-FA displacement was determined from before to 3.1 hours after either: no smoking, smoking a denicotinized cigarette, or smoking a low-nicotine cigarette. Analysis of this PET data revealed that smoking a denicotinized and a low-nicotine cigarette resulted in 26% and 79% $\alpha_4\beta_2^*$ nAChR occupancies, respectively, across three regions of interest. The EC₅₀ determined from this dataset was 0.75 ng/ml. Given the consistency of findings between our previous study with regular cigarettes and the present study, nicotine inhalation during smoking appears to be solely responsible for $\alpha_4\beta_2^*$ nAChR occupancy, with other factors (if present at all) having either short-lived or very minor effects. Furthermore, smoking a denicotinized cigarette resulted in substantial nAChR occupancy.

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Introduction

Nicotine is the constituent of cigarette smoke that is most closely linked to tobacco dependence (Henningfield and Fant, 1999). The binding of nicotine to nicotinic acetylcholine receptors (nAChRs) is the

presumed pharmacological event mediating the feeling of reward (Leshner and Koob, 1999), reduced irritability and anxiety (West and Shiffman, 2001), heightened attentional performance (Newhouse et al., 2004), and improved reaction times (Ernst et al., 2001) that occur when a smoker smokes a cigarette. However, cigarette smoking is a behaviour that involves much more than the delivery of nicotine. Cigarette smoking includes the inhalation of thousands of constituents of tobacco smoke other than nicotine (Green and Rodgman, 1996) and results in a wide range of

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physical sensations (such as the taste, smell, and feel of a cigarette). Thus, there are many aspects of cigarette smoking other than nicotine inhalation that could affect nAChR occupancy either directly or through the release of endogenous acetylcholine. The central goal of the present study was to determine if factors associated with smoking other than nicotine inhalation result in nAChR occupancy.

Recently, denicotinized cigarettes (0.05–0.1 mg nicotine yield) have come into wider use for research and clinical purposes. For research, denicotinized cigarettes have been compared to regular cigarettes to differentiate the effects of nicotine inhalation from all other aspects of cigarette smoking. Smoking denicotinized cigarettes has been shown to relieve withdrawal to a similar degree as smoking regular cigarettes (Buchhalter et al., 2001; Dallery et al., 2003; Donny et al., 2007; Pickworth et al., 1999); however, denicotinized cigarettes provide less satisfaction (Butschky et al., 1995; Gross et al., 1997) and reward (Donny et al., 2007; Naqvi and Bechara, 2005) than regular cigarettes, and do not produce all of the physical manifestations of regular cigarettes (Buchhalter et al., 2001; Pickworth et al., 1999; Schuh et al., 2001). For clinical use, denicotinized cigarettes have been found to reduce craving in smokers attempting to quit (Rezaishiraz et al., 2007), and are currently being tested as an adjunct to standard smoking cessation treatments (Donny et al., 2007). Because of the expanding role of denicotinized cigarettes in both research studies and clinical treatment, it is important to understand the effects of these cigarettes on brain nAChR occupancy.

Our group recently examined the effects of smoking varying amounts of regular nicotine-containing cigarettes (1.2–1.4 mg nicotine) on brain $\alpha_4\beta_2^*$ nAChR occupancy. For that study, we used the radiotracer 2-[^{18}F]fluoro-3-(2(*S*)azetidylmethoxy)pyridine (2-FA-85380, abbreviated as 2-FA), which was developed recently for the in-vivo imaging of $\alpha_4\beta_2^*$ nAChRs with positron emission tomography (PET) (Chefer et al., 2003; Gallezot et al., 2005; Koren et al., 1998). 2-FA was injected and infused during PET scanning, and the extent of its displacement from smoking was determined. We found that the effective dose of a cigarette needed to occupy 50% of available $\alpha_4\beta_2^*$ nAChRs (ED_{50}) at 3.1 h after smoking was 13% and that the effective venous plasma nicotine concentration needed to occupy 50% of these nAChRs was 0.87 ng/ml [much less than the 10–50 ng/ml achieved during the day in typical daily smokers (Benowitz et al., 1990)]. Because subjects in this previous study smoked different amounts of regular nicotine-containing cigarettes, the

study did not rule out the possibility that smoking-related factors other than nicotine might result in receptor occupancy. If such factors are responsible for nAChR occupancy, our previous study would have overestimated the effect of nicotine on nAChR occupancy and underestimated the value for EC_{50} .

To address this issue in the study presented here, we determined the percentage occupancy of $\alpha_4\beta_2^*$ nAChRs from smoking a denicotinized (0.05 mg nicotine yield) and a low-nicotine (0.6 mg yield) cigarette. Based on our previous determination of ED_{50} using regular cigarettes and the equation

$$\text{receptor occupancy} = \text{dose}/(\text{dose} + \text{ED}_{50}),$$

we predicted that smoking a denicotinized cigarette would result in a substantial 22–24% occupancy of $\alpha_4\beta_2^*$ nAChRs and smoking a low-nicotine cigarette would result in 77–79% occupancy of $\alpha_4\beta_2^*$ nAChRs, if nicotine inhalation from cigarette smoking is solely responsible for this occupancy. We also hypothesized that the EC_{50} would be the same for smoking low-nicotine cigarettes as it was for smoking regular cigarettes (i.e. 0.87 ng/ml), if nicotine inhalation alone is responsible for nAChR occupancy.

Method

General study design

Tobacco-dependent cigarette smokers underwent the following sequence of procedures (described in detail below): (1) screening over the telephone and in person, (2) two bolus + continuous infusion 2-FA PET sessions (at least 1 wk apart), with either no smoking, smoking of a denicotinized cigarette (0.05 mg nicotine), or smoking of a low-nicotine cigarette (0.6 mg nicotine) during a break in the middle of scanning, and (3) a structural magnetic resonance imaging (MRI) scan of the brain within 1 wk of PET scanning to aid in localization of regions on the PET scans.

Subject screening

Fifteen otherwise healthy tobacco-dependent smokers (≥ 15 cigarettes/d) were recruited through newspaper advertisements and the internet. Initial screening consisted of an anonymous telephone interview in which smoking, medical, psychiatric, and substance use histories were obtained. Qualified subjects who wished to participate were then assessed in person using screening questions from the Structured Clinical Interview for DSM-IV (SCID; First et al., 1995) 2 d prior to PET scanning. The central inclusion criterion was the DSM-IV diagnosis of nicotine dependence,

while any history of an Axis I diagnosis other than nicotine dependence was exclusionary. Other exclusion criteria were pregnancy, the use of medications or history of a medical condition that might affect the central nervous system during scanning, and the use of more than the caffeine equivalent of two cups of coffee per day. Women of childbearing potential had a urine pregnancy test, and all subjects had a urine toxicology screen to verify subject reports. After a complete description of the study to subjects, written informed consent was obtained using forms approved by the local institutional review board.

During the initial visit, additional screening data were obtained, including the Smoker's Profile Form (which includes smoking history), the Fagerström Test for Nicotine Dependence (FTND; Fagerström, 1978; Heatherton et al., 1991), the Hamilton Depression Rating Scale (17-item HAMD; Hamilton, 1967), the Hamilton Anxiety Rating Scale (HAMA; Hamilton, 1969), and the Shiffman–Jarvik Withdrawal Scale (SJWS; Shiffman and Jarvik, 1976). An exhaled carbon monoxide (CO) level was obtained using the Micro-Smokerlyzer (Bedfont Scientific Ltd, Kent, UK) to verify smoking status [subjects were considered to be active smokers if a CO ≥ 8 parts per million (ppm) was obtained].

Because we were primarily interested in the effects of smoking a denicotinized cigarette on nAChR occupancy and because dosimetry concerns limited us to PET scanning subjects only twice, subjects were alternately assigned to receive either a no-smoking and a denicotinized cigarette PET session ($n=7$) or a low-nicotine and a denicotinized cigarette PET session ($n=8$). We did not assign subjects to the third possibility of having a no-smoking and a low-nicotine cigarette PET session, because our previous results with low dosages of regular cigarettes strongly indicated that smoking a low-nicotine cigarette (0.6 mg nicotine) would result in significant occupancy compared to no smoking. Due to the challenging two-night abstinence period and the ~ 9 h PET scanning sessions (described below), six subjects did not complete both PET sessions to which they were assigned. Specifically, for subjects assigned to the no-smoking and denicotinized cigarette conditions ($n=7$), four completed both PET conditions, while three completed only the no-smoking condition. For subjects assigned to the low-nicotine and denicotinized cigarette conditions ($n=8$), five completed both PET sessions, while two completed only the low-nicotine and one completed only the denicotinized cigarette condition. Thus, a total of 24 PET sessions were used for this study ($n=7$ for the no-smoking condition, $n=10$ for

the denicotinized cigarette condition, and $n=7$ for the low-nicotine cigarette condition).

Abstinence period and PET protocol

After the initial screenings, participants began smoking/nicotine abstinence at 18:00 hours two nights prior to PET scanning. They reported to our laboratory at 13:00 hours the day after initiating abstinence, and a brief clinical interview and an exhaled CO measurement were obtained. Participants were deemed to be compliant with the study protocol if they reported no smoking/nicotine since 18:00 hours the previous night and had an exhaled CO level of ≤ 8 ppm. Subjects were seen the following day for PET scanning, and were required to report continuous abstinence since two nights previously and have an exhaled CO level of ≤ 4 ppm in order to undergo PET scanning. Subjects were given financial incentives to maintain this abstinence.

PET scanning followed the same general protocol as in our previous report (Brody et al., 2006), except that either a denicotinized or low-nicotine cigarette was smoked (along with the no-smoking control condition) in this study. At 12:00 hours on the day of scanning, subjects arrived at the VA Greater Los Angeles Healthcare System PET Center, and abstinence was verified as described above. Each participant then had an intravenous line placed at 12:45 hours in a room adjacent to the scanner. At 13:00 hours, bolus + continuous infusion of 2-FA was initiated. The amount of 2-FA administered as a bolus was equal to the amount infused over 500 min ($K_{\text{bolus}}=500$ min) (Carson et al., 1993; Carson, 2000; Kimes et al., 2008). The K_{bolus} of 500 min was chosen based on a study which demonstrated this value as optimal for reaching steady state between blood and brain within 6–8 h (Kimes et al., 2008). Consistent with this paradigm, 147 MBq (3.98 ± 0.06 mCi) of 2-FA was administered as an intravenous bolus. This same amount of 2-FA (147 MBq) was also diluted in 60 ml saline, and 58 ml (141 MBq) was infused over the next 480 min (7.3 ml/h). This bolus + continuous infusion method has been validated vs. the bolus-only method (Kimes et al., 2008). PET scans were obtained as series of 10-min frames.

After initiation of the 2-FA bolus + continuous infusion, subjects remained seated in a room adjacent to the PET scanner for the next 3 h to allow the radio-tracer to reach a relatively steady state. At 16:00 hours, brain scanning commenced and continued for 60 min. At 17:00 hours, subjects had a 10-min break in scanning, during which they smoked either a low-nicotine cigarette (Quest 1 brand: 0.6 mg nicotine yield, $n=7$

PET sessions), a denicotinized cigarette (Quest 3 brand: 0.05 mg nicotine yield, $n=10$ PET sessions), or did not smoke ($n=7$ PET sessions). To maintain a blind for the smoking conditions, a low-nicotine or denicotinized cigarette was de-identified by a research assistant, who covered brand markings. This de-identified cigarette was given to a study investigator (A.L.B. or R.E.O.) who gave it to the participant to smoke during the break in scanning. Participants were observed throughout this break. Subjects were then scanned for 3 h 50 min more with three scheduled 10- or 15-min breaks. Scanning ended at 21:00 hours.

Blood samples (5 ml) for assay of venous plasma nicotine levels were drawn at six time-points: at the beginning of the first break in scanning (immediately before smoking for the scans with cigarette smoking), at the end of this break (immediately after smoking for the scans with cigarette smoking) and at 10, 30, 60, and 180 min after the first break in scanning. These samples were drawn from a separate catheter placed for this purpose. For four of the denicotinized cigarette scans, we were unable to obtain a sufficient number of samples for plasma nicotine levels due to poor venous access. Samples were centrifuged, and concentrations of venous plasma nicotine were determined in the laboratory of Dr P. Jacob (University of California, San Francisco) using a GC-MS/MS system (lower limit of detection 0.2 ng/ml).

Withdrawal symptoms were monitored immediately before and after smoking, and at each post-smoking break in scanning. Cigarette craving was monitored with the Urge to Smoke (UTS) Scale (Brody et al., 2002; Jarvik et al., 2000), an analogue scale (range 0–6) with 10 craving-related questions. Mood (from 'sad' to 'happy') and nervousness were measured with analogue rating scales (range 0–6) with single questions asking subjects to rate their levels of these two states.

PET scans were obtained on a General Electric Advance NXi scanner (General Electric Medical Systems, Milwaukee, WI, USA) with 35 slices in 3D mode, transaxial resolution FWHM 5.2–7.7 mm (Dhawan et al., 1998). Attenuation correction scanning was performed with the germanium rotating rod source built into the scanner for 5 min at the end of the scanning session, and this attenuation correction was applied to all scans. 2-FA was prepared using a previously published method (Dolle et al., 1998).

MRI scanning

An MRI scan of the brain was obtained for each subject within a week of their first PET scanning session, with

the following specifications: three-dimensional Fourier-transform (3D FT) spoiled-gradient-recalled acquisition with TR=30 ms, TE=7 ms, 30° flip angle, two acquisitions, 256 × 192 view matrix. The acquired volume was reconstructed as roughly 90 contiguous 1.5-mm-thick transaxial slices.

Region of interest (ROI) placement

PET-to-MRI co-registration was performed using an automated image registration method (Woods et al., 1993) within MEDx 3.3 (Sensor Systems Inc., Sterling, VA, USA). Each summed PET scan (lasting 30–60 min) was co-registered to the MRI separately, and the individual 10-min time-frames within each scan were co-registered to MRI scans using the transformation matrix parameters from the co-registration of the summed images.

ROIs were drawn on MRI within MEDx and transferred to the co-registered PET scans. ROIs included the thalamus, brainstem, and cerebellum, which were drawn as whole structures. These regions were chosen based on previous reports indicating a range of nAChR densities in these regions (Fujita et al., 2003; London et al., 1985; Valette et al., 1999). ROI placement on each PET scan frame was visually inspected to minimize the effects of co-registration errors and movement issues, with the procedure being repeated if there was a noticeable problem. Mean volumes for the thalamus, brainstem, and cerebellum were 8 ± 1 , 15 ± 3 , and 92 ± 18 cm³, respectively.

Demographic and rating-scale data analysis

Means \pm standard errors of the mean (S.E.M.) were determined for demographic and rating-scale data. For withdrawal-symptom rating-scale data, changes from before to after the smoking break in scanning were evaluated using paired Student's *t* tests (one-tailed, given the unidirectional hypotheses that withdrawal symptoms would be alleviated with smoking).

Determination of $\alpha_4\beta_2^$ nAChR occupancy from smoking a denicotinized and a low-nicotine cigarette*

For the determination of $\alpha_4\beta_2^*$ nAChR occupancies from smoking a denicotinized cigarette and a low-nicotine cigarette, the general method was that the fraction of total radioactivity displaced from smoking (corrected for the imperfect steady state determined from the no-smoking scans) to the maximum fractional displacement of radioactivity (from our previous study, Brody et al., 2006) was determined. This ratio is equal to the fraction of specifically bound

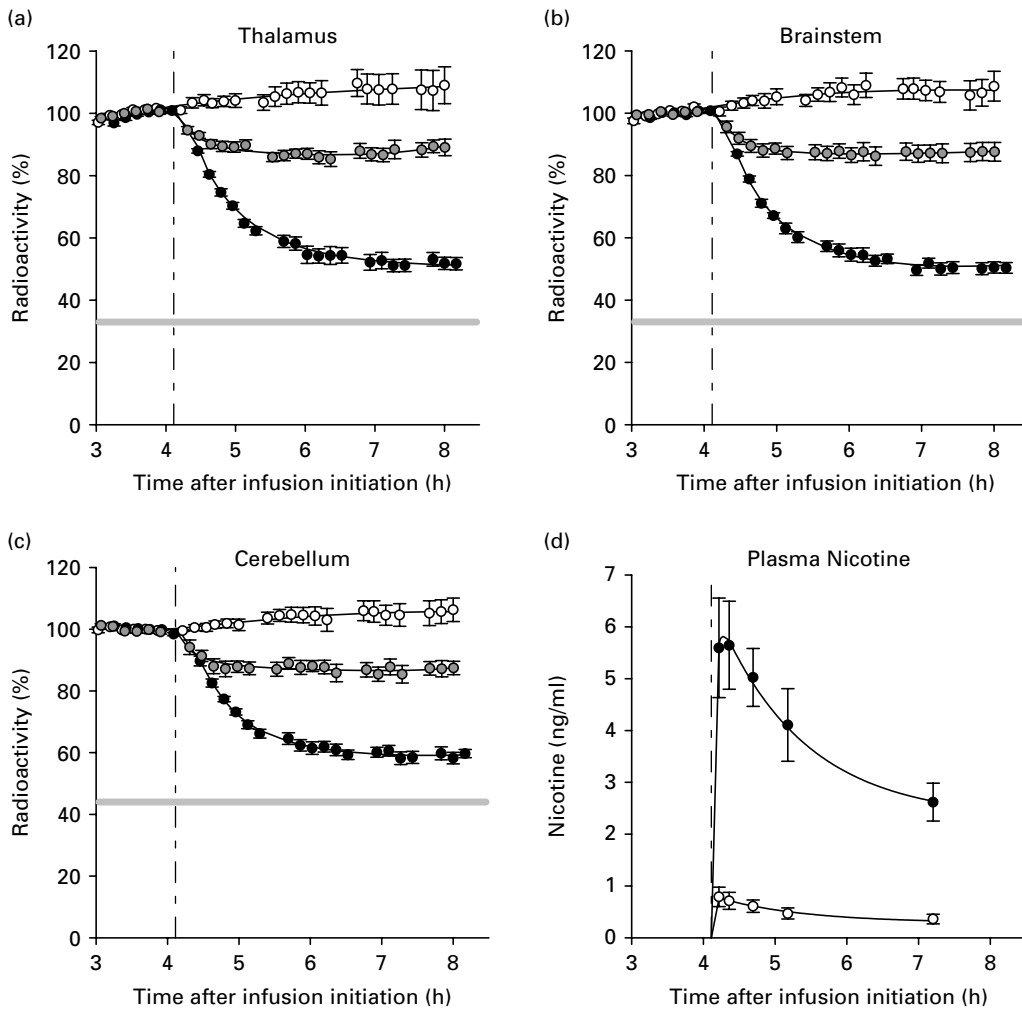


Figure 1. (a)–(c) Time–activity curves demonstrating changes in percent radioactivity for the three regions of interest (thalamus, brainstem, and cerebellum) over time following the initiation of bolus + infusion of 2-FA. The white, grey, and black circles represent the mean values (\pm S.E.M.) for the scans with no smoking ($n=7$), smoking of a denicotinized cigarette (0.05 mg nicotine yield) ($n=10$), and smoking of a low-nicotine cigarette (0.6 mg nicotine yield) ($n=7$), respectively. The horizontal grey lines in panels (a)–(c) represent the percent of maximum fractional displaceable radioactivity for each region, which were obtained from the asymptotic portions of the curves in figure 4 of our previous study in which subjects smoked regular cigarettes to satiety (Brody et al., 2006). (d) Changes in mean plasma nicotine levels (ng/ml) with time in subjects who smoked a low-nicotine (●) or denicotinized (○) cigarette.

radioactivity that was displaced from smoking, and is equal to the fractional occupancy of $\alpha_4\beta_2^*$ nAChRs from smoking.

As for the details of this analysis, the mean radioactivity for each 10-min PET frame for each condition was converted to a percent of total mean radioactivity at baseline for each ROI and plotted vs. time (Figure 1). The total concentration of tissue radioactivity before (C_T) and after (C'_T) the smoking break in scanning was determined by averaging data from the six 10-min frames (1 h) prior to the break in scanning [180–240 min (mean 210 min) after bolus injection] and

the last seven 10-min frames of the PET scanning session [395–480 min including a 15-min break (mean 438 min) after bolus injection] for each ROI. The displacement of total radioactivity ($C_T - C'_T$ for each cigarette-smoking condition (no smoking, denicotinized cigarette, and low-nicotine cigarette) was then calculated as a percent of total concentration of tissue radioactivity (C_T). For each ROI, the percent total radioactivity displacement from the two smoking conditions was corrected for the imperfect steady state (+2.5% per h) determined from the no-smoking condition scans. This corrected percent radioactivity

Table 1. Demographic and rating-scale variable

Variable	Total group (n = 15)	No smoking (n = 7)	Denicotinized cigarette (n = 10)	Low-nicotine cigarette (n = 7)
Age (yr)	35.7 ± 3.1	35.3 ± 5.0	33.5 ± 3.2	38.6 ± 3.6
Female/male	6/9	2/5	5/5	3/4
Cigarettes/d	18.7 ± 0.5	18.8 ± 0.9	17.8 ± 0.6	18.7 ± 0.7
Years smoking	13.5 ± 2.6	15.1 ± 3.7	12.2 ± 2.9	13.1 ± 4.2
Education (yr)	13.5 ± 0.3	13.9 ± 0.4	13.8 ± 0.4	13.3 ± 0.6
FTND	5.7 ± 0.3	5.7 ± 0.5	5.9 ± 0.4	5.9 ± 0.6
HAMD	2.2 ± 0.4	2.7 ± 0.5	1.7 ± 0.5	2.0 ± 0.6
HAMA	2.3 ± 0.4	3.0 ± 0.6	2.3 ± 0.7	2.0 ± 0.6
SJWS per item	3.9 ± 0.1	3.8 ± 0.3	3.7 ± 0.2	3.9 ± 0.2

FTND, Fagerström Test for Nicotine Dependence; HAMD, Hamilton Depression Rating Scale; HAMA, Hamilton Anxiety Rating Scale; SJWS, Shiffman-Jarvik Withdrawal Scale.

Values are stated as mean (± standard error of the mean); *p* values (Student's *t* tests) for demographic and rating scale variables (and a χ^2 test for female/male ratio) between the subjects assigned to the three conditions (no smoking, denicotinized cigarette, and low-nicotine cigarette) were all non-significant (range -0.3 to 1.0).

displacement was then divided by the percent of maximum displaceable radioactivity for each ROI, as determined from the asymptotic portions of the saturation curves in our previous study with smoking various amounts of regular cigarettes (see table 1 and figure 4a in Brody et al., 2006). The percentages of maximum displaceable radioactivity in the previous study for thalamus, brainstem, and cerebellum were 67%, 67% and 56%, respectively. These percent displaceable radioactivities are equal to 100% minus the non-displaceable radioactivity (thereby accounting for both non-specifically bound and free radiotracer). The ratio of percent total radioactivity displaced from smoking to percent of maximum displaceable radioactivity is equal to the percent of specifically bound radiotracer that is displaced, which is the percent $\alpha_4\beta_2^*$ nAChR occupancy. The percent $\alpha_4\beta_2^*$ nAChR occupancies for both the denicotinized and low-nicotine cigarette conditions were then compared to the predicted values of 22–24% and 77–79%, respectively, which were calculated from our previous data with regular cigarettes as described above.

Comparison of $\alpha_4\beta_2^$ nAChR occupancy between the no-smoking, denicotinized cigarette, and low-nicotine cigarette conditions*

To compare $\alpha_4\beta_2^*$ nAChR occupancy between the three conditions, separate analyses were performed for the three potential comparisons (no smoking vs. low-nicotine cigarette, no smoking vs. denicotinized

cigarette, and denicotinized vs. low-nicotine cigarette). Separate analyses were used because the no smoking vs. low-nicotine cigarette comparison had no subject overlap, while the other two comparisons had partial subject overlap (due to some subjects not completing the study).

For comparison of the no-smoking vs. low-nicotine cigarette conditions (no subject overlap), unpaired *t* tests were performed for the ROI data between conditions. Bonferroni correction was applied to account for the fact that three brain ROIs were tested (thalamus, brainstem, cerebellum).

For the comparisons of both the no-smoking vs. denicotinized cigarette conditions and the denicotinized cigarette vs. low-nicotine cigarette conditions, two different (but overlapping) analyses were performed, due to the unusual circumstance of partial overlap in the subject pool for the conditions being compared. First, an unpaired *t* test was performed for both comparisons using subjects from each condition who did not complete both conditions. And second, two independent sequential tests were performed, namely a paired *t* test between each pair of conditions for the subjects who underwent both conditions, followed by an unpaired *t* test for the remaining subjects who received only one scanning session. For this second analysis, the overall *p* value is given by $p_1 p_2 [1 - \ln(p_1 p_2)]$ where *p*₁ and *p*₂ are the *p* values for the two sequential tests. For the independent samples in these sequential tests, multiplying the *p* values by each other and then by a correction factor was felt to be

a conservative approach (see Eadie et al., 1971). Bonferroni correction was applied for both analyses to account for the fact that three ROIs were tested.

Determination of EC_{50}

To determine the venous plasma nicotine level sufficient to result in 50% occupancy of $\alpha_4\beta_2^*$ nAChRs (EC_{50}), we used a similar method to that in our previous report (Brody et al., 2006). Percent $\alpha_4\beta_2^*$ nAChR occupancy for each ROI was plotted against plasma nicotine concentration at the break in scanning corresponding to the middle of the post-smoking radioactivity measurements used for calculations. The plotted data were fitted using nonlinear regression with SigmaStat software (Systat Software Inc.) to determine the EC_{50} .

Correlations between $\alpha_4\beta_2^*$ nAChR occupancy and cigarette withdrawal symptoms

To determine associations between $\alpha_4\beta_2^*$ nAChR occupancy and alleviation of cigarette withdrawal, correlation coefficients for the entire study group (corrected for condition) were determined between percent $\alpha_4\beta_2^*$ nAChR occupancy and change in the three primary withdrawal rating scales (craving/UTS, mood, nervousness) from before to 3.1 h after smoking.

Results

Clinical results

The subject population consisted of adults (mean age \pm S.E.M., 35.7 ± 3.1 yr), was mostly male (nine males, six females), and smoked moderately (18.7 ± 0.5 cigarettes/d) for many years (13.5 ± 2.6 yr). Subjects were moderately nicotine-dependent (mean total FTND scores: 5.7 ± 0.3), had low levels of depression and anxiety (HAMD and HAMA scores: 2.2 ± 0.4 and 2.3 ± 0.4 , respectively), and experienced moderate withdrawal during the initial visit (mean per item SJWS score = 3.9 ± 0.1). All subjects reported compliance with the abstinence protocol, and exhaled CO levels at the time of scanning (2.8 ± 0.4 ppm) were consistent with these subject reports. The groups that underwent the three scanning conditions did not significantly differ in demographic or rating scale variables (Table 1).

From before to after the smoke break, subjects who smoked a cigarette had significant reductions in craving (UTS scores: 4.8 ± 0.6 to 2.4 ± 0.4 and 5.0 ± 0.5 to 3.6 ± 0.6 for the low-nicotine and denicotinized

cigarette conditions, respectively, paired Student's *t* tests, $p=0.03$ and $p=0.04$, respectively). Although the reduction in mean UTS score was more pronounced in the low-nicotine cigarette group than the denicotinized cigarette group (UTS score change: -2.4 vs. -1.4 , respectively), this difference did not reach statistical significance. The no-smoking group had no change in mean UTS score from before to after the break in scanning (UTS scores: 2.5 ± 0.5 to 2.5 ± 0.5 , paired Student's *t* test, n.s.). For the last break in scanning (which corresponded to the middle of the post-smoking PET scanning frames used for data analysis, 3.1 h after smoking), mean UTS scores returned towards baseline for both the low-nicotine and the denicotinized cigarette groups (3.8 ± 0.7 and 4.2 ± 0.7 , respectively), and were no longer significantly different from baseline (paired Student's *t* tests, n.s.).

Nervousness and mood scores did not change significantly from the beginning to end of the smoke break for the three conditions (nervousness: 4.4 ± 0.8 to 3.7 ± 0.7 , 3.0 ± 0.6 to 3.4 ± 0.5 , and 1.7 ± 0.3 to 1.4 ± 0.3 for the low-nicotine cigarette, denicotinized cigarette, and no-smoking conditions, respectively; paired Student's *t* tests, n.s.; mood: 3.9 ± 0.7 to 3.9 ± 0.4 , 4.7 ± 0.4 to 3.7 ± 0.5 , and 4.3 ± 0.7 to 4.6 ± 0.6 for the three groups, respectively; paired Student's *t* tests, n.s.). [As an aside, subjects undergoing the no-smoking condition had significantly lower UTS and nervousness scores prior to the break in scanning than those undergoing the two smoking conditions (Student's *t* tests, $p=0.002$ for both comparisons for UTS scores and $p<0.05$ for both comparisons for nervousness scores).]

Determination of nAChR occupancy from smoking a denicotinized and a low-nicotine cigarette

Time-activity curves for the three ROIs (Figure 1a-c) demonstrated that displacement of 2-FA was consistent with the amount of nicotine in the cigarette smoked. Compared to the no-smoking control condition (which had a mean 2.5% per h increase in radioactivity), smoking a denicotinized cigarette resulted in $17 \pm 1\%$, $16 \pm 2\%$, and $16 \pm 2\%$ displacement of total radioactivity in the thalamus, brainstem, and cerebellum, respectively. Smoking a low-nicotine cigarette resulted in $52 \pm 2\%$, $53 \pm 2\%$, and $43 \pm 2\%$ displacement of total radioactivity in these three brain regions, respectively.

Receptor occupancy from smoking a denicotinized cigarette (displacement of specifically bound radio-tracer) was $25 \pm 2\%$, $25 \pm 3\%$, and $28 \pm 4\%$ (mean 26%) for the thalamus, brainstem, and cerebellum,

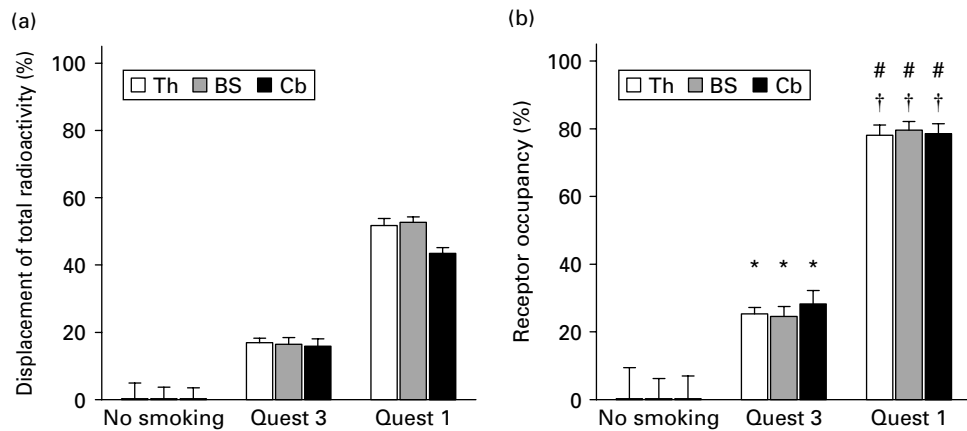


Figure 2. (a) The mean (\pm S.E.M.) percent displacements of total radioactivity for the three smoking conditions [no smoking, Quest 3 (denicotinized cigarette), Quest 1 (low-nicotine cigarette)] for the three regions of interest (Th, thalamus, BS, brainstem, Cb, cerebellum) from before to 3.1 h after smoking. (b) The mean (\pm S.E.M.) receptor occupancies for the three smoking conditions for the three regions of interest (ROIs). The * symbol indicates a significance level of $p < 0.05$ for Student's *t* test comparisons (following Bonferroni correction for the three ROIs) between the denicotinized cigarette and the no-smoking conditions. The # and † symbols indicate significance levels of $p < 0.0001$ for Student's *t* test comparisons (following Bonferroni correction for the three ROIs) between the low-nicotine cigarette condition and the no-smoking and denicotinized cigarette conditions.

respectively, while receptor occupancy from smoking a low-nicotine cigarette was $78 \pm 3\%$, $80 \pm 3\%$, and $79 \pm 3\%$ (mean 79%) for the three brain regions, respectively. These receptor occupancies were similar to the predicted values of 22–24% and 77–79%, respectively, and indicate that nicotine inhalation is primarily responsible for $\alpha_4\beta_2^*$ nAChR occupancy from smoking. Plasma nicotine levels were also consistent with the amount of nicotine in the cigarette smoked (Figure 1d).

Comparison of $\alpha_4\beta_2^$ nAChR occupancy between the no-smoking, denicotinized cigarette, and low-nicotine cigarette conditions*

For the comparison of $\alpha_4\beta_2^*$ nAChR occupancy between the no-smoking and low-nicotine cigarette conditions, all three brain regions had significantly different occupancies (unpaired Student's *t* test, two-tailed, $p < 0.0001$, for the three regions, following Bonferroni correction) (Figures 2 and 3). These results indicate that smoking a low-nicotine cigarette results in significant receptor occupancy compared to no smoking.

For the comparison of $\alpha_4\beta_2^*$ nAChR occupancy between the no-smoking and denicotinized cigarette conditions, all three brain regions were significantly different with both statistical methods applied (for the first analysis, unpaired Student's *t* tests, two-tailed, $p < 0.01$ for the three regions; for the second analysis using sequential tests, $p = 0.01$, 0.03, and 0.04 for the

thalamus, brainstem, and cerebellum, respectively; all results are Bonferroni-corrected) (Figures 2 and 3). These results indicate that smoking a denicotinized cigarette results in significant receptor occupancy compared to no smoking.

For the comparison of $\alpha_4\beta_2^*$ nAChR occupancy between the low-nicotine and denicotinized cigarette conditions, all three brain regions were significantly different with both statistical analysis methods (for the first analysis, unpaired Student's *t* tests, two-tailed, $p < 0.0001$, for the three regions; for the second analysis using sequential tests, $p < 0.0001$ for the three regions; all results are Bonferroni-corrected) (Figures 2 and 3). These results indicate that smoking a low-nicotine cigarette results in significantly greater receptor occupancy than smoking a denicotinized cigarette.

Determination of EC_{50}

The effective venous plasma concentration of nicotine needed to occupy 50% of $\alpha_4\beta_2^*$ nAChRs (EC_{50}) was 0.79 ± 0.08 , 0.77 ± 0.10 , and 0.68 ± 0.10 ng/ml for the thalamus, brainstem, and cerebellum, respectively (Figure 4a–c), with the mean EC_{50} being 0.75 ± 0.08 ng/ml (Figure 4d).

Correlations between $\alpha_4\beta_2^$ nAChR occupancy and cigarette withdrawal symptoms*

There were no significant correlations between receptor occupancy and change in withdrawal symptoms

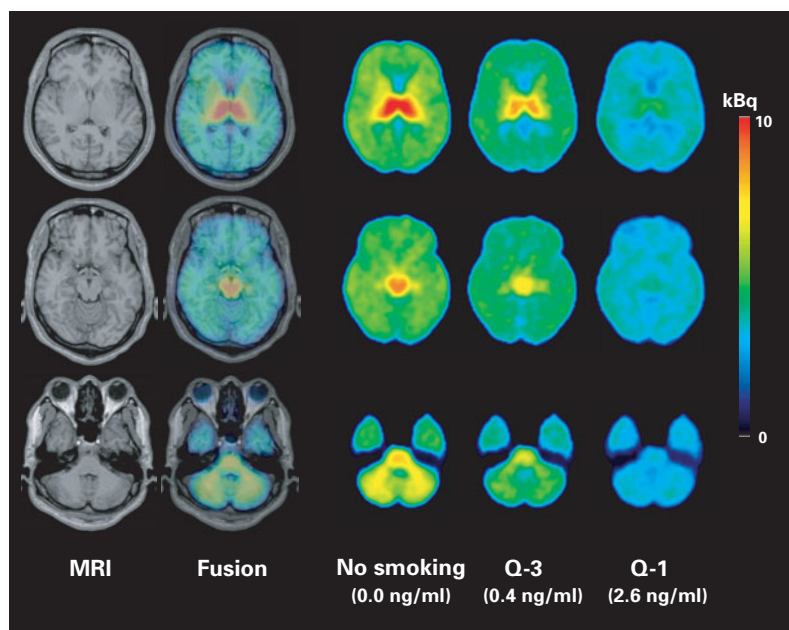


Figure 3. Radioactivity levels on mean positron emission tomography (PET) images 3.1 h after the smoke break in scanning. Column 1 shows a magnetic resonance imaging (MRI) scan with representative slices from three levels of interest for this study (row 1 at the level of the thalamus, row 2 at the level of the brainstem, and row 3 at the level of the cerebellum). Column 2 demonstrates a PET/MRI fusion used for transfer of drawn regions of interest from MRI to PET. Columns 3–5 show mean PET images from the three study groups [no smoking, Q-3 (denicotinized cigarette), Q-1 (low-nicotine cigarette)] 3.1 h after the smoking break in scanning, demonstrating radiotracer displacement for the two active smoking conditions, but not for the no-smoking condition. Mean plasma nicotine levels at the 3.1 h time-point are shown below columns 3–5.

(correlation values -0.27 to -0.13 , d.f. = 13, p values >0.05), indicating that factors other than nicotine binding to $\alpha_4\beta_2^*$ nAChRs are at least partly responsible for the improvement in withdrawal symptoms seen with smoking either low-nicotine or denicotinized cigarettes, or (alternatively) that the sample size here was too small to detect such correlations.

Discussion

The present findings indicate that inhalation of nicotine during cigarette smoking is solely responsible for occupancy of brain $\alpha_4\beta_2^*$ nAChRs. The results which support this conclusion are that: (1) the receptor occupancy after smoking an entire denicotinized cigarette (26%) was very similar to the predicted values (22–24%) calculated from our previous study using regular cigarettes (Brody et al., 2006), and (2) the EC_{50} found here (0.75 ng/ml) was compatible with the previously determined EC_{50} (0.87 ng/ml). The slight discrepancies between results of the present study and the predictions based on our previous study are probably due to small errors in measurement. Although it is possible that the slightly higher receptor occupancy (2–4%) found here with smoking a

denicotinized cigarette is real (perhaps due to intake of the other constituents of tobacco smoke or behavioural factors affecting endogenous acetylcholine levels), these effects would be very minor (if present at all) compared to the effects of nicotine. In our previous study, smoking a full regular cigarette and smoking to satiety resulted in 88% and 95% receptor occupancy, respectively, so that any small effect of factors other than nicotine would be insignificant in daily cigarette smokers compared to the effects of their regular smoking.

The present study also demonstrated significant $\alpha_4\beta_2^*$ nAChR occupancy from smoking a denicotinized cigarette, which has implications for the basic assumption both in research and clinical practice that denicotinized cigarettes provide little pharmacological effect of nicotine. While the present study demonstrated a mean 26% $\alpha_4\beta_2^*$ nAChR occupancy 3.1 h after smoking a denicotinized cigarette, we can infer that the percent $\alpha_4\beta_2^*$ nAChR occupancy would be higher sooner after smoking, since nicotine plasma levels were twice as large within the first hour after smoking (Figure 1d). Therefore, researchers, clinicians, and smokers themselves should consider the fact that smoking denicotinized cigarettes results in substantial

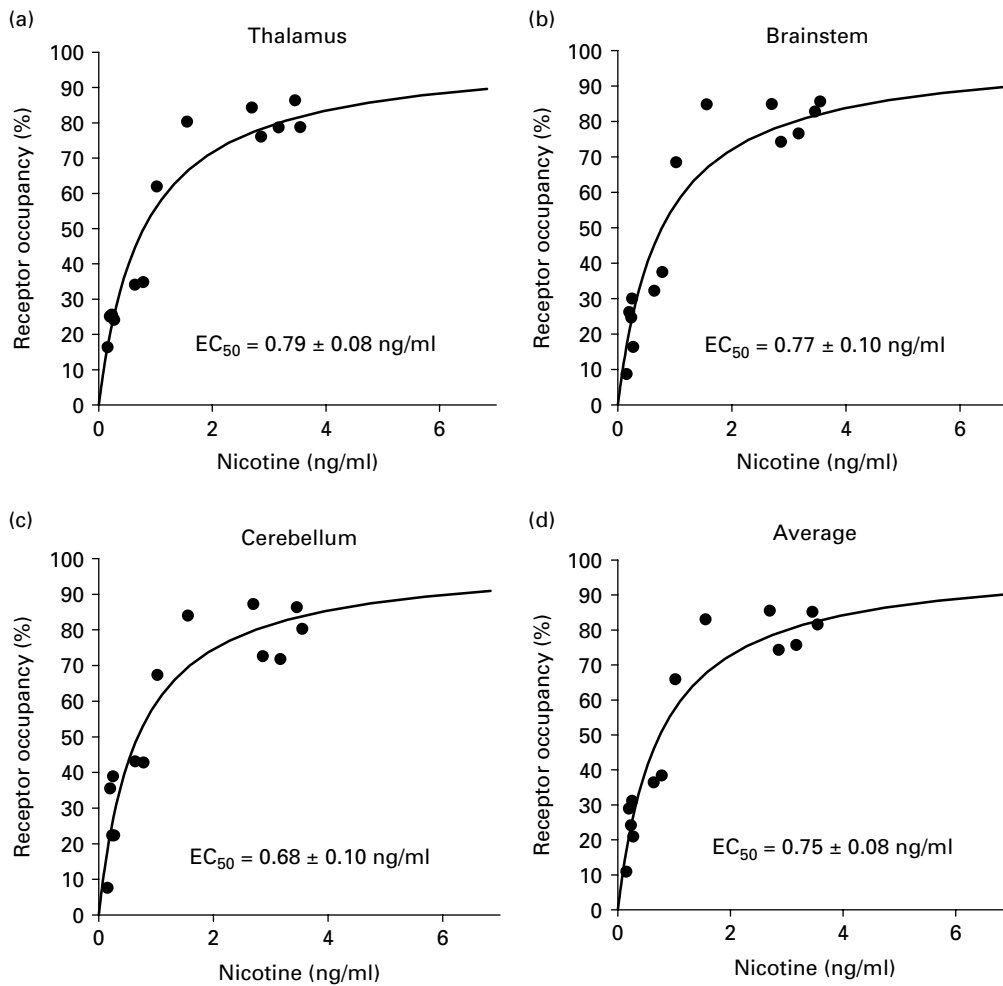


Figure 4. (a)–(c) Graphs showing percent $\alpha_4\beta_2^*$ nicotinic acetylcholine receptor occupancy as a function of plasma nicotine concentration (ng/ml) for the three regions of interest and (d) for the mean of these three regions. From these graphs, the effective plasma concentration of nicotine needed to occupy 50% of available receptors (EC_{50}) was determined for the three regions separately and for the mean of the regions.

$\alpha_4\beta_2^*$ nAChR occupancy, even if this effect is much smaller than that of smoking a regular cigarette.

This study also complements other lines of research indicating that factors other than nicotine binding to $\alpha_4\beta_2^*$ nAChRs are responsible for withdrawal alleviation from smoking. In the present study, there were no significant correlations between changes in withdrawal symptoms and $\alpha_4\beta_2^*$ nAChR occupancy, and craving was returning to baseline levels during the post-smoking PET time-points of interest despite PET scanning evidence of continued $\alpha_4\beta_2^*$ nAChR occupancy. While the absence of correlations with withdrawal symptoms and the return of craving with continued $\alpha_4\beta_2^*$ nAChR occupancy do not prove that there is no relationship between $\alpha_4\beta_2^*$ nAChR occupancy and withdrawal, these findings are consistent

with the wealth of literature demonstrating that factors other than nicotine intake are important for withdrawal alleviation. This literature includes studies demonstrating that denicotinized cigarettes alleviate cigarette craving, often to a comparable degree as smoking regular cigarettes (Buchhalter et al., 2001; Dallery et al., 2003; Donny et al., 2007; Pickworth et al., 1999), and studies of nicotine replacement therapies (Gross and Stitzer, 1989; Muramoto et al., 2003; Rose et al., 1990; West and Shiffman, 2001), which demonstrate incomplete withdrawal alleviation with these medications. Thus, our study results support the theory that factors other than nicotine binding to brain $\alpha_4\beta_2^*$ nAChRs are at least partly responsible for relief of craving (and other withdrawal symptoms) from smoking.

A limitation of this study is the slow brain kinetics of 2-FA, requiring assessment of receptor occupancy >3 h after smoking. If smoking-induced receptor occupancy through non-nicotine mechanisms is short-lasting and disappears in the first minutes after smoking, it would not be detected with our method. New radioligands with faster kinetics will be needed to determine such effects. Nonetheless, even if there are short-lasting effects on nAChR occupancy from non-nicotine aspects of smoking, the low values for plasma EC₅₀ found in both of our studies suggest that brain $\alpha_4\beta_2^*$ nAChRs in smokers are almost completely occupied by nicotine and are probably in the inactive (desensitized) state (Quick and Lester, 2002) throughout the day.

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Statement of Interest

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