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β_2-Nicotinic Acetylcholine Receptor Availability during Acute and Prolonged Abstinence from Tobacco Smoking

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Abstract

Context—β_2*-nicotinic acetylcholine receptor (β_2*-nAChR) availability is higher in recently abstinent smokers compared to never smokers. Variations in β_2*-nAChR availability over the course of abstinence may be related to the urge to smoke, the extent of nicotine withdrawal and successful abstinence.

Objective—To examine changes in β_2*-nAChR availability during acute and prolonged abstinence from tobacco smoking and to determine how changes in β_2*-nAChR availability were related to clinical features of tobacco smoking.

Design—Tobacco smokers participated in up to 4 [^{123}I]5-IA-85380 ([^{123}I]5-IA) single photon emission computed tomography (SPECT) scans during abstinence: 1 day (n=7), 1 week (n=17), 2 weeks (n=7), 4 weeks (n=11), and 6-12 weeks (n=6). Age-matched nonsmokers participated in 1 [^{123}I]5-IA SPECT scan. All subjects completed 1 magnetic resonance imaging study.

Setting—Academic imaging center.

Participants—Tobacco smokers (n=19) and an age-matched nonsmoker comparison group (n=20).

Main Outcome Measure—[^{123}I]5-IA SPECT images were converted to distribution volume and were analyzed using regions of interest.

Results—Compared to nonsmokers, β_2*-nAChR availability in the striatum, cortex and cerebellum of smokers was not different at one day of abstinence, significantly higher at 1week of abstinence and not different at 4 or 6-12 weeks of abstinence. In smokers, at 6-12 weeks of abstinence, β_2*-nAChR availability was significantly lower in the cortex and cerebellum compared to 1 week of abstinence. Additionally, cerebellar β_2*-nAChR availability at 4 weeks of abstinence was positively correlated with craving on the day of scan.

Conclusions—These data suggest higher β_2*-nAChR availability persists up to 1 month of abstinence, and normalizes to nonsmoker levels by 6-12 weeks of abstinence from tobacco smoking. These marked and persistent changes in β_2*-nAChR availability may contribute to difficulties with tobacco cessation.

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A wealth of evidence from postmortem[1-3] and preclinical[4-7] studies demonstrate smoking- and nicotine-induced elevations in nicotinic acetylcholine receptors (nAChRs) throughout the brain. Previously, we demonstrated *in vivo* higher β_2-subunit containing nAChR (β_2*-nAChR) availability in recently abstinent tobacco smokers compared to nonsmokers[8]. Higher β_2*-nAChR availability in smokers may be due to a variety of molecular changes including increased assembly of α_4 and β_2 subunits in the endoplasmic reticulum[9], increased transport of receptors to the membrane[10], decreased receptor turnover[11], and/or the presence of nicotine promoting intracellular maturation of the $\alpha_4\beta_2$-nAChR to a high-affinity conformation[12]. Higher β_2*-nAChR availability is thought to functionally reflect higher numbers of desensitized receptors[13, 14]. Additionally, smoking one cigarette led to greater than 88% receptor occupancy[13] suggesting that smokers maintain saturation of β_2*-nAChRs over the day. Thus, a chronic tobacco smoker who maintains persistently elevated nicotine levels may experience repeated cycles of nAChR activation and desensitization over each day[14].

Preclinical studies have demonstrated that nAChR levels return to control levels after termination of nicotine exposure, but with great variability in the time course[15-17]. A postmortem human study indicated that individuals who had quit smoking at least 2 months prior to their death (range 2 months-30 years) had nicotine binding levels similar to control nonsmokers[2]. A recent *in vivo* study in a small number of male smokers demonstrated a trend for a normalization of the β_2-nAChR by 21 days of smoking cessation[18]. However, these data need to be confirmed in a larger, more heterogeneous population during prolonged abstinence.

The exact subunit combination of nAChR that upregulate in response to nicotine is emerging. NAChRs that contain α_4 and β_2 subunits [19, 20] are the most abundant nAChRs in brain, and nicotine demonstrates the highest affinity for these receptors [reviewed by 21]. In recent years evidence has emerged that the β_2*-nAChRs are a critical neural substrate mediating the effects of nicotine in brain. Much of this information has been derived from studies in β_2 knockout mice. These studies have demonstrated that the β_2-subunit is critical for self-administration [22], conditioned place preference[23], discriminative stimulus and taste aversion[24], dopamine release [22, 25, 26], dopamine-dependent locomotor activation[27] and enhancement of incentive aspects of motivation[28] of nicotine. Studies in wild-type animals further confirm the β_2-subunit is critical to the reinforcing properties of nicotine[29]. The β_2-subunit also determines the sensitivity to nicotine[30-32], but does not play a critical role in nicotine withdrawal symptoms in rodents[33]. Importantly, nicotine induced increases in nAChR, termed “upregulation”, are conferred by a specific microdomain that is in the β_2-subunit [34] and thus this upregulation is confined to nAChR that contain the β_2-subunit[4, 19, 31, 35-38].

β_2*-nAChR availability can be measured *in vivo* with [^{123}I]-5-IA-85380 ([^{123}I]5-IA) and single photon emission computed tomography (SPECT). [^{123}I]5-IA is a nicotinic agonist that binds with high affinity to the nicotine binding site on nAChR that contain the β_2-subunit[39]. This ligand demonstrates low nonspecific binding[40] and has acceptable dosimetry in human subjects with high brain uptake[41, 42] and good test-retest reproducibility[43]. Because [^{123}I]5-IA is administered at a “trace” dose (<1% occupancy) for SPECT imaging it does not interfere with receptor and cell function. Imaging with [^{123}I]5-IA SPECT in nonhuman primate and human subjects results in a binding pattern that is consistent with the established regional distribution of β_2-nAChR and is highest in the thalamus and intermediate throughout the cortex and cerebellum[43, 44].

The primary goal of the present study was to evaluate the time course of change in β_2*-nAChR availability over prolonged abstinence using [^{123}I]5-IA SPECT. A secondary goal was to explore the relationships between β_2*-nAChR availability and behavioral features of tobacco smoking and withdrawal. We hypothesized that during acute abstinence, at 1 day of withdrawal, β_2*-nAChR availability would be lower compared to 1 week of abstinence due to the presence

of nicotine in the brain, which would block radiotracer binding. Consistent with our previous study[8], we hypothesized that compared to control nonsmokers, β_2*-nAChR availability would be higher at 1 week of withdrawal, and then would progressively decline over prolonged abstinence.

Methods

Subjects

Nineteen healthy tobacco smokers (9 men, 10 women; 41.1±9.0 years; 13 Caucasian, 5 African American, 1 Hispanic) and 20 age-matched healthy controls (9 men, 11 women; 42.4±9.8 years; 10 Caucasian, 7 African American, 2 Hispanic, 1 Asian) participated in this study. Smokers participated in up to four [^{123}I]5-IA SPECT scans and one magnetic resonance imaging (MRI) study. Subjects were grouped into the following time points of abstinence: 1 day (1.0±0 days; mean ± SD, n=7), 1 week (7.7±1.4 days, n=17), 2 weeks (17.9±3.0 days, n=7), 4 weeks (30.5±4.3 days, n=11), and 6-12 weeks (69.0±23.5 days, n=6). Subjects were grouped as described due to the difficulty in retaining subjects who remained abstinent over long periods and due to the challenges in having subjects complete time consuming (e.g., >8 h day) scans on multiple days. Subjects were scanned during a range of days at each time point due to scheduling constraints. Nonsmoker controls (n=20) participated in one [^{123}I]5-IA SPECT scan and one MRI study.

This study was approved by the Yale University School of Medicine Human Investigation Committee, the West Haven Veterans Administration Human Subjects Subcommittee, and the Radiation Safety Committee. The use of the radiotracer, [^{123}I]5-IA, was approved by the Food and Drug Administration. Subjects were recruited by word of mouth, posters, and newspaper advertisements from the community. Eligibility was determined as follows. All subjects had a medical examination by a study physician to exclude any major medical issues or neurological disorders. This included a physical examination, electrocardiogram, serum chemistries, thyroid function studies, complete blood count, urinalysis, and urine toxicology screening. Subjects were given structured interviews using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (SCID) to rule out any Axis I Disorder except for Nicotine Dependence. All tobacco smokers had to smoke ≥10 cigarettes per day for at least one year. Smoking status was confirmed by plasma cotinine levels >150 ng/mL, urine cotinine levels >100 ng/mL and carbon monoxide levels >11 on the day of intake. Smokers were helped to quit smoking using Clinical Practice Guidelines and contingency management[45-47]. Briefly, contingency management is a behavioral therapy in which reinforcement is provided contingent upon a successful response. In this study, monetary reinforcement was provided contingent upon abstinence from smoking measured by urine cotinine and breath carbon monoxide levels. Breath carbon monoxide and urine cotinine levels were monitored daily for the first 8 days of smoking cessation and a minimum of twice weekly thereafter. Subjects were instructed that they could not use any form of nicotine replacement therapy or medication throughout the study. All control subjects were nonsmokers (defined as <100 cigarettes in lifetime) and had no history of significant medical illness or major head trauma. Nonsmoking status, was confirmed by plasma cotinine levels <15 ng/mL, urine cotinine levels <100 ng/mL and carbon monoxide levels <11 ppm on the day of intake and day of scan. All women were required to have a negative pregnancy test during the screening process and prior to radiotracer injection on each study day. Plasma nicotine and cotinine levels were measured as previously described[8]. Urine cotinine levels were measured with either Accutest NicoMeter™ cotinine test strips (Jant Pharmacal, Encino, CA) or NicAlert™ cotinine test strips (Nymox Pharmaceutical, Hasbrouck Heights, NJ).

The severity of nicotine dependence was assessed at intake with the Fagerström Test for Nicotine Dependence (FTND)[48], and craving and nicotine withdrawal symptoms were

assessed with the Urge to Smoke Questionnaire (QSU)[49] and the Minnesota Nicotine Withdrawal Scale (MNWS)[50], respectively, at baseline, e.g., on the day of their intake prior to quitting smoking, and on each day of their participation in [^{123}I]5-IA scans. The QSU has two main factors, the intention/desire to smoke (QSU-Intent) and relief of negative affect and withdrawal (QSU-Relief).

MR Imaging

MRI studies were obtained on a 1.5 Tesla Siemens camera in a standard orientation (TE=5-7 ms; TR=24ms; a 256×192 matrix; 1 NEX; FOV 30 cm; 124 contiguous slices with 1.2 mm thickness) and were used for coregistration to the SPECT images to provide an anatomical guide for placement of regions of interest.

[^{123}I]5-IA SPECT scan

All subjects received a 0.6-g saturated solution of potassium iodide, to protect their thyroid from possible exposure to radioactive iodide, in the hour prior to radiotracer administration. [^{123}I]5-IA was synthesized as previously described [51] and administered as a bolus to constant infusion at a ratio of 7.0 for 8 hours. Subjects were injected with equivalent doses of a bolus (mean±SD, MBq) and constant infusion (mean±SD, MBq/h) as follows: control nonsmokers (157.9±14.6 MBq, 22.8±2.1 MBq/h), 1 day abstinent smokers (134.5±24.6 MBq, 20.5±3.8 MBq/h), 1 week abstinent smokers (154.3±15.9 MBq, 22.2±2.6 MBq/h), 2 week abstinent smokers (159.4±5.6 MBq, 23.3±0 MBq/h), 4 week abstinent smokers (158.9±7.4 MBq, 22.6 ±1.7 MBq/h) and 6-12 week abstinent smokers (141.5±30.5 MBq, 21.9±3.5 MBq/h). Three consecutive 30-min emission scans and one 15-min simultaneous transmission and emission protocol scan (STEP) were obtained between hours 6 and 8 of the [^{123}I]5-IA infusion on a Picker PRISM 3000 XP (Cleveland, OH) SPECT camera. The PRISM 3000 XP is a 3-headed camera equipped with a low energy, ultra-high resolution fanbeam collimator (photopeak window, 159 keV ± 10%; matrix 128×128) with a uniform sensitivity across the field of view. A ^{57}Co-distributed source was measured with each experiment to control for day-to-day variation in camera sensitivity. The axial resolution (full width at half maximum) is 12.2 mm, measured with a ^{123}I line source in water in a cylindrical phantom. Blood was drawn prior to injection and at the beginning and end of the emission scans for analysis of plasma total parent and free fraction of parent tracer in plasma (f_P, free fraction). The chemical fate of [^{123}I]5-IA post injection was assessed in plasma as previously described[51]. Briefly, plasma total parent was assessed by acetonitrile protein denaturation, while free fraction was determined by ultrafiltration using Centrifree units.

Image analysis and outcome measures

Images were reconstructed and analyzed as previously described including a nonuniform attenuation correction [43] with one exception. Specifically, in subjects who had more than one SPECT scan, the second and subsequent SPECT scans were coregistered to the same position as the first scan in order to apply the same region of interest template for that subject. MRIs were coregistered to the SPECT images to provide an anatomical guide for placement of the regions of interest using Medx (version 3.4) software (Medical Numerics, Inc., MD). Regions of interest chosen were those known to contain β_2-nAChRs and included frontal, parietal, anterior cingulate, temporal and occipital cortices, thalamus, striatum (an average of caudate and putamen) and cerebellum. Regions-of-interest are corrected to account for differences in size. Two raters conducted the analysis. Variability between raters was <12 % across regions-of-interest. The mean of the analysis from the two raters is reported.

The outcome measure V_T/f_P (regional activity divided by free plasma parent between 6 and 8 hours), was used to correct for possible differences in radiotracer metabolism or plasma protein binding between groups and subjects. Specifically, V_T/f_P equals [^{123}I]5-IA uptake in a region-

of-interest (kBq/cc) / free plasma parent (kBq/mL)[52]. We refer to V_T/f_P as β_2*-nAChR availability because we are only measuring receptors that are "available" to be bound by radiotracer. Receptors that are already occupied, e.g., by residual nicotine, a pharmacologically active metabolite (cotinine or nornicotine) or by endogenous neurotransmitter (acetylcholine) are not available. V_T/f_P is proportional to the binding potential (BP, mL/g=B_{max}/K_D), which is proportional to the receptor number (Bmax) at equilibrium, given the assumptions that there is no change in affinity (K_D) and that nondisplaceable (nonspecific and free) uptake does not differ between subjects or comparison groups. As described previously[43], there is no appropriate reference region for this radiotracer, so nondisplaceable [^{123}I]5-IA uptake could not be measured. The measures of total plasma parent (kBq/mL), f_P and free plasma parent (kBq/mL) which is defined as total parent * f_P were compared between groups to determine differences in radiotracer metabolism or protein binding.

Statistical Analyses

Data were analyzed using SAS version 9.1 (SAS Institute Inc, Cary, NC). Differences in blood measures (total and free parent, and f_P) and regional β_2*-nAChR availability, V_T/f_P, between subjects in the nonsmoker control group and each of the 1 day, 1 week, 2 week, 4 week, and 6-12 week abstinent smoker groups were assessed using two-sample t-tests. Differences in regional brain V_T/f_P and behavioral measures of tobacco smoking and withdrawal between subjects in the abstinent smoker groups were assessed using repeated measures, mixed-effects regression models with group as a fixed effect and compound symmetry covariance structure across repeated measurements. These between group differences were evaluated using repeated measures, mixed-effects models in order to account for the observations between abstinent smoker groups that were not entirely independent, given that some abstinent smokers contribute observations to more than one group. For models examining between-group differences for which there was a significant effect of group, four planned post hoc between-group comparisons were conducted, i.e., between 1 week abstinent smokers and 1 day, 2 week, 4 week, and 6-12 week abstinent smokers. For these post hoc tests, p-values were Bonferroni corrected for multiple comparisons, and statistical significance was considered at $p \leq 0.00625$. Correlational analyses for the associations between receptor availability and smoking assessments were conducted using SPSS version 16.0 (SPSS Inc. Headquarters, Chicago, IL). Correlations between β_2*-nAChR availability, V_T/f_P, at each time point and clinical variables (smoking, craving, withdrawal) were assessed with Spearman's rho correlation coefficients. Different slopes for each abstinence time point were estimated to illustrate the relationship between significant clinical variables and β_2*-nAChR availability. Due to multiple comparisons, statistical significance was considered at $p \leq 0.01$.

Results

Clinical population

Nineteen tobacco smokers and 20 age-matched nonsmokers were included in the study. Smokers who participated in scans at different times since the last cigarette were equivalent in age, level of nicotine dependence (as assessed by the FTND at intake), cigarettes smoked per day, and years of smoking (Table 1). Plasma cotinine and nicotine levels and carbon monoxide measurements were negligible in nonsmokers and in smokers were highest at 1 day of abstinence and decreased over time, confirming abstinence from smoking (Table 1).

β_2*-nAChR availability during acute and prolonged abstinence

There were no differences between groups in injected dose, bolus to infusion ratio, or time of scan (data not shown). Concentrations of [^{123}I]5-IA activity in the blood (kBq/mL) were measured in order to correct for potential differences between groups in radiotracer metabolism or protein binding. There were significant differences in total parent (kBq/mL) between

nonsmokers and 1 day abstinent smokers and in f_P between nonsmokers and 2 week abstinent smokers (Table 2). There were no differences in total parent (kBq/mL) or f_P between groups at other time points, or between groups at any time point in free parent (kBq/mL). There was also variability between smokers who participated in multiple scans in changes in β_2*-nAChR availability over time with some abstinent smokers showing dramatic changes in β_2*-nAChR availability (up to 48% change in the cortex) and others showing barely any difference, e.g., less than 5%, over time (individual data not shown).

Regional β_2*-nAChR availability, reflected by V_T/f_P, was compared between nonsmokers and each of the abstinent smoker groups and between the abstinent smoker groups (Table 3 and Figures 1 and 2). In 1 day abstinent smokers as compared to nonsmokers, V_T/f_P was significantly reduced in the thalamus. In 1 week abstinent smokers as compared to nonsmokers, V_T/f_P was significantly higher in the striatum, cerebellum and throughout the cortex. In 4 week abstinent smokers as compared to nonsmokers, V_T/f_P was significantly higher in the occipital cortex (Table 3 and Figures 1 and 2).

Among the abstinent smoker groups, there were significant between group differences in V_T/f_P in the thalamus ($F[4,25]=4.12$, $p=0.011$), parietal cortex ($F[4,25]=2.95$, $p=0.040$), frontal cortex ($F[4,25]=2.88$, $p=0.043$), anterior cingulate ($F[4,25]=3.75$, $p=0.016$), occipital cortex ($F[4,25]=3.42$, $p=0.023$), and cerebellum ($F[[4,25]=4.00$, $p=0.012$). Compared to 1 week abstinent smokers, 1 day abstinent smokers had significantly lower V_T/f_P in the thalamus ($t[25]=-3.51$, corrected $p=0.007$) and cerebellum ($t[25]=-3.02$, corrected $p=0.023$). Compared to 1 week abstinent smokers, 6-12 week abstinent smokers had significantly lower V_T/f_P in the parietal cortex ($t[25]=-2.87$, corrected $p=0.033$), frontal cortex ($t[25]=-2.80$, corrected $p=0.039$), anterior cingulate ($t[25]=-3.21$, corrected $p=0.014$), occipital cortex ($t[25]=-3.10$, corrected $p=0.019$) and cerebellum ($t[25]=-3.17$, corrected $p=0.016$). There were no significant differences in V_T/f_P between 1 week abstinent smokers compared to smokers at 2 or 4 weeks of abstinence (Table 3 and Figures 1 and 2).

Relationship between β_2*-nAChR availability and clinical features

There were significant differences in QSU-Intent ($F[4,25]=7.41$, $p<0.015$) and QSU Relief ($F[4,25]=5.63$, $p=0.002$), but not in MNWS scores ($F[4,25]=0.33$, $p=0.857$), between groups of abstinent smokers (Table 4). Specifically, overall group differences in QSU-Intent and QSU-Relief were attributable to significantly higher levels of each of these measures on the day of the scan in the 1 day abstinent smoker group as compared to smokers at other time points.

There were significant correlations between regional β_2*-nAChR availability and clinical features and assessment scores at baseline, e.g., at intake prior to quitting smoking (Table 5). Specifically, baseline QSU-Intent scores correlated negatively with β_2*-nAChR availability at 1 day of abstinence in the thalamus ($rho=-0.90$, $p=0.006$) and parietal cortex ($rho=-0.88$, $p=0.008$). There were also significant correlations between regional β_2*-nAChR availability and assessment scores taken at each abstinent time point (Table 6). Specifically, a positive correlation was observed between cerebellar β_2*-nAChR availability and craving on both the QSU-Intent ($rho=0.74$, $p=0.01$) and QSU-Relief ($rho=0.74$, $p=0.01$) at 4 weeks of abstinence. There were no significant correlations between baseline smoking variables, e.g., FTND, number of years smoked, or number of cigarettes smoked per day with β_2*-nAChR availability at any time point (data not shown).

Comment

The present study examined the time course of changes in β_2*-nAChR availability during acute and prolonged abstinence in tobacco smokers compared to nonsmokers using [^{123}I]5-IA SPECT. The present findings demonstrate higher β_2*-nAChR availability in the striatum,

cerebellum and cerebral cortex in tobacco smokers at 1 week of abstinence compared to nonsmokers, but similar or lower β_2*-nAChR availability to nonsmokers smokers at 1 day and 6-12 weeks of abstinence. While there is not a significant difference between β_2*-nAChR availability in smokers at 2 and 4 weeks of abstinence compared to nonsmokers, there remains a robust difference, e.g., higher β_2*-nAChR availability in smokers at 2 weeks (16-23%) and 4 weeks (14-18%) of abstinence in the striatum, cerebellum and cortex compared to nonsmokers, that does not return to nonsmoker levels until 6-12 weeks of abstinence (-4-5% difference). There are two primary implications to these results. First, at 1 day of abstinence there is still residual nicotine, or a pharmacologically active metabolite of nicotine, such as cotinine or nornicotine present in the brain that interferes with radiotracer binding, thus leading to the appearance of lower β_2*-nAChR availability. Second, the normalization of the β_2*-nAChR is prolonged, requiring up to 6-12 weeks of abstinence to fully return to nonsmoker levels.

Interestingly, in the smokers at 1 day of abstinence, the levels of total parent of the radiotracer were significantly lower, but normalized quickly, by 1 week of abstinence. This highlights the impact of nicotine or another chemical in tobacco smoke on metabolism, e.g., because nicotine was still present, it may have changed the metabolism of the radiotracer, resulting in lower total parent at 1 day of abstinence. Cytochrome P450 (CYP 2A6) is primarily responsible for the metabolism of nicotine to its main metabolite cotinine[53]. There is evidence that nicotine is metabolized faster in smokers than in nonsmokers and there are genetically-mediated differences in the metabolism of nicotine in smokers[54]. Additionally, nicotine can interfere with metabolism of other drugs[55]. Consistently, we expect that [^{123}I]5-IA is metabolized in the liver by enzymes in the cytochrome P450 family, such as CYP2A6, which acts on nicotine, and CYP2B6 and CYP2D6, which catalyze the dealkylation of aromatic ethers[56, 57]. In radiotracer imaging studies it is imperative that brain uptake is corrected for radiotracer metabolism, since differences in metabolism of the radiotracer determine how much radiotracer is available to the brain, e.g., fast metabolizers will have less radiotracer available to brain, while slow metabolizers will have more radiotracer available to brain for a given dose. By using the outcome measure V_T/f_P, we correct for individual differences in radiotracer metabolism and protein binding.

In the present study we report a negative correlation between baseline craving scores, e.g., prior to quitting smoking, and β_2*-nAChR availability at 1 day of abstinence in the thalamus and parietal cortices. Because receptor availability is defined as receptors that are available to be bound by the radiotracer, at 1 day of abstinence subjects with lower receptor availability have more nicotine present in the brain occupying receptors and blocking the radiotracer from binding to the receptor. Thus, we believe that subjects who reported high baseline craving, likely smoked more cigarettes immediately prior to their quit day and had lower receptor availability at 1 day of abstinence. However, the experience of craving in the presence of nicotine occupancy of the β_2*-nAChR is not unusual. Smokers experience craving within 2 h of their last cigarette despite continued occupancy of the receptor by nicotine[13]. Dopamine release has been associated with the feeling of craving[58], and both nicotine[59] and cotinine[60] have been shown to facilitate dopamine release, thus the prolonged partial occupancy of the receptor by nicotine or cotinine, may contribute to the feelings of craving that are reported 2 h after the last cigarette and throughout the first week of abstinence.

We also report a positive correlation between craving on the day of scan at 4 weeks of abstinence and cerebellar β_2*-nAChR availability at 4 weeks of abstinence. Thus, those individuals with higher cerebellar β_2*-nAChR availability at 4 weeks of abstinence report a greater urge to smoke on that day. Associations between nicotine and craving have previously been identified in the thalamus[61, 62] and generally in areas associated with emotion and reward, and those with high densities of nAChRs (see[63] for review). Interestingly, a previous study

found associations between craving and regions subserving motor functions including the primary motor cortex, premotor cortex and supplementary motor area[64] which require input from the cerebellum. Additionally, when smokers are told to actively resist craving during cigarette cue exposure, the motor cortex is deactivated[65]. Our finding of a link between the cerebellum and craving further suggests that craving has a motor component, so that craving for cigarettes may elicit preparation for action or voluntary movement, goal directed actions (e.g., lighting a cigarette, bringing a cigarette up to the mouth) and/or motor imagery which are linked to the motor system[66, 67]. It can be estimated that over the course of 20 years, a one pack per day smoker (assuming 11 puffs on average per cigarette68) may perform the action of bringing a lit cigarette to the mouth over 1.5 million times. Thus, the physical action of smoking a cigarette is likely to be critically tied to craving during abstinence.

In addition to the role of the cerebellum in motor functions, there is increasing interest in the involvement of the cerebellum in cognition. Specifically, activation of the cerebellum has been associated with tasks requiring explicit, episodic memory, e.g., recall of autobiographical events[69, 70]. With regard to drug abuse, imaging studies have determined that the cerebellum is activated in response to smoking-related cues[71], and is associated with cue-induced craving in cocaine abusers[72, 73] and recently abstinent alcoholics[74]. Activity in the cerebellum has also been linked to executive dysfunction in cocaine users[75]. Taken together, these studies and the present findings highlight the cerebellum as a brain region that is critically linked to craving by both motor and cognitive functions.

We report no significant correlations between nicotine withdrawal and β_2*-nAChR availability. This is consistent with the preclinical literature suggesting that the β_2*-subtype does not play a critical role in the physical symptoms of nicotine withdrawal[33, 76]. In this study, subjects reported a mild-moderate level of nicotine withdrawal symptoms at baseline and over the course of the study, thus these results require replication in a larger sample with a greater range of nicotine withdrawal symptoms. Additionally, we did not obtain significant correlations between β_2*-nAChR availability at 1 week of abstinence and clinical features. We previously found a negative correlation between the urge to smoke to relieve withdrawal symptoms and β_2*-nAChR availability in the sensorimotor cortex at approximately 1 week of abstinence[8]. This discrepancy may be due to differences in correlational analysis methods, e.g., voxel-based analyses in the previous study versus spearman's rho correlations with regions-of-interest in the current study. Voxel-based analyses may be more sensitive to detecting significance in smaller brain regions, but that was beyond the scope of the current study. The lack of additional correlations at 1 week of abstinence was previously discussed[8].

Consistent with our previous study[8] we report significantly higher β_2*-nAChR availability in smokers at one week of abstinence in the cortex, striatum and cerebellum, but not thalamus compared to nonsmokers. The difference between recently abstinent smokers and never smokers in the previous study[8] was of a greater magnitude, e.g., 26-36% in the cerebral cortex and 27% in the striatum, than in the current study, e.g., 21-29% in the cerebral cortex and 22% in the striatum. This may be due to the older average age (approximately 5 years) of subjects in the current study, since β_2*-nAChR availability has been shown to decrease with age in nonsmokers[77]. This study and previous *in vivo* PET[78] and SPECT[8, 18] studies report no upregulation of thalamic β_2*-nAChR availability during acute abstinence, which conflicts with postmortem[2] and animal[8, 79] studies. In general, this may be due to differences in methodology or the higher relative dose of nicotine in the postmortem and animal studies. However, two smokers in the current study exhibited increased β_2*-nAChR availability in the thalamus compared to nonsmokers, highlighting the role of individual differences in receptor regulation.

This study is also consistent with a previous study[18] demonstrating that higher β_2*-nAChR availability in recently abstinent tobacco smokers compared to nonsmokers is temporary. In

the previous study in men, β_2*-nAChR availability decreased to nonsmoker levels in some subjects by 21 days of abstinence. Specifically, compared to nonsmokers, smokers had significantly lower β_2*-nAChR availability at 4 hr of abstinence, significantly higher β_2*-nAChR availability at 10 days of abstinence, and similar β_2*-nAChR availability at 21 days of abstinence. Additionally, they reported significantly lower β_2*-nAChR availability at 21 days of abstinence compared to 10 days of abstinence. One difference is that the previous study[18] had significantly lower β_2*-nAChR availability in all regions at 4 hr of abstinence compared to the nonsmokers, while the current study reports lower thalamic but similar β_2*-nAChR availability in the striatum, cerebellum and throughout the cortex compared to the nonsmoker group at 1 day of abstinence. This is interesting and likely due to high levels of residual nicotine or metabolites present in the brain at 4 hr of abstinence (versus ~24 hours in the current study) resulting in lower β_2*-nAChR availability. Also, while the current study did not find a significant decrease, on average, in β_2*-nAChR availability by 4 weeks of abstinence compared to 1 week of abstinence, in some subjects normalization did occur by this point. The high heterogeneity of the subject population in the current study compared to the previous study in men only[18] likely accounts for the high individual variability with regard to receptor changes during prolonged abstinence. Thus, the present study contributes to the literature with a larger, more heterogeneous subject group, a more prolonged period of abstinence, and additional assessments of behavioral features of tobacco smoking.

Previous preclinical studies demonstrated that in chronically nicotine treated animals, nAChR levels returned to levels observed in control animals after termination of nicotine, but with variable timing ranging between 1 and 3 weeks[15, 80, 81]. The previous human study[18] indicated a return to control levels within 21 days of abstinence; and, the current study indicates that on average, β_2*-nAChR availability in recently abstinent tobacco smokers does not normalize until between 4-12 weeks, although, this is highly variable between individuals. The differences in the time course changes may be due to differences in dosing regimen, chronicity of nicotine, route of administration, metabolism between species, specificity of the radioligand, or sex and/or genetic differences in nAChR subunit expression and composition of nicotinic agonist binding sites, but taken together the results consistently highlight a return to control levels or "normalization" of the β_2*-nAChR after termination of chronic nicotine. The preclinical studies support a process of prolonged normalization when we consider that 1-3 weeks is substantial in the life span of a rodent. Additionally, this prolonged normalization of the receptor is in line with the protracted withdrawal symptoms reported by tobacco smokers. For example, while withdrawal symptoms such as anger, anxiety, depression and difficulty concentrating tend to peak within the first week of quitting smoking they continue up to 4 weeks after the quit attempt[82]. We are currently examining variables that may be associated with the rate of normalization.

One limitation of this study is that [^{123}I]5-IA measures the availability of the β_2-subunit of the nAChR, primarily the $\alpha_4\beta_2$-subunit, but other subunits also contribute to the regulation of the nAChR by tobacco smoking. Notably, the β_2-subunit has been linked primarily to the reinforcing effects of nicotine[22, 83, 84] and combines with the $\alpha_{3\text{-}6}$ subunits. Importantly, different β_2*-subunit combinations appear to be differentially regulated by nicotine, with α_3/α_6 and $\alpha_6\alpha_4\beta_2$* and α5α4β2* not upregulating or decreasing in response to nicotine[38, 85-88] while $\alpha_6\beta_2$ (nonα_4) nAChR increase in response to nicotine[88]. Notably, nAChR subunit expression varies regionally, and differential regulation of these distinct subunit combinations that are measured by a single nicotinic agonist ligand such as [^{123}I]5-IA will result in regional differences in the degree of upregulation in smokers and the degree of receptor normalization during abstinence. Thus, we are likely measuring the β_2-subunit in combination with $\alpha_{3\text{-}6}$ subunits leaving the *in vivo* measurement of the β_3-subunit and α_7-subunit for the future, with further radiotracer development. Both the α_7- and β_3-subunit have been implicated in modulating dopamine release[14, 89] and thus, may also play a role in the rewarding properties

of tobacco smoke. While the α_7-subunit does not upregulate in response to chronic nicotine[31], it is critically involved along with the β_2-subunit in mediating desensitization/inactivation of the neuronal nAChRs in response to chronic nicotine[90].

In summary, our data extend the findings of previous studies[8, 18] which showed that the upregulation of the β_2*-nAChR in recently abstinent tobacco smokers was temporary and could be measured with [^{123}I]5-IA SPECT imaging. Specifically, we demonstrate that the normalization of upregulated β_2*-nAChRs in tobacco smokers during smoking cessation is prolonged. This is consistent with the clinical course of tobacco smoking in which craving, withdrawal symptoms, and risk for relapse are prolonged. The variation between individuals in the magnitude of upregulation and rates of normalization may ultimately be used to delineate subgroups based on genetics, sex, and comorbid mental illness and thus to target treatment medications.

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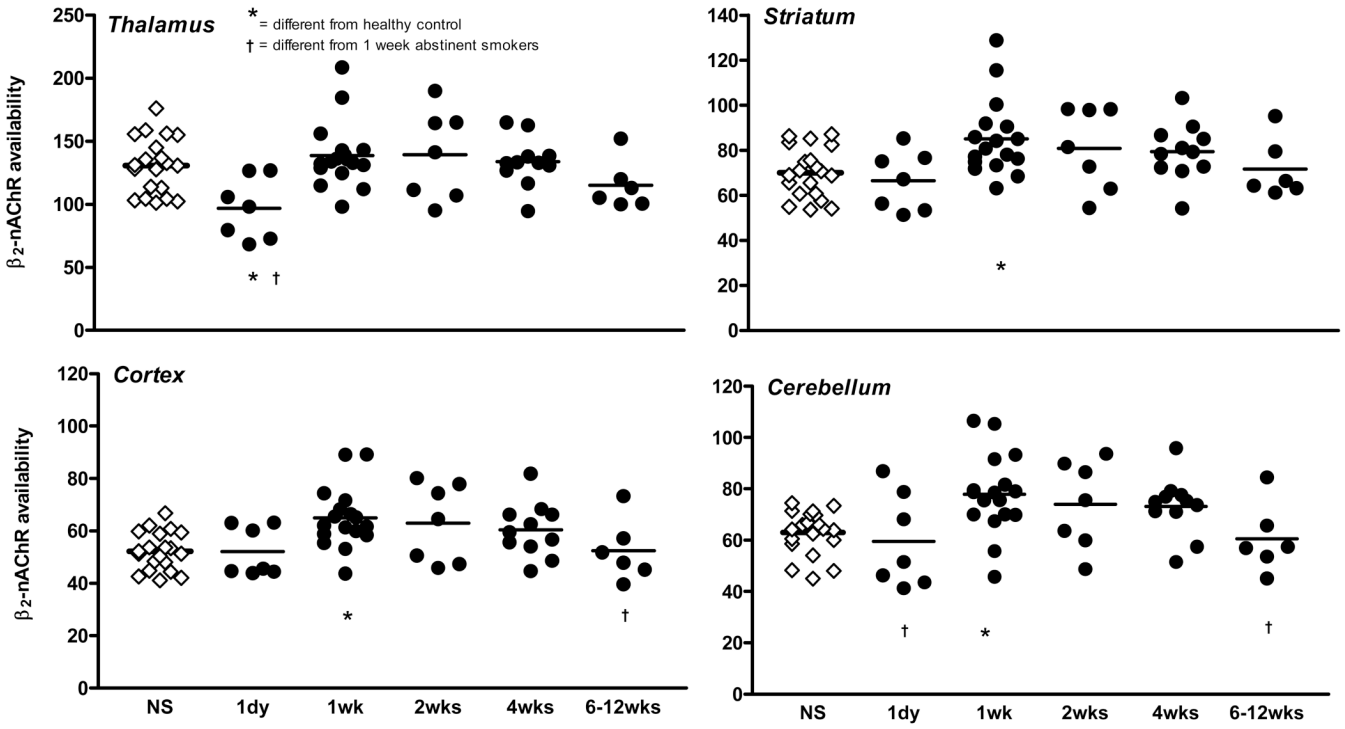

Figure 1.

β_2*-nAChR availability (V_T/f_P) is shown in individual nonsmokers (open diamonds) and tobacco smokers (filled circles) at 1 day, 1 week, 2 weeks, 4 weeks, and 6-12 weeks of abstinence in the thalamus, striatum (average of caudate and putamen), cortex (average of cortical regions including parietal, frontal, anterior cingulate, temporoinsular, and occipital cortex), and cerebellum. The line in each scatter plot represents the mean value of those subjects. * indicates significant difference from control nonsmokers after Bonferroni's correction using two-sample t-tests. † indicates significant difference from 1 week abstinent smokers after Bonferroni's correction using planned post-hoc between-group comparisons subsequent to the analysis of repeated measures mixed-effects regression models including the overall effect of abstinent smoker group.

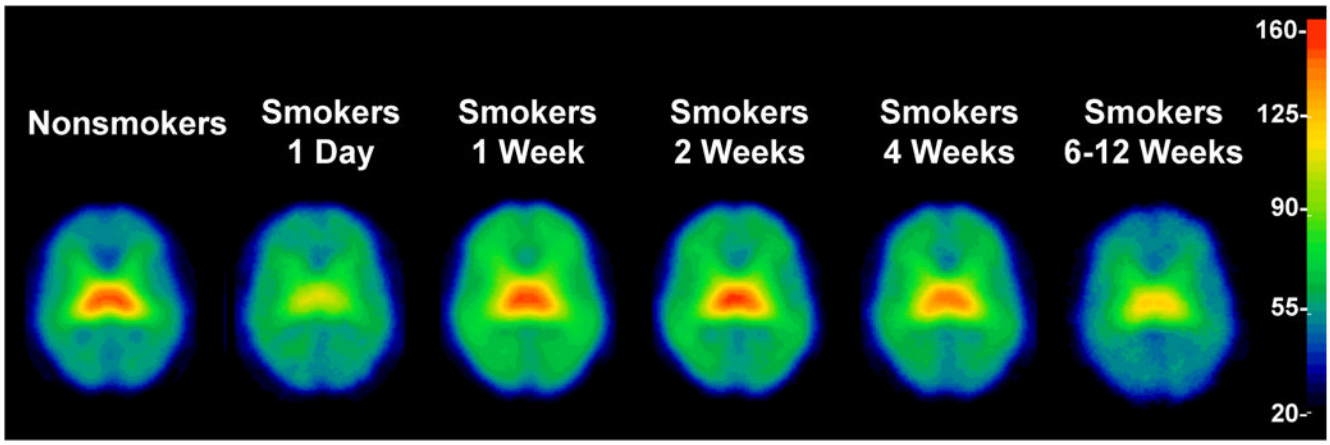

Figure 2.

Mean parametric images illustrating β_2*-nAChR availability in nonsmokers and tobacco smokers at 1 day, 1 week, 2 weeks, 4 weeks, and 6-12 weeks of abstinence at similar transaxial levels of brain. The color scale is shown with red, yellow, green and blue corresponding to V_T/f_P values.

Table 1

Demographics and Smoking Characteristics of Nonsmoker Controls and Smokers during Acute and Prolonged Abstinence

Characteristics	Nonsmoker Control (n=20)	Smoker 1 day (n=7)	Smoker 1 week (n=17)	Smoker 2 weeks (n=7)	Smoker 4 weeks (n=11)	Smoker 6-12 weeks (n=6)
Age, years	42.4±9.8	42.7±8.2	41.7±9.4	43.4±11.7	43.9±7.5	38.7±7.0
FTND	0	5.9±2.8	5.5±2.6	5.4+2.8	4.9±2.6	6.8±2.3
Cigarettes/Day	0	19.9±10.2	19.7±8.5	22.7±7.9	14.7±3.6	20.3±10.3
Years Smoked	0	21.8±6.3	19.9±7.6	22.0±7.4	20.8±7.7	21.2±6.6
Plasma Cotinine (ng/mL)	<15	370.3±185.6	21.1±46.3	<15	<15	<15
Plasma Nicotine (ng/mL)	<4	3.6±5.2	<4	<4	<4	<4
Carbon Monoxide	2.2±3.1	12.0±7.2	3.2±2.4	2.9±1.9	2.9±2.2	4.2±3.3

Data are presented as mean ± SD.

Table 2

Blood [^{123}I]5-IA Measures of Nonsmoker Controls and Smokers during Acute and Prolonged Abstinence

Measure	Nonsmoker Control (n=20)	Smoker 1 day (n=7)	Smoker 1 week (n=17)	Smoker 2 weeks (n=7)	Smoker 4 weeks (n=11)	Smoker 6-12 weeks (n=6)
Total Parent (kBq/mL)	0.333±0.103	0.236±0.032*	0.302±0.081	0.310±0.088	0.305±0.089	0.281±0.088
Free Parent (kBq/mL)	0.112±0.036	0.080±0.018	0.108±0.032	0.123±0.043	0.100±0.024	0.101±0.045
f_p	0.342±0.039	0.334±0.073	0.358±0.049	0.398±0.056*	0.337±0.052	0.356±0.052

Data are presented as mean ± SD.

* Indicates significantly different after Bonferroni's correction from nonsmoker controls.

Table 3

β_2*-nAChR availability (V_T/f_P) throughout the Brain in Nonsmoker Controls and Smokers during Acute and Prolonged Abstinence

	Nonsmoker	Smoker 1 day			Smoker 1 week			Smoker 2 weeks			Smoker 4 weeks			Smoker 6-12 weeks		
Region	**V_T/f_P***	**V_T/f_P**	**% Diff****	***P*†**	**V_T/f_P**	**% Diff**	***P***	**V_T/f_P**	**% Diff**	***P***	**V_T/f_P**	**% Diff**	***P***	**V_T/f_P**	**% Diff**	***P***
Thalamus	130.6±22.2	96.9±24.4	-26%	.002	138.5±25.9	+6%	.322	139.2±35.6	+7%	.461	133.8±19.3	+2%	.694	115.2±19.7	-12%	.140
Striatum	70.0±11.0	66.5±13.1	-5%	.492	85.1±16.8	+22%	.002	80.9±18.2	+16%	.070	79.5±12.6	+14%	.037	71.7±13.3	+2%	.760
Parietal Cortex	46.0±7.8	44.4±7.5	-3%	.659	56.6±9.9	+23%	.001	56.4±16.2	+23%	.144	52.3±10.9	+14%	.071	44.5±9.6	-3%	.714
Frontal Cortex	51.0±8.6	47.9±10.8	-6%	.450	61.8±13.0	+21%	.005	60.9±15.9	+19%	.158	58.3±13.8	+14%	.078	49.1±12.9	-4%	.685
Anterior Cingulate	52.5±7.8	54.8±7.7	+4%	.511	67.5±12.2	+29%	<.001	63.9±15.1	+22%	.098	61.4±10.0	+17%	.010	55.3±11.6	+5%	.500
Temporal Cortex	57.7±8.7	57.4±10.4	-1%	.941	70.2±12.8	+22%	.001	67.5±15.3	+17%	.048	66.1±9.7	+15%	.021	58.2±11.6	+1%	.908
Occipital Cortex	54.2±7.7	56.3±11.7	-4%	.587	68.6±13.4	+27%	.001	66.3±14.3	+22%	.067	64.1±10.3	+18%	.005	55.5±13.9	+2%	.771
Cerebellum	62.9±8.5	59.5±18.4	-5%	.649	77.9±15.5	+24%	.002	74.0±17.0	+18%	.143	73.2±11.5	+16%	.009	60.6±13.5	-4%	.605

* V_T/f_P equals regional [^{123}I]5-IA uptake (kBq/cc)/free plasma parent (kBq/mL);

** Percent Difference from Nonsmokers = ((Smoker-Nonsmoker)/Nonsmoker)*100;

† p-values <0.0625 are significant after Bonferroni's correction

Table 4

Clinical Characteristics of Smokers during Acute and Prolonged Abstinence

Measure	Smoker 1 day (n=7)	Smoker 1 week (n=17)	Smoker 2 weeks (n=7)	Smoker 4 weeks (n=11)	Smoker 6-12 weeks (n=6)
MNWS	11.2±3.2	10.5±2.5	11.7±3.2	12.8±2.8	12.6±3.3
QSU Intent	26.0±2.7*	16.3±2.1	16.0±2.7	12.3±2.3	14.8±2.8
QSU Relief	20.1±2.4*	10.6±1.8	10.4±2.4	10.2±2.0	8.3±2.5

Data are presented as least-squares mean ± SE.

* Indicates significantly different after Bonferroni's correction compared to smokers at other time points.

Table 5

ations between β_2^*-nAChR Availability and Baseline Assessments

	Smoker 1 day (n=7)			Smoker 1 week (n=10)**			Smoker 2 weeks (n=7)†			Smoker 4 weeks (n=11)			Smoker 6-12 weeks (n=6)†		
	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief
s	.16	-.90*	-.56	.06	.29	.17	-.31	-	-	.22	-.34	-.05	.83	-	-
	-.05	-.76	-.56	-.01	.16	.17	-.31	-	-	.17	-.29	-.29	.43	-	-
tex	.27	-.88*	-.56	-.15	.45	-.20	.02	-	-	-.03	-.49	-.67	.66	-	-
ex	.09	-.78	-.49	.06	-.31	-.18	-.31	-	-	.08	-.53	-.43	.89	-	-
ulate	-.22	-.63	-.51	.02	.46	.18	-.20	-	-	.17	-.35	-.39	.77	-	-
rtex	-.34	-.67	-.63	.07	.46	.23	-.34	-	-	.25	-.37	-.25	.89	-	-
rtex	-.11	-.67	-.32	.22	.12	.59	-.45	-	-	.48	-.73	.05	.89	-	-
n	-.02	-.85	-.52	.11	-.09	.12	-.34	-	-	.64	-.61	.21	.77	-	-

ed)

QSU baseline data, correlations were only conducted in 10 of 17 subjects.

QSU baseline data, Spearman's correlations could not be conducted.

Table 6

ations between β_2^*-nAChR Availability and Assessments on Day of Scan

	Smoker 1 day (n=7)			Smoker 1 week (n=17)			Smoker 2 weeks (n=7)			Smoker 4 weeks (n=11)			Smoker 6-12 weeks (n=6)		
	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief	MNWS	QSU Intent	QSU Relief
s	-.72	-.14	-.52	-.12	.21	-.03	-.18	.06	-.26	-.06	.36	.54	.09	.44	.40
	-.52	.18	-.67	-.31	.03	-.15	-.18	0.6	-.26	-.41	.15	.32	.03	.79	.39
tex	-.45	.14	-.36	-.28	-.10	-.36	.25	.46	.10	-.60	-.19	.23	.03	.18	.13
tex	-.52	.00	-.36	-.36	.04	-.17	-.18	.06	-.26	-.63	-.16	.26	-.03	.44	.39
ulate	-.31	.21	-.60	-.01	.01	-.08	.07	.11	-.12	-.34	.10	.19	-.09	.56	.39
rtex	-.56	.00	-.74	.04	.12	-.08	-.04	.11	-.12	-.33	.18	.34	-.03	.44	.39
rtex	-.38	-.25	-.34	.01	.16	.08	-.07	.00	-.02	-.25	.33	.41	-.03	.44	.39
n	-.74	-.32	-.61	-.23	.12	-.05	-.07	-.27	.04	.14	.74*	.74*	-.09	.56	.39

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