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ARTICLE

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Cholinergic system adaptations are associated with cognitive function in people recently abstinent from smoking: a (-)-[^{18}F] flubatine PET study

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The cholinergic system is a critical mediator of cognition in animals. People who smoke cigarettes exhibit cognitive deficits, especially during quit attempts. Few studies jointly examine the cholinergic system and cognition in people while trying to quit smoking. We used positron emission tomography (PET) brain imaging with the β_2-subunit containing nicotinic acetylcholine receptor (β_2*-nAChR) partial agonist radioligand (-)-[^{18}F]flubatine and the acetylcholinesterase inhibitor physostigmine to jointly examine the cholinergic system, smoking status, and cognition. (-)-[^{18}F]Flubatine scans and cognitive data were acquired from twenty people who recently stopped smoking cigarettes (aged 38 ± 11 years; 6 female, 14 male; abstinent 7 ± 1 days) and 27 people who never smoked cigarettes (aged 29 ± 8 years; 11 female, 16 male). A subset of fifteen recently abstinent smokers and 21 never smokers received a mid-scan physostigmine challenge to increase acetylcholine levels. Regional volume of distribution (V_T) was estimated with equilibrium analysis at "baseline" and post-physostigmine. Participants completed a cognitive battery prior to (-)-[^{18}F]flubatine injection and physostigmine administration assessing executive function (Groton Maze Learning test), verbal learning (International Shopping List test), and working memory (One Back test). Physostigmine significantly decreased cortical (-)-[^{18}F]flubatine V_T, consistent with increased cortical acetylcholine levels reducing the number of β_2*-nAChR sites available for (-)-[^{18}F]flubatine binding, at comparable magnitudes across groups (p values < 0.05). A larger magnitude of physostigmine-induced decrease in (-)-[^{18}F]flubatine V_T was significantly associated with worse executive function in people who recently stopped smoking (p values < 0.05). These findings underscore the role of the cholinergic system in early smoking cessation and highlight the importance of neuroscience-informed treatment strategies.

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INTRODUCTION

Tobacco cigarette smoking remains a leading cause of preventable disease and death [1]. Although 55% of people who smoked tobacco in 2018 made an attempt to quit, only 8% reported successful attempts [2]. Relapse during early quit attempts is often a consequence of smoking to relieve adverse effects of early abstinence, including cognitive deficits (e.g., adaptations in attention, inhibitory control, memory, etc.) [3–5]. Notably, cognitive dysfunction during quit attempts is predictive of poorer long-term smoking cessation success [6]. It is critical therefore to examine the brain during early abstinence and identify neural mechanisms associated with cognitive function to inform smoking cessation treatment strategies.

The cholinergic system is a critical mediator of cognitive function in animal models. Nicotine, a primary addictive constituent of cigarettes, can modulate this relationship in complex ways [7]. People who smoke cigarettes exhibit cognitive deficits compared to people who never smoked cigarettes and demonstrate worsened cognitive performance in early nicotine withdrawal [5, 8]. In the human brain, the frontal cortex is an important hub for cognitive function [9–11]. Aside from work on cognitive dysfunction in neurodegenerative disorders, there is a surprising dearth of molecular imaging studies that investigate relationships between the cholinergic system and cognition in people [12]. It is clinically imperative to examine this relationship to better understand how cholinergic interventions may influence cognition during smoking cessation.

Nicotine enacts its reinforcing properties by binding to and activating β_2-subunit containing nicotinic acetylcholine receptors (β_2*-nAChRs) on ventral tegmental area neurons to acutely facilitate downstream dopamine release in the ventral striatum [13–15]. Nicotine also binds to β_2*-nAChRs, and other related subtypes, across various subcortical and cortical brain regions [16]. Animal studies demonstrate that chronic nicotine can saturate, desensitize, and elevate the number of β_2*-nAChRs [17, 18], and thus may disrupt activity of the endogenous β_2*-nAChR ligand

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Table 1. Subject and (-)-[^{18}F]flubatine Injection Information.

	People who never smoked cigarettes ($n = 27$)	People who smoke cigarettes ($n = 20$)
Female, Male (*n*)	11 F, 16 M	6 F, 14 M
Age (years)	29 ± 8	38 ± 11
Race and ethnicity	2 A, 1 BH, 10 BNH, 5 HL, 1 M, 2 WH, 6 WNH	1 A, 6 BNH, 1 HL, 1 M, 1 WH, 10 WNH
Body mass index (kg/m^2)[a]	25.8 ± 4.0	28.2 ± 4.7
FTCD[a]	–	5 ± 3
Cigarettes smoked per day[a]	–	16 ± 7
Years smoked[a]	–	16 ± 11
Pack-years smoking[a]	–	14 ± 12
Days abstinent on PET scan day	–	7 ± 1
(-)-[^{18}F]flubatine injected MBq/kg	3.4 ± 1.1	3.0 ± 0.8
(-)-[^{18}F]flubatine injected μg/kg	0.001 ± 0.001	0.001 ± 0.001

Data presented as mean ± one standard deviation.
[a]Smoking characteristics collected at intake prior to abstinence.
n sample size, *F* female, *M* male, *FTCD* Fagerström Test for Cigarette Dependence, *A* Asian, *BH* Black Hispanic, *BNH* Black not Hispanic, *HL* Hispanic and/or Latino, *M* multiracial and/or multiethnic, *WH* White Hispanic, *WNH* White not Hispanic.

acetylcholine [19]. Human in vivo molecular imaging studies that utilize single photon emission computed tomography (SPECT) or positron emission tomography (PET) reveal that people who smoke cigarettes exhibit higher β_2*-nAChR availability than people who never smoked cigarettes [20–24], and that higher upregulation of β_2*-nAChR availability is predictive of worse smoking cessation outcomes [25, 26]. As cognitive dysfunction and upregulated β_2*-nAChR availability are each independently predictive of smoking cessation success, examination of links between the cholinergic system and cognition may lend insight into treatment strategies that decrease relapse likelihood [4, 25, 26].

In this study, PET imaging was conducted with the β_2*-nAChR-specific partial agonist radioligand (-)-[^{18}F]flubatine and combined with administration of physostigmine. Physostigmine inhibits acetylcholinesterase function, thereby elevating extracellular acetylcholine levels. Physostigmine administration in people reduces radiotracer V_T estimates as shown in previous studies [27–29], likely reflecting increased acetylcholine levels that compete with the radiotracer to reduce the number of β_2*-nAChRs available for radiotracer binding. This work leveraged this study design to investigate relationships between cholinergic system function, smoking status, and cognition. We hypothesized that people who recently stopped smoking would exhibit altered physostigmine-induced reductions in (-)-[^{18}F]flubatine V_T compared to people who never smoked, and the magnitude of these changes in the frontal cortex would be associated with cognitive function in early abstinence.

PATIENTS AND METHODS

Study participants

Twenty people who recently stopped smoking cigarettes for 7 ± 1 days (abstinent smokers, AS; aged 38 ± 11 years; 6 female, 14 male; 10 individuals with some college education or higher) and 27 people who never smoked cigarettes (never smokers, NS; aged 29 ± 8 years; 11 female, 16 male; 23 individuals with some college education or higher) participated in one (-)-[^{18}F]flubatine PET scan (data presented as mean ± standard deviation) (Table 1). Six never smokers and 7 people who recently stopped smoking reported cannabis use. Although people who recently stopped smoking were older and had higher body mass index (BMI) values than people who never smoked, these factors did not significantly influence the effect of smoking status on baseline β_2*-nAChR availability (Supplementary Information). A subset of 15 people who recently stopped smoking cigarettes and 21 people who never smoked cigarettes also received physostigmine during the (-)-[^{18}F]flubatine PET scan. Smokers received smoking cessation counselling and were imaged one week after their last cigarette. Structural magnetic resonance imaging (MRI) scans were acquired for delineation of regions of interest (ROIs). Cigarette use history was obtained prior to participants quitting smoking. Cognitive measures were collected early on PET scan day, prior to the (-)-[^{18}F]flubatine injection and subsequent administration of physostigmine.

Participants were recruited from the community and administered the Structured Clinical Interview for DSM-IV or DSM-5 and a medical exam. Participants provided demographic information, substance use and psychiatric history, electrocardiogram, and urine samples for toxicology and pregnancy testing. Inclusion criteria for people who smoke cigarettes included smoking ≥5 cigarettes/day for ≥1 year, recent smoke inhalation and nicotine exposure indicated by carbon monoxide (CO) breath output >11 ppm [30] and urine cotinine (nicotine metabolite) concentration >150 ng/mL, respectively, and agreement to quit smoking for up to two weeks. Inclusion criteria for people who never smoked cigarettes included <100 cigarettes smoked in lifetime, CO < 8 ppm, and urine cotinine 0–30 ng/mL. Participants who never smoked cigarettes reported no other use of nicotine-containing products. Exclusion criteria for all participants included presence or history of serious medical or neurological illness, current major psychiatric diagnosis or non-nicotine substance use disorder, anxiolytic or antidepressant use, uncontrolled hypertension or electrocardiogram abnormalities, positive drug toxicology except for 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (11-COOH-THC) reflecting recent cannabis use, MRI contraindications, and pregnancy or lactating. Participants provided written informed consent after review of the study protocols, approved by the Yale-New Haven Hospital Radiation Safety Committee and the Yale University Human Investigation Committee. This study was registered with ClinicalTrials.gov (Identifier: NCT02008292), and the analyses presented pertain to the registered study goal to examine the cholinergic system in people who either smoke or never smoked cigarettes.

Cigarette use characteristics, cessation counselling, and cognitive measures

Prior to quitting, people who smoked reported 16 ± 7 cigarettes per day for 16 ± 11 years or 14 ± 12 pack-years smoking, and moderate cigarette dependence (Fagerström Test for Cigarette Dependence, or FTCD, total score: 5.4 ± 2.5) [31, 32]. They also received daily cessation counselling for one week prior to PET scanning with the Smoking Cessation Clinical Practice Guideline [33] and were compensated $10 per daily visit contingent on breath CO < 11 ppm. On PET scan day, urine cotinine levels were acquired to confirm abstinence. Six people who recently stopped smoking had urine cotinine levels >600 ng/mL on scan day, indicative of a 'lapse' in smoking abstinence. Individuals with evidence of a "lapse" are referred to as "high-cotinine" participants, while remaining participants who recently stopped smoking for a least one week are referred to as "low-cotinine" participants.

On PET scan day, prior to (-)-[^{18}F]flubatine injection and physostigmine administration, all participants completed a computerized cognitive battery (CogState Ltd.), except one person who recently stopped smoking was not able to complete the battery due to time constraints. A subset of three cognitive tasks were analyzed based on relevance in studies of cognition in cigarette use and withdrawal [3–5]. The three tasks were the Groton Maze Learning Test (executive function), the International Shopping List Test (verbal learning), and the One Back Test (working memory) [34]. One additional person who recently stopped smoking did not complete the working memory task. Statistical outliers in cognitive performance were defined as ≥1.5 standard deviations from the nearest neighbouring data point, were excluded from analyses, and are noted in the results.

PET and MR scanning and physostigmine administration

T1-weighted structural magnetic resonance (MR) scans were acquired from participants prior to PET scanning with a Siemens 3.0 T scanner and a 64-channel head coil to yield a high-resolution anatomical map for PET data coregistration. A sagittal gradient-echo MPRAGE sequence (FOV: 256 × 256 mm^2, 176 slices (1 mm thickness), TE: 2.77 ms, TR: 2530 ms, TI: 1100 ms, FA: 7°) was used.

On PET scanning days, CO breath output and urine cotinine levels were measured in people who recently stopped smoking to confirm abstinence from cigarettes. Negative pregnancy and urine toxicology (except 11-COOH-THC) results were required to scan. PET scan data were acquired on the High Resolution Research Tomograph (Siemens/CTI). Participants wore a Vicra cap for motion correction and underwent a 6-min transmission scan for attenuation correction. (-)-[^{18}F]Flubatine was synthesized at high molar activity as previously described [35] and administered with a computer-controlled pump (Harvard Apparatus) as a bolus plus constant infusion (B/I) (K_{Bol} = 360 min; NS: 248.6 ± 56.6 MBq, 0.09 ± 0.08 μg; AS: 258.9 ± 54.6 MBq, 0.09 ± 0.09 μg) for at least 120 min for baseline measurements, and up to 240 min for individuals who received a physostigmine challenge. Six people who never smoked (3 female, 3 male) and 5 people who recently stopped smoking (3 female, 2 male) did not participate in the physostigmine challenge due to a physostigmine supply shortage (n = 9), a sinus arrhythmia (n = 1), and an arterial line malfunction (n = 1). The remaining 21 people who never smoked and 15 people who recently stopped smoking received physostigmine. First, to reduce nausea symptoms 200 μg glycopyrrolate was administered intravenously over 1 min starting at 120 min of (-)-[^{18}F]flubatine infusion. Next, 1.5 mg physostigmine was administered as a bolus plus infusion over 60 min, starting after 125 min of (-)-[^{18}F]flubatine infusion as previously described [27]. There were no significant differences between participant groups in injected (-)-[^{18}F]flubatine radioactivity or mass, independent of or relative to body weight. Emission data were acquired either 90–240 min or 90–120 min after B/I initiation for individuals who did or did not receive physostigmine, respectively. Arterial blood was collected in either 15-min intervals 90–240 min, or 10-min intervals 90–120 min, after (-)-[^{18}F]flubatine injection to measure the metabolite-corrected (-)-[^{18}F]flubatine parent input function in individuals with or without physostigmine administration, respectively, as previously described [27].

PET image processing

List-mode PET data were binned into 5 min frames and reconstructed with corrections for normalization, attenuation, randoms, scatter, deadtime, and head motion using the MOLAR algorithm [36] and an optical detector (Vicra, NDI Systems). Participants' MR scans were transformed nonlinearly into Montreal Neurological Imaging template space, co-registered to early summed kinetic PET images generated with Vicra motion-corrected and transmission data, and parcellated into ROIs with Anatomic Automatic Labelling [37]. (-)-[^{18}F]Flubatine PET time-activity curves were generated for a selection of grey matter subcortical and cortical cerebral regions involved in tobacco use and cognition and/or with measurable [^{18}F]flubatine activity [27], including the caudate, frontal cortex, hippocampus, insula, occipital cortex, parietal cortex, putamen, and temporal cortex.

PET data analysis

(-)-[^{18}F]Flubatine PET data were modelled with an equilibrium analysis to estimate volume of distribution (V_T), the ratio of mean (-)-[^{18}F]flubatine activity in tissue to parent (-)-[^{18}F]flubatine activity in arterial plasma at equilibrium, proportional to β_2*-nAChR availability [27, 38–40]. Baseline and post-physostigmine V_T estimates were estimated during the established equilibrium periods of 90–120 min and 180–210 min post-injection, respectively [27]. V_T estimates additionally featured a tissue clearance correction to account for potential inter-subject or -group variance in (-)-[^{18}F]flubatine kinetics or metabolism and to reduce consequent bias in V_T estimation [41]. The free plasma fraction of (-)-[^{18}F]flubatine (f_P) was only measured prior to physostigmine administration, and results from the baseline-only PET analyses were comparable with or without correction for f_P. Therefore, regional V_T was the selected PET outcome measure for both baseline-only and physostigmine-related analyses. Regional baseline (-)-[^{18}F]flubatine V_T estimates with and without correction for f_P are summarized in Supplementary Table S1.

Physostigmine, as an acetylcholinesterase inhibitor, inhibits the breakdown of acetylcholine and thus increases levels of acetylcholine. Physostigmine administration in people reduces radiotracer V_T estimates as shown in previous studies [27–29], likely reflecting increased acetylcholine levels that compete with the radiotracer to reduce the number of β_2*-nAChRs available for radiotracer binding. Consequently, physostigmine-induced percent change in regional (-)-[^{18}F]flubatine V_T was calculated as [1-($V_{T:Physostigmine}$/$V_{T:Baseline}$)]*100 as an indirect measure of elevation in acetylcholine levels, such that higher, more positive values reflect higher elevation of acetylcholine levels after physostigmine.

Statistical analyses

Statistical analyses were performed with R (The R Foundation for Statistical Computing). Overall, two-sample tests (e.g., t tests, Welch's t tests, or Wilcoxon rank sum tests) were selected according to data normality and variance and were conducted to evaluate differences between people who never smoked and people who recently stopped smoking in demographics, PET injection information, PET outcome measures, and PET scan day cognitive performance. Pearson's correlation coefficients (r) were computed to assess relationships between PET outcome measures and cognitive performance metrics. All associative analyses focused on people who recently stopped smoking a priori as the clinical population of interest. A two-tailed $\alpha = 0.05$ threshold was used to determine significance across analyses, and p values were adjusted with a false discovery rate (FDR) correction for multiple comparisons when appropriate and as described below.

Primary analyses examined the magnitude of, and group difference in, physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T across regions. Statistical significance was FDR-corrected for multiple regions of interest (excluding the frontal cortex as our a priori region of interest; n = 7) within each diagnostic group. Primary analyses also examined relationships between cognitive performance metrics and frontal cortex estimates of physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T in people who recently stopped smoking. Statistical significance results were FDR-corrected for multiple cognitive tasks (n = 3). Secondary analyses were conducted to investigate if this relationship was specific to the frontal cortex or specific to diagnosis. Here, associations between cognitive performance metrics and physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T in the other 7 ROIs were computed, in both people who never smoked and people who recently stopped smoking. Statistical significance results for these secondary analyses were FDR-corrected for multiple cognitive tasks (n = 3).

Although primary analyses focused on physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T, there was potential for insights to be gleaned from analysis of baseline (-)-[^{18}F]flubatine V_T in light of previous work examining the β_2*-nAChR system [20–26]. To build upon this work, secondary analyses were conducted to examine group differences in baseline (-)-[^{18}F]flubatine V_T and relationships of baseline (-)-[^{18}F]flubatine V_T with cognitive performance. Statistical significance results were FDR-corrected in the same manner as described for the physostigmine-related analyses. High-cotinine people who recently stopped smoking were excluded from baseline-related analyses, as nicotine was not cleared from the system and therefore may have affected (-)-[^{18}F]flubatine binding to β_2*-nAChR sites relative to low-cotinine people who recently stopped smoking [22]. High-cotinine participants were included for physostigmine-related analyses because the outcome of interest was a within-subject change, and baseline (-)-[^{18}F]flubatine V_T was not significantly associated with physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T (Supplementary Table S2).

RESULTS

Physostigmine administration lowers β_2*-nAChR availability across groups

(-)-[^{18}F]Flubatine V_T estimates were significantly lower post-physostigmine than at baseline in the a priori frontal cortex of

people who never smoked cigarettes (NS, or 'never smokers') ($p_{uncorr} < 0.001$), and "trend"-level lower post-physostigmine than at baseline in the frontal cortex of people who recently stopped smoking cigarettes (AS, or "abstinent smokers") ($p_{uncorr} = 0.068$) (Fig. 1, Table 2). (-)-[^{18}F]Flubatine V_T estimates were significantly lower post-physostigmine than at baseline in both NS and AS in the occipital (NS: $p_{corr} = 0.006$, AS: $p_{corr} = 0.04$) and parietal (NS: $p_{corr} = 0.004$, AS: $p_{corr} = 0.047$) cortices. (-)-[^{18}F]Flubatine V_T estimates were significantly lower post-physostigmine than at baseline in the temporal cortex of NS ($p_{corr} = 0.004$), and "trend"-level lower post-physostigmine than at baseline in the temporal cortex of AS ($p_{corr} = 0.076$) (Fig. 1, Table 2). The magnitude of physostigmine's effect on (-)-[^{18}F]flubatine V_T, calculated as the physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T (% ΔV_T), was on average lower in AS compared to NS, but group differences were not significantly different in any region (p_{uncorr}-values>0.05) (Fig. 1, Table 2). Additionally, no significant differences in physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T were detected between high-cotinine and low-cotinine recently abstinent smokers (Supplementary Table S3).

Worse executive function is associated with higher physostigmine-induced elevation of acetylcholine levels in people who recently stopped smoking

The number of errors made during the executive function 'Groton Maze Learning' task was not significantly different between groups on (-)-[^{18}F]flubatine scan day, when including all task-completers (NS: 48 ± 15 errors; AS: 57 ± 32 errors; NS vs. AS $p_{corr} = 0.46$) or only task-completers who received physostigmine (NS: 49 ± 15 errors; AS: 48 ± 21 errors; NS vs. AS $p_{corr} = 0.90$) (Fig. 2). A higher number of errors made (i.e., worse executive function performance) was significantly associated with higher physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T estimates in the frontal cortex of people who recently stopped smoking ($r = 0.73$, $p_{corr} = 0.009$) (Fig. 2), and not in people who never smoked ($r = 0.16$, $p_{corr} = 0.73$). Significant associations were also observed in the caudate, insula, and occipital and temporal cortices of people who recently stopped smoking (Supplementary Table S4). There were no other significant associations between physostigmine-induced changes in (-)-[^{18}F]flubatine V_T and cognitive performance.

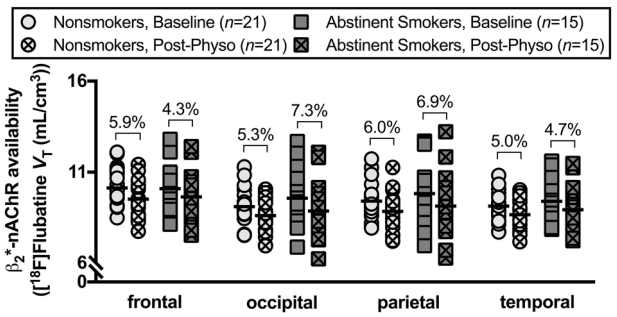

Fig. 1 Physostigmine-induced percent change in β_2*-nAChR availability in people who never smoked cigarettes and people who recently stopped smoking cigarettes. In the frontal cortex, physostigmine significantly decreases estimates of β_2*-nAChR availability ((-)-[^{18}F]flubatine V_T) in people who never smoked cigarettes (light circles) ($p_{uncorr} < 0.001$) and 'trend'-level decreases estimates in people who recently stopped smoking cigarettes (dark squares) ($p_{uncorr} = 0.068$). Physostigmine significantly decreases estimates of β_2*-nAChR availability in the occipital, parietal, and temporal cortices of people who never smoked cigarettes (p_{corr}-values<0.05), and in the occipital and parietal cortices of people who recently stopped smoking cigarettes (p_{corr}-values<0.05). The magnitude of physostigmine-induced percent change in β_2*-nAChR availability is not significantly different between groups in any regions. Mean V_T (bars) and group mean values of physostigmine-induced percent change in β_2*-nAChR availability (percentages) are presented. *n*: sample size.

Worse working memory is associated with higher β_2*-nAChR availability in people who recently stopped smoking

Baseline (-)-[^{18}F]Flubatine V_T estimates were significantly higher in low-cotinine people who recently stopped smoking compared to people who never smoked in all regions (p_{corr}-values<0.05) except the caudate ($p_{corr} = 0.09$) and hippocampus ($p_{corr} = 0.15$) (Supplementary Table S5). Performance speed on the working memory "One Back" task was not significantly different between groups on (-)-[^{18}F]flubatine scan day, when including all task-completers recently abstinent from smoking (NS: 2.9 ± 0.1 $\log_{10}$ ms; AS: 2.9 ± 0.1 $\log_{10}$ ms; NS vs. AS $p_{corr} = 0.46$) or only task-completers who were low-cotinine and recently abstinent from smoking (NS: 2.9 ± 0.1 $\log_{10}$ ms; AS: 2.9 ± 0.1 $\log_{10}$ ms; NS vs. AS $p_{corr} = 0.74$) (Fig. 3). Slower performance speed (i.e., worse performance) was significantly associated with higher baseline (-)-[^{18}F]flubatine V_T estimates (i.e., higher β_2*-nAChR availability) in the frontal cortex of low-cotinine people who recently stopped smoking ($r = 0.59$, $p_{uncorr} = 0.045$) (Fig. 3), and not in people who never smoked ($r = 0.19$, $p_{uncorr} = 0.33$), without correction for multiple cognitive tasks. After statistical correction for 3 cognitive tasks, the relationship observed in low-cotinine abstinent smokers was not significant ($r = 0.59$, $p_{corr} = 0.14$). Significant associations were also observed in the hippocampus and putamen of low-cotinine people who recently stopped smoking (Supplementary Table S6). There were no other significant associations between (-)-[^{18}F]flubatine V_T and cognitive performance.

DISCUSSION

This study demonstrated that in people who recently stopped smoking, a larger reduction in cortical (-)-[^{18}F]flubatine V_T after physostigmine is significantly associated with worse executive function. These results suggest a link of cholinergic function with cognitive performance in people who recently stopped smoking. Given that cognitive deficits in early withdrawal can precipitate smoking relapse, these data highlight the cholinergic system as a target for early quit attempts to mitigate cognitive changes and promote long-term cessation.

Physostigmine administration in this study significantly decreased cortical (-)-[^{18}F]flubatine V_T, consistent with increased cortical acetylcholine levels reducing the number of β_2*-nAChR sites available for (-)-[^{18}F]flubatine binding, at comparable magnitudes across participant groups. This builds on previous work that established the physostigmine imaging protocol [27–29] by innovatively extending this paradigm to a larger sample with a clinical focus on people who recently stopped smoking. Nicotine continuously saturates and desensitizes β_2*-nAChRs in the brains of people who smoke [42], which can disrupt endogenous acetylcholine activity long-term and potentially contribute to cognitive dysfunction in early cigarette withdrawal. It was therefore surprising to not detect a significant difference between groups in physostigmine-induced elevation of acetylcholine levels. Detection of group differences may have been precluded by the small response to physostigmine (approximate average of 4–7% change in cortical β_2*-nAChR availability) across groups as measured with (-)-[^{18}F]flubatine V_T. However, if an estimated (-)-[^{18}F]flubatine V_{ND} of ~6.5 mL/cm^3 is assumed uniform throughout the brain [43, 44], a 4-7% reduction in (-)-[^{18}F]flubatine V_T translates to 10–20% reduction in (-)-[^{18}F]flubatine BP_{ND}, comparable to differences observed with amphetamine challenge [45, 46]. The physostigmine effects on (-)-[^{18}F]flubatine V_T were most robust in cortical brain regions but more muted in hippocampus and striatum (see Table 2). Interestingly, this regional pattern matches regions with relatively higher basal levels of acetylcholine that occupy a larger fraction of β_2*-nAChRs

Table 2. Effects of physostigmine on β_2*-nAChR availability ((-)-[^{18}F]flubatine V_T) in people who never smoked and people who recently stopped smoking.

	People who never smoked ($n = 21$)			People who recently stopped smoking ($n = 15$)			Two-sample tests (p values)		
	'pre' V_T (mL/cm^3)	'post' V_T (mL/cm^3)	% ΔV_T (%)	'pre' V_T (mL/cm^3)	'post' V_T (mL/cm^3)	%ΔV_T (%)	NS: 'pre' vs. 'post'[a]	AS: 'pre' vs. 'post'[a]	%ΔV_T: NS vs. AS[b]
caudate	10.0 ± 1.1	10.0 ± 1.3	0.2 ± 8.1	9.9 ± 1.5	9.7 ± 1.5	2.0 ± 7.8	0.964	0.489	0.511
frontal cortex	10.1 ± 1.0	9.5 ± 1.0	5.9 ± 6.5	10.1 ± 1.4	9.6 ± 1.4	4.3 ± 8.4	***0.000	0.068	0.522
hippocampus	9.7 ± 0.7	9.8 ± 0.9	–1.1 ± 7.3	9.3 ± 1.2	9.3 ± 1.2	–0.5 ± 9.2	0.732	0.964	0.831
insula	9.7 ± 0.8	9.5 ± 0.9	2.2 ± 7.0	9.5 ± 1.1	9.2 ± 1.1	3.3 ± 8.3	0.278	0.235	0.690
occipital cortex	9.1 ± 1.0	8.6 ± 0.9	5.3 ± 6.8	9.6 ± 1.6	8.9 ± 1.6	7.3 ± 7.9	*0.012	*0.021	0.374
parietal cortex	9.4 ± 1.0	8.8 ± 1.0	6.0 ± 6.5	9.8 ± 1.8	9.1 ± 1.9	6.9 ± 8.9	**0.007	*0.038	0.899
putamen	10.7 ± 0.8	10.7 ± 1.1	0.1 ± 7.0	10.6 ± 1.2	10.6 ± 1.4	0.2 ± 10.6	0.964	0.964	0.974
temporal cortex	9.1 ± 0.8	8.7 ± 0.8	5.0 ± 6.0	9.4 ± 1.3	8.9 ± 1.3	4.7 ± 7.7	**0.007	0.076	0.892

(-)-[^{18}F]Flubatine V_T estimates pre- and post-physostigmine, as well as physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T estimates (%ΔV_T), per region labelled in the left column are presented as group mean ± one standard deviation for people who never smoked and people who recently stopped smoking. n: sample size.

[a]FDR-corrected p value results (except in a priori ROI frontal cortex, uncorrected) from two-sample tests described in subheadings are presented.

[b]Uncorrected p value results are presented. *$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$.

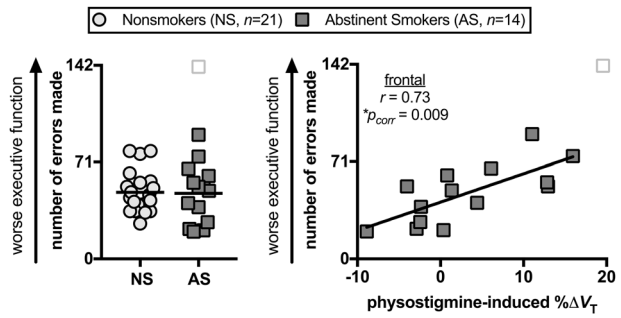

Fig. 2 Executive function and physostigmine-induced percent change in β_2*-nAChR availability in people who recently stopped smoking cigarettes. People who recently stopped smoking cigarettes ('AS,' or 'abstinent smokers,' dark squares) do not significantly differ from people who never smoked cigarettes ('NS,' light circles) in number of errors made on the 'Groton Maze Learning' executive function task (NS: 49 ± 15 errors; AS: 48 ± 21 errors; $p_{corr} = 0.90$) (left panel). Higher number of errors made (i.e., worse executive function) is associated with higher physostigmine-induced percent change in (-)-[^{18}F]flubatine V_T (%ΔV_T) estimates (i.e., higher magnitude increase in acetylcholine levels) in the frontal cortex of people who recently stopped smoking ($r = 0.73$, $p_{corr} = 0.009$) (right panel). Plots include task-completing individuals who received physostigmine. The plots are generated with distributions, sample sizes, and statistical analyses which exclude one statistical outlier who recently stopped smoking, included as a hollow marker for transparency. n: sample size. Horizontal bars (left panel): group mean task performance speed. r: Pearson correlation coefficient. Significance: *$p < 0.05$.

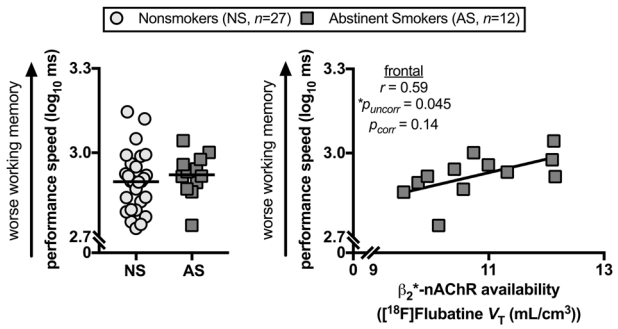

Fig. 3 Working memory and β_2*-nAChR availability in low-cotinine people who recently stopped smoking cigarettes. Low-cotinine people who recently stopped smoking cigarettes ('AS,' or 'abstinent smokers,' dark squares) do not significantly differ from people who never smoked cigarettes ('NS,' light circles) in performance speed on the 'One-Back' working memory task (NS: 2.9 ± 0.1 $\log_{10}$ ms; AS: 2.9 ± 0.1 $\log_{10}$ ms; $p = 0.49$) (left panel). Slower task performance (i.e., worse working memory) is associated with higher (-)-[^{18}F]flubatine V_T estimates (i.e., higher β_2*-nAChR availability) in the frontal cortex of low-cotinine people who recently stopped smoking ($r = 0.59$, $p_{uncorr} = 0.045$), without correction for multiple cognitive tasks (right panel). This relationship becomes statistically non-significant after correction for multiple cognitive tasks ($p_{corr} = 0.14$). n: sample size. Horizontal bars (left panel): group mean task performance speed. r: Pearson correlation coefficient. Significance: *$p < 0.05$.

[47, 48], potentially altering the physostigmine effect in these regions. This study also reproduced results from prior studies that demonstrated higher global baseline β_2*-nAChR availability in people who recently stopped smoking than in people who never smoked [20–24]. Since β_2*-nAChR upregulation is detected at one week of abstinence and most individuals resume use within two weeks of quit attempt initiation [49], it is clinically imperative for smoking cessation success to mitigate withdrawal effects through the weeks or months needed for β_2*-nAChR normalization [22]. Ultimately, this study innovatively combined physostigmine administration with (-)-[^{18}F]flubatine PET imaging to investigate cholinergic system function in early cigarette withdrawal.

The a priori region of interest, the frontal cortex, as well as the cholinergic system broadly, have demonstrated roles in cognitive function [50–53]. Indeed, 3–4 week interventions that either increase acetylcholine levels or target the β_2*-nAChR system specifically demonstrated pro-cognitive effects in people who smoke [54, 55]. The present work builds on these findings with evidence that a larger magnitude of acute physostigmine-induced decrease in (-)-[^{18}F]flubatine V_T (consistent with higher magnitude elevation of acetylcholine levels) was significantly associated with worse executive function in people who recently stopped smoking cigarettes, but were otherwise untreated. Taken together, this raises the hypothesis that recently abstinent smokers with larger underlying cholinergic dysfunction may exhibit a larger neurobiological response to an acute pro-cholinergic

physostigmine challenge. Relatedly, individuals with larger underlying cholinergic dysfunction would be expected to exhibit worsened cognitive performance, given the role of acetylcholine in cognitive function. This could indicate a brain-behaviour phenotype most likely to reap the pro-cognitive benefits of extended acetylcholinesterase inhibitor treatment. While additional studies would be needed to test this hypothesis, the current study may lend mechanistic understanding to pro-cognitive effects of acetylcholinesterase inhibitors.

Limitations of this study include no evaluation of cognitive performance after physostigmine, which precluded our ability to capture whether acute pro-cognitive effects of physostigmine were linked to pre-treatment measures of the cholinergic system. Second, the groups in this study did not differ in cognitive performance, despite larger studies repeatedly showing links between worse cognitive performance and cigarette use and withdrawal [5, 8, 56, 57]. It is possible, therefore, that we did not recruit a representative sample of cigarette smokers in early withdrawal. Finally, the clinical significance of these findings may be limited by the modest sample sizes of individuals who completed all study tasks, and the uneven distribution of cannabis use across diagnostic groups, both of which may impact (-)-[^{18}F]flubatine V_T and cognitive performance. In contrast, strengths of this study include use of the radiotracer (-)-[^{18}F]flubatine, a second-generation PET radioligand targeting β_2*-nAChRs with fast kinetics, favourable metabolism [44]. PET imaging also occurred at the optimal timepoint of one week of abstinence: early enough to observe persistent neurobiological adaptations, and late enough to ensure nicotine clearance from the brain [21, 22, 58]. Most innovatively, this study uniquely investigated and detected associations between physostigmine-induced effects on (-)-[^{18}F]flubatine V_T, baseline (-)-[^{18}F]flubatine V_T, and cognitive performance in healthy people who recently stopped smoking cigarettes. Few in vivo human PET imaging studies have jointly examined cholinergic and cognitive function, most of which focus on populations with neurodegenerative diseases [12, 59]. Given that cognitive dysfunction in withdrawal predicts poorer smoking cessation outcomes [7], these results highlight the importance of further investigating pro-cognitive effects of cholinergic smoking cessation aids.

In summary, this study jointly examined the cholinergic system and cognitive performance during early abstinence from cigarette use. The results revealed evidence for significant associations between larger physostigmine-induced elevation of acetylcholine levels and worse executive function in people who recently stopped smoking. Altogether, these findings underscore the importance of normalization of the cholinergic system and the development of neuroscience-informed treatment strategies to mitigate the consequences of cigarette withdrawal and promote smoking cessation success.

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AUTHOR CONTRIBUTIONS

KPC designed the study. KCC, ATH, JA, BL, SRB, GAA, DM, and KPC supervised recruitment and participation of human volunteers. SRB, GAA, and DM were study physicians. MK, MZ, and YH were study radiochemists. ATH performed image analysis. KCC analyzed clinical data and performed statistical analyses of imaging and clinical data. KCC and KPC drafted the initial manuscript. All authors contributed to editing this article.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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