YALE UNIVERSITY
HUMAN INVESTIGATION COMMITTEE

e-Application to Involve Human Subjects in Biomedical Research
100 FR 1e (2012-1)

For use with Electronic Protocol Submissions Only

HIC Protocol Number: 1210010989

Title of Research Project: Acetylcholine, Tobacco smoking, Genes and Nicotinic Receptors	
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Investigator Interests:

Does the principal investigator, or do any research personnel who are responsible for the design, conduct or reporting of this project or any of their family members (spouse or dependent child) have an incentive or interest, financial or otherwise, that may affect the protection of the human subjects involved in this project, the scientific objectivity of the research or its integrity? Note: The Principal Investigator (Project Director), upon consideration of the individual's role and degree of independence in carrying out the work, will determine who is responsible for the design, conduct, or reporting of the research.

See Disclosures and Management of Personal Interests in Human Research
http://www.yale.edu/hrpp/policies/index.html#COI

o Yes x No

Do you or does anyone on the research team who is determined by you to be responsible for the design, conduct or reporting of this research have any patent (sole right to make, use or sell an

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invention) or copyright (exclusive rights to an original work) interests related to this research protocol?

o Yes x No

If yes to either question above, list names of the investigator or responsible person:

The Yale University Principal Investigator, all Yale University co-investigators, and all Yale University individuals who are responsible for the design, conduct or reporting of research must have a current financial disclosure form on file with the University's Conflict of Interest Office. Yale New Haven Hospital personnel who are listed as con-investigators on a protocol with a Yale University Principal Investigator must also have a current financial disclosure form on file with the University's Conflict of Interest Office. If this has not been done, the individual(s) should follow this link to the COI Office Website to complete the form: http://www.yale.edu/coi/

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SECTION I: GENERAL INFORMATION

1. **Performing Organizations:** Identify the hospital, in-patient or outpatient facility, school or other agency that will serve as the location of the research. Choose all that apply:

a. Internal Location[s] of the Study:

- ☒ Magnetic Resonance Research Center (MR-TAC)
- ☐ Yale Cancer Center/Clinical Trials Office (CTO)
- ☐ Yale Cancer Center/Smilow
- ☒ Yale-New Haven Hospital
- ☐ Specify Other Yale Location:
- ☒ Yale University PET Center
- ☐ YCCI/Church Street Research Unit (CSRU)
- ☐ YCCI/Hospital Research Unit (HRU)
- ☒ YCCI/Keck Laboratories
- ☐ Cancer Data Repository/Tumor Registry

b. External Location[s]:

- ☐ APT Foundation, Inc.
- ☒ Connecticut Mental Health Center
- ☒ Clinical Neuroscience Research Unit (CNRU)
- ☐ Haskins Laboratories
- ☐ John B. Pierce Laboratory, Inc.
- ☐ Veterans Affairs Hospital, West Haven

☐ Other Locations, Specify: ☐ International Research Site (Specify location(s)):

2. **Probable Duration of Project:** State the expected duration of the project, including all follow-up and data analysis activities.

   9 years

3. **Research Type/Phase: (Check all that apply)**
   a. **Study Type**
   ☒ Single Center Study
   ☐ Multi-Center Study
   Does the Yale PI serve as the PI of the multi-site study? Yes ☐ No ☐
   ☐ Coordinating Center/Data Management
   ☐ Other:

   b. **Study Phase** ☒ **N/A**
   ☐ Pilot ☐ Phase I ☐ Phase II ☐ Phase III ☐ Phase IV

4. Is this study a clinical trial? Yes ☒ No ☐

NOTE the current ICMJE (International Committee of Medical Journal Editors) definition of a clinical trial: "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes." Health-related interventions include any intervention used to modify a biomedical or health-related outcome (for example, drugs, surgical procedures, devices, behavioral treatments, dietary interventions, and process-of-care changes). Health outcomes include any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events"

If yes, where is it registered?
Clinical Trials.gov registry ☒
Other (*Specify*)

*Registration of clinical trials **at their initiation** is required by the FDA, NIH and by the ICMJE.*

If this study is registered on clinicaltrials.gov, there is new language in the consent form and compound authorization that should be used.

For more information on registering clinical trials, including whether your trial must be registered, see the YCCI webpage, http://ycci.yale.edu/researchers/ors/registerstudy.aspx or contact YCCI at 203.785.3482)

5. Will this study have a billable service as defined by the Billable Service Definition?
Yes ☐ No ☒
If you answered "yes", this study will need to be set up in Patient Protocol Manager (PPM) http://medicine.yale.edu/ymg/systems/ppm/index.aspx

6. Are there any procedures involved in this protocol that will be performed at YNHH or one of its affiliated entities? Yes _ __ No X *If Yes, please answer questions a through c and note*

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instructions below. If No, proceed to Section II.

a. Does your YNHH privilege delineation currently include the **specific procedure** that you will perform?

b. Will you be using any new equipment or equipment that you have not used in the past for this procedure?

c. Will a novel approach using existing equipment be applied?

If you answered "no" to question 6a, or "yes" to question 6b or c, please contact the YNHH Department of Physician Services (688-2615) for prior approval before commencing with your research protocol.

SECTION II: RESEARCH PLAN

1. **Statement of Purpose:** State the scientific aim(s) of the study, or the hypotheses to be tested.

The acetylcholinergic (ACh) system is critically involved in learning, memory, arousal and attention, functions that are substantially impaired in many neuropsychiatric conditions. For example, Alzheimer's disease (AD) is associated with decreased cholinergic function, whereas recent evidence suggests increased and decreased cholinergic function in major depression and schizophrenia, respectively. Cholinergic function is also fundamentally impaired in nicotine addiction. *In vivo* molecular imaging studies of muscarinic and nicotinic receptors have provided substantial contributions to our understanding of these disorders, but these contributions were limited by the lack of a method suitable to measure fluctuations in synaptic ACh level.
We recently developed and tested an innovative molecular imaging method in humans to detect changes in brain synaptic ACh levels, using single photon emission computed tomography (SPECT), the beta2-nicotinic acetylcholine receptor (β_2-nAChR) radioligand [^{123}I]5-IA-85380 (5-IA) and the acetylcholinesterase (AChE) inhibitor, physostigmine, challenge. Results suggest elevated ACh levels induced by physostigmine lead to a reduction in the availability of nicotinic receptors for the binding of the radioligand. If validated, this paradigm might provide a very useful method to interrogate presynaptic cholinergic function in health and disease. To date, receptor imaging of the ACh system at Yale has been limited to 5-IA and SPECT. SPECT is known to have poorer resolution and less reliable quantification compared to positron emission tomography (PET). Furthermore, the slow kinetic of this radiotracer requires lengthy imaging sessions, which is not well tolerated in impaired populations. [^{18}F]NCFHEB (NCFHEB) has been recently introduced as a superior β_2-nicotinic PET imaging agent (1, 2). This proposal outlines a series of studies aiming at testing the suitability of PET and NCFHEB to examine genetic polymorphisms that modulate nAChR availability and to detect changes in ACh synaptic levels, as well as to determine the effect of nicotine on nAChR availability.
<u>This innovative paradigm will lay the groundwork for future studies that can directly interrogate ACh function in the living human brain. This is critical to the advancement of our understanding of ACh involvement in psychiatric and medical disorders</u>.

Additionally, a substantial literature body demonstrates that nAChRs and the cholinergic system dynamically control the mesolimbic DA system by enhancing, inhibiting, and filtering striatal DA release. We have preliminary data suggesting tobacco smokers at 2 weeks of abstinence have blunted DA release compared to nonsmokers. We propose in Aim 7 to determine 1)

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whether there is reduced DA release in smokers at 2 weeks of withdrawal vs. nonsmokers, 2) if the magnitude of DA release is correlated with alterations in ACh levels measured in Aim 4 and 3) if alterations in DA and ACh predict relapse vulnerability.

Aim 1: To determine the test-retest reproducibility of NCFHEB binding parameters in 10 nonsmoking subjects. β_2-nAChRs will be quantified twice in nonsmoking subjects. Based on preclinical evidence, we hypothesize within 20% difference of binding parameters between test and retest scans.

CLOSED Aim 2: To determine if β_2-nAChR availability is genetically predisposed in never smokers and smokers We propose to determine if the ANKK1 genotype (rs4938015/hCV38879) or CHRNA4 genotype determines β_2-nAChR availability in adult never smokers. Specifically, 10 never smokers and 10 smokers at one week of abstinence will be imaged using NCFHEB PET and blood samples for DNA extraction will be collected from all subjects.
Hypothesis*:* Homozygotes (TT) will have higher β_2-nAChR availability compared to age-, and sex matched heterozygotes that carry the C allele in the ANKK1 gene (rs4938015/ hCV38879).

CLOSED Aim 3: To determine if the adaptive increase in β_2-nAChR availability and change in β_2-nAChR availability over the first month of abstinence in smokers is genetically predisposed. We will determine if ANKK1 or CHRNA4 genotypes are associated with changes in β_2-nAChR availability in smokers over time recruited in aim 2. The same smokers from Aim 2 will be supported to maintain abstinence for 4-8 weeks. Smokers that successfully abstain for 4-8 weeks (~ 50%; e.g. 5 smokers) will be imaged a second time using NCFHEB and PET. The % change in β_2-nAChR availability will be determined as the difference in NCFHEB binding [(V_T/f_p @7days of abstinence $-V_T/f_p$ 4 wks abstinence)/V_T/f_p @7 days abstinence]. Blood samples for DNA extraction will be collected from all subjects.
Hypothesis: Based on our preliminary data we hypothesize that smokers that carry the A allele for hCV16178933/rs2273504 or the G allele for hCV15953820/rs2236196 genotype will have higher β_2-nAChR availability at 7-9 days of abstinence compared to never smokers matched for age and sex and genotype. Whereas smoker homozygotes (GG and AA respectively for hCV16178933/rs2273504 or for hCV15953820/rs2236196*)* will demonstrate similar β_2-nAChR availability compared to never smokers. We also hypothesize that CC homozygotes for the CHRNA4 rs2273502 will show a pronounced decrease in β_2-nAChR availability while carriers of the T allele show no change in β_2-nAChR availability will demonstrate the greatest change or normalization over 6 weeks of abstinence.

Aim 4: To measure the sensitivity of NCFHEB binding to changes in endogenous ACh levels in smoking and nonsmoking subjects. After baseline quantification of β_2-nAChR availability, physostigmine will be administered as previously described (HIC#09100005837) and subjects will be scanned again with NCFHEB. We hypothesize there will be greater increase in ACh level (or greater reduction in radio tracer binding) in smoking as compared to nonsmoking subjects. These results will be correlated with changes in mood and cognition associated with physostigmine administration. Up to 50 smoking and 50 nonsmoking subjects will participate. Smokers that are able to remain abstinent will have a second set of baseline and post-physostigmine scans with NCFHEB 4-8 weeks later. This will allow us to determine whether ACh function changes over the course of abstinence.

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CLOSED Aim 5: To measure the sensitivity of NCFHEB binding to changes in endogenous ACh levels in smoking and nonsmoking subjects with schizophrenia. After baseline quantification of β_2-nAChR availability, at 1 week of smoking abstinence, physostigmine will be administered as previously described (HIC#09100005837) and subjects will be scanned again with NCFHEB. We hypothesize there will be greater increase in ACh level (or greater reduction in radio tracer binding) in smoking as compared to nonsmoking subjects with schizophrenia, but the extent of this change will be different than in controls. These results will be correlated with changes in mood, negative and positive symptoms, and cognition associated with physostigmine administration. Up to 10 smoking and 10 nonsmoking subjects with schizophrenia will participate.

Aim 6: To determine the efficacy of a bolus to infusion scan versus a bolus scan of NCFHEB with physostigmine challenge. We will invite back never smokers and smokers who completed bolus scans in Aim 4 to complete one bolus to infusion scan with NCFHEB with physostigmine administration. Smokers will complete the scan at approximately one week of abstinence from smoking. We will compare the results from this scan to their Aim 4 bolus scans to determine which administration better suits a physostigmine challenge. Smokers who completed all 4 scans in Aim 4 will not be asked to return within the same year.

CLOSED Aim 7. To determine amphetamine-induced DA release in tobacco smokers during acute withdrawal and in nonsmokers. Smoker and nonsmoker subjects from Aim 4 will participate. Each subject will participate in up to 2 [^{11}C]PHNO PET scans (ideally, the two PET scans will be carried out in the same day). Up to three hours before the second PET scan, amphetamine (0.5 mg/kg, PO) will be administered. In smokers, the scan will occur at 10-30 days of smoking abstinence. We hypothesize that at 10-30 days of withdrawal amphetamine-induced DA release will be blunted compared to healthy nonsmokers.

CLOSED Aim 8. To determine the degree of occupancy of the β_2*-nAChRs by nicotine after use of an e-cig compared to a tobacco cigarette using [^{18}F]NCFHEB PET neuroimaging. Given recent evidence that experienced e-cig users can achieve similar plasma nicotine levels compared to tobacco smoking [6, 7] , we hypothesize there will not be a significant difference in β_2*-nAChR occupancy by nicotine after use of an e-cig as compared to a regular cigarette. We will also examine arterial plasma nicotine levels and compare to nicotine occupancy of the nAChRs between groups.

2. **Background:** Describe the background information that led to the plan for this project. Provide references to support the expectation of obtaining useful scientific data.

The cholinergic system, and the beta2-nicotinic acetylcholine receptors (β_2-nAChRs) specifically, have been implicated in many neuropsychiatric conditions, such as addiction, depression, schizophrenia and AD.(3-6) This involvement has been supported by molecular imaging studies of the β_2-nAChR, including studies performed by our group at Yale.(7-13) The β_2-nAChRs are the most abundant nAChRs in the brain. Until very recently, β_2-nAChR SPECT and PET radiotracers available for human use displayed slow kinetics, requiring prolonged scanning sessions(14, 15), and practically eliminated our ability to study severely impaired patients.(16, 17) The recently introduced radiolabeled antagonist (-)-2-(6-(18)F-fluoro-2,3'-bipyridin-5'-yl)-7-methyl-7-azabicyclo[2.2.1]heptane ([^{18}F]NCFHEB or NCFHEB) has been

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shown to have high binding potential at the β_2-nAChRs and rapid kinetics (1) and is thus a very promising new radioligand. The reproducibility of NCFHEB V_T measurement in humans and the sensitivity of NCFHEB V_T to changes in extracellular Ach concentrations have not yet been reported. Here, we propose to test the reproducibility of NCFHEB *in vivo* binding in humans and to examine its vulnerability to changes in synaptic ACh. These critical studies will answer a fundamental question regarding use of NCFHEB and PET in the study of cholinergic systems and will have broad implications to the future studies of severe neuropsychiatric conditions (nicotine addiction, mood disorder, schizophrenia, AD), with high relevance to individual and public health. If validated, such an imaging tool would have enormous potential to facilitate the development of innovative medicines aimed at modulating the cholinergic system.

Furthermore, since nicotine binds with high affinity to the β_2-nAChRs and there is a high prevalence of smoking in the US, we will examine this system and its response to increases in endogenous ACh in healthy smokers and the associated cognitive and genetic factors.

Evidence for Genetic Inheritance of Tobacco Smoking A family history of tobacco smoking is a powerful predictor of smoking risk. Children of ever- or current- smokers are more likely to smoke, compared to children of never smokers (18-22). Smokers with a family smoking history are more likely to be persistent smokers than those with no history (23-26). Regular stable smoking in adolescence is related to having a current parent smoker relative to a former or never smoker parent (27). Failure to quit smoking is linked to having parents that smoked daily (19, 28, 29). Parental smoking is related to a greater likelihood of developing nicotine dependence, higher numbers of cigarettes smoked per day, higher anxiety levels and a trend towards depression (24, 30). While all smokers experience anxiety in response to stressor, only family history positive smokers experience heightened craving for cigarettes (31). *These data suggest that there is a biological difference between family history positive smokers and family history negative smokers that is likely transmitted between generations through shared genetics.*

There is strong evidence to support genetic transmission of smoking between family members. Ever-smoking segregates in families following expectations consistent with a dominant Mendelian genetic factor with a frequency of 0.02. Studies of monozygotic and dizygotic twins reared together and apart suggest that there is 50-72% inheritance rate (26, 32-44). The concordance rate amongst monozygotic twins is higher than dizygotic twins, regardless of whether they were raised together or separately [reviewed by (45)]. Genetics contributes to the initiation of smoking behavior (25, 26, 34, 43) with heritability estimates of 0.11 and 0.78 whereas shared and unique environmental effects range between 0.00 and 0.59 and 0.07 and 0.36 respectively [reviewed by (46, 47)]. Persistence of smoking is also determined by genetics with heritability estimates between 0.04 and 0.86 [reviewed by (46, 47)]. An analysis of 17,500 monozygotic and dizygotic twin pairs from 14 studies, estimated that genetics accounted for 56%, whereas shared and unique environmental factors accounted for 24 and 20% of the liability for smoking initiation, and genetics accounted for 67%, whereas shared and unique environmental factors accounted for 2 and 31% of the liability for smoking persistence.

Smoking behavior has been linked to chromosomes 1, 2, 4, 5, 6, 9, 10, 11, 14, 17, 18 and 21 (48-51). A recent genome-wide association study of nicotine dependent individuals showed that single nucleotide polymorphisms (SNPs) nominate candidate genes coding for cell adhesion, enzymes, transcriptional regulators, neurotransmitters, and receptors and the regulation of DNA, RNA, and proteins are associated with successful abstinence from smoking (52). In marker based genetic studies, associations between smoking behavior and several candidate genes have been

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evaluated including cytochrome p450, dopamine receptor (D_2) dopamine transporter, serotonin transporter and the nicotinic acetylcholine receptor [reviewed by (46)]. Variants in genes occur due to insertions, deletions, variable numbers of repeats, and single nucleotide polymorphisms (SNPs) in the DNA sequence. SNPs are the most common type of polymorphism occurring as frequently as once for every 200-1000 base pairs in different regions of the genome. SNPs have been targeted because they may be used either as synonymous markers for linkage, and linkage disequilibrium mapping, or they may represent functional mutations themselves. This limitation may be dealt with by doing a haplotype analysis of multiple SNPs. Haplotypes provide greater statistical power compared to SNPs (53).

Nicotinic Acetylcholine Receptor (nAChR) Nicotine initiates its effects in brain through the nicotinic acetylcholine receptor (nAChR). Neuronal nAChR belong to a receptor family of ligand gated ion channel receptors that include the $GABA_A$ and $GABA_C$, glycine and 5-HT_3 receptors. Neuronal nAChR are comprised of 5 subunits (54). Each subunit spans the membrane 4 times such that the second transmembrane domain forms the wall of the ionic pore that transports Ca^{2+}. To date, 12 genes coding for subunits associated with the neuronal nAChR have been identified from the mammalian genome including $\alpha_{\square}\square\alpha_{\square\square}\square\square\beta_{\square}\square\beta_{\square,}$ [reviewed by (55)]. Neuronal nAChR comprised of α_7 and α_9 are functional as monomeric receptors which are pharmacologically characterized by low affinity for nicotinic agonists, and high affinity for α-bungarotoxin, while all other □ subunits (e.g. $\alpha_{\square}\square\square\alpha_{\square}\square\square\alpha_{\square}\square\square\alpha_{\square}\square\square\alpha_{\square}$) require co-expression of α and β pairs and are distinguished by their high affinity for nicotinic agonists and low affinity for α-bungarotoxin (56, 57). Binding sites for the endogenous neurotransmitter, acetylcholine, are highly conserved between subunit types whereas nicotine has higher affinity for the α_4 and α_3 subunits versus the α_2, α_3, α_4, or α_6 and β_2 or β_4 subunits (58). Acetylcholine binds to heteromeric nAChRs at the interface between α_2, α_3, α_4, or α_6 and β_2 or β_4 subunit (59). The fifth accessory subunit, which is not involved in acetylcholine binding may be β_2, or β_4, or α_5 or β_3 (59). The β_3 and α_5 subunits are likely partners with α_6 subunits in nAChR forming a pentamer of $\alpha_6\alpha_4\alpha_3\alpha_5$ that are localized to dopaminergic cell bodies (60). NAChR $\alpha_4\beta_2$ receptors demonstrate 4-fold lower amplitude whole cell currents, and slower acute desensitization and functional rundown as compared to α_7-containing nAChR. Beta subunits influence the sensitivity of α_4 to functional inactivation. The second major intracellular loop influences the acute desensitization of nAChR (61). Nicotinic agonists have higher functional potency at α_4 containing versus β_2 containing but they have higher binding affinity at β_2 containing nAChR(62). High and low affinity $\alpha_4\beta_2$nAChR result from the assembly of α_4 and β_2 into two distinct stoichiometric arrangements $(\alpha_4)_2(\beta_2)_3$ high affinity; and $(\alpha_4)_3(\beta_2)_2$, low affinity that differ significantly in their functional and pharmacological properties [reviewed by(63)]. The $(\alpha_4)_2(\beta_2)_3$ stoichiometry is more sensitive to activation and upregulation by nicotine and desensitizes more slowly. Importantly, agonist binding to nAChR likely reflects multiple states, including the resting and desensitized states (62).

Role of nAChR Subunits in Nicotine Dependence

The reinforcing properties of nicotine are mediated primarily the heteropentameric $\boldsymbol{\alpha_{\square}\beta_{\square}}$-nAChR and the homomeric $\boldsymbol{\alpha_7}$-nAChR. Both subtypes have been localized to GABA neurons in the ventral tegmental area, the brain region where mesolimbic dopaminergic neurons believed to be part of the final common pathway for drug reward originate. These GABAergic neurons synapse onto the dopaminergic neurons that also have $\boldsymbol{\alpha_7}$-nAChR and $\boldsymbol{\alpha_{\square}\beta_{\square}}$-nAChR that are likely also associated with $\boldsymbol{\alpha_5}$ and/or $\boldsymbol{\alpha_6}$ subunits (64). Importantly, mice without the β_2 subunit do not self-

administer nicotine and also have no high affinity nicotine binding sites (65). In keeping, restoration of□β_2 subunits in VTA causes reinstatement of nicotine self-administration (66). Interestingly, the β_2 but not the α_7-subunit is required for nicotine-conditioned place preference (67). Since α_4 subunits predominantly co-localize with the β_2 subunit, these studies suggest that the $\alpha_{\square}\beta_{\square}$-nAChR is a principal nAChR responsible for the reinforcing properties of nicotine. Although evidence is accumulating demonstrating that the α_5, α_6 and β_3 subunits also co-localize with α_4 and β_2 subunits and form part of the heteropentamer. In mice with α_4 nAChR subunits containing a single point mutation in the pore-forming M2 domain that renders α_4* receptors hypersensitive to nicotine, that selective activation of α_4* with low nicotine doses is sufficient to induce reinforcement, tolerance and sensitization to nicotine, suggesting that the $\alpha_{\square}$ subunit plays a critical role in nicotine dependence.

Other nAChR subunits are also implicated in nicotine dependence. α_4 nAChR may influence nicotine consumption and relapse due to its relationship to the expression of anxiety related behaviors (68). Nicotine withdrawal is precipitated by drugs with preferences for $\alpha_{\square}\beta_{\square}$, $\square_{\square}\beta_2$, or α_7 (69). $\square_4$ knockout mice demonstrated decreased nicotine withdrawal symptoms whereas $\square_2$ knockouts do not (70).□□ The α_7 nAChR in the VTA have also been linked to nicotine withdrawal symptoms (71, 72). Nicotine treated α_7 knockout mice demonstrate no nicotine withdrawal symptoms (73). Further evidence supporting a role for $\alpha_{\square}$nAChR in nicotine dependence includes the ability of the □α_7 receptor antagonist methylcaconitine to attenuate the ability of nicotine to reduce ICSS (intracranial self-stimulation) threshold (74).

Effects of Chronic Nicotine Exposure on nAChR It is well established in the preclinical literature that chronic nicotine exposure causes an adaptive increase in β_2-nAChR expression (75-80). In postmortem human brain nicotinic agonist binding is higher in the gyrus rectus (Brodman area 11), hippocampus, thalamus, midbrain (81, 82), striatum, entorhinal cortex , cerebellum (83) and prefrontal and temporal cortex (84) of smokers versus never smokers. Increased β_2-nAChR expression after chronic nicotine was recently demonstrated in nonhuman primates using SPECT and [^{123}I]5-IA (85) [Staley et al., 2006] and also in human tobacco smokers abstinent for 4-9 days [Staley et al., 2006].

In humans, nicotine binding in ex-smokers (> 2 months) is similar to that of the non-smokers suggesting that the nicotine-mediated upregulation of agonist binding to nAChR is reversible (81-83). ***Our ongoing studies evaluating the normalization of agonist binding to β_2-nAChR availability within-subject in living human smokers have revealed inter-individual differences in this normalization with some subjects showing decreases of 14-17% over the first month and others showing no change < 4%. We have hypothesized that these differences in receptor normalization are genetically determined. We now have preliminary data suggesting that this difference in normalization is related to the CHRNA4 rs2273502 SNP.***

Genes Associated with Nicotine Dependence

CHRNA4 The □□$_{\square}$ nAChR subunit is encoded by the CHRNA4 gene, that, has been mapped to chromosome 20q13.2-13.3 (86). The CHRNA4 gene [MIM118504] [National Center for Biotechnology Information, locus ID 1137] has 6 exons and is approximately 17kb in size (87). To date, 30 single nucleotide polymorphisms (SNPs) have been identified on the CHRNA4 gene, of which, 14 were polymorphic (88). The relationship between 6 SNPs [see Table 2] and nicotine dependence defined as two siblings plus at least one parent or another sibling with an FTND score $\geq$ 8 demonstrated that rs1044396 (T allele) and rs1044397 (A allele), in exon 5 of the CHRNA4 gene are protective against nicotine dependence (88). Moreover, the family based

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association test suggested that these 6 SNPs meet criteria for a haplotype block that is significantly associated with nicotine dependence and age-adjusted FTND (Fagerstrom Test for Nicotine Dependence Score). Thus, persons with the GCTATA haplotype of the CHRNA4 gene are protected from developing nicotine dependence.

An ethnic and gender-specific association of the CHRNA4 gene with nicotine dependence (89) such that in European Americans (EA) rs2273504 and rs 1044396 are significantly associated with smoking quantity and severity of nicotine dependence as determined by the Fagerstrom Test for Nicotine Dependence (FTND), whereas, SNPs rs3787137 and rs2236196 were associated with smoking quantity, FTND score and heaviness of smoking index (only rs2236196). Hutchison and colleagues have recently evaluated the CHRNA4 SNPs rs612249 and rs609387 located in the 5' promoter UTR region and also the 3'UTR SNP rs2236196 and the relationship to CHRNA4 mRNA, epibatidine binding in postmortem tissue (90), and nicotine sensitivity in living humans (91). In brief, these studies demonstrated that individuals with the AG (vs AA) genotype to RS2236196 demonstrated higher epibatidine binding [which agrees with our preliminary data [see section C.7] and greater sensitivity to the physiological and cognitive effects of smoking suggesting that CHRNA4 genotypes influence the etiology of nicotine dependence. And, individuals with GG RS612249 SNP reported greater subjective physiological effects after smoking three cigarettes and greater baseline CO. Recent studies have suggested an association between CHRNA4 rs 1044396 and nicotine-mediated attentional network function in the anterior cingulate cortex and parietal cortex (92) and CHRNA4 rs3746372 has been linked to the number of cigarettes smoked in schizophrenic smokers (93).

While there is some debate on how SNPs in the human genome translate to the rodent genome, it is interesting to note that a naturally occurring SNP (1587A to G) results in an alanine to threonine variation at the amino acid position 529 on the nascent α_4 subunit in the last 1/3 of the cytoplasmic loop between the TM3 and TM4. This SNP alters nAChR function including increased vulnerability to nicotine-induced seizures; ethanol withdrawal seizures; the effects of alcohol and nicotine on locomotor activity and is associated with lower nicotine consumption and preference (94). There were no differences in cytisine binding between variants, however cytisine is nonselective and labels multiple nAChRs, compared to 5-IA, which labels only β_2-nAChRs. This variant also leads to a difference in the ratio of high and low affinity nAChR such that SS (AA or A carriers) mice have a greater fractions of desensitized α_4- nAChR compared to the LS (GG) mice. Importantly, this variant is common in European Americans and uncommon in Asians and Africans (94-97).

CHRNB2. The β_2 subunit is encoded by the CHRNB2 [MIM 118507] [NCBI locus ID 1141] gene and has been mapped to chromosome 1q21.3 (98). The CHRNB2 gene has 6 exons with a G-C rich promoter region that includes a presumptive neural-restrictive silencer element, an

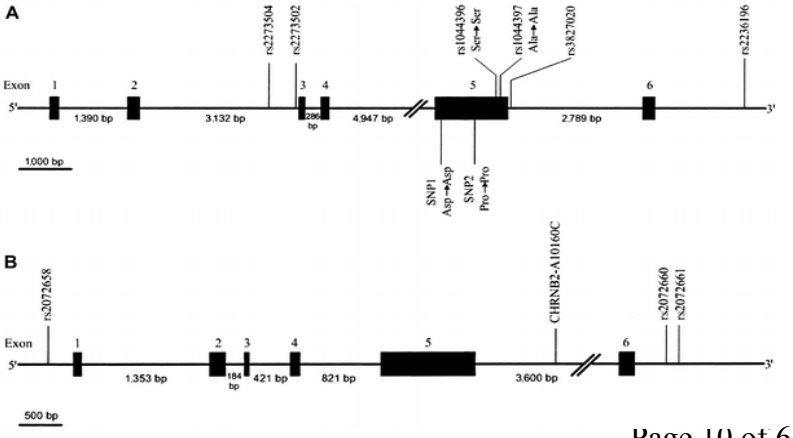

Table 2: Single Nucleotide Polymorphisms in Genes Encoding α4β2 nAChR

CHRNA4 SNPs	Gene Location	Base Change	Amino Acid Change
rs1044396	Exon 5	C -> T	Ser -> Ser
rs1044397	Exon 5	G -> A	Ala -> Ala
rs2273504	Intron	G -> A	
rs2273502	Intron	C -> T	
rs3827020	Intron	T -> C	
rs2236196	3' UTR	A -> G	
CHRNB2 SNPs			
rs2072658	5' UTR	G -> A	
A10160C	Intron 5		
rs207660	3' UTR	C -> T	
rs2072661	3' UTR	G -> A	

Obtained from Feng et al., Am J Hum Genet 75:112-121, 2004

Fig 1. *A*, Schematic representation of the six SNPs located on the *CHRNA4* gene, showing their locations according to the information from the chromosome 20 genomic contig (GenBank accession number NT_011333). SNP1 and SNP2 in exon 5 are synonymous SNPs revealed by direct DNA sequencing. *B*, Schematic representation of the four SNPs located on the *CHRNB2* gene, showing their locations according to the information from the chromosome 1 genomic contig (GenBank accession number NT_004668): From Feng et al., Am J Hum Genet 75:112-121, 2004.

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unusually long 3' untranslated region and is approximately 12 kb (99). To date 24 single nucleotide polymorphisms (SNPs) have been identified for the CHRNB2 gene although only 4 have been found to be polymorphic (88). Two studies that have assessed the relationship between CHRNB2 gene variants and nicotine dependence have suggested that there is no association (99, 100). However, Feng and colleagues recently demonstrated that while individual SNPs were not associated with nicotine dependence the family based association test suggested that 4 SNPs (Table 2) meet criteria for a haplotype block that demonstrates marginally significant correlation with the age –adjusted FTND score (p = .0.065) (88). Specifically, persons with the GCCG haplotype may be protected from developing nicotine dependence.

CHRNA5 The CHRNA5, CHRNA3, and CHRNB4 genes are clustered on chromosome 15q24(101). A recent study has demonstrated a strong association between a non-synonymous SNP in the □5 -nAChR subunit gene (rs16969968) and the risk for nicotine dependence. This variant results in a change in amino acid 398 from asparagine encoded by the G allele, to aspartic acid, encoded by the A allele, which alters the charge of the amino acid in the second intracellular loop of the □5 -nAChR subunit. Individuals homozygous for the A allele demonstrate a 2-fold higher risk of developing nicotine dependence once exposed to cigarette smoking (102). Importantly, □5 -nAChR subunits commonly fill the fifth position of the heteropentamer most commonly comprised of α_4 and β_2 receptors (103)

CHRNB3 The CHRNB3 gene is located on chromosome 8 p11.2. To date 47 SNPs have been identified. In brain □3 can take the fifth position in the pentamer of β_2-nAChR, although it does not contribute to the acetylcholine binding site it does alter channel function and influences agonist potency because they participate in the conformational changes that go along with activation of the receptor and desensitization (104). This gene has recently been associated with a higher risk for developing nicotine dependence (102). And CHRNB3 (rs 4950 and rs 1380604) has been linked to subjective responses to tobacco smoking (105).

DRD2 TaqIA The relationship of the DRD2 TaqIA polymorphism with tobacco smoking has been extensively studied. A meta-analysis suggests that there is a higher prevalence of the DRD_2 Taq1A polymorphism, in European American smokers versus never smokers (106). Moreover this relationship seems to transcend across multiple ethnic groups with an association of the A1/A1 genotype with current smoking status in Mexican-Americans (107) versus a higher association of the A2/A2 in Japanese ever-smokers (108). Chinese smokers with the A2/A2 genotype smoked a greater number of cigarettes/day than smokers with at least one A1 allele (109). DRD2 Taq1A showed no association with smoking status or quantity in the UK population (110) or in African Americans (107).In European Americans, there appears to be no overall association between the DRD_2 Taq1A polymorphism and smoking cessation (111). However, Japanese women smokers with the A1 allele are less likely to quit smoking than the A2/A2 genotype (112). Carriers of the DRD2-A1 allele are more likely to present tobacco withdrawal syndrome, shorter latency periods prior to relapse and demonstrate greater response to the nicotine patch (113-115). While there appears to be an association between the DRD_2 TaqA1 allele and tobacco smoking, the functional relevance of this association is still not understood. The Taq1A polymorphism is located ~ 10 kb downstream and up to 25 kb away from the DRD2 gene and is unlikely to alter DRD2 activity directly. However, the DRD_2 TaqA1 allele has been associated with reduced D_2 receptor binding affinity and low striatal D_2 receptor densities (113, 116, 117). Furthermore, a functional relationship between D_2 and nAChR has recently been elucidated. Both receptors are known to modulate dopamine release in the striatal reward pathways such that acetylcholine binding to nAChR hetero-autoreceptors enhances DA

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release, while dopamine interactions with presynaptic D_2 autoreceptors decrease DA release. A recent study has demonstrated that D_2 autoreceptors and nAChR heteroreceptors form heteromeric dopamine autoreceptor complexes to modulate the efficacy of DA release (118). Knockout studies suggest that there may be up to six different nAChR subunit combinations that mediate DA release including $\alpha_6\beta_2$; $\alpha_6\ \beta_2(\beta_3)$; $\alpha_4\beta_2$; $\alpha_4\alpha_5\alpha_6\beta_2$ $\alpha_4\alpha_6\beta_2(\beta_3)$ and $\alpha_4\alpha_5\ \beta_2$ (119)

ANKK1 Polymorphism. Recent studies have revealed that the DRD2 TaqI A (Rs1800497) site is located in a gene adjacent to DRD2 known as the ankryin repeat and kinase domain containing ANKK1 (120). The ankyrin repeat is one of the most common protein-protein interaction motifs in nature. Repeated modules of about 33 amino acids occur in a large number of functionally diverse proteins, including transcriptional initiators, cell cycle regulators, cytoskeletal proteins, ion transporters, and signal transducers. The repeats typically have negative charged surface so a polymorphism that changes from negative to neutral or positive change may change the functional role (121). The Taq1A polymorphism in ANKK1 may be in linkage disequilibrium with upstream polymorphism in DRD2 and lie within the same haplotype block that spans the overlap between the two genes (121).

The ANKK1 gene codes a protein that has been likened to the tyrosine kinase like genes (TKL) that resemble tyrosine and serine kinases and are similar to receptor interacting protein kinases (RIPK) and the leucine rich repeat kinases. Phosphorylation represents the major post-translational modification regulating numerous ligand gated ion channels including the nAChR (122). Subunit phosphorylation can alter the affinity state of the receptor for agonist and the functional characteristics of the channel. The DRD2 TaqI A system causes an amino acid substitution within the 11^{th} ankrin repeat of this gene (Glu713Lys), that may alter substrate binding specificity(120). In keeping, Taq1A (A1 carriers) are associated with decreased D_2 autoreceptor density, and corresponding decrease in autoreceptor function (measured by ^{18}F DOPA) (121).

Our colleague, Dr Joel Gelernter has recently demonstrated that ANKK1 SNPs are strongly associated to nicotine dependence in European Americans and African Americans. Specifically the ANKK1 is in linkage disequilibrium with other variants that are associated with nicotine dependence as defined by DSM-IV criteria(123). This phenotype is particularly broad and thus may be related to one or more of smoking behaviors including, tolerance and withdrawal to name a few (123). The relationship between the ANKK1 gene and β2-nAChR availability that we have observed in our pilot analyses is not entirely clear. However, it may be hypothesized that it may play a role in determining the phosphorylation state of nAChR subunits, which may modulate desensitization and recovery of the nAChR receptor in the presence and absence of nicotine (124). Tyrosine kinases regulate all nAChR but the functional consequences by the same kinase family are specific for each subtype and location [reviewed by (125)]. The $\square_4$ subunit has been shown to be phosphorlated *in situ* by c AMP dependent protein kinase, (PKA) and protein kinase C (122). PKA mediated phosphorylation of the $\square_4$ subunit enhances its affinity for the 14-3-3 chaperone protein resulting in increased expression of the $\square_4\beta_2$ receptor(126) (127). The phosphorylation state of neuronal nAChRs appears to regulate their rate of recovery from desensitization and, receptors rely on balance between phosphorylation and dephosphorylation state to regain their agonist sensitivity(128). While it is known that the $\square_4$ subunit exists in phosphorylated and dephosphorylated states, the relationship between these states and receptor function are not yet known (122). Additional work remains to elucidate the function of ANKK1 including more extensive expression profiling and characterization of the functional significance and localization at the protein levels(120) and its relationship to β2-nAChR availability.

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In the present proposal we will explore the relationship of these and other SNPs (Aim 2 and 3) that have been linked to nicotine dependence with β_2-nAChR availability in never smokers and smokers to determine if these SNPs also convey genetic liability to β_2-nAChR availability in relation to the regulatory effects of nicotine as well as relapse rates.

Nicotinic receptors and schizophrenia

Schizophrenia is associated with very high rates of tobacco addiction (80%) relative to the general population (20%) and other psychiatric disorders (129, 130). Compared to typical smokers, individuals with schizophrenia are reported to extract higher amounts of nicotine per cigarette and higher rates of smoking-related cardiovascular disease, pulmonary disease and associated mortality (131, 132). The high rate of tobacco smoking may reflect an attempt to "self-medicate" the negative symptoms, cognitive dysfunction, and antipsychotic-related side-effects associated with schizophrenia (133-135). Therefore, understanding the basis for high rates smoking in this population, a modifiable risk factor, might lead to increased life expectancy and quality of life, and may also provide the basis for developing drugs to target the symptoms of schizophrenia.

Nicotine, the primary addictive and reinforcing constituent in cigarettes, has high affinity for the beta2-subunit containing nicotinic acetylcholine receptors (β_2*-nAChRs). Evidence from postmortem (82), preclinical (10), and clinical (8, 9) studies demonstrates that chronic administration of nicotine and tobacco smoking increases the number of β_2*-nAChRs in most brain regions (9, 136), which is commonly referred to as "upregulation". However, post- mortem study suggests that smokers with schizophrenia fail to upregulate to the same extent as comparison smokers. A study that controlled for smoking status in postmortem samples of smokers and nonsmokers with and without schizophrenia shows that nonsmokers with and without schizophrenia have similar binding of [^{3}H]-nicotine (symbolizing similar availability of β_2*-nAChRs), but smokers with schizophrenia have lower [^{3}H]-nicotine binding than smokers without schizophrenia. This suggests that smokers with schizophrenia do not upregulate nAChRs to the same extent as smokers without schizophrenia (137). We confirmed *in vivo* the postmortem findings of Breese et al. that smokers with schizophrenia had lower β_2*-nAChR availability relative to smokers without schizophrenia (138). Furthermore, we showed that β_2*-nAChR availability in individuals with schizophrenia correlated with negative symptoms (138). We followed up this evaluation with smokers and nonsmokers with schizophrenia and detected that although there is lower β_2*-nAChR availability in schizophrenia, smokers with schizophrenia do upregulate as compared to nonsmokers. The study also showed that those smokers with schizophrenia who had lower β_2*-nAChR availability, reported more negative symptoms and scored lower on tests of executive control. Given these recent developments, and the fact that the pharmaceutical companies are developing cholinergic medications for treatment of tobacco addiction, and possibly for treatment of cognitive deficits, it is first imperative to understand the cholinergic involvement in schizophrenia. Conducting an acetylcholine challenge via administration of an acetylcholinesterase inhibitor is an excellent way to examine whether there is cholinergic compromise in schizophrenia beyond lower receptor availability. We will correlate these results with measures of schizophrenia symptoms and cognitive performance in order to understand whether increasing endogenous acetylcholine may have effects on these variables.

Interaction of the Cholinergic and Dopaminergic Systems

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A substantial literature body demonstrates that nAChRs and the cholinergic system dynamically control the mesolimbic DA system by enhancing, inhibiting, and filtering striatal DA release. Nicotine releases DA by binding to β2*-nAChRs located on the mesolimbic DA neurons in the ventral tegmental area, resulting in neuronal firing and DA release in the nucleus accumbens and dorsal caudate. β2*-nAChRs are also critical for the reinforcing and motivational effects of nicotine, e.g., tying cues to drug consumption. Nicotine also "filters" DA release by modifying the sensitivity of DA synapses through its desensitization of the nAChRs. As reviewed in Exley and Cragg (2008), nicotine promotes DA release through both burst and tonic activity. Nicotine functions as an agonist and also as an antagonist via its desensitization/blocking actions and both may ultimately enhance DA neurotransmission. Thus, β2*-nAChRs are responsible for controlling the dynamic range of DA release. A recent preclinical study reported that chronic nicotine administration or deletion of the β2*-nAChRs (i.e., β2* knockout) reduced acute DA release. This suggests that chronic smokers may have a blunted stimulus-induced DA release compared to nonsmokers; however, this has not been examined. Several PET studies have demonstrated blunted amphetamine-induced DA release in the ventral striatum in both cocaine and alcohol dependent populations at approximately 2 weeks of withdrawal vs. controls. In the cocaine-dependent subjects, those with a more blunted drug induced DA release responded less well to a behavioral treatment that incorporated positive reinforcement. Their findings suggest that individuals with dysfunctional DA transmission are not able to "switch" well from drug-reinforced behavior to more natural alternative rewards and this is supported by a preclinical study in which rats with a lesioned nucleus accumbens displayed an inability to choose greater magnitude delayed rewards vs. immediate rewards of lesser value. We have preliminary data suggesting tobacco smokers at 2 weeks of abstinence have blunted DA release compared to nonsmokers. We propose in Aim 7 to determine 1) whether there is reduced DA release in smokers at 2 weeks of withdrawal vs. nonsmokers, 2) if the magnitude of DA release is correlated with alterations in ACh levels measured in Aim 4 and 3) if alterations in DA and ACh predict relapse vulnerability.

As mentioned, DA regulates the motivational properties of reinforcers including salience of stimuli and cue-reactivity. In landmark studies, Dr. Schultz illustrated that after learning the association between a cue and a reward, the DA neurons respond to the cue that occurs prior to the reward in anticipation of a future reward. This highlights the nature of cues because the cues themselves become reinforcing. In a smoker, the DA that is initially released as a result of smoking takes on the role of determining salience and connecting the smokers' actions and rewarding feelings to environmental cues, e.g., the cigarette, lighter, ashtray, rest area where smoking occurs. A pack per day 20-year smoker has had 1,500,000 learning trials (puffs on a cigarette) that reinforce cues as stimuli for the physiological and rewarding effects of smoking. These associated cues elicit craving, and the magnitude of the reactivity to cues (craving, increase in heart rate, brain activation) may predict smoking cessation outcomes. Those who experience more craving in response to cues have a harder time quitting smoking. Functional magnetic resonance imaging (MRI) studies have found that during acute abstinence (24 h) there are increases in brain activations to smoking-related cues compared to when smoking as usual. PET studies have shown smoking-induced DA release that correlates with craving during acute withdrawal. While these studies confirm increased craving shortly after the last cigarette that is associated with DA release, few studies have systematically examined cue-induced reactivity over longer abstinence periods and none have examined the association between DA signaling and cue-induced reactivity and the relationship to relapse in human smokers. While many withdrawal related behaviors including significant mood impairment may improve over the first week of abstinence, cue-induced reactivity is long-lasting and may increase with abstinence. A

preclinical study demonstrated an incubation of nicotine-seeking behavior (i.e., cue-induced reinstatement) after extended nicotine self-administration. After self-administration, animals with a 7-day vs. 1-day enforced abstinence took longer to extinguish drug-seeking behavior and responded at significantly higher levels during the cue-induced reinstatement, and these behavioral changes were associated with dopaminergic molecular changes. Incubation of cue-induced craving has also been shown in human tobacco smokers with different durations of abstinence (35 vs. 7 days). Critically, absolute craving levels in smokers do decrease over time, but cue-induced craving appears to increase. It is also known that DA D2 receptor drugs block cue-induced reinstatement in rodents and D3 receptor-specific drugs are being studied as treatments for smoking specifically for their potential to reduce relapse. We will determine relationships between drug-induced DA release and cue-reactivity during early phase abstinence and whether the magnitude of DA release predicts relapse vulnerability or change in cue reactivity. We predict that individuals who exhibit blunted drug-induced DA release will have a shorter latency to relapse and that their cue-reactivity will not be significantly reduced over time. While the focus in Aim 2 is on uncovering relationships between DA and cue-reactivity in smokers, DA also plays a powerful role in regulating mood and affect. Smoking is highly comorbid with Major Depressive Disorders, and many otherwise healthy smokers report significant changes in mood during acute withdrawal. Thus, in addition to cue-reactivity, we will examine relationships between DA transmission, mood and affect, and relapse vulnerability.

E-cigarettes

Tobacco smoking and secondhand smoke exposure has caused >20 million estimated premature deaths since 1965 and is the number one preventable cause of disease[1]. Although dependence rates have stabilized at ~18% for the general population, quit rates continue to be low[2, 3]. Nicotine is the primary addictive ingredient in tobacco smoke and binds with high affinity to the nicotinic acetylcholine receptors (β_2*-nAChRs). Inhalation is the quickest way a drug can reach the brain and provide neurological effects; thus, when smoked, tobacco delivers nicotine to the pulmonary circulation and reaches the arterial circulation and brain within seconds after inhalation, leading to a rapid neurologic effect and a high level of addiction[12, 13]. Nicotine replacement products designed to help with smoking abstinence or to be used as replacement of smoked tobacco, have been developed in the form of patches, gum, lozenges, nasal spray, and inhaler. However, they deliver nicotine more slowly compared to smoked tobacco and may not be as rewarding to smokers[11]. Recently, electronic cigarettes (e-cigs) have gained popularity among seasoned tobacco smokers as well as tobacco-naïve users[4]. However, due to quick growth and popularity of these products and ever-changing design, it has proved difficult to apply regulatory process to e-cigs. The present study aims to add to the small pool of controlled studies examining biological and physiological effects of e-cigs.

E-cigs are battery-operated devices that heat and aerosolize a liquid that typically contains nicotine. Although early studies of e-cigs suggested that nicotine delivery was ineffective[14, 15], later studies found that nicotine can be delivered to the blood in significant amounts [6, 7, 16, 17]. Further, advances in product technology have led to the development of more sophisticated e-cigs (i.e. personal vaporizers) with larger batteries and tank systems that are capable of producing larger amounts of aerosol and greater nicotine delivery as compared to earlier e-cig products [6, 7]. Although there is little available data informing the addictive nature of e-cigs, the few available published studies suggest that e-cigs have lower addictive potential compared to smoked tobacco[16, 18-20].

Receptor imaging has been successfully utilized to examine mechanisms of tobacco addiction through evaluation of β_2*-nAChR availability as a consequence of tobacco smoking[21-

[23] as well as occupancy by nicotine in multiple previous studies[8, 10, 24]. Findings suggest that typical smokers maintain near complete receptor occupancy throughout the day, and that lower receptor occupancy levels lead to craving. As an example of our work, we compared β_2*-nAChR occupancy between the Nicotrol nicotine inhaler and a regular tobacco cigarette using [^{123}I]5-IA and SPECT imaging. We determined that using the inhaler leads to an average 55.9 ± 6.4% occupancy by nicotine of the β_2*-nAChRs, which was significantly less compared to the 67.6 ± 14.1% occupancy after use of a tobacco cigarette[11]. Further, we detected that arterial plasma nicotine concentration was significantly lower after use of the inhaler as compared to a regular cigarette. We observed a significant reduction in withdrawal but not craving symptoms associated with the use of the inhaler. These findings suggest that the nicotine occupancy after use of the inhaler may be at the degree of attenuating nicotine withdrawal; however, greater saturation of the receptors may be required to relieve nicotine craving symptoms. These data also suggest the nicotine inhaler has lower potential for abuse and addiction compared to a regular cigarette.

In the present study, we will use [^{18}F]NCFHEB, a radioligand that has high affinity for the β_2*-nAChRs and has been shown to be sensitive to increases in nicotine[25], and PET to conduct a critical study of nicotine's occupancy of the β_2*-nAChRs after use of an e-cig and relate to clinical data to better elucidate the molecular actions and addictive potential of e-cigs. Furthermore, we propose to conduct a novel examination of nicotine concentration in arterial plasma after use of an e-cig and a regular cigarette and relate these findings to the brain and clinical data. While the prior findings suggest differences in addictiveness between e-cigs and tobacco, the amount of nicotine delivered to the brain from an e-cig is not known and the relationship to tobacco smoking craving and withdrawal is not elucidated. Identifying e-cigs' mechanisms of action in the brain, potential addictive nature, and the relationship between mechanism and behavior is thus the critical step in evaluating these products for potential regulation by the FDA.

[^{18}F]NCFHEB PET imaging.

The most commonly used β_2-nAChRs ligands to date, 5-IA for SPECT and 2-[^{18}F]-F-A-85380 for PET, have high affinity for this receptor but their utility for challenge studies in psychiatric populations is likely to be sub-optimal due to slow tracer kinetics. For example, 6 hrs are required for 5-IA to reach equilibrium in the brain, followed by 2 additional hours of scanning. For challenge studies, a 16+ hr scan day is required. Recent advancements have made possible the synthesis of NCFHEB for use in PET. NCFHEB activity uptake is similar to β2-nAChRs distribution in the brain, with higher uptake in the thalamus (Fig 3) and is superior to the previously discussed radioligands - it has similar affinity for the β_2-nAChRs and it displays faster kinetics making it possible to conduct a single imaging study in 90mins (139).This radioligand is safe for use in humans *in vivo* (140), and the Yale PET Center has initiated its production.

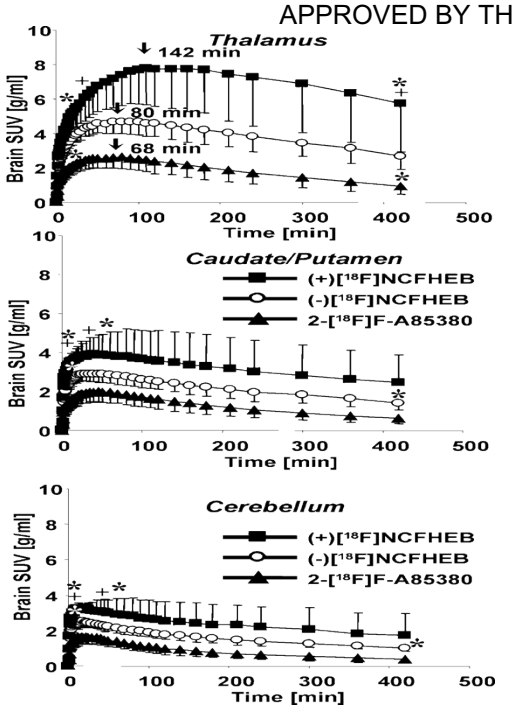

Fig. 3. Kinetics in of [18F]NCFHEB, [18F]NCFHEB and 2-[18F]F-A85380 in left and right thalamus, caudate/putamen, and cerebellum of the porcine brain. Data represent standardized uptake values, SUV, (**6**SD). For the thalamus the time of peak uptake is given. * and denote significant differences between the SUV for the particular time points in between. *P < 0.05 vs. 2-[18F]F-A85380,P < 0.05 vs. [18F]NCFHEB.

In the current proposal, we propose the first steps at assessing NCFHEB binding measure reproducibility in humans and sensitivity to drug-induced changes in endogenous ACh in order to establish the first paradigm to interrogate ACh function *in vivo* in humans. We predict that we can replicate and improve upon the findings obtained with 5-IA SPECT, which will enable future studies to include psychiatric populations through the use of a better tracer (NCFHEB) and a higher resolution method with superior quantification (PET).

Preliminary data

β_2-nAChR involvement in mood disorder. We conducted a preliminary evaluation of β_2-nAChR involvement in MDD in living human depressed and control subjects, as well as in postmortem tissue (141). Twenty–three nonsmokers with MDD (8 acutely depressed and 15 remitted) and 23 age and sex-matched controls each participated in one 5-IA SPECT scan. Receptor availability was lowest in the acute MDD group (amygdala, p=0.02, hippocampus p=0.01, thalamus p=0.04 and mean cortex p=0.04). The remitted MDD group also showed lower receptor availability (p=0.01). However, in post-mortem data where endogenous ACh is not present, there appears to be no difference in receptor availability between depressed (n=15) and age- and sex-matched control (n=15) subjects. Therefore, it is hypothesized that high ACh levels associated with depressed mood competed with 5-IA binding at the β_2-nAChRs, and we thus quantified lower β_2-nAChR availability. Further experimentation is required to confirm these findings.

We are presently conducting a similar evaluation of β_2-nAChR involvement in patients with bipolar disorder (BD). Fifteen 15 depressed BD nonsmokers (5 unmedicated, 10 medicated (41.6 ± 13.1yrs)) and 15 controls (40.8 ± 13.1yrs) participated in one 5-IA SPECT. We found a significant effect of diagnosis (Hotelling's Trace F=4.7, p=0.002) with an average 29% lower β_2-nAChR availability in BD as compared to control subjects in frontal, parietal, anterior cingulate, temporal, and occipital cortices, thalamus, striatum, and cerebellum. There was a negative correlation between symptoms of depression and β_2-nAChR (p=.04) only in unmedicated BD individuals. These baseline receptor availability studies provide important information on the neurochemistry of mood disorders, but limit our interpretation and future directions.

β_2-nAChR involvement in schizophrenia. Smoking and nonsmoking subjects with schizophrenia (n=31) and age-, smoking- and sex-matched comparison subjects (n=31) participated in one [^{123}I]5-IA-85380 single photon emission computed tomography (SPECT) scan to quantify β_2*-nAChR availability. Psychiatric, cognitive, nicotine craving and mood assessments were obtained during active smoking as well as smoking abstinence. There were no differences in smoking characteristics between smokers with and without schizophrenia. Subjects with schizophrenia had lower β_2*-nAChR availability relative to comparison group, and nonsmokers had lower β_2*-nAChR availability relative to smokers. However, there was no smoking by diagnosis interaction. Relative to nonsmokers with schizophrenia, smokers with schizophrenia had higher β_2*-nAChR availability in limited brain regions. In smokers with schizophrenia, higher β_2*-nAChR availability was associated with fewer negative symptoms of schizophrenia and better performance on tests of executive control. Chronic exposure to antipsychotic drugs was not associated with changes in β_2*-nAChR availability in schizophrenia. Conclusions: Although subjects with schizophrenia have lower β_2*-nAChR availability as compared to comparison group, smokers with schizophrenia appear to upregulate in the cortical regions. Lower receptor availability in smokers with schizophrenia in the cortical regions is associated with a higher number of negative symptoms and worse performance on tests of executive function; suggesting smoking subjects with schizophrenia who upregulate to a lesser degree may be at risk for poorer outcomes.

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5-IA SPECT imaging of β_2-nAChR involvement in MCI and early AD. Our colleagues(11) conducted an evaluation of β_2-nAChR involvement in early AD and mild cognitive impairment (MCI). They detected a global decrease in β_2-nAChR as a function of group. No region-specific differences were detected. Due to poor resolution of SPECT, they were constrained to evaluation of larger brain regions and were not able to attain correct information in regions such as the hippocampus. The availability of NCFHEB would considerably increase our ability to measure regional change and to correlate with the clinical state. Furthermore, the availability of a method to quantify the acute response to AChE administration would provide a more functional evaluation of ACh function compared to the static measurement of baseline receptor availability. Imaging ACh changes in the brain in humans. Competition between neurotransmitter and radioligands has provided a very useful method to assess synaptic changes in dopamine, but such an approach has not been applied to ACh. We examined whether 5-IA SPECT is sensitive to increases in extracellular levels of ACh in human subjects, when induced by the administration of physostigmine. Such sensitivity has been previously demonstrated in non-human primates by Fujita and colleagues, who detected a significant displacement of 5-IA in the thalamus after a physostigmine challenge(142). In our study, six healthy subjects participated in one 5-IA SPECT study. We used a bolus plus constant infusion method to establish and maintain a state of binding equilibrium at the level of the receptors. After three 30-min baseline scans at 6h (early interval), physostigmine (1.5mg) was administered IV over 60 min, and nine additional 30-min scans were collected during the next 6h (late interval). We observed a significant reduction in BP_F ($=B_{max}/K_D$; specific binding) after physostigmine (25 ± 15% reduction in cortical regions, 15 ± 11% thalamus, 16 ± 14% in striatum, and 35 ± 34% in cerebellum; $p<.05$; Figures 4 and 5). It has been previously established that, in the absence of challenge, no significant change in 5-IA BP_F is observed during these time intervals (143). These data

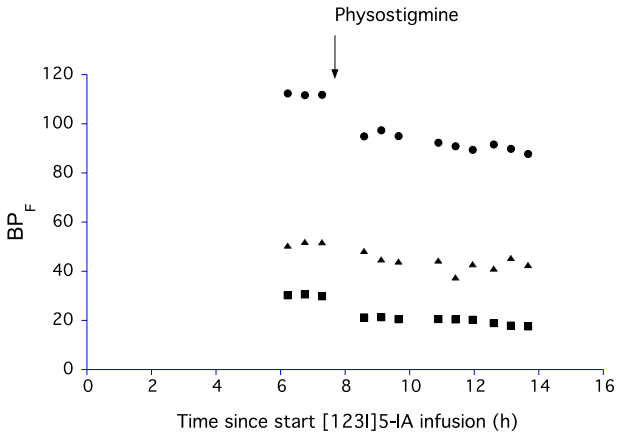

Figure 4. Regional [^{123}I]5-IA-85380 binding potential (BP_F) in thalamus (circles), striatum (triangles) and prefrontal cortex (squares) measured during [^{123}I]5-IA-85380 ([^{123}I]5-IA) constant infusion in healthy volunteers. The first three scans were obtained starting at 6 hours post beginning of tracer infusion, when a state of equilibrium is achieved, and provided the baseline binding potential. Following completion of the baseline scans, the AChE inhibitor physostigmine was administered i.v. (1.5 mg over 1 h, arrow). At the end of the physostigmine infusion, scanning was resumed up to 14 h. BP_F values measured after the physostigmine infusion were significantly reduced compared to the baseline values. As physostigmine has negligible affinity for β_2-nAChRs, the decrease in β_2-nAChR availability might be due to physostigmine induced elevation in ACh in the vicinity of β_2-nAChR receptors and binding competition or allosteric modulation reducing the binding availability of the receptors for the radiotracer.

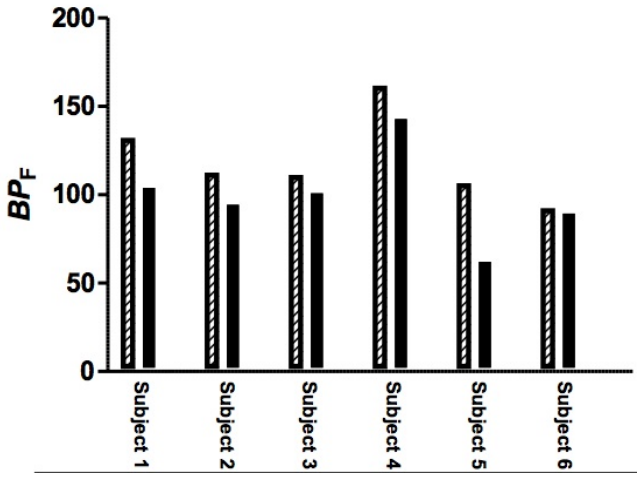

Figure 5. Thalamic β_2-nAChR availability (BP_F, equal to B_{max}/K_D) before (shaded bars) and after (solid bars) physostigmine injection for each subject. Percent displacement of 5-IA for subjects 1-6 was 21%, 18%, 9%, 11%, 41%, 2%, respectively

replicate in humans the previously published observation in baboon (142) and suggest that physostigmine-induced increases in ACh compete with 5-IA for binding to β_2-nAChRs.

Several limitations of these studies should be acknowledged. 1) While bolus plus constant infusion studies have demonstrated no systematic changes in V_T between the early and late intervals in the absence of pharmacological challenges, suboptimal equilibrium conditions have been observed with this slow radiotracer during long scans, which is a source of noise in the evaluation of the physostigmine effect (143); 2) The limited resolution of SPECT and the low count rate toward the end of the infusion is a source of noise. Both of these limitations will be addressed by moving to PET (better resolution, more accurate quantification), by using a fast radiotracer NCFHEB, and a single bolus injection; 3) The physostigmine effect on 5-IA V_T might be due to factors other than increase in synaptic ACh. Although physostigmine has no appreciable affinity for β_2-nAChRs(144) (making a direct displacement by physostigmine unlikely), we cannot rule out that a brain-penetrant metabolite of physostigmine might directly interact with this receptor, or that some other nonspecific factors might be at play. Thus, it is imperative to verify that the effect can also be observed with an AChE inhibitor not chemically related to physostigmine.
It should be clarified that other validations studies will be required before proposing that this paradigm provides a measure of changes in synaptic ACh. Studies in nonhuman primates will be needed to assess the correlation between changes in extracellular ACh measured with microdialysis and decrease in NCFHEB BP. Our collaborators Drs. Laruelle(145) and Carson(146) have demonstrated that in nonhuman primates the magnitude of changes in extracellular DA measured with microdialysis following an amphetamine challenge are correlated with the magnitude of the decrease in striatal DA D_2 receptors BP measured with PET or SPECT, respectively. These experiments provided a critical validation of the molecular imaging measurement of DA tone (147). In the current study, we propose to replicate and extend the human 5-IA SPECT physostigmine data with NCFHEB PET. In future extensions of this work, we will propose combined PET/microdialysis studies in nonhuman primates.

[^{11}C]PHNO PET brain imaging. [^{11}C]PHNO has been developed as a dopamine $D_{2/3}$ agonist ligand for PET brain imaging. Its novelty lies in the measurement of the high affinity, functionally active D_2 receptor, whereas most $D_{2/3}$ ligands are antagonists and thus measure both the high and low affinity D_2 receptors. Dopamine is expected to bind preferentially to the high affinity state D_2 receptors, which are thought to be the functionally important receptors. Typically, the most widely used ligand to measure endogenous dopamine release with PET has been [^{11}C]raclopride, which is an antagonist, and thus measures both high and low affinity D_2 receptors. Differences in brain uptake between the two ligands have been examined with both ligands binding in areas with high $D_{2/3}$ receptors (e.g., caudate and putamen). In addition, [^{11}C]PHNO showed uptake in the ventral striatum and globus pallidus, while [^{11}C]raclopride had uptake in dorsal striatum. Recently, a first report was published on the success of measuring d-amphetamine-induced dopamine release using [^{11}C]PHNO PET and the authors suggest this tracer may have some improvements in measuring endogenous dopamine release over the typical $D_{2/3}$ ligands. They reported a 25% change in striatal BP after amphetamine administration. This is a significantly greater change than obtained with amphetamine-induced dopamine release measured with [^{11}C]raclopride where average changes in striatal BP are between 10-15%.

Amphetamine-induced DA release. Due to the difficulty of obtaining nicotine- or tobacco smoking-induced dopamine release, the majority of PET studies examining changes in synaptic DA levels have relied on amphetamine. Amphetamine, as opposed to nicotine, acts directly at

the DA-ergic system and is a potent reuptake inhibitor of dopamine and a dopamine releaser. While nicotine results in 200% increases in dopamine release as measured with microdialysis, amphetamine results in up to 1000% increases in dopamine release. Therefore, there is a much larger signal to detect, which has been reliably measured in many PET studies. In the current study, we will use amphetamine-induced DA release to measure DA neurotransmission.

3. **Research Plan:** Summarize the study design and research procedures using non-technical language that can be readily understood by someone outside the discipline. Be sure to distinguish between standard of care vs. research procedures when applicable, and include any flowcharts of visits specifying their individual times and lengths.

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3.1 Overall plan:

Aim 1: To evaluate the test-retest reproducibility of NCFHEB binding paramenters in 10 healthy subjects.

CLOSED Aim 2: To determine if β_2-nAChR availability is genetically predisposed in 10 never smokers and 10 smokers at one week of abstinence with NCFHEB.

CLOSED Aim 3: To determine if the adaptive increase in β_2-nAChR availability and change in β_2-nAChR availability over the first month of abstinence in smokers is genetically predisposed (the same 10 smokers from Aim 2) with NCFHEB.

Aim 4: To measure the sensitivity of NCFHEB binding to changes in endogenous ACh levels in up to 50 smoking and 50 nonsmoking subjects.

CLOSED Aim 5: To measure the sensitivity of NCFHEB binding to changes in endogenous ACh levels in up to 10 smoking and 10 nonsmoking subjects with schizophrenia.

Aim 6: To determine the efficacy of a bolus to infusion scan versus a bolus scan of NCFHEB with physostigmine challenge.

CLOSED Aim 7: To determine amphetamine-induced DA release in tobacco smokers during acute withdrawal and in nonsmokers with [^{11}C] PHNO.

CLOSED Aim 8. To determine the degree of occupancy of the β_2*-nAChRs by nicotine after use of an e-cig compared to a tobacco cigarette using [^{18}F]NCFHEB PET neuroimaging in 10 subjects.

3.2 Subject recruitment.
We have an established program to recruit healthy controls. For the present study, subjects will be recruited through our program as well as flyers, public advertisement (newspaper, radio, internet postings), and word of mouth. Dr. Cyril D'Souza has an established program to recruit subjects with schizophrenia. He will recruit them through nearby treatment programs, CMHC, clinicians, word of mouth and advertisements.

3.3 Screening for eligibility.
After completing the informed consent process, subjects will have a physical and neurological examination. The following lab tests will be performed at screening to exclude medical illnesses:

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complete blood count (CBC) and differential, chemistries, kidney function tests (creatinine, BUN, urinalysis), liver function tests, and TSH. A urine drug screen and a pregnancy test (for women) will be done at screening and before radiotracer administration. The psychiatric assessment will include a psychiatric history, a structured clinical interview (SCID), and assessment of subsyndromal depression with the Center for Epidemiological Studies Depression Scan – CES-D and subsyndromal anxiety with Speilberger State-Trait Inventory (STAI).

Aim 1, Aim 2, Aim 4/5, Aim 6, and Aim 7 (nonsmokers)

- smoked < 40 cigarettes in lifetime
- urinary cotinine levels 0-30 ng/mL both at intake evaluation and on scan day

Aims 2, 3 Aim 4/5, Aim 6, Aim 7, and Aim 8 (smokers)

- have a Fagerstrom Test for Nicotine Dependence (FTND) rating of at least 3
- have been smoking cigarettes on a daily basis for at least 1 year
- carbon monoxide levels > 8 ppm during intake evaluation
- plasma nicotine levels > 10 ng/mL during intake evaluation
- plasma cotinine levels of > 50 ng/mL during intake evaluation
- wish or willing to quit smoking for up to 8 weeks (except for Aim 8 for which only nontreatment seeking smoking will be asked to participate who are willing to abstain from smoking for up to 1 week to do the study)

Aim 5 (subjects with schizophrenia)

- have been on stable antipsychotic monotherapy treatment for at least 1 month
- if smoker, willing to abstain from smoking for about 1 week
- if on anxiolytics, willing to abstain on PET scan days

3.4 Assessments:

All participants will be screened initially using a telephone screen that will include questions to evaluate medical history, personal and familial psychiatric and smoking history.

3.4a. General Intake Assessments

1. Demographic Questionnaire This questionnaire will obtain: (1) basic demographic information including age, gender, marital status, employment status, occupation, (2) alcohol/drug history, (3) family history of alcohol/drug use, depression, anxiety, and smoking history.
2. Medical History This questionnaire will obtain a basic medical history (personal and family) including past or current conditions such as neurological, endocrine, cardiovascular, renal, liver, and thyroid pathology. Current body weight and current medications will also be assessed.
3. Medical Assessments will include a physical exam by a state licensed physician (overseen by Dr. David Matuskey), a complete blood count, blood urea nitrogen, creatinine, fasting blood sugar, electrolytes, liver function tests, thyroid function tests (including T_3, T_4, T_3RU, estimated free T4), thyroid stimulating hormone levels, urine toxicology, EKG, and urinalysis**.** Female subjects will have serum pregnancy tests. All EKGs are read by a state licensed cardiologist and all abnormal MRIs will be reviewed by a state licensed neuroradiologist.
4. Illicit Drug/Pregnancy Screen A urine sample will be collected to determine current illicit drug use (for all potential subjects). In addition, urine samples will be collected for the intake

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visit and on each MR and PET scan day to confirm that the subject is not pregnant. Note: the urine pregnancy test will not be required prior to the MRI if the serum pregnancy test was done within 1 week prior to the MR imaging session.
5. Structured Clinical Interview for DSM-IV Axis I Disorders The psychotic screening and depression sections of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID) will be used to determine whether subjects meet exclusion criteria for diagnosis of psychotic disorders and major depression (148).

3.4b. Mood Measures

We may of mood and anxiety at intake and also on the PET scan day including the following.
1. Center for Epidemiological Studies Depression Scale (CES-D) The CES-D (149) is a 20-item self-report instrument, which has been extensively used in both clinical and nonclinical populations to measure the frequency and severity of depressive symptoms over the past week. The CES-D, which has been used to document the severity of depressive symptoms in adults (150) and has been shown to be a sensitive measure of negative affect in smokers (151), will be used in the proposed studies to exclude for presence of major depression, and to measure level of mild depressive symptoms commonly noted in tobacco smokers.
2. Anxiety: The State-Trait Anxiety Inventory (152) is a 40-item, self-report measure, comprised of two subscales. The State-Anxiety scale is 20 items and assesses transitory states characterized by feelings of tension, apprehension, and heightened autonomic reactivity. The Trait-Anxiety scale is 20 items and assesses stable individual differences in anxiety proneness.
3. Impulsivity: Barratt Impulsiveness Scale (BIS; (153)) is a 30 item self-report instrument designed to assess the personality/behavioral construct of impulsiveness.

3.4c. Smoking Measures

We may obtain smoking measures at intake and also on the PET scan day, which may include the following.

1.Fagerstrom Test for Nicotine Dependence (FTND).(154) This will be used to measure the severity of nicotine dependence. It is a 6-item scale with an internal consistency of .61 and its total score is closely related to biochemical measures of intensity of smoking. A score of at least 3 is necessary for inclusion in the study.
2. Smoking History. This questionnaire will assess basic smoking status and history such as number of years smoked, number and length of quit attempts, reasons for quitting, and second hand smoke exposure.
3. Nicotine Withdrawal Checklist.(155) This measures the severity of eight withdrawal symptoms on 5-point Likert scales.
4. Tiffany Questionnaire of Smoking Urges (QSU).(156) The QSU-brief is a 10-item questionnaire that evaluates the structure and function of smoking urges. Subjects indicate on a likert-type scale how strongly they agree or disagree with each statement with a score of 1 (strongly agree) to 7 (strongly disagree). This characterizes 'urges to smoke' into a negative affect related to relief of withdrawal symptoms and positive affect related to expectancy of reinforcement.
5. GLMS – set of questionnaires in which subjects rate sensations related to smoking such as craving on a scale from "no sensation" to "strongest imaginable."
6. LHS – set of questionnaires in which subjects rate hedonic reaction to sensations from smoking on a scale from "most disliked sensation imaginable" to "most liked sensation imaginable."

3.4d Cognitive Measures
We may obtain these measures at baseline and up to two times on the PET days.

1. Cogstate Battery (30 minutes) – This computerized test battery will assess memory and cognition. The tasks may include:

a. International Shopping List Task – a computerized task to assess verbal learning and memory.
b. Groton Maze Learning Task – a computerized task to assess executive function and spatial problem solving.
c. Detection Task – a computerized task to assess psychomotor function and speed of processing.
d. Identification Task – a computerized task to assess visual attention and vigilance.
e. One Card Learning Task – a computerized task to assess visual learning and memory.
f. One Back Task – a computerized task to assess attention and working memory

2. **Probabilistic Reward Task**: The PRT has been successfully used to assess reward responsiveness (166-168). In each trial, subjects choose which of two difficult-to-differentiate stimuli was presented. Stimuli consist of simple cartoon faces (diameter: 25 mm; eyes: 7 mm) presented in the center of the monitor. At the beginning of the trial, the face has no mouth. After a given delay, either a straight mouth of 11.5 mm ("short mouth") or 13 mm ("long mouth") is presented for 100 ms. Subjects are instructed to press an appropriate button to decide whether a long or small mouth had been presented. Unbeknownst to subjects, correct identification of one stimulus ("rich stimulus") is rewarded three times more frequently (*"Correct! You won 20 cents"*) than the other ("lean") stimulus. In healthy controls, this reinforcement schedule leads to a response bias (i.e., a preference for the more frequently rewarded stimulus). The degree of response bias toward the more frequently reinforced alternative will be used for operationalizing sensitivity to reward.

3. **Conditioned Hallucinations Task**: participants' thresholds for detection (75% likelihood of detection) of a tone embedded in white noise will be determined using the maximum-likelihood-based QUEST62 method, with detection and non-detection signaled by button-press. Target auditory stimuli will be of the following easily-discernible63 frequencies: 500, 1000, 1500, 2000, and 2500 Hz. All target auditory stimuli will be paired with a unique visual pattern (colors: white, blue, orange, red, green, matched for luminance and contrast) present for the duration of the auditory stimulus presentation. All auditory and visual stimuli will be randomized across participants and across assessments. Given participant responses, individualized psychometric functions will be estimated, corresponding to likelihood of detection as a function of target stimulus intensity, and from this, estimations of the intensities at which individuals will report detection of the target stimulus at rates of 50% and 25% will be determined. Over twelve blocks, participants will be presented first with stimuli played at their detection threshold, and then increasingly with sub-threshold and absent stimuli. This is meant to foster implicit learning and subsequent testing of the association between light and tone. Participants will respond by button-press, and during the train/test period will hold down response buttons to indicate degree of confidence in their answer, guided by an on-screen visual-analog scale. Conditioned Hallucination trials will be taken to be those during which participants report the presence of the target tone despite its absence.

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4. Cold Pressor Task

Subjects may be asked to participate in the Cold Pressor Task. The cold pressor task (CPT) is a stress task used to measure pain sensitivity and pain tolerance. This task will be used to determine alterations in pain thresholds as a result of nicotine use. Participants will immerse their hand (up to the wrist) for up to 3 minutes in the experimental (ice-cold temperature 0-4°C)) and control (room temperature (20°C)) conditions. Physiological measure (heart rate, blood pressure and subject responses (stress, mood) will be collected 5 minutes before, 1 minute into, and immediately after the CPT.

3.4.e Schizophrenia assessments

Scale for the Assessment of Negative Symptoms (SANS), the Positive and Negative Symptom Scale (PANSS), the Abnormal Involuntary Movements Scale (AIMS), the Montgomery-Asberg Depression Scale (MADRS) for depression to assess any mood or behavioral changes

3.5 Experimental Methods

MRI.

Within approximately 6 months of the PET study, anatomical MRIs will be acquired at the Yale University MRI Center. Subjects will be taken through a ferromagnetic metal detector before entering the scan room. The purpose of the MRI scan is to direct the region of interest placement on the lower resolution PET images. The T1 weighted images will be acquired on a 3 Tesla Siemens Scanner. There will also be an additional resting state scan with subjects in the scanner, eyes open, fixating on a cross. Smokers who participate in Aims 4 and 7 may return for a second MRI with resting state at 4-8 weeks of smoking abstinence.

Physostigmine challenge (same paradigm as HIC# 09100005837)

Physostigmine will be administered as follows. Glycopyrrolate, a cholinergic antagonist that does not have central side-effects, will be administered prior to physostigmine challenge to block physostigmine peripheral side-effects (e.g. nausea). Subjects will receive 200 mcg /ml x 1 ml of glycopyrrolate through an IV. Physostigmine administered i.v. has a short half life of 20 min with peak plasma levels 20-30 min post administration. The same dose of physostigmine will be administered as in the preliminary data: 1.5 mg/hr for 1 hr. Vital signs, including systolic and diastolic blood pressure, heart rate and respiration rate, will be monitored before physostigmine and then at 10, 20, 30, 60 min after the beginning of the infusion and then hourly until the end of the study day. Subjects will be questioned before, during, and after physostigmine challenge about potential adverse reactions typical to this medication (nausea, upset stomach, etc., as in Risks section). [If there is a significant and persistent drop in subjects' heart rate (>15% for at least 1 min), the IV physostigmine infusion will be stopped but the subject will continue to be monitored and will be discharged at the discretion of the study doctor. In emergency situation, PET center protocol will be followed accordingly (on file with HIC).

Amphetamine challenge

Dextroamphetamine sulfate is the dextro isomer of the compound d,l-amphetamine sulfate, a sympathomimetic amine of the amphetamine group. It is an FDA approved drug available for the treatment of narcolepsy and attention deficit hyperactivity disorder (maximum approved total

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daily dose of 5-60 mg). After the first [^{11}C]PHNO scan, dextro-amphetamine (0.5 mg/kg, PO) will be given in increments of 5 or 10 mg, as it is available in 5 mg and 10 mg tablets, and will be rounded down to approximate 0.5 mg/kg total dose without exceeding it. Total dextro-amphetamine dose will not exceed 50 mg per scan. A second transmission and emission scan will be acquired approximately 2.5-3 hours post amphetamine identical to the methods outlined previously. The timing of the second [^{11}C]PHNO administration and subsequent PET scanning (i.e., 2.5-3 hours) corresponds to the time of maximum plasma concentration of amphetamine as stated in the respective FDA-approved product labeling. EKG and frequent BP monitoring will occur throughout the study and until the vital signs are within normal limits. Supplemental oxygen will be provided via nasal cannula if necessary. If the systolic BP reaches or exceeds 200 mmHg for more than 5 minutes, the study doctor will take the appropriate clinical measures in order to lower the BP, which may include phentolamine administration (5 mg IV over 10 min) or other appropriate measures.

Following the post-amphetamine PET scan, subjects will be assessed (EKG and vital signs) by one of the research nurses. Subjects will be discharged when vital signs are within normal limits and when behavioral changes (if any) are found to be not clinically significant by the MD attending to the subject at the PET Center. If subjects experience any adverse events, they will be treated until they become asymptomatic, prior to discharge.

Behavioral response to amphetamine will be measured by self-ratings with a simplified version of the Amphetamine Interview Rating Scale (van Kammen and Murphy, 1975). Four items will be investigated: euphoria ("feel good"), alertness ("feel energetic"), restlessness ("feel like moving") and anxiety ("feel anxious"). Self ratings will be obtained by analog scales at the following times relative to the d-amphetamine administration: -5 minutes, 0, and hourly thereafter until end of the second scan.

Cigarette challenges (same procedures as in protocol HIC 0804003655)

Subjects will participate in one or more PET scans during which they will participate in 4 possible smoking challenges: Smoking an e-cig with no nicotine, an e-cig with a low dose of nicotine (6mg/mL-10mg/mL), an e-cig with a high dose of nicotine (24 mg/mL-36mg/mL), or smoking a regular cigarette. Subjects will participate in a different challenge for each of their PET scans. For the e-cig challenges, subjects will not be aware of the amount of nicotine in the e-cig. Subjects will abstain from smoking for ~1 week prior to each challenge day. After each PET scan, subjects will return to smoking for at least 1 week. They will then need to abstain from smoking for ~1 week prior to participating in their next PET scan. Because of this off-and-on smoking cessation, only non-treatment-seeking smokers will be asked to participate in this study. We have employed this procedure previously for protocol HIC 0804003655.

Use of e-cig: Based on the findings of a recent study that showed use of e-cigs led to similar plasma nicotine levels as tobacco smoking within 5 minutes of use[7], we plan to use an e-Go type e-cig battery (3.3 V, 1000 mAh) with 1.5 ohm dual-coil 510-style cartomizer and a 70/30 propylene glycol vegetable glycerin e-liquid with nicotine concentrations of approximately 0mg/mL, 6mg/mL-10mg/mL, and 24mg/mL-36mg/mL. Subjects will be instructed to puff on the e-cig once every 30 seconds in a manner as they normally would puff on a cigarette for 10 puffs total. A mouthpiece to measure puff topography may be used.

Use of regular cigarette: To standardize research methodology, Camels brand cigarettes will be used for all subjects. This brand is not prevalent with the smokers who participate in our studies and any subject who has used this brand prior will be excluded. Subjects will be instructed to

puff every 30 sec for a total of 10 puffs via the puff device (CreSS from Plowshare Technologies, USA), which provides measures of depth of puff inhalation and volume.
Blood Samples: On PET scan days, blood samples will be collected prior to radiotracer administration and at multiple time points after e-cig and tobacco cigarette use to determine nicotine concentrations in the blood, which will be assayed using reversed-phase HPLC [49].

PET
PET scans may be performed on the High Resolution Research Tomograph (HRRT, 2-3 mm resolution) or another similar camera. Venous catheters will be used for i.v. administration of the radiotracers, venous blood sampling of AChE activity, for the administration of glycopyrrolate and physostigmine, and for venous blood sampling of physostigmine and amphetamine PKs. A radial artery catheter will be inserted by an experienced physician before the NCFHEB PET scans to draw arterial blood samples for metabolite analysis and for determination of the fraction of plasma radioactivity unbound to protein. At the beginning of scan, the subject's head will be immobilized and a transmission scan will be obtained for attenuation correction. PET scans will be acquired using bolus or bolus to infusion administration of up to 8 mCi of [^{18}F]NCFHEB or up to 10 mCi of [^{11}C]PHNO and subjects will be scanned for up to 4 hours. Dynamic images of radioactivity concentration are reconstructed with corrections for attenuation, normalization, random events, scatter, and deadtime. Subject motion is corrected automatically on an event-by-event basis with the Vicra motion tracking system. Vital signs (blood pressure, pulse and respiration) are collected prior to and during each PET scan. Urine pregnancy test will be again administered on the PET scan day prior to the initiation of any imaging procedures. Smoking abstinence, when appropriate, will also be confirmed for smoking subjects prior to PET scanning.
PET scanning will then proceed as following for each aim:

Aim 1. Subjects will be asked to come to the PET center on two separate days to participate in one NCFHEB PET scan each time to assess test retest reproducibility of binding parameters measured with the radiotracer.

CLOSED Aim 2. Subjects will participate in one NCFHEB PET scan day.

CLOSED Aim 3. Subjects from Aim 2 who are able to continue smoking abstinence will be asked to come back for another NCFHEB PET scan after about 4-8 weeks of smoking abstinence.

Aim 4. Baseline NCFHEB PET imaging will be conducted followed by administration of physostigmine. Preferably, this will be done on the same day. However, at times there is not enough radiotracer or subject is not able to tolerate a longer scan day. Therefore, some subjects may complete the study over two separate days (preferably within 1 month apart based on the availability of PET scanning times and subject's schedule).

CLOSED Aim 5. Baseline NCFHEB PET imaging will be conducted followed by administration of physostigmine. Preferably, this will be done on the same day. However, at times when there is not enough radiotracer or when a subject is not able to tolerate a longer scan day. Therefore, some subjects may complete the study over two separate days (preferably within 1 month apart based on the availability of PET scanning times and subject's schedule).

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Aim 6. Subjects from Aim 4 may be asked to come back for another PET scan with physostigmine administration. Up to 8mCi of NCFHEB will be administered as a bolus plus constant infusion for the duration of the scan (up to 4 hours). Once equilibrium is reached (approximately two hours into the scan) glycopyrrolate and physostigmine will be administered and subjects will be scanned for approximately two more hours. A transmission scan will be acquired.

CLOSED Aim 7. Subjects from Aim 4 will be asked to come back for two [^{11}C]PHNO scans with amphetamine administration. Up to 10 mCi of PHNO will be administered. After the first scan, subjects will take amphetamine (0.5mg/kg) by mouth, and will be imaged again with PHNO. If technical difficulties arise the second PET scan will be scheduled as soon as possible (within 5 weeks). In that case, subjects may be asked to re-take amphetamine on the day of the rescheduled PHNO scan, approximately 3 hrs prior to the scan. Amphetamine dose will not exceed 50mg per scan. Subjects will not be allowed to drive home.

CLOSED Aim 8. Subjects who previously participated in Aim 4 or 6, or new subjects may complete this portion of the protocol as long as their yearly radiation dose allows. For Aim 8, subjects will participate in one or more PET scans during which they will participate in 4 possible smoking challenges: Smoking an e-cig with no nicotine, an e-cig with a low dose of nicotine (6mg/mL-10mg/mL), an e-cig with a high dose of nicotine (24 mg/mL-36mg/mL, or smoking a regular cigarette. Subjects will participate in a different challenge for each of their PET scans.. For each scan, up to 8mCi of NCFHEB will be administered as a bolus plus constant infusion for the duration of the scan (up to 4 hours). Once equilibrium is reached (approximately two hours into the scan), subjects will use a cigarette or e-cigarette (order will be predetermined ahead of time and subjects will be told what they will be using for each scan. However, if subjects are using the e-cig they will not be told which nicotine concentration they will receive). A transmission scan will be acquired.

Control subjects may participate in more than 1 aim. For example, nonsmoking subjects may complete Aim 1 and if they choose, participate in Aim 4. Thus, subjects may participate in up to 4 NCFHEB PET scans and up to 2 PHNO PET scans for this protocol per year. Subjects who complete 4 NCFHEB and 2 PHNO scans may return after 365 days to complete more scans under additional aims in this protocol.

3.6 Image analysis.

NCFHEB time activity curves will be extracted for each subject in the following brain regions: amygdala, caudate, cerebellum gray matter, cerebellum white matter, corpus callosum, anterior cingulate, posterior cingulate, frontal cortex, hippocampus, occipital cortex, pallidum, parietal cortex, putamen, temporal cortex, and thalamus. Sabri and colleagues found that NCFHEB distribution volume (VT) can be estimated in humans using the one-tissue compartmental model. (139) We will perform and compare several quantification methods (such as one and two tissue compartment model, Logan, and multilinear analysis) on the test/retest dataset to define the optimal method for V_T derivation. The selected method will be used for the specific aims 2 studies.

β_2-nAChRs are present in all brain regions, so that no practical region of reference can be used to define nonspecific binding. The definition of the nonspecific binding would require displacement studies, which are not proposed in this application. In the thalamus, where the density of B2-nAChR is the most abundant, the contribution of nonspecific binding to the total NCFHEB VT is

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small in rhesus monkey (20%) (unpublished data), so that significant changes in thalamic NCFHEB VT can be reasonably ascribed to changes in specific binding. In other regions such as cortex, the contribution of the nonspecific binding to VT is larger (about 60%, unpublished data), thus global VT changes in these regions should be interpreted carefully.

For **PHNO** scans, The primary outcome measure is the binding potential (BP_{ND}), which in turn is proportional to the available receptor concentration (B_{avail}), given that there is no change in affinity (K_D) and that nondisplaceable (nonspecific and free) uptake does not differ between subjects and studies. We will examine the regions-of-interest listed below with the cerebellum used as a reference region because it is devoid of $D_{2/3}$ receptors.

We obtain an MRI (as previously described) to use as a guide to place our regions of interest. This is because we can define anatomical regions of interest on the MRI, which provides structural information and then we can apply these regions of interest to the PET scans. The PET scans alone are not sufficient to identify brain regions. The PET image sets are aligned and resliced to yield images in the same planes and spatial system as the MRI images using AAL template. Primary ROIs will be caudate, putamen, globus pallidus, substantia nigra, midbrain, thalamus, hippocampus, cingulate, frontal and occipital cortices, as well as raphe and amygdala. Other ROIs will be examined *post hoc* to assess radiotracer binding. Cerebellum will be the reference region.

3.7 Statistical analysis.

3.7 a Power Analysis in Never Smokers: Assuming Hardy-Weinberg equilibrium, the allele frequency predicts that 30 will be homozygous (CC); 25 will be heterozygous (CT) and 5 will be homozygous (TT) for the ANKK1 alleles, and 11 will be homozygous (AA) 29 will be heterozygous (AG) and 20 will be homozygous (GG) for the CHRNA5 alleles. In a one-way ANOVA analysis, sample sizes of 30, 25, and 5 are obtained from the three genotypes of ANKK1 whose means are to be compared. The total sample of 30 subjects achieves 0.96 power to detect differences among the means versus the alternative of equal means using an F test with a 0.05 significance level for thalamus. The size of the variation in the means is represented by their standard deviation which is 17.49. The common standard deviation within a group is assumed to be 32.30. The other powers are 0.95, 0.87, and 0.99 for ANKK1 for the brain regions of striatum, cerebellum and cortex, respectively. We have enough power to detect the effect with the proposed sample size. We aim to recruit at least 10 subjects that are homozygous for TT; if we do not achieve our goals for the rarer genotype by the end of year 3; we will screen subjects prior to imaging them in year 4.

***3.7.b Power Analysis in Smokers*:** The group means of □$_2$-nAChR availability for the heterozogotes of hCV16178933/rs2273504 or hCV15953820/rs2236196 are higher in smokers than non-smokers in the brain regions of striatum, cerebellum and mean cortex, but not thalamus. We can achieve 0.81 power to detect a difference of 11.7 in striatum between the null hypothesis that both group means are 97.5 and the alternative hypothesis that the mean of non-smoking group is 85.8 with estimated group standard deviations of 14.9 and 10.8 and with a significance level (alpha) of 0.05000 using a one-sided two-sample t-test for hCV16178933/rs2273504. The other powers are 0.97, 0.95 for hCV16178933/rs2273504 and for cerebellum and mean cortex, respectively. The differences do not present in the homozygote subjects. We can also achieve 0.98 to 0.99 for hCV15953820/rs2236196 in the brain regions of striatum, cerebellum and mean cortex.

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Genotype quality control – genotyping error and missing genotype We will check the genotypes for the SNPs via the method of departures from Hardy-Weinberg equilibrium (HWE) developed by (157), and excluded genotyping errors as a likely cause of the disequilibrium. Genotyping errors and missing genotype/allele calls will be carefully examined. Those with genotyping errors will be assigned to be missing.

3.8a Contingency Reinforcement for Smoking Cessation (healthy controls)
We plan to image smokers whom have been abstinent from smoking for approximately 1 week (with and without schizophrenia), 2 weeks (controls only) and 6 weeks (controls only). Contingency management techniques have been successfully used to reduce CO levels in non-treatment seeking smokers by a number of investigators (158-160).

Subjects will set a quit date and prior to the quit date they meet with the research staff who will provide them with brief advice on quitting smoking based on AHCPR guidelines (The Smoking Cessation and Clinical Practice Guideline Panel and Staff, 1996). They will be advised about the risks and benefits of quitting smoking and told that they will be monitored daily to ensure abstinence. Subjects will be informed of payment schedules (see below) for CO levels indicating abstinence and also given information on how quit rates in the first week of smoking cessation predicted sustained abstinence. We use CO levels < 11 ppm to define abstinence from cigarettes. During the first week of abstinence we will obtain CO levels from subjects up to twice daily. In addition, we will obtain urine samples once daily to measure cotinine levels (a nicotine metabolite).

For each contingency management appointment, subjects will get $10 if their CO levels are less than 11ppm and urine cotinine levels are less than 100 ng/ml.

3.8b Contingency Reinforcement for Smoking Cessation (subjects with schizophrenia)
Because it is more difficult for smokers with schizophrenia to quit smoking, they may choose to participate in this part of the study as an inpatient at CNRU. This was previously done in our HIC27532. Therefore, smokers with schizophrenia consenting to the study will be admitted to the CNRU, for approximately 1 week prior to PET scanning to ensure abstinence from smoking, this is Day 1 of the study. All antipsychotic medications will be continued. While we will not ask subjects to change antipsychotic medications, anticholinergics agents like cogentin, that interfere with radioligand binding will be suspended for a duration of the study with the approval of the subject's non-research clinician. Patients will not be responsible for paying the costs associated with this admission, whether the subject completes the study or discontinues early. Ratings of psychiatric symptoms and a Battery of Neuropsychological Assessments will be obtained by the research staff from screening until the SPECT scan. In addition, a research staff member will visit the subjects daily for behavioral counseling to assist with smoking cessation while inpatient. Expired air carbon monoxide (CO<10ppm) and daily dipstick-urinary cotinine levels will be monitored to confirm abstinence from smoking. However, in case CO is found >10ppm or positive urine cotinine is detected on the day of PET scan, which means that subject has smoked a cigarette, the scan may be cancelled and subject may be discharged from the study.

3.9 Genotyping

3.9.a Source of DNA DNA will be extracted from blood using standard "salting out" methods. Using these kits we expect to obtain from 10-30 μg of DNA from each sample, with a failure rate of about 3%. Each genotype will require between 1 and 15 ng of DNA, so this expected yield

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will be sufficient for the genotyping described here. Using this method, we have obtained DNA of suitable quality for SNP, STR, and VNTR genotyping. DNA yields from blood are at least an order of magnitude higher than saliva.

3.9.b SNP Genotyping Methods The ABI TaqMan system (model 7900 detection device) will be used for SNP genotyping. This instrument uses probes with two dyes on opposite ends of a target sequence oligonucleotide to recognize SNP polymorphisms. One dye is a reporter dye, the other a quencher. When the probe is intact, the quencher suppresses fluorescence from the reporter; when the quencher and reporter are separated, the reporter emits a fluorescence signal. When the probe hybridizes exactly to its complement, the 5' exonuclease activity of Taq polymerase cleaves the probe and allows the signal to be detected. The Taqman system uses two probes to detect a SNP, one complementary to each allele. An advantage of the Taqman system is that ABI offers detection reagents for many polymorphic systems pre-synthesized and tested, "on demand." Detection reagents for other variants are ordered "on demand" through a user-friendly WWW interface.

3.9.c Quality Assurance for Genotyping In each 96-well DNA set, we include two blank lanes as contamination controls and a CEPH control of known genotype. In addition, at least 8% of genotypes will be repeated and if any discrepancies are noted, the entire cohort will be regenotyped.

3.10 Future directions

As discussed above, the successful demonstration of a significant reduction in NCFHEB V_T by both AChE inhibitors in humans follows preclinical studies in nonhuman primates, aiming at further validation of this paradigm as a noninvasive and functional measure of pre-synaptic ACh function. The availability of this paradigm at Yale and in other centers has the potential to greatly extend our understanding of ACh function in health and disease.

4. **Genetic Testing N/A☐**

A. Describe

i. the types of future research to be conducted using the materials, specifying if immortalization of cell lines, whole exome or genome sequencing, genome-wide association studies, or animal studies are planned

- One 10 mL tube of blood will be collected for DNA for testing of polymorphisms in genes of interest to nicotine dependence including ANKK1 and CHRNA4.

ii. the plan for the collection of material or the conditions under which material will be received

-as above, a blood sample will be collected at intake to test polymorphisms of interest for nicotine dependence.

iii. the types of information about the donor/individual contributors that will be entered into a database

- the genetic polymorphism results will be entered. The results of this testing will be confidential, will not be entered into the subject's medical record, and will not

be made available to the subject.

iv. the methods to uphold confidentiality

- The results of genetic testing in locked file cabinets and by separating the personal identifying information of the subjects from the genetic information.

B. What are the conditions or procedures for sharing of materials and/or distributing for future research projects?

No sharing is planned.

C. Is widespread sharing of materials planned?

No.

D. When and under what conditions will materials be stripped of all identifiers?

It will not.

E. Can donor-subjects withdraw their materials at any time, and/or withdraw the identifiers that connect them to their materials? Yes.
   i. How will requests to withdraw materials be handled (e.g., material no longer identified: that is, anonymized) or material destroyed)?

      Subjects will be informed that their material has been anonymized.

F. Describe the provisions for protection of participant privacy

Risks associated with genetic testing will be minimized by keeping the results of genetic testing in locked file cabinets and by separating the personal identifying information of the subjects from the genetic information.

G. Describe the methods for the security of storage and sharing of materials

The results of genetic testing in locked file cabinets and by separating the personal identifying information of the subjects from the genetic information.

5. Subject Population Provide a detailed description of the targeted population of human subjects for this research project.

Healthy controls and healthy smokers will be recruited from the community through advertisements as approved by the Yale University Human Investigations Committee (HIC). Interested individuals contacting the clinic by phone in response to advertisements are told that the information they give over the phone is written down and discussed by the research team. They are advised that if they do not enroll in research with the clinic the information is destroyed, and that if they do, it becomes part of their research chart. A phone screen is completed after they give verbal authorization. If an individual appears to meet enrollment criteria and is interested in participating, a face-to-face interview is conducted. A release of

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information is obtained for review of any available historical and clinical data. A written authorization form is also obtained from each subject, permitting the research team to use, create, or disclose the subject's PHI for research purposes. The nature of the project, procedures, relative risks and benefits, and alternatives to participation in the project are discussed with the individual. Following this discussion, the individual is given a copy of the consent form to review, and any questions are answered. We will seek written consent from all participants.

Subjects with Schizophrenia will be recruited who are clinically stable, and meet criteria for schizophrenia according to the DSM-IV, and who are between 18-60 years of age, on stable monotherapy antipsychotic treatment and who are able to give written informed consent will be included.

6. Subject Classifications: Will subjects who may require additional safeguards or other considerations be enrolled in the study? If so, identify the population of subjects requiring special safeguards and provide a justification for their involvement.

a. Is this research proposal designed to enroll children who are wards of the state as potential subjects? ☐ Yes ☒ No *(If yes, see Instructions section VII #4 for further requirements)*

7. Inclusion/Exclusion Criteria: What are the criteria used to determine subject inclusion or exclusion?

General inclusion criteria:

- men and women, aged 18-60 years
- who are able to read and write
- who are able to give voluntary written informed consent
- have no current uncontrolled medical condition such as neurological, cardiovascular, endocrine, renal, liver, or thyroid pathology
- have no history of a neurological or psychiatric disorder (DSMIV Axis 1 and 2) other than schizophrenia in schizophrenia subgroup
- have not regularly used any prescription, herbal or illegal psychotropic medications (e.g. antidepressants, antipsychotics, anxiolytics, ecstasy) in the past 6 months (controls) that in the PI's determination puts the subject at increased risk or interferes with the study outcome.
- Subjects with schizophrenia have not used any herbal or illegal substances in the past 6 months (medication inclusion listed below in Aim 5)
- drink less than <21 drinks/week for women and less than <35 drinks per week for men
- have not used marijuana in the past 30 days and have not met criteria for dependence in the past 2 years
- If female, not pregnant or breast feeding
- If female of childbearing age, must use an acceptable method of birth control, as determined by the principal investigator
- do not suffer from claustrophobia or any MR contradictions
- willing to donate blood for genetic studies
- willing to be followed up monthly after study participation via phone or email contact

General exclusion criteria:

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- Presence of acute or unstable medical or neurological illness. Subjects will be excluded from the study if they present with any history of serious medical or neurological illness or if they show signs of a major medical or neurological illness on examination or lab testing including history of seizures, head injury, brain tumor, heart, liver or kidney disease, eating disorder, diabetes.
- Presence of an Axis I diagnosis other than nicotine dependence and schizophrenia (for schizophrenia subgroup) in the past 2 years
- Regular use of any psychotropic drugs including anxiolytics and antidepressants and other over-the-counter medications and herbal products within the last year, and none within the last month for healthy controls per the PI's discretion. The PI will take a number of factors into consideration on a case-by-case basis including type of psychotropic drug used, frequency, and dose.
- For subjects with Schizophrenia, use of SSRI's (Paxil, Prozac Zoloft, Lexapro and Celexa) and use of tricyclic anti-depressants, except for a minimal dose used to treat anything other than depression, per the Investigator's discretion.
- Pregnancy/Breast feeding
- Subjects with a pacemaker or other ferromagnetic material in body.
- Subjects with a sitting pulse rate >100 bpm will be excluded
- Subjects with hypertension defined as sitting systolic blood pressure of >160 mmHg and/or sitting diastolic blood pressure of >100 mmHg will be excluded. Those individuals with hypertension that is well controlled by medication (e.g., within the above mentioned range) are not excluded
- Specifically, we will exclude subjects who have any active clinically significant deviation from the normal range in their electrocardiogram (EKG). However, subjects who have abnormalities in their EKG but the condition has been present for a while and the study cardiologist has evaluated and feels comfortable with the condition, would not be excluded on the basis of their cardiac condition. Examples of conditions that may meet these criteria (e.g., condition has been present for a while) include but are not limited to T-wave abnormalities, atrial fibrillation, prolonged PR interval, and right bundle branch block.
- Subjects with an allergy to salicylates
- Subjects with history of prior radiation exposure for research purposes within the past year such that participation in this study would place them over FDA limits for annual radiation exposure. This guideline is an effective dose of 5 rem received per year.
- Subjects with current, past or anticipated exposure to radiation in the work place
- Blood donation within eight weeks of the start of the study.
- History of a bleeding disorder or are currently taking anticoagulants (such as Coumadin, Heparin, Pradaxa, Xarelto).

8. How will **eligibility** be determined, and by whom?

Eligibility to participate will be determined by the PI of this study after completion of the medical and psychiatric evaluation of the potential participant.

9. Risks: Describe the reasonably foreseeable risks, including risks to subject privacy,

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discomforts, or inconveniences associated with subjects' participation in the research.

Risks from this study include 1) risks associated with radiation exposure, 2) risks associated with MRI, 3) intravenous lines and blood drawing, 4) arterial catheter, 5) nicotine withdrawal symptoms, 6) genetic testing, 7) IV physostigmine, 8) administration of physostigmine to subjects with schizophrenia 9) Risks from D-Amphetamine 10) Risk from E-Cigarettes 11) Risk from Cognitive Testing

1. Risks Associated with Radiation
The Yale University Radioactive Drug Research Committee (YURDRC) will review the use of radiation in this research study, and no subjects will be enrolled until RDRC approval is obtained. This research study involves exposure to radiation from [^{18}F]NCFHEB and [^{11}C]PHNO PET scanning. This radiation exposure is not necessary for medical care and is for research purposes only.

For each individual [^{18}F]NCFHEB PET scan, subjects will receive up to ≤ 8 mCi of [^{18}F]NCFHEB, plus transmission scans. This is equal to an effective dose equivalent of 0.635 rem per injection. For each individual [^{11}C]PHNO PET scan, subjects will receive up to ≤ 10 mCi of [^{11}C]PHNO, plus transmission scans. This is equal to an effective dose equivalent of 0.27 rem per injection.

The maximum amount of radiation per year an individual subject will receive in this study is from up to 4 injections of ≤ 8 mCi each of [^{18}F]NCFHEB, up to 2 injections of ≤ 10mCi each of [^{11}C]PHNO, plus transmission scans. This includes the fact that some subjects may have to come back on a separate day for baseline and physostigmine scans. Four [^{18}F]NCFHEB injections and two [^{11}C]PHNO injections is the maximum each subject may receive from participation in this study.

Although each organ will receive a different dose, the maximum amount of radiation exposure subjects will receive per year from this study is equal to an effective dose equivalent of 3.08 rem for a total of up to 32 mCi of [^{18}F] NCFHEB in 4 injections of ≤ 8mCi each and 20 mCi of [^{11}C] PHNO in 2 injections of ≤ 10mCi each. This calculated value is used to relate the dose received by each organ to a single value.

The amount of radiation subjects will receive in this study is below the dose guidelines established by the FDA and monitored by the Yale University Radioactive Drug Research Committee for research subjects. This guideline sets an effective dose limit of 5 rem per year.

2. MRI
MR carries a risk for subjects who have pacemakers, metal pieces, aneurysm clips, or other contraindications for MR.

Magnetic resonance (MR) is a technique that uses magnetism and radio waves, not x-rays, to take pictures and measure chemicals of various parts of the body. The United States Food and Drug Administration (FDA) has set guidelines for magnet strength and exposure to radio waves, and we carefully observe those guidelines.

Subjects will be watched closely throughout the MR study. Some people may feel uncomfortable or anxious. If this happens, the subject may ask to stop the study at any time and

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we will take them out of the MR scanner. On rare occasions, some people might feel dizzy, get an upset stomach, have a metallic taste or feel tingling sensations or muscle twitches. These sensations usually go away quickly but we will ask subjects to tell the research staff if they have them.

There are some risks with an MR study for certain people. If subjects have a pacemaker or some metal objects inside their body, they may not be in this study because the strong magnets in the MR scanner might harm them. Another risk is the possibility of metal objects being pulled into the magnet and hitting a subject. To reduce this risk we require that all people involved with the study remove all metal from their clothing and all metal objects from their pockets. We also ask all people involved with the study to walk through a detector designed to detect metal objects. It is important to know that no metal can be brought into the magnet room at any time. Also, once subjects are in the magnet, the door to the room will be closed so that no one from outside accidentally goes near the magnet.

We want subjects to read and answer very carefully the questions on the MR Safety Questionnaire related to their personal safety. We will be sure that subjects have read the MR Safety Questionnaire and tell us any information they think might be important.

This MR study is for research purposes only and is not in any way a clinical examination. The scans performed in this study are not designed to find abnormalities. The primary investigator, the lab, the MR technologist, and the Magnetic Resonance Research Center are not qualified to interpret the MR scans and are not responsible for providing a diagnostic evaluation of the images. If a worrisome finding is seen on a subject's scan, a radiologist or another physician will be asked to review the relevant images. Based on his or her recommendation (if any), the primary investigator or consulting physician will contact the subject, inform them of the finding, and recommend that they seek medical advice as a precautionary measure. The decision for additional examination or treatment would lie solely with the subject and their physician. The investigators, the consulting physician, the Magnetic Resonance Research Center, and Yale University are not responsible for any examination or treatment that a subject receives based on these findings. The images collected in this study are not a clinical MR exam and for that reason, they will not be made available for diagnostic purposes.

3. Blood Drawing and IV line Insertion
Drawing blood and inserting an intravenous line (IV) into an arm vein are safe and standard medical procedures. Sometimes a bruise will occur at the puncture site and rarely a blood clot or infection will occur in the vein. Certain individuals may feel light-headed during venipuncture. The volume of blood collected during this study, may include screening laboratories, MRI-and PET scans, will be approximately 32 tablespoons. This is not expected to have any serious negative effects on a study participant.

4. **Arterial Catheter**
On the NCFHEB PET scan days a radial arterial catheter will be inserted. Arterial sampling may be associated with mild-to-moderate pain, hematoma, inflammation, or bruising at the puncture site. If this occurs, these signs and symptoms will dissipate over time, usually 24 to 72 hours after the event. In rare instances blocking of the artery, poor healing, or infection at the catheter insertion site may occur. Certain individuals may feel light-headed during arterial catheter placement.

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5. Nicotine Withdrawal

Smokers that quit smoking may experience symptoms of nicotine withdrawal such as craving cigarettes, mild anxiety, restlessness, irritability, difficulty concentrating, loss of energy, and excessive hunger. These are typical symptoms that people experience when they stop smoking and they can be uncomfortable but they are not life threatening.

6. Genetic Testing

Under some circumstances, it can be a risk for genetic information about the subject to be known. Variation in some genes is known to be directly related to risk for certain illnesses and drug dependence, or may in the future be shown to be related to illness. Since the results of these genetic tests may allow prediction of risk of illness in some cases, we will keep the results confidential (only scientists working on this research project will know the results). We will not make any of our laboratory results available to the subject, nor will we add them to their medical record. (If the participant wants to know their risk for genetic diseases, we will refer them to a genetic counselor.) The DNA samples will not have the subject's name on them.

7. IV Physostigmine

IV physostigmine is a cholinergic agent. The dose proposed in this protocol is less than that used for treatment.

Side-effects from physostigmine include nausea, vomiting, diarrhea, anorexia, dizziness, headache, stomach pain, sweating and dyspepsia, pain, itching, burning, or swelling, or a lump under the skin where the shot is given. Rarely, more serious side-effects may include allergic reaction such as itching or hives, swelling in the face or hands, swelling or tingling in the mouth or throat, chest tightness, trouble breathing; increased watering in the mouth, severe nausea, or vomiting; increase in volume or frequency of urination, or severe diarrhea; seizures; slow heartbeat, dizziness, or fainting. In order to reduce these effects, we will administer glycopyrrolate, prior to physostigmine administration and control food intake. Furthermore, physostigmine will be administered in the presence of a doctor who will monitor side-effects, including reduced heart rate, and will be available throughout the study. In the case of serious side-effects, the study may be terminated and appropriate measures, which may include call to 911, will be taken.

8. Administration of physostigmine and glycopyrrolate to subjects with schizophrenia

There are few studies with Physostigmine in schizophrenia patients (161, 162). It has been used primarily as a probe of cholinergic function to study schizophrenia, tardive dyskinesia and mania. Overall, it appears that physostigmine either improves or has no effects on symptoms in schizophrenia. In a more recent study with the related acetylcholine esterase inhibitor Rivastigmine, there appeared to be no benefit or worsening of either cognitive deficits or symptoms in schizophrenia patients (163, 164). In contrast to the studies with physostigmine and Rivastigmine, Rowntree et al. (165) observed that Diisopropylfluorophosphonate, a centrally active cholinesterase inhibitor, produced worsened symptoms among a 30% of schizophrenia patients studies. The increase in symptoms in contrast to a lowering of symptoms has been interpreted as a rebound phenomenon. In summary, the evidence available suggests that physostigmine will either have no effects or may improve symptoms in schizophrenia patients. If schizophrenia patients do experience any worsening of symptoms we will manage those symptoms as we usually do: with a range of supportive and pharmacological measures.

Since patients with schizophrenia may already be on an anticholinergics e.g., benztropine or triphenhexydl, for the treatment of antipsychotic-induced motor side effects making them more sensitive to the peripheral anticholinergic effects of glycopyrrolate, we will withhold standing

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anticholinergics for 24 hrs prior to each physostigmine test day in those patients who are taking anticholinergics. Withholding standing anticholinergics for 24 hours is likely to be well-tolerated.

9. Risks of oral d-amphetamine
Risks of amphetamine administration include both medical and psychiatric risks.

The frequent somatic side effects of d-amphetamine administration are cardiovascular (hypertension, palpitations, tachycardia, bradycardia, orthostasis). General effects such as sweating, feeling warm or cold, nausea, diarrhea, muscle and abdominal cramping, have been reported frequently. Behavioral effects in this dose range are increased level of alertness, talkativeness, restlessness, agitation, mood changes (usually euphoria) and anxiety. In our experience, these effects are generally transient and well tolerated. This dose of amphetamine has not been reported to induce psychotic symptoms in non-schizophrenic subjects. Infrequently blurred vision, headaches and chest tightness, and changes in EKG have been reported. There is a rare risk of permanent neurological damage and death as a result of cardiac arrest or stroke.

Psychiatric or behavioral side effects: General behavioral effects of amphetamine in this dose range are increased level of alertness, talkativeness, restlessness, agitation, mood changes (usually euphoria) and anxiety. In our experience, these effects are generally transient and well tolerated. This dose of amphetamine has not been reported to induce psychotic symptoms in non schizophrenic subjects and we confirm this observation.

10. Risks of E-Cigarette Use
The physiologic effects of e-cigarette use have been evaluated in human subjects in 9 studies. The following physiologic effects were associated with acute exposure to e-cigarette aerosols:

-Mouth and throat irritation and dry cough at initial use (which decreased in severity with continued use),
-No change in the following biomarkers: complete blood count (CBC), lung function, cardiac function, inflammatory markers, carbon monoxide (CO) level, plasma nicotine level, and heart rate

-Reduced fractional exhaled nitric oxide (FeNO) and increase in respiratory impedance and respiratory flow resistance

Based on available data regarding the short term risks of using e-cigarettes, these products appear to be less harmful than tobacco cigarettes. They will be administered to tobacco smokers only, who are already dependent on tobacco cigarettes.

10. Risk from Cognitive Testing

The risk from Cognitive Testing is very minimal, there is a small risk for subjects who have a history of seizures due to flashing lights in the Conditioned Hallucination Task, however a history of seizures is an exclusion criteria.

11. Minimizing Risks: Describe the manner in which the above-mentioned risks will be minimized.

1. The dose of radiation will be submitted for approval to the **Yale University Radioactive Drug Research Committee (YURDRC)**. All scans will be done in the presence of medical supervision and trained staff in an institution specifically designed to support imaging studies. In the event of serious medical complications, the PET scan facilities have immediate access to or consultation with specialized medical units at the Yale-New Haven Hospital. Preparation of radiopharmaceuticals and performance of PET scans will be by radiochemists, physicians, and technologists of the Department of Diagnostic Radiology, Yale University School of Medicine. These professionals are qualified by training and experience in the safe use and handling of radiopharmaceuticals. Subjects will be asked about their previous radiation exposure and those who have had research exposure within the past year will be excluded if their cumulative annual exposure (including the present study) exceeds FDA limits. The information on the previous radiation exposure of study subjects will be notified to the study doctor.

No PET studies will be performed on pregnant or potentially pregnant women, as confirmed by pregnancy testing during evaluation and on each scan day before initiation of any scan procedures. If subjects are breastfeeding they will not be able to participate in this research study.

2. The risks of bruising, clotting, and infection will be minimized by having venipuncture performed by trained and experienced personnel using aseptic technique. To avoid injury due to fainting, the venous catheters will be inserted when the subjects are recumbent. The blood draws during PET scanning sessions will be obtained from the already inserted catheter, to minimize discomfort.

3. Risks of radial artery cannulation are minimized by having the procedure performed by an experienced physician. Pain is minimized by local anesthesia. Bleeding is prevented by local pressure applied for a minimum of 15 minutes after catheter removal. Subjects will have their hand and finger blood supply examined after arterial cannulation and again following catheter removal. Also, subjects will be asked to abstain from using aspirin or other NSAIDs for 7-10 days prior to arterial line insertion and 7-10 days following arterial line removal. Subjects will be provided a 24 hour emergency physician telephone number to call if they encounter pain, discoloration, numbness, tingling, coolness, hematoma, inflammation, or any other unusual symptoms in the wrist or hand, or fever, chills or drainage from the vascular puncture sites, following the procedure. In addition, if an emergency arises at the time of cannulation or scanning, 911 will be called, and the subject will be sent to the Emergency Department for evaluation and treatment. Nurses will provide the subjects an instruction sheet documenting problems to watch for and procedures to follow should such problems occur. Infection is avoided by adequate cleansing of the skin prior to intravascular line insertion.

4. All subjects will be screened for any metallic objects other MR contraindications that they may be holding or have implanted in their bodies using a questionnaire and all potential subjects with contraindications for MR will be excluded. This questionnaire will be repeated immediately before each measurement to insure that no metallic materials are brought into close proximity of the magnet, where they might be pulled toward the magnet. For additional security, subjects will be taken through a ferromagnetic metal detector immediately before going to the scan room.

Effective screening to exclude subjects who would be placed at a greater risk. This includes medical history, physical examination, and screening studies (blood, urine and ECG) performed before starting studies. A state-licensed physician or an Advanced Practice Registered Nurses

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(APRN's) will conduct all medical screenings.

In case of an adverse event, the PI, under the advice of the study collaborators Drs. Matuskey and Carson, will evaluate the adverse event and determine whether the adverse event affects the Risk/ Benefit ratio of the study and whether modifications to the protocol (at Risks to Subjects) or consent form (at Risks and Inconveniences) are required.

5. **For risks associated with oral d-amphetamine administration**

Medical side effects: Subjects will be screened for absence of significant medical history and current medical conditions with a complete medical history, physical examination, routine blood tests, urine toxicology and EKG. Inclusion in the study will be limited to individuals who are between the ages of 18-55. Patients will be excluded if they have any h/o severe medical or neurological illness, any clinically significant brain abnormality, insulin dependent diabetes, a history of cardiovascular disease, or hypertension. Patients will be excluded if they have recently donated blood. Administration of oral d-amphetamine will take place at the PET center by a research nurse, with a physician on site. The research nurse will report vital signs to the physician prior to administering the amphetamine.

If several of the subject's blood pressure readings are recorded at >100 or <60 for diastolic BP or >160 or <90 for systolic BP while at rest, they will be evaluated by the MD. The study may be cancelled at the discretion of the MD after evaluation. Any automated blood pressure results that are abnormal will be repeated manually. The manual reading will be the official reading. Constant EKG and frequent BP monitoring will occur until the vital signs are within normal limits. If the systolic BP reaches or exceeds 200 mmHg for more than 5 minutes, an infusion of phentolamine (5 mg IV, over 10 min) or other appropriate measures may be initiated to control the blood pressure response. The study physician will be notified if those parameters are reached and he/she will supervise the treatment.

In case of chest pain, chest tightness or other symptoms suggestive of cardiac ischemia, the experiment may be cancelled and an EKG will be obtained to rule out angina (ST segment elevation or depression as compared to the baseline EKG). Appropriate treatment will be initiated.

Upon discharge, patients will be given the phone numbers of the study physicians and will not be allowed to drive home. They will either arrange a ride or a taxi.

For Data and Safety Monitoring Plan templates, see
http://www.yale.edu/hrpp/forms-templates/biomedical.html

Data Safety Monitoring Plan:

1. Personnel responsible for the safety review and its frequency:

The principal investigator will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator (monitor) will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment. Either the principal investigator, the IRB or Safety Monitoring Committee (DSMC) have the authority to stop or suspend the study or require modifications.

2. The risks associated with the current study are deemed moderate for the following reasons:

1. We do not view the risks associated with the __ radiotracers [18F]NCFHEB and [11C]PHNO_ as minimal.
2. We do not view the risks associated with the combined use of _____and_____ as minimal.
3. Given the now established safety and validity of the current _____ in our prior work, we do not view the proposed studies as high risk.
4. Given our experience with the combined co-administration_____, we do not view the proposed studies as high risk.

Although we have assessed the proposed study as one of moderate risk, the potential exists for anticipated and/or unanticipated adverse events, serious or otherwise, to occur since it is not possible to predict with certainty the absolute risk in any given individual or in advance of first-hand experience with the proposed study methods. Therefore, we provide a plan for monitoring the data and safety of the proposed study as follows:

3. Attribution of Adverse Events:
Adverse events will be monitored for each subject participating in the study and attributed to the study procedures / design by the principal investigator Kelly Cosgrove, Ph.D. according to the following categories:

a.) Definite: Adverse event is clearly related to investigational procedures(s)/agent(s).
b.) Probable: Adverse event is likely related to investigational procedures(s)/agent(s).
c.) Possible: Adverse event may be related to investigational procedures(s)/agent(s).
d.) Unlikely: Adverse event is likely not to be related to the investigational procedures(s)/agent(s).
e.) Unrelated: Adverse event is clearly not related to investigational procedures(s)/agent(s).

4. Plan for Grading Adverse Events:

The following scale will be used in grading the severity of adverse events noted during the study:

1. Mild adverse event
2. Moderate adverse event
3. Severe

5. Plan for Determining Seriousness of Adverse Events:

Serious Adverse Events:
In addition to grading the adverse event, the PI will determine whether the adverse event meets the criteria for a Serious Adverse Event (SAE). An adverse event is considered serious if it:

1. is life-threatening OR
2. results in in-patient hospitalization or prolongation of existing hospitalization OR
3. results in persistent or significant disability or incapacity OR
4. results in a congenital anomaly or birth defect OR
5. results in death OR
6. based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition, OR

7. adversely affects the risk/benefit ratio of the study

An adverse event may be graded as severe but still not meet the criteria for a Serious Adverse Event. Similarly, an adverse event may be graded as moderate but still meet the criteria for an SAE. It is important for the PI to consider the grade of the event as well as its "seriousness" when determining whether reporting to the HIIRB is necessary.

6. Plan for reporting serious AND unanticipated AND related adverse events, anticipated adverse events occurring at a greater frequency than expected, and other unanticipated problems involving risks to subjects or others to the IRB

The investigator will report the following types of adverse events to the IRB: a) serious AND unanticipated AND possibly, probably or definitely related events; b) anticipated adverse events occurring with a greater frequency than expected; and c) other unanticipated problems involving risks to subjects or others.

These adverse events or unanticipated problems involving risks to subjects or others will be reported to the IRB within 48 hours of it becoming known to the investigator, using the appropriate forms found on the website.

7. Plan for reporting adverse events to co-investigators on the study, as appropriate the protocol's research monitor(s), e.g., industrial sponsor, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), Protocol Review Committee (PRC), DSMBs, study sponsors, funding and regulatory agencies, and regulatory and decision-making bodies.

For the current study, the following individuals, funding, and/or regulatory agencies will be notified:

☐ All Co-Investigators listed on the protocol.

☐ Safety Monitoring Committee (DSMC)

☐ National Institutes of Health

The principal investigator Kelly Cosgrove, Ph.D. will conduct a review of all adverse events upon completion of every study subject. The principal investigator will evaluate the frequency and severity of the adverse events and determine if modifications to the protocol or consent form are required.

12. Statistical Considerations: Describe the targeted number of subjects and the statistical analyses that support the study design.

Never Smokers: In a one-way ANOVA analysis, sample sizes of 30, 25, and 5 are obtained from the three genotypes of ANKK1 whose means are to be compared. The total sample of 30 subjects achieves 0.96 power to detect differences among the means versus the alternative of equal means using an F test with a 0.05 significance level for thalamus. The size of the variation in the means

is represented by their standard deviation which is 17.49. The common standard deviation within a group is assumed to be 32.30. The other powers are 0.95, 0.87, and 0.99 for ANKK1 for the brain regions of striatum, cerebellum and cortex, respectively. We have enough power to detect the effect with the proposed sample size. We aim to recruit at least 10 subjects that are homozygous for TT; if we do not achieve our goals for the rarer genotype by the end of year 3; we will screen subjects prior to imaging them in year 4.

***Smokers*:** The group means of b_2-nAChR availability for the heterozogotes of hCV16178933/rs2273504 or hCV15953820/rs2236196 are higher in smokers than non-smokers in the brain regions of striatum, cerebellum and mean cortex, but not thalamus. We can achieve 0.81 power to detect a difference of 11.7 in striatum between the null hypothesis that both group means are 97.5 and the alternative hypothesis that the mean of non-smoking group is 85.8 with estimated group standard deviations of 14.9 and 10.8 and with a significance level (alpha) of 0.05000 using a one-sided two-sample t-test for hCV16178933/rs2273504. The other powers are 0.97, 0.95 for hCV16178933/rs2273504 and for cerebellum and mean cortex, respectively. The differences do not present in the homozygote subjects. We can also achieve 0.98 to 0.99 for hCV15953820/rs2236196 in the brain regions of striatum, cerebellum and mean cortex.

Genotype quality control – genotyping error and missing genotype We will check the genotypes for the SNPs via the method of departures from Hardy-Weinberg equilibrium (HWE) developed by (157), and excluded genotyping errors as a likely cause of the disequilibrium. Genotyping errors and missing genotype/allele calls will be carefully examined. Those with genotyping errors will be assigned to be missing.

SECTION III: RESEARCH INVOLVING DRUGS, BIOLOGICS, RADIOTRACERS, PLACEBOS AND DEVICES

If this section (or one of its parts, A or B) is not applicable, state N/A and delete the rest of the section.

. DRUGS, BIOLOGICS and RADIOTRACERS

13. Identification of Drug ,Biologic or Radiotracer: What is (are) the **name(s)** of the drug(s), biologic(s) or radiotracer(s) being used? Identify whether FDA approval has been granted and for what indication(s).

[^{18}F]NCFHEB, IV, radioactivity dose of no more than 8 millicuries for one injection and PET. RDRC approval to use [^{18}F]NCFHEB to image the nAChRs will be obtained from the Yale University RDRC for this protocol.

[^{11}C]PHNO, IV, radioactivity does of no more than 10 millicuries for one injection. [^{11}C]PHNO has been used in humans and has been shown to be *safe and well tolerated after its administration to healthy subjects or patients.* No serious adverse effects are expected from tracer doses, which is one thousand fold ≤ the pharmacological used during the therapeutic trials of PHNO. The Yale RDRC has approved its use at the Yale University PET Center.

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Physostigmine is a parasympathomimetic, specifically, a reversible cholinesterase inhibitor which effectively increases the concentration of acetylcholine at the sites of cholinergic transmission. Its mechanism is to prevent the hydrolysis of acetylcholine by acetylcholinesterase at the transmitted sites of acetylcholine. This inhibition enhances the effect of acetylcholine, making it useful for the treatment of cholinergic disorders and myasthenia gravis. More recently, physostigmine has been used to improve the memory of Alzheimer's patients due to its potent anticholinesterase activity.

d-Amphetamine, dose 0.5 mg/kg, PO to healthy controls so no IND necessary per 21 CFR 312.2(b).

All protocols which utilize a drug, biologic or radiotracer **not** approved by, but regulated by, the FDA must provide the following information:

a. What is the Investigational New Drug (IND) **number** assigned by the FDA?

b. Who holds the IND?

c. All protocols which utilize a radiotracer not approved by, but regulated by the FDA must provide the IND number: _____
Alternatively, use of the investigational radiotracer may be under RDRC/RSC oversight: (check if appropriate)_____

For all investigational radiotracers, attach a copy of the RDRC/RSC application (for radioisotopes used in the PET Center, PET Center personnel may complete this step)
Go to http://rsc.med.yale.edu/login.asp?url=myApps.asp. When you have logged in, complete the application and attach a copy to this submission.

Alternatively, an **exemption from IND filing requirements** may be sought for a clinical investigation of a drug product that is lawfully marketed in the United States. If there is no IND and an exemption is being sought, review the following categories and complete the category that applies (*and delete the inapplicable categories)*:

Exempt Category 1
The clinical investigation of a drug product that is lawfully marketed in the United States can be exempt from IND regulations if all of the following are yes:

i. The intention of the investigation is NOT to report to the FDA as a well-controlled study in support of a new indication for use or to be used to support any other significant change in the labeling for the drug. ☐ Yes ☐ No
ii. The drug that is undergoing investigation is lawfully marketed as a prescription drug product, and the intention of the investigation is NOT to support a significant change in the advertising for the product. ☐ Yes ☐ No
iii. The investigation does NOT involve a route of administration or dosage level or use in populations or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product. ☐ Yes ☐ No
iv. The investigation will be conducted in compliance with the requirements for institutional (HIC) review and with the requirements for informed consent of the FDA regulations (21 CFR Part 50 and 21 CFR Part 56). ☐ Yes ☐ No

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v. The investigation will be conducted in compliance with the requirements regarding promotion and charging for investigational drugs. ☐ Yes ☐ No

Exempt Category 2 (all items i, ii, and iii must be checked to grant a category 2 exemption)

☐ i. The clinical investigation is for an *in vitro* diagnostic biological product that involves one or more of the following (check all that apply):
- ☐ Blood grouping serum
- ☐ Reagent red blood cells
- ☐ Anti-human globulin

☐ ii. The diagnostic test is intended to be used in a diagnostic procedure that confirms the diagnosis made by another, medically established, diagnostic product or procedure; and

☐ iii. The diagnostic test is shipped in compliance with 21 CFR §312.160.

Exempt Category 3

☐ The drug is intended solely for tests in vitro or in laboratory research animals if shipped in accordance with 21 CFR 312.60

Exempt Category 4

☐ A clinical investigation involving use of a placebo if the investigation does not otherwise require submission of an IND.

1. **Background Information:** Provide a description of previous human use, known risks, and data addressing dosage(s), interval(s), route(s) of administration, and any other factors that might influence risks. If this is the first time this drug is being administered to humans, include relevant data on animal models.

[^{18}F]NCFHEB has been administered I.V. to human subjects previously by collaborator Osama Sabri, Ph. D., at the Department of Radiopharmacy, Institute of Interdisciplinary Isotope Research, Leipzig, Germany.

Whole body dosimetry of [18F]-(-)-NCFHEB was performed in volunteers. The subjects were sequentially imaged up to 7h post i.v. injection of 353.7±10.2 MBq of[18F]- (-)-NCFHEB on a SIEMENS Biograph16 PET/CT-system with 9 bed positions (BP) per frame,1.5-6min/BP, CT-attenuation correction and iterative reconstruction. All relevant organs were defined by volumes of interest. Exponential curves were fitted to the time-activity-data. The ODs were calculated using the adult male model with OLINDA. The ED was calculated using tissue weighing factors as published in the ICRP 103/2007. The highest OD was received by the urinary bladder (80.2±37.8), followed by liver (44.7±5.4) and kidneys (38.6±5.1).

Preclinical Characterization of [^{11}C]PHNO
The radiosynthesis of [^{11}C]-(+)-PHNO ([^{11}C]PHNO) has been described in detail. The specificity of [^{11}C]PHNO binding was demonstrated in preclinical studies that included *ex vivo* and *in vivo* studies in rodents and cats. Ex vivo biodistribution studies in rat brain demonstrated that [^{11}C]PHNO crossed the blood-brain barrier readily and had an appropriate regional brain

distribution for a radiotracer that maps dopamine D2 receptors. Recent in vivo imaging studies showed that [^{11}C]PHNO is a D_2/D_3 agonist, but D_3 preferring, and it displayed unusually high binding in globus pallidus (GP), which is not observed with [^{11}C]raclopride (a D_2-like antagonist with the highest binding in dorsal striatum). Most recently, studies showed that 95% of [^{11}C]PHNO binding in substantia nigra (SN) is due to the binding to D3, not to D2. Furthermore, [^{11}C]PHNO showed marked and appropriate sensitivity to both increases and decreases in levels of endogenous dopamine.

PET Imaging with [^{11}C]PHNO in Monkeys at Yale University PET Center

We have successfully prepared [^{11}C]PHNO with high radiochemical purity and high specific activity at the Yale University PET Center radiochemistry laboratory. One test scan and two control-preblock studies were performed in three different rhesus monkeys using [^{11}C]PHNO (Table 1). For the control-preblock studies, injections of [^{11}C]PHNO were delivered as a 2-minute bolus followed by 118 minutes of constant infusion. In the preblock studies, 2 mg/kg of SB-277011, a DA D_3 receptor antagonist, was infused for a total of 3 hours, starting 1 hour before the second scan. Images for the baseline and preblock studies are shown in Fig 1. Changes in radiotracer concentration following the preblock were measured in cerebellum, basal ganglia, and thalamus brain regions. Equilibrium was reached by about 60 minutes post-injection and BP_{ND} was calculated by the equilibrium ratio of regions to cerebellum averaged from 60 to 90 minutes (Table 1). These results indicate that [^{11}C]PHNO is a DA D_3 receptor-preferring radiotracer. Respiratory and cardiovascular functions were closely monitored throughout the PET scans, and no noticeable effects were observed on the respiratory or cardiovascular functions of the animals,

Table 1. Binding potential results from control-preblock experiments of [^{11}C]PHNO

Study	Region	$BP_{control}$*	$BP_{preblock}$*	% Blockade
[^{11}C]PHNO #1	Caudate	4.03	3.18	20%
	Putamen	4.35	4.43	-2%
	Pallidum	3.68	2.32	35%
	Thalamus	1.02	0.39	65%
[^{11}C]PHNO #2	Caudate	3.91	2.72	30%
	Putamen	3.92	3.35	14%
	Pallidum	4.12	1.79	56%
	Thalamus	0.79	0.26	68%

*BP values are calculated from 60 to 90 minutes post-injection

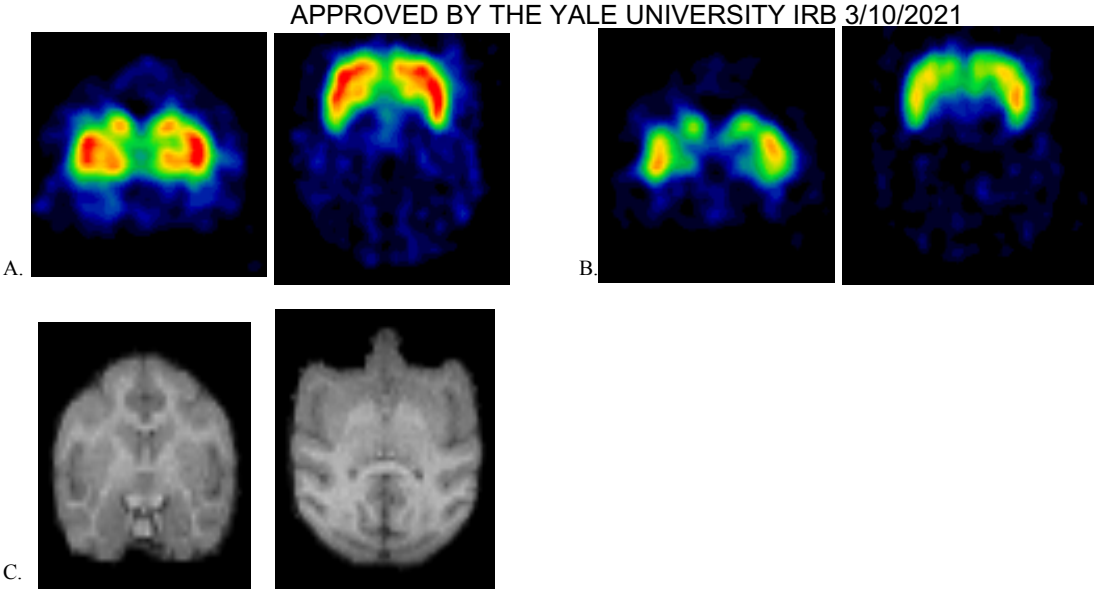

Figure 1. [^{11}C]PHNO uptake summed from 60 to 90 minutes post-injection (A) at baseline and (B) after preblock with the D_3 receptor antagonist SB277011;(C) Corresponding MRI slices are displayed for anatomical reference. The left panels show slices at the level of the striatum and globus pallidus in coronal orientation, while the right panels show the striatum and globus pallidus in transverse orientation. Brain regions with the highest concentrations of [^{11}C]PHNO are shown in red, with progressively lower concentrations displayed in yellow, green, and blue, respectively.

2.2.Dosimetry Studies of [^{11}C]PHNO in Monkeys at Yale PET Center

Radiation dosimetry for [^{11}C]PHNO has been estimated (using the standard MIRD absorption fractions program MIRDOSE 3.1) based on PET scans with a tracer dose of [^{11}C]PHNO on four Rhesus monkeys (2 females and 2 males) to obtain organ concentration and residence time. The percentage of the injected dose per cm^3 was calculated from ROIs drawn on the PET images, and average organ weights for female and male Rhesus monkeys were used to derive the amount of activity per organ as a function of time. The scans were carried out to 120 minutes and the residence times calculated from a trapezoidal approximation of the integral.

Based on these numbers we estimated that the maximum allowable injection dose for [^{11}C]PHNO is 55 mCi per single injection. We tabulated the dosimetry below (all entries refer to scan data from monkeys).

	Absorbed Dose (rad/mCi)		RDRC MAX DOSE	
	mean	SD	max rad/study	
Brain	1.84E-02	2.08E-03	5	2.72E+02
Breasts	6.64E-03	4.08E-04	5	7.53E+02
Gallbladder Wall	4.63E-02	2.66E-02	5	1.08E+02
LLI Wall	9.72E-03	4.12E-04	5	5.14E+02
Small Intestine	2.95E-02	4.77E-03	5	1.70E+02
Stomach Wall	1.32E-02	2.45E-03	5	3.80E+02
ULI Wall	1.20E-02	4.11E-04	5	4.18E+02
Heart Wall	1.43E-02	1.26E-03	5	3.50E+02
Kidneys	9.07E-02	2.83E-02	5	5.51E+01

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Liver	5.56E-02	9.17E-03		5	9.00E+01
Lungs	2.86E-02	7.17E-03		5	1.75E+02
Muscle	7.79E-03	4.49E-04		5	6.42E+02
Ovaries	1.06E-02	4.24E-04		5	4.73E+02
Pancreas	3.21E-02	7.45E-03		3	9.34E+01
Red Marrow	1.10E-02	5.45E-04		5	4.57E+02
Osteogenic Cells	1.23E-02	6.85E-04		3	2.43E+02
Skin	5.89E-03	5.34E-04		5	8.49E+02
Spleen	1.71E-02	3.81E-03		5	2.92E+02
Testes	7.03E-03	6.25E-04		5	7.11E+02
Thymus	7.77E-03	4.70E-04		3	3.86E+02
Thyroid	6.90E-03	7.79E-04		5	7.25E+02
Urinary Bladder Wall	7.01E-02	1.88E-02		5	7.13E+01
Uterus	1.37E-02	2.32E-03		5	3.65E+02
Total Body	1.04E-02	0.00E+00		5	4.81E+02
Effective Dose Equivalent	2.69E-02	4.72E-03			
Effective Dose (rem/mCi)	1.92E-02	2.57E-03	Critical organ	Max Dose (mCi)	
ED with Stom Adjust	2.08E-02	2.81E-03	Kidneys	5.51E+01	

The maximum allowable dose for a single injection is 3000 mR to the whole body, active blood-forming organs, lens of the eye and gonads. The dose to any other organ cannot exceed 5000 mR.
The maximum allowable dose for one year is 5000 mR to the whole body, active blood-forming organs, lens of the eye and gonads. The dose to any other organ cannot exceed 15000 mR.

Critical Organ(s): kidneys

For comparison, the average person in the United States receives a radiation exposure of 0.3 rem (or 300 mrem) per year from natural background sources, such as from the sun, outer space, and from radioactive materials that are found naturally in the earth's air and soil. The dose that a subject will receive from participation in this research study would be less than that obtained in one year from natural sources.

2.3. Clinical Characterization and Safety profile of [^{11}C]PHNO

[^{11}C]PHNO has been used in several PET centers. It was initially developed at the PET Center, University of Toronto, and the safety of its use in humans has been demonstrated.

It states that oral or intravenous administration of **therapeutic doses** of PHNO (0.2 up to 60 mg) produces side effects commonly seen with other dopamine agonists. In addition to orthostasis, the most frequently reported side effects were dystonia and nausea. Drop-outs in these studies were most commonly due to somnolence or orthostatic reactions. None of the studies reported significant drug- related hematological, biochemical, or electrocardiographic changes. In the first 18 healthy subjects studied with **tracer doses** of [^{11}C]PHNO, there were no changes in vital signs, EKG, or biochemical markers.

The most common adverse event reported has been self-limiting transient nausea shortly after injection (well before the peak brain uptake). To date, they have carried out 331 PET scans with

[^{11}C]PHNO (≤ 20 mCi for each injection) in healthy controls and in patients with schizophrenia at the Toronto CAMH PET Centre. Of those, 12% experienced nausea, 1% emesis and 3% reported other effects such as dizziness, headache or a warm sensation. The occurrence of these side effects had no relation with the injected mass (2.3 ± 0.4 μg/kg; range 1.0–5.6 μg/kg), subject's sex, body mass or age.

No serious adverse effects are expected from tracer doses of [^{11}C]PHNO, with a recommended maximum mass less 0.5 nmoles/kg body weight, which is one thousand fold less than the pharmacological dose used during the therapeutic trials of PHNO.

[^{11}C]PHNO has been used in humans at the Yale University PET center in several studies including HIC 0910005822.

Previous studies involving IV physostigmine in humans
At least 2 human studies employing same paradigm as described above have been conducted in human subjects without adverse side effects from the low dose of IV physostigmine administration.

1. Koeppe et al (1999) conducted a PET brain imaging study in 23 healthy volunteers to evaluate a new radiotracer. A subgroup of these individuals received physostigmine at a dose of 1.5mg/hr for one hour. They were pre-treated with glycopyrrolate. No unexpected adverse effects were reported. One subject was reported to feel nauseated but was able to complete the study.
2. Esterlis et al (2013) conducted a SPECT imaging study in 7 healthy volunteers to evaluate whether [^{123}I]5IA radioligand is susceptible to increases in endogenous ACh. Subjects underwent the same physostigmine paradigm as described in #1 above and in the current protocol. One subject vomited as consequence of physostigmine administration. No serious or unexpected side effects were noted.

<u>d-Amphetamine:</u>
Amphetamine is administered to measure changes in [^{11}C]PHNO binding due to dopamine release. This dose was chosen because it is expected to produce a quantifiable displacement of the radiotracer. The risks are outlined in Section VI. Oral and IV d-amphetamine administration to healthy humans and individuals with psychiatric disorders has been safely used in many PET imaging studies. We will use the oral route due to the greater safety and ease of administration.

3. **Source:** a) Identify the source of the drug or biologic to be used.

[^{18}F]NCFHEB and [^{11}C]PHNO will be synthesized at the Yale University PET Center radiochemistry Laboratory. Physostigmine, glycopyrrolate, and d-amphetamine will be provided by the YNHH pharmacy.

b) Is the drug provided free of charge to subjects? ☒ Yes ☐ No
If yes, by whom? PET center

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4. **Storage, Preparation and Use:** Describe the method of storage, preparation, stability information, and for parenteral products, method of sterilization and method of testing sterility and pyrogenicity.

[^{18}F]NCFHEB

Preparation of [^{18}F]-(-)-NCFHEB will be carried out in accordance with procedures and quality specifications contained in local Drug Master File. Briefly, [^{18}F]-(-)-NCFHEB is synthesized by reacting [^{18}F]fluoride with the radiolabeling precursor in an appropriate reaction solvent followed by deprotection of the Boc protecting group. Purification of the radiolabeled product is performed by semi-preparative HPLC, followed by removal of the HPLC eluent via solid-phase extraction, formulation in sterile saline containing ethanol and sterile filtration to yield the ready-for-injection radiotracer [^{18}F]-(-)-NCFHEB in saline solution containing <10% ethanol.
The radioactive product is stored at room temperature and is stable for at least 8 hours after preparation. Pyrogen test is performed for each batch of product. Sterility is achieved by passing the product through a membrane filter for terminal sterilization as the last step in the preparation process, and confirmed by sterility test performed after administration.

[^{11}C]PHNO

The starting material for the production of [^{11}C]PHNO, des-N-propyl-PHNO, is purchased from ABX Advanced Biochemicals. We have successfully prepared [^{11}C]PHNO with high radiochemical purity and high specific activity at the Yale University PET Center radiochemistry laboratory. We have also conducted several PET studies in non-human primates (see the preliminary data section). Radiosynthesis of [^{11}C]PHNO was performed at the Yale University PET Center radiochemistry lab using a four-step synthetic sequence starting from [^{11}C]CO_2. A few minutes before the end of bombardment (EOB), Grignard solution (prepared by dissolving 1 M EtMgBr with an appropriate amount of anhydrous ethyl ether) was introduced into a vessel previously flushed with argon. Purified [^{11}C]CO_2 was swept into the Grignard vessel under stream of argon until activity peaked in the vessel. Then phthaloyl dichloride was introduced and the mixture was allowed to stand for ~20 seconds. 2,6-di-*tert*-butylpyridine was then added and the resulting solution was heated under a stream of argon to distill 11C-propionyl chloride into ice cold solution of the precursor and *N,N*-diisopropylethylamine until activity peaked. The resulting solution was heated at ~80° C for a few minutes. After cooling in ice bath, LAH solution in THF was added to the reaction vial and the mixture was heated for ~5 minutes at ~100° C under a stream of argon to remove THF. After cooling in ice bath, diluted HCl(aq) was introduced and the solution was heated for ~4 minutes at ~100° C. The resulting solution was cooled, diluted with deionized water, then purified by semipreparative HPLC. The product fraction was collected and diluted with ~50 mL deionized water, then passed through a C18 Sep-Pak (Waters). The Sep-Pak was washed with 10 mL of diluted HCl(aq) and eluted with 1 mL ethanol followed by 3 mL of saline. The combined ethanol and saline solution was then passed through a sterile 0.2 □m filter and collected into a vented sterile vial containing 7 mL saline. Purity and specific activity were determined by analytical HPLC.

The radiochemical yield was 19.0 ± 4.2 mCi. The average synthesis time was 66 min. The specific activity was 705 ± 205 Ci/mmol at end of synthesis (EOS). The final formulated product was >99% pure radiochemically. The radioligand is produced according to the local Standard Manufacturing Procedure and to local quality control procedures in effect at the Yale University PET Center. This tracer has been validated at Yale University PET Center Radiochemistry Lab and has been approved for human studies.

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Physostigmine, glycopyrrolate, and amphetamine will be stored and prepared at Yale New Haven Hospital pharmacy.

Check applicable Investigational Drug Service utilized:

☒ **YNHH IDS** ☐ **Yale Cancer Center**
☐ **CMHC Pharmacy** ☐ **West Haven VA**
☒ **PET Center** ☐ **None**
☐ **Other:**

Note: If the YNHH IDS (or comparable service at CMHC or WHVA) will not be utilized, explain in detail how the PI will oversee these aspects of drug accountability, storage, and preparation.

5. **Use of Placebo:** ☒ **Not applicable to this research project**
   If use of a placebo is planned, provide a justification which addresses the following:
   a. Describe the safety and efficacy of other available therapies. If there are no other available therapies, state this.
   b. State the maximum total length of time a participant may receive placebo while on the study.
   c. Address the greatest potential harm that may come to a participant as a result of receiving placebo.
   d. Describe the procedures that are in place to safeguard participants receiving placebo.

6. **Use of Controlled Substances:**
   Will this research project involve the use of controlled substances in human subjects?
   ☒ Yes ☐ No *See HIC Application Instructions to view controlled substance listings*.

   If yes, is the use of the controlled substance considered:
   ☐ Therapeutic: The use of the controlled substance, within the context of the research, has the potential to benefit the research participant.
   ☒ Non-Therapeutic: *Note, the use of a controlled substance in a non-therapeutic research study involving human subjects may require that the investigator obtain a Laboratory Research License. Examples include controlled substances used for basic imaging, observation or biochemical studies or other non-therapeutic purposes. See Instructions for further information.*

Dextro-amphetamine (0.5 mg/kg) will be given by mouth to each subject 3 hours prior to the second [^{11}C]PHNO PET scan in Aim 3.

7. **Continuation of Drug Therapy After Study Closure** ☒ **Not applicable to this project**
   Are subjects provided the opportunity to continue to receive the study drug(s) after the study has ended?
   ☐ Yes If yes, describe the conditions under which continued access to study drug(s) may apply as well as conditions for termination of such access.

   ☐ No If no, explain why this is acceptable.

SECTION IV: RECRUITMENT/CONSENT AND ASSENT PROCEDURES

1. **Targeted Enrollment:** Give the number of subjects

a. targeted for enrollment at Yale for this protocol 150

b. If this is a multi-site study, give the total number of subjects targeted across all sites___

5. **Indicate recruitment methods below.** Attach copies of any recruitment materials that will be used.

☒ Flyers
☒ Posters
☐ Letter
☐ Medical Record Review
☐ Departmental/Center Newsletters
☒ YCCI Recruitment Database
☐ Other (describe):

☒ Internet/Web Postings
☐ Mass E-mail Solicitation
☒ Departmental/Center Website
☒ Departmental/Center Research Boards
☐ Web-Based Clinical Trial Registries
☒ Clinicaltrials.gov Registry (do not send materials to HIC)

☒ Radio
☐ Telephone
☒ Television
☒ Newspaper

6. **Recruitment Procedures:**
   a. Describe how potential subjects will be identified.
   b. Describe how potential subjects are contacted.
   c. Who is recruiting potential subjects?

Subjects will be recruited through flyers, public advertisement (newspaper, radio, internet posting), by word of mouth, contact with community service groups, and clinics and local treatment facilities (the VA Hospital, West Haven, CMHC, the Yale Psychiatric Hospital, Mood Disorders Research Program, the Yale Depression Research Program). The subjects will be asked to call us if they are interested in participating in the research study. The PI, in collaboration with study investigators, is responsible for subject recruitment.

7. **Screening Procedures**

a. Will email or telephone correspondence be used to screen potential subjects for eligibility prior to the potential subject coming to the research office? ☒ Yes ☐ No

b. If yes, identify any health information and check off any of the following HIPAA identifiers to be collected and retained by the research team during this screening process.

HEALTH INFORMATION TO BE COLLECTED:

HIPAA identifiers:

☒ Names
☒ All geographic subdivisions smaller than a State, including: street address, city, county, precinct, zip codes and their equivalent geocodes, except for the initial three digits of a zip code if, according to the current publicly-available data from the Bureau of the Census: (1) the geographic unit formed by combining all zip codes with the same three initial digits contains more than 20,000 people, and (2) the initial three digits of a zip code for all such geographic units containing 20,000 or fewer people is changed to 000.
☒ Telephone numbers
☐ Fax numbers
☒ E-mail addresses
☒ Social Security numbers
☐ Medical record numbers
☐ Health plan beneficiary numbers
☐ Account numbers

☒ All elements of dates (except year) for dates related to an individual, including: birth date, admission date, discharge date, date of death, all ages over 89 and all elements of dates (including year) indicative of such age, except that such ages and elements may be aggregated into a single category of age 90 or older
☐ Certificate/license numbers
☐ Vehicle identifiers and serial numbers, including license plate numbers
☐ Device identifiers and serial numbers
☐ Web Universal Resource Locators (URLs)
☐ Internet Protocol (IP) address numbers
☐ Biometric identifiers, including finger and voice prints
☐ Full face photographic images and any comparable images
☐ Any other unique identifying numbers, characteristics, or codes

8. **Assessment of Current Health Provider Relationship for HIPAA Consideration:** Does the Investigator or any member of the research team have a direct existing clinical relationship with any potential subject?
   ☐ Yes, all subjects
   ☐ Yes, some of the subjects
   ☒ No

   If yes, describe the nature of this relationship.

9. **Request for waiver of HIPAA authorization:** (When requesting a waiver of HIPAA Authorization for either the entire study, or for recruitment purposes only. Note: if you are collecting PHI as part of a phone or email screen, you must request a HIPAA waiver for recruitment purposes.)

   Choose one: For entire study: _____ For recruitment purposes only: ___X___
   i. Describe why it would be impracticable to obtain the subject's authorization for use/disclosure of this data;
   ii. If requesting a waiver of **signed** authorization, describe why it would be impracticable to obtain the subject's signed authorization for use/disclosure of this data;

   By signing this protocol application, the investigator assures that the protected health information for which a Waiver of Authorization has been requested will not be reused or disclosed to any person or entity other than those listed in this application, except as required by law, for authorized oversight of this research study, or as specifically approved for use in another study by an IRB.

Researchers are reminded that unauthorized disclosures of PHI to individuals outside of the Yale HIPAA-Covered entity must be accounted for in the "accounting for disclosures log", by subject name, purpose, date, recipients, and a description of information provided. Logs are to be forwarded to the Deputy HIPAA Privacy Officer.

10. **Required HIPAA Authorization:** If the research involves the creation, use or disclosure of protected health information (PHI), separate subject authorization is required under the HIPAA Privacy Rule. Indicate which of the following forms are being provided:
    ☒ Compound Consent and Authorization form
    ☐ HIPAA Research Authorization Form

11. **Consent Personnel**: List the names of all members of the research team who will be obtaining consent/assent:

Kelly Cosgrove, Ph.D., Irina Esterlis, Ph.D., Stephen Baldassari, M.D., Nicole DellaGioia, Jon Mikael Anderson, Ansel Hillmer, Ph.D., Shivani Bhatt, Sophie Holmes, Ph.D., Sarah O'Grady, Yasmin Zakiniaeiz, Halle Thurnauer, Kim Bielen, Grai Bluez, Gina Creatura, Michael Kleinberg, and Emma Deaso

12. **Process of Consent/Assent:** Describe the setting and conditions under which consent/assent will be obtained, including parental permission or surrogate permission and the steps taken to ensure subjects' independent decision-making.

The consent process is a multistep process, whereby information about the risks and benefits of the study will be provided to potential subjects across several sessions. The number of sessions over which this information will be provided will depend on how well the subject understands and retains the information. The process begins with the subject initiating contact via telephone. The research staff will provide a brief description of the study following which the subject is screened by a member of the research team. Thereafter, potentially eligible candidates are scheduled for a face-to-face interview. The study procedures will be described as a research tool with potential to enhance our knowledge about the brain. Subjects will also be informed of all potential risks of participation. Subjects will be required to read the informed consent form and the investigator will additionally describes the risks and discomforts.

To ensure that the study subject understands the study, the subject will be asked questions about the study procedures and the risks associated with participation. If any concern arises that the study subject did not fully understand the study, the principal investigator (PI) may decide that the subject is not suitable for participation. This process generally takes about one hour. If the subject is still interested after all questions have been answered, the PI or staff member consenting, will ask the subject to sign the informed consent form. Any subject who appears incapable of providing informed consent will be excluded. Subjects will be informed that they can decline to participate in the study without penalty and given the opportunity to withdraw from the study prior to analysis of their data. Following the resolution of any questions, the subjects will be asked to sign the consent form if he/she agrees to participate.

The decision not to participate will not affect an individual's eligibility to participate in future studies, to receive treatment at Yale-New Haven Hospital, or to receive treatment on a private basis from a referring clinician. A copy of the signed consent form will be provided to all participating subjects. For subjects who are not eligible, all PHI will be destroyed.

13. **Evaluation of Subject(s) Capacity to Provide Informed Consent/Assent:** Indicate how the personnel obtaining consent will assess the potential subject's ability and capacity to consent to the research being proposed.

In cases in which capacity is in doubt, the PI will assess the subject's understanding of the study and the subject's capacity to decide to participate.

14. **Documentation of Consent/Assent:** Specify the documents that will be used during the consent/assent process. Copies of all documents should be appended to the protocol, in the same format that they will be given to subjects.

15. **Non-English Speaking Subjects:** Explain provisions in place to ensure comprehension for research involving non-English speaking subjects. Translated copies of all consent materials must be submitted for approval prior to use.

Non-English speaking subjects will not be invited to participate in the studies. All of our materials are in English only, and staff members are fluent in English. Furthermore, cognitive testing is validated in English-speaking subjects only.

16. **Consent Waiver: In certain circumstances, the HIC may grant a waiver of signed consent, or a full waiver of consent, depending on the study.** If you will request either a waiver of consent, or a waiver of signed consent for this study, complete the appropriate section below.
☒ **Not Requesting a consent waiver**
☐ **Requesting a waiver of signed consent**
☐ **Requesting a full waiver of consent**

A. Waiver of **signed** consent: (Verbal consent from subjects will be obtained. **If PHI is collected, information in this section must match Section IV, Question 6**)
☐ **Requesting a waiver of signed consent for <u>Recruitment/Screening</u> only**

If requesting a waiver of signed consent, please address the following:
a. Would the signed consent form be the only record linking the subject and the research?
☐ Yes ☐ No
b. Does a breach of confidentiality constitute the principal risk to subjects?
☐ Yes ☐ No

OR

c. Does the research activity pose greater than minimal risk?
☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** Please note: Recruitment/screening is generally a minimal risk research activity
☐ No

AND

d. Does the research include any activities that would require signed consent in a non-research context? ☐ Yes ☐ No

☐ **Requesting a waiver of signed consent for the <u>Entire Study</u> (**Note that an information sheet may be required.)

If requesting a waiver of signed consent, please address the following:
a. Would the signed consent form be the only record linking the subject and the research?
☐ Yes ☐ No
b. Does a breach of confidentiality constitute the principal risk to subjects?
☐ Yes ☐ No

OR

c. Does the research pose greater than minimal risk? ☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** ☐ No

AND

d. Does the research include any activities that would require signed consent in a non-research context? ☐ Yes ☐ No

B. **Full waiver** of consent: (No consent from subjects will be obtained for the activity.)

☐ **Requesting a waiver of consent for Recruitment/Screening only**

a. Does the research activity pose greater than minimal risk to subjects?

☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** Please note: Recruitment/screening is generally a minimal risk research activity

☐ No

b. Will the waiver adversely affect subjects' rights and welfare? ☐ Yes ☐ No

c. Why would the research be impracticable to conduct without the waiver?

d. Where appropriate, how will pertinent information be returned to, or shared with subjects at a later date?

☐ **Requesting a full waiver of consent for the Entire Study (Note: If PHI is collected, information here must match Section IV, question 6.)**

If requesting a full waiver of consent, please address the following:

a. Does the research pose greater than minimal risk to subjects? ☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** ☐ No

b. Will the waiver adversely affect subjects' rights and welfare? ☐ Yes ☐ No

c. Why would the research be impracticable to conduct without the waiver?

d. Where appropriate, how will pertinent information be returned to, or shared with subjects at a later date?

SECTION V: PROTECTION OF RESEARCH SUBJECTS

Confidentiality & Security of Data:

a. What protected health information (medical information along with the HIPAA identifiers) about subjects will be collected and used for the research?

Required private identifiable information about individuals, such as their medical history, current medications, psychiatric problems, and family history, will be collected by research staff and be used for research purposes and charting after consent is obtained.

b. How will the research data be collected, recorded and stored?

The data are collected and recorded by trained research personnel. The data will be recorded on Excel spreadsheets that will be saved onto a server or will be in the form of questionnaires that are filled out by the subject or the researcher. These paper research materials containing confidential information are stored in locked filing cabinets. Additional brain data is collected during the brain imaging scans by trained technologists and is stored on password-protected and encrypted computers with identifying information carefully in compliance with HIPAA regulations.

c. How will the digital data be stored? ☐ CD ☐ DVD ☐ Flash Drive ☐ Portable Hard Drive ☒ Secured Server ☐ Laptop Computer ☐ Desktop Computer ☐ Other

d. What methods and procedures will be used to safeguard the confidentiality and security of the identifiable study data and the storage media indicated above during and after the subject's participation in the study?

Do all portable devices contain encryption software? ☐ Yes ☐ No
If no, see http://hipaa.yale.edu/guidance/policy.html

All staff members that come into contact with the data are fully trained to the current HIPAA regulations and are informed as to the proper use of all data.

Identifiable paper information is kept in locked file drawers and password protected computer files. Results are published as group data without the use of characteristics that would identify individual subjects. We quote information only by number in conference discussions, scientific reports, or publications, in order to maintain anonymity.

Identifiable research data, including recruitment and screening information and code keys, are stored on a secure database located on the internal PET Center Network. The PET network is protected by a Cisco PIX firewall operated by ITS. All research data are backed up nightly to a Dell PV-136T library wit 4 IBM Ultrium-TD2 tape drives using the backup software Legato Networker 7.3 from EMC. Human subjects enrolled in the study are assigned a subject-specific random identifier. Subject identifiers and the means to link the subject names and codes with the research data are stored in separate locations within the database. The software of the database limits the ability to connect the random identifier to the actual subject identification information to research team members only. Access to the database is password protected and each research team member is required to have a unique ID and password to gain access to the database. Authorized users employ their netid and authentication is performed using Yale's central authentication server. Users always access research data through the random identifier only. Direct identifiers belonging to subjects who withdraw from the study, will be stripped from the key.

e. What will be done with the data when the research is completed? Are there plans to destroy the identifiable data? If yes, describe how, by whom and when identifiers will be destroyed. If no, describe how the data and/or identifiers will be secured.

The data will be stored in locked filing cabinets and on the password-protected secure database on the internal Yale University PET Center Network for at least 7 years, accessed only by authorized personnel.

f. Who will have access to the protected health information (such as the research sponsor, the investigator, the research staff, all research monitors, FDA, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), SSC, etc.)? (please distinguish between PHI and de-identified data)

The investigator and research staff (e.g., PET center nuclear technologists, recruiters) will have access to the PHI only on as needed to know basis. The FDA may also have access to the PHI.

g. If appropriate, has a Certificate of Confidentiality been obtained?

NA

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h. Are any of the study procedures likely to yield information subject to mandatory reporting requirements? (e.g. HIV testing – reporting of communicable diseases; parent interview - incidents of child abuse, elderly abuse, etc.). Please verify to whom such instances will need to be reported.

No.

SECTION VI: POTENTIAL BENEFITS

Potential Benefits: Identify any benefits that may be reasonably expected to result from the research, either to the subject(s) or to society at large. *(Note: Payment of subjects is not considered a benefit in this context of the risk-benefit assessment.)*

There are no direct benefits to the nonsmoking subjects for participating in this study. Smoking subjects receive smoking cessation support to abstain from tobacco smoking. This research will benefit scientific knowledge by contributing to the understanding of the use of PET imaging in tobacco addiction and schizophrenia. This may have clinical application in the future.

SECTION VII: RESEARCH ALTERNATIVES AND ECONOMIC CONSIDERATIONS

1. **Alternatives:** What alternatives are available to the study subjects outside of the research?

The alternative to participation in this research protocol is to not participate. Subjects will be informed that they are free to choose not to participate and, if they do agree to become a subject, they will be free to withdraw from the study at any time during its course. They will also be informed that if they choose not to participate or if they withdraw, it will not adversely affect their relationship with their doctors or the hospital (see attached Consent Form).

2. **Payments for Participation (Economic Considerations):** Describe any payments that will be made to subjects and the conditions for receiving this compensation.

The subjects will be compensated for their time commitment and inconveniences necessary for completing the study. Subjects will have no financial responsibilities for any portion of the study. For all Aims compensation may be $550 for each NCFHEB PET scan, $350 for each PHNO PET scan, $50 for each arterial line placement and $50 for each MRI scan and $40 for cognitive testing at baseline. Subjects who participate in the Probabilistic Reward Task may also be compensated for the amount that they "win" during the task, up to $60. Subjects may also receive an extra $10 for each Cold Pressor Task that they participate in. Subjects who complete the Conditioned Hallucinations Task (Hearing Task in the Consent) will receive $75. Subjects will be paid either by check, and are advised to allow 4-6 weeks for receipt of payment, or they will be given a credit card or cash. In addition, subjects will be provided with a light meal, at the end of the PET imaging day. Reasonable transportation costs will be reimbursed. Receipts must be submitted. If participation in the PET Scan has already begun, then compensation will be based on involvement in the study, and will be up to the discretion of the PI.
Smokers will also receive $10 for each contingency management appointment at which their carbon monoxide and urine cotinine levels show that they have not smoked. Subjects can decide if they want to receive this amount at each appointment in cash, or receive it all at the end of the study in the form of a credit card or check or cash. Smokers will receive an additional $100

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bonus for completing the study. Aim 6 subjects will receive an additional $100 on PET day because of the longer scan day they will encounter as a result of the bolus to infusion scan. Subjects with schizophrenia: Because smoking subjects with schizophrenia will be asked to participate in the smoking cessation part of the study as inpatient, a different payment schedule was devised (as in HIC 27532). Subjects will be paid $25 for first day of smoking cessation, $50 for second, $75 for third, etc (increments of $25 for each successful day, for up to $525). Thus, smokers with schizophrenia may earn up to $1325 for smoking cessation, PET and MRI scans. Nonsmokers with schizophrenia may earn up to $800.

Cancellations: If a PET scan should get cancelled for a reason outside of the subject's control (i.e. radiotracer synthesis failure) the subject will be paid $50 minimum, or a higher amount not to exceed the payment for a full scan day. The amount of the payment for cancellation will be based on the subject's length of participation on that scan day prior to the cancellation, and will be up to the discretion of the PI.

3. **Costs for Participation (Economic Considerations):** Clearly describe the subject's costs associated with participation in the research, and the interventions or procedures of the study that will be provided at no cost to subjects.

There will be no costs to subjects related to participation in this research intervention.

4. **In Case of Injury:** This section is required for any research involving more than minimal risk.
    a. Will medical treatment be available if research-related injury occurs?
    b. Where and from whom may treatment be obtained?
    c. Are there any limits to the treatment being provided?
    d. Who will pay for this treatment?
    e. How will the medical treatment be accessed by subjects?

Medical treatment will be offered to the subjects for any physical injuries that they receive as a result of participating in this research. However, the subject or his/her insurance company is responsible for the cost. Federal regulations require that subjects be told that if they are physically injured, no additional financial compensation is available.

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YALE UNIVERSITY
HUMAN INVESTIGATION COMMITTEE

e-Application to Involve Human Subjects in Biomedical Research
100 FR 1e (2012-1)

For use with Electronic Protocol Submissions Only

HIC Protocol Number: 2000023470

Title of Research Project: Imaging Tobacco Smoking Withdrawal using [^{11}C]PHNO	
Principal Investigator: Kelly Cosgrove, Ph.D.	**Yale Academic Appointment:** Associate Professor
E-mail: Kelly.cosgrove@yale.edu	
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E-mail:	
Campus phone:	
Faculty Advisor: *(required if PI is a student, resident, fellow or other trainee)*	
Yale Academic Appointment:	
Email:	
Campus Phone:	

Investigator Interests:

Does the principal investigator, or do any research personnel who are responsible for the design, conduct or reporting of this project or any of their family members (spouse or dependent child) have an incentive or interest, financial or otherwise, that may affect the protection of the human subjects involved in this project, the scientific objectivity of the research or its integrity? Note: The Principal Investigator (Project Director), upon consideration of the individual's role and degree of independence in carrying out the work, will determine who is responsible for the design, conduct, or reporting of the research.

See Disclosures and Management of Personal Interests in Human Research
http://www.yale.edu/hrpp/policies/index.html#COI

☐ Yes x No

Do you or does anyone on the research team who is determined by you to be responsible for the design, conduct or reporting of this research have any patent (sole right to make, use or sell an

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invention) or copyright (exclusive rights to an original work) interests related to this research protocol?

☐ Yes x No

If yes to either question above, list names of the investigator or responsible person:

The Yale University Principal Investigator, all Yale University co-investigators, and all Yale University individuals who are responsible for the design, conduct or reporting of research must have a current financial disclosure form on file with the University's Conflict of Interest Office. Yale New Haven Hospital personnel who are listed as con-investigators on a protocol with a Yale University Principal Investigator must also have a current financial disclosure form on file with the University's Conflict of Interest Office. If this has not been done, the individual(s) should follow this link to the COI Office Website to complete the form: http://www.yale.edu/coi/

NOTE: The requirement for maintaining a current disclosure form on file with the University's Conflict of Interest Office extends primarily to Yale University and Yale-New Haven Hospital personnel. **Whether or not they are required to maintain a disclosure form with the University's Conflict of Interest Office, all investigators and individuals deemed otherwise responsible by the PI who are listed on the protocol are required to disclose to the PI any interests that are specific to this protocol.**

Billing Information: IRB Review fees are charged for projects funded by Industry or Other For-Profit Sponsors. If this study is funded by Industry or Other For-Profit Sponsor, provide the Name and Address of the Sponsor Representative to whom the invoice should be sent. ***Note: the PI's home department will be billed if this information is not provided.***

Send IRB Review Fee Invoice To:

Name:
Company:
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SECTION I: GENERAL INFORMATION

1. **Performing Organizations:** Identify the hospital, in-patient or outpatient facility, school or other agency that will serve as the location of the research. Choose all that apply:

a. Internal Location[s] of the Study:

☒ Magnetic Resonance Research Center (MR-TAC)
☐ Yale Cancer Center/Clinical Trials Office (CTO)
☐ Yale Cancer Center/Smilow
☒ Yale-New Haven Hospital
☐ Specify Other Yale Location:
☒ Yale University PET Center
☐ YCCI/Church Street Research Unit (CSRU)
☐ YCCI/Hospital Research Unit (HRU)
☒ YCCI/Keck Laboratories
☐ Cancer Data Repository/Tumor Registry

b. External Location[s]:

☐ APT Foundation, Inc.
☐ Connecticut Mental Health Center
☒ Clinical Neuroscience Research Unit (CNRU)
☐ Haskins Laboratories
☐ John B. Pierce Laboratory, Inc.
☐ Veterans Affairs Hospital, West Haven

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☐ Other Locations, Specify: ☐ International Research Site (Specify location(s)):

2. **Probable Duration of Project:** State the expected duration of the project, including all follow-up and data analysis activities.

   5 years

3. **Research Type/Phase: (Check all that apply)**
   a. **Study Type**
   ☒ Single Center Study
   ☐ Multi-Center Study
   Does the Yale PI serve as the PI of the multi-site study? Yes ☐ No ☐
   ☐ Coordinating Center/Data Management
   ☐ Other:

   b. **Study Phase** ☒ **N/A**
   ☐ Pilot ☐ Phase I ☐ Phase II ☐ Phase III ☐ Phase IV

4. Is this study a clinical trial? Yes ☒ No ☐

NOTE the current ICMJE (International Committee of Medical Journal Editors) definition of a clinical trial: "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes." Health-related interventions include any intervention used to modify a biomedical or health-related outcome (for example, drugs, surgical procedures, devices, behavioral treatments, dietary interventions, and process-of-care changes). Health outcomes include any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events"

If yes, where is it registered?
Clinical Trials.gov registry ☒
Other (*Specify*)

*Registration of clinical trials **at their initiation** is required by the FDA, NIH and by the ICMJE.*

If this study is registered on clinicaltrials.gov, there is new language in the consent form and compound authorization that should be used.

For more information on registering clinical trials, including whether your trial must be registered, see the YCCI webpage, http://ycci.yale.edu/researchers/ors/registerstudy.aspx or contact YCCI at 203.785.3482)

5. Will this study have a billable service as defined by the Billable Service Definition?
Yes ☐ No ☒
If you answered "yes", this study will need to be set up in Patient Protocol Manager (PPM) http://medicine.yale.edu/ymg/systems/ppm/index.aspx

6. Are there any procedures involved in this protocol that will be performed at YNHH or one of its affiliated entities? Yes _ __ No X *If Yes, please answer questions a through c and note*

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instructions below. If No, proceed to Section II.

a. Does your YNHH privilege delineation currently include the **specific procedure** that you will perform?

b. Will you be using any new equipment or equipment that you have not used in the past for this procedure?

c. Will a novel approach using existing equipment be applied?

If you answered "no" to question 6a, or "yes" to question 6b or c, please contact the YNHH Department of Physician Services (688-2615) for prior approval before commencing with your research protocol.

SECTION II: RESEARCH PLAN

1. **Statement of Purpose:** State the scientific aim(s) of the study, or the hypotheses to be tested. Tobacco smoking is one of the only leading causes of death that is 100% preventable. While most smokers express a desire to quit smoking, only ~6% achieve abstinence in a given year and the majority relapse in the first 2 weeks. This highlights the acute withdrawal period as the critical window in maintaining abstinence. Nicotine is the primary addictive chemical in tobacco smoke and exerts its initial reinforcing effects through the mesolimbic dopamine (DA) system(1). Specifically, nicotine binds to beta2 subunit-containing nicotinic acetylcholine receptors (beta2*-nAChRs) located throughout the brain and this leads to widespread changes in neurotransmitter levels including dopamine (DA), and upregulation, i.e., increase in number, of beta2*-nAChRs. beta2*-nAChRs are also responsible for controlling the dynamic range of DA release, with chronic nicotine administration or deletion of the beta2*-nAChRs resulting in reduced DA release(2). This suggests that chronic smokers may have blunted DA release compared to nonsmokers and that changes in DA may be tied to other neurochemical alterations in the brains of smokers. However, the basic dopaminergic mechanisms involved in withdrawal and relapse in tobacco smokers remain unknown.

We have a radiotracer at the Yale PET center that will allow us to measure dopaminergic neurotransmission in the brains of smokers. The radiotracer is called [^{11}C]PHNO and it has advantages over other dopamine D2 receptor ligands because it is an agonist and measures the high affinity, functionally active D2 receptors and not the low affinity D2 receptors. This has been recently shown to produce an increased sensitivity over other PET ligands to measure changes in synaptic dopamine levels and thus provides a novel paradigm to investigate drug-induced dopamine release, such as amphetamine-induced dopamine release. We have preliminary data suggesting tobacco smokers at approximately 2 weeks of abstinence have blunted DA release compared to nonsmokers. The overall goal of the study is to measure amphetamine-induced DA release during early and prolonged withdrawal in smokers (vs. nonsmokers) and to examine relationships to relapse and other clinical correlates of tobacco smoking.

Aim 1. To determine amphetamine-induced DA release in nonsmokers and in tobacco smokers during acute and prolonged withdrawal. We will scan 50 Healthy Smokers and 50

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Non Smoker healthy controls. Each tobacco smoking subject will participate in up to 4 [^{11}C]PHNO PET scans, one pair of scans during the first 2 weeks of smoking abstinence and, for those who remain abstinent, the option to have another pair of scans at 2-8 weeks abstinence. We aim to have at least one week in between sets of scans. Ideally, two PET scans will be carried out in the same day. We first obtain a baseline scan, then approximately three hours before the second scan, amphetamine (0.5 mg/kg, PO) will be administered. Nonsmokers will only be asked to participate in one set of scans. We hypothesize that smokers at 1-2 weeks of withdrawal will have amphetamine-induced DA release that is blunted compared to healthy nonsmokers. We also hypothesize that the smokers who are scanned again at 2-8 weeks withdrawal will exhibit some 'normalization' of the dopamine system and will have a larger amphetamine-induced DA release compared to their first scan. We hypothesize that smokers with amphetamine-induced DA release similar to nonsmokers, or that increases over time, will have better abstinence outcomes.

2. **Background:** Describe the background information that led to the plan for this project. Provide references to support the expectation of obtaining useful scientific data.

Tobacco Smoking: There are enormous costs to society and to individuals from tobacco smoking addiction and related disease, e.g., ~80% of smokers will die from a smoking related disease. Smokers are clearly aware of the negative consequences; but 20% of the adult American population continues to smoke. Most smokers want to quit smoking and make yearly quit attempts, but only 6% are successful in a given year and the vast majority cannot quit for more than 2 weeks, *even with all of the currently available treatments*. <u>We do not know how to help smokers remain abstinent.</u> The behavioral manifestations of withdrawal symptoms are clear – cognitive dysfunction, intense craving, irritability, anxiety, and bad mood – but because tobacco smoking is also a brain-based addiction, it is imperative that we uncover the molecular mechanisms that drive these symptoms during acute and prolonged withdrawal. Understanding the time course of the molecular changes that occur over withdrawal and determining relationships between neurochemistry, clinical symptoms, and their predictive relationship to relapse will achieve several goals: 1) provide to the public a greater understanding of the neurobiological basis of tobacco smoking withdrawal, e.g., it is not just a "bad habit"; 2) provide the public and clinicians neurochemical data on how long the brain needs to recover from the addiction; 3) inform the field about directions we should take in terms of targeting new drug development and in creating more easily obtained biomarkers or "proxies" of dysfunctional neurotransmission over the first few months of withdrawal, to personalize treatment selection consistent with a smoker's individual neurochemistry.

How does the dopaminergic system regulate tobacco smoking and withdrawal? A substantial literature demonstrates that nAChRs and the cholinergic system dynamically control the mesolimbic DA system by enhancing, inhibiting and filtering striatal DA release(3, 4). Nicotine releases DA by binding to β_2*-nAChRs located on the mesolimbic DA neurons in the ventral tegmental area, resulting in neuronal firing and DA release in the nucleus accumbens and dorsal caudate **(5)**. β_2*-nAChRs are also critical for the reinforcing(6) and motivational effects of nicotine, e.g., tying cues to drug consumption(7). Nicotine also "filters" DA release by modifying the sensitivity of DA synapses through its desensitization of the nAChRs. As reviewed in Exley and Cragg (2008)(3), nicotine promotes DA release through both burst and tonic activity. Nicotine functions as an agonist and also as an antagonist via its desensitization/blocking actions and both may ultimately enhance DA neurotransmission. Thus, β_2*-nAChRs are responsible for controlling the dynamic range of DA release. A recent

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preclinical study reported that chronic nicotine administration or deletion of the β_2*-nAChRs (i.e., β_2* knockout) reduced acute DA release(2). This suggests that chronic smokers may have a blunted stimulus-induced DA release compared to nonsmokers; however, this has not been examined. Several PET studies have demonstrated blunted amphetamine-induced DA release in the ventral striatum in both cocaine and alcohol dependent populations at approximately 2 weeks of withdrawal vs. controls(8-10). In the cocaine-dependent subjects, those with a more blunted drug-induced DA release responded less well to a behavioral treatment that incorporated positive reinforcement(10). Their findings suggest that individuals with dysfunctional DA transmission are not able to "switch" well from drug-reinforced behavior to more natural alternative rewards(8, 10), and this is supported by a preclinical study in which rats with a lesioned nucleus accumbens displayed an inability to choose greater magnitude delayed rewards vs. immediate rewards of lesser value(11). We have preliminary data suggesting tobacco smokers at approximately 2 weeks of abstinence have blunted DA release compared to nonsmokers.

Imaging amphetamine-induced DA release in smokers vs. nonsmokers measured with [^{11}C]PHNO and PET. Amphetamine administration results in a reliable and robust increase in extracellular DA and has been widely used in brain imaging studies as a marker of DA function in psychiatric and healthy populations(8, 9, 12-16). We have chosen oral amphetamine based on its robust and reliable DA signal and because it is routinely used in PET studies(17-23) and by our group to probe DA neurotransmission. The amphetamine robustly increases synaptic levels of DA. The increased DA competes with the radiotracer to bind at the DA receptor, thus an increase in DA results in a decrease in radiotracer binding compared to baseline. This allows calculation of the "occupancy" of the receptors by DA or a change in binding potential (BP), and is an indirect measure of DA release based on the "occupancy model"(13). We have obtained preliminary data that demonstrates blunted DA release in smokers at 2-3 weeks of abstinence vs. controls and is similar to what has previously been found in cocaine- and alcohol- dependent individuals(8, 9). While the previous studies(8, 9) used [^{11}C]raclopride which measures striatal D2/3 receptor binding, we propose to use [^{11}C]PHNO which measures striatal and extrastriatal (substantia nigra and globus pallidum) D2/3 binding(24). Importantly, blunted DA release in cocaine-dependent individuals was associated with the choice to self-administer cocaine over money(8) and with treatment failure(10). In the current study, we will determine whether smokers have blunted DA release compared to nonsmokers and if the degree of DA release in smokers is associated with time to relapse.

3. Research Plan: Summarize the study design and research procedures using non-technical language that can be readily understood by someone outside the discipline. Be sure to distinguish between standard of care vs. research procedures when applicable, and include any flowcharts of visits specifying their individual times and lengths.

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3.1 Overall plan for Aim 1: To determine amphetamine-induced DA release in nonsmokers and tobacco smokers during acute and prolonged nicotine withdrawal. We will image up to 50 smokers and 40 nonsmokers.

Smoker-subjects: Subjects that qualify for the study will be asked to participate in the following:

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a) One MRI scan, which will take approximately 30-60 minutes. This will take place at the Anlyan Center at 300 Cedar St.
b) Up to 8 weeks of smoking abstinence- meet with staff multiple times a week to ensure abstinence. For the first week the subject will meet with staff every day (barring an unforeseen circumstance that prevents the subject from meeting). Meetings will occur either at 2 Church St South, the Yale PET center, or a meeting point midway between the subject and Yale due to distance. As the weeks proceed, meetings will taper down.
c) Two sets of two PET scans with [^{11}C]PHNO approximately 1-2 weeks after quitting smoking and again at 2-8 weeks with at least a week break in between scan sets. Both scans of each set will take about 2 hours each and should be done on the same day. Starting up to 3 hours prior to the second [^{11}C]PHNO scan of the set, subjects will be asked to take a drug called amphetamine by mouth. These will take place at the Yale PET Center.

Sequence of procedures

Order	Study procedure	How Long	When
1.	MRI scan/Day 0 appointment: This includes, mri, computer testing, cold pressor task, and withdrawal guidelines.	2 hours	After screening
2.	Smoking abstinence for about 2-8 weeks w/ daily appts for the first week tapering down as the weeks proceed..	2-8 weeks	After MRI scan
3.	Set of 2 PHNO PET scans with amphetamine challenge (with computer task and cold pressor task)	All Day	At ~1-2 weeks of smoking abstinence
4.	MRI scan(with possible computer task and Cold pressor task)	1 hour	At ~ 2-8 weeks of smoking abstinence
5.	Set of 2 PHNO PET scans with amphetamine challenge (with computer task and cold pressor task)	All Day	At ~2-8 weeks of smoking abstinence
6.	May be called followed up with Monthly to check in on smoking status	15 mins	monthly

Non-Smoker subjects:

Subjects that qualify for this study will be asked to participate in the following:
a) One MRI scan, which will take approximately 30-60 minutes. This will take place at the Anlyan Center at 300 Cedar St.
b) One set of two PET scans with [^{11}C]PHNO. Both scans of the set will take about 2 hours each and should be done on the same day. Starting up to 3 hours prior to the second [^{11}C]PHNO scan of the set, subjects will be asked to take a drug called amphetamine by mouth. This will take place at the Yale PET Center.

Sequence of procedures

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Order	Study procedure	How Long	When
1.	MRI scan/Day 0 appointment(with computer task and cold pressor task)	2 hours	After screening
2.	Set of 2 PHNO PET scans with amphetamine challenge(with computer task and cold pressor task)	All Day	After screening

3.2 Subject recruitment.
We have an established program to recruit healthy controls. For the present study, subjects will be recruited through our program as well as flyers, public advertisement (newspaper, radio, internet postings), and word of mouth.

3.3 Screening for eligibility.
After completing the informed consent process, subjects will have a physical and neurological examination. The following lab tests will be performed at screening to exclude medical illnesses: complete blood count (CBC) and differential, chemistries, kidney function tests (creatinine, BUN, urinalysis), liver function tests, and TSH. A urine drug screen and a pregnancy test (for women) will be done at screening and before radiotracer administration on each PET scan day. The psychiatric assessment will include a psychiatric history, a structured clinical interview (SCID), and assessment of subsyndromal depression with the Center for Epidemiological Studies Depression Scan – CES-D and subsyndromal anxiety with Speilberger State-Trait Inventory (STAI).

Aim 1 Healthy nonsmokers
- smoked < 100 cigarettes in lifetime
- urinary cotinine levels 0-30 ng/mL both at intake evaluation and on scan day

Aims 1 Healthy Smokers
- have been smoking cigarettes on a daily basis for at least 1 year
- carbon monoxide levels > 8 ppm during intake evaluation
- plasma nicotine levels > 10 ng/mL during intake evaluation
- plasma cotinine levels of > 50 ng/mL during intake evaluation
- wish or willing to quit smoking for at least 1 week and up to 8 weeks

3.4 Assessments:
All participants will be screened initially using a telephone screen that will include questions to evaluate medical history, personal and familial psychiatric and smoking history.

3.4a. General Intake Assessments
1. Demographic Questionnaire This questionnaire will obtain: (1) basic demographic information including age, gender, marital status, employment status, occupation, (2)

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alcohol/drug history, (3) family history of alcohol/drug use, depression, anxiety, and smoking history.
2. Medical History This questionnaire will obtain a basic medical history (personal and family) including past or current conditions such as neurological, endocrine, cardiovascular, renal, liver, and thyroid pathology. Current body weight and current medications will also be assessed.
3. Medical Assessments will include a physical exam by a state licensed physician (overseen by Dr. David Matuskey), a complete blood count, blood urea nitrogen, creatinine, fasting blood sugar, electrolytes, liver function tests, thyroid function tests (including T_3, T_4, T_3RU, estimated free T4), thyroid stimulating hormone levels, urine toxicology, EKG, and urinalysis**.** Female subjects will have serum pregnancy tests. All EKGs are read by a state licensed cardiologist and all abnormal MRIs will be reviewed by a state licensed neuroradiologist.
4. Illicit Drug/Pregnancy Screen A urine sample will be collected to determine current illicit drug use (for all potential subjects), positive results other than THC may be cause for exclusion from the study, however positive results will not be kept on file due to the sensitive nature of this information. In addition, urine samples will be collected for the intake visit and on each MR and PET scan day to confirm that the subject is not pregnant. Note: the urine pregnancy test will not be required prior to the MRI if the serum pregnancy test was done within 1 week prior to the MR imaging session.
5. Structured Clinical Interview for DSM-IV Axis I Disorders The psychotic screening and depression sections of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID) will be used to determine whether subjects meet exclusion criteria for diagnosis of psychotic disorders and major depression (25).

3.4b. Mood Measures

We may of mood and anxiety at intake and also on the PET scan day including the following.
1. Center for Epidemiological Studies Depression Scale (CES-D) The CES-D (26) is a 20-item self-report instrument, which has been extensively used in both clinical and nonclinical populations to measure the frequency and severity of depressive symptoms over the past week. The CES-D, which has been used to document the severity of depressive symptoms in adults (27) and has been shown to be a sensitive measure of negative affect in smokers (28), will be used in the proposed studies to exclude for presence of major depression, and to measure level of mild depressive symptoms commonly noted in tobacco smokers.
2. Anxiety: The State-Trait Anxiety Inventory (29) is a 40-item, self-report measure, comprised of two subscales. The State-Anxiety scale is 20 items and assesses transitory states characterized by feelings of tension, apprehension, and heightened autonomic reactivity. The Trait-Anxiety scale is 20 items and assesses stable individual differences in anxiety proneness.
3. Impulsivity: Barratt Impulsiveness Scale (BIS; (30)) is a 30 item self-report instrument designed to assess the personality/behavioral construct of impulsiveness.

3.4c. Smoking Measures

We will obtain smoking measures at intake and also on the PET scan day, which may include the following.

1.Fagerstrom Test for Nicotine Dependence (FTND).(31) This will be used to measure the severity of nicotine dependence. It is a 6-item scale with an internal consistency of .61 and its total score is closely related to biochemical measures of intensity of smoking.

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2. Smoking History. This questionnaire will assess basic smoking status and history such as number of years smoked, number and length of quit attempts, reasons for quitting, and second hand smoke exposure.

3. Nicotine Withdrawal Checklist.(32) This measures the severity of eight withdrawal symptoms on 5-point Likert scales.

4. Tiffany Questionnaire of Smoking Urges (QSU).(33) The QSU-brief is a 10-item questionnaire that evaluates the structure and function of smoking urges. Subjects indicate on a likert-type scale how strongly they agree or disagree with each statement with a score of 1 (strongly agree) to 7 (strongly disagree). This characterizes 'urges to smoke' into a negative affect related to relief of withdrawal symptoms and positive affect related to expectancy of reinforcement.

3.4d Cognitive Measures

We will obtain these measures at baseline and up to two times on the PET days.

1. Cogstate Battery: This computerized test battery will assess memory and cognition. The tasks may include:

a. International Shopping List Task – a computerized task to assess verbal learning and memory.
b. Groton Maze Learning Task – a computerized task to assess executive function and spatial problem solving.
c. Detection Task – a computerized task to assess psychomotor function and speed of processing.
d. Identification Task – a computerized task to assess visual attention and vigilance.
e. One Card Learning Task – a computerized task to assess visual learning and memory.
f. One Back Task – a computerized task to assess attention and working memory

2. Probabilistic Reward Task: The PRT has been successfully used to assess reward responsiveness (166-168). In each trial, subjects choose which of two difficult-to-differentiate stimuli was presented. Stimuli consist of simple cartoon faces (diameter: 25 mm; eyes: 7 mm) presented in the center of the monitor. At the beginning of the trial, the face has no mouth. After a given delay, either a straight mouth of 11.5 mm ("short mouth") or 13 mm ("long mouth") is presented for 100 ms. Subjects are instructed to press an appropriate button to decide whether a long or small mouth had been presented. Unbeknownst to subjects, correct identification of one stimulus ("rich stimulus") is rewarded three times more frequently (*"Correct! You won 20 cents"*) than the other ("lean") stimulus. In healthy controls, this reinforcement schedule leads to a response bias (i.e., a preference for the more frequently rewarded stimulus). The degree of response bias toward the more frequently reinforced alternative will be used for operationalizing sensitivity to reward.

3. Cold Pressor Task: Subjects may be asked to participate in the Cold Pressor Task. The cold pressor task (CPT) is a stress task used to measure pain sensitivity and pain tolerance. This task will be used to determine alterations in pain thresholds as a result of nicotine use. Participants will immerse their hand (up to the wrist) for up to 3 minutes in the experimental (ice-cold temperature 0-4°C)) and control (room temperature (20°C)) conditions. Physiological measure (heart rate, blood pressure and subject responses (stress, mood) will be collected 5 minutes before, 1 minute into, and immediately after the CPT.

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3.5 Experimental Methods

MRI.
Within approximately 6 months of the PET study, anatomical MRIs will be acquired at the Yale University MRI Center. Subjects will be taken through a ferromagnetic metal detector before entering the scan room. The purpose of the MRI scan is to direct the region of interest placement on the lower resolution PET images. The T1 weighted images will be acquired on a 3 Tesla Siemens Scanner. There will also be an additional resting state scan with subjects in the scanner, eyes open, fixating on a cross. Smokers who participate in the 2-8 week PET scan set may return to have a second MRI with resting state at 2-8 weeks withdrawal A member of the research team will accompany the subject and remain for the duration of the scan.

Prisma Scan sequence: 64 Channel

Series 1: 3 plane localize
Series 2: Sag 3d tfl; 256fov; 1mm thick slices; 176 slices total; TE 2.77; TR 2530; TI 1100; FA 7; 256X256 1 average.
Series 3: Resting state: Ep2d bold; 210fov; 2.5mm thick slices; TE 30; TR 3400; FA 85; 84x84 (Run twice).
Series 4: Diffusion Tensor Imaging (DTI) sequence

MR images provide a matching anatomical atlas for creating individualized region-of-interest templates for each subject. We will also examine functional connectivity at rest.

Amphetamine challenge
Dextroamphetamine sulfate is the dextro isomer of the compound d,l-amphetamine sulfate, a sympathomimetic amine of the amphetamine group. It is an FDA approved drug available for the treatment of narcolepsy and attention deficit hyperactivity disorder (maximum approved total daily dose of 5-60 mg). After the first [^{11}C]PHNO scan, dextro-amphetamine (0.5 mg/kg, PO) will be given in increments of 5 or 10 mg, as it is available in 5 mg and 10 mg tablets, and will be rounded down to approximate 0.5 mg/kg total dose without exceeding it. Total dextro-amphetamine dose will not exceed 50 mg per scan. A second transmission and emission scan will be acquired approximately 2.5-3 hours post amphetamine identical to the methods outlined previously. The timing of the second [^{11}C]PHNO administration and subsequent PET scanning (i.e., 2.5-3 hours) corresponds to the time of maximum plasma concentration of amphetamine as stated in the respective FDA-approved product labeling. EKG and frequent BP monitoring will occur throughout the study and until the vital signs are within normal limits. Supplemental oxygen will be provided via nasal cannula if necessary. If the systolic BP reaches or exceeds 200 mmHg for more than 5 minutes, the study doctor will take the appropriate clinical measures in order to lower the BP, which may include phentolamine administration (5 mg IV over 10 min) or other appropriate measures.

Following the post-amphetamine PET scan, subjects will be assessed (EKG and vital signs) by one of the research nurses. Subjects will be discharged when vital signs are within normal limits

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and when behavioral changes (if any) are found to be not clinically significant by the MD attending to the subject at the PET Center. If subjects experience any adverse events, they will be treated until they become asymptomatic, prior to discharge.

Behavioral response to amphetamine will be measured by self-ratings with a simplified version of the Amphetamine Interview Rating Scale (van Kammen and Murphy, 1975). Four items will be investigated: euphoria ("feel good"), alertness ("feel energetic"), restlessness ("feel like moving") and anxiety ("feel anxious"). Self-ratings will be obtained by analog scales at the following times relative to the d-amphetamine administration: -5 minutes, 0, and hourly thereafter until end of the second scan.

PET

PET scans may be performed on the High Resolution Research Tomograph (HRRT, 2-3 mm resolution) or another similar camera. Venous catheters will be used for i.v. administration of the radiotracers, venous blood sampling of activity, and for venous blood sampling of amphetamine plasma levels and hormone levels. At the beginning of scan, the subject's head will be immobilized and a transmission scan will be obtained for attenuation correction. PET scans will be acquired using bolus or bolus to infusion administration of up to 10 mCi of [^{11}C]PHNO and subjects will be scanned for up to 2 hours. Dynamic images of radioactivity concentration are reconstructed with corrections for attenuation, normalization, random events, scatter, and deadtime. Subject motion is corrected automatically on an event-by-event basis with the Vicra motion tracking system. Vital signs (blood pressure, pulse and respiration) are collected prior to and during each PET scan. Urine pregnancy test will be again administered on the PET scan day prior to the initiation of any imaging procedures. Smoking abstinence, when appropriate, will also be confirmed for smoking subjects prior to PET scanning.

PET scanning will then proceed as following for each aim:

Aim 1. Smoking subjects will be asked to come in for two [^{11}C]PHNO scans with amphetamine administration. The first scan will be during the first 2 weeks of withdrawal with the option to come back again at 2-8 weeks of withdrawal. Nonsmoking subjects will be asked to come in for one set of two [^{11}C]PHNO scans with amphetamine administration. Up to 10 mCi of PHNO will be administered per scan. After the first scan, subjects will take amphetamine (0.5mg/kg) by mouth, and then will be imaged again with PHNO. If technical difficulties arise, the second PET scan of the set may have to be re-scheduled and will be scheduled as soon as possible. In that case, subjects may be asked to re-take amphetamine on the day of the rescheduled PHNO scan, approximately 3 hrs prior to the scan. Amphetamine dose will not exceed 50mg per scan. Subjects will not be allowed to drive home.

In the rare event that a scan would fail post injection, subjects may repeat each scan set up to a total of 8 PHNO scans for smokers and 4 PHNO scans for nonsmokers.

3.6 Image analysis.

The primary outcome measure is the binding potential (BP_{ND}), which in turn is proportional to the available receptor concentration (B_{avail}), given that there is no change in affinity (K_D) and that nondisplaceable (nonspecific and free) uptake does not differ between subjects and studies. We will examine the regions-of-interest listed below with the cerebellum used as a reference region because it is devoid of $D_{2/3}$ receptors.

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We obtain an MRI (as previously described) to use as a guide to place our regions of interest. This is because we can define anatomical regions of interest on the MRI, which provides structural information and then we can apply these regions of interest to the PET scans. The PET scans alone are not sufficient to identify brain regions. The PET image sets are aligned and resliced to yield images in the same planes and spatial system as the MRI images using AAL template. Primary ROIs will be caudate, putamen, ventral striatum, globus pallidus, and substantia nigra. Cerebellum will be the reference region.

3.7 Statistical analysis.
Demographics between smokers and nonsmokers will be compared using *t* tests or Chi-Square tests, as appropriate. Scores on psychological tests such as depressive symptoms at baseline will be compared between groups and may be used as covariates in subsequent analyses. All outcomes will be summarized descriptively and assessed for normality prior to analysis using normal probability plots and Kolmogorov test statistics. Transformations or nonparametric analyses will be performed as necessary. All tests will be two-sided and considered statistically significant at alpha = 0.05. Post-hoc comparisons will be conducted as necessary with significance levels adjusted for multiple comparisons basing the adjustment on the number of conceptually related statistical tests within each hypothesis. All statistical analyses will be conducted using SAS version 9.3 (Cary, NC).

Aim 1. **To determine if amphetamine-induced DA release is blunted during abstinence in tobacco smokers compared to nonsmokers.** Hypothesis 1. We hypothesize that amphetamine-induced striatal DA release will be blunted in smokers compared to nonsmokers. Power Calculations for Hypothesis 1: Our preliminary data revealed blunted amphetamine-induced DA release (d=0.91) in the ventral striatum among smokers (-21%±12.0) compared to nonsmokers (-29%±6.3). Assuming a two-sided t-test with α=0.05, 40 nonsmokers and 50 smokers assessed after 14-21 days of abstinence will provide 80% statistical power to detect effect sizes as small as d=0.63, which compares favorably with the effects observed above.

Regional [^{11}C]PHNO uptake for each subject will be quantified as BP_{ND} described above for each ROI and will represent the primary outcome measure for the proposed experiments. Repeated measures ANOVA will be used to evaluate the change between scans (deltaBP) for brain measures for the scans. Delta BP will be computed as [($BP_{baseline}$-$BP_{condition}$)/$BP_{baseline}$ X 100]. Analysis will be conducted using SAS version 9.1 (Cary, NC). N=20 subjects per group is necessary to detect differences between smokers and nonsmokers. Potential associations, e.g., relationship between magnitude of dopamine release and time to relapse, will be examined using correlation analysis with Type I error corrected for multiple comparisons. Exploratory analyses of the other cognitive and behavioral measures and their relationship to relapse will also be performed.

3.8 Contingency Reinforcement for Smoking Cessation (for healthy smokers)
We plan to image smokers during the first 8 weeks of abstinence. Contingency management techniques have been successfully used to help smokers quit smoking by a number of investigators including our group for many years (34-36).

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We will help the subjects set a quit date and prior to the quit date they will meet with the research staff who will provide them with brief advice on quitting smoking based on AHCPR guidelines (The Smoking Cessation and Clinical Practice Guideline Panel and Staff, 1996). They will be advised about the risks and benefits of quitting smoking and told that they will be monitored daily to ensure abstinence. Subjects will be informed of payment schedules (see below) for CO levels indicating abstinence and also given information on how quit rates in the first week of smoking cessation predicted sustained abstinence. We use CO levels < 11 ppm to define abstinence from cigarettes. During the first week of abstinence we will obtain CO levels during contingency management meetings from subjects up to twice daily. In addition, we will obtain urine samples once daily to measure cotinine levels (a nicotine metabolite). These meeting will occur either in the research office, the Yale PET center, or in a midway point if subjects travel a far distance.

For each contingency management appointment, subjects will get $10 if their CO levels are less than 11ppm and urine cotinine levels are less than 100 ng/ml.

4. **Genetic Testing N/A☒**

5. Subject Population Provide a detailed description of the targeted population of human subjects for this research project.

Target numbers for participation are 40 Healthy controls and 50 Healthy Smokers. Healthy controls and healthy smokers will be recruited from the community through advertisements as approved by the Yale University Human Investigations Committee (HIC). Interested individuals contacting the clinic by phone in response to advertisements are told that the information they give over the phone is written down and discussed by the research team. They are advised that if they do not enroll in research with the clinic the information is destroyed, and that if they do, it becomes part of their research chart. A phone screen is completed after they give verbal authorization. If an individual appears to meet enrollment criteria and is interested in participating, a face-to-face interview is conducted. A release of information is obtained for review of any available historical and clinical data. Written authorization is also obtained from each subject, permitting the research team to use, create, or disclose the subject's PHI for research purposes. The nature of the project, procedures, relative risks and benefits, and alternatives to participation in the project are discussed with the individual. Following this discussion, the individual is given a copy of the consent form to review, and any questions are answered. We will obtain written consent from all participants.

6. Subject Classifications: Will subjects who may require additional safeguards or other considerations be enrolled in the study? If so, identify the population of subjects requiring special safeguards and provide a justification for their involvement.

a. Is this research proposal designed to enroll children who are wards of the state as potential subjects? ☐ Yes ☒ No *(If yes, see Instructions section VII #4 for further requirements)*

7. Inclusion/Exclusion Criteria: What are the criteria used to determine subject inclusion or exclusion?

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General inclusion criteria:

- men and women, aged 18-55 years
- who are able to read and write
- who are able to give voluntary written informed consent
- have no current uncontrolled medical condition such as neurological, cardiovascular, endocrine, renal, liver, or thyroid pathology
- drink less than <21 drinks/week for women and less than <35 drinks per week for men
- If female, not pregnant or breast feeding
- If female of childbearing age, must use an acceptable method of birth control, as determined by the principal investigator
- do not suffer from claustrophobia or any MR contradictions
- willing to be followed up monthly after study participation via phone or email contact

General exclusion criteria:

- Presence of acute or unstable medical or neurological illness. Subjects will be excluded from the study if they present with any history of serious medical or neurological illness or if they show signs of a major medical or neurological illness on examination or lab testing including history of seizures, head injury, brain tumor, heart, liver or kidney disease, eating disorder, diabetes.
- Presence of an Axis I diagnosis other than nicotine dependence
- Regular use of any psychotropic drugs including anxiolytics and antidepressants and other over-the-counter medications and herbal products in the past six months other than THC, per the PI's discretion. The PI will take a number of factors into consideration on a case-by-case basis including type of psychotropic drug used, frequency, and dose.
- Pregnancy/Breast feeding
- Subjects with a pacemaker or other ferromagnetic material in body.
- Subjects with a sitting pulse rate >100 bpm will be excluded
- Subjects with hypertension defined as sitting systolic blood pressure of >160 mmHg and/or sitting diastolic blood pressure of >100 mmHg will be excluded. Those individuals with hypertension that is well controlled by medication (e.g., within the above mentioned range) are not excluded
- Specifically, we will exclude subjects who have any active clinically significant deviation from the normal range in their electrocardiogram (EKG). However, subjects who have abnormalities in their EKG but the condition has been present for a while and the study cardiologist has evaluated and feels comfortable with the condition, would not be excluded on the basis of their cardiac condition. Examples of conditions that may meet these criteria (e.g., condition has been present for a while) include but are not limited to T-wave abnormalities, atrial fibrillation, prolonged PR interval, and right bundle branch block.
- Subjects with history of prior radiation exposure for research purposes within the past year such that participation in this study would place them over FDA limits for annual radiation exposure. This guideline is an effective dose of 5 rem received per year.
- Subjects with current, past or anticipated exposure to radiation in the work place
- Blood donation within eight weeks of the start of the study.

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- History of a bleeding disorder or are currently taking anticoagulants (such as Coumadin, Heparin, Pradaxa, Xarelto).

8. How will **eligibility** be determined, and by whom?

Eligibility to participate will be determined by the PI of this study after completion of the medical and psychiatric evaluation of the potential participant.

9. Risks: Describe the reasonably foreseeable risks, including risks to subject privacy, discomforts, or inconveniences associated with subjects' participation in the research.

Risks from this study include 1) risks associated with radiation exposure, 2) risks associated with MRI, 3) intravenous lines and blood drawing, 4) nicotine withdrawal symptoms, 5) amphetamine

1. Risks Associated with Radiation

The Yale New Haven Radiation Safety Committee and the FDA will review the use of radiation in this research study as the radiotracer [^{11}C]PHNO PET will be under IND # **134138**, This research study involves exposure to radiation from [^{11}C]PHNO PET scanning. This radiation exposure is not necessary for medical care and is for research purposes only.

For each individual [^{11}C]PHNO PET scan, subjects will receive up to ≤ 10 mCi of [^{11}C]PHNO, plus transmission scans. This is equal to an effective dose equivalent of 0.2146 rem per injection.

The maximum amount of radiation per year an individual subject will receive in this study is from up to 8 injections of ≤ 10mCi each of [^{11}C]PHNO, plus transmission scans. The intended amount is from up to 4 injections of ≤ 10mCi each of [^{11}C]PHNO for smokers and up to 2 injections of ≤ 10mCi each of [^{11}C]PHNO in nonsmokers, however in case of scan failures post injection we have included up to 8 injections if scan sets need to be repeated within the study time frame (i.e. 1-2 wks withdrawal and 2-8 wks withdrawal). Nonsmokers maximum number of injections including re-scans would be 4 scans total. Should the second scan of the set fail, we will make every effort to reschedule the scan for repeat within 1 wk, however if scheduling does not permit this, we may ask the subject to repeat the whole set due to timing related data.

Although each organ will receive a different dose, the maximum intended amount of radiation exposure subjects will receive per year from this study is equal to an effective dose equivalent of 0.858400001 rem for a total of up to 40 mCi of [^{11}C] PHNO in 4 injections of ≤ 10mCi each for smokers and 0.4292 rem for a total of up to 20 mCi [^{11}C] PHNO in 2 injections of ≤ 10mCi each for non smoker. This calculated value is used to relate the dose received by each organ to a single value.
However, if scans should need to be repeated the maximum dose for smokers would be equal to an effective dose of 1.716800002 rem from up to 8 injections and for non smokers would be 0.858400001 rem from up to 4 injections. We have every intention to limiting radiation exposure to subjects and avoiding re-scans if possible.

2 injections PHNO	0.4292 rem

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4 injections PHNO	0.8584 rem
8 injections PHNO	1.7168 rem

The amount of radiation subjects will receive in this study is below the dose guidelines established by the FDA and monitored by the Yale University Radioactive Drug Research Committee for research subjects. This guideline sets an effective dose limit of 5 rem per year.

2. MRI

MR carries a risk for subjects who have pacemakers, metal pieces, aneurysm clips, or other contraindications for MR.

Magnetic resonance (MR) is a technique that uses magnetism and radio waves, not x-rays, to take pictures and measure chemicals of various parts of the body. The United States Food and Drug Administration (FDA) has set guidelines for magnet strength and exposure to radio waves, and we carefully observe those guidelines.

Subjects will be watched closely throughout the MR study. Some people may feel uncomfortable or anxious. If this happens, the subject may ask to stop the study at any time and we will take them out of the MR scanner. On rare occasions, some people might feel dizzy, get an upset stomach, have a metallic taste or feel tingling sensations or muscle twitches. These sensations usually go away quickly but we will ask subjects to tell the research staff if they have them.

There are some risks with an MR study for certain people. If subjects have a pacemaker or some metal objects inside their body, they may not be in this study because the strong magnets in the MR scanner might harm them. Another risk is the possibility of metal objects being pulled into the magnet and hitting a subject. To reduce this risk we require that all people involved with the study remove all metal from their clothing and all metal objects from their pockets. We also ask all people involved with the study to walk through a detector designed to detect metal objects. It is important to know that no metal can be brought into the magnet room at any time. Also, once subjects are in the magnet, the door to the room will be closed so that no one from outside accidentally goes near the magnet.

We want subjects to read and answer very carefully the questions on the MR Safety Questionnaire related to their personal safety. We will be sure that subjects have read the MR Safety Questionnaire and tell us any information they think might be important.

This MR study is for research purposes only and is not in any way a clinical examination. The scans performed in this study are not designed to find abnormalities. The primary investigator, the lab, the MR technologist, and the Magnetic Resonance Research Center are not qualified to interpret the MR scans and are not responsible for providing a diagnostic evaluation of the images. If a worrisome finding is seen on a subject's scan, a radiologist or another physician will be asked to review the relevant images. Based on his or her recommendation (if any), the primary investigator or consulting physician will contact the subject, inform them of the finding, and recommend that they seek medical advice as a precautionary measure. The decision for additional examination or treatment would lie solely with the subject and their physician. The investigators, the consulting physician, the Magnetic Resonance Research Center, and Yale University are not responsible for any examination or treatment that a subject receives based on

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these findings. The images collected in this study are not a clinical MR exam and for that reason, they will not be made available for diagnostic purposes.

3. Blood Drawing and IV line Insertion
Drawing blood and inserting an intravenous line (IV) into an arm vein are safe and standard medical procedures. Sometimes a bruise will occur at the puncture site and rarely a blood clot or infection will occur in the vein. Certain individuals may feel light-headed during venipuncture. The volume of blood collected during this study, may include screening laboratories, MRI-and PET scans, will be approximately 32 tablespoons. This is not expected to have any serious negative effects on a study participant.

4. Nicotine Withdrawal
Smokers that quit smoking may experience symptoms of nicotine withdrawal such as craving cigarettes, mild anxiety, restlessness, irritability, difficulty concentrating, loss of energy, and excessive hunger. These are typical symptoms that people experience when they stop smoking and they can be uncomfortable but they are not life threatening.

5. Risks of oral d-amphetamine
Risks of amphetamine administration include both medical and psychiatric risks.

The frequent somatic side effects of d-amphetamine administration are cardiovascular (hypertension, palpitations, tachycardia, bradycardia, orthostasis). General effects such as sweating, feeling warm or cold, nausea, diarrhea, muscle and abdominal cramping, have been reported frequently. Behavioral effects in this dose range are increased level of alertness, talkativeness, restlessness, agitation, mood changes (usually euphoria) and anxiety. In our experience, these effects are generally transient and well tolerated. This dose of amphetamine has not been reported to induce psychotic symptoms in non-schizophrenic subjects. Infrequently blurred vision, headaches and chest tightness, and changes in EKG have been reported. There is a rare risk of permanent neurological damage and death as a result of cardiac arrest or stroke.

Psychiatric or behavioral side effects: General behavioral effects of amphetamine in this dose range are increased level of alertness, talkativeness, restlessness, agitation, mood changes (usually euphoria) and anxiety. In our experience, these effects are generally transient and well tolerated. This dose of amphetamine has not been reported to induce psychotic symptoms in non schizophrenic subjects and we confirm this observation.

10. Minimizing Risks: Describe the manner in which the above-mentioned risks will be minimized.

1. The dose of radiation will be submitted for approval to the **Yale New Haven Hopsital Radiation Safety Committee.** All scans will be done in the presence of medical supervision and trained staff in an institution specifically designed to support imaging studies. In the event of serious medical complications, the PET scan facilities have immediate access to or consultation with specialized medical units at the Yale-New Haven Hospital. Preparation of radiopharmaceuticals and performance of PET scans will be by radiochemists, physicians, and technologists of the Department of Diagnostic Radiology, Yale University School of Medicine.

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These professionals are qualified by training and experience in the safe use and handling of radiopharmaceuticals. Subjects will be asked about their previous radiation exposure and those who have had research exposure within the past year will be excluded if their cumulative annual exposure (including the present study) exceeds FDA limits. The information on the previous radiation exposure of study subjects will be notified to the study doctor.

No PET studies will be performed on pregnant or potentially pregnant women, as confirmed by pregnancy testing during evaluation and on each scan day before initiation of any scan procedures. If subjects are breastfeeding they will not be able to participate in this research study.

2. Minimizing risks with MRI: All subjects will be screened for any metallic objects other MR contraindications that they may be holding or have implanted in their bodies using a questionnaire and all potential subjects with contraindications for MR will be excluded. This questionnaire will be repeated immediately before each measurement to insure that no metallic materials are brought into close proximity of the magnet, where they might be pulled toward the magnet. For additional security, subjects will be taken through a ferromagnetic metal detector immediately before going to the scan room.

3. Minimizing risks with blood draws: The risks of bruising, clotting, and infection will be minimized by having venipuncture performed by trained and experienced personnel using aseptic technique. To avoid injury due to fainting, the venous catheters will be inserted when the subjects are recumbent. The blood draws during PET scanning sessions will be obtained from the already inserted catheter, to minimize discomfort.

4. Minimizing risks with nicotine withdrawal: Subjects will meet with research staff and be counseled prior to the quit attempt to provide them with skills to help ease the withdrawal symptoms.

5. Minimizing risks with oral d-amphetamine administration: *Medical side effects:* Subjects will be carefully screened for absence of significant medical history and current medical conditions with a complete medical history, physical examination, routine blood tests, urine toxicology and EKG. Inclusion in the study will be limited to individuals who are between the ages of 18-55. Patients will be excluded if they have any h/o severe medical or neurological illness, any clinically significant brain abnormality, insulin dependent diabetes, a history of cardiovascular disease, or hypertension. Administration of oral d-amphetamine will take place at the PET center by a research nurse, with a physician on site. The research nurse will report vital signs to the physician prior to administering the amphetamine.

If several of the subject's blood pressure readings are recorded at >100 or <60 for diastolic BP or >160 or <90 for systolic BP while at rest, they will be evaluated by the MD. The study may be cancelled at the discretion of the MD after evaluation. Any automated blood pressure results that are abnormal will be repeated manually. The manual reading will be the official reading. Constant EKG and frequent BP monitoring will occur until the vital signs are within normal limits. If the systolic BP reaches or exceeds 200 mmHg for more than 5 minutes, an infusion of phentolamine (5 mg IV, over 10 min) or other appropriate measures may be initiated to control the blood pressure response. The study physician will be notified if those parameters are reached and he/she will supervise the treatment.
In case of chest pain, chest tightness or other symptoms suggestive of cardiac ischemia, the

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experiment may be cancelled and an EKG will be obtained to rule out angina (ST segment elevation or depression as compared to the baseline EKG). Appropriate treatment will be initiated.
Upon discharge, patients will be given the phone numbers of the study physicians and will not be allowed to drive home. They will either arrange a ride or a taxi.

For Data and Safety Monitoring Plan templates, see
http://www.yale.edu/hrpp/forms-templates/biomedical.html
Data Safety Monitoring Plan:

1. Personnel responsible for the safety review and its frequency:

The principal investigator will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency which must be conducted at a minimum of every 6 months (including when re-approval of the protocol is sought). During the review process, the principal investigator (monitor) will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment. Either the principal investigator, the IRB or Safety Monitoring Committee (DSMC) have the authority to stop or suspend the study or require modifications.

2. The risks associated with the current study are deemed moderate for the following reasons:

1. We do not view the risks associated with the radiotracer [11C]PHNO as minimal.
2. We do not view the risks associated with the combined use of _____and_____ as minimal.
3. Given the now established safety and validity of the current _____ in our prior work, we do not view the proposed studies as high risk.
4. Given our experience with the combined co-administration_____, we do not view the proposed studies as high risk.

Although we have assessed the proposed study as one of moderate risk, the potential exists for anticipated and/or unanticipated adverse events, serious or otherwise, to occur since it is not possible to predict with certainty the absolute risk in any given individual or in advance of first-hand experience with the proposed study methods. Therefore, we provide a plan for monitoring the data and safety of the proposed study as follows:

3. Attribution of Adverse Events:
Adverse events will be monitored for each subject participating in the study and attributed to the study procedures / design by the principal investigator Kelly Cosgrove, Ph.D. according to the following categories:

a.) Definite: Adverse event is clearly related to investigational procedures(s)/agent(s).
b.) Probable: Adverse event is likely related to investigational procedures(s)/agent(s).
c.) Possible: Adverse event may be related to investigational procedures(s)/agent(s).
d.) Unlikely: Adverse event is likely not to be related to the investigational procedures(s)/agent(s).

e.) Unrelated: Adverse event is clearly not related to investigational procedures(s)/agent(s).

4. Plan for Grading Adverse Events:

The following scale will be used in grading the severity of adverse events noted during the study:

1. Mild adverse event
2. Moderate adverse event
3. Severe

5. Plan for Determining Seriousness of Adverse Events:

Serious Adverse Events:
In addition to grading the adverse event, the PI will determine whether the adverse event meets the criteria for a Serious Adverse Event (SAE). An adverse event is considered serious if it:

1. is life-threatening OR
2. results in in-patient hospitalization or prolongation of existing hospitalization OR
3. results in persistent or significant disability or incapacity OR
4. results in a congenital anomaly or birth defect OR
5. results in death OR
6. based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition, OR
7. adversely affects the risk/benefit ratio of the study

An adverse event may be graded as severe but still not meet the criteria for a Serious Adverse Event. Similarly, an adverse event may be graded as moderate but still meet the criteria for an SAE. It is important for the PI to consider the grade of the event as well as its "seriousness" when determining whether reporting to the HIIRB is necessary.

6. Plan for reporting serious AND unanticipated AND related adverse events, anticipated adverse events occurring at a greater frequency than expected, and other unanticipated problems involving risks to subjects or others to the IRB

The investigator will report the following types of adverse events to the IRB: a) serious AND unanticipated AND possibly, probably or definitely related events; b) anticipated adverse events occurring with a greater frequency than expected; and c) other unanticipated problems involving risks to subjects or others.

These adverse events or unanticipated problems involving risks to subjects or others will be reported to the IRB within 48 hours of it becoming known to the investigator, using the appropriate forms found on the website.

7. Plan for reporting adverse events to co-investigators on the study, as appropriate the protocol's research monitor(s), e.g., industrial sponsor, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), Protocol Review Committee (PRC), DSMBs, study sponsors, funding and regulatory agencies, and regulatory and decision-making bodies.

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For the current study, the following individuals, funding, and/or regulatory agencies will be notified:

☐ All Co-Investigators listed on the protocol.

☐ Safety Monitoring Committee (DSMC)

☐ National Institutes of Health

The principal investigator Kelly Cosgrove, Ph.D. will conduct a review of all adverse events upon completion of every study subject. The principal investigator will evaluate the frequency and severity of the adverse events and determine if modifications to the protocol or consent form are required.

11. Statistical Considerations: Describe the targeted number of subjects and the statistical analyses that support the study design.

Demographics between smokers and nonsmokers will be compared using *t* tests or Chi-Square tests, as appropriate. Scores on psychological tests such as depressive symptoms at baseline will be compared between groups and may be used as covariates in subsequent analyses. All outcomes will be summarized descriptively and assessed for normality prior to analysis using normal probability plots and Kolmogorov test statistics. Transformations or nonparametric analyses will be performed as necessary. All tests will be two-sided and considered statistically significant at alpha = 0.05. Post-hoc comparisons will be conducted as necessary with significance levels adjusted for multiple comparisons basing the adjustment on the number of conceptually related statistical tests within each hypothesis. All statistical analyses will be conducted using SAS version 9.3 (Cary, NC).

Aim 1. **To determine if amphetamine-induced DA release is blunted during abstinence in tobacco smokers compared to nonsmokers.** Hypothesis 1. We hypothesize that amphetamine-induced striatal DA release will be blunted in smokers compared to nonsmokers. Power Calculations for Hypothesis 1: Our preliminary data revealed blunted amphetamine-induced DA release (d=0.91) in the ventral striatum among smokers (-21%±12.0) compared to nonsmokers (-29%±6.3). Assuming a two-sided t-test with α=0.05, 40 nonsmokers and 50 smokers assessed after 14-21 days of abstinence will provide 80% statistical power to detect effect sizes as small as d=0.63, which compares favorably with the effects observed above.

Regional [^{11}C]PHNO uptake for each subject will be quantified as BP_{ND} described above for each ROI and will represent the primary outcome measure for the proposed experiments. Repeated measures ANOVA will be used to evaluate the change between scans (deltaBP) for brain measures for the scans. Delta BP will be computed as [($BP_{baseline}$-$BP_{condition}$)/$BP_{baseline}$ X 100]. Analysis will be conducted using SAS version 9.1 (Cary, NC). N=20 subjects per group is necessary to detect differences between smokers and nonsmokers. Potential associations, e.g., relationship between magnitude of dopamine release and time to relapse, will be examined using correlation analysis with Type I error corrected for multiple comparisons. Exploratory analyses of the other cognitive and behavioral measures and their relationship to relapse will also be performed.

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SECTION III: RESEARCH INVOLVING DRUGS, BIOLOGICS, RADIOTRACERS, PLACEBOS AND DEVICES

If this section (or one of its parts, A or B) is not applicable, state N/A and delete the rest of the section.

DRUGS, BIOLOGICS and RADIOTRACERS

12. Identification of Drug, Biologic or Radiotracer: What is (are) the **name(s)** of the drug(s), biologic(s) or radiotracer(s) being used? Identify whether FDA approval has been granted and for what indication(s).

[^{11}C]PHNO, IV, radioactivity dose of no more than 10 millicuries for one injection. [^{11}C]PHNO has been used in humans and has been shown to be *safe and well tolerated after its administration to healthy subjects or patients.* No serious adverse effects are expected from tracer doses, which is one thousand fold ≤ the pharmacological used during the therapeutic trials of PHNO. This will be used under the PET Center's approved IND#**134138** for the radiotracer [^{11}C]PHNO.

d-Amphetamine, dose 0.5 mg/kg, PO by study physician to participants so no IND necessary per 21 CFR 312.2(b).

All protocols which utilize a drug, biologic or radiotracer **not** approved by, but regulated by, the FDA must provide the following information:

a. What is the Investigational New Drug (IND) **number** assigned by the FDA? 134138

b. Who holds the IND? Yale PET Center

c. All protocols which utilize a radiotracer not approved by, but regulated by the FDA must provide the IND number:
Alternatively, use of the investigational radiotracer may be under RDRC/RSC oversight: (check if appropriate)_____

For all investigational radiotracers, attach a copy of the RDRC/RSC application (for radioisotopes used in the PET Center, PET Center personnel may complete this step)
Go to http://rsc.med.yale.edu/login.asp?url=myApps.asp. When you have logged in, complete the application and attach a copy to this submission.

Alternatively, an **exemption from IND filing requirements** may be sought for a clinical investigation of a drug product that is lawfully marketed in the United States. If there is no IND and an exemption is being sought, review the following categories and complete the category that applies

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(*and delete the inapplicable categories)*:

Exempt Category 1 (d-Amphetamine)
The clinical investigation of a drug product that is lawfully marketed in the United States can be exempt from IND regulations if all of the following are yes:

i. The intention of the investigation is NOT to report to the FDA as a well-controlled study in support of a new indication for use or to be used to support any other significant change in the labeling for the drug. ☒ Yes ☐ No
ii. The drug that is undergoing investigation is lawfully marketed as a prescription drug product, and the intention of the investigation is NOT to support a significant change in the advertising for the product. ☒ Yes ☐ No
iii. The investigation does NOT involve a route of administration or dosage level or use in populations or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product. ☒ Yes ☐ No
iv. The investigation will be conducted in compliance with the requirements for institutional (HIC) review and with the requirements for informed consent of the FDA regulations (21 CFR Part 50 and 21 CFR Part 56). ☒ Yes ☐ No
v. The investigation will be conducted in compliance with the requirements regarding promotion and charging for investigational drugs. ☒ Yes ☐ No

Exempt Category 2 (all items i, ii, and iii must be checked to grant a category 2 exemption)

☐ i. The clinical investigation is for an *in vitro* diagnostic biological product that involves one or more of the following (check all that apply):
- ☐ Blood grouping serum
- ☐ Reagent red blood cells
- ☐ Anti-human globulin

☐ ii. The diagnostic test is intended to be used in a diagnostic procedure that confirms the diagnosis made by another, medically established, diagnostic product or procedure; and

☐ iii. The diagnostic test is shipped in compliance with 21 CFR §312.160.

Exempt Category 3

☐ The drug is intended solely for tests in vitro or in laboratory research animals if shipped in accordance with 21 CFR 312.60

Exempt Category 4

☐ A clinical investigation involving use of a placebo if the investigation does not otherwise require submission of an IND.

1. **Background Information:** Provide a description of previous human use, known risks, and data addressing dosage(s), interval(s), route(s) of administration, and any other factors that might influence risks. If this is the first time this drug is being administered to humans, include relevant data on animal models.

Previous human use of [^{11}C]PHNO

The use of [^{11}C]PHNO in PET imaging in human was first reported in 2006 (37). No relevant abnormalities in blood pressure, heart rate, or ECG were reported at any time in the study. There also were no relevant findings in physical or neurological exams or in routine blood and urine analyses during the study. However nausea was reported approximately 2–3 min after tracer injection, which subsided rapidly 2–3 min later. This effect was attributed to the transient high [^{11}C]PHNO concentration resulting from the bolus injection may have been responsible for the reported transient self-limiting nausea (37).

[^{11}C]PHNO has been used extensively used in many clinical trials. A survey of the *ClinicalTrials.gov* website shows 12 completed trials, 5 recruiting trials, and 1 active clinical trial with [^{11}C]PHNO investigating a variety of behaviors and disorders: alcohol drinking, addictive behavior, behavioral symptoms, cocaine-related disorders, compulsive behavior, depression, depressive disorder, drinking behavior, impulsive behavior, mental disorders, mood disorders, obsessive-compulsive disorder, psychotic disorders, schizophrenia studies, schizophrenia spectrum and other psychotic disorders, psychological stress, substance-related disorders, and tobacco use disorder.

[^{11}C]PHNO has been used in humans at the Yale University PET center in several studies including HIC 0910005822. To date, more than 250 human [^{11}C]PHNO scans have been conducted at Yale PET Center. All injected doses were limited to ≤ 0.03 μg/kg. These studies included smokers, cocaine users, pathological gamblers, schizophrenia subjects, and healthy controls. Adverse events were observed in studies which involved administration of amphetamine in addition to [^{11}C]PHNO. The typical protocol involved a morning [^{11}C]PHNO administration, followed by oral amphetamine, followed by a second PHNO injection, starting 3 hours post-amphetamine. Because nausea is attributed to the transient high [^{11}C]PHNO concentration 2–3 min after the bolus injection of the tracer (37), the Yale PET Center administers [^{11}C]PHNO as a bolus over a period of 5 minutes, *i.e.*, at a PHNO dose rate of 7 ng/min for the average 70 kg subject. Administration of [^{11}C]PHNO dose at this rate is about 5 times slower than lowest rate reported in the clinical study involving i.v. administration of PHNO (38).

Proposed single study dose limit

[^{11}C]PHNO will be administered intravenously.

The proposed radioactivity dose of 10 mCi per single administration is below the 21 CFR 361.1 estimated dose limit of 75.5 mCi for a 70 kg Hermaphrodite Male (77.7 mCi for female). The estimated dose limit calculations are based upon the liver as the critical organ; 5 rem per single study limit: 0.0662 rem per mCi to the combined male and female livers.

The maximum allowable dose for a single injection is 3000 mR to the whole body, active blood-forming organs, lens of the eye and gonads. The dose to any other organ cannot exceed 5000 mR.

The maximum allowable dose for one year is 5000 mR to the whole body, active blood-forming organs, lens of the eye and gonads. The dose to any other organ cannot exceed 15000 mR.

For comparison, the average person in the United States receives a radiation exposure of 0.3 rem (or 300 mrem) per year from natural background sources, such as from the sun, outer space, and from radioactive materials that are found naturally in the earth's air and soil. The dose that a subject will receive from participation in this research study would be less than that obtained in one year from natural sources.

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As previously mentioned, the maximum amount of radiation per year an individual subject will receive in this study is from up to 8 injections of ≤ 10mCi each of [^{11}C]PHNO, plus transmission scans. The intended amount is from up to 4 injections of ≤ 10mCi each of [^{11}C]PHNO for smokers and up to 2 injections of ≤ 10mCi each of [^{11}C]PHNO in nonsmokers, however in case of scan failures post injection we have included up to 8 injections if scan sets need to be repeated within the study time frame (i.e. 1-2 wks withdrawal and 2-8 wks withdrawal). Nonsmokers maximum number of injections including re-scans would be 4 scans total. Should the second scan of the set fail, we will make every effort to reschedule the scan for repeat within 1 wk, however if scheduling does not permit this, we may ask the subject to repeat the whole set due to timing related data.

2 injections PHNO	0.4292 rem
4 injections PHNO	0.8584 rem
8 injections PHNO	1.7168 rem

<u>d-Amphetamine:</u>
Amphetamine is administered to measure changes in [^{11}C]PHNO binding due to dopamine release. This dose was chosen because it is expected to produce a quantifiable displacement of the radiotracer. The risks are outlined in Section VI. Oral and IV d-amphetamine administration to healthy humans and individuals with psychiatric disorders has been safely used in many PET imaging studies. We will use the oral route due to the greater safety and ease of administration.

3. **Source:** a) Identify the source of the drug or biologic to be used.

[^{11}C]PHNO will be synthesized at the Yale University PET Center radiochemistry Laboratory under the supervision of Drs. Henry Huang & Nabeel Nabulsi.
d-amphetamine will be provided by the YNHH pharmacy.

b) Is the drug provided free of charge to subjects? ☒ Yes ☐ No
If yes, by whom? PET center

4. **Storage, Preparation and Use:** Describe the method of storage, preparation, stability information, and for parenteral products, method of sterilization and method of testing sterility and pyrogenicity.

[^{11}C]PHNO

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Due to the short half-life, PET drugs are prepared and formulated immediately before administration, and therefore there are no issues with storage or stability. PET drug products are stored at room temperature and are stable for at least 60 min after preparation.

The preparation of sterile PET drug products is validated prior to human use. Sterility is achieved by passing the PET drug product through a 0.22 micron membrane filter during the last step of the formulation process. Prior to release for administration, a bubble point test is performed on the membrane filter used for terminal sterilization in order to validate and verify its integrity during the filtration process. Due to the short half-life, a sample of the PET drug product is tested for sterility after administration for further confirmation.

The level of endotoxin in each batch of the final PET drug product is determined quantitatively prior to release for administration using the FDA approved Charles River Laboratory's Portable Testing System (Endosafe®-PTS).

[^{11}C]PHNO will be prepared at the Yale PET Center in accordance with local Chemistry Manufacturing & Control (CMC) procedures and quality specifications described in local FDA-approved Drug Master File (IND No. 134138). Briefly, [^{11}C]propionyl chloride is prepared by reaction of [^{11}C]CO_2 with ethylmagnesium bromide, followed by treatment with phthaloyl dichloride. [^{11}C]propionyl chloride then reacts with 9-hydroxynaphthoxazine to generate a [^{11}C]-amide, which is subsequently reduced by lithium aluminium hydride to yield [^{11}C]PHNO. The resulting PET drug product is purified first by semi-preparative HPLC, followed by solid-phase extraction to remove the HPLC buffer mixture. Finally [11C]PHNO is formulated in <10% ethanolic saline solution (USP), and the resulting PET drug product is then passed through a 0.22 micron sterile membrane filter for terminal sterilization and collected in a sterile pyrogen free collection vial to afford a formulated I.V. solution ready for dispensing and administration.

Amphetamine
Amphetamine will be stored and prepared at Yale New Haven Hospital pharmacy.

Check applicable Investigational Drug Service utilized:

☒ **YNHH IDS**	☐ **Yale Cancer Center**
☐ **CMHC Pharmacy**	☐ **West Haven VA**
☒ **PET Center**	☐ **None**
☐ **Other:**	

Note: If the YNHH IDS (or comparable service at CMHC or WHVA) will not be utilized, explain in detail how the PI will oversee these aspects of drug accountability, storage, and preparation.

5. **Use of Placebo: ☒ Not applicable to this research project**
   If use of a placebo is planned, provide a justification which addresses the following:
   a. Describe the safety and efficacy of other available therapies. If there are no other available therapies, state this.
   b. State the maximum total length of time a participant may receive placebo while on the study.
   c. Address the greatest potential harm that may come to a participant as a result of receiving placebo.
   d. Describe the procedures that are in place to safeguard participants receiving placebo.

6. **Use of Controlled Substances:**

Will this research project involve the use of controlled substances in human subjects?
☒ Yes ☐ No *See HIC Application Instructions to view controlled substance listings*.

If yes, is the use of the controlled substance considered:
☐ Therapeutic: The use of the controlled substance, within the context of the research, has the potential to benefit the research participant.
☒ Non-Therapeutic: *Note, the use of a controlled substance in a non-therapeutic research study involving human subjects may require that the investigator obtain a Laboratory Research License. Examples include controlled substances used for basic imaging, observation or biochemical studies or other non-therapeutic purposes. See Instructions for further information.*

Dextro-amphetamine (0.5 mg/kg) will be given by mouth to each subject 3 hours prior to the second [^{11}C]PHNO PET scan.

7. **Continuation of Drug Therapy After Study Closure** ☒ **Not applicable to this project**
Are subjects provided the opportunity to continue to receive the study drug(s) after the study has ended?
☐ Yes If yes, describe the conditions under which continued access to study drug(s) may apply as well as conditions for termination of such access.

☐ No If no, explain why this is acceptable.

SECTION IV: RECRUITMENT/CONSENT AND ASSENT PROCEDURES

1. **Targeted Enrollment:** Give the number of subjects

a. targeted for enrollment at Yale for this protocol: 100
b. If this is a multi-site study, give the total number of subjects targeted across all sites___

2. **Indicate recruitment methods below.** Attach copies of any recruitment materials that will be used.

☒ Flyers	☒ Internet/Web Postings	☒ Radio
☒ Posters	☐ Mass E-mail Solicitation	☐ Telephone
☐ Letter	☒ Departmental/Center Website	☒ Television
☐ Medical Record Review	☒ Departmental/Center Research Boards	☒ Newspaper
☐ Departmental/Center Newsletters	☐ Web-Based Clinical Trial Registries	
☒ YCCI Recruitment Database	☒ Clinicaltrials.gov Registry (do not send materials to HIC)	
☐ Other (describe):		

3. **Recruitment Procedures:**
   a. Describe how potential subjects will be identified.
   b. Describe how potential subjects are contacted.
   c. Who is recruiting potential subjects?

Subjects will be recruited through flyers, public advertisement (newspaper, radio, internet posting), by word of mouth, contact with community service groups, and clinics and local

treatment facilities (the VA Hospital, West Haven, CMHC, the Yale Psychiatric Hospital, Mood Disorders Research Program, the Yale Depression Research Program). The subjects will be asked to call us if they are interested in participating in the research study. The PI, in collaboration with study investigators, is responsible for subject recruitment.

4. **Screening Procedures**

a. Will email or telephone correspondence be used to screen potential subjects for eligibility prior to the potential subject coming to the research office? ☒ Yes ☐ No

b. If yes, identify any health information and check off any of the following HIPAA identifiers to be collected and retained by the research team during this screening process.

HEALTH INFORMATION TO BE COLLECTED: We will ask about general health, neurological disorders, past surgeries, past injuries especially to the head, psychological history, immediate family psychological history.

HIPAA identifiers:

☒ Names

☒ All geographic subdivisions smaller than a State, including: street address, city, county, precinct, zip codes and their equivalent geocodes, except for the initial three digits of a zip code if, according to the current publicly-available data from the Bureau of the Census: (1) the geographic unit formed by combining all zip codes with the same three initial digits contains more than 20,000 people, and (2) the initial three digits of a zip code for all such geographic units containing 20,000 or fewer people is changed to 000.

☒ Telephone numbers

☐ Fax numbers

☒ E-mail addresses

☐ Social Security numbers

☐ Medical record numbers

☐ Health plan beneficiary numbers

☐ Account numbers

☒ All elements of dates (except year) for dates related to an individual, including: birth date, admission date, discharge date, date of death, all ages over 89 and all elements of dates (including year) indicative of such age, except that such ages and elements may be aggregated into a single category of age 90 or older

☐ Certificate/license numbers

☐ Vehicle identifiers and serial numbers, including license plate numbers

☐ Device identifiers and serial numbers

☐ Web Universal Resource Locators (URLs)

☐ Internet Protocol (IP) address numbers

☐ Biometric identifiers, including finger and voice prints

☐ Full face photographic images and any comparable images

☐ Any other unique identifying numbers, characteristics, or codes

5. **Assessment of Current Health Provider Relationship for HIPAA Consideration:**

Does the Investigator or any member of the research team have a direct existing clinical relationship with any potential subject?

☐ Yes, all subjects

☐ Yes, some of the subjects

☒ No

If yes, describe the nature of this relationship.

6. **Request for waiver of HIPAA authorization:** (When requesting a waiver of HIPAA Authorization for either the entire study, or for recruitment purposes only. Note: if you are

collecting PHI as part of a phone or email screen, you must request a HIPAA waiver for recruitment purposes.)

Choose one: For entire study: _____ For recruitment purposes only: ___X___

i. Describe why it would be impracticable to obtain the subject's authorization for use/disclosure of this data;
ii. If requesting a waiver of **signed** authorization, describe why it would be impracticable to obtain the subject's signed authorization for use/disclosure of this data;

By signing this protocol application, the investigator assures that the protected health information for which a Waiver of Authorization has been requested will not be reused or disclosed to any person or entity other than those listed in this application, except as required by law, for authorized oversight of this research study, or as specifically approved for use in another study by an IRB.

Researchers are reminded that unauthorized disclosures of PHI to individuals outside of the Yale HIPAA-Covered entity must be accounted for in the "accounting for disclosures log", by subject name, purpose, date, recipients, and a description of information provided. Logs are to be forwarded to the Deputy HIPAA Privacy Officer.

7. **Required HIPAA Authorization:** If the research involves the creation, use or disclosure of protected health information (PHI), separate subject authorization is required under the HIPAA Privacy Rule. Indicate which of the following forms are being provided:
   ☒ Compound Consent and Authorization form
   ☐ HIPAA Research Authorization Form

8. **Consent Personnel**: List the names of all members of the research team who will be obtaining consent/assent:

Located in IRES

9. **Process of Consent/Assent:** Describe the setting and conditions under which consent/assent will be obtained, including parental permission or surrogate permission and the steps taken to ensure subjects' independent decision-making.

The consent process is a multistep process, whereby information about the risks and benefits of the study will be provided to potential subjects across several sessions. The number of sessions over which this information will be provided will depend on how well the subject understands and retains the information. The process begins with the subject initiating contact via telephone. The research staff will provide a brief description of the study following which the subject is screened by a member of the research team. Thereafter, potentially eligible candidates are scheduled for a face-to-face interview. The study procedures will be described as a research tool with potential to enhance our knowledge about the brain. Subjects will also be informed of all potential risks of participation. Subjects will be required to read the informed consent form and the investigator will additionally describe the risks and discomforts.

To ensure that the study subject understands the study, the subject will be asked questions about the study procedures and the risks associated with participation. If any concern arises that the study subject did not fully understand the study, the principal investigator (PI) may decide

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that the subject is not suitable for participation. This process generally takes about one hour. If the subject is still interested after all questions have been answered, the PI or staff member consenting, will ask the subject to sign the informed consent form. Any subject who appears incapable of providing informed consent will be excluded. Subjects will be informed that they can decline to participate in the study without penalty and given the opportunity to withdraw from the study prior to analysis of their data. Following the resolution of any questions, the subjects will be asked to sign the consent form if he/she agrees to participate.

The decision not to participate will not affect an individual's eligibility to participate in future studies, to receive treatment at Yale-New Haven Hospital, or to receive treatment on a private basis from a referring clinician. A copy of the signed consent form will be provided to all participating subjects. For subjects who are not eligible, all PHI will be destroyed.

10. **Evaluation of Subject(s) Capacity to Provide Informed Consent/Assent:** Indicate how the personnel obtaining consent will assess the potential subject's ability and capacity to consent to the research being proposed.

In cases in which capacity is in doubt, the PI will assess the subject's understanding of the study and the subject's capacity to decide to participate.

11. **Documentation of Consent/Assent:** Specify the documents that will be used during the consent/assent process. Copies of all documents should be appended to the protocol, in the same format that they will be given to subjects.

Compound Authorization Form

12. **Non-English Speaking Subjects:** Explain provisions in place to ensure comprehension for research involving non-English speaking subjects. Translated copies of all consent materials must be submitted for approval prior to use.

Non-English speaking subjects will not be invited to participate in the studies. All of our materials are in English only, and staff members are fluent in English. Furthermore, cognitive testing is validated in English-speaking subjects only.

13. **Consent Waiver: In certain circumstances, the HIC may grant a waiver of signed consent, or a full waiver of consent, depending on the study.** If you will request either a waiver of consent, or a waiver of signed consent for this study, complete the appropriate section below.

☐ **Not Requesting a consent waiver**
☒ **Requesting a waiver of signed consent**
☐ **Requesting a full waiver of consent**

A. Waiver of **signed** consent: (Verbal consent from subjects will be obtained. **If PHI is collected, information in this section must match Section IV, Question 6**)

☒ **Requesting a waiver of signed consent for <u>Recruitment/Screening</u> only**

If requesting a waiver of signed consent, please address the following:

a. Would the signed consent form be the only record linking the subject and the research?
☐ Yes ☐ No

b. Does a breach of confidentiality constitute the principal risk to subjects?
☐ Yes ☐ No

OR

c. Does the research activity pose greater than minimal risk?
☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** Please note: Recruitment/screening is generally a minimal risk research activity
☒ No

AND

d. Does the research include any activities that would require signed consent in a non-research context? ☐ Yes ☒ No

☐ **Requesting a waiver of signed consent for the <u>Entire Study</u> (**Note that an information sheet may be required.)

If requesting a waiver of signed consent, please address the following:
a. Would the signed consent form be the only record linking the subject and the research?
☐ Yes ☐ No
b. Does a breach of confidentiality constitute the principal risk to subjects?
☐ Yes ☐ No

OR

c. Does the research pose greater than minimal risk? ☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** ☐ No

AND

d. Does the research include any activities that would require signed consent in a non-research context? ☐ Yes ☐ No

B. **Full waiver** of consent: (No consent from subjects will be obtained for the activity.)

☐ **Requesting a waiver of consent for <u>Recruitment/Screening</u> only**

a. Does the research activity pose greater than minimal risk to subjects?
☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** Please note: Recruitment/screening is generally a minimal risk research activity
☐ No
b. Will the waiver adversely affect subjects' rights and welfare? ☐ Yes ☐ No
c. Why would the research be impracticable to conduct without the waiver?
d. Where appropriate, how will pertinent information be returned to, or shared with subjects at a later date?

☐ **Requesting a full waiver of consent for the <u>Entire Study</u> (Note: If PHI is collected, information here must match Section IV, question 6.)**

If requesting a full waiver of consent, please address the following:

a. Does the research pose greater than minimal risk to subjects? ☐ Yes ***If you answered yes, stop. A waiver cannot be granted.*** ☐ No
b. Will the waiver adversely affect subjects' rights and welfare? ☐ Yes ☐ No
c. Why would the research be impracticable to conduct without the waiver?
d. Where appropriate, how will pertinent information be returned to, or shared with subjects at a later date?

SECTION V: PROTECTION OF RESEARCH SUBJECTS

Confidentiality & Security of Data:

a. What protected health information (medical information along with the HIPAA identifiers) about subjects will be collected and used for the research?

Required private identifiable information about individuals, such as their medical history, current medications, psychiatric problems, and family history, will be collected by research staff and be used for research purposes and charting after consent is obtained. Identifiers collected may include name, birthdate, and social security number.

b. How will the research data be collected, recorded and stored?

The data are collected and recorded by trained research personnel. The data will be recorded on Excel spreadsheets that will be saved onto a server or will be in the form of questionnaires that are filled out by the subject or the researcher. These paper research materials containing confidential information are stored in locked filing cabinets. Additional brain data is collected during the brain imaging scans by trained technologists and is stored on password-protected and encrypted computers with identifying information carefully in compliance with HIPAA regulations.

c. How will the digital data be stored? ☐ CD ☐ DVD ☐ Flash Drive ☐ Portable Hard Drive ☒ Secured Server ☐ Laptop Computer ☐ Desktop Computer ☐ Other

d. What methods and procedures will be used to safeguard the confidentiality and security of the identifiable study data and the storage media indicated above during and after the subject's participation in the study?

Do all portable devices contain encryption software? ☐ Yes ☐ No
If no, see http://hipaa.yale.edu/guidance/policy.html

All staff members that come into contact with the data are fully trained to the current HIPAA regulations and are informed as to the proper use of all data.

Identifiable paper information is kept in locked file drawers and password protected computer files. Results are published as group data without the use of characteristics that would identify individual subjects. We quote information only by number in conference discussions, scientific reports, or publications, in order to maintain anonymity.

Identifiable research data, including recruitment and screening information and code keys, are stored on a secure database located on the internal PET Center Network. The PET network is protected by a Cisco PIX firewall operated by ITS. All research data are backed up nightly to a Dell PV-136T library wit 4 IBM Ultrium-TD2 tape drives using the backup software Legato Networker 7.3 from EMC. Human subjects enrolled in the study are assigned a subject-specific random identifier. Subject identifiers and the means to link the subject names and codes with the research data are stored in separate locations within the database. The software of the database limits the ability to connect the random identifier to the actual subject identification information to research team members only. Access to the database is password protected and each research team member is required to have a unique ID and password to gain access to the database. Authorized users employ their netid and authentication is performed using Yale's

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central authentication server. Users always access research data through the random identifier only. Direct identifiers belonging to subjects who withdraw from the study, will be stripped from the key.

e. What will be done with the data when the research is completed? Are there plans to destroy the identifiable data? If yes, describe how, by whom and when identifiers will be destroyed. If no, describe how the data and/or identifiers will be secured.

The data will be stored in locked filing cabinets and on the password-protected secure database on the internal Yale University PET Center Network for at least 7 years, accessed only by authorized personnel.

f. Who will have access to the protected health information (such as the research sponsor, the investigator, the research staff, all research monitors, FDA, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), SSC, etc.)? (please distinguish between PHI and de-identified data)

The investigator and research staff (e.g., PET center nuclear technologists, recruiters) will have access to the PHI only on as needed to know basis. The FDA may also have access to the PHI.

g. If appropriate, has a Certificate of Confidentiality been obtained?

This protocol is funded by NIH. As such, according to the NIH policy issues in October 2017, the information collected from subjects is automatically protected by a Certificate of Confidentiality (CoC).

h. Are any of the study procedures likely to yield information subject to mandatory reporting requirements? (e.g. HIV testing – reporting of communicable diseases; parent interview - incidents of child abuse, elderly abuse, etc.). Please verify to whom such instances will need to be reported.

No.

SECTION VI: POTENTIAL BENEFITS

Potential Benefits: Identify any benefits that may be reasonably expected to result from the research, either to the subject(s) or to society at large. *(Note: Payment of subjects is not considered a benefit in this context of the risk-benefit assessment.)*

There are no direct benefits to the nonsmoking subjects for participating in this study. Smoking subjects receive smoking cessation support to abstain from tobacco smoking. This research will benefit scientific knowledge by contributing to the understanding of the neurochemical changes that occur during the recovery from tobacco smoking. This may have clinical application in the future.

SECTION VII: RESEARCH ALTERNATIVES AND ECONOMIC CONSIDERATIONS

1. **Alternatives:** What alternatives are available to the study subjects outside of the research?

The alternative to participation in this research protocol is to not participate. Subjects will be informed that they are free to choose not to participate and, if they do agree to become a subject, they will be free to withdraw from the study at any time during its course. They will also be informed that if they choose not to participate or if they withdraw, it will not adversely affect their relationship with their doctors or the hospital (see attached Consent Form).

2. **Payments for Participation (Economic Considerations):** Describe any payments that will be made to subjects and the conditions for receiving this compensation.

The subjects will be compensated for their time commitment and inconveniences necessary for completing the study. Subjects will have no financial responsibilities for any portion of the study. For Aim 1 payment would be $350 for each PHNO PET scan, $50 for each MRI scan, and $40 for cognitive testing at baseline. Subjects who participate in the Probabilistic Reward Task may also be compensated for the amount that they "win" during the task, up to $50. Subjects may also receive an extra $10 for each Cold Pressor Task that they participate in. Subjects will be paid either by check, and are advised to allow 4-6 weeks for receipt of payment, or they will be given a credit card or cash. In addition, subjects will be provided with a light meal, at the end of the PET imaging day. Reasonable transportation costs will be reimbursed. Receipts must be submitted. If participation in the PET Scan has already begun, then compensation will be based on involvement in the study, and will be up to the discretion of the PI.
Smokers will also receive $10 for each contingency management appointment at which their carbon monoxide and urine cotinine levels show that they have not smoked. Subjects can decide if they want to receive this amount at each appointment in cash, or receive it all at the end of the study in the form of a credit card or check or cash. Smokers will receive an additional $100 bonus for completing the study.

Cancellations: If a PET scan should get cancelled for a reason outside of the subject's control (i.e. radiotracer synthesis failure) the subject will be paid $50 minimum, or a higher amount not to exceed the payment for a full scan day. The amount of the payment for cancellation will be based on the subject's length of participation on that scan day prior to the cancellation, and will be up to the discretion of the PI.

3. **Costs for Participation (Economic Considerations):** Clearly describe the subject's costs associated with participation in the research, and the interventions or procedures of the study that will be provided at no cost to subjects.

There will be no costs to subjects related to participation in this research intervention.

4. **In Case of Injury:** This section is required for any research involving more than minimal risk.
    a. Will medical treatment be available if research-related injury occurs?
    b. Where and from whom may treatment be obtained?
    c. Are there any limits to the treatment being provided?
    d. Who will pay for this treatment?
    e. How will the medical treatment be accessed by subjects?

Medical treatment will be offered to the subjects for any physical injuries that they receive as a result of participating in this research. However, the subject or his/her insurance company is

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responsible for the cost. Federal regulations require that subjects be told that if they are physically injured, no additional financial compensation is available.

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