Tobacco smoking interferes with GABA$_A$ receptor neuroadaptations during prolonged alcohol withdrawal

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Understanding the effects of tobacco smoking on neuroadaptations in GABA$_A$ receptor levels over alcohol withdrawal will provide critical insights for the treatment of comorbid alcohol and nicotine dependence. We conducted parallel studies in human subjects and nonhuman primates to investigate the differential effects of tobacco smoking and nicotine on changes in GABA$_A$ receptor availability during acute and prolonged alcohol withdrawal. We report that alcohol withdrawal with or without concurrent tobacco smoking/nicotine consumption resulted in significant and robust elevations in GABA$_A$ receptor levels over the first week of withdrawal. Over prolonged withdrawal, GABA$_A$ receptors returned to control levels in alcohol-dependent nonsmokers, but alcohol-dependent smokers had significant and sustained elevations in GABA$_A$ receptors that were associated with craving for alcohol and cigarettes. In nonhuman primates, GABA$_A$ receptor levels normalized by 1 mo of abstinence in both groups—that is, those that consumed alcohol alone or the combination of alcohol and nicotine. These data suggest that constituents in tobacco smoke other than nicotine block the recovery of GABA$_A$ receptor systems during sustained alcohol abstinence, contributing to alcohol relapse and the perpetuation of smoking.

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Alcohol dependence and tobacco smoking are highly comorbid (1). Alcohol-dependent smokers who quit drinking but continue smoking may have a reduced severity of alcohol withdrawal and relapse risk (2) compared with alcohol-dependent smokers who stop smoking and drinking at the same time (3–5). This has led to some complacency in the field about treating the addiction to nicotine in alcohol-dependent smokers, and few treatment settings provide any systematic tobacco treatment (6). However, a large part of the morbidity and mortality from alcohol dependence can be attributed to concurrent tobacco smoking (7), and a large number of alcohol-dependent individuals in treatment express a desire to quit smoking (8). Understanding the involvement of tobacco smoking in the neuroadaptations and behavioral changes that occur during alcohol withdrawal will provide critical insights to direct treatment strategies.

Given the multiple molecular targets for alcohol in the brain and numerous constituents of tobacco smoke, it is likely that the neurobiology of this comorbidity is complex. However, the γ-aminobutyric acid (GABA) system may be an important point of convergence of the effects of tobacco smoke and alcohol in the brain. For example, nicotine reinforcement has been critically linked to activation of GABA neurons (9), and alcohol appears to both directly stimulate extrasynaptic GABA$_A$ receptors with relatively high affinity (10) and to indirectly stimulate the release of GABA and neurosteroids (11), such as allopregnanolone, that also stimulate extrasynaptic GABA$_A$ receptors (12, 13). Alcohol and neurosteroids can act at synaptic GABA$_A$ receptors, but the affinity is low, the response is variable, and the dose of alcohol that would facilitate signaling at these synaptic receptors would induce a coma in humans (14, 15).

Studies in both animals and humans have yielded a tentative model about the convergence of the codependency produced by smoking and alcohol consumption, as has been reviewed (16). In the absence of smoking, chronic alcohol administration produces an adaptive down-regulation of synaptic GABA$_A$ receptor function by altering GABA$_A$ receptor subunit composition and subtly shifting subpopulations of receptors from a relative predominance of low-affinity high Cl– conductance type to greater numbers of a high-affinity low Cl– conductance subtype, characteristic of extrasynaptic GABA$_A$ receptors (15). In early recovery, there is a transitional phase, during which deficits in GABA$_A$ receptor signaling are thought to contribute to withdrawal-related cortical hyperexcitability and low-affinity high-conductance receptors are recruited to reestablish the cortical balance of excitation and inhibition. The recruitment of the additional GABA$_A$ receptors was demonstrated by a transient increase in ligand binding over the first week of alcohol withdrawal (17). In this same cross-sectional study (17), a subset of smokers did not show similar time-dependent alterations during early recovery. Moreover, GABA$_A$ receptor availability was positively correlated with alcohol withdrawal symptoms in nonsmokers but not smokers, suggesting that smoking may have suppressed withdrawal

Significance

Alcohol dependence and tobacco smoking are highly comorbid. Although continued smoking during alcohol withdrawal may reduce relapse risk, most of the morbidity associated with alcohol dependence is due to tobacco smoking and many individuals in alcohol treatment express a desire to quit smoking. We conducted a parallel study in alcohol-dependent humans and nonhuman primates to identify the impact of tobacco smoke and nicotine on the neuroadaptations in the GABA-ergic system that occur during alcohol withdrawal. Our findings show that tobacco smoking, but not nicotine consumption, blocks the recovery of GABA$_A$ receptors during extended alcohol withdrawal and that sustained elevations in GABA$_A$ receptor levels in alcohol-dependent smokers are associated with alcohol and cigarette cravings, possibly contributing to continued smoking.

symptoms by preventing alcohol-related neuroadaptations in GABA$_A$ receptors.

The goal of the current study was to systematically examine the effect of tobacco smoking on alcohol withdrawal-related neuroadaptations in GABA$_A$ levels. The first study was designed to extend the previous cross-sectional findings to determine differences in GABA$_A$ receptor levels in alcohol-dependent smokers versus nonsmokers at multiple times during early withdrawal and during extended abstinence. Additionally, tobacco smoke consists of over 4,000 chemicals. Many of these chemicals may play a role in influencing alcohol-related withdrawal symptoms; however, nicotine, the primary addictive chemical in tobacco smoke, has been the most widely studied tobacco constituent and has been associated with GABA system regulation (9, 18). Thus, a second parallel study was conducted in nonhuman primates that were randomized to self-administer alcohol with or without concurrent access to a nicotine solution rather than tobacco smoke to determine the role of nicotine per se on alcohol withdrawal-related neuroadaptations.

Results

Clinical Characteristics. Twenty-seven alcohol-dependent individuals (5 women; 17 smokers, 10 nonsmokers) and 25 healthy non–alcohol-dependent comparison subjects (5 women; 15 smokers, 10 nonsmokers) were included in the study (Table S1). Alcohol-dependent subjects were admitted to the Clinical Neuroscience Research Unit (CNRU) for up to 1 mo and were imaged with [^{123}I]iomazenil single photon emission computed tomography (SPECT) up to three times (at approximately day 3, 10, and 30) during alcohol withdrawal to measure GABA$_A$ receptor availability. Healthy smokers and nonsmokers were imaged once. Alcohol-dependent smokers had significantly higher levels of craving for alcohol ($P \leq 0.05$) at all three time points during withdrawal compared with alcohol-dependent nonsmokers (Table S2). Craving for alcohol was significantly lower at 4 wk compared with 3 d of withdrawal in alcohol-dependent smokers ($P \leq 0.05$).

Smoking Alters the Time Course of Changes in GABA$_A$ Receptor Availability During Alcohol Withdrawal. GABA$_A$ receptor availability (measured as [^{123}I]iomazenil V$_T$) in alcohol-dependent subjects compared with smoking status-matched controls was significantly different by brain region and by duration of withdrawal (Figs. 1 and 2 and Table S3). A voxel-wise analysis was performed to localize differences in GABA$_A$ receptor availability

between groups. Analyses of t statistic maps demonstrated significant differences ($P < 0.05$) between alcohol-dependent non-smokers and healthy nonsmokers at all time points, with variation by brain region and time. Specifically, alcohol-dependent nonsmokers had significantly higher, but regionally restricted, GABA$_A$ receptor availability at ~3 d of withdrawal compared with healthy nonsmokers in the posterior cingulate, cuneus, and middle occipital gyrus. At ~10 d of withdrawal, differences in GABA$_A$ receptor availability in alcohol-dependent nonsmokers compared with healthy nonsmokers were more widespread, reaching significance in bilateral medial frontal gyri (BA 9 and 10), bilateral insula, and posterior thalamic nuclei, and in addition, remained significantly higher in the posterior cingulate, cuneus, and middle occipital gyrus observed at 3 d of withdrawal. At 4 wk of withdrawal, there was significantly higher but regionally restricted GABA$_A$ receptor availability in alcohol-dependent nonsmokers versus healthy nonsmokers in comparable clusters observed after 3 d of withdrawal, including portions of posterior cingulate, caudate, and thalamic nuclei (Fig. 1). The magnitude of these changes, in the statistically significant voxels, with higher GABA$_A$ receptor availability in alcohol-dependent nonsmokers versus healthy nonsmokers was 6% at 3 d, 38% at 10 d, and back to 6% by 4 wk. The pattern of changes in GABA$_A$ receptor availability is consistent with our previous cross-sectional study (17).

The time course of changes in alcohol-dependent smokers was distinctly different from that in the alcohol-dependent nonsmokers. Analyses of the t statistic maps demonstrated statistically significant differences ($P < 0.05$) in GABA$_A$ receptor availability in alcohol-dependent smokers compared with healthy smokers at each time point that remained strikingly similar in spatial extent over time (Fig. 2). Specifically, alcohol-dependent smokers had widespread and significantly higher GABA$_A$ receptor availability compared with healthy smokers in the medial frontal gyri, anterior cingulate, insular cortex, and medial occipital cortex at ~3 d (25% higher), 10 d (48% higher), and 4 wk (51% higher) of withdrawal. Notably, the brain regions associated with these increases were nearly identical across the three time points (Fig. 2). Thus, in alcohol-dependent smokers, tobacco smoking appears to contribute to elevated GABA$_A$ receptor availability and to prevent the decline observed in the alcohol-dependent nonsmokers with extended abstinence (Fig. 1).
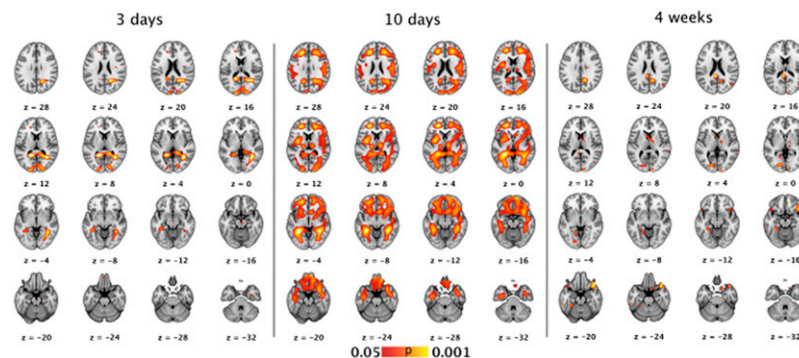

Fig. 1. Higher GABA$_A$ receptor availability in alcohol-dependent nonsmokers compared with healthy nonsmokers peaks at 10 d of withdrawal and recovers by 4 wk of abstinence. The t statistic SPECT images of alcohol-dependent nonsmokers minus age and sex-matched healthy nonsmokers at three time points after cessation of drinking. The color bar indicates significant differences between alcohol-dependent patients and controls at $P < 0.05$. At 3 d of withdrawal (*Left*), alcoholic patients were significantly different from controls in orbito-frontal cortex, posterior cingulate cortex, and the peri-calcarine area of the visual cortex. At 10 d of withdrawal (*Middle*), differences were more widespread; alcoholic patients significantly differed from controls in the same regions as at 3 d of withdrawal, and in addition, there were differences in the middle frontal gyrus and subgyral limbic regions. By 4 wk of withdrawal (*Right*), significant differences were very similar to those at the 3 d withdrawal time point, with less overall differences compared with controls. This indicates that alcohol-dependent patients have undergone substantial recovery in GABA$_A$ receptor availability by 1 mo of abstinence.
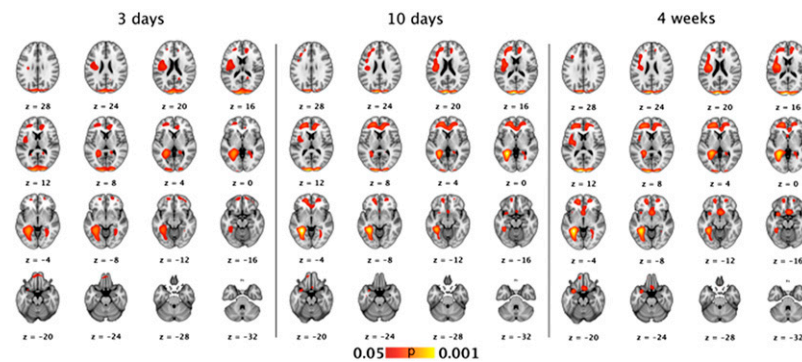
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Fig. 2. Higher GABA$_A$ receptor availability in alcohol-dependent smokers compared with healthy smokers is evident at 3 d of withdrawal and does not change over 4 wk of abstinence. The t statistic SPECT images of alcohol-dependent smokers minus age and sex-matched healthy smokers at three time points after cessation of drinking: 3 d of withdrawal (*Left*), 10 d of withdrawal (*Middle*), and 4 wk of withdrawal (*Right*). Alcohol-dependent smokers had significantly higher GABA$_A$ receptor availability compared with healthy smokers in the following regions from rostral to caudal: bilateral medial frontal gyri (BA 9 and 10), bilateral anterior cingulate (BA 24 and 25), left insular cortex (BA 13), and bilateral medial occipital cortex (BA 18 and 19). These regions are significant and widespread at 3 d and change in magnitude but are remarkably spatially consistent throughout 4 wk of abstinence.

GABA$_A$ Receptor Availability Is Related to Alcohol and Tobacco Smoking Craving in Alcohol-Dependent Smokers but Not Nonsmokers. We examined within-group relationships between five clinical correlates of interest [drinks per day and Tiffany Urge to Smoke Questionnaire (QSU) craving subscales for "Desire" and "Relief" of alcohol and smoking] and the average GABA$_A$ receptor availability at each of the three time points from the group of significant voxels shown in Figs. 1 and 2 in both groups of subjects (24 correlations total; Tiffany QSU for smoking was not examined in the alcohol-dependent nonsmokers). There were no significant correlations between clinical correlates and GABA$_A$ receptor availability in alcohol-dependent nonsmokers at any of the three withdrawal time points. We also investigated if there were correlations between the change in alcohol craving from time 1 to time 2 and GABA$_A$ receptor availability at time 2, which is the peak elevation in GABA$_A$ receptor availability in the alcohol-dependent nonsmokers. There were no significant relationships found. However, in alcohol-dependent smokers, there were significant positive correlations between craving for alcohol and cigarettes and GABA$_A$ receptor availability during the first week of withdrawal. Higher GABA$_A$ receptor availability at 3 d of withdrawal was associated with greater craving to smoke ($r = 0.65$; $P < 0.05$), specifically the desire to smoke to relieve withdrawal symptoms. At 10 d of withdrawal, higher GABA$_A$ receptor availability was associated with greater craving for alcohol ($r = 0.74$; $P < 0.05$), specifically desire to drink for the positive effects of alcohol. Thus, alcohol-dependent smokers have significantly higher levels of craving for alcohol at all time points compared with alcohol-dependent nonsmokers, and higher levels of craving were associated with higher levels of GABA$_A$ receptor availability during early withdrawal.

Preclinical Model of Alcohol and Nicotine Dependence. Thirteen rhesus monkeys were imaged with [^{11}C]flumazenil and positron-emission tomography (PET) to image GABA$_A$ receptor availability at baseline—that is, when drug naïve—and then up to three times during alcohol withdrawal. [^{11}C]flumazenil and [^{123}I]iomazenil both measure cortical GABA$_A$ receptors, and the outcome measures are highly correlated (19, 20). One group of animals ($n = 7$) consumed only alcohol for up to 20 wk (alcohol-only group) before alcohol withdrawal, and a separate group of animals ($n = 6$) consumed alcohol and nicotine for up to 20 wk and then continued to consume nicotine over the course of alcohol withdrawal (alcohol + nicotine group). Animals consumed clinically relevant amounts of alcohol: 4.25 ± 1.03 and 4.18 ± 1.28 g/kg/d over the 20 wk in the alcohol-only and alcohol + nicotine

groups, respectively. Considering 0.25 g/kg to be a standard drink, the animals were averaging 16 drinks per day, which is comparable to the amount consumed by the alcohol-dependent patients. Some animals exhibited increased levels of autonomic arousal during alcohol withdrawal compared with baseline. This is consistent with the clinical presentation of alcohol dependence in that arousal-related withdrawal symptoms do not typically appear initially but worsen with repeated withdrawal cycles.

Nicotine Does Not Alter the Time Course of Alcohol Withdrawal-Induced Changes in GABA$_A$ Receptor Availability in Rhesus Monkeys. There were no significant differences between the alcohol-only and alcohol + nicotine group in changes in GABA$_A$ receptor availability (measured as [^{11}C]flumazenil BP_{ND}) during alcohol withdrawal, suggesting that nicotine did not interfere with adaptations in GABA$_A$ receptors over extended abstinence. Thus, we combined the groups for further analysis. There was a main effect of time in all regions examined (all $P < 0.001$), including frontal, temporal, and occipital cortices and cerebellum. Post hoc analyses indicated statistically significant increases ($P \leq 0.05$) in GABA$_A$ receptor availability in all regions from baseline to 1 d of withdrawal (8–12% increases from baseline across regions) and 8 d of withdrawal (13–15% increases from baseline across regions) but no significant difference between baseline and 4 wk of withdrawal (–5% to 3% changes from baseline across regions) (Fig. 3). Additionally, in the occipital and temporal cortices and cerebellum, GABA$_A$ receptor availability was significantly higher ($P \leq 0.05$) at 8 d of withdrawal compared with 4 wk of withdrawal. These preclinical data bear a striking resemblance to both the temporal pattern and magnitude of changes observed in the alcohol-dependent nonsmokers, with significant increases from baseline during acute withdrawal and an apparent return to baseline or control levels by 4 wk of abstinence.

Discussion

The current study provided, to our knowledge, the first longitudinal evidence in humans and nonhuman primates that alcohol dependence is associated with a rapid up-regulation in GABA$_A$ receptor availability during the initial week of abstinence and a gradual return to baseline levels over the subsequent 3 wk. GABA$_A$ receptor availability is inferred from measurement of [^{123}I]iomazenil V_T in the human subjects and [^{11}C]flumazenil BP_{ND} in the nonhuman primates. Specifically, we found that alcohol-dependent nonsmokers have significantly higher GABA$_A$ receptor availability at 3 d of withdrawal compared with healthy
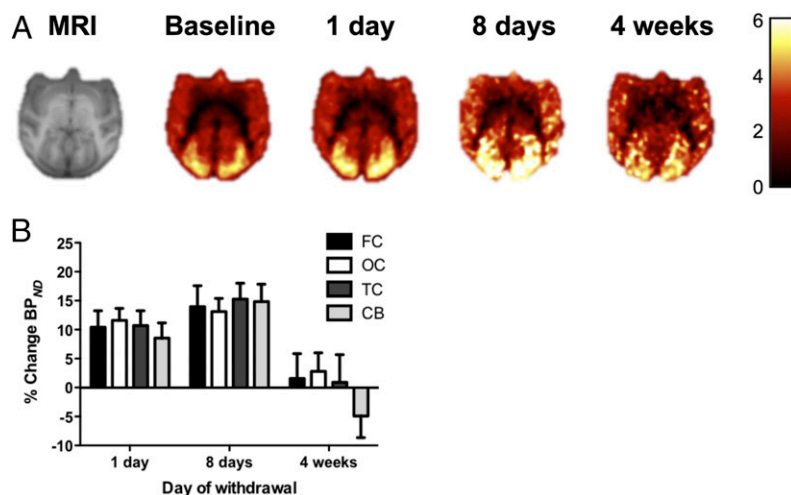
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Fig. 3. Increased GABA$_A$ receptor availability during acute alcohol withdrawal normalized by 4 wk and was not changed by nicotine consumption. The top row (*A*) depicts a representative monkey MRI followed by parametric images of GABA$_A$ receptor availability in the same animal at baseline and at 1 d, 8 d, and 4 wk of withdrawal. The color bar to the right depicts corresponding BP_{ND} values. (*B*) The percent change in BP_{ND} from each animal's baseline to 1 d (*n* = 13), 8 d (*n* = 13), and 4 wk (*n* = 4) of withdrawal in the frontal cortex (FC), occipital cortex (OC), temporal cortex (TC), and cerebellum (CB).

nonsmokers, which increases and peaks at ∼10 d of withdrawal and then by 4 wk of abstinence returns to levels observed at 3 d of withdrawal. The nonhuman primates had a similar temporal pattern and magnitude of change in GABA$_A$ receptor availability after cessation of alcohol administration. Thus, this study extended the findings of an earlier study that inferred an up-regulation of this receptor population during acute withdrawal on the basis of cross-sectional data (17). Further, the similarity of the findings in humans and nonhuman primates supports the attribution of GABA$_A$ receptors to the pharmacologic effects of alcohol, as opposed to other clinical characteristics of the patient population.

Previous studies in patients with more severe alcohol use disorders found evidence of reductions in GABA$_A$ receptors at more prolonged abstinence time points (1–3 mo), which may reflect neural atrophy due to the more severe population (21–23). Similarly, the lack of correlation between GABA$_A$ receptor levels and withdrawal symptoms in the current study may have reflected the relatively low levels of withdrawal symptoms consistent with relatively low levels of severity and chronicity of their alcohol use disorders. However, it is notable that most patients are prophylactically treated with benzodiazepines before it is clear that they will ever have any symptoms that would justify medication. In studies of the treatment of withdrawal that use conservative criteria (i.e., low thresholds for medication that leave a wide safety margin), application of these thresholds dramatically reduces the amount of medications prescribed (for example, see ref. 24). Most of the patients who entered our study were safely monitored throughout detoxification without benzodiazepine administration (25 of 27), increasing the generalizability of our sample to the general population of alcohol-dependent patients.

The temporal pattern of changes was remarkably different in the alcohol-dependent smokers, who did not show time-dependent changes in GABA$_A$ receptor availability associated with the onset of sobriety. This group had significantly higher GABA$_A$ receptor availability at 3 d of withdrawal compared with healthy smokers that remained elevated to a similar and even more pronounced degree at subsequent time points. This pattern in humans contrasted with findings in the nonhuman primates that were administered both alcohol and nicotine. These animals did not differ from nonhuman primates administered only alcohol in the recovery time course of their GABA$_A$ receptors following cessation of alcohol administration. Overall, these findings suggest

two possible interpretations: (*i*) that a constituent of tobacco smoke other than nicotine impedes the recovery of GABA$_A$ receptors after the onset of sobriety or (*ii*) that patients who are at risk for both drinking and smoking differ from patients who only abuse alcohol with respect to their GABA$_A$ receptor regulation.

Tobacco smoke contains a number of substances other than nicotine that might be relevant to the current findings. For example, tobacco smoke contains high levels of the harmala alkaloids harman and norharman, also known as beta-carbolines, which are monoamine oxidase inhibitors and act as inverse agonists at the GABA$_A$ benzodiazepine receptor site (25). Other substances, such as carbon monoxide (26), influence the balance of excitatory and inhibitory neurotransmission and might be expected to directly or indirectly modify GABA$_A$ regulation. Additionally, tobacco smoking is a complex behavior, and people smoke for many reasons, including mood and stress regulation, social bonding, habit, in response to cues, and to alleviate withdrawal. Thus, although it appears that the nicotine per se does not directly affect GABA$_A$ receptor levels, we cannot exclude the possibility that other behavioral components of tobacco smoking are also relevant to our findings. Based on previous work demonstrating lower GABA$_A$ receptor levels in individuals with anxiety disorders versus healthy controls (27) and our previous finding that smoking appears to disrupt the negative relationship between anxiety symptoms and GABA$_A$ receptor levels in healthy controls (28), we might speculate that continued smoking impacts changes in GABA$_A$ receptor levels during alcohol withdrawal by modulating anxiety at a behavioral and a molecular level. Interestingly, a recent study reports higher levels of GABA$_A$ receptors in the amygdala and nucleus accumbens in ex-smokers compared with those who never smoked (29). Of note, the radiotracer used in that study is selective for GABA$_A$ receptors containing the alpha 5 subunit and suggests the effects of smoking on GABA$_A$ receptors are persistent and widespread in the brain. From a clinical perspective, the current data do not raise concerns about the safety of nicotine replacement treatments used to reduce smoking in patients with alcohol use disorders.

The maladaptive nature of the GABA$_A$ receptor up-regulation in alcohol-dependent smokers is suggested by the association with higher levels of craving for alcohol and cigarettes. Generally speaking, alcohol cravings tracked with the severity of alcohol withdrawal, peaking within 3 d of their last drink and declining

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over the subsequent month. However, alcohol-dependent smokers had significantly higher levels of craving for alcohol at all time points than nonsmoking patients, and their cravings were positively correlated with GABA$_A$ receptor availability. Because craving levels tend to predict later patterns of smoking or drinking, these findings raise the possibility that persistent elevations in GABA$_A$ receptors increase relapse risk, whereas normalization of these receptors facilitates recovery.

The current data are consistent with a model that has emerged from both basic and clinical research reviewed in refs. 15–17. In this model, the rise in ligand binding to GABA$_A$ receptors associated with the initial phase of alcohol withdrawal signals an adaptive response to deficits in signaling via these receptors that develops as a consequence of alcohol dependence and is reflective of changes in extrasynaptic versus synaptic receptors. During chronic alcohol exposure, there is an increase in extrasynaptic and a reduction in synaptic GABA$_A$ receptors (30). Extrasynaptic GABA$_A$ receptors typically contain alpha 4 and alpha 6 subunits, whereas intrasynaptic GABA$_A$ receptors typically contain alpha 1–3 subunits (reviewed in ref. 31). During early phase withdrawal, physiological GABAergic disruptions lead to a transient increase in total numbers of GABA$_A$ receptors because extrasynaptic GABA$_A$ receptors remain elevated and the synaptic GABA$_A$ receptors are being recruited. We propose that the peak of GABA$_A$ receptor availability in the alcohol-dependent nonsmokers at ~10 d of abstinence reflects the height of this transition in GABA$_A$ receptors and also signals the end of the early phase withdrawal period. After this approximate 10-d mark, protracted withdrawal begins and the extrasynaptic GABA$_A$ receptors and the overall pool of available receptors returns to baseline levels over time. A limitation of the current study is that we are restricted to measuring changes in GABA$_A$ receptor availability, but it is likely that changes in endogenous GABA tone are occurring in concert with the receptor normalization. Future studies could probe GABA neurotransmission over the course of withdrawal by measuring the effects of a tiagabine challenge with [^{11}C]flumazenil PET.

The radiotracers used in this study, [^{123}I]iomazenil and [^{11}C]flumazenil, are both GABA$_A$ antagonists, and [^{123}I]iomazenil also has weak inverse agonist properties (32, 33). They have similar pharmacological specificity, and both bind to alpha 1 through alpha 6 subunits and thus measure all GABA$_A$ receptors in the brain. Although we cannot distinguish between subunits, it is known that the alpha 1 subunit is a component of ~50% of GABA$_A$ receptors and is usually expressed in synaptic GABA$_A$ receptors (31); thus, it is likely that we are measuring a transient up-regulation of alpha 1 subunit-containing synaptic GABA$_A$ receptors during acute alcohol withdrawal. Further, there are several endogenous benzodiazepine ligands that could compete with [^{123}I]iomazenil and [^{11}C]flumazenil at the binding site; however, the sensitivity of these radiotracers to those ligands has not been documented in vivo. Additionally, prior work has argued against fluctuations in endogenous inverse agonist levels in humans during alcohol withdrawal (34).

In conclusion, GABA$_A$ receptor levels undergo adaptive changes during alcohol withdrawal. In the current study, numbers of GABA$_A$ receptors robustly increased during the first week and normalized by the fourth week of alcohol abstinence. However, continued tobacco smoking during withdrawal interfered with the subsequent normalization of the GABA$_A$ receptors and was associated with higher levels of craving, which may increase relapse risk. These data suggest that chemicals in tobacco smoke other than nicotine impede the adaptive recovery of GABA$_A$ receptors during extended alcohol abstinence and may contribute to alcohol relapse and the perpetuation of smoking. Thus, the GABAergic system is an important mediator of the relationship between comorbid alcohol dependence and tobacco smoking and should be targeted in future therapeutic strategies.

Materials and Methods

Human Subjects. This study was approved by the Yale University School of Medicine Human Investigation Committee, the West Haven Veterans Administration Human Subjects Subcommittee, and the Radiation Safety Committee. Twenty-seven alcohol-dependent (17 smokers, 10 nonsmokers) men ($n = 22$) and women ($n = 5$; 2 smokers, 3 nonsmokers) and 25 age- and sex-matched healthy controls (15 smokers, 10 nonsmokers) participated in up to three [^{123}I]iomazenil SPECT and one magnetic resonance imaging (MRI) scan. Alcohol-dependent subjects were admitted to the Connecticut Mental Health Center Clinical Neuroscience Unit and imaged at the following time points: 2.5 ± 1.1 ($n = 24$), 9.8 ± 2.2 ($n = 25$), and 29.4 ± 3.3 ($n = 21$) d of abstinence. They were assessed for alcohol withdrawal symptoms using the Clinical Institute Withdrawal Assessment every 6 h until the scores were 0. Subjects with Clinical Institute Withdrawal Assessment scores ≥ 10 were evaluated for needing benzodiazepine treatment. Alcohol-dependent subjects requiring benzodiazepine treatment during the first week were not imaged while they were taking benzodiazepines, but they were encouraged to complete remaining scans because benzodiazepine treatment does not alter GABA$_A$ receptor levels (35). Two alcohol-dependent subjects required benzodiazepine treatment, with one subject missing scan 1 and the second subject missing scans 2 and 3. Subjects were recruited and deemed eligible according to the same criteria used previously (17) (*SI Materials and Methods*).

[^{123}I]iomazenil SPECT Imaging. [^{123}I]iomazenil was prepared and administered as previously described (17) with a total dose of 211 ± 24 MBq that did not differ between groups. One MRI, SPECT emission scans, and i.v. blood samples were acquired as previously described (17).

Human Image and Statistical Analysis. Emission data were reconstructed with a nonuniform attenuation correction as described previously (17). For each subject, a mean image was made from the three [^{123}I]iomazenil scans scaled to the total volume of distribution (V_T/f_P, milliliters per cubic centimeter). Values of f_p did not differ significantly within a group over time or between groups. The primary intent of this study was to locate and quantitate regional GABA$_A$ receptor availability changes. Global (whole-brain) fluctuations do occur both inter- and intrasubject and can be due to individual-specific physiology or technical causes. To prevent the confounding of focal changes with effectively random global effects, regional GABA$_A$ receptor levels were analyzed after normalization that negated both inter- and intrasubject global fluctuations. This normalization consisted of multiplying every voxel of each scan by a normalization factor, calculated as the mean gray matter GABA$_A$ receptor availability across this study divided by the individual-specific GABA$_A$ receptor availability for each scan. This normalization is conservative in that it causes a minor but systematic underestimation of receptor availability and follows standard protocol for the detection of region-specific tracer uptake (36). Percent changes were calculated within significant regions displayed in Figs. 1 and 2. Specifically, the proportion of increased signal in the test group (alcohol-dependent smoker or alcohol-dependent nonsmoker) relative to the control group (healthy smoker or healthy nonsmoker, respectively) reflects the proportion of increased receptor availability. One-tailed t tests were used to test the hypothesis that GABA$_A$ receptor availability increased as a function of time in both alcohol-dependent nonsmokers and alcohol-dependent smoker groups after cessation of alcohol use. Notably, no minimum volume threshold was applied to significant clusters (Figs. 1 and 2), as significant voxels clustered naturally and did not require such enhancement (37). All tests were considered significant at the $\alpha = 0.05$ threshold. The false discovery rate was controlled at 5% using standard methods (38). Relationships between significant voxels of interest and clinical correlates were evaluated with correlational analysis and were considered significant at the $\alpha = 0.05$ threshold, not corrected for multiple correlations.

Animals. The animal protocol was approved by the Yale University Institutional Animal Care and Use Committee and is in compliance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Thirteen gonadally intact adolescent (3–4 y, 4.2–6.9 kg) male rhesus macaques (*Macacca mulatta*) were used. Animals had two baseline [^{11}C]flumazenil PET scans—that is, before administration of alcohol and nicotine—and then were imaged at 1 d, 8 d, and 4 wk after removal of alcohol (details on drug self-administration in *SI Materials and Methods*). During the first 8 d of withdrawal, water and food consumption were recorded and behavior was monitored with a video recorder and rated for withdrawal-associated behaviors. A research assistant rated the animals' behaviors for

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15 min from the video at 30 min after filming started for fearful agitation (e.g., teeth chattering, eye blinks, yawns), general agitation (cage bangs, pacing behavior), anxiety (e.g., scratches, foot licks, cage licks), and abdominal distress (e.g., vomiting, dry heaves).

[^{11}C]flumazenil PET Imaging and Analysis. Animal PET imaging was conducted as previously described (39). Animals were imaged under isoflurane anesthesia, which was maintained at 2% (range of 1.75–2.25%) for the duration of each scan.

[^{11}C]flumazenil was administered as a bolus plus constant infusion (197 ± 67 MBq), and the mass of unlabeled flumazenil was 0.13 ± 0.09 μg/kg. Data were reconstructed and analyzed as previously described (39), with one exception. Specifically, binding potential (BP_{ND}) was calculated using the simplified reference tissue model (40, 41), with pons as the reference region (41). The following ROIs were delineated on the parametric BP_{ND} image: frontal cortex, occipital cortex, temporal cortex, and cerebellum. We note that there is some evidence for specific binding in the pons, which we used here as the reference region for BP_{ND} calculations. However, if pons-specific binding increased along with cortical-specific binding during withdrawal, our measured increases would in fact underestimate the changes in the cortex.

Statistical Analysis. Alcohol withdrawal-induced changes in GABA$_A$ receptor availability in rhesus monkeys were assessed separately for each region using linear mixed models with group (alcohol-only, alcohol + nicotine) as a between-subjects factor and time (baseline, 24 h, 8 d, and 4 wk of withdrawal) as a within-subjects factor. The interaction between group and time was modeled, and the best fitting variance–covariance structure was chosen based on information criteria. Post hoc linear contrasts were estimated to evaluate the nature of main and interactive effects. Levels of fearful and general agitation were highly skewed and could not be sufficiently normalized with transformation. Thus, these outcomes were analyzed, including the same factors described above, using the nonparametric approach for repeated measures data (42), where the data were first ranked, and then fitted using a mixed-effects model with an unstructured variance–covariance matrix and P values adjusted for ANOVA-type statistics. All tests were considered significant at the α = 0.05 threshold.

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