ACS Chemical Neuroscience

Letter

pubs.acs.org/chemneuro

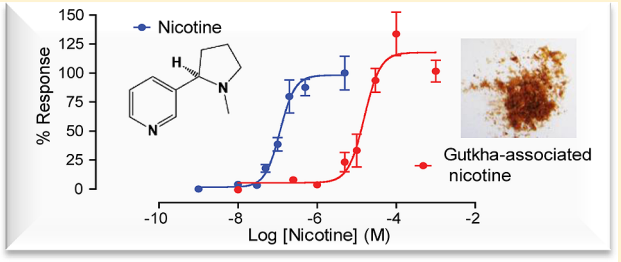
Striatal Dopamine Release Regulation by the Cholinergic Properties of the Smokeless Tobacco, Gutkha

Brian O'Neill,[†,∥] Dana Lauterstein,[§,∥] Jyoti C. Patel,[†] Judith T. Zelikoff,[*,§] and Margaret E. Rice[*,†,‡]

[†]Department of Neurosurgery, and [‡]Department of Neuroscience and Physiology, New York University School of Medicine, New York, New York 10016, United States

[§]Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York 10987, United States

ABSTRACT: Tobacco products influence striatal dopamine (DA) release primarily through the actions of nicotine, an agonist of nicotinic acetylcholine receptors (nAChR). Gutkha is a smokeless tobacco product that contains not only nicotine, but also includes the habit-forming areca nut and other plant-based constituents that contribute muscarinic acetylcholine receptor (mAChR) agonists and other cholinergic agents. Thus, the net influence of the cholinergic agents in gutkha on striatal DA release is difficult to predict. This study investigated the influence of gutkha extract on evoked DA release in mouse striatal slices using fast-scan cyclic voltammetry. The potency of a given concentration of nicotine in the gutkha extract was found to be significantly lower than that of a comparable concentration of nicotine alone. Atropine, a mAChR antagonist, increased the potency of gutkha-associated nicotine; however, other experiments suggested that this was mediated in part by direct effects of atropine at nAChRs. Overall, these results suggest that the unique constituents of gutkha work together to oppose the influence of gutkha-associated nicotine on evoked striatal DA release.



KEYWORDS: *Nicotine, brain slices, dorsal striatum, fast-scan cyclic voltammetry, nAChR, atropine*

Gutkha is a form of smokeless tobacco that includes powdered tobaccos, areca nut, slake lime, spices, sweeteners, seeds, and catechu.[1] In the United States, gutkha is most commonly used by individuals who have emigrated from South Asia,[2,3] which has the largest number of smokeless tobacco users worldwide.[4] Although users apply gutkha to the buccal lining of the mouth, this tobacco product can have both local and systemic consequences after extraction of constituents into saliva, and subsequent absorption into the bloodstream. Public health consequences of gutkha use include high rates of oral and other cancers, as well as elevated rates of pulmonary and cardiovascular disease.[3,5−7] Currently, South Asians are the second largest and most rapidly growing immigrant subgroup in the United States,[3] with a corresponding increase in gutkha use in this country.[2,3] United States national surveys suggest that the prevalence of tobacco use by this ethnic group is ∼7−12%,[8,9] and that the majority of South Asian tobacco users in the United States are using ethnically linked smokeless tobacco products (e.g., paan, paan masala, and gutkha).[9,10]

Although gutkha is known to be addictive,[9,11] few studies have examined its effects in the CNS. The gutkha constituent most commonly associated with addiction is nicotine, acting at nicotinic acetylcholine receptors (nAChRs), although additional components may also contribute to its addictive potential.[9] Chief among these components is areca nut, which can cause dependence, with or without concurrent use of tobacco.[12] Areca nut contains the alkaloid arecoline,[13] a potent muscarinic ACh receptor (mAChR) agonist,[11,14] which might also contribute to the addictive properties of gutkha.[15]

Nicotine and other drugs of abuse act on brain reward pathways, with most having a net enhancing effect on dopamine (DA) transmission.[16−21] As an agonist of nAChRs, nicotine can enhance DA release; however, at the level of striatal DA axons, nicotine exposure rapidly causes nAChR desensitization,[22] especially when coupled with electrically evoked release of endogenous ACh.[17,23,24] Desensitization of nAChRs on DA axons leads to a decrease in DA release evoked by local, single-pulse electrical stimulation or by low frequency pulse-train stimulation, but amplifies DA release evoked by burst-like stimulation, thereby increasing signal-to-noise in striatal DA signaling.[17,24,25] In the present study, fast-scan cyclic voltammetry (FCV) was used to compare the effect of nicotine with that of nicotine in an extract of water-soluble components of gutkha (gutkha-associated nicotine) on electrically evoked extracellular DA concentration ($[DA]_o$) in dorsal striatum of ex vivo forebrain slices from drug-naïve mice. Based on our initial finding that the potency of gutkha-associated nicotine was lower than that of comparable concentrations of nicotine alone, we then tested the hypothesis that this decreased potency was a consequence of the presence of mAChR agonists in the gutkha extract.

Published: March 22, 2015

DOI: 10.1021/cn500283b
ACS Chem. Neurosci. 2015, 6, 832−837

Table 1. Major Chemical Classes and Relative Abundance of Gutkha Constituents Identified by LC-HRTOFMS, with Reported CNS Effects

class	estimated abundance by dry mass	major constituents	proportion of class	reported CNS effects
alkaloids	7−9%	nicotine	98.7%	nAChR agonist[a]
		arecoline	1.3%	mAChR agonist[b]
benzopyrones	<1%	coumarin	26%	inhibitor of acetylcholinesterase[c]
		scopoletin	74%	inhibitor of acetylcholinesterase[d]
		dihydrocoumarin	<0.1%	none
flavonoids	3−5%	catechin	100%	regulates acetylcholine/acetylcholine esterase cycle[e]

[a]References 17, 19 and 23−25. [b]References 15 and 16. [c]References 33 and 34. [d]References 35 and 36. [e]Reference 37.

RESULTS AND DISCUSSION

As described in Methods, water-soluble constituents of gutkha were extracted, filtered, lyophilized, and frozen. Chemical analysis of the lyophilized product provided an estimated abundance by dry mass for nicotine (Table 1). Nicotine content was found to be ∼8% of the lyophilized gutkha product, or 0.08 g nicotine/g gutkha lyophilysate, which was used to calculate the concentration of nicotine after reconstitution in artificial cerebrospinal fluid (aCSF) for application to striatal slices.

As in previous studies,[17,23,24] $[DA]_o$ evoked by a single electrical stimulus pulse (1 p) in dorsolateral striatum was suppressed by concentrations of nicotine that are sufficient to cause nAChR desensitization when combined with locally evoked ACh release (Figure 1A). For example, in the presence of 300 nM nicotine, 1 p evoked $[DA]_o$ was decreased to 23% ± 7% of control ($p < 0.01$, $n = 3$, unpaired t test with Welch's correction). Suppression of DA release when nAChRs are desensitized reflects the loss of release facilitation by concurrently released ACh, which can drive axonal DA release even in the absence of DA axon stimulation.[26,27] Strikingly, however, gutkha-associated nicotine had no effect at such submicromolar concentrations, but rather required a 100-fold higher concentration to suppress evoked $[DA]_o$ (Figure 1B), with a decrease to 38% ± 8% of control ($p < 0.05$, $n = 3$, unpaired t test with Welch's correction) seen with 30 μM gutkha-associated nicotine.

Under control conditions, evoked $[DA]_o$ in the dorsolateral striatum in ex vivo slices shows little frequency dependence, with a similar peak $[DA]_o$ evoked by 5 pulses (5 p) delivered at 100 Hz, to that evoked by 1 p; thus, the 5 p/1 p ratio is typically ∼1.[17,24,28] However, when cholinergic drive is removed as a consequence of nAChR blockade or desensitization,[17,24] or by the absence of concurrent ACh release,[27,28] 1 p evoked $[DA]_o$ is not only suppressed (Figure 1B), but the contrast in $[DA]_o$ evoked by low versus high stimulation frequencies becomes enhanced.[17,24,25,27,28] Indeed, with brief pulse-train stimulation (up to 5 pulses) at short interpulse intervals (10 ms), each stimulus pulse evokes roughly the same increase in $[DA]_o$, leading to an enhanced 5 p/1 p ratio of ∼4−5 in the absence of nAChR activation.[17,24,27,28]

Here, a concentration of nicotine or gutkha-associated nicotine that was sufficient to suppress 1 p evoked $[DA]_o$ (Figure 1A, B), also increased the 5 p/1 p ratio of evoked $[DA]_o$ (Figure 1C). Thus, the 5 p/1 p ratio can be used as a biomarker for nAChR desensitization, and therefore as an index of nicotine potency or efficacy.

Using this 5 p/1 p ratio, we determined full concentration−response curves for nicotine vs gutkha-associated nicotine. The maximal increase in 5 p/1 p ratio was ∼4 for either nicotine alone or gutkha-associated nicotine (data shown normalized,

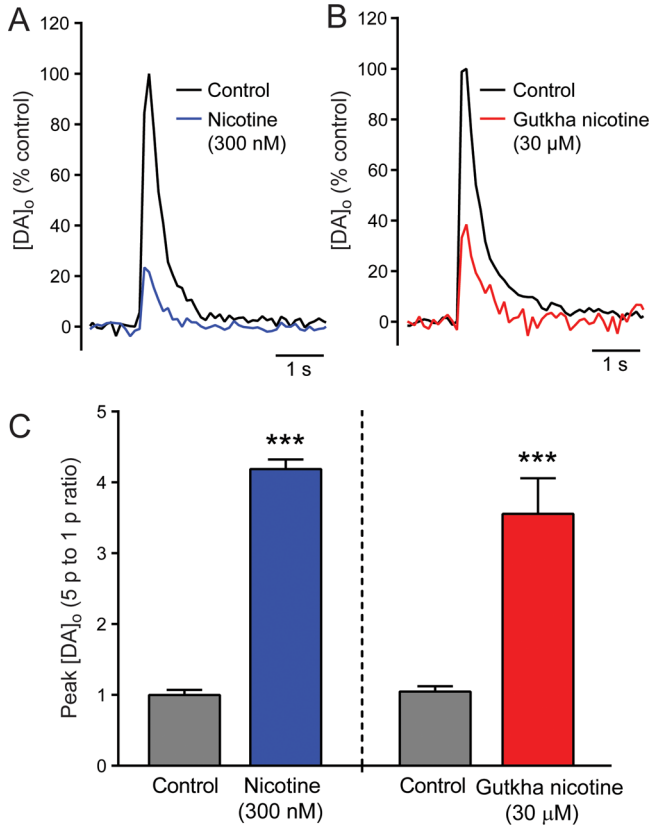


Figure 1. Nicotine and gutkha-associated nicotine suppress single pulse (1 p) evoked $[DA]_o$ and amplify 5 p/1 p ratio. (A) Representative concentration−time profiles of $[DA]_o$ evoked by 1 p local electrical stimulation in dorsolateral striatum ex vivo in the presence and absence of nicotine (300 nM) in aCSF. (B) Representative concentration−time profiles of $[DA]_o$ evoked by 1 p stimulation in dorsolateral striatum ex vivo in the presence and absence of gutkha-associated nicotine (gutkha nicotine, 30 μM) in aCSF. (C) Summary of the amplification of 5 p/1 p ratio for evoked $[DA]_o$ at the effective concentrations required for suppression of evoked $[DA]_o$ in (A) and (B) (***$p < 0.001$ vs control for each condition; mean ± SEM, $n = 3$ per mean; one-way ANOVA with Bonferroni's multiple comparison of selected groups).

Figure 2). However, the concentration that produced a half-maximal increase in 5 p/1 p ratio in mouse dorsolateral striatum (EC_{50}) differed markedly for the two agents. The EC_{50} for nicotine was calculated to be 116 nM (95% confidence interval: 82−163 nM), which is consistent with concentrations found to induce nAChR desensitization previously.[17,24,25] In sharp contrast, the EC_{50} for gutkha-associated nicotine was 16 μM (95% confidence interval: 10−24 μM), implying a 140-fold

DOI: 10.1021/cn500283b
ACS Chem. Neurosci. 2015, 6, 832−837

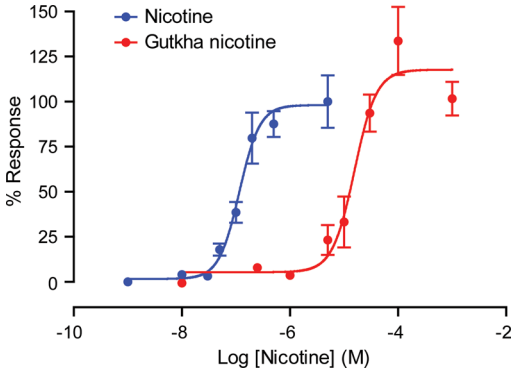


Figure 2. Decreased potency of gutkha-associated nicotine versus nicotine alone. Nonlinear regression using a sigmoidal (variable slope) model for the concentration–response (ratio of 5 p/1 p evoked $[DA]_o$) for nicotine and gutkha-associated nicotine (gutkha nicotine) confirmed progressive nAChR desensitization for both agents, but a marked difference in potency - indicated by a significant difference in EC_{50} values ($p < 0.001$ nicotine vs gutkha nicotine; $n = 3$ per point; unpaired t test with Welch's correction).

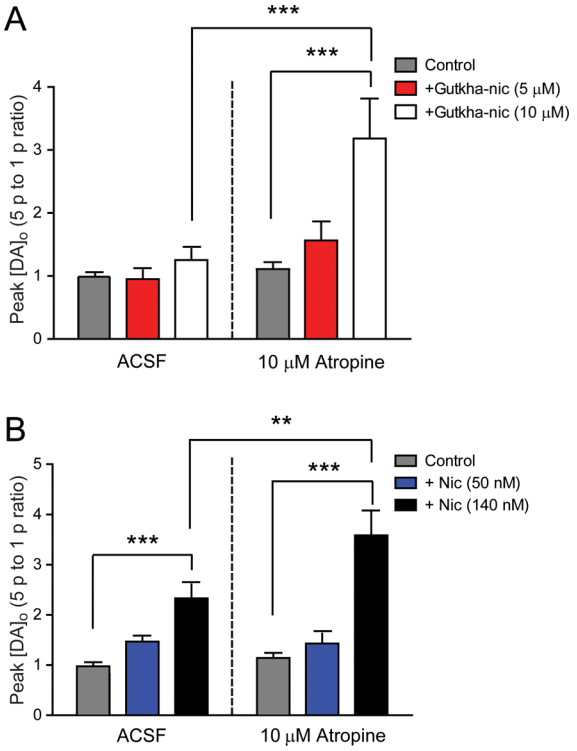


Figure 3. mAChR antagonism alters nAChR desenstization by gutkha-associated nicotine, but also by nicotine. (A) At a calculated concentration of 10 μM, gutkha-associated nicotine (gutkha-nic, n = 6) had no effect on the ratio of 5 p/1 p evoked $[DA]_o$ versus control (n = 12). An antagonist of mAChRs (atropine, 10 μM) also had no effect on 5 p/1 p ratio (n = 12). However, when this ineffective concentration of gutkha nicotine was applied in the presence of atropine (n = 6), a significant increase in 5 p/1 p ratio was induced (***$p < 0.001$ vs either gutkha-nic or atropine alone; mean ± SEM two-way ANOVA with Bonferroni's multiple comparison of selected groups). There were no significant effects of 5 μM gutkha-nicotine in either condition (n = 3–6; $p > 0.05$). (B) Effect of 10 μM atropine on changes in evoked $[DA]_o$ by 50 nM (n = 3) and 140 nM (n = 12) nicotine (Nic). Nicotine alone produced a concentration-dependent increase in the 5 p/1 p ratio (***$p < 0.001$ for 140 nM nicotine). Atropine potentiated the increase 5 p/1 p ratio at 140 nM nicotine (n = 9; ***$p < 0.001$), but not 50 nM nicotine (n = 12; **$p < 0.01$ vs 140 nM nicotine alone).

shift in relative potency ($p < 0.001$, n = 3, unpaired t test with Welch's correction).

Given the presence of mAChR agonists in gutkha, including arecoline,[13−15] we initially hypothesized that these constituents might be responsible for this observed difference by opposing the actions of gutkha-associated nicotine. We tested this by comparing the effect of gutkha-associated nicotine in the presence or absence of atropine, a mAChR antagonist. As in concentration–response studies (Figure 2), either 5 or 10 μM gutkha-associated nicotine had little effect on the 5 p/1 p ratio ($p > 0.05$, n = 6) (Figure 3A). Atropine (10 μM) had no effect on 1 p evoked $[DA]_o$, consistent with previous findings[29] (data not shown), or on the 5 p/1 p ratio ($p > 0.05$, n = 12) (Figure 3A), confirming minimal tonic regulation of DA release by mAChRs in ex vivo striatal slices. The presence of atropine did not alter the limited effect of 5 μM gutkha-associated nicotine on 5 p/1 p ratio (Figure 3A). However, when slices were exposed to 10 μM gutkha-associated nicotine in the presence of atropine, a significant increase in 5 p/1 p ratio was induced ($p <$ 0.001 vs atropine alone, n = 6; two-way ANOVA followed by Bonferroni posthoc tests) (Figure 3A). This amplified 5 p/1 p ratio also differed significantly from that observed in 10 μM gutkha-associated nicotine ($p < 0.001$).

A potential confounding factor in these studies, suggested by previous findings in *Xenopus* oocytes, is that atropine can have a direct influence on nAChR desensitization.[30] We tested this in parallel experiments using two concentrations of nicotine: 50 nM, which produced little change in 5 p/1 p ratio; and 140 nM nicotine, near the nicotine EC_{50} (Figure 2) to facilitate detection of either an increase or decrease in 5 p/1 p ratio with atropine. The presence of atropine (10 μM) did not alter the 5 p/1 p ratio seen with 50 nM nicotine (Figure 3B). However, the desensitization seen with 140 nM nicotine was enhanced in the presence of atropine, with a significant increase in 5 p/1 p ratio ($p < 0.01$ vs 140 nM nicotine; two-way ANOVA with Bonferroni tests). These data suggest that atropine does not cause nAChR desensitization on its own, but that its interactions with nAChRs exacerbate desensitization when the process has been initiated by nicotine. Importantly, these findings demonstrate that previous results from transfected frog oocytes[30] also pertain to endogenous nAChRs in mammalian brain tissue.

In isolation, these results with atropine would confound, but not necessarily falsify, our hypothesis that decreased ACh release by mAChR activation could increase the concentration of nicotine required for desensitization. We therefore also tested whether a mAChR agonist found in gutkha, arecoline, could decrease the desensitizing effect of a low, but effective nicotine concentration. In pilot studies, we tried various combinations, but low arecoline concentrations did not alter nicotine's effect, whereas higher levels (10 μM) directly induced an increase in 5 p/1 p ratio (data not shown), as seen when ACh release is curtailed in the presence of other mAChR agonists.[29]

Overall, the data reported here provide support for the hypothesis that multiple mAChR agonists or other classes of cholinergic agents in gutkha contribute to the decreased potency of gutkha-associated nicotine. Indeed, Smulders and colleagues[30] found that a variety of cholinergic agents can alter

DOI: 10.1021/cn500283b
ACS Chem. Neurosci. 2015, 6, 832–837

Table 2. Major Chemical Classes and Relative Abundance of Gutkha Constituents Identified by SPME-GC-MS, with Reported CNS Effects

class	estimated abundance by dry mass	major constituents	proportion of class	reported CNS effects
terpenoids	70−72%	menthol	94%	nAChR modulator (suppressor/inhibitor)[a]
		camphor	<1%	nAChR inhibitor[b]
		citronellol	4%	inhibitor of acetylcholinesterase[c]
		geraniol	2%	inhibitor of acetylcholinesterase[c]
terpene	2−3%	borneol	54%	nAChR inhibitor[d]
		rose oxide	8%	none
		safranal	38%	inhibitor of acetylcholinesterase[e]
monoterpene alcohol	3−4%	terpineol	71%	inhibitor of acetylcholinesterase[f]
		linalool	29%	inhibitor of acetylcholinesterase[g]
phenylpropene	<1%	eugenol	100%	inhibitor of acetylcholinesterase[h]

[a]References 38−40 [b]References 41 and 42. [c]Reference 43. [d]Reference 44. [e]Reference 45. [f]Reference 46. [g]References 46−48. [h]Reference 49.

both the activation and desensitization of nAChRs by nicotine. Given the wealth of cholinergic agents in gutkha (Tables 1 and 2), it is plausible that one or more of these agents could limit nAChR activation and desensitization by gutkha-associated nicotine, thereby influencing the rewarding properties and addictive potential compared to tobacco alone.

METHODS

Gutkha Lyophilysate Preparation. Preparation of gutkha for extraction of water-soluble constituents required grinding intact gutkha pellets (Manikchand, Fazalpur, Gujarat, India), using a mortar and pestle. The resulting powder was combined with 10 mL of sterile, filtered water and then incubated at 37 °C for 30 min to allow the liberation of water-soluble components into solution. The resulting water-soluble extract was filtered twice, first with a Büchner funnel and then using a sterile filter (0.25 μm pore size) to ensure sterility of the product and removal of undissolved material. The extract was frozen at −80 °C and lyophilized 24 h later at 4 °C. The lyophilysate was then stored at −20 °C until reconstitution in aCSF immediately before experimentation.

Determination of Gutkha Constituents. The constituents contained in the lyophilized gutkha product used in this study were characterized by nontargeted chemical profiling using solvent extracts of the lyophilized gutkha conducted by contract with British American Tobacco (London, U.K.). Lyophilized gutkha samples were solubilized with 50:50 (v/v) methanol/water for instrumental analysis. Nontargeted screening was achieved using liquid chromatography high-resolution time-of-fight-mass spectrometry (LC-HRTOFMS); additional screening with solid phase microextraction−gas chromatography mass spectrometry (SPME-GC-MS) allowed identification of more volatile compounds. For LC-HRTOFMS analysis, a Bruker maXis impact Q-TOF instrument with electrospray ionization was used. Data processing was conducted using the Bruker Dissect algorithm, which detects all of the LC-MS components based on chromatographic profiles of the detected ions. Molecular formulas of constituents identified by LC-HRTOFMS analysis were confirmed by the composition of an accurate mass library of relevant compounds. The main classes of chemicals and major constituents identified via LC-HRTOFMS, and their potential CNS effects relevant to this study are summarized in Table 1. Data obtained through SPME-GC-MS analysis were processed using Agilent Chemstation version E.02.02.1431. Mass spectra of compounds identified through SPME-GC-MS analysis (that were not found in LC-HRTOFMS analysis) were compared against commercially available mass spectral libraries. The main classes of chemicals and major constituents identified via SPME-GC-MS and their potential CNS effects relevant to this study are summarized in Table 2.

Fast-Scan Cyclic Voltammetry. Brain slices were prepared from 6−10 week old male C57Bl/6J mice (Jackson Laboratories; Bar Harbor, ME), as described previously.[28] Briefly, mice were deeply anesthetized with pentobarbital (60 mg/kg in saline, intraperitoneal injection), decapitated, and the brains removed into ice cold sucrose-based aCSF containing the following (in mM): sucrose (225), $NaHCO_3$ (28), D-glucose (7), $MgCl_2$ (7), sodium pyruvate (3), KCl (2.5), NaH_2PO_4 (1.25), ascorbic acid (1), $CaCl_2$ (0.5). Coronal striatal slices (300 μm thickness) were cut on a Leica VT1200S vibrating blade microtome (Leica Microsystems; Bannockburn, IL) and then transferred to a holding chamber at room temperature (20−22 °C) for at least 1 h in modified aCSF containing (in mM): NaCl (120), $NaHCO_3$ (20), D-glucose (10), HEPES acid (6.6), KCl (5), HEPES sodium (3.3), $CaCl_2$ (2), $MgSO_4$ (2). Individual slices were transferred to a recording chamber and superfused at 2 mL/min with aCSF containing (in mM): NaCl (124.2), $NaHCO_3$ (26), D-glucose (10), KCl (3.76), $CaCl_2$ (2.4), $MgSO_4$ (1.33), KH_2PO_4 (1.23), saturated with 95% O_2/5% CO_2 at 32 °C.[28,31,32]

Release of DA was evoked by single electrical pulses (300 μA, 0.1 ms duration) or by trains of 5 pulses (300 μA, 0.1 ms duration, 100 Hz) using a bipolar concentric stimulating electrode (FHC Inc. Bowdoin, Maine). Evoked $[DA]_o$ was monitored using FCV with carbon-fiber microelectrodes that were fabricated in-house from 7 μm carbon fibers.[31,32] Evoked $[DA]_o$ was quantified from postexperiment calibration in the recording chamber at 32 °C in all media used in a given experiment. A triangular voltage waveform of −0.7 V/+1.3 V/0.7 V (vs Ag/AgCl) was applied at 10 Hz using a Millar voltammeter (available from Julian Millar; University of London, UK). The scan rate was 800 V/s, with no applied potential between scans, which minimizes DA adsorption. Timing of the voltage sweeps and stimulation was controlled by using a Master-8 pulse generator and optical stimulus isolator (A.M.P.I. Jerusalem, Israel). Background-subtracted data were collected using a DigiData 1200B A/D board coupled to a PC running Clampex 7.0 software and were analyzed in real time. To minimize rundown of evoked $[DA]_o$ in the presence of nicotine, several recording sites were tested in the dorsolateral striatum of each slice under each condition. At each site, a stimulation series of 1 p, 5 p, and 1 p, (each separated by 5 min intervals) was applied. The first and last 1 p evoked $[DA]_o$ were averaged for calculation of 5 p/1 p ratio for each site.

Drugs and Chemicals. All drugs were applied in aCSF by superfusion in the recording chamber after collection of control evoked $[DA]_o$. All chemicals, except for gutkha, were purchased from Sigma-Aldrich (St. Louis, MO) and were prepared in aCSF immediately before use.

Data Analysis and Statistics. All statistical analyses were performed using GraphPad Prism 6.0. Unpaired Student's *t* tests were used for two-group comparisons; Welch's correction was used where there were unequal variances. Two-way ANOVA followed by Bonferroni's post hoc tests were used for three groups or more when there were two factors, such as nicotine dose and the presence of atropine (Figure 3). Specific tests for illustrated data are indicated in the corresponding figure legends.

DOI: 10.1021/cn500283b
ACS Chem. Neurosci. 2015, 6, 832−837

AUTHOR INFORMATION

Corresponding Authors

*(M.E.R.) Telephone: 212-263-5438. Fax: 212-689-0334. E-mail: margaret.rice@nyu.edu.

*(J.T.Z.) Telephone: 845-731-3528. E-mail: judith.zelikoff@nyumc.org.

Author Contributions

[‖]B.O. and D.L. contributed equally to the work. B.O. and D.L. conducted all experiments; B.O., J.C.P, and M.E.R. contributed to experimental design; J.T.Z. initiated the project and obtained the analysis of gutkha; J.C.P., B.O., and D.L. conducted statistical analyses; D.L., B.O., and M.E.R. wrote the paper with input from all authors.

Funding

This work was supported by NIH/NIDA Grants R01 DA033811 (M.E.R.) and T32 DA007254 (B.O.), and Memorial Sloan Kettering Cancer Center Pilot Program (J.T.Z.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Special thanks to Marianna Gaca in the analytical science department at British American Tobacco for conducting the chemical characterization of the lyophilized gutkha product, and to Dr. Francesca Gany for piquing our interest in gutkha, and for many insightful discussions and support.

REFERENCES

(1) Willis, D., Popovech, M., Gany, F., and Zelikoff, J. T. (2012) Toxicology of smokeless tobacco: implications for immune, reproductive, and cardiovascular systems. *J. Toxicol. Environ. Health, Part B 15*, 317−331.

(2) Banerjee, S. C., Ostroff, J. S., Bari, S., D'Agostino, T. A., Khera, M., Acharya, S., and Gany, F. (2014) Gutka and Tambaku Paan use among South Asian immigrants: a focus group study. *J. Immigr. Minor Health 16*, 531−539.

(3) Mukherjea, A., Morgan, P. A., Snowden, L. R., Ling, P. M., and Ivey, S. L. (2012) Social and cultural influences on tobacco-related health disparities among South Asians in the USA. *Tob. Control 21*, 422−428.

(4) International Agency for Research on Cancer (IARC), and World Health Organization (WHO) (2007) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Smokeless Tobacco and Some Tobacco-Specific N-Nitrosamines*, Vol. *89*, WHO Press, Geneva, Switzerland.

(5) Gupta, P. C., Ray, C. S., Sinha, D. N., and Singh, P. K. (2011) Smokeless tobacco: A major public health problem in the SEA region: A review. *Indian J. Public Health 55*, 199−209.

(6) Prasad, D. S., Kabir, Z., Dash, A. K., and Das, B. C. (2009) Smoking and cardiovascular health: a review of the epidemiology, pathogenesis, prevention and control of tobacco. *Indian J. Med. Sci. 63*, 520−533.

(7) Kakde, S., Bhopal, R. S., and Jones, C. M. (2012) A systematic review on the social context of smokeless tobacco use in the South Asian population: Implications for public health. *Public Health 126*, 635−645.

(8) Barnes, P. M., Adams, P. F., and Powell-Griner, E. (2008) Health characteristics of the Asian adult population: United States, 2004−2006. *Adv. Data 394*, 1−22.

(9) Willis, D. N., Popovech, M. A., Gany, F., Hoffman, C., Blum, J. L., and Zelikoff, J. T. (2014) Toxicity of gutkha, a smokeless tobacco product gone global: is there more to the toxicity than nicotine? *Int. J. Environ. Res. Public Health 11*, 919−933.

(10) National Cancer Institute, Centers for Disease Control and Prevention, and Stockholm Centre of Public Health (2002) Smokeless tobacco fact sheets. In *Proceedings Third International Conference on Smokeless Tobacco*, Stockholm, Sweden (http://cancercontrol.cancer.gov/brp/tcrb/stfact_sheet_combined10-23-02.pdf).

(11) Winstock, A. (2002) Areca nut-abuse liability, dependence and public health. *Addict. Biol. 7*, 133−138.

(12) Mirza, S. S., Shafique, K., Vart, P., and Arain, M. I. (2011) Areca nut chewing and dependency syndrome: Is the dependence comparable to smoking? A cross sectional study. *Subst. Abuse Treat. Prev. Policy 6*, 23.

(13) Farnworth, E. R. (1976) Betel nut—Its composition, chemistry and uses. *Sci. New Guinea 4*, 85−90.

(14) Chu, N. S. (2001) Effects of Betel chewing on the central and autonomic nervous systems. *J. Biomed. Sci. 8*, 229−236.

(15) Lee, C. H., Chiang, S. L., Ko, A. M., Hua, C. H., Tsai, M. H., Warnakulasuriya, S., Ibrahim, S. O., Sunarjo, Zain, R. B., Ling, T. Y., Huang, C. L., Lane, H. Y., Lin, C. C., and Ko, Y. C. (2014) Betel-quid dependence domains and syndrome associated with betel-quid ingredients among chewers: An Asian multi-country evidence. *Addiction 109*, 1194−1204.

(16) Schultz, W. (1986) Responses of midbrain dopamine neurons to behavioral trigger stimuli in the monkey. *J. Neurophysiol. 56*, 1439−1461.

(17) Rice, M. E., and Cragg, S. J. (2004) Nicotine amplifies reward-related dopamine signals in striatum. *Nat. Neurosci. 7*, 583−584.

(18) Exley, R., and Cragg, S. J. (2008) Presynaptic nicotinic receptors: A dynamic and diverse cholinergic filter of striatal dopamine neurotransmission. *Br. J. Pharmacol. 153* (Suppl 1), S283−S297.

(19) Livingstone, P. D., and Wonnacott, S. (2009) Nicotinic acetylcholine receptors and the ascending dopamine pathways. *Biochem. Pharmacol. 78*, 744−755.

(20) Wanat, M. J., Willuhn, I., Clark, J. J., and Phillips, P. E. M. (2009) Phasic dopamine release in appetitive behaviors and drug addiction. *Curr. Drug Abuse Rev. 2*, 195−213.

(21) Wise, R. A. (2013) Dual roles of dopamine in food and drug seeking: The drive-reward paradox. *Biol. Psychiatry 73*, 819−826.

(22) Wang, H., and Sun, X. (2005) Desensitized nicotinic receptors in brain. *Brain Res. Rev. 48*, 420−437.

(23) Zhou, F. M., Liang, Y., and Dani, J. A. (2001) Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. *Nat. Neurosci. 4*, 1224−1229.

(24) Zhang, H., and Sulzer, D. (2004) Frequency-dependent modulation of dopamine release by nicotine. *Nat. Neurosci. 7*, 581−582.

(25) Wang, L., Shang, S., Kang, X., Teng, S., Zhu, F., Liu, B., Wu, Q., Li, M., Liu, W., Xu, H., Zhou, L., Jiao, R., Dou, H., Zuo, P., Zhang, X., Zheng, L., Wang, S., Wang, C., and Zhou, Z. (2014) Modulation of dopamine release in the striatum by physiologically relevant levels of nicotine. *Nat. Commun. 5*, 3925.

(26) Ding, J. B., Guzman, J. N., Peterson, J. D., Goldberg, J. A., and Surmeier, D. J. (2010) Thalamic gating of corticostriatal signaling by cholinergic interneurons. *Neuron 67*, 294−307.

(27) Threlfell, S., Lalic, T., Platt, N. J., Jennings, K. A., Deisseroth, K., and Cragg, S. J. (2012) Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons. *Neuron. 75*, 58−64.

(28) Patel, J. C., Rossignol, E., Rice, M. E., and Machold, R. P. (2012) Opposing regulation of dopaminergic activity and exploratory motor behavior by forebrain and brainstem cholinergic circuits. *Nat. Commun. 3*, 1172.

(29) Threlfell, S., Clements, M. A., Khodai, T., Pienaar, I. S., Exley, R., Wess, J., and Cragg, S. J. (2010) Striatal muscarinic receptors promote activity dependence of dopamine transmission via distinct receptor subtypes on cholinergic interneurons in ventral versus dorsal striatum. *J. Neurosci. 30*, 3398−3408.

(30) Smulders, C. J., Zwart, R., Bermudez, I., van Kleef, R. G., Groot-Kormelink, P. J., and Vijverberg, H. P. (2005) Cholinergic drugs potentiate human alpha4beta2 acetylcholine receptors by a competitive mechanism. *Eur. J. Pharmacol. 509*, 97−108.

(31) Patel, J. C., and Rice, M. E. (2006) Monitoring dopamine release in brain slices. In *Encyclopedia of Sensors* (Grimes, C. A., Dickey,

DOI: 10.1021/cn500283b
ACS Chem. Neurosci. 2015, 6, 832−837

E. C., and Pishko, M. V., Eds.), Vol. *6*, pp 313−334, American Scientific Publishers, Stevenson Ranch, CA.
(32) Patel, J. C., and Rice, M. E. (2013) Monitoring axonal and somatodendritic dopamine release using fast-scan cyclic voltammetry in brain slices. *Methods Mol. Biol. 964*, 243−273.
(33) Fallarero, A., Oinonen, P., Gupta, S., Blom, P., Galkin, A., Mohan, C. G., and Vuorela, P. M. (2008) Inhibition of acetylcholinesterase by coumarins: The case of coumarin 106. *Pharmacol. Res. 58*, 215−221.
(34) Anand, P., Singh, B., and Singh, N. (2011) A review on coumarins as acetylcholinesterase inhibitors for Alzheimer's disease. *Bioorg. Med. Chem. 20*, 1175−1180.
(35) Hornick, A., Lieb, A., Vo, N. P., Rollinger, J. M., Stuppner, H., and Prast, H. (2011) The coumarin scopoletin potentiates acetylcholine release from synaptosomes, amplifies hippocampal long-term potentiation and ameliorates anticholinergic- and age-impaired memory. *Neuroscience 197*, 280−292.
(36) Rollinger, J. M., Hornick, A., Langer, T., Stuppner, H., and Prast, H. (2004) Acetylcholinesterase inhibitory activity of scopolin and scopoletin discovered by virtual screening of natural products. *J. Med. Chem. 47*, 6248−6254.
(37) Srividhya, R., Gayathri, R., and Kalaiselvi, P. (2012) Impact of epigallo catechin-3-gallate on acetylcholine-acetylcholine esterase cycle in aged rat brain. *Neurochem. Int. 60*, 517−522.
(38) Hans, M., Wilhelm, M., and Swandulla, D. (2012) Menthol suppresses nicotinic acetylcholine receptor functioning in sensory neurons via allosteric modulation. *Chem. Senses. 37*, 463−469.
(39) Kabbani, N. (2013) Not so Cool? Menthol's discovered actions on the nicotinic receptor and its implications for nicotine addiction. *Front. Pharmacol. 4*, 95.
(40) Ashoor, A., Nordman, J. C., Veltri, D., Yang, K. H., Al Kury, L., Shuba, Y., Mahgoub, M., Howarth, F. C., Sadek, B., Shehu, A., Kabbani, N., and Oz, M. (2013) Menthol binding and inhibition of α7-nicotinic acetylcholine receptors. *PLoS One 8*, e67674.
(41) Kichko, T. I., Lennerz, J., Eberhardt, M., Babes, R. M., Neuhuber, W., Kobal, G., and Reeh, P. W. (2013) Bimodal concentration-response of nicotine involves the nicotinic acetylcholine receptor, transient receptor potential vanilloid type 1, and transient receptor potential ankyrin 1 channels in mouse trachea and sensory neurons. *J. Pharmacol. Exp. Ther. 347*, 529−539.
(42) Park, T. J., Seo, H. K., Kang, B. J., and Kim, K. T. (2001) Noncompetitive inhibition by camphor of nicotinic acetylcholine receptors. *Biochem. Pharmacol. 61*, 787−793.
(43) Senol, F. S., Orhan, I. E., Kurkcuoglu, M., Khan, M. T. H., Altintas, A., Sener, B., and Baser, K. H. C. (2013) A mechanistic investigation on anticholinesterase and antioxidant effects of rose (*Rosa damascena* Mill.). *Food Res. Int. 53*, 502−509.
(44) Park, T. J., Park, Y. S., Lee, T. G., Ha, H., and Kim, K. T. (2003) Inhibition of acetylcholine-mediated effects by borneol. *Biochem. Pharmacol. 65*, 83−90.
(45) Geromichalos, G. D., Lamari, F. N., Papandreou, M. A., Trafalis, D. T., Margarity, M., Papageorgiou, A., and Sinakos, Z. (2012) Saffron as a source of novel acetylcholinesterase inhibitors: Molecular docking and in vitro enzymatic studies. *J. Agric. Food Chem. 60*, 6131−6138.
(46) Perry, N. S., Houghton, P. J., Theobald, A., Jenner, P., and Perry, E. K. (2000) In-vitro inhibition of human erythrocyte acetylcholinesterase by salvia lavandulaefolia essential oil and constituent terpenes. *J. Pharm. Pharmacol. 52*, 895−902.
(47) Re, L., Barocci, S., Sonnino, S., Mencarelli, A., Vivani, C., Paolucci, G., Scarpantonio, A., Rinaldi, L., and Mosca, E. (2000) Linalool modifies the nicotinic receptor-ion channel kinetics at the mouse neuromuscular junction. *Pharmacol. Res. 42*, 177−182.
(48) Lopez, M. D., and Pascual-Villalobos, M. J. (2010) Mode of inhibition of acetylcholinesterase by monoterpenoids and implications for pest control. *Ind. Crops Prod. 31*, 284−288.
(49) Dalai, M. K., Bhadra, S., Chaudhary, S. K., Bandyopadhyay, A., and Mukherjee, P. K. (2014) Anti-cholinesterase activity of the standardized extract of *Syzygium aromaticum* L. *Pharmacogn. Mag. 10*, 276−282.

DOI: 10.1021/cn500283b
ACS Chem. Neurosci. 2015, 6, 832−837