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

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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 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 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.

Graphical Abstract

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Introduction

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 sub-group 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 has been shown to cause dependence, with or without concurrent use of tobacco.¹² Areca nut contains the alkaloid arecoline,¹³ a potent muscarinic ACh receptor (mAChRs) 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 an 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 single-pulse local 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 the decreased potency of gutkha-associated nicotine was a consequence of the presence of mAChR agonists in the gutkha extract.

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 in the presence of 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 *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 and high frequency becomes enhanced.^{17,24,25,27,28} Indeed, with brief pulse-train stimulation (up to 5 pulses) at short inter-pulse 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 2). However, the concentration that produced a half-maximal increase in 5 p/1 p ratio in mouse dorsolateral striatum (EC₅₀) differed markedly for the two agents. The EC₅₀ 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 EC₅₀ for gutkha-associated nicotine was 16 μM (95% confidence interval: 10–24 μM), implying a 140-fold 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 μ M gutkha-associated nicotine had little effect on the 5 p/1 p ratio ($p > 0.05$, $n = 6$) (Figure 3A). Atropine (10 μ M) had no effect on 1 p evoked $[DA]_o$, consistent with previous findings²⁹ (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 μ M gutkha-associated nicotine on 5 p/1 p ratio (Figure 3A). However, when slices were exposed to 10 μ M 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 post-hoc tests) (Figure 3A). This amplified 5 p/1 p ratio also differed significantly from that observed in 10 μ M gutkha-associated nicotine alone ($p < 0.001$).

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 studies 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 μ M) did not alter the 5 p/1 p ratio seen with 50 nM nicotine alone (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 alone; 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 oocytes³⁰ 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 μ M) 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 colleagues³⁰ found that a variety of cholinergic agents can alter 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 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 hours 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 non-targeted 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. Non-targeted screening was achieved using liquid chromatography high-resolution time-of-flight-mass spectrometer (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), NaHCO₃ (28), D-glucose (7), MgCl₂ (7), sodium pyruvate (3), KCl (2.5), NaH₂PO₄ (1.25), ascorbic acid (1), CaCl₂ (0.5). Coronal striatal slices (300 µm thickness) were cut on a Leica VT1200S vibrating blade microtome (Leica Microsystems; Bannockburn, IL) 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), NaHCO₃ (20), D-glucose (10), HEPES acid (6.6),

KCl (5), HEPES sodium (3.3), CaCl₂ (2), MgSO₄ (2). Individual slices were transferred to a recording chamber, and superfused at 2 mL/min with aCSF containing (in mM): NaCl (124.2), NaHCO₃ (26), D-glucose (10), KCl (3.76), CaCl₂ (2.4), MgSO₄ (1.33), KH₂PO₄ (1.23), saturated with 95% O₂/5% CO₂ 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, ME). 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 post-experiment 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 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.

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Appendix

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Conflict of interest: The authors declare no competing financial interest.

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Figure 1. Nicotine and gutkha-associated nicotine suppress single pulse (1 p) evoked $[DA]_0$ and amplify 5 p/1 p ratio

A. Representative concentration-time profiles of $[DA]_0$ 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 $[DA]_0$ evoked by 1 p stimulation in dorsolateral striatum *ex vivo* in the presence and absence of gutkha-associated nicotine (gutkha nicotine, 30 μ M) in aCSF. **C.** Summary of the amplification of 5 p/1 p ratio for evoked $[DA]_0$ at the effective concentrations required for suppression of evoked $[DA]_0$ in A and B (***) $p < 0.001$ vs. control for each condition; mean $\pm$ SEM, $n = 3$ per mean; one-way ANOVA with Bonferroni's multiple comparison of selected groups).

Figure 2. Decreased potency of gutkha-associated nicotine vs. nicotine alone

Nonlinear regression using a sigmoidal (variable slope) model for the concentration-response (ratio of 5 p/1 p evoked $[DA]_0$) for nicotine and gutkha-associated nicotine (guttika nicotine) confirmed progressive nAChR desensitization for both agents, but a marked difference in potency - indicated by a significant difference in EC₅₀ 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 desensitization 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 $[DA]_0$ 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-associated 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 $\pm$ SEM two-way ANOVA with Bonferroni's multiple comparison of selected groups). There were no significant effects of 5 μM gutkha-nicotine in either context ($n = 3-6$; $p > 0.05$). **B.** Effect of 10 μM atropine on changes in evoked $[DA]_0$ by 50 nM ($n = 3$) and 140 nM ($n = 12$) nicotine (Nic). Nicotine also produced a concentration-dependent increase in the 5 p/1 p ratio for these doses in the absence of atropine ($***p < 0.001$ for 140 nM nicotine). Atropine potentiated the increased 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).

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

^aReferences 17, 19, and 23–25

^bReferences 15 and 16

^cReferences 33 and 34

^dReferences 35 and 36

^eReference 37

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) ^f
		Camphor	<1%	nAChR inhibitor ^g
		Citronellol	4%	Inhibitor of acetylcholinesterase ^h
		Geraniol	2%	Inhibitor of acetylcholinesterase ^h
Terpene	2–3%	Borneol	54%	nAChR inhibitor ⁱ
		Rose oxide	8%	None
		Safranal	38%	Inhibitor of acetylcholinesterase ^j
Monoterpene Alcohol	3–4%	Terpineol	71%	Inhibitor of acetylcholinesterase ^k
		Linalool	29%	Inhibitor of acetylcholinesterase ^l
Phenylpropene	<1%	Eugenol	100%	Inhibitor of acetylcholinesterase ^m

^fReferences 38–40

^gReferences 41 and 42

^hReference 43

ⁱReference 44

^jReference 45

^kReference 46

^lReference 46–48

^mReference 49