



# Effects of nicotine, nornicotine and cotinine, alone or in combination, on locomotor activity and ultrasonic vocalization emission in adult rats

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## Abstract

**Rationale** The behavioral effects of the nicotine metabolites nornicotine and cotinine have not been investigated extensively.

**Objectives** To evaluate the effects of nicotine, cotinine, and nornicotine, given alone or in combination, on locomotor activity and emission of ultrasonic vocalizations in male adult rats.

**Methods** Rats were first given home cage nicotine injections to make them tolerant to the drug's locomotor depressant effects. On subsequent days, locomotor activity (LMA) and ultrasonic vocalizations were recorded in an open field, for 60 min after challenge injection, using repeated measures designs. In single-drug experiments, subjects were tested with nicotine 0.05–0.4 mg/kg, cotinine 0.03–3 mg/kg, or nornicotine 0.1–10 mg/kg. In drug-combination experiments, saline or nicotine 0.2 mg/kg challenge was preceded by cotinine (0, 0.3, 3 mg/kg) or nornicotine (0, 0.1, 0.3, 1, 3 mg/kg) injection.

**Results** High doses of nornicotine increased LMA and blunted the locomotor stimulant effect of nicotine. Less consistently, nicotine and high doses of nornicotine decreased the 50-kHz call rate, with no clear evidence of a nornicotine × nicotine interaction. Cotinine, given alone or before nicotine injection, altered neither LMA nor the call rate. No drug altered the relative prevalence of flat vs. trill 50-kHz call subtypes, except that the highest dose of nornicotine promoted flat calls over trills. No drug evoked 22-kHz calls.

**Conclusion** Nornicotine can exert an acute anti-nicotine effect in vivo, as previously reported in vitro. The finding that nicotine did not detectably alter the 50-kHz call profile appears consistent with this drug's mild subjective effects in human subjects.

**Keywords** Nicotine · Nornicotine · Cotinine · Ultrasonic vocalization · Locomotion · Rat behavior

## Introduction

Nornicotine is a tobacco constituent and minor metabolite of nicotine, whereas cotinine is a major nicotine metabolite and a commonly used biomarker of smoking status (Hukkanen et al. 2005). Thus, in smokers, less than 1% of nicotine is metabolized to nornicotine, with a similar amount derived from inhaled tobacco smoke, whereas around 70% of nicotine is metabolized to cotinine (Benowitz and Jacob, III 1994; Benowitz et al. 1994). Nornicotine and cotinine possess longer elimination half-lives than nicotine (Hukkanen et al. 2005;

Kyerematen et al. 1990b), and rodent studies additionally suggest that both metabolites accumulate in the brain during prolonged exposure to nicotine (Ghosheh et al. 2001). Thus, it has been proposed that both agents may reach pharmacologically relevant concentrations in the brains of smokers (Crooks and Dwoskin 1997; Ghosheh et al. 1999; Ghosheh et al. 2001; Miksys et al. 2000).

Nornicotine and cotinine activate neuronal nicotinic cholinergic receptors (nAChRs), although often only at higher doses than nicotine (Crooks and Dwoskin 1997; Dwoskin et al. 1999b). For example, nornicotine is reported to act less potently than nicotine in evoking neurotransmitter release in vitro (e.g., Dwoskin et al. 1995; Green et al. 2001; Lu et al. 1998), and also in several rodent behavioral assays (Bardo et al. 1999; Dwoskin et al. 1999a; Goldberg et al. 1989; Stolerman et al. 1995; Takada et al. 1989). Nornicotine nevertheless activates certain recombinant nAChR subtypes more potently than nicotine (Papke et al.

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2007). Cotinine typically interacts with nAChR agonists only at very high concentrations (Briggs and McKenna 1998; Dwoskin et al. 1999b; Terry et al. 2015; Vainio et al. 2000) and has additional, nAChR-independent, actions via molecular targets that remain largely obscure (Buccafusco and Terry 2003; Goldberg et al. 1989; Terry et al. 2015).

Numerous *in vitro* studies suggest that nornicotine and cotinine can also *desensitize* CNS nAChRs (Briggs and McKenna 1998; Dwoskin et al. 1999b, 2001; Green et al. 2001; Lu et al. 1999; Oliver et al. 2007; Teng et al. 1997; Terry et al. 2015; Vainio et al. 2000), and in addition, nornicotine has been identified as a partial agonist at certain recombinant nAChR subtypes (Papke et al. 2007). However, nicotine-opposing actions of these compounds have been little investigated *in vivo*. Notably, acute pretreatment with nornicotine, which did not inhibit the nicotine cue (Caine et al. 2014), suppressed intravenous nicotine self-administration in rodents (Caine et al. 2014; Green et al. 2000; Stairs et al. 2007). However, with regard to the latter finding, it is unclear whether nornicotine substituted for nicotine or directly inhibited nicotine's actions at nAChRs.

A major aim of the present study was therefore to identify possible anti-nicotine effects of nornicotine and cotinine *in vivo*. The main measure used for this purpose was nicotine-induced stimulation of locomotor activity (LMA). This behavioral effect was chosen because it occurs through a direct activation of brain nAChRs and is stable across repeated testing (Clarke and Kumar 1983a; Clarke and Kumar 1983b; Stolerman et al. 1995). During the locomotor test sessions, we also determined the acute effects of nicotine, nornicotine, and cotinine on the emission of ultrasonic vocalizations (USVs). Adult rats emit two broad classes of USVs: 22-kHz “alarm” calls, and 50-kHz calls (Brudzynski 2015; Wohr and Schwarting 2013). Among the plethora of identifiable 50-kHz call subtypes (Wright et al. 2010), “trill” and “flat” calls are of particular interest. In particular, rewarding drugs, as well as anticipation of play, preferentially promote trill calls over flat calls (Best et al. 2017; Burke et al. 2017; Simola and Brudzynski 2018; Wright et al. 2012b; Wright et al. 2010), whereas acute morphine withdrawal produced the opposite shift (Lin et al. 2018). On this basis, the relative prevalence of trill and flat calls appear to signal positive vs. negative affect.

Based on these considerations, we predicted that nicotine, which is only weakly euphoriant in humans (Dar et al. 2007; Kalman 2002), would, at best, mildly promote trills over flat calls. Previous published studies have not detected such an effect, but low overall rates of calling may have hindered detection (Simola et al. 2012; Simola et al. 2014; Swalve et al. 2019; Swalve et al. 2016). Possible mood-modifying effects of nornicotine have apparently not been studied in

human subjects, whereas cotinine appears not to improve mood and may possibly be mildly anxiogenic (Benowitz et al. 1983; Hatsukami et al. 1998; Keenan et al. 1994). Since drug-induced anxiety does not appear to produce a consistent change in USV emission (Willadsen et al. 2018), cotinine would be expected not to affect USV emission, whereas nornicotine would mimic nicotine, albeit less potently, based its nicotine-like activity in other *in vitro* and behavioral assays (see above). In the present study, we also analyzed the *rate* of 50-kHz call emission, predicting that since our rats had a history of nicotine exposure, this drug would likely promote 50-kHz calling (Swalve et al. 2016), potentially providing an additional measure with which to assess drug-drug interactions.

The present study therefore sought to address several questions. First, what are the locomotor effects of nornicotine and cotinine, given alone and in combination with nicotine? Second, does acute pretreatment with nornicotine or cotinine blunt acute behavioral effects of nicotine? Lastly, how do these three drugs affect the emission 50-kHz USV subtypes, especially those proposed to reflect affect? To address these questions, we initially tested a range of doses of nicotine, nornicotine and cotinine, each drug given alone. Subsequently, to test the effects of nicotine after acute nornicotine or cotinine pretreatment, we selected a dose of nicotine (0.2 mg/kg) that produced robust locomotor stimulation. In these drug-combination experiments, we included nornicotine and cotinine doses that appeared behaviorally inactive, since nicotinic agonists tend to desensitize nAChRs even at concentrations lower than required for activation (Rollema and Hurst 2018).

## Methods

### Animals

Subjects were adult male Long-Evans rats (Charles River Laboratories, Kingston, NY, USA). At the start of testing, body weights were as follows (for experiments 1–6, respectively): 242–303, 275–290, 265–320, 265–311, 240–263, and 410–540 g. The rats were housed two per cage in a humidity- and temperature-controlled colony room at the McGill University Animal Resources Center. Home cage bedding consisted of laboratory-grade Beta Chip® (NEPCO, Warrensburg, NY). Rats were maintained on a reverse 12:12-h light/dark cycle with the lights off at 0700 h, and all testing was performed during the dark phase of the cycle. Food and water were available *ad libitum* except during testing. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

## Drugs

The following drugs were used: (S)-(–)-nicotine hydrogen ditartrate (Sigma-Aldrich, ON, Canada), (R,S)-(±)-nornicotine and (S)-(–)-cotinine (catalog numbers N757000 and C725000, both of 98% purity, from Toronto Research Chemicals, ON, Canada). All drugs were dissolved in 0.9% sterile saline. Nicotine and nornicotine solutions were pH-neutralized with dilute NaOH, to pH  $7.2 \pm 0.2$  and pH 6.8–7.3, respectively. Injections were given subcutaneously (SC) into the flank in a volume of 1 ml/kg. Control injections were of saline. Nicotine doses are expressed as base.

The choice of racemic nornicotine and the (S)-(–)- stereoisomer of cotinine was based on the following considerations. Both nornicotine enantiomers are present in tobacco smoke, with the (S)-isomer being more prevalent (Liu et al. 2008). Nornicotine is also formed from nicotine, both in the periphery and potentially to a significant extent in the brain (Crooks et al. 1997; Ghosheh et al. 2001). This N-demethylation step has been shown to favor the (R)-(+)-nornicotine enantiomer in hamsters (Kyerematen et al. 1990a), but whether this is the case in rats or humans appears uninvestigated. With regard to cotinine, the (S)-(–)- isomer appears to predominate in cigarette smokers, since nicotine metabolism is generally stereospecific (Kyerematen and Vesell 1991) and since tobacco smoke contains mainly the (S)-(–)- isomer of nicotine (Hukkanen et al. 2005).

## Testing boxes and locomotor activity

Four rectangular test boxes were used (58 cm long, 29 cm wide, and 53 cm high). Each comprised four vertical wooden white-painted walls, covered by an 8-mm-thick clear Plexiglas™ lid, attached by a hinge. The four test boxes were placed in a quadrant configuration, 2.5 cm apart, on a layer of Beta Chip® bedding which was changed between experiments but not between individual sessions. The boxes were lit with Kodak GBX-2 safelight filters (Vistek, Toronto, Ontario, Canada) providing far-red (wavelength > 650 nm) illumination. A video camera (WV-BP330 CCTV, Panasonic, USA) was positioned overhead, and software (MediaCruise, Canopus Corp., San Jose, CA, USA) created an MPEG video file of each session. The video recording was tracked offline using the EthoVision v. 3.0 software (Noldus Information Technology, Leesburg, VA, USA) in order to measure locomotor activity (LMA, i.e., total horizontal distance moved).

## Acquisition and acoustic analysis of ultrasonic vocalizations

Broadband recordings and acoustic analysis were performed as detailed in our recent publications (e.g., Best et al. 2017),

but adapted to our locomotor test boxes. Thus, each box was equipped with an ultrasonic condenser microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany), inserted through a small (9 cm × 6 cm) recess cut halfway along one long side of the lid, and connected to an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics). The sampling rate was 250-kHz with 16-bit resolution. Spectrograms were generated by fast Fourier transform (512 points, 75% overlap, FlatTop window, 100% frame size) using Avisoft SASLab Pro (v. 5.2.09). All calls in a given session were manually selected from spectrograms by a single individual who was blinded to the treatment conditions. All calls were analyzed, except in experiments 4 and 5, where a few rats with unusually high call rates (i.e., >1200 calls/h) were time-sampled; for these subjects, only calls emitted in every fifth minute were analyzed (i.e., minutes 3, 8, 13, etc.). 22-kHz calls were counted, and 50-kHz calls were categorized according to our 14-subtype scheme (Wright et al. 2010), as indicated in the Fig. 2 legend (below).

## General testing procedure (all experiments)

The rats were initially naïve to drugs and to the experimental apparatus. They were handled once daily for 5 min for 3 days, and then, on 3 consecutive days, they received twice-daily home cage injections of nicotine (0.4 mg/kg SC, at least 10 h apart). This regimen served to produce tolerance to nicotine's initial locomotor depressant effect, revealing a stronger stimulant effect (Louis and Clarke 1998). On the next day, rats were individually habituated to their test boxes for 15 min. A given rat was always exposed to the same test box throughout each experiment. The first test day followed 24 h later. On each test day, rats were transported from the colony room, weighed, and left in their home cages for 15 min to habituate. In some experiments (see Table 1), rats were tested immediately after a single injection of drug, whereas in other experiments subjects received a pretreatment injection and were returned to their home cage for 30 min, prior to acute challenge with nicotine (0.2 mg/kg SC) or saline. Immediately after the acute challenge injection, LMA and USVs were recorded for 60 min. The order of drug presentations was counterbalanced as far as possible within each experiment, based on a modified Williams square.

The timing of drug injections and test days is justified as follows. The 30-min interval between pretreatment-challenge injections was chosen because: (1) cotinine is reported to peak in the brain 30–60 min after SC cotinine injection (Crooks et al. 1997), (2) although the rate of brain entry after SC nornicotine injection has not apparently been reported, behavioral effects are evident within 10–20 min when nornicotine is administered at relevant doses (Dwoskin et al. 1999a; Goldberg et al. 1989; Green et al. 2002; Stairs et al. 2007; Stolerman et al. 1995). The spacing of test days took account

of the respective brain half-lives reported for nicotine, cotinine, and nornicotine in adult rats, i.e., 52, 333, and 166 min (Ghosheh et al. 1999). Accordingly, cotinine was tested at 2–3-day intervals whereas nicotine and nornicotine were both tested at 1–2-day intervals.

## Statistical analysis

Drug solutions were coded so that experimenters were blind to drug conditions. Data were analyzed using Systat software (v. 11, SPSS, Chicago, IL) and figures were generated using Prism 4 (GraphPad Software, La Jolla, CA). Primary measures of interest were locomotor activity (LMA, i.e., horizontal distance traveled), the 50-kHz call rate, and the percentages of flat and trill calls. 50-kHz call profiles were defined by the proportional contributions of all 14 call subtypes (Wright et al. 2010). Data were averaged between any test sessions performed under the same drug conditions, in which case each call (rather than each session) was given equal weight in the call profile calculation. For statistical analysis, call profiles were considered unreliable in test sessions yielding less than 5 calls (i.e., in 33 out of the 500 sessions). In such cases, values for the percent flat and trill prevalence were set as missing, except that in experiments 1–3 (dose-response), these values were interpolated from the two nearest-dose values, and the error degrees of freedom were reduced accordingly. Parametric tests were used unless underlying assumptions were violated. ANOVA factors were all within-subject, as follows: DOSE (i.e., all doses including saline), NIC (nicotine), COT (cotinine), NOR (nornicotine), and TIME (5-min time bins). Fifty-kilohertz call rates were positively skewed and hence analyzed using non-parametric tests (i.e., Friedman or Wilcoxon rank-sum). In experiment 1, two rats each received an incorrect nicotine dose on one occasion, and data for these sessions was interpolated from adjacent doses. No adjustment was made for multiple comparisons, but instead, the alpha level was set at 1% (two-tailed) throughout.

## Results

The main findings of all experiments are summarized in Table 2. In all six experiments, 50-kHz calls were much more prevalent than 22-kHz calls. Thus, the total number of 22-kHz and 50-kHz calls emitted in experiments 1–6 were, respectively: 1672 and 16,979; 205 and 16,795; 229 and 12,602; 47 and 7253; 3033 and 13,805; 277 and 16,260. The 22-kHz calls were emitted in only a minority of rats and in only a subset of test sessions within a given experiment. Thus, for experiments 1–6, the number of test sessions containing any 22-kHz calls was, respectively: 10/72, 3/96, 8/96, 3/72, 21/96, and 7/80. There was no clear relation between drug condition and 22-

kHz USV emission, hence 22-kHz call data are not presented further.

## Experiment 1: Nicotine dose-response

**LMA** Nicotine dose-dependently stimulated LMA and this effect was not detectably time-dependent (Fig. 1a, b; DOSE  $F_{4,44} = 10.47$ ,  $p = 0.0000$ ; DOSE  $\times$  TIME  $F_{44,484} = 1.39$ ,  $p = 0.1429$ ). Significant increases occurred at doses of 0.1, 0.2, and 0.4 mg/kg ( $t$  tests, respectively:  $t_{11} = 3.16$ ,  $p = 0.0091$ ;  $t_{11} = 4.97$ ,  $p = 0.0004$ ;  $t_{11} = 6.80$ ,  $p = 0.0000$ ).

**50-kHz USVs** Nicotine tended to reduce the 50-kHz call rate, but not significantly (Friedman  $Q_4 = 9.20$ ,  $p = 0.0563$ ), even at the three highest doses (Wilcoxon tests:  $p = 0.0229$ – $0.0281$ ). Median (IQR) 50-kHz calls per session were as follows (for 0, 0.05, 0.1, 0.2 and 0.4 mg/kg, respectively): 260 (155–387), 240 (24–329), 54 (19–98), 68 (3–157), and 61 (18–111). Nicotine also did not significantly alter the percentage of flat calls or trill calls (Fig. 2a–e: DOSE main effects, respectively:  $F_{4,40} = 0.46$ ,  $p = 0.7655$ ;  $F_{4,40} = 0.81$ ,  $p = 0.4785$ ).

## Experiment 2: Cotinine dose-response

**LMA** Cotinine (tested at 0.03–3 mg/kg) did not significantly alter LMA (Fig. 1c, d; DOSE  $F_{5,55} = 1.25$ ,  $p = 0.2992$ ; DOSE  $\times$  TIME  $F_{55,605} = 1.06$ ,  $p = 0.3800$ ). In contrast, nicotine (0.2 mg/kg, positive control) increased this measure by approximately 35% ( $t_{11} = 4.88$ ,  $p = 0.0005$ ).

**50-kHz USVs** The call rate was not significantly altered by cotinine (Friedman  $Q_5 = 1.64$ ,  $p = 0.8960$ ), but was reduced by nicotine (Wilcoxon  $Z = 2.98$ ,  $p = 0.0029$ ). Median (IQR) call rates were as follows (saline, cotinine 0.03–3 mg/kg, and nicotine 0.2 mg/kg, respectively): 50 (14–172), 51 (19–163), 54 (29–139), 62 (16–135), 61 (25–110), 30 (4–198) and 5 (3–19) calls/60 min. The corresponding call profiles are shown in Fig. 2f–i. Neither cotinine nor nicotine detectably changed the percentage of flat or trill calls (respectively: for cotinine, Friedman  $Q_5 = 6.62$ ,  $p = 0.2502$  and  $Q_5 = 2.18$ ,  $p = 0.8237$ ; for nicotine, Wilcoxon  $Z = 0.52$ ,  $p = 0.6002$  and  $Z = 0.34$ ,  $p = 0.7353$ ).

## Experiment 3: Nornicotine dose-response

**LMA** Nicotine (0.2 mg/kg) increased LMA, as expected ( $t_{11} = 4.01$ ,  $p = 0.0021$ ). Nornicotine (tested at 0.1–10 mg/kg) exerted dose- and time-dependent effects on LMA, as shown in Fig. 1e, f (DOSE  $F_{5,55} = 5.89$ ,  $p = 0.0004$ ; DOSE  $\times$  TIME  $F_{55,605} = 2.19$ ,  $p = 0.0000$ ). Nornicotine significantly increased whole-session LMA only at 3 mg/kg ( $t_{11} = 3.18$ ,  $p = 0.0087$ ), and this stimulant effect was apparent from 15 to 50 min (Fig. 1f). The highest dose of nornicotine (10 mg/kg),

**Table 1** Summary of experimental conditions

Experiment	<i>n</i>	Pretreatment (mg/kg)		Challenge (mg/kg)	
1	12			NIC	0 (twice), 0.05, 0.1, 0.2, 0.4
2	12			COT	0 (twice), 0.03, 0.1, 0.3, 1, 3
				NIC	0.2
3	12			NOR	0 (twice), 0.1, 0.3, 1, 3, 10
				NIC	0.2
4	12	COT	0, 0.3, 3	NIC	0, 0.2
5	12	NOR	0, 0.1, 0.3, 1	NIC	0, 0.2
6	10	NOR	0, 0.3, 1, 3	NIC	0, 0.2

NIC, nicotine; NOR, nornicotine; COT, cotinine

while not significantly altering whole-session LMA, appeared to inhibit LMA in the first 15 min of the session (Fig. 1f).

**50-kHz USVs** The call rate was significantly reduced by the two highest doses of nornicotine (3 and 10 mg/kg) and also by nicotine (respectively: Wilcoxon  $Z = 2.90, 2.82$ , and  $3.06$ ,  $p = 0.0037, 0.0047$  and  $0.0022$ ). Median (IQR) call rates (i.e., calls/60-min session) were as follows (saline, nornicotine 0.1–10 mg/kg, and nicotine 0.2 mg/kg, respectively): 102 (35–155), 97 (11–251), 55 (17–183), 35 (11–164), 17 (9–65), 26 (12–58), and 20 (3–60). Call profiles are shown in Fig. 2m–s. Nornicotine altered the call profile only at the highest dose (10 mg/kg, Fig. 2r), significantly increasing the percentage of flats ( $t_9 = 3.36$ ,  $p = 0.0084$ ) and tending to inhibit trills ( $t_9 = 3.10$ ,  $p = 0.0128$ ). Nicotine affected neither flat or trill call prevalence (respectively:  $t_6 = 0.16$ ,  $p = 0.8767$ ;  $t_6 = 0.94$ ,  $p = 0.3852$ ; Fig. 2s).

#### Experiment 4: Cotinine combined with nicotine

**LMA** The locomotor stimulant effect of nicotine (Fig. 3a–c) was not altered by cotinine pretreatment (COT  $\times$  NIC  $F_{2,22} = 2.17$ ,  $p = 0.1381$ ), irrespective of time within session (3-way interaction  $F_{22,242} = 0.92$ ,  $p = 0.5378$ ). Cotinine, given alone, tended to increase LMA at the lower dose (Fig. 3a, 0.3 mg/kg;  $t_{11} = 3.06$ ,  $p = 0.0108$ ).

**50-kHz USVs** Results are shown in Table 3. Nicotine did not detectably reduce the call rate when it was given alone (Wilcoxon test:  $Z = 1.18$ ,  $p = 0.2393$ ), but it did significantly decrease this measure when the data were pooled across pretreatment conditions (Wilcoxon test:  $Z = 3.06$ ,  $p = 0.0022$ ). Cotinine had no overall effect on call rate (Friedman  $Q_2 = 1.17$ ,  $p = 0.5580$ ), and did not significantly alter the depressant effect of nicotine, as analyzed by nicotine-saline difference

**Table 2** Summary of main findings

Experiment	Drug/doses (mg/kg)	Locomotor activity	50-kHz call rate	Percent flat	Percent trill
1	NIC 0, 0.05, 0.1, 0.2, 0.4	↑ at 0.1, 0.2 and 0.4 mg/kg	↓? at 0.1, 0.2 and 0.4 mg/kg	↔	↔
2	COT 0, 0.03, 0.1, 0.3, 1, 3	↔	↔	↔	↔
2	NIC 0.2	↑	↓	↔	↔
3	NOR 0, 0.1, 0.3, 1, 3, and 10	↑ at 3 mg/kg	↓ at 3 and 10 mg/kg	↑ at 10 mg/kg	↓? at 10 mg/kg
3	NIC 0.2	↑	↓	↔	↔
4	COT 0, 0.3, or 3	↑? at 0.3 mg/kg	↔	↔	↔
4	NIC 0.2	↑	↓	↔	↔
4	COT $\times$ NIC	No interaction	No interaction	No interaction	No interaction
5	NOR 0, 0.1, 0.3, 1	↑ at 1 mg/kg	↔	↔	↔
5	NIC 0.2	↑	↓?	↔	↔
5	NOR $\times$ NIC	Significant at NOR 1 mg/kg, trend at NOR 0.3 mg/kg	Interaction?	↔	↔
6	NOR 0, 0.3, 1, 3	↑ at 3 mg/kg	↔	Not analyzed	Not analyzed
6	NIC 0.2	↑	↔	Not analyzed	Not analyzed
6	NOR $\times$ NIC	Significant at NOR 3 mg/kg	No interaction	Not analyzed	Not analyzed

The significance level (i.e., alpha) was set at 1%. Arrows refer to effects of drugs when given alone: ↑ significant increase, ↓ significant decrease, ↔ no significant effect. Trends refer to  $p = 0.05–0.01$ , and are shown by “?” (i.e., ↑?, ↓?). COT, cotinine; NIC, nicotine; NOR, nornicotine. Overall, the emission of 22-kHz calls was sporadic, with no clear relation to drug condition; these data are presented briefly at the start of the “Results” section

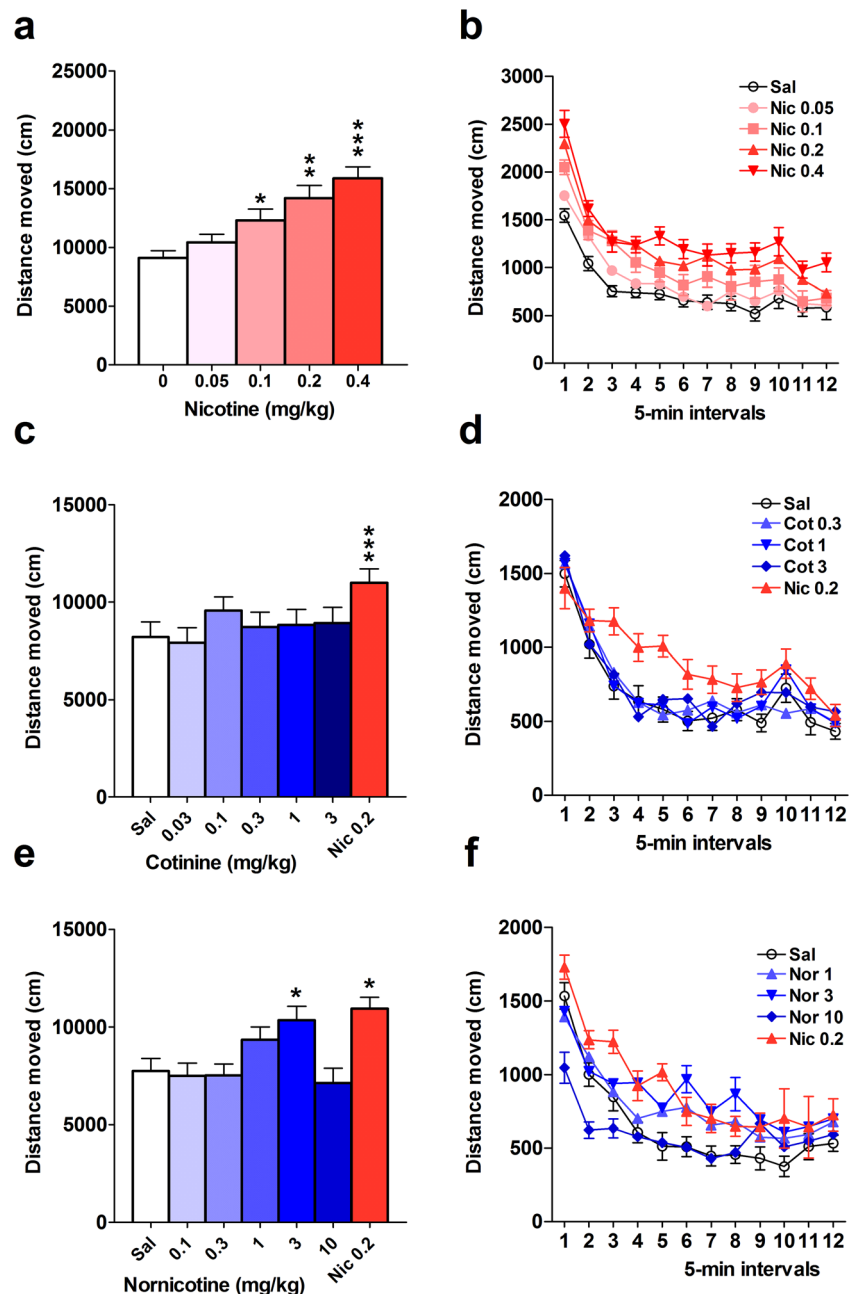
scores (Friedman  $Q_2 = 3.17$ ,  $p = 0.2053$ ). Neither nicotine nor cotinine, given alone or in combination, appeared to alter the percentage of flat or trill calls (Table 3;  $p > 0.05$  for all comparisons).

### Experiment 5: Nornicotine combined with nicotine

**LMA** The stimulant effect of nicotine, and its interaction with nornicotine, appeared time-dependent (Fig. 3e, f; NIC  $\times$  TIME  $F_{11,121} = 3.97$ ,  $p = 0.0003$ ; 3-way interaction  $F_{33,363} = 1.50$ ,  $p = 0.0458$ ). Nicotine, given alone, did not detectably increase LMA in the second half of the session (Fig. 3e, 30–

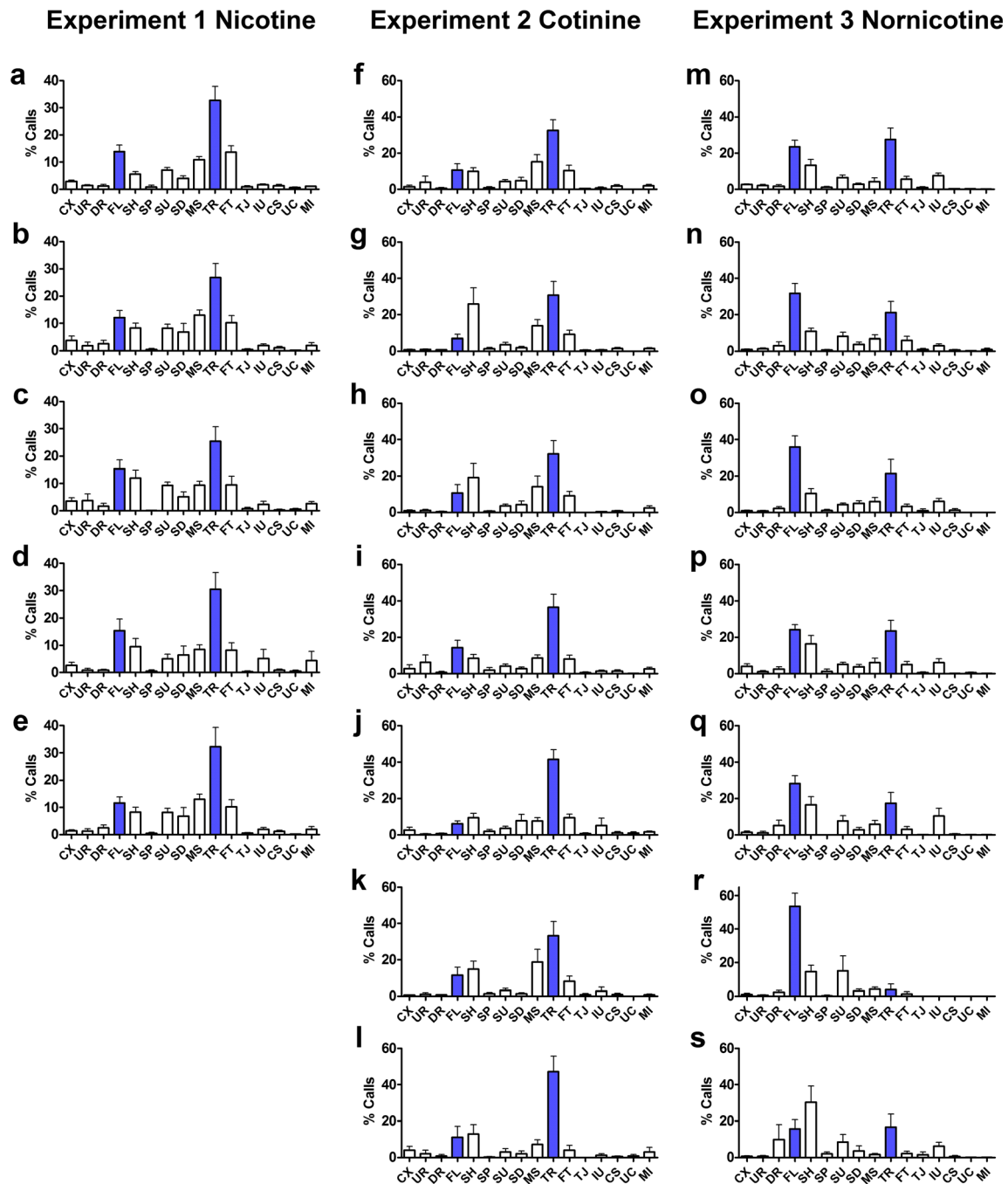
60 min:  $t_{11} = 1.48$ ,  $p = 0.1679$ ), hence only the 0–30 min time interval was analyzed further. During this 0–30 min period (Fig. 3d), the highest nornicotine dose (1 mg/kg) increased LMA when given alone (38% increase:  $t_{11} = 4.10$ ,  $p = 0.0018$ ). In addition, nornicotine dose-dependently inhibited the locomotor stimulant effect of nicotine (NOR  $\times$  NIC  $F_{3,33} = 4.77$ ,  $p = 0.0090$ ). This antagonistic effect was significant at the highest nornicotine dose of 1 mg/kg (NOR  $\times$  NIC  $F_{1,11} = 17.00$ ,  $p = 0.0017$ ), with a similar trend at 0.3 mg/kg ( $F_{1,11} = 7.54$ ,  $p = 0.0190$ ). However, nicotine still tended to increase LMA even after pretreatment with the highest dose of nornicotine ( $t_{11} = 2.24$ ,  $p = 0.0469$ ).

**Fig. 1** Locomotor activity as a function of dose of nicotine, cotinine or nornicotine (experiments 1, 2, and 3, respectively). The mean  $\pm$  SEM distance moved (cm) is shown for the entire 60-min session (panels a, c, and e), and in consecutive 5-min time bins (panels b, d, and f). Within each experiment, each rat ( $n = 12$ ) was tested under all conditions. For clarity, some SEM bars are omitted from the time course panels, and lower doses of cotinine and nornicotine are omitted in panels d and f, respectively. Asterisks are shown only for the whole-session data. \* $p < 0.05$  (i.e., trend), \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. saline (paired  $t$  tests, 2-tailed alpha set at 1%)



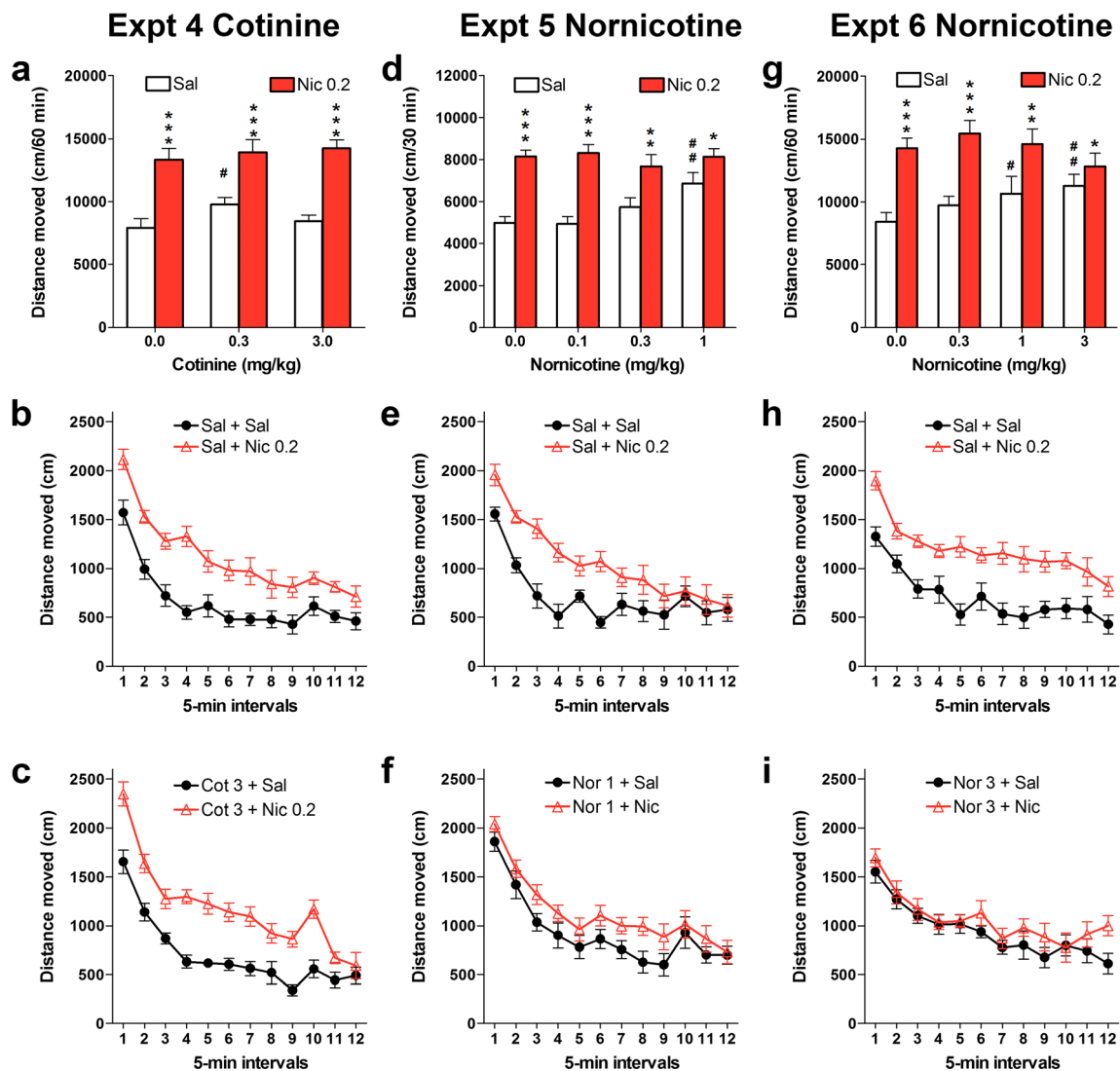
**50-kHz USVs** When tested alone, nornicotine (0.1–1 mg/kg) did not discernably alter the 50-kHz call rate (Table 3; Friedman  $Q_3 = 0.1000$ ,  $p = 0.9918$ ). Nicotine (0.2 mg/kg) tended to reduce the 50-kHz call rate when given alone (Wilcoxon  $Z = 2.27$ ,  $p = 0.0229$ ), but tended to increase this

measure when combined with the higher doses of nornicotine (Table 3). To further assess the possibility of a nornicotine  $\times$  nicotine interaction, a one-way ANOVA was performed on nicotine-saline difference scores, revealing a non-significant trend ( $F_{3,33} = 3.80$ ,  $p = 0.0192$ ).



**Fig. 2** 50-kHz call subtype profiles: nicotine, cotinine, and nornicotine (experiments 1, 2, and 3, respectively). Panels **a–e** represent nicotine (0, 0.05, 0.1, 0.2, and 0.4 mg/kg, respectively). Panels **f–i** show, respectively, cotinine 0, 0.03, 0.1, 0.3, 1, and 3 mg/kg and nicotine 0.2 mg/kg. Panels **m–s** show, respectively, nornicotine 0, 0.1, 0.3, 1, 3, and 10 mg/kg and nicotine 0.2 mg/kg. Y-axes show the relative prevalence of each call subtype expressed as the mean  $\pm$  SEM percentage of all 50-kHz calls.

Flat (FL) and trill (TR) call data are shown by blue bars. Call subtype abbreviations: CX complex, UR upward ramp, DR downward ramp, FL flat, SH short, SP split, SU step-up, SD step-down, MS multi-step, TR trill, FT flat-trill, TJ trill with jumps, IU inverted-U, CS composite, UC unclear, MI miscellaneous. Within a given experiment, each rat ( $n = 12$ ) was tested under all conditions



**Fig. 3** Nicotine-induced locomotor activity after pretreatment with cotinine (left: experiment 4) or nornicotine (middle and right, respectively: experiments 5 and 6). The top panels show the distance moved across the entire 60-min session (panels a and g) or in the first 30 min (panel d). The remaining panels illustrate the time course following acute challenge with saline or nicotine 0.2 mg/kg, after pretreatment either with saline (panels b, e, h) or with the highest pretreatment dose of

cotinine (panel c) or nornicotine (panels f and i). Note that a higher dose range of nornicotine was used in experiment 6 vs. 5. Mean  $\pm$  SEM are shown. Within a given experiment, each rat ( $n = 12$ ) was tested under all conditions. Asterisks are shown only for the top panels: \* $p < 0.05$  (i.e., trend), \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , nicotine vs. saline at a given dose of pretreatment drug. # $p < 0.05$  (i.e., trend), ### $p < 0.01$ , pretreatment drug alone vs. saline (paired  $t$  tests, 2-tailed alpha set at 1%)

Neither nicotine nor nornicotine significantly altered the percentage of flat or trill calls, and there was no significant drug interaction for either measure (Table 3; flat and trill, respectively: NOR  $F_{3,33} = 2.20$  and  $1.82$ ,  $p = 0.1070$  and  $0.1673$ ; NIC  $F_{1,11} = 4.12$  and  $0.14$ ,  $p = 0.0673$  and  $0.7129$ ; interactions  $F_{3,33} = 0.26$  and  $1.35$ ,  $p = 0.8413$  and  $0.2751$ ).

### Experiment 6: Nornicotine combined with nicotine (partial replication)

**LMA** A higher nornicotine dose range was used than in experiment 5 (i.e., 0.3–3 mg/kg instead of 0.1–1 mg/kg). Neither the locomotor stimulant effect of nicotine nor its interaction with

nornicotine was time-dependent (Fig. 3h, i; 2-way and 3-way interactions, respectively:  $F_{11,99} = 1.22$ ,  $p = 0.2923$  and  $F_{33,297} = 0.87$ ,  $p = 0.6596$ ). When tested alone, both nicotine and the highest nornicotine dose (3 mg/kg) significantly increased whole-session LMA (Fig. 3g;  $t$  tests, respectively:  $t_9 = 6.49$ ,  $p = 0.0001$  and  $t_9 = 3.52$ ,  $p = 0.0065$ ), with the trend at nornicotine 1 mg/kg ( $t_9 = 2.27$ ,  $p = 0.0490$ ). As in experiment 5, nornicotine dose-dependently inhibited the locomotor stimulant effect of nicotine (NOR  $\times$  NIC  $F_{3,27} = 7.88$ ,  $p = 0.0006$ ); this interaction was significant at 3 mg/kg ( $F_{1,9} = 12.88$ ,  $p = 0.0058$ ) but not at 1 mg/kg ( $F_{1,9} = 5.08$ ,  $p = 0.0507$ ). Blockade appeared near-total at the highest nornicotine dose (residual nicotine effect:  $t_9 = 2.38$ ,  $p = 0.0412$ ).

**Table 3** 50-kHz call rate, and prevalence of flat and trill 50-kHz call subtypes in rats treated with cotinine or nornicotine in combination with nicotine (experiments 4–6)

	COT (mg/kg)	50-kHz calls/60 min		Percent flat		Percent trill	
		Median (IQR)		Mean $\pm$ SEM		Mean $\pm$ SEM	
		Saline	Nicotine	Saline	Nicotine	Saline	Nicotine
Expt 4	0	61 (20–370)	23 (5–145)	16.0 $\pm$ 4.0	9.2 $\pm$ 2.8	41.7 $\pm$ 7.6	36.9 $\pm$ 6.7
	0.3	89 (31–467)	28 (6–113)	10.4 $\pm$ 1.5	10.8 $\pm$ 6.3	47.8 $\pm$ 4.6	49.5 $\pm$ 9.4
	3	98 (42–205)	14 (4–50)	13.0 $\pm$ 4.2	19.2 $\pm$ 9.0	48.0 $\pm$ 5.8	33.7 $\pm$ 10.6
Expt 5	NOR (mg/kg)	Saline	Nicotine	Saline	Nicotine	Saline	Nicotine
	0	98 (36–269)	47 (11–150)	29.2 $\pm$ 4.9	25.6 $\pm$ 5.5	25.7 $\pm$ 4.2	16.3 $\pm$ 5.0
	0.1	115 (40–163)	49 (29–315)	25.5 $\pm$ 3.5	19.4 $\pm$ 5.2	24.4 $\pm$ 4.8	31.6 $\pm$ 5.6
	0.3	127 (34–184)	159 (43–378)	23.8 $\pm$ 4.5	13.3 $\pm$ 3.9	29.4 $\pm$ 5.2	32.7 $\pm$ 5.8
Expt 6	1	123 (37–224)	146 (41–223)	20.3 $\pm$ 4.1	15.7 $\pm$ 3.5	30.1 $\pm$ 6.2	32.5 $\pm$ 5.1
	NOR (mg/kg)	Saline	Nicotine	Saline	Nicotine	Saline	Nicotine
	0	73 (13–607)	60 (9–130)	–	–	–	–
	0.3	194 (46–441)	128 (15–353)	–	–	–	–
	1	156 (12–344)	118 (32–360)	–	–	–	–
	3	99 (32–282)	47 (5–407)	–	–	–	–

The 50-kHz call rate is expressed as median and interquartile range (IQR). The prevalence of flat or trill calls is expressed as a percentage of all 50-kHz calls emitted. COT, cotinine; NOR, nornicotine.  $n = 11$ –12, 12 and 9–10 rats (experiments 4–6, respectively)

**50-kHz USVs** The call rate (Table 3) was not significantly altered by either drug when given alone (nicotine and nornicotine, respectively: Wilcoxon  $Z = 1.58$ ,  $p = 0.1282$ ; Friedman  $Q_3 = 5.18$ ,  $p = 0.1590$ ). Furthermore, no nornicotine  $\times$  nicotine interaction was detected, based on one-way ANOVA using nicotine-saline difference scores as the dependent variable ( $F_{3,24} = 0.75$ ,  $p = 0.5275$ ).

## Discussion

Novel findings are as follows. In nicotine-experienced rats, acute challenge with locomotor stimulant doses of nicotine tended to reduce the 50-kHz call rate and did not alter the relative prevalence of flat and trill calls. Nornicotine administration, which produced mild locomotor stimulation, decreased 50-kHz call rate at the highest dose tested, while promoting flat calls over trill calls. Nornicotine also countered the locomotor stimulant effect of nicotine, including at some lower doses. In contrast, cotinine affected neither locomotor activity (LMA) nor any of the 50-kHz call measures. Finally, no drugs evoked 22-kHz calls.

## Pharmacokinetic aspects

In interpreting any high-dose effects of nornicotine or cotinine administration, two potential caveats must be considered: trace contamination by nicotine (see Goldberg et al. 1989;

Oliver et al. 2007; Takada et al. 1989), and in vivo conversion to nicotine. In the present study, cotinine was without detectable effect, which is consistent with reports that cotinine is not significantly converted to nicotine in adult rats (Crooks et al. 1997; Sepkovic et al. 1984). Behavioral effects observed after nornicotine administration are unlikely to reflect trace amounts of nicotine, for two reasons. First, nornicotine appeared only about 10-fold less potent than nicotine, as shown in Fig. 1. Second, nornicotine was supplied as 98% pure, and its synthesis route did not include nicotine; accordingly, nicotine was undetectable by proton NMR (data supplied by Toronto Research Chemicals). Lastly, we are unaware of any reports indicating that nornicotine can be converted to nicotine in vivo.

Both cotinine and nornicotine would be expected to reach the brain quite rapidly after SC injection. Thus, brain cotinine levels have been reported to peak at 30–60 min after SC cotinine injection (Crooks et al. 1997). Although comparable data appear unavailable for administered nornicotine, behavioral effects are seen within 10–20 min of SC injection. By way of comparison, after SC injection of nicotine, brain cotinine and nornicotine levels reached half-maximal by 30–60 min (Ghosheh et al. 1999; Vieira-Brock et al. 2013). Extrapolating from literature reports, it is possible to estimate approximate peak plasma and brain concentrations of cotinine and nornicotine that would occur during behavioral test sessions, following injections of either our standard nicotine dose (0.2 mg/kg SC) or cotinine. The following estimates are based

on the assumption that concentrations are directly proportional to dose administered:

After nicotine injection, we estimate maximal plasma levels of around 20–60 ng/ml for cotinine (Craig et al. 2014; Turner 1975; Vieira-Brock et al. 2011) and 0.1–1.5 ng/ml for nornicotine (Craig et al. 2014; Ghosheh et al. 2001; Vieira-Brock et al. 2013). Corresponding values for brain tissue would be approximately 10–25 ng/g for cotinine (Ghosheh et al. 1999; Vieira-Brock et al. 2011) and 2.5–10 ng/g for nornicotine (Ghosheh et al. 1999; Vieira-Brock et al. 2013).

After cotinine administration, the wide dose range used (0.03–3 mg/kg SC) would have provided estimated peak plasma cotinine levels ranging from 40 to 4000 ng/ml and peak brain cotinine levels of 25 to 2500 ng/g (Li et al. 2012; Terry et al. 2012). On this basis, our 0.2 mg/kg nicotine dose could be expected to produce cotinine levels corresponding to the lowest tested dose of cotinine.

For nornicotine administration, there appear to be no relevant published studies on which to base an estimate.

### Effects of drugs administered alone on locomotor activity

In the present study, all rats first received home cage injections of nicotine to render them tolerant to the locomotor depressant effects of the drug. As expected, the acute locomotor stimulant effect of nicotine was detected at 0.1–0.4 mg/kg and the dose-response relation was monotonic (Clarke and Kumar 1983b; Stolerman et al. 1995). Under the same conditions, cotinine did not detectably alter LMA. To our knowledge, acute locomotor effects of cotinine have only been reported in drug-naïve rats, with only equivocal signs of locomotor depression (Marusich et al. 2017; Wiley et al. 2015).

Nornicotine acutely increased LMA, albeit within a narrow dose range (i.e., 1–3 mg/kg), with an inverted-U dose-response relationship. These results appear consistent with previous reports suggesting that nornicotine acutely stimulates LMA in rats that have been subchronically treated with either nicotine (Stolerman et al. 1995) or nornicotine (Dwoskin et al. 1999a; Green et al. 2002; Stolerman et al. 1995). Although actions of nornicotine show some stereoselectivity (Dwoskin et al. 1999a; Green et al. 2001; Risner et al. 1988), the locomotor effects we observed using racemic nornicotine are in broad agreement with earlier findings obtained with either the racemate (Green et al. 2002), or the R(+)- isomer (Stolerman et al. 1995), or each isomer individually (Dwoskin et al. 1999a).

### Effects of drugs alone on call rate and call profile

The absence of nicotine-induced 22-kHz calls in our experiments is consistent with two literature reports (Simola et al.

2012; Simola et al. 2014), but runs counter to a third report in which spectrographically complex 22-kHz vocalizations were observed after nicotine administration (Swalve et al. 2016). In previous work, the 50-kHz call rate tended to be reduced by acute nicotine administration in drug-naïve rats (Simola et al. 2012; Simola et al. 2014; Swalve et al. 2016), whereas after repeated nicotine testing, the drug either did not significantly alter the call rate (Simola et al. 2014) or increased it (Swalve et al. 2016). In our nicotine-experienced rats, nicotine instead tended to decrease the 50-kHz call rate, although statistical significance (with the alpha level set at 1%) was reached in only 3 of the 6 experiments. The finding that nicotine concurrently reduced the 50-kHz call rate while increasing LMA adds to evidence that these two measures can be dissociated (Burke et al. 2017; Engelhardt et al. 2017; Laplagne and Elias 2016; Wright et al. 2012a).

Here, we describe the acute effects of nicotine on the full range of 50-kHz call subtypes identified by Wright et al. (2010). This level of detail was previously available only in nicotine-naïve animals (Simola et al. 2012), as reports in nicotine-experienced rats were limited to trill calls (Simola et al. 2014) or to composite, multistep and upward ramp subtypes (Swalve et al. 2016). The low call rates occurring in these previous studies could potentially have obscured effects on call subtype prevalence, whereas in the present study, the majority (i.e., 93%) of test sessions yielded enough 50-kHz calls to derive a call profile with reasonable confidence.

Although nicotine decreased the 50-kHz call rate, it did not appear to alter the prevalence of any call subtype; in particular, it left unchanged the relative proportions of flat and trill calls subtypes. When administered to human subjects, nicotine produces both positive and negative subjective effects and is at best only mildly euphoriant unless administered in a high dose as a rapid intravenous bolus (Dar et al. 2007; Kalman 2002). In contrast, drugs that are strongly euphoriant in humans (i.e., amphetamine, cocaine, and morphine) have all been found to promote trill calls over flat 50-kHz calls (Best et al. 2017; Pereira et al. 2014; Wright et al. 2012b; Wright et al. 2010), and the opposite shift has been seen during acute morphine withdrawal (Lin et al. 2018). Hence, our findings support the proposal that trill calls, either considered alone or in relation to flat calls, distinguish positive vs. negative affect (Best et al. 2017; Lin et al. 2018; Wright et al. 2012b; Wright et al. 2010). As such, 50-kHz calls may prove useful in modeling affective disorders that occur in human subjects (Simola and Granon 2018).

Among nicotine metabolites, only cotinine appears to have been studied for its subjective effects in human subjects. In this context, cotinine was administered during tobacco abstinence (Benowitz et al. 1983; Hatsukami et al. 1998; Keenan et al. 1994), resulting in increased restlessness and anxiety in one study (Keenan et al. 1994). The effects of these drugs on rodent USV emission have not been previously reported.

Here, nornicotine exerted significant effects at the highest dose tested (10 mg/kg), reducing the 50-kHz call rate while increasing the proportion of flat calls relative to trills (Fig. 2, panel m vs. r). This call profile shift suggests that high-dose nornicotine may be aversive, but whether nornicotine also produces a conditioned place or taste aversion appears unreported. Cotinine is reportedly neutral in the conditioned place preference/aversion assay (Fudala and Iwamoto 1986; Marusich et al. 2017), so its lack of effect on call profile (present study) is perhaps to be expected.

### Drug interactions with nicotine

Since we were primarily attempting to detect possible inhibitory, nAChR-desensitizing, effects of cotinine and nornicotine (experiments 5–6), we chose a nicotine test dose (0.2 mg/kg SC) that robustly increased LMA. This dose was likely near-maximal in terms of nicotine-induced locomotor stimulation (Clarke and Kumar 1983a; Clarke and Kumar 1983b; Stolerman et al. 1995), making it sub-optimal for detecting potential synergistic effects between the drugs. More specifically, if cotinine or nornicotine had rendered nicotine more potent (i.e., shifting the nicotine dose-response curve to the left), the resultant increase in nicotine-induced locomotion might have been too small to detect, even with a repeated measures design. If, instead, either pretreatment drug had made nicotine more efficacious (upward curve shift), there would be no such ceiling effect since nicotine, when administered alone, is only a weak locomotor stimulant.

In experiment 4, acute cotinine pretreatment did not detectably alter LMA when given alone, nor did it significantly alter nicotine-induced locomotion. Although cotinine has been reported to *increase* nicotine-induced locomotion in rats (Clemens et al. 2009), it should be noted that in the latter study the possible effects of cotinine alone were apparently not controlled for. To our knowledge, the effect of acute *nornicotine* pretreatment has been tested against only two *in vivo* effects of nicotine: cue properties and reinforcing effects. The nicotine cue, tested in mice, was not significantly attenuated by nornicotine (Caine et al. 2014), whereas nornicotine pretreatment did reduce intravenous nicotine self-administration in rats (Caine et al. 2014; Green et al. 2000; Stairs et al. 2007). In the latter case, however, it is unclear whether nornicotine inhibited, or substituted for, a reinforcing effect of nicotine, and interpretation is further complicated by the finding that nornicotine markedly reduced sucrose-reinforced responding in one study (Stairs et al. 2007).

To our knowledge, only one LMA study has previously investigated interactions between nornicotine and nicotine (Dwoskin et al. 1999a). In this previous report, nornicotine pretreatment comprised multiple injections spread over several days, ending 48 h before the nicotine challenge. In the present study, in contrast, each

nornicotine pretreatment comprised a single injection given 30 min in advance of nicotine. The latter pretreatment resulted in a dose-dependent inhibition of nicotine-induced locomotion, with a near-total block occurring at the highest nornicotine dose (3 mg/kg). Importantly, this interaction was not due to a ceiling effect, since after nicotine challenge the rats showed only moderate locomotor stimulation, for example, less than would be seen after administration of amphetamine (Louis and Clarke 1998; Simola et al. 2014).

In summary, our findings indicate that nornicotine can inhibit nicotine's ability to activate behaviorally relevant nAChRs, which possibly include  $\alpha 4\beta 2^*$  isoforms (Stolerman et al. 1997). Potential mechanisms include nAChR desensitization (see Introduction for references), or else partial agonism by nornicotine, as identified on a range of recombinant nAChR subtypes (Papke et al. 2007).

### Possible relation to tobacco smoking

In experiment 1, a nicotine dose range was chosen so as to bracket the range of plasma nicotine concentrations seen in typical habitual smokers. In subsequent experiments, a dose of 0.2 mg/kg was used, expected to produce peak within-session blood levels of around 50 ng/ml, compared with typical trough (i.e., between-cigarette) venous nicotine concentrations of 15–20 ng/ml in daily smokers (see Constantin and Clarke 2018).

It has been suggested that, in tobacco smokers, nornicotine and cotinine potentially achieve brain levels sufficient to activate some nAChR populations (Crooks and Dwoskin 1997; Ghosheh et al. 1999; Ghosheh et al. 2001; Miksys et al. 2000). Although the present study revealed only high-dose effects of nornicotine, these effects are conceivably of relevance to tobacco smoking, as follows. Although plasma levels of nornicotine are low in smokers ( $\sim 2$  ng/ml or  $\sim 0.01$   $\mu$ M) (Earla et al. 2014; Moyer et al. 2002), rodent studies indicate that *brain* nornicotine levels may be considerably higher. Thus, in adult rats, nornicotine persisted in the brain much longer than nicotine itself (Ghosheh et al. 1999) and accumulated further during chronic nicotine administration, with some nornicotine potentially formed locally in the brain (Ghosheh et al. 2001).

### Study limitations

The main study limitations are as follows. First, without consistent nicotine effects on USV emission, only the drug's locomotor stimulant effect could be used to evaluate interactions with nornicotine and cotinine; the use of a single behavioral measure clearly does not reflect the diversity of nicotinic receptors in the CNS. Second, animals received multiple home cage injections of nicotine before the start of behavioral

testing. This is a common procedure for inducing tolerance to the drug's depressant effects, but potentially reduces generalizability. Related to this, home cage nicotine pretreatment would likely have increased the density of brain nAChRs, but it is unclear to what extent this additional receptor pool would have been functional (Picciotto et al. 2008; Wu and Lukas 2011). Third, cotinine and nornicotine are both metabolized further in smokers (Hukkanen et al. 2005), and if their downstream metabolites are centrally active, they may not have had time to form during the 60-min test sessions. Fourth, as indicated above, our test dose of nicotine was not optimal for detecting possible synergistic effects. Lastly, the exclusive use of male rats leaves open the possibility of sex differences.

## Conclusion

The present findings indicate that nornicotine administration can result in nicotine-opposing effects in vivo, as reported in vitro. At present, any possible relevance to tobacco smoking is unclear. Given that nicotine is at best only weakly euphoriant, its lack of detectable effect on ultrasonic call profiles supports the use of trill vs. flat call prevalence as a potential marker of affect.

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**Compliance with ethical standards** All experiments comply with the current laws of Canada. All research procedures were reviewed and approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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