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Menthol enhances nicotine-induced locomotor sensitization and in vivo functional connectivity in adolescence

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Abstract

Mentholated cigarettes capture a quarter of the US market, and are disproportionately smoked by adolescents. Menthol allosterically modulates nicotinic acetylcholine receptor function, but its effects on the brain and nicotine addiction are unclear. To determine if menthol is psychoactive, we assessed locomotor sensitization and brain functional connectivity. Adolescent male Sprague Dawley rats were administered nicotine (0.4 mg/kg) daily with or without menthol (0.05 mg/kg or 5.38 mg/kg) for nine days. Following each injection, distance traveled in an open field was recorded. One day after the sensitization experiment, functional connectivity was assessed in awake animals before and after drug administration using magnetic resonance imaging. Menthol (5.38 mg/kg) augmented nicotine-induced locomotor sensitization. Functional connectivity was compared in animals that had received nicotine with or without the 5.38 mg/kg dosage of menthol. Twenty-four hours into withdrawal after the last drug administration, increased functional connectivity was observed for ventral tegmental area and retrosplenial cortex with nicotine+menthol compared to nicotine-only exposure. Upon drug re-administration, the nicotine-only, but not the menthol groups, exhibited altered functional connectivity of the dorsal striatum with the amygdala. Menthol, when administered with nicotine, showed evidence of psychoactive properties by affecting brain activity and behavior compared to nicotine administration alone.

Keywords

Smoking, tobacco, nicotine, menthol, adolescence

Introduction

Efforts in tobacco control and education have reduced the smoking rate substantially (Brown, 2010), yet between 2004 and 2010 the use of mentholated cigarettes increased by 2.5% in 18–25 year olds (Substance Abuse and Mental Health Services, 2011). Menthol cigarettes account for roughly 25% of the cigarette market in the USA, but are used disproportionately by younger people (Fallin et al., 2015; Giovino et al., 2015; Hickman et al., 2014). The sale of all flavored tobacco products except menthol was banned under the Family Smoking Prevention and Tobacco Control Act of 2009, but a ban against it is already in place, legislated, or proposed in several countries across the globe (European Union, 2014; Tobacco Control Legal Consortium, 2015). In a study by the US Food and Drug Administration (FDA), 39% of menthol smokers reported they would quit smoking if menthol cigarettes were banned (Hartman/US FDA, 2011) and the FDA has such a ban under consideration.

Many people smoke at least once in their lifetime, but only a subset continue to smoke and become addicted (de Wit et al., 1986). There is concern that menthol might enhance the addictiveness of nicotine. Compared to non-menthol smokers, menthol smokers smoke sooner after arising (Fagan et al., 2010; Muscat et al., 2012; Rosenbloom et al., 2012), a measure of physical

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dependence (DiFranza et al., 2013). On one hand, menthol smokers may have a harder time quitting compared to non-menthol smokers, and have a higher chance of relapse (Gundersen et al., 2009; Levy et al., 2011; Pletcher et al., 2006). On the other hand, menthol smokers consume the same number or fewer cigarettes per day than non-menthol smokers, and are less likely to be daily smokers ((Frost-Pineda et al., 2014; Lawrence et al., 2010), but the evidence is mixed (Fagan et al., 2010)). It is also known that genetic and environmental effects interact to contribute to an individual's propensity to nicotine addiction, including hyperactivity, stress, and anxiety (Comeau et al., 2001; Kassel et al., 2003; Milberger et al., 1997). It is thus further noted that individuals experiencing at least moderate psychological stress are more likely to smoke menthol cigarettes (Hickman et al., 2014).

Menthol can reduce the metabolic rate and/or the clearance of nicotine (Benowitz et al., 2004; Fagan et al., 2015; MacDougall et al., 2003). It is well-absorbed and rapidly reaches the brain (Clegg et al., 1982; Pan et al., 2012). In men, menthol promotes brain nicotine accumulation (Zuo et al., 2015). In mice, menthol dampens brain activity (Pezzoli et al., 2014). Thus, it is plausible that menthol may have psychoactive pharmacological effects.

Previous studies found that oral and intraperitoneal menthol administration facilitated intravenous nicotine self-administration in rats (Biswas et al., 2016; Wang et al., 2014). A different paradigm in mice found that chronic menthol pre-exposure reduced the rewarding properties of nicotine (Henderson et al., 2016). In animal models of substance use, sensitization to drugs of abuse such as nicotine, morphine, alcohol, cocaine, amphetamine, and methamphetamine is evidenced by increased locomotor activity in response to repeated exposure to the same dose of the drug (Babbini et al., 1975; Chaudhry et al., 1988; DiFranza and Wellman, 2007; Heidbreder et al., 1996; Itzhak and Martin, 1999), an outcome associated with drug rewarding properties (Horger et al., 1990; Lett, 1989; Piazza et al., 1989). We sought to determine if the co-administration of nicotine and menthol, in proportions similar to those found in tobacco products would affect the development of locomotor sensitization.

We could find no reports on the effects of simultaneous, co-administration of these drugs during adolescence, and no reports on their effects on in vivo brain network function. In order to address these gaps in the literature, we sensitized adolescent rats to nicotine alone and in combination with three doses of menthol. The effects of this exposure were assessed in relation to locomotor activity and functional connectivity as measured by magnetic resonance imaging. It was hypothesized that repeated nicotine and menthol co-administration would enhance locomotor sensitization, and that this behavioral effect would be accompanied by enhanced functional connectivity involving the nucleus accumbens and the ventral tegmental area, circuits previously identified as affected by nicotine in our laboratory (Huang et al., 2015; Li et al., 2008).

Material and methods

Animals

Male Sprague-Dawley rats from Harlan Laboratories were housed two per cage in a temperature- and humidity-controlled room on a 12-hour reverse light-dark cycle (lights off at 08:00), with food and water readily available. Animals arrived at the facility either in early adolescence at post-natal day (PND) 21±1, or in adulthood (PND≥90, 250–260 g).

After an initial acclimation period, the animals were handled with their cage mate and given subcutaneous (s.c.) saline (1 mg/kg, s.c.) once daily for two days prior to the start of locomotor testing. All open-field experiments were performed during the dark phase of the light-dark cycle. All procedures were in accordance with National Institutes of Health (NIH) guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

Timeline

Adolescent animals arrived at PND21. They were gently handled from PND23–PND27. Magnetic resonance imaging (MRI) acclimation proceeded from PND28 through PND36. Daily drug injections were administered from PND41 through PND49. MRI scanning occurred on PND50 (see Figure 1). Adult animals arrived at PND90 and followed an identical schedule.

Drugs

(-)-Nicotine hydrogen tartrate (Sigma, St Louis, Missouri, USA) was administered at a concentration of 0.4 mg/kg (base). This concentration reflects human cotinine plasma levels after smoking (Matta et al., 2007). Since the menthol content of cigarettes within the same brand family can range over three orders of magnitude, we selected nicotine-to-menthol ratios over the same range (Ai et al., 2015). (-)Menthol (Sigma) was dissolved with 0.05% ethanol in 0.9% saline for delivery (1 mL/kg) at a dosage of 0.05 mg/kg or 5.38 mg/kg (base). Nicotine was thus was prepared with this same vehicle. The menthol/nicotine ratio for the lowest menthol concentration is in the same order of magnitude as that used in vitro studies (Ashoor et al., 2013; Talavera et al., 2009), and aligns with the ratio of a popular brand of mentholated cigarettes (Benowitz et al., 2004). The highest concentration is equivalent to that used in a prior study with the Sprague-Dawley strain (Biswas et al., 2016). Solutions were pH adjusted to 7.0 with NaOH or HCl. The ethanol dose was two orders of magnitude below that previously found to produce behavioral sensitization, to affect self-administration behavior, or to alter neurotransmitter release (Hoshaw and Lewis, 2001; Weiss et al., 1996).

Assessment of locomotor activity

Animals were acclimated to an open field arena (black Plexiglas, 121 cm^2) for 15 min, followed by a 30-minute assessment of baseline locomotor activity. Activity was captured with a Canon ZR100 color digital video camera (Canon, New York, USA) mounted 1 m above the arena with a red light source and video tracking software (EthoVision 6.0, Noldus Information Technology, Wageningen, The Netherlands). Each rat was placed in the open field on consecutive days immediately after receiving an injection.

As rats that are more active in a novel environment are more prone to develop nicotine sensitization (Kayir et al., 2011), the animals were ranked by baseline locomotion and semi-randomly assigned for balanced nicotine-menthol and nicotine-vehicle treatment groups.

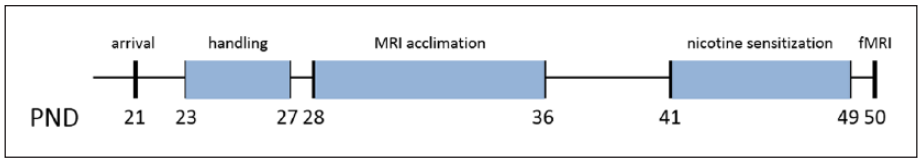

Figure 1. Timeline of study involving behavioral sensitization and neuroimaging in late adolescent rats. PND: post-natal day.

Behavioral study design

The awake neuroimaging protocol requires that animals be acclimated to restraint in the scanner environment prior to the experiment (which is described below). To determine if the restraint acclimation might have influenced locomotor sensitization, additional groups (adolescents and adults) underwent locomotor sensitization without first being subjected to restraint acclimation.

Locomotor sensitization in adolescents with prior restraint acclimation. Following awake neuroimaging restraint acclimation (PND28–36) over nine days, late adolescent rats (PND41–49) were administered either nicotine (0.4 mg/kg) with the menthol vehicle (n=18), nicotine with menthol (0.05 mg/kg, n=10; 5.38 mg/kg, n=10), or menthol with the nicotine vehicle (n=8). Locomotor activity was monitored after each dose. On PND50 the animals were imaged. Hereafter, it should be understood that the menthol-only and nicotine-only groups also received an injection of the vehicle for the other drug.

Locomotor sensitization in adolescents without prior restraint acclimation. From PND28 through PND36, brief handling was substituted for the daily restraint acclimation procedure. This experiment compared two groups: nicotine-only, and nicotine with menthol 5.38 mg/kg (n=10/group).

Locomotor sensitization in adults without prior restraint acclimation. Following the same handling procedure as the prior experiment, adult rats received seven daily injections with either nicotine-only (n=12), menthol-only (n=8), nicotine with menthol 0.05 mg/kg (n=11), or nicotine with menthol 5.38 mg/kg (n=12).

Statistical analysis of the locomotor data

Linear mixed models with each rat as the unit of analysis were used to examine the distance traveled over the repeated trials. This approach has superior statistical power and increased ability to account for uneven groups, missing values, or correlations between a subject's repeated data (Gueorguieva and Krystal, 2004; Willett, 1989; Willett et al., 1998). This approach has been use in prior substance exposure studies (e.g. Adkins et al., 2013; Grebenstein et al., 2013; Hahn and Stolerman, 2002; Welge and Richtand, 2002). Drug treatment (menthol concentration), Time (day), and relevant interactions were treated as fixed effects. To examine the potential non-linear effect of Time, a quadratic pattern was also tested using Time^2 (i.e. squared values) as a fixed effect. Finally, in order to account for potential variability in individual slopes, Time and Time^2 were also included as random effects. A formal top-down approach was adopted, starting with a fully-loaded model, reduced by removing non-significant parameters on the basis of likelihood ratio tests (West et al., 2014). Restricted maximum likelihood estimation was used, except for fixed effects model comparisons where maximum likelihood was used instead. Analyses were conducted using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, New York, USA), with alpha set at 0.05. The impact of acclimation was next directly statistically explored. Although treated similarly, these studies from different cohorts of animals exhibited substantially different baseline levels of locomotor activity, and thus normalized locomotion on the final day was used for comparison (Day 9/Day 1).

Functional magnetic resonance imaging (fMRI) procedures

On the day following the open-field experiment (PND50±1), subsets of previously acclimated adolescent animals from two groups (nicotine-only, n=6, and nicotine with 5.38 mg/kg menthol, n=6) underwent fMRI imaging as previously described (Huang et al., 2015; Li et al., 2008; Liang et al., 2012a,b).

fMRI acclimation procedure. Anesthesia can impact fMRI, diminishing neuronal metabolism and cerebral blood flow, affecting signal intensity (Lahti et al., 1999; Sicard et al., 2003). In spite of general network similarities between anesthetized and awake imaging (Liang et al., 2012b), anesthesia produces qualitatively different functional connectivity patterns (Bettinardi et al., 2015; Liang et al., 2012a) and substantially dampens the effects of nicotinic agonists (Chin et al., 2008). For these reasons, fMRI was conducted with awake animals using a validated procedure to acclimate rats to restraint and noise (King et al., 2005).

Starting on PND28±1, rats began an eight-day restraint acclimation procedure. Once daily, rats were anesthetized briefly with isoflurane. EMLA cream (lidocaine 2.5% and prilocaine 2.5% Cream, Hi-Tech Pharmacal Co., Inc.) was applied to the ears to minimize discomfort. Animals were then secured in a Plexiglass stereotaxic head holder using plastic ear bars. They were then placed into a black opaque tube "mock scanner" and exposed to recorded scanner noises. The duration of restraint acclimation began with 15 min and increased by 15 min/day, holding steady at 90 min for days 6, 7, and 8.

Animal preparation. The animal was briefly anesthetized using isoflurane, EMLA cream was applied to the ears, and the animal's head was fitted into a restrainer with a built-in coil, with the incisors secured over a bite bar. The nose was secured with a nose clamp, and ear bars were positioned inside the head holder with adjustable screws. Before the body was placed into a body restrainer, a winged infusion needle was inserted subcutaneously to allow for injection while in the magnet. After setting up,

isoflurane administration was discontinued and the restraint apparatus was placed in the magnet for imaging in an awake state. Following signal optimization, imaging sessions began approximately 15 min after positioning in the magnet.

fMRI data acquisition. MRI experiments were performed on a 4.7T/40 cm horizontal magnet equipped with a Biospec Bruker console (inner diameter 12 cm). A surface coil (inter-diameter 2.3 cm) was used for brain imaging. For each rat, anatomical images were obtained using rapid acquisition relaxation enhanced (RARE) sequence with TR (relaxation time)=3000 ms, RARE factor=8, TE (echo time)=12 ms, resolution matrix=256×256, FOV (field of view)=32 mm×32 mm, slice number=18, slice thickness=1 mm. Functional images were acquired using echo-planar imaging (EPI) with the same FOV and slice thickness, TR=1 s, TE=30 ms, flip angle=60°, and resolution matrix=64×64. Three EPI scans were performed, such that the first (EPI1), was acquired at "resting state" for 20 min (1200 repetitions), where no drug was administered. The second EPI (EPI2) took 30 min (1800 repetitions), in which a 1 min of baseline period was followed by subcutaneous 5 s drug administration, followed by 29 min of continuous data acquisition. The third EPI (EPI3) was acquired for 20 min (1200 repetitions) to examine the residual effects of the drug.

Functional connectivity analysis of mesocorticolimbic areas. Seed-based functional connectivity analysis was carried out according to previously detailed procedures (Colon-Perez et al., 2016) on 20 regions of interest (ROIs), which included the anterior cingulate cortex, infralimbic cortex, orbital cortex, bed nucleus of the stria terminalis, dorsal striatum, the hippocampus, ventral tegmental area, substantia nigra, insular cortex, retrosplenial cortex, along with other brain regions, such as perirhinal cortex, parietal cortex, and primary somatosensory subregions. Briefly, time series fMRI signals were extracted from each region of interest (ROI) based on the atlas-guided seed location. Signals were averaged from voxels in each ROI (Colon-Perez et al., 2016). Voxel-wise cross correlations were then carried out to create correlation coefficient (Pearson *r*) maps. The first nine images in each functional time series were not used in the cross-correlation step. Pearson *r* maps were then subjected to a voxelwise *z*-transformation. Two correlation maps were averaged per subject to generate a single correlation map subsequently used for statistical mapping. Statistical composite maps were thresholded at $p<0.05$ (uncorrected).

Network analysis. In a separate analysis similar to that previously reported (Colon-Perez et al., 2016), we assessed functional connectivity across 150 total areas, equally divided in left and right representations of each region. This additional dataset was used to determine the effects of nicotine and menthol on various connectomic metrics. Putative brain functional networks were analyzed using the Brain Connectivity Toolbox for Matlab (Rubinov and Sporns, 2010). Matrices with a total 11,175 entries were organized in Matlab and *z* score values were thresholded for each subject to create matrices with equal densities (e.g. *z* values in the top 15% of all possible correlation coefficients). Matrix *z* values were normalized, such that all matrices had edge weight values ranging from 0–1. Node strength, clustering coefficient, average shortest path length, and small worldness were calculated for weighted graphs (Boccaletti et al., 2006; Newman, 2003; Saramaki et al., 2007). Connectivity maps were visualized using BrainNet (Xia et al., 2013). The 3D networks were generated with undirected edges weights Eundir ≥0.3. The node size and color in these maps scaled by node strength and edges by *z*-scores. Unless otherwise specified, data were analyzed by a mixed analysis of variance (ANOVA), with Drug treatment as between-subjects and Time as repeated factors (significance at $p<0.05$). Follow-up tests were adjusted for multiple testing using Tukey honest significant difference tests.

Determination of menthol presence in brain

Menthol was derivatized using dimethylglycine (DMG) similarly to a previously described method (Pan et al., 2012). For each animal analyzed (adolescent, $n=4$/group, 5.38 mg/kg menthol vs vehicle), one hemisphere of the brain was homogenized in 3 mL phosphate-buffered saline (in silanized borosilicate round bottom glass test tubes using a rotary tissue homogenizer on high for 30 s (Omni International). Three volumes of hexanes were added to each sample and vortexed on high for 30 s before inducing phase separation by centrifugation at 2000×g. The organic layer was removed to a silanized borosilicate glass conical bottom reaction vial, and the extraction repeated. The cumulative organic layers were dried under nitrogen stream at room temperature (RT), the residue resuspended in 1 mL chloroform (amylene stabilized), and transferred to silanized borosilicate glass autosampler vials. The samples were then dried under nitrogen (RT) and resuspended in 160 µL chloroform (amylene stabilized) containing 93.75 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 46.875 mM DMG HCl, and 187.5 mM 4-dimethylaminopyridine (DMAP). The reaction was incubated at 37°C overnight, dried under nitrogen (RT), and resuspended in 400 µL of buffer (1/2.25/0.75 water/methanol/acetonitrile+0.1% (v/v) acetic acid) for liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis. DMAP, DMG, and EDC were all purchased from Sigma Aldrich.

LC-MS/MS analyses were performed on a Thermo Fisher Scientific UltiMate 3000 binary UPLC coupled to a Thermo TSQ Quantiva triple quadrupole mass spectrometer equipped with a heated electrospray ionization probe operating in positive ion mode. Using an injection volume of 10 µL, samples were separated on a 50×2.1 mm Kinetex (Phenomenex) C18 column (1.7 µm, 100Å), using the following gradient program: 10–100% B over 10 min at 200 µL/min, 100% B for 1 min at 200 µL/min, 100% B for 1 min at 500 µL/min, 10% B for 3 min at 500 µL/min (A: 0.1% formic acid (v/v) in water, and B: 0.1% formic acid (v/v) in acetonitrile). Menthol was detected by monitoring the transition from m/z 242 to m/z 104 at 20 V CE. The method was validated using negative control and menthol-spiked control mouse brain tissue homogenates over a menthol concentration range of 10 pg–1 µg per mg tissue. All data was processed in Thermo Xcalibur (version 3.0.63).

Results

Locomotor sensitization in adolescents with prior restraint acclimation

Ambulation distance increased with Time (day) with a quadratic time course (($F_{1,70.460}=17.4$, $p<0.001$); note linear effect ($F_{1,82.1}=41.9$, $p<0.001$)). As seen in Figure 2, drug condition significantly affected the distance traveled ($F_{3,272.1}=5.6$, $p=0.001$). In comparison to the menthol-only group, all nicotine groups exhibited enhanced

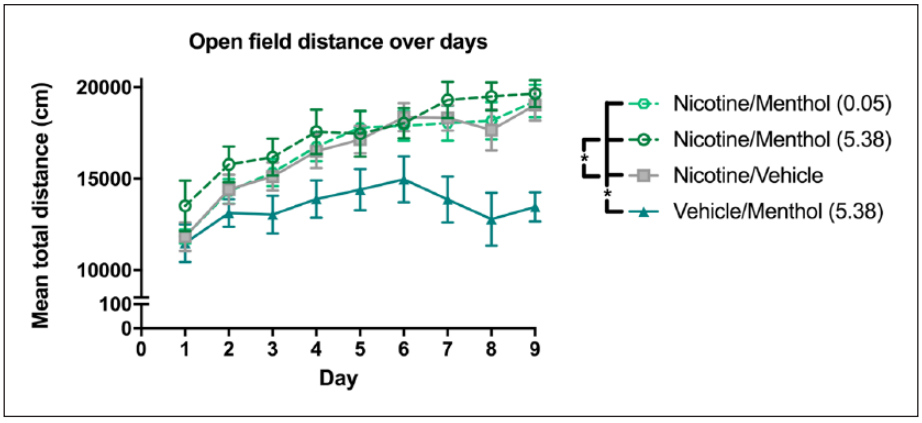

Figure 2. Locomotor activity in conjunction with repeated daily drug administration (Days 1–9). All groups receiving nicotine were more active than the menthol-only group (all $p<0.05$). The distance traveled was greater in the group receiving nicotine with menthol 5.38 mg/kg in comparison to the nicotine-only group ($p<0.05$). Error bars represent the standard error of the mean. $n=18$, 10, and 10 for nicotine only, nicotine with menthol 0.05 mg/kg, and nicotine with menthol 5.38 mg/kg groups respectively. *$p<0.05$.

locomotion (Nicotine+Menthol 5.38 mg/kg, $t_{273.7}=4.1$, $p<0.001$; Nicotine+Menthol 0.05 mg/kg, $t_{273.4}=2.1$, $p=0.04$; Nicotine only, $t_{271.6}=2.2$, $p=0.03$). In comparison to the Nicotine-only group, enhanced locomotion was observed only with the group receiving the highest dose of menthol (5.38 mg/kg, $t_{271.3}=2.5$, $p=0.013$). Activity in the group that received nicotine in combination with low (0.05 mg/kg menthol) did not differ significantly from that of the group that received nicotine alone ($p>0.9$).

Locomotor sensitization in adolescents without prior restraint acclimation

As restraint acclimation is stressful, the experiment was repeated omitting the acclimation procedure. As only the highest dose of menthol affected locomotion in the preceding experiment, only this dose was tested in this follow-on experiment. As seen in Figure 3, the main effect of Time (day) was apparent ($F_{1,18.0}=16.7$, $p=0.001$; note quadratic effect of Time, $F_{1,16.5}=4.5$, $p=0.051$). However, there was no main effect of Drug ($F<1$), nor interaction between Time and Drug ($F<1$) indicating that the addition of menthol 5.38 mg/kg did not alter the effect of nicotine when the animals had not been exposed to restraint acclimation. Statistical comparison of the Day 9/Day 1 ratio between Nicotine+Menthol (5.38 mg/kg) adolescent groups revealed a significantly reduced with acclimation (Mann–Whitney $U=4$, $p=0.003$, two-tailed; Supplementary Material, Figure S1). This observation further supports the contention that such an environmental factor can affect the modulation by menthol of the behavioral impact of nicotine.

Locomotor sensitization in adults without prior restraint acclimation

As seen in Figure 4, using the same linear mixed model analysis as used for the adolescent experiments, a quadratic effect of Time on distance traveled was noted ($F_{1,63.4=}20.2$, $p< 0.001$; note linear effect, $F_{1,65.1=}65.6$, $p<0.001$). There was an effect of Drug condition ($F_{3,228.8}=14.1$, $p< 0.001$), and an interaction of Drug and Time ($F_{3,40.2}=6.5$, $p=0.001$). Follow-up analyses of this interaction revealed that the only significant group effect was that the menthol-only group differed from all three nicotine groups: nicotine-only ($t_{39.9=}4.3$, $p<0.001$); nicotine with menthol 0.05 mg/kg, $t_{39.2=}3.3$, $p=0.002$; and nicotine with menthol 5.38 mg/kg, $t_{39.0}=3.2$, $p=0.003$. None of the nicotine with menthol groups differed from the nicotine only group (all $p\geq0.2$) indicating that menthol demonstrated no behavioral effect in adult rats that had not been exposed to restraint acclimation.

Menthol reaches the brain

In order to determine the potential for central site of action of menthol in the present study, the presence of menthol in the brain was examined. Menthol was successfully detected in the brain of rats of the same age as the adolescent groups (Figure 5).

Functional connectivity

As only the combination of nicotine with menthol at 5.38 mg/kg demonstrated a significant difference from nicotine alone in the behavioral experiments, brain functional connectivity was examined for this dosage (Figure 6, according to each significant seed region of interest). Three time points were examined: (a) 24 h after the last drug exposure during the behavioral experiment (EPI1), (b) 10 min after drug injection (re-exposure to either nicotine alone, nicotine with menthol, or menthol without nicotine, EPI2), and (c) 30 min after that injection (EPI3). Subjects retained for analysis numbered $n=6$ for Nicotine+Menthol group, $n=5$ for Nicotine-only group, and $n=5$ for Menthol-only group. The addition of menthol to nicotine induced functional connectivity alterations, detailed in Table 1 and illustrated in Figure 7. Significant effects of Drug condition and interactions with Time point are. Repeated drug exposure led to enhanced coupling of the ventral tegmental area with the (caudal) retrosplenial cortex (Figure 7(a)) in the Nicotine+Menthol than the Nicotine-only group. Following re-exposure, both nicotine groups exhibited elevated coupling of these regions in comparison to the Vehicle/Menthol group. In contrast, at that time this retrosplenial cortex segment was functionally coupled with the substantia nigra only in the Nicotine-only group, which was significantly different to the Menthol-only group, with co-exposure in the Nicotine+Menthol group producing an intermediate

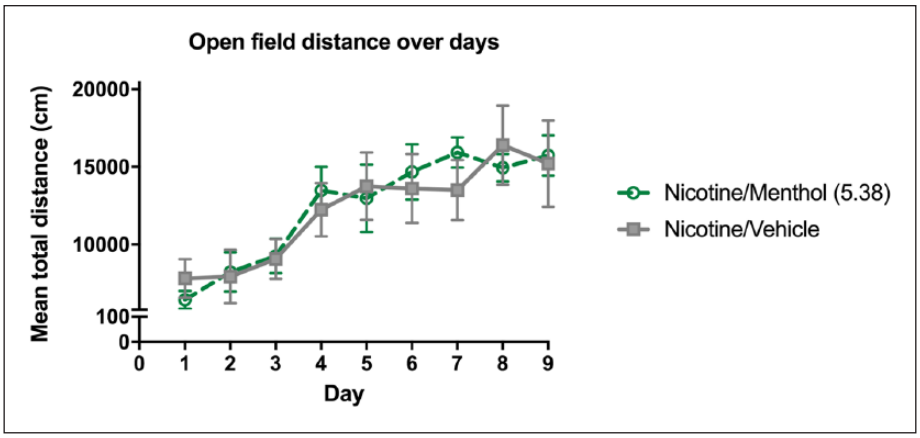

Figure 3. Menthol co-administration with nicotine did not alter nicotine sensitization in adolescent rats not exposed to restraint acclimation. Mean±standard error of the mean (SEM), *n*=6/group.

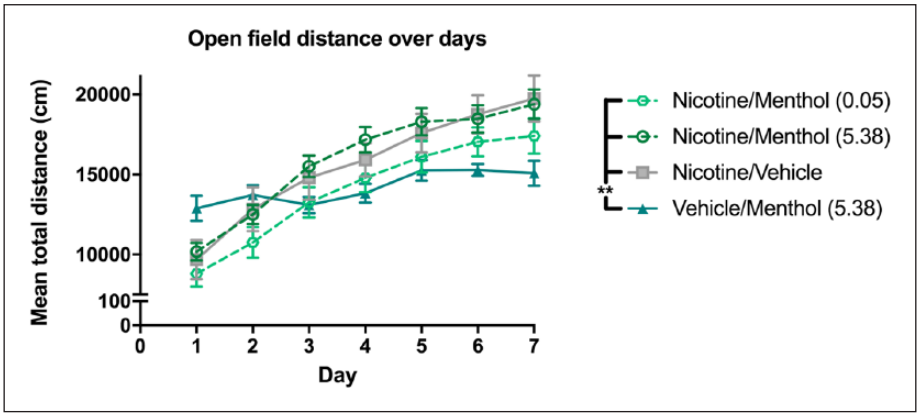

Figure 4. Menthol co-administration with nicotine does not enhance sensitization in adult rats that had not been exposed to restraint acclimation. Means (±standard error of the mean (SEM)), *n*=12, 11, 12, 8 for nicotine-only, nicotine with menthol 0.05 mg/kg, nicotine with menthol 5.38 mg/kg, and menthol 5.38 mg/kg with vehicle, respectively. Locomotor sensitization was seen in all nicotine groups ($p<0.001$), but not in the group receiving only menthol. **Effect of time on menthol vs nicotine groups, $p<0.005$.

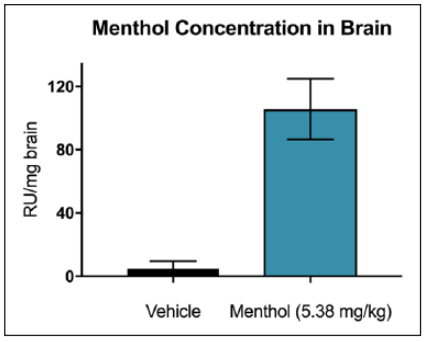

Figure 5. Menthol can be found in the brain following peripheral administration. Menthol (5.38 mg/kg) was administered and brain levels were compared to vehicle administration (*n*=4 each) using liquid chromatography tandem-mass spectrometry (LC-MS/MS) approach. RU, relative units.

level (Figure 7(b)). In the rostral portion of the retrosplenial cortex, prior to the injection a significant difference was seen in the Vehicle/Menthol compared to the Nicotine+Menthol group (Figure 7(c)). Interactions were also found for dorsal striatum with both amygdala and infralimbic cortex (Figure 7(d) and (e)), but no adjusted *p*-values were significant for follow-up simple effects analyses. Meanwhile, following up the significant interactions, effects of Time were revealed for caudal retrosplenial cortex (RSCc)-ventral tegmental area (VTA) for both groups exposed to menthol, and for RSCr-bed nucleus of the stria terminalis (BNST) only with Menthol-only rats. As for the Nicotine-only group, it was the only one exhibiting an effect of Time for dorsal striatum coupling with amygdala. Finally, coupling of the dorsal hippocampus and of the infralimbic cortex was different between both menthol groups throughout (main effect of Drug condition and significant post-hoc test, see Table 1).

Considering the whole-brain network analyses, no significant differences were detected in the clustering coefficient, path length, or small worldness (Supplementary Material, Figure S2).

Discussion

Prior research has established that menthol enters the brain and interacts with nicotinic receptors. In the current study, menthol (dosed at

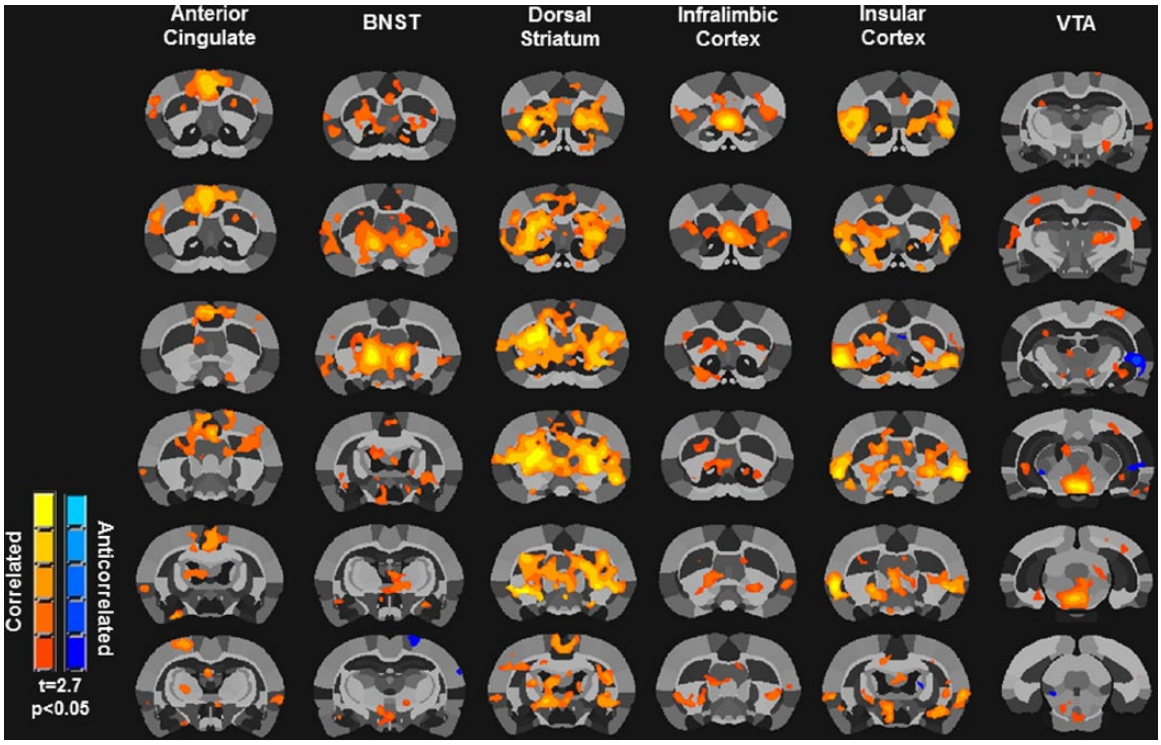

Figure 6. Functional connectivity according to seed regions of interest. Statistical composite maps based on correlation matrix, thresholded at $p<0.05$ (uncorrected), and overlaid onto an atlas segmentation of the brain. BNST: bed nucleus of the stria terminalis; VTA: ventral tegmental area.

Table 1. Effect of nicotine and menthol on resting state functional connectivity.

ROI pair	ANOVA results	Treatment effect
RSCc-VTA	Treatment: n.s. Time: n.s. Interaction: $F(4,26)=5.2$, $p=0.003$	Nicotine/Vehicle<Nicotine/Menthol (pre injection) Vehicle/Menthol<Nicotine/Menthol (injection) $Nicotine/Menthol: Post<Pre and Inj. &Vehicle/Menthol: Pre>Inj
RSCc-SN	Treatment: n.s. Time: n.s. Interaction: $F(4,26)=3.3$, $p=0.02$	Nicotine/Vehicle>Vehicle/Menthol (injection)
RSCr-BNST	Treatment: n.s. Time: $F(2,26)=11.1$, $p=0.0003$ Interaction: $F(4,26)=4.0$, $p=0.03$	Nicotine/Menthol<Vehicle/Menthol (pre-injection) &Vehicle/Menthol, Pre->Injection and Post-injection n.s. Nicotine/Vehicle, Injection vs Post-Injection, $p=0.07$
dSTR-AMY	Treatment: n.s. Time: n.s. Interaction: $F(4,26)=3.2$, $p=0.029$	n.s. Pre-injection, Nicotine/Menthol vs Nicotine/Vehicle, $p=0.07$ @Nicotine/Vehicle: Pre<Inject
dSTR-IL	Treatment: n.s. Time: n.s. Interaction: $F(4,26)=3.0$, $p=0.036$	n.s. Nicotine/Menthol vs Vehicle/Menthol, Pre-injection, $p=0.09$
dHPC-IL	Treatment: $F(2,13)=3.9$, $p=0.048$ Time: $F(2,26)=3.7$, $p=0.037$ Interaction: n.s.	*Nicotine/Menthol>Vehicle/Menthol

ANOVA results are for Treatment, Imaging time point effects, or Interactions. Follow-up analyses were conducted for significant Drug treatment main effect or interaction, adjusted for multiple comparisons with application of Tukey honest significant difference tests. *Comparisons between drug conditions at each time point, whereas comparisons across time points for each drug condition are indicated with alternate symbols: $, Nicotine+Menthol; @, Nicotine-only; and &, Menthol-only.
AMY: amygdala; ANOVA: analysis of variance; BNST: bed nucleus of stria terminalis; dHPC: dorsal hippocampus; dSTR: dorsal striatum; ROI: region of interest; RSCr/RSCc: rostral/caudal retrosplenial cortex; SN: substantia nigra; VTA: ventral tegmental area.

5.38 mg/kg) was detected in the brain, augmented locomotor sensitization to nicotine in adolescent rats, and impacted functional connectivity of brain regions implicated in addiction processes, the ventral tegmental area and the striatum, among other regions. These findings suggest that menthol has psychoactive properties when it is administered with nicotine during adolescence.

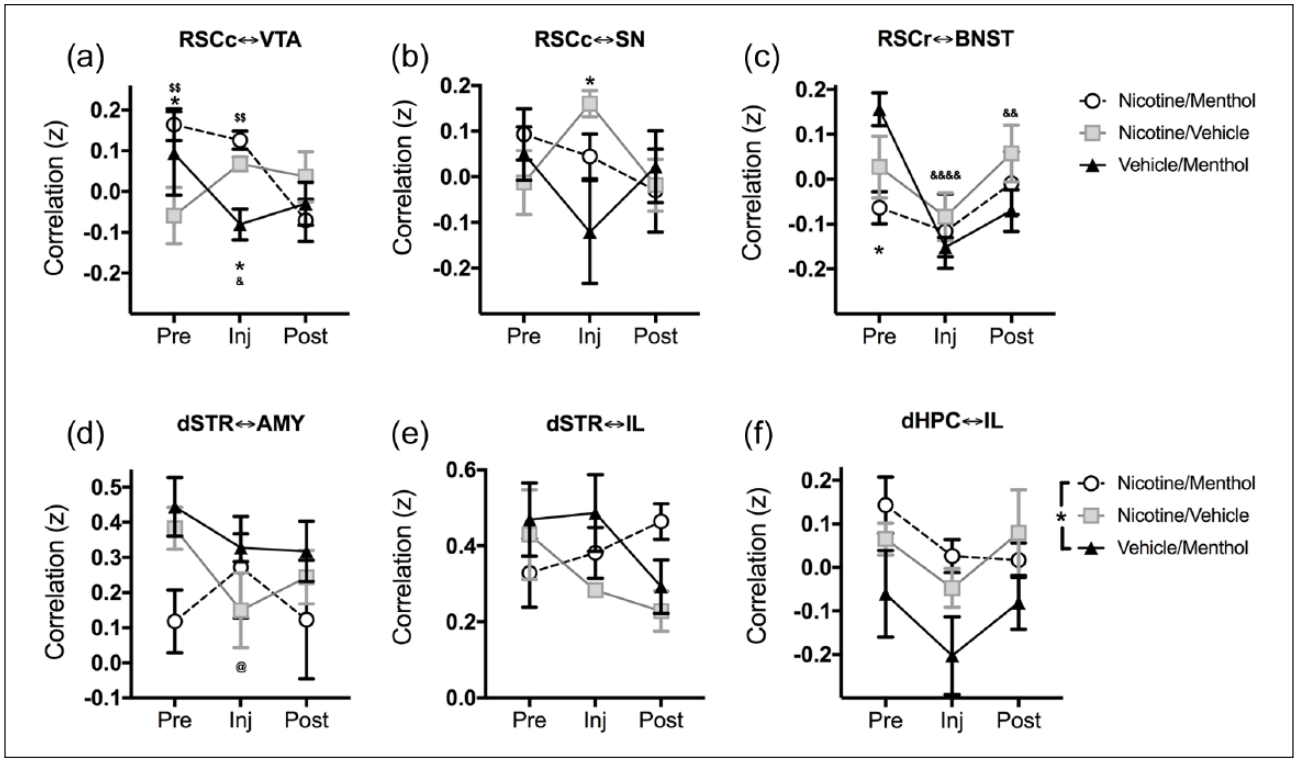

Figure 7. Functional connectivity according to seed regions of interest. between groups of animals that received either nicotine alone, nicotine with menthol 5.38 mg/kg, or menthol (5.38 mg/kg without nicotine (respectively, n=5, n=6, and 5), 24 h after last exposure (or pre re-exposure (Pre)), or 10 (Injection) and 30 min after re-exposure (Post). Mean correlation coefficients z-scores (±standard error of the mean (SEM)) are given for each brain region pair with a significant main effect of Drug Treatment or interaction of Drug and Time. *p<0.05; **p<0.01; ****p<0.0001; *comparisons between drug conditions at each time point, whereas comparisons across time points for each drug condition are indicated with alternate symbols: $, Nicotine+Menthol; @, Nicotine-only; and &, Menthol-only. AMY: amygdala; BNST: bed nucleus of the stria terminalis; dHPC: dorsal hippocampus; dSTR: dorsal striatum; IL: infralimbic cortex; inj.: injection; INS: insula: RSCr: rostral retrosplenial cortex: RSCc: caudal retrosplenial cortex; SN: substantia nigra; VTA: ventral tegmental area.

Locomotor sensitization, associated with drug reward (Horger et al., 1990; Lett, 1989; Piazza et al., 1989), is accompanied by the up-regulation of brain nicotinic acetylcholine receptors (nAChRs) (Ksir et al., 1985; Marks et al., 1985). In adult rats repeated administration of menthol alone at a dose of 5.38 mg/kg had no acute effect on locomotion and did not produce locomotor sensitization. The same result had been reported with a single dose and four daily doses of 400 mg/kg in rats (Ruskin et al., 2007). Studies of menthol-only exposures may fail to detect effects that occur when menthol is combined with nicotine as it is in menthol cigarettes. It has been proposed that, with nicotine exposure, transient nAChR upregulation and long-term potentiation consequently produce long-lasting sensitization of midbrain dopaminergic neuron reactivity (Vezina et al., 2007). Such a mechanism could plausibly be also implicated in a menthol enhancement of the nicotine-induced functional connectivity.

Our study showed selective brain region functional coupling alterations in the drug, unaccompanied in differences in the clustering coefficient, path length, or small worldness. Functional connectivity of one particular pair of brain regions exhibited differential activation from nicotine with the addition of menthol. Coupling of the retrosplenial cortex with the ventral tegmental area prior to drug re-exposure, i.e. 24 h after the last injection, was greater in the Nicotine/Menthol than the Nicotine/Vehicle group, the only such difference between the Nicotine and the Nicotine and Menthol groups. Furthermore, dynamic coupling that was seen between the amygdala and the dorsal striatum was not observed for either menthol group, a finding with potential implications for understanding nicotine withdrawal and craving mechanisms (Sweitzer et al., 2016) and their modulation by the mentholation of tobacco products, with implications for cessation efforts.

The brain structures differentially affected in the present study, including the ventral tegmental area, are mostly widely implicated in addiction processes (Changeux, 2010; Koob and Volkow, 2009). The retrosplenial cortex in the context of nicotine is implicated in cue associations or emotional responses (Brody et al., 2007; Gehricke et al., 2009; Nestor et al., 2011). Its activation and functionally connectivity is also observed in animal models of nicotine exposure, based on our findings and those of others (Hsu et al., 2007; Huang et al., 2015; Poirier et al., 2017). Nicotine increases the firing rate of dopamine neurons in the striatum, as well as nucleus accumbens (Bahk et al., 2002; De Biasi and Dani, 2011; Rasmussen and Czachura, 1995). Parts of the striatum receive excitatory innervation from the prefrontal cortex and hippocampus (Haber et al., 2000). Additionally, a trend in the present for alterations in the functional coupling of the dorsal striatum with the prefrontal cortex may be worth further investigating for the recruitment of that circuitry has been reported to have predictive value for treatment outcomes (Wilcox et al., 2017).

Although menthol actions outside of the brain are dependent on the "cool receptor", the transient receptor potential cation channel subfamily M member 8 (TRPM8) (Liu et al., 2013; Mandadi et al., 2009; Proudfoot et al., 2006), there is diverging evidence concerning the effects of menthol in the brain. Studies in juvenile rodents suggest independent effects of this substance in the brain (Lau et al., 2014; Pezzoli et al., 2014). In vitro menthol dampens neuronal activity and reduces neuronal recruitment in the HP and SN (Pezzoli et al., 2014). Menthol was reported to target gamma-aminobutyric acid receptors in the hippocampus (Zhang et al., 2008) and in the midbrain (Henderson et al., 2016). On non-neural nAChRs, there is accumulating evidence that menthol acts as a negative allosteric modulator at the α3β4 (Ton et al., 2015), α4β2 (Hans et al., 2012), and α7 nAChRs (Ashoor et al., 2013), prompting proposals of equivalent effects in the brain (Kabbani, 2013; Wickham, 2015), yet little remains known in the latter (Alsharari et al., 2015; Henderson et al., 2016). Brain regions expressing α3β4-containing nAChRs have been shown to mediate nicotine withdrawal (Changeux, 2010; Jackson et al., 2013). Notably, menthol co-application with nicotine increases desensitization of α3β4 nAChRs and prolongs the amount of time that the receptor remains desensitized (Ton et al., 2015).

In the adolescent rodent brain, examination of α4 and β2 nAChR subunit proteins in mice (serially) exposed daily to nicotine (12 mg/kg) and menthol (100 mg/kg) revealed that the only differential impact of co-exposure to these drugs was a summative effect for β2 in the prefrontal cortex, producing increases beyond that of each single drug (Alsharari et al., 2015). In humans, mentholated cigarette smokers exhibited higher $\alpha 4\beta 2$ nAChR densities than non-menthol smokers across several regions, including the prefrontal cortex, corpus callosum, cerebellum, and brain stem (Brody et al., 2013). The literature supports a conclusion that menthol impacts the brain. Our study extends that research by demonstrating that some of that impact manifests as changes in patterns of functional connectivity. One implication of this is that functional connectivity patterns should be examined in smokers to determine if menthol alters brain function in humans.

Our results should be interpreted with the following caveats in mind. Due to species differences, including in nicotine pharmacokinetics (Craig et al., 2014), it is difficult to know what dose of menthol in the rat might have the same effect as the dose of menthol delivered to a smoker. Thus, although in the current study, only the highest dose of menthol affected locomotor sensitization, suggesting that menthol effects in humans might also be dose-dependent, we cannot conclude based on these results that menthol affects humans in the same way.

Although our results indicate that menthol affects function in structures previously identified as being involved in "reward" circuits, further studies will help determine whether and under which parameters menthol might increase or decrease reward, unpleasant side effects, the severity of nicotine withdrawal, or the addictiveness of nicotine.

Another caveat is that menthol enhanced locomotor sensitization only in adolescent rats that had been subjected to restraint acclimation. Menthol had no observed behavioral effect in this paradigm on adolescent or adult rats that had not been subjected to the stress of restraint acclimation. The acclimation procedure dampens animal stress responses during subsequent neuroimaging as evidenced by reduced corticosterone and movement (King et al., 2005), yet it must be assumed that although hypothalamic–pituitary–adrenal axis activation is decreased, it remains unpleasant to some degree. It is well-known that early life stress is a risk factor for many forms of drug abuse (reviewed in Andersen and Teicher, 2009), but the mechanism of the association between stress and nicotine addiction are incompletely understood. In one nicotine exposure paradigm, locomotor sensitization was induced in adults but not in adolescents unless they were repeatedly stressed by physical restraint (Zago et al., 2012). In the current study, locomotor sensitization was observed in all groups of adolescent rats, but relative to nicotine an augmentation of locomotor sensitization by menthol was observed only in those adolescent rats that were stressed. Our restraint acclimation protocol has been validated as a method to reduce the stress of imaging, not as a source of stress. Therefore, we would caution that experiments should be conducted to specifically investigate the effects of stressors prior to drawing conclusions regarding an interaction between menthol and stress, and encourage further examination of this phenomenon, including a disentangling of acute and chronic effects. Furthermore, even though here no behavioral alterations were observed in unacclimated adolescents and adults, it will also be important to examine other relevant behaviors, and evaluate whether brain activation changes are observed under these conditions. Nevertheless, it is interesting to note that in one study, individuals who reported increased lifetime psychological distress were more likely to smoke menthol cigarettes (Hickman et al., 2014).

Strengths of this study include the combination of behavioral and imaging experiments using awake animals, and the use of a range of menthol doses in the behavioral studies. In conclusion, we found evidence that menthol enhances behavioral activity as measured by locomotor sensitization in adolescents, and evidence that menthol impacts brain activity in addiction circuits as measured by functional connectivity. Added to what is already known about menthol's actions on nicotinic receptors, our data indicate that menthol may be considered a psychoactive agent until proven otherwise.

Declaration of conflicting interests

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