

Menthol Enhances Nicotine Reward-Related Behavior by Potentiating Nicotine-Induced Changes in nAChR Function, nAChR Upregulation, and DA Neuron Excitability

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Understanding why the quit rate among smokers of menthol cigarettes is lower than non-menthol smokers requires identifying the neurons that are altered by nicotine, menthol, and acetylcholine. Dopaminergic (DA) neurons in the ventral tegmental area (VTA) mediate the positive reinforcing effects of nicotine. Using mouse models, we show that menthol enhances nicotine-induced changes in nicotinic acetylcholine receptors (nAChRs) expressed on midbrain DA neurons. Menthol plus nicotine upregulates nAChR number and function on midbrain DA neurons more than nicotine alone. Menthol also enhances nicotine-induced changes in DA neuron excitability. In a conditioned place preference (CPP) assay, we observed that menthol plus nicotine produces greater reward-related behavior than nicotine alone. Our results connect changes in midbrain DA neurons to menthol-induced enhancements of nicotine reward-related behavior and may help explain how smokers of menthol cigarettes exhibit reduced cessation rates.

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INTRODUCTION

Menthol cigarettes are used by 1 out of 3 smokers and >85% of African-American smokers (McCarthy *et al*, 1995). Smokers of menthol cigarettes are less likely to quit compared to smokers of non-menthol cigarettes (Ahijevych and Garrett, 2010). Youth smokers of menthol cigarettes are twice as likely to become lifelong smokers compared to youth smokers of non-menthol cigarettes (D'Silva *et al*, 2012). With e-cigarettes, consumption rates of flavored products (including menthol) are rising, especially among young smokers (Singh *et al*, 2016). Thus, it is important that we understand how menthol and other tobacco flavorants alter nicotine reward.

VTA neurons containing $\alpha 4$, $\alpha 6$, and $\beta 2$ nAChR subunits ($\alpha 4\beta 2$, $\alpha 4\alpha 6\beta 2$, $\alpha 6\beta 2\beta 3$) mediate aspects of nicotine addiction (Tapper *et al*, 2004; Pons *et al*, 2008). Upregulation of these nAChRs is considered a biomarker for addiction. Smokers of menthol cigarettes exhibit greater upregulation of brain nAChRs compared to smokers of non-menthol cigarettes (Brody *et al*, 2013). Menthol enhances nicotine withdrawal (Alsharari *et al*, 2015) and nicotine intravenous

self-administration (IVSA) in rats (Wang *et al*, 2014; Biswas *et al*, 2016). We have previously reported that menthol alone upregulates $\alpha 4$ -containing ($\alpha 4^*$) and $\alpha 6^*$ nAChRs in the VTA and substantia nigra pars compacta (SNc) (Henderson *et al*, 2016). Also, menthol alone alters nAChR function on midbrain DA neurons (Henderson *et al*, 2016). This suggests that menthol directly alters midbrain neurons, in addition to any sensory and metabolic actions that may increase nicotine exposure in the brain.

Here we demonstrate that menthol plus nicotine enhances nicotine reward-related behavior and nicotine's actions on midbrain neurons. We suggest that menthol's ability to enhance nicotine-induced changes in nAChR function, nAChR number, and DA neuron excitability are important for menthol's enhancement of nicotine reward-related behavior.

MATERIALS AND METHODS

Menthol Dose Selection

We estimated the pharmacologically relevant dose of menthol by analogy with nicotine doses used in mouse studies. Typical menthol cigarettes contain 1–5 mg of menthol (Ai *et al*, 2015) and ~1 mg of nicotine (Rodgman and Perfetti, 2009). Therefore, menthol is 1–5 times that of nicotine. Steady state and peak concentrations of nicotine in human smokers are replicated in mice using 0.4 and 2.0 mg/kg/h doses of nicotine, respectively (Matta *et al*, 2007). CPP

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assays use 0.5 mg/kg nicotine (Tapper *et al*, 2004). Assays for *in vivo* upregulation use 2.0 mg/kg/h nicotine (Henderson *et al*, 2014). We selected 1 mg/kg and 2 mg/kg/h menthol for CPP and *in vivo* upregulation assays, respectively. Both dose selections fall within the 1–5 menthol-to-nicotine ratio of menthol cigarettes.

We previously discussed our menthol dose selection for cultured cells and neurons (Henderson *et al*, 2016). In preliminary assays to determine the concentration of menthol in a mouse brain, our chronic dosing methods (2 mg/kg/h, osmotic pump) produced concentrations of menthol at 0.5–2.5 μ M. Thus, 500 nM menthol is appropriate in studying cultured neurons and cells and is consistent with previous investigations (Henderson *et al*, 2016).

All material and methods are described in detail in the Supplementary Material.

RESULTS

Menthol Enhances Nicotine Reward-Related Behavior

Mice administered with saline or menthol alone (1.0–10 mg/kg) did not exhibit CPP (Figure 1a). Nicotine and menthol plus nicotine produced a significant effect on CPP (Figure 1b, one-way ANOVA, $F_{(4, 83)} = 14.3$, $p < 0.001$). 0.5 mg/kg nicotine produced CPP ($p < 0.05$) (Figure 1b), similar to previous observations (Tapper *et al*, 2004; Henderson *et al*, 2016). Menthol (1.0 mg/kg) was administered with either 0.25 mg/kg or 0.5 mg/kg nicotine (Figure 1b). In both pairs, menthol plus nicotine produced a significant increase in CPP compared to respective nicotine-only doses ($p < 0.05$, Figure 1b). This suggests that menthol enhances nicotine reward-related behavior and agrees with previous nicotine IVSA assays (Wang *et al*, 2014; Biswas *et al*, 2016).

Menthol Plus Nicotine Alters Baseline Midbrain Neuron Firing Frequency

Using midbrain cultures from tyrosine hydroxylase (TH)-eGFP mice, we identified TH+/DA and TH-/non-DA (putative GABA) neurons. Cultured midbrain neurons were

treated with control medium (control), 200 nM nicotine, or 500 nM menthol plus nicotine for 10 days. Control TH+/DA neurons exhibited a firing frequency of 4.1 ± 0.7 Hz (Figure 2a1–a3). Drug treatments produced a significant change in firing frequency for TH+/DA (one-way ANOVA, $F_{(2,61)} = 13.07$, $p < 0.001$) and TH-/non-DA neurons (one-way ANOVA, $F_{(2,29)} = 8.69$, $p = 0.002$). Nicotine treatment significantly decreased TH+/DA neuron firing frequency (1.5 ± 0.3 Hz, $p < 0.005$). Menthol plus nicotine decreased TH+/DA neuron firing frequency significantly more than nicotine treatment alone (1.2 ± 0.2 Hz, $p < 0.05$). TH-/non-DA neurons exhibited a baseline firing frequency of 8.1 ± 1.2 Hz (Figure 2b1–b3). Nicotine treatment increased firing frequency (13.9 ± 1.0 Hz, not significant) but menthol plus nicotine increased firing frequency more than nicotine alone (22.4 ± 2.1 Hz, $p < 0.005$).

Menthol Enhances nAChR-Stimulated TH+/DA Neuron Excitability

Midbrain TH+/DA neurons exhibit transient increases in firing frequency following nAChR activation, and nicotine's ability to alter tonic and phasic firing of these TH+/DA neurons is necessary for nicotine reward (Mansvelder *et al*, 2002). Thus, we examined menthol's effect on TH+/DA neuron transient increases in firing frequency following nAChR activation.

Neurons were treated for 10 days with control, 200 nM nicotine, or 500 nM menthol plus 200 nM nicotine and were recorded in current clamp mode before and after a brief application of ACh (Figure 2c1–c3). Drug treatments produced a significant effect on TH+/DA neuron excitability (one-way ANOVA, $F_{(2,29)} = 9.01$, $p = 0.001$). After ACh application, control TH+/DA neurons exhibited a transient, ~2-fold increase in firing frequency to 8.0 ± 1.1 Hz (Figure 2c1–c3). Nicotine-treated TH+/DA neurons exhibited a ~5-fold increase in firing frequency, with a maximum of 13.6 ± 2.5 Hz (Figure 2c1–c3). TH+/DA neurons treated with menthol plus nicotine exhibited an ~8-fold increase in firing frequency with a maximum of 19.6 ± 3.1 Hz (Figure 2c1–c3). Thus, we observed that menthol plus nicotine increased firing frequency more than nicotine alone ($p < 0.05$).

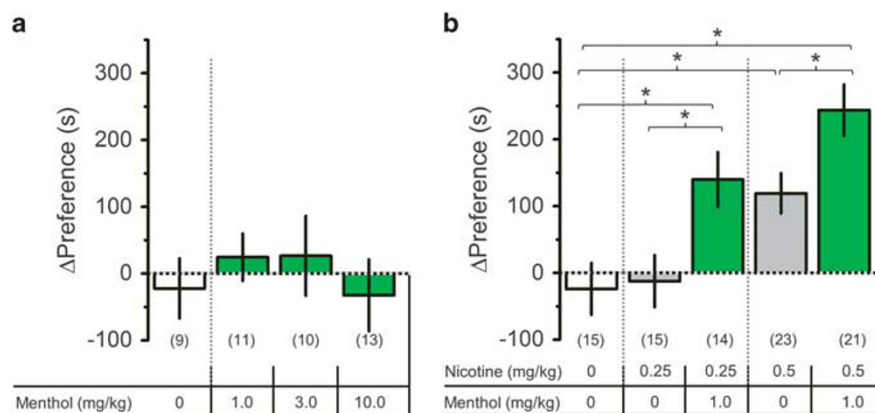


Figure 1 Menthol alone is not rewarding but enhances nicotine reward-related behavior. (a and b) Mice were administered saline, menthol, nicotine, or menthol plus nicotine at doses indicated. The data are mean \pm SEM. * $p < 0.05$, one-way ANOVA, post hoc Tukey. Numbers in parentheses represent number of mice per group.

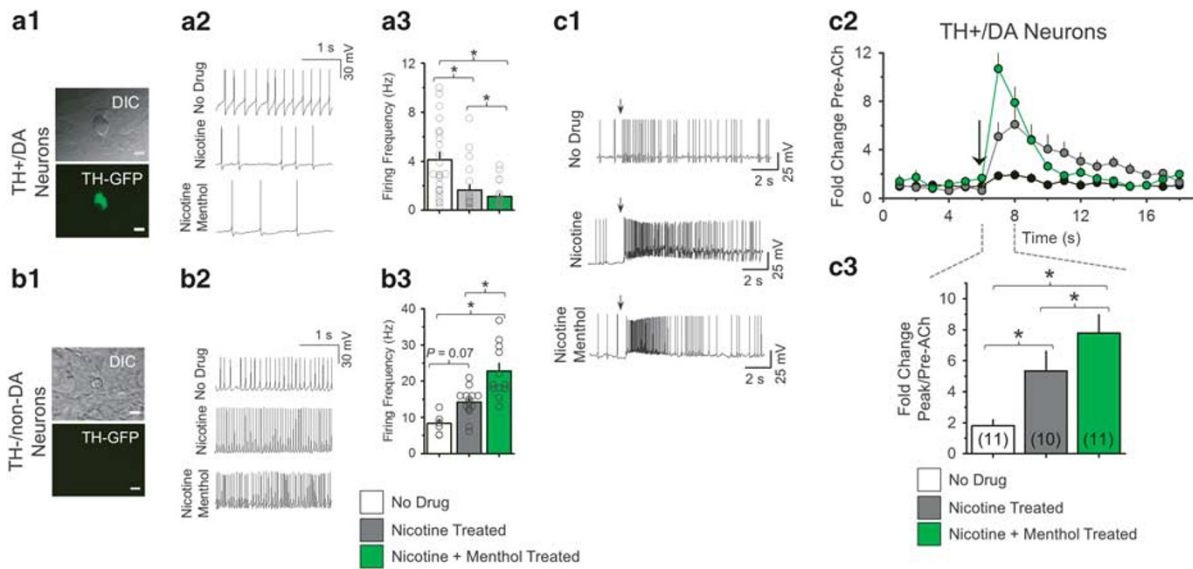


Figure 2 Menthol enhances nicotine-induced changes in midbrain neuron firing frequency. (a1, b1) Neurons were identified as TH+/DA or TH-/non-DA using TH-eGFP fluorescence. Bars, 20 μ m. (a2–3, b2–3, c1–3) Neurons were treated 10 days with control, 200 nM nicotine, or 500 nM menthol plus 200 nM nicotine and firing frequencies were recorded in current clamp mode. (a3, b3) Mean firing frequency of neurons ($n = 20$ –22 for TH+/DA neurons and 9–13 for TH-/non-DA neurons). (c1) Current-clamp recordings of TH+/DA neurons before and after ACh puff (100 ms, 300 μ M at arrows). (c2) Mean firing frequency over time before and after ACh application. (c3) Mean 'peak' firing frequency during 2 s post-ACh puff. For (c1–3), number of neurons recorded is indicated in parenthesis of (c3). The data are mean \pm SEM; *, $p < 0.05$, one-way ANOVA, post hoc Tukey.

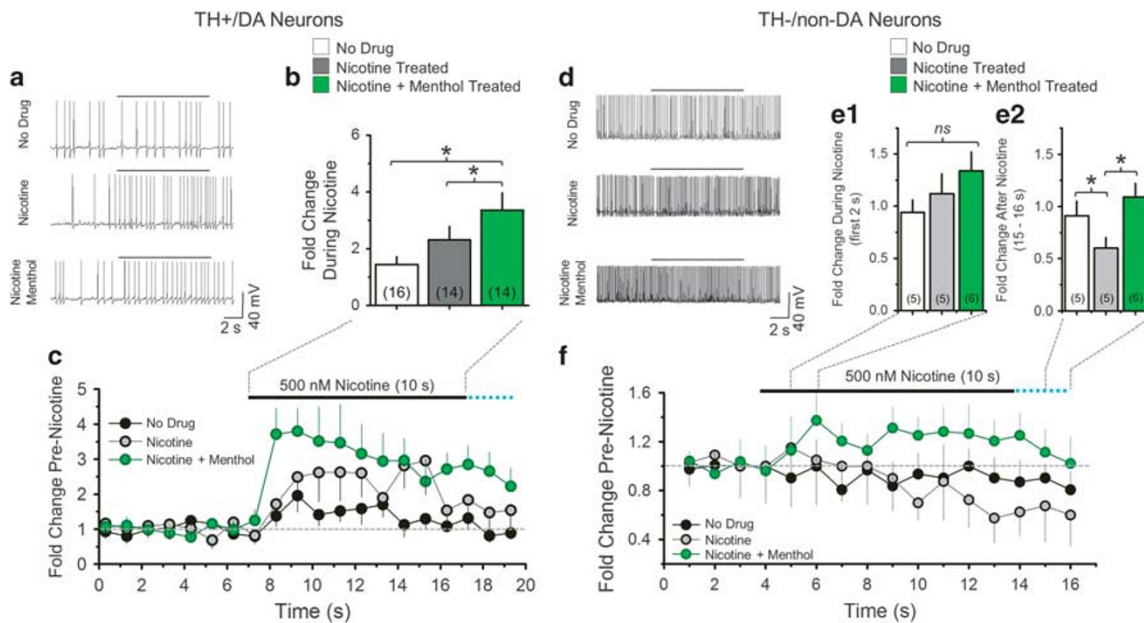


Figure 3 Menthol plus nicotine alters neuron excitability during acute exposure to smoking-relevant nicotine concentrations. Neurons were treated 10 days with control, nicotine, or menthol plus nicotine. (a and d) Representative current-clamp traces from TH+/DA and TH-/non-DA neurons puffed with nicotine. (b) Mean change in TH+/DA neuron firing frequency during 10 s nicotine applications. (c) and (f), mean firing frequency over time for TH+/DA and TH-/non-DA neurons, respectively. (e1–2) Mean fold-change in TH-/non-DA firing frequency following the first 2 s of nicotine puff (e1) and 2 s following end of nicotine puff (e2). For (c) and (b), number of individual neurons recorded is indicated in parenthesis in (b). For (f) and (e1–2), number of individual neurons recorded is indicated in parenthesis in (e1–2). The data are mean \pm SEM. *, $p < 0.05$, one-way ANOVA, post hoc Tukey. Bars indicate 10 s, 500 nM nicotine application and (c, f) dotted blue lines indicate nicotine remains briefly after the end of puff due to perfusion rate.

In humans, brain nicotine concentrations peak at ~ 500 nM seconds after a puff on a cigarette (Matta *et al*, 2007). Accordingly, we investigated how TH+/DA neuron firing frequency changed during a 10 s exposure to 500 nM nicotine after 10-day treatment with control, nicotine, or

menthol plus nicotine (Figure 3). Drug treatment produced a significant effect (one-way ANOVA, $F_{(2,41)} = 7.29$, $p = 0.002$). Control TH+/DA neurons exhibited a transient, 1.4-fold increase in firing frequency during nicotine application. Nicotine-treated TH+/DA neurons exhibited a transient,

2.3-fold increase in firing frequency during nicotine application. TH+/DA neurons treated with menthol plus nicotine exhibited a larger, slowly decaying increase in firing frequency during nicotine application that peaked at 4.0-fold over baseline and was significantly greater than control-treated ($p < 0.005$) or nicotine-treated ($p < 0.05$, Figure 3) TH+/DA neurons.

We completed similar assays on TH-/non-DA neurons (Figure 3d–f). Control-treated TH-/non-DA neurons exposed to acute nicotine (10 s, 500 nM) exhibited no change in firing frequency. Nicotine-treated TH-/non-DA neurons exposed to acute nicotine exhibited an initial increase in firing frequency followed by a significant decrease as the nicotine puff continued (Figure 3e1–e2 and f) (one-way ANOVA, $F_{(2,27)} = 9.21$, $p < 0.005$, Figure 3e2). TH-/non-DA neurons treated with menthol plus nicotine exposed to acute nicotine exhibited a non-significant increase in firing frequency that returned to baseline. We have previously reported that acute applications of menthol (up to 1 μM) do not activate nAChRs or potentiate nAChR currents (Henderson *et al*, 2016). We also stress that 500 nM menthol failed to alter firing frequency or stimulate inward currents in TH+/DA or TH-/non-DA neurons (Supplementary Figure S1). Thus, the effects we observed result from chronic and not acute actions of menthol.

Menthol Enhances Nicotine-Induced Upregulation of $\alpha 4^*$ But Not $\alpha 6(\text{non-}\alpha 4)^*$ nAChRs

Midbrain DA neurons contain $\alpha 4\beta 2(\text{non-}\alpha 6)^*$, $\alpha 6\beta 2(\text{non-}\alpha 4)^*$, and $\alpha 4\alpha 6\beta 2^*$ nAChRs, while midbrain GABA neurons contain $\alpha 4\beta 2(\text{non-}\alpha 6)^*$ nAChRs (Champtiaux *et al*, 2003). These nAChR subtypes are most sensitive to nicotine and are vital for nicotine reward (Pons *et al*, 2008). To study $\alpha 4^*$ and $\alpha 6^*$ nAChR upregulation, we used $\alpha 4$ -mCherry/ $\alpha 6$ -GFP mice that were treated for 10 days with vehicle, nicotine (2 mg/kg/h), or menthol plus nicotine (2 mg/kg/h, each). Similar to previous studies (Henderson *et al*, 2014, 2016), increases in integrated density of GFP or mCherry fluorescence was used to determine change in nAChR number. In midbrain, $\alpha 6^*$ nAChRs are selectively expressed in DA neurons (Mackey *et al*, 2012), so DA neurons of the SNc and VTA were identified by the presence of $\alpha 6$ -GFP fluorescence (Figure 4a and b1–b2).

We observed a significant effect of drug treatment on $\alpha 4$ -mCherry and $\alpha 6$ -GFP fluorescence intensity in the VTA (one-way ANOVA: $F_{(2,12)} = 24.1$, $p < 0.001$ and $F_{(2,12)} = 5.43$, $p = 0.017$, $\alpha 4$ -mCherry and $\alpha 6$ -GFP, respectively) and SNc (one-way ANOVA: $F_{(2,12)} = 4.14$, $p = 0.043$ and $F_{(2,12)} = 7.87$, $p = 0.007$, $\alpha 4$ -mCherry and $\alpha 6$ -GFP respectively) and substantia nigra pars reticulata (SNr) (one-way ANOVA: $F_{(2,12)} = 8.68$, $p = 0.005$, $\alpha 4$ -mCherry). Nicotine treatment robustly increased $\alpha 4^*$ nAChR number in VTA DA neurons and SNr GABA neurons (Figure 4c1–c3). Menthol plus nicotine produced an increase in $\alpha 4^*$ nAChRs in these same regions that was significantly greater than nicotine alone ($p < 0.05$). In SNc DA neurons, $\alpha 4^*$ nAChR number was increased by nicotine ($p < 0.05$) and the addition of menthol yielded no difference. As observed previously (Henderson *et al*, 2014), nicotine increased $\alpha 6^*$ nAChR number in VTA and SNc DA neurons (Figure 4c1–c2). In VTA neurons, menthol plus nicotine increased $\alpha 6^*$ nAChR number

but was not different from nicotine treatment alone. In SNc DA neurons, menthol plus nicotine did not increase $\alpha 6^*$ nAChR number. These data suggest that menthol enhances only $\alpha 4^*$ nAChR upregulation in VTA DA and SNr GABA neurons, and does not enhance $\alpha 6^*$ nAChR upregulation.

We observed a similar trend in functional upregulation of nAChRs: nicotine alone upregulates $\alpha 4^*$ and $\alpha 6^*$ nAChR function, but menthol plus nicotine provides a further increase in only $\alpha 4^*$ nAChR function (Supplementary Figure S2). *In vitro*, we observed that menthol enhanced nicotine-induced upregulation of $\alpha 4^*$ and not $\alpha 6^*$ nAChRs (Supplementary Figure S3) and the upregulated $\alpha 4^*$ nAChRs were of the high-sensitivity stoichiometry (Supplementary Figure S4). These data further suggest that menthol may selectively enhance upregulation of $\alpha 4^*$ and not $\alpha 6^*$ nAChRs. Using RNA-seq, we found that nAChR upregulation by menthol plus nicotine was not accompanied by any changes in mRNA (Supplementary Figure S3G). Full descriptions of these results can be found in the Supplementary Results section of the Supplementary Material.

Menthol Enhances Nicotine-Induced Upregulation of $\alpha 4\alpha 6^*$ nAChRs

Using pixel-based FRET methods (Henderson *et al*, 2014), we identified regions where $\alpha 4$ -mCherry and $\alpha 6$ -GFP nAChR subunits co-assembled to form $\alpha 4\alpha 6^*$ nAChRs (Figure 4b1–b2). Drug treatment produced a significant effect on $\alpha 4\alpha 6^*$ nAChR number in the VTA (one-way ANOVA: $F_{(2,12)} = 14.4$, $p < 0.001$) and SNc (one-way ANOVA: $F_{(2,12)} = 8.23$, $p = 0.006$). In the VTA, $\alpha 4\alpha 6^*$ nAChR number did not significantly increase following nicotine treatment, but menthol plus nicotine significantly increased $\alpha 4\alpha 6^*$ nAChR number (Figure 4c1). In the SNc, nicotine and menthol plus nicotine increased $\alpha 4\alpha 6^*$ nAChR number to a similar extent (Figure 4c2).

Next, we examined whether menthol potentiated nicotine-induced nAChR upregulation on VTA DA neurons under the same drug exposure conditions as our CPP assays. $\alpha 4$ -mCherry/ $\alpha 6$ -GFP mice were given injections of saline, nicotine, or menthol plus nicotine, with dosing identical to CPP assays (presented in Figure 1). Because VTA DA neurons are critical for nicotine reward (Pons *et al*, 2008), we examined only VTA DA neurons (Figure 4d1–d3). We observed a significant effect on $\alpha 4^*$ nAChRs ($F_{(2,15)} = 3.90$, $p = 0.04$, Figure 4d1). Nicotine produced 20.1% increase in $\alpha 4^*$ nAChR number (not significant), while menthol plus nicotine produced a 41.3% increase ($p < 0.05$). We observed no change in $\alpha 6^*$ nAChR number (Figure 4d2). These less-robust observations with alternating daily injections, compared to osmotic pumps, agreed with previous observations of nAChR upregulation.

We observed a significant effect on $\alpha 4\alpha 6^*$ nAChR number ($F_{(2,15)} = 15.7$, $p < 0.001$, Figure 4d3). Nicotine produced a 54.7% increase ($p < 0.05$), while menthol plus nicotine produced a 117.6% increase ($p < 0.005$). The increased $\alpha 4\alpha 6^*$ nAChR number in VTA neurons is accounted for by an increased number of FRET pixels, rather than by increased fluorescence intensity. This was consistent in both osmotic pump and injected preparations.

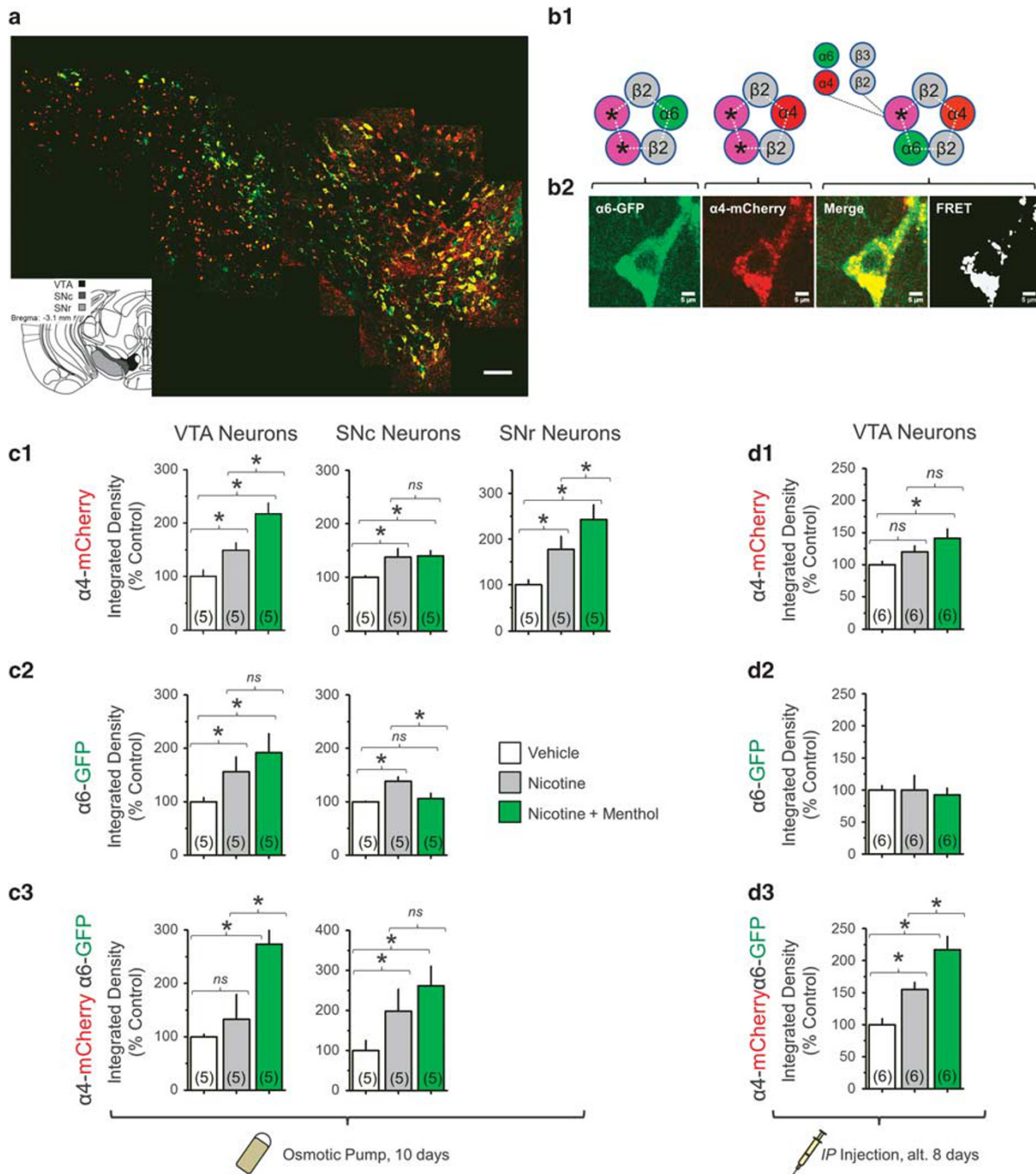


Figure 4 Menthol enhances nicotine-induced upregulation. (a) Representative coronal slice (bregma = 3.1) with $\alpha 4$ -mCherry/ $\alpha 6$ -GFP-labeled neurons in the VTA, SNc, and SNr. Bar, 100 μ m. (b1) Potential assemblies of midbrain nAChRs revealed by GFP and mCherry fluorescence. The subunits indicated by * are uncertain ($\alpha 4$, $\alpha 6$, $\beta 2$, or $\beta 3$). (b2) Representative $\alpha 4$ -mCherry/ $\alpha 6$ -GFP* neurons. (c1–3) $\alpha 4$ -mCherry*, $\alpha 6$ -GFP*, or $\alpha 4\alpha 6$ * nAChR-integrated density. The data are mean \pm SEM, normalized to vehicle. (c1–3) Chronic treatments were 10 days: vehicle, 2 mg/kg/h nicotine, or 2 mg/kg/h nicotine with 2 mg/kg/h menthol. (d1–3) Mice were given drug dosing identical to CPP assays. Number of mice for each treatment is indicated in parenthesis. * p < 0.05, one-way ANOVA, post hoc Tukey.

DISCUSSION

Individual deletions of $\alpha 4$, $\alpha 6$, or $\beta 2$ nAChR subunits prevent self-administration of nicotine in mice; and selective re-expression of these deleted subunits in the VTA (not SNc or SNr) re-instates self-administration (Pons *et al*, 2008). Selective activation of $\alpha 6\beta 2$ * or $\alpha 4\beta 2$ * nAChRs by smoking-relevant concentrations of nicotine stimulates

depolarization and elevates firing frequency of VTA DA neurons (Liu *et al*, 2012; Engle *et al*, 2013). Both $\alpha 4$ and $\alpha 6$ nAChR subunits are necessary for this as preparations lacking either $\alpha 4$ or $\alpha 6$ nAChR subunits failed to stimulate depolarization or elevate VTA DA neuron firing in response to nicotine. Together, these data highlight VTA $\alpha 4\alpha 6\beta 2$ * nAChRs as the primary targets of smoking-relevant concentrations of nicotine.

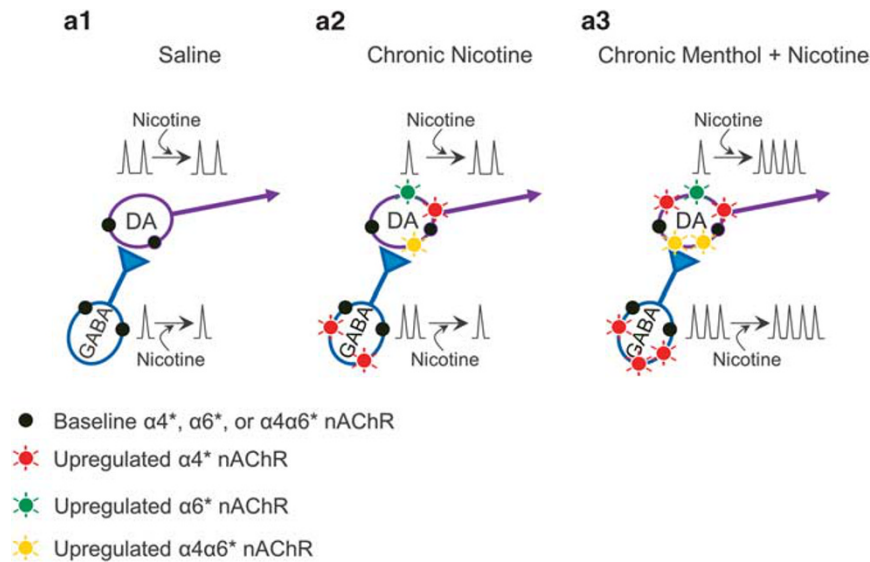


Figure 5 Summary. Simplified circuit diagram showing GABAergic neurons (blue) projecting to VTA DA neurons (purple). Arrows indicate acute applications of nicotine and its effect on firing frequency. See discussion for complete description.

We observed a significant increase in $\alpha4\alpha6\beta2^*$ nAChR number following menthol plus nicotine treatment, greater than the increase observed with nicotine alone (Figure 4c3 and d3). In assays using alternating daily injections, we observed little or no increase in total $\alpha4^*$ or $\alpha6^*$ nAChRs (Figure 4d1–d2). Considering the increase in $\alpha4\alpha6^*$ nAChRs, detected primarily by an increase in FRET pixels, this suggests that nicotine and menthol plus nicotine selectively upregulate the rather small, highly nicotine-sensitive subpopulation of nAChRs that contain both $\alpha4$ and $\alpha6$ subunits. Note that our brain slice microscopy method measures both intracellular and plasma membrane nAChRs and is not a direct measurement of functional nAChRs.

If $\alpha4\alpha6\beta2^*$ nAChRs are the primary targets of smoking-relevant concentrations of nicotine, their enhanced upregulation by menthol plus nicotine may underlie the enhancement in reward-related behavior (Figure 1b). The enhancement in TH+/DA-neuron firing frequency that we observed with menthol plus nicotine (Figure 3c) also supports this. Liu *et al* (2012) found that 300 nM nicotine applications elevated VTA DA neuron firing in a biphasic manner where $\alpha4(\text{non-}\alpha6)\beta2^*$ nAChRs mediated a rapid, desensitizing response and $\alpha4\alpha6\beta2^*$ mediated a sustained elevation in firing. We did observe robust, sustained increases in TH+/DA-neuron firing with similar nicotine applications. This may be attributed to the upregulated $\alpha4\alpha6\beta2^*$ nAChRs. GABAergic inputs on VTA neurons also play an important role in nicotine's actions. Following chronic exposure to nicotine, $\alpha4^*$ nAChRs are upregulated on SNr GABAergic neurons (Figure 4)(Nashmi *et al*, 2007). Acute nicotine exposure enhances GABAergic activity transiently, followed by sustained depression due to the upregulated $\alpha4^*$ nAChRs' rapid desensitization, leading to increased DA neuron excitability (Mansvelder *et al*, 2002). In TH-/non-DA neurons, we did measure transient increases in firing, followed by a maintained depression in activity (Figure 3f) in agreement with Mansvelder *et al*. TH-/non-DA neurons treated with menthol plus nicotine did not decrease in firing frequency during nicotine applications. We

have previously observed that menthol (alone) reduces $\alpha4\beta2^*$ nAChR desensitization (Henderson *et al*, 2016). This may explain how TH-/non-DA neurons may fail to desensitize during nicotine applications as they have only $\alpha4(\text{non-}\alpha6)\beta2^*$ nAChRs. These observations are summarized in Figure 5.

Comparing menthol alone (Henderson *et al*, 2016) to menthol plus nicotine (studied here), we observe a distinct difference. Menthol alone upregulates low-sensitivity $\alpha4^*$ and $\alpha6^*$ nAChRs, suppresses DA neuron excitability, and suppresses nicotine reward-related behavior. Menthol plus nicotine enhances DA neuron excitability and nicotine reward-related behavior (Figures 1,2,3). The likely molecular basis is that menthol plus nicotine increases the number of high-sensitivity $\alpha4^*$ and $\alpha6^*$ nAChRs, but menthol alone upregulates low-sensitivity $\alpha4^*$ and $\alpha6^*$ nAChRs (Supplementary Figure S4 and Henderson *et al*, 2016).

We note that smokers experience odorant and tastant effects of menthol. It was shown that menthol decreases oral nicotine aversion in mice through a TRPM8-dependent mechanism (Fan *et al*, 2016). We stress that our results presented here use a drug exposure paradigm that avoids tastant effects and the neurons in our preparations (slices or cultures) do not contain TRPM8 receptors (Henderson *et al*, 2016). We suggest that tastant and sensory effects act in addition to the direct, neuron-mediated effects we observed.

We provide a cellular basis for how menthol may enhance nicotine reward-related behavior. Our findings, combined with the knowledge that menthol may enhance nicotine withdrawal (Alsharari *et al*, 2015) and nicotine IVSA (Wang *et al*, 2014; Biswas *et al*, 2016), may provide insight into why smokers of menthol cigarettes report lower quit rates.

FUNDING AND DISCLOSURE

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