

## **Menthol Enhances the Desensitization of Human $\alpha 3\beta 4$ Nicotinic Acetylcholine Receptors**

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**Running title:** Menthol inhibits  $\alpha 3\beta 4$  Nicotinic Acetylcholine Receptors

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**Text pages:** 16

**Figures:** 9

**References:** 42

**Abstract:** 244

**Introduction:** 488

**Discussion:** 1138

**Non-standard abbreviations:** nAChR, nicotinic acetylcholine receptor; TRPM8, Transient receptor potential channel, subfamily M, member 8; TRPA1, Transient receptor potential channel, subfamily A, member 1.

## Abstract

The  $\alpha 3\beta 4$  nicotinic acetylcholine receptor (nAChR) subtype is widely expressed in the peripheral and central nervous systems, including in airway sensory nerves, which transduce the irritant effects of nicotine in tobacco smoke, and in certain brain areas that may be involved in nicotine addiction and/or withdrawal. Menthol, a widely used additive in cigarettes, is a potential analgesic and/or counterirritant at sensory nerves and may also influence nicotine's actions in the brain. We examined menthol's effects on recombinant human  $\alpha 3\beta 4$  nAChRs and native nAChRs in mouse sensory neurons. Menthol markedly decreased nAChR activity as assessed by  $\text{Ca}^{2+}$  imaging,  $^{86}\text{Rb}^{+}$  efflux and voltage-clamp measurements. Co-application of menthol with acetylcholine or nicotine increased desensitization, demonstrated by an increase in the rate and magnitude of the current decay and a reduction of the current integral. These effects increased with agonist concentration. Pretreatment with menthol followed by its washout did not affect agonist-induced desensitization, suggesting that menthol must be present during the application of agonist to augment desensitization. Notably, menthol acted in a voltage-independent manner and reduced the mean open time of single channels without affecting their conductance, arguing against a simple channel blocking effect. Further, menthol slowed or prevented the recovery of nAChRs from desensitization indicating that it probably stabilizes a desensitized state. Moreover, menthol at concentrations up to 1 mM did not compete for the orthosteric nAChR binding site labeled by [ $^3\text{H}$ ]-epibatidine. Taken together, these data indicate that menthol promotes desensitization of  $\alpha 3\beta 4$  nAChRs by an allosteric action.

## Introduction

Menthol is a monoterpene alcohol widely used in consumer products. Most notably, menthol is extensively used in cigarettes. More than 90% of tobacco products contain some amount of menthol; the majority, ~75%, has only low levels of l-menthol as an additive (.03%), while 25% contain higher levels (0.1 - 0.45%) and are designated mentholated cigarettes. Menthol may be added to tobacco to deliver a distinct oral sensation, as it imparts a characteristic cooling sensation via activation of TRPM8 ion channels expressed in a population of thermosensory nerves (McKemy et al., 2002; Peier et al., 2002; Bautista et al., 2007). In turn, signaling downstream of TRPM8 can lead to analgesia through undefined mechanisms (Willis et al., 2011). In addition, menthol may produce analgesic/counterirritant effects through activation and desensitization of the nociceptive TRPA1 channel (Macpherson et al., 2006; Karashima et al., 2007; Xiao et al., 2008) or by direct antagonism of the TRPA1 channel (Karashima et al., 2007; Xiao et al., 2008). Indeed, in the context of smoking, the analgesic effects of menthol may be desirable for the smoker. Cigarette smoke contains numerous noxious compounds that may be irritants to the airway. Further, nicotine itself can be noxious by activating nicotinic acetylcholine receptors (nAChRs) located on pulmonary sensory neurons; in fact, nicotine may be the primary mediator of airway irritation and cough evoked by smoked cigarettes (Lee et al., 2007). Menthol, may therefore reduce the harshness of cigarette smoke and nicotine, and thereby increase the tolerability and/or palatability of smoking. This might be particularly important to the person just beginning to smoke.

Interestingly, two recent studies revealed that menthol inhibits ACh and nicotine-stimulated currents in heterologously expressed  $\alpha 4\beta 2$  (Hans et al., 2012) and  $\alpha 7$  (Ashoor et al., 2013) nAChRs, subtypes predominantly expressed in the brain, and produces a slow, time-

dependent inhibition of ACh- and nicotine-evoked currents in cells from the trigeminal ganglia (Hans et al., 2012). In each case, menthol appeared to act allosterically. These data suggest that nAChRs may be additional pharmacological targets of menthol. However, the effects of menthol on  $\alpha 3\beta 4$  nAChRs, the major nicotinic subtype expressed in sensory nerves, and the potential mechanisms of menthol's inhibition of nAChRs are unknown. Understanding the effects of menthol on  $\alpha 3\beta 4$  nAChRs in particular is relevant to nociceptive signaling in the airways arising from cigarette smoke and nicotine. In addition,  $\alpha 3\beta 4$  nAChRs are highly expressed in the habenula and intrapeduncular nuclei, brain regions implicated in reward processing and possibly addiction to and/or withdrawal from nicotine (Salas et al., 2004; McCallum et al., 2012). Here we show that menthol acts allosterically to inhibit the function of  $\alpha 3\beta 4$  nAChRs by increasing the rate and extent of agonist-induced desensitization. We propose that this mechanism may contribute to the analgesic actions of menthol in the bronchial airways in the presence of tobacco smoke, as well as to some of the effects of nicotine in the brain. Both of these actions may contribute to nicotine addiction.

## Materials and Methods

All experimental procedures involving mice were approved by the Georgetown University Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

(-)-Menthol and other chemicals were purchased from Sigma Chemical Co (St. Louis, MO) unless otherwise indicated. [ $^3\text{H}$ ]-Epibatidine ([ $^3\text{H}$ ]-EB) and  $^{86}\text{RbCl}$  were purchased from Perkin-Elmer Life Sciences (Boston, MA).

**Cell culture.** HEK cells stably expressing human  $\alpha 3\beta 4$  nAChRs were cultured in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% 100X MEM non-essential amino acids and 1% penicillin/streptomycin (HyClone, Utah, USA) at 37° C in a water-saturated atmosphere containing 5% CO<sub>2</sub>. Cell cultures were seeded in a culture flask (25cm<sup>2</sup>) (Sarstedt Inc., NC, USA) and sub-cultured twice a week. For Ca<sup>2+</sup> imaging and electrophysiology, cells were plated on poly-D-lysine-coated coverslips and used for experiments within 1-3 days.

**Nodose ganglia neurons.** For experiments with nodose ganglia neurons, adult C57BL/6J mice (25-30 g) were killed by CO<sub>2</sub>/decapitation and the nodose ganglia were dissected, digested with collagenase, and cultured in Neurobasal medium plus 2% B-27 (Invitrogen), 0.1% L-glutamine, and 1% penicillin/streptomycin on poly-D-lysine-coated glass coverslips at 37°C in 5% CO<sub>2</sub>. Neurons were used within 24 –36 h of culture.

**Ca<sup>2+</sup> imaging.** Ca<sup>2+</sup> imaging was performed using the dye Fluo4-AM. The cells were loaded with 1  $\mu$ M Fluo4-AM (Invitrogen, CA, USA) in a standard buffer containing 140 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 10 mM HEPES, 5 mM glucose, pH=7.3. The dye was excited at 480  $\pm$  15 nm. Emitted fluorescence was filtered with a 535  $\pm$  25 nm bandpass filter, captured by a SPOT RT digital camera (Diagnostic Instruments, PA, USA) and read into a computer. Analysis was performed offline by using Simple PCI software (Compix Inc., University of New South Wales). Solutions were applied via a valve-controlled gravity-fed perfusion system with a 200  $\mu$ m diameter outlet. The bathing solution contained 5  $\mu$ M atropine to block muscarinic acetylcholine receptors.

**[<sup>3</sup>H]-Epibatidine binding competition assays.** Binding competition assays were performed to determine menthol's affinity for human  $\alpha 3\beta 4$  nAChRs. Cell membrane homogenates were prepared as described previously (Xiao and Kellar, 2004). Briefly, cells were washed, suspended in 50 mM Tris-HCl buffer and homogenized with a Polytron homogenizer. The homogenate was centrifuged at 33,000 x g for 10 min at 4°C, the supernatant was discarded and the pellet was then suspended in fresh buffer. This was repeated two more times before the final pellet was resuspended in 50 mM Tris-HCl buffer and used in subsequent assays. The membranes were incubated for 2 hours with ~0.5 nM [<sup>3</sup>H]-EB in the absence or presence of increasing concentrations of menthol or nicotine (for comparison). The membrane homogenates were filtered through Whatman GF/C filters treated with 0.5% polyethylenimine and then counted in a Beckman Scintillation counter. The data were analyzed by nonlinear least square regression analysis in GraphPad Prism 5 (San Diego, CA).

**Rubidium efflux assays.** Menthol's effect on  $\alpha 3\beta 4$  nAChR function was initially examined by assessing <sup>86</sup>Rb<sup>+</sup> efflux through the receptor channel, as described previously (Xiao et al., 1998; Meyer et al., 2001). Cells were first loaded with <sup>86</sup>RbCl by incubating them for ~2 hours with 0.5 ml media containing ~100,000 dpm <sup>86</sup>Rb<sup>+</sup>. To test menthol's agonist activity, the cells were rinsed gently 4 times with 1 ml buffer over 10 min and then either buffer alone, buffer containing 100  $\mu$ M nicotine or 100  $\mu$ M menthol was added for 2 mins. To test for menthol's antagonist activity, cells were incubated for 2 min with 100  $\mu$ M nicotine in the absence or presence of increasing concentrations of menthol. In some experiments, menthol was added 10 min before and maintained during the two-min nicotine stimulation. In all cases, the background efflux was determined in the cells incubated in buffer alone, while maximal response was defined as the efflux elicited by 100  $\mu$ M nicotine. The <sup>86</sup>Rb<sup>+</sup> efflux from the cells into the media was assessed

using Cherenkov counting on a Beckman-Coulter LS6500 Scintillation Counter. After subtracting background efflux, stimulated efflux was calculated as the  $^{86}\text{Rb}^+$  in the media over the sum of the  $^{86}\text{Rb}^+$  in the media plus that in the cells. Results are expressed as the percent of efflux elicited by 100  $\mu\text{M}$  nicotine, which elicits a maximal response (Meyer et al., 2001).

**Electrophysiology.** Whole-cell and cell-attached voltage-clamp recordings were performed by using an EPC8 patch-clamp amplifier (HEKA Electronics) that was controlled by the program Pulse (version 8.65, HEKA Electronics). Data was collected at 5 KHz and low-pass filtered at 3 KHz. Single channel data was analyzed by Channel2 software. The bath solution contained 140 mM NaCl, 4 mM KCl, 1 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{CaCl}_2$ , 10 mM Hepes, 5 mM glucose, pH 7.3. The pipette solution contained 140 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM Mg-ATP, pH 7.3. For cell-attached recordings the pipette solution was the same as the bath solution and included 10  $\mu\text{M}$  ACh. Solutions were applied via a valve-controlled gravity-fed perfusion system with an outlet (200  $\mu\text{m}$  diameter) positioned  $\sim 50$   $\mu\text{m}$  from the cell of interest. The solution exchange time constant (Tau) was  $\sim 1$  s.

To characterize desensitization, currents were evoked by ACh or nicotine in the absence or presence of menthol for 40 s and the time constant was measured by fitting the current decays with one or two exponential equations using Origin 8.0 software. The fraction of the current remaining at the end of the 40 s drug application was designated as D and was used to quantify the extent of desensitization. All values were normalized to those produced by ACh for each individual cell.



## Results

### **Menthol inhibits human $\alpha 3\beta 4$ nAChR activity in a non-competitive manner.**

Sensory and autonomic ganglia neurons predominantly express the  $\alpha 3\beta 4$  subtype of nAChR. To examine potential effects of menthol at these ion channels we performed  $\text{Ca}^{2+}$  imaging and  $^{86}\text{Rb}^{+}$  efflux measurements in HEK293 cells stably expressing human  $\alpha 3\beta 4$  nAChRs. As shown in figure 1A and 1B, successive applications of 30  $\mu\text{M}$  ACh (with an interstimulus interval of 5 min) evoked  $\text{Ca}^{2+}$  transients of similar magnitude (mean  $\Delta\text{F}/\text{F}_0$  application 1 =  $3.49 \pm 0.18$  vs application 2 =  $3.45 \pm 0.20$ ,  $n = 39$  cells). However, co-application of menthol (100  $\mu\text{M}$ ) inhibited the ACh-induced peak  $\text{Ca}^{2+}$  signal by 47% ( $\Delta\text{F}/\text{F}_0 = 3.30 \pm 0.16$  vs  $1.90 \pm 0.18$ ,  $n = 37$  cells) (Figs. 1A and 1B).

We next examined menthol's effect on nAChR channel function by measuring nicotine-stimulated  $^{86}\text{Rb}^{+}$  efflux in these cells. Menthol co-applied with 100  $\mu\text{M}$  nicotine inhibited nicotine-stimulated  $^{86}\text{Rb}^{+}$  efflux in a concentration-dependent manner, with an  $\text{IC}_{50}$  of  $100 \pm 8$   $\mu\text{M}$  (Fig. 1C). Pre-incubation with menthol for 10 min before addition of nicotine decreased menthol's  $\text{IC}_{50}$  only slightly, to  $69 \pm 8$   $\mu\text{M}$  (Fig. 1D).

To determine if menthol acted at the agonist binding site of  $\alpha 3\beta 4$  nAChRs, we examined its competition for nAChR binding sites labeled by [ $^3\text{H}$ ]-EB in cell membrane homogenates, and compared it to nicotine. As shown in figure 2, in contrast to nicotine, which competed effectively for binding with a  $K_i$  of  $\sim 250$  nM, menthol at concentrations up to 1 mM was completely ineffective.

### **Menthol enhances the apparent desensitization of human $\alpha 3\beta 4$ nAChR activity.**

To further explore how menthol inhibits the function of  $\alpha 3\beta 4$  nAChRs we performed whole-cell voltage-clamp recordings. Application of 30 or 100  $\mu$ M ACh to cells expressing  $\alpha 3\beta 4$  nAChRs evoked inward currents with a rapid onset and minimal desensitization (Figs. 3A and 3B). However, co-application of menthol (100  $\mu$ M) with ACh markedly increased the rate and magnitude of desensitization without appreciably affecting the peak response to ACh (Figs. 3A and 3B). These effects were nearly completely reversed 60 s after removal of menthol (Fig. 3A, right trace).

Menthol could potentially act as an agonist or partial agonist to increase receptor desensitization; however, consistent with its lack of binding to the receptor, we found that application of menthol alone (1-1000  $\mu$ M) did not elicit inward currents (Fig. 3C). Further, a 5-minute treatment with 100  $\mu$ M menthol alone followed by washout did not affect a subsequent ACh-evoked response (Fig. 3D). Neither, the peak ACh-evoked current nor the level of desensitization was affected by menthol pretreatment (Figs. 3 D and E). These data indicate that menthol alone produces little to no desensitization, and that its effects thus require the presence of ACh.

### **The inhibitory effects of menthol are dependent on the concentration of ACh.**

Next, we tested the effect of menthol (300  $\mu$ M) on inward currents evoked by different concentrations of ACh ranging from 3 - 300  $\mu$ M. We analyzed these currents to determine the level of desensitization (D), and the time constant ( $\tau$ ) or weighted time constant ( $\tau_w$ ) for current decay, obtained by the best fits to single- or double-exponential functions. As shown in Figure

4A, menthol had no measurable effect on currents evoked by a low concentration of ACh (3 $\mu$ M), which exhibited a very slow rate of desensitization ( $\tau \sim 50$ s; Fig. 4B) and a low level of desensitization ( $\sim 25\%$ ; Fig. 4C) with or without menthol. However, at ACh concentrations of 30 and 300  $\mu$ M, menthol markedly increased the rate and extent of desensitization (Fig. 4A). Thus, as shown in figures 4B and 4C, at 30  $\mu$ M ACh, menthol decreased the  $\tau_w$  from 35 s to 2.4 s and increased D from 35% to 91%; and at 300  $\mu$ M ACh, menthol decreased the  $\tau_w$  from 21 s to 0.9 s and increased D from 74% to 94%. At 300  $\mu$ M ACh, the  $\tau_w$  approaches the time for activation of  $\sim 1$ s (limited by the solution exchange time). Consequently, at these higher concentrations of ACh, the peak currents evoked in the presence of menthol were very much decreased (Fig. 4A). These results demonstrate the importance of the ACh concentration in the actions of menthol at  $\alpha 3\beta 4$  nAChRs. Moreover, the fact that menthol had little effect on desensitization parameters at the lowest ACh concentration used here but a large effect as the ACh concentrations were increased and open channel probability increased, indicates that menthol acts in a state-dependent manner; that is, menthol acts preferentially on the open or desensitized state of the channel.

### **Menthol inhibits in a voltage-independent manner and without affecting single channel conductance**

The observation that the effects of menthol depend on ACh concentration raised the possibility of an open-channel block mechanism. To explore this hypothesis we tested for voltage-dependent effects of menthol. Figure 5A shows the current-voltage relationship (elicited by 200 ms voltage ramps) during the peak response to ACh, and 20 s after addition of menthol (100  $\mu$ M). Both traces exhibit characteristic inward rectification that is relieved at high positive

membrane potentials. The inset reveals the fractional current (menthol/control) at different voltages and shows that menthol similarly reduced the current by ~50 % at all potentials. Therefore, menthol inhibits nAChRs in a voltage-independent manner. In addition, these data show that menthol acts independently of the direction of net current flow, which is inward and outward respectively at negative and positive potentials. Next, we tested for use-dependent effects that are characteristic of many open channel blockers. Figure 5B&C shows the response of repeated, 5 s applications of ACh with or without continued presence of 200  $\mu$ M menthol. The relatively brief application duration was chosen to minimize desensitization. In both cases the peak responses exhibited a marginal decrease with successive ACh applications (Fig. 5C) but there was no difference between control and menthol treatments. Thus, menthol does not produce a rapid, use-dependent block, although we cannot exclude the possibility that menthol binds very slowly to the pore ( $\gg 5$  s), and therefore failed to significantly inhibit current during brief applications of ACh used here. Finally, we tested the effects of menthol on single channel activity. Figure 5D shows representative current traces from a cell-attached recording before and after application of 200  $\mu$ M menthol. Under control conditions channel activity consisted of bursts of openings separated by long and variable closed times. Menthol markedly reduced the mean open time from  $32.4 \pm 6.4$  ms to  $5.7 \pm 1.1$  ms (Fig. 5D&F) without affecting the single channel conductance (Fig. 5E; slope conductance 30.9 versus 30.3 pS). Taken together, these data suggest that menthol inhibits  $\alpha 3\beta 4$  nAChRs in an allosteric manner by altering gating rather than simply blocking the channel pore.

**Increasing menthol concentration enhances the desensitization of the ACh-induced currents.**

We next examined the concentration dependent effects of menthol on currents evoked by 30  $\mu$ M ACh. Figure 6A shows that both the rate and extent of desensitization increases with increasing menthol concentration. In assessing the desensitization parameters,  $\tau_w$  and D, values were normalized to data obtained from the same cell in menthol-free conditions. Menthol increased the rate of desensitization in a concentration dependent manner, and at 300  $\mu$ M almost completely desensitized currents within 1s of ACh/menthol application (Fig. 6A and B). The extent of desensitization, D, also increased with increasing menthol concentration, and at the end of the 40 s application of ACh in the presence of 300  $\mu$ M menthol the extent of desensitization was more than twice as great as in the absence of menthol (Fig. 6C). Figure 6D shows the current integral (the total current passed during the 40 s application of agonist) as a function of menthol concentration. Half-maximal inhibition (obtained by the best-fit to a Hill equation) occurred at 43  $\mu$ M, similar to the values obtained by the  $^{86}\text{Rb}^+$  efflux measurements (see Fig. 1C and D). Notably, these values are also similar to the  $\text{EC}_{50}$  for menthol activity at TRPM8 receptors of ~55 to 80  $\mu$ M at room temperature (McKemy et al., 2002; Premkumar et al., 2005) and ~30  $\mu$ M for activation of human TRPA1 (Xiao et al., 2008). Thus, the concentration of menthol needed to drive its sensory perception (cooling sensation and pungency) in airway C fibers is also sufficient to decrease channel activity through  $\alpha 3\beta 4$  nAChRs by promoting desensitization.

### **Menthol enhances desensitization of currents evoked by nicotine.**

To corroborate and further explore the effects of menthol on nAChRs, we determined if these effects extended to nicotine, the addictive component of tobacco. Similar to its effect on the ACh-induced currents, we observed that menthol enhanced the apparent desensitization of the nicotine-induced currents by reducing the time constant and increasing current decay during a 40 s concomitant application of nicotine and 100  $\mu$ M menthol. Figure 7A-D illustrate the

currents induced by nicotine alone at concentrations of 1 to 100  $\mu$ M (left column); the combination of nicotine and 100  $\mu$ M menthol (middle); and a second application of nicotine alone after a 5 min washout of the menthol (right). At a low concentration of nicotine (1  $\mu$ M), menthol had no measurable effect on the currents. At concentrations of 3 and 10  $\mu$ M nicotine, the decay of current during the 40 s nicotine exposure was fit best to a single exponential with a time constant of 18.1 and 15.9 s, respectively, and the current desensitized by 26 and 22%. In the presence of menthol, the time constants of the current decay were decreased to 9.5 and 10.8 s, respectively, and there was a nearly two-fold increase in the extent of desensitization at both concentrations of nicotine. After the 5 min washout, the time constant and the degree of desensitization to 3  $\mu$ M nicotine nearly fully recovered to the initial control levels. The time constant to 10  $\mu$ M nicotine did not fully recover, but the degree of desensitization did. At a nicotine concentration of 100  $\mu$ M, the decay current during the 40 s nicotine exposure was fit best to a single exponential with a time constant of 9.4 s, and the current desensitized by 60%. In the presence of menthol, the decay current was best fit to two exponentials with time constants of 5.1 s and 1.9 s, which yielded a weighted time constant,  $\tau_w$ , of 3.2 s; the current in the presence of menthol desensitized by 78%. After the 5 min washout, the decay constant again fit best to a single exponential but had only partially recovered to the original control value. Similarly, the degree of desensitization had not recovered completely. Taken together, these findings indicate menthol enhances the apparent desensitization of human  $\alpha 3\beta 4$  nAChRs to nicotine.

### **Menthol traps $\alpha 3\beta 4$ nAChRs in the desensitized state.**

Next, we examined whether continued presence of menthol would hinder the receptor's recovery from desensitization. We stimulated receptors with 30  $\mu$ M ACh in the presence of menthol, to promote desensitization, and subsequently measured the time-course for recovery

(over 1- 5 min), in either the presence or absence of 100  $\mu$ M menthol. Figure 8 shows that in the absence of menthol the  $\alpha 3\beta 4$  receptors recovered rapidly from desensitization with ACh (Fig. 8A), with  $83 \pm 5 \%$  (n=3) recovery after 1 min and  $97 \pm 2 \%$  (n=5) recovery after 5 min (Fig. 8B). In contrast, in the presence of menthol, recovery from desensitization was greatly attenuated (Fig. 8A), with only  $20 \pm 3\%$  (n=3),  $26 \pm 0.6$  (n=3) and  $33 \pm 5 \%$  (n=5) recovery after 1, 3, and 5 min, respectively (Fig. 8B). As shown in Figure 3D, treating non-desensitized  $\alpha 3\beta 4$  receptors with menthol had minimal effect on subsequent recovery, suggesting that menthol acts mainly on the desensitized state. To further discriminate actions of menthol at open or desensitized states we tested effects of menthol on  $\alpha 3\beta 4$  nAChRs that were already fully desensitized by a high concentration (300  $\mu$ M) of ACh. Figure 8C shows that menthol (100  $\mu$ M) almost fully prevented recovery of these receptors from desensitization; however following removal of menthol, channel activity was nearly fully restored (n=3). Taken together, these data suggest that menthol traps  $\alpha 3\beta 4$  nAChRs preferentially in their desensitized state(s), thereby markedly slowing their recovery from desensitization. Thus, menthol may both augment initial agonist-induced desensitization and prolong it.

### **Menthol inhibits ACh-evoked currents in nodose ganglion neurons.**

To test whether menthol similarly affects native  $\alpha 3\beta 4$  nAChRs, we examined responses in cultured nodose ganglion neurons. These neurons send vagal projections to the lung and predominantly express the  $\alpha 3\beta 4$  nAChR subtype (Mao et al., 2006). Figure 9 shows that ACh evoked a slowly desensitizing current in a voltage-clamped sensory neuron. Co-application of menthol markedly reduced the peak current and increased the extent of desensitization from ~50% to ~90%. The current responses to ACh nearly fully recovered following washout of the menthol. These responses in nodose neurons are therefore consistent with menthol increasing

the speed and magnitude of desensitization and mirror the responses observed with recombinant  $\alpha 3\beta 4$  nAChRs.

## Discussion

The studies presented here demonstrate that menthol attenuates signaling through human  $\alpha 3\beta 4$  nAChRs. This was shown with three different kinds of measurements: ACh-stimulated  $\text{Ca}^{2+}$  signaling, nicotine-stimulated  $^{86}\text{Rb}^{+}$  efflux, and both ACh- and nicotine-stimulated currents measured by whole-cell and single channel voltage-clamp recordings. Our data also demonstrate that menthol does not bind to the orthosteric site, indicating that its actions are via an allosteric mechanism. Importantly, these effects of menthol at nAChRs occurred at pharmacologically relevant concentrations similar to those required to activate TRPM8 and TRPA1 receptors.

Previous studies also found that menthol attenuated nAChR functions. Hans et al. (2012) found that menthol at concentrations similar to those used here decreased nicotine-stimulated currents through unidentified nAChRs in trigeminal ganglia cells, and reduced single channel currents in cells heterologously expressing  $\alpha 4\beta 2$  nAChRs. Their data also suggested that menthol acted as a negative allosteric modulator. Ashoor et al. (2013) found that menthol attenuated function of  $\alpha 7$  nAChRs expressed in *Xenopus* oocytes but did not compete for  $\alpha 7$  receptor binding sites, again suggesting a negative allosteric effect.

Our studies extend these findings to human  $\alpha 3\beta 4$  nAChRs and, importantly, identify augmented desensitization as the mechanism by which menthol attenuates this receptor's function. This conclusion is based on the following observations: First, addition of menthol to



cells for 5 min and its removal immediately before addition of agonist did not alter the response of  $\alpha 3\beta 4$  nAChRs to ACh, indicating that menthol did not produce a long-lasting effect in the absence of an agonist. Second, the effects of menthol on channel function are minimal or absent in the presence of low concentrations of agonist, but become prominent as the agonist concentration is increased to the level where desensitization begins to occur. This indicates that menthol acts preferentially on the open state or desensitized state(s) of the channel. Third, the effects of menthol are independent of the membrane voltage and the net current direction, two parameters that can affect the actions of open channel blockers. Furthermore, menthol did not produce a rapid, use-dependent inhibition nor reduce the single channel conductance characteristic of open channel blockade. Rather, menthol reduced the open probability and mean open time of single  $\alpha 3\beta 4$  nAChR channels consistent with alterations in channel gating. Interestingly, Hans et al. (2012) reported similar effects of menthol on single channel properties of  $\alpha 4\beta 2$  nAChRs. Fourth, menthol markedly delayed recovery from desensitization; in particular, menthol prevented the recovery of receptors already fully desensitized by a high concentration of ACh. This suggests that menthol binds and traps the receptor in a desensitized conformation(s). Importantly, these data argue strongly against the possibility that menthol acts as a slow, open-channel blocker. Instead, menthol appears to speed and magnify nAChR desensitization and stabilize a desensitized conformation. Thus, we propose that menthol augments and facilitates the normally weak desensitizing effect of ACh and low concentrations of nicotine, with the result that menthol, acting via an allosteric site, markedly decreases the current carried by the receptor.

Allosteric modulators, both positive and negative, of nAChRs have been studied previously (for review, see Pandya and Yakel, 2011; Williams et al., 2011). These modulators

include metal ions (Vernino et al., 1992; Zwart et al., 1995; Hsiao et al., 2001), steroid hormones (Valera et al., 1992; Ke and Lukas, 1996; Paradiso et al., 2000; Curtis et al., 2002) and small synthetic ligands (Bertrand and Gopalakrishnan, 2007; Moaddel et al., 2007; Henderson et al., 2010). In fact, menthol has recently been reported to allosterically inhibit currents mediated by  $\alpha 4\beta 2$  (Hans et al., 2012) and  $\alpha 7$  (Ashoor et al., 2013) nAChRs. However, to our knowledge menthol is the first example of a drug demonstrated to act allosterically to augment desensitization of a neuronal nAChR without activating or even binding to the receptor's orthosteric site.

nAChRs are desensitized immediately after or even during their activation by high concentrations of ACh or by nicotine and other nicotinic agonists and partial agonists. Moreover, this desensitization usually lasts much longer than the brief agonist-induced activation (Katz and Thesleff, 1957; Sharp and Beyer, 1986; Hulihan-Giblin et al., 1990). We do not yet know whether menthol's effect to augment desensitization extends to other nAChR subtypes, but interestingly, the  $\alpha 3\beta 4$  nAChR subtype is one of the slowest to desensitize and fastest to resensitize (Cachelin and Jaggi, 1991; Fenster et al., 1997; Quick and Lester, 2002); thus, the effect of menthol to augment desensitization at this receptor may be especially important.

The suppression of  $\alpha 3\beta 4$  nAChR activity by menthol has potentially important implications for its analgesic effects in sensory nerves and in airways. Rat trigeminal ganglia neurons innervating the mouth and throat express predominantly  $\alpha 3\beta 4$  nAChRs (Flores et al., 1996) as do nodose ganglia neurons innervating the airways (Mao et al., 2006). Bronchial epithelial cells express this subtype, as well as other nAChRs (Maus et al., 1998; Wang et al., 2001). Notably, compared to other subtypes, the  $\alpha 3\beta 4$  nAChR is more resistant to agonist-

induced desensitization (Olale et al., 1997); thus, menthol may augment the desensitization effects of nicotine at these receptors. Indeed, we found that menthol markedly inhibited ACh-evoked currents in nodose sensory neurons apparently by increasing the speed and magnitude of desensitization. The consequences of this effect are not known with certainty, but one possibility is that it could offset the irritant effects of nicotine in the airways, allowing cigarette smoke to be inhaled deeper into the lungs and held there for a longer time. Thus, the known analgesic effects of menthol, acting via sensory nerve TRPM8 (Willis et al., 2011) and TRPA1 channels, may be augmented by enhanced desensitization of sensory nerve nAChRs.

Interestingly, desensitization of brain  $\alpha 3\beta 4$  nAChRs may also be an important component of nicotine addiction. For example, mice null for the  $\beta 4$  nAChR subunit display fewer signs of withdrawal from nicotine (Salas et al., 2004). Accordingly, by augmenting desensitization of brain  $\alpha 3\beta 4$  nAChRs, menthol may similarly delay or blunt signs and symptoms of nicotine withdrawal. Additionally, one hypothesis supporting an underlying mechanism of nicotine addiction predicts that the drive to smoke a cigarette is prompted by a cyclical need to desensitize overactive brain nAChRs, some of which are up-regulated by chronic administration of nicotine (Dani and Heinemann, 1996; Hussmann et al., 2012). In both of these cases, menthol's effect to increase desensitization of the nAChRs underlying addiction and/or withdrawal may actually result in less nicotine being needed to desensitize the overactive receptors and to blunt withdrawal effects. If menthol were to modulate nAChRs in the CNS, then sufficient levels of menthol must accumulate in the brain along with nicotine. Although precise concentrations of menthol in the brains of smokers are unknown the results of an animal study show that menthol can readily penetrate CNS. For example, Pan et al. (2012) showed that

menthol can reach high levels in the brains of mice within 5 minutes of a bolus i.p. injection and was still measurable 60 min after injection.

In conclusion, we have shown that menthol in the presence of ACh or nicotine acts allosterically to augment desensitization of  $\alpha 3\beta 4$  nAChRs, resulting in decreased agonist-induced intracellular  $\text{Ca}^{2+}$  and currents. In addition, menthol appears to prolong the time that the receptor resides in a desensitized state.

## Acknowledgements

We thank Phan Thieu for assistance with cell culture.

## Author contributions

*Participated in research design:* Ton, Kellar and Ahern

*Conducted experiments:* Ton, Smart, Aguilar, Olson and Ahern

*Contributed new reagents or analytic tools:* N/A

*Performed data analysis:* Ton, Kellar and Ahern

*Wrote or contributed to the writing of the manuscript:* Ton, Aguilar, Kellar and Ahern

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## Footnotes

This study was supported by the National Institutes of Health National Institute on Drug Abuse [Grant DA012976] and National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant U01DK101040].



## Figure legends

**Figure 1. Effects of menthol on human  $\alpha 3\beta 4$  nAChR-mediated  $[Ca^{2+}]$  influx and nicotine-stimulated  $^{86}Rb^{+}$  efflux.** (A) Representative Fluo-4 fluorescence images of  $\alpha 3\beta 4$ -expressing HEK293 cells captured during control, repeated application of 30  $\mu M$  ACh (upper images) or application of 30  $\mu M$  ACh alone and ACh plus 100  $\mu M$  menthol (lower images). (B upper trace) Mean Fluo-4 fluorescence during repeated application of ACh (n=39 cells) or (B lower trace) ACh and ACh plus menthol (n=37 cells). The interstimulus interval was 5 min. (C) Inhibition of nicotine-stimulated  $^{86}Rb^{+}$  efflux in the presence of menthol or (D) 10 min preincubation with menthol, n = 3 independent assays.

**Figure 2. Menthol does not compete for the nAChR agonist binding site labeled by [ $^3H$ ]-epibatidine.** Membranes from HEK cells expressing  $\alpha 3\beta 4$  nAChRs were incubated for 2 hours with ~0.5 nM [ $^3H$ ]-EB in the absence or presence of increasing concentrations of menthol or nicotine. The membranes were then filtered and counted. Data were analyzed by nonlinear least square regression analysis. The  $K_i$  of nicotine in these studies was 256 nM. Menthol at concentrations up to 1 mM did not compete for these receptors. Data shown are the mean  $\pm$  of 5 independent assays.

**Figure 3. Menthol enhances the decay of ACh-evoked currents.** Representative inward currents in HEK293 cells expressing  $\alpha 3\beta 4$  nAChRs in response to (A) 30  $\mu M$  ACh and (B) 100  $\mu M$ , with or without menthol (100  $\mu M$ ) and after 60 s washout (A). The holding potential was -50 mV. (C) Menthol (100  $\mu M$ ) alone evokes no current. (D) A 5-minute treatment with menthol (100  $\mu M$ ) alone does not affect the subsequent current evoked by ACh (30  $\mu M$ ). (E) Summary of the peak current evoked by ACh and amount of current decay following 5 minute pretreatment with either control or menthol (n= 3).

**Figure 4. Menthol modulation of  $\alpha 3\beta 4$  nAChRs is dependent on the concentration of ACh.** (A) Representative whole-cell inward currents in response to different concentrations of ACh in the absence (left) and in the presence (right) of 300  $\mu M$  menthol in  $\alpha 3\beta 4$  nAChR expressing cells. The current decay during desensitization was best fit to one or two exponential functions, yielding the indicated time constants. (B) Mean weighted average time constants for decay and (C) fraction of desensitization of the ACh-mediated current obtained in the absence of menthol (open circle) and in the presence of 300  $\mu M$  of menthol (filled circle). Data are mean  $\pm$  SEM, n = 3-5.

**Figure 5. Menthol inhibits  $\alpha 3\beta 4$  nAChRs in a voltage- and use-independent manner and without affecting single channel conductance.** (A) Current voltage relationship for peak response to ACh (30  $\mu$ M) and after 20 s application of menthol (100  $\mu$ M). The background current in the absence of ACh is subtracted. The inset shows the fraction of the menthol versus control current at indicated potentials. (B) Responses to repeated, 5 s stimulation with ACh (30  $\mu$ M) under control conditions (upper trace) or in the presence of 200  $\mu$ M menthol (lower trace). Scale bars, 1 nA and 20 s. (C) Mean peak responses to ACh from experiments depicted in (B) (n=3). (D) Representative single channel currents from a cell-attached patch ( $V_M$ , -110 mV, 10  $\mu$ M ACh in pipette) under control conditions (upper trace) and in the presence of 200  $\mu$ M menthol. (E) All-points histograms constructed from 2 seconds of continuous data. The smooth lines represent best-fits to Gaussian functions yielding similar conductances of 30.9 and 30.3 pS respectively. (F) Mean open time measured from data in E (measured from >50 events).

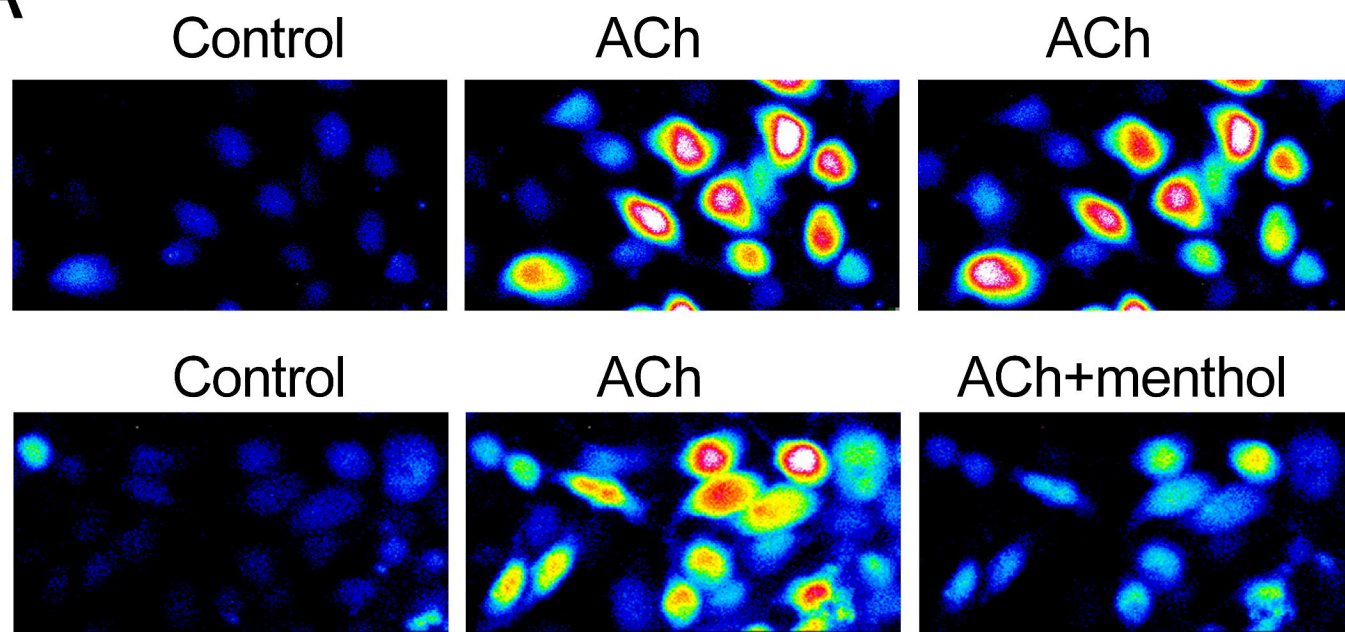
**Figure 6. Concentration-dependent effects of menthol.** (A) Representative currents activated by ACh (30 $\mu$ M) and ACh plus menthol (30  $\mu$ M and 300  $\mu$ M); (B) Bar graph of time constant; (C) The fraction of desensitized receptors; and (D) The current integrals in response to the concentration of menthol from 10 to 300  $\mu$ M, which was co-applied with 30  $\mu$ M ACh. Data are the normalized means  $\pm$  SEM. of 3-6 experiments.

**Figure 7. Menthol enhances the desensitization of the currents evoked by nicotine.** Representative inward currents induced by (A) 1  $\mu$ M, (B) 3  $\mu$ M, (C) 10  $\mu$ M and (D) 100  $\mu$ M nicotine in the absence of menthol (left), presence of 100  $\mu$ M menthol (middle) and 5 min after washout of the menthol (right). Time constant ( $\tau$ ) and the extent of desensitization (D) were used to characterize the desensitization.

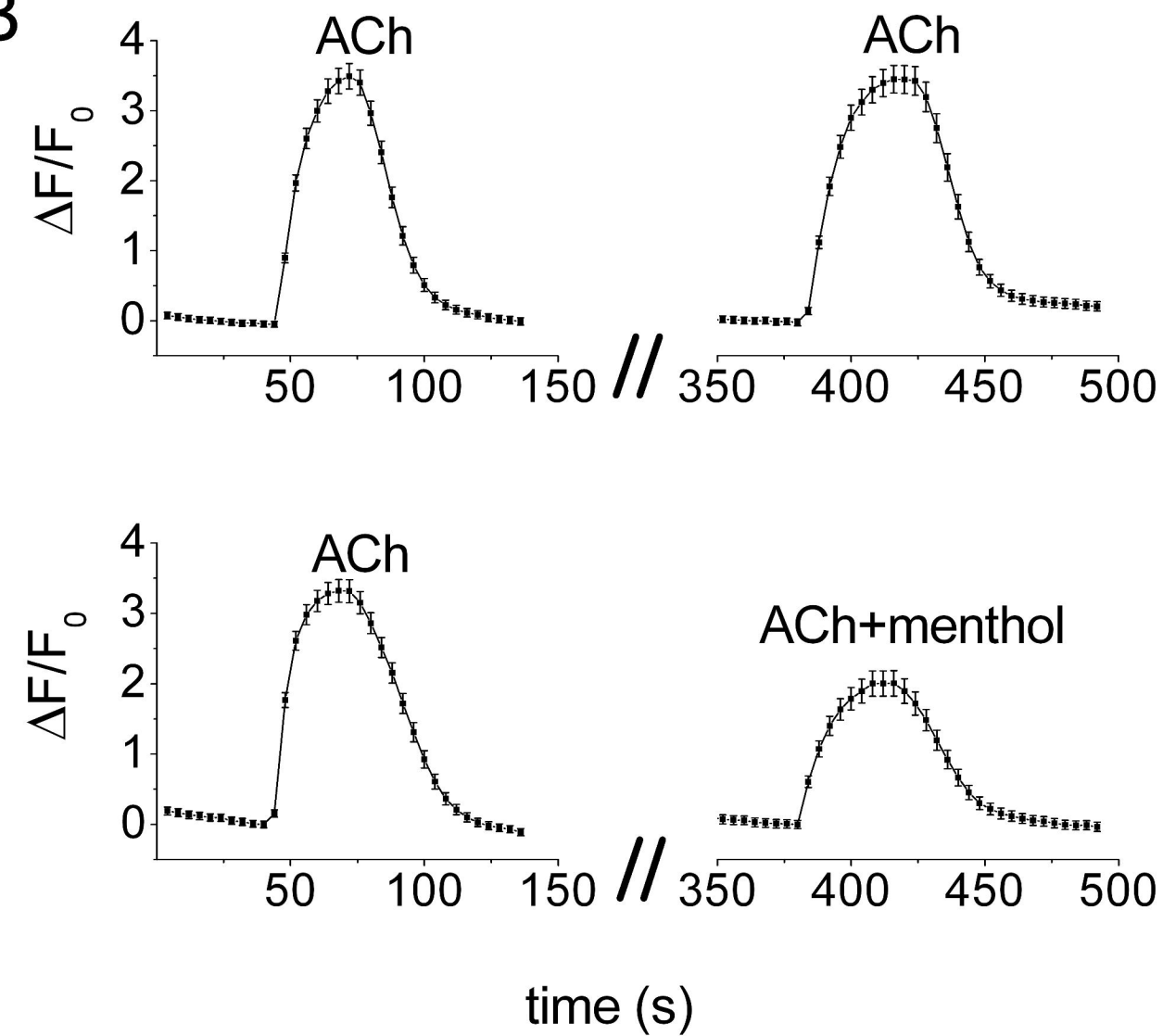
**Figure 8. Menthol hinders the recovery of  $\alpha 3\beta 4$  nAChRs from desensitization.** (A) Representative inward currents showing desensitization induced by co-applied 30  $\mu$ M ACh and 100  $\mu$ M menthol and the subsequent response to ACh following a 5 min wash in either control bath solution (upper traces) or 100  $\mu$ M menthol (lower traces). (B) Time course for recovery following the treatment described in (A), control (open circles) and menthol (closed circles). Data are the means  $\pm$  SEM. of 3-5 experiments. (C) Menthol (100  $\mu$ M) prevents recovery of  $\alpha 3\beta 4$  nAChRs following desensitization with 300  $\mu$ M ACh but receptors almost fully recover after 1 min washout with control solution (n=3).

**Figure 9. Menthol inhibits ACh-evoked currents in nodose ganglion neurons.** Representative current traces in a voltage-clamped neuron evoked by ACh (30  $\mu$ M) and ACh plus menthol (100  $\mu$ M). Note that menthol increases the speed and extent of desensitization. The cell was washed for 60 s between ACh applications.

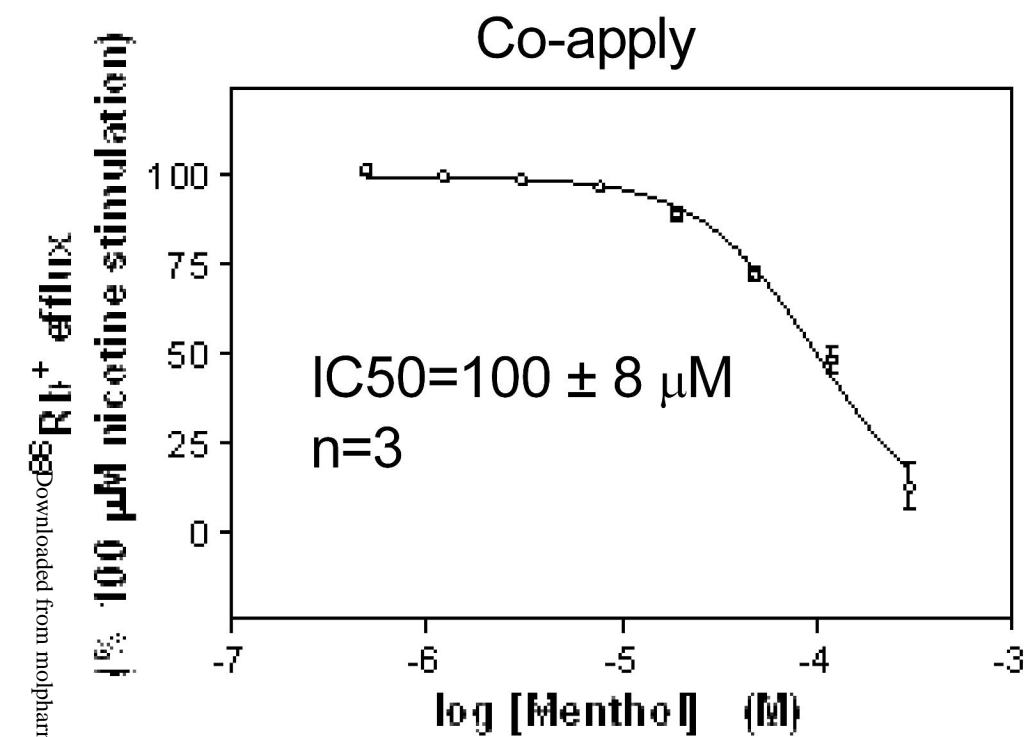
A



B



C



D

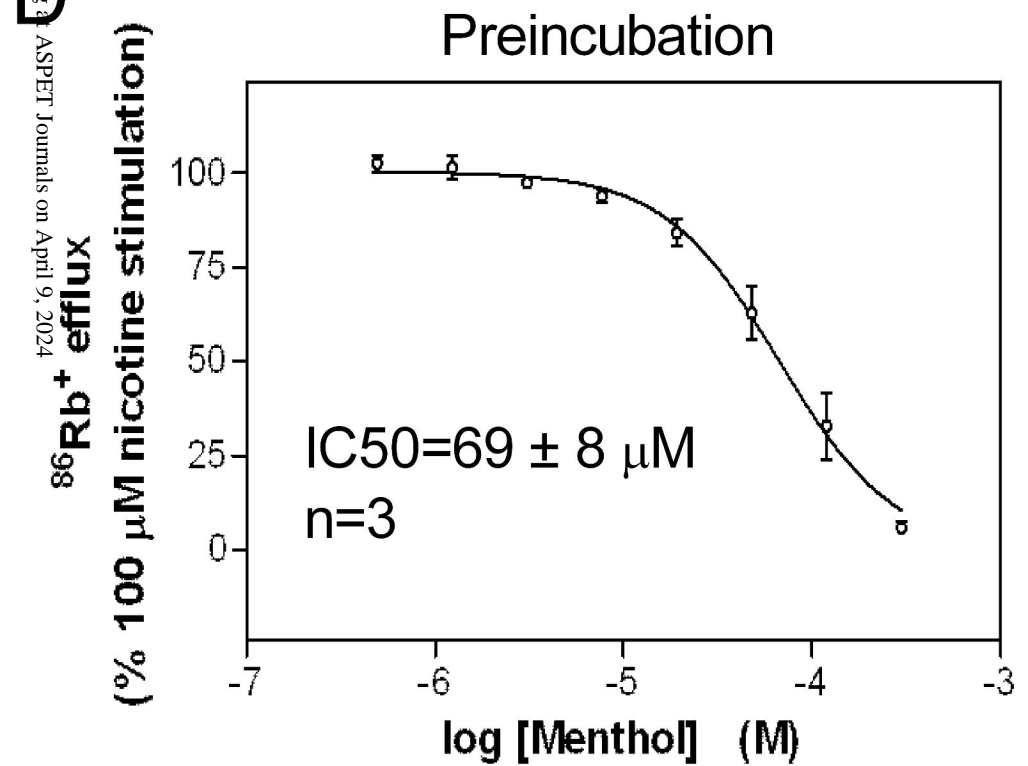


Figure 1

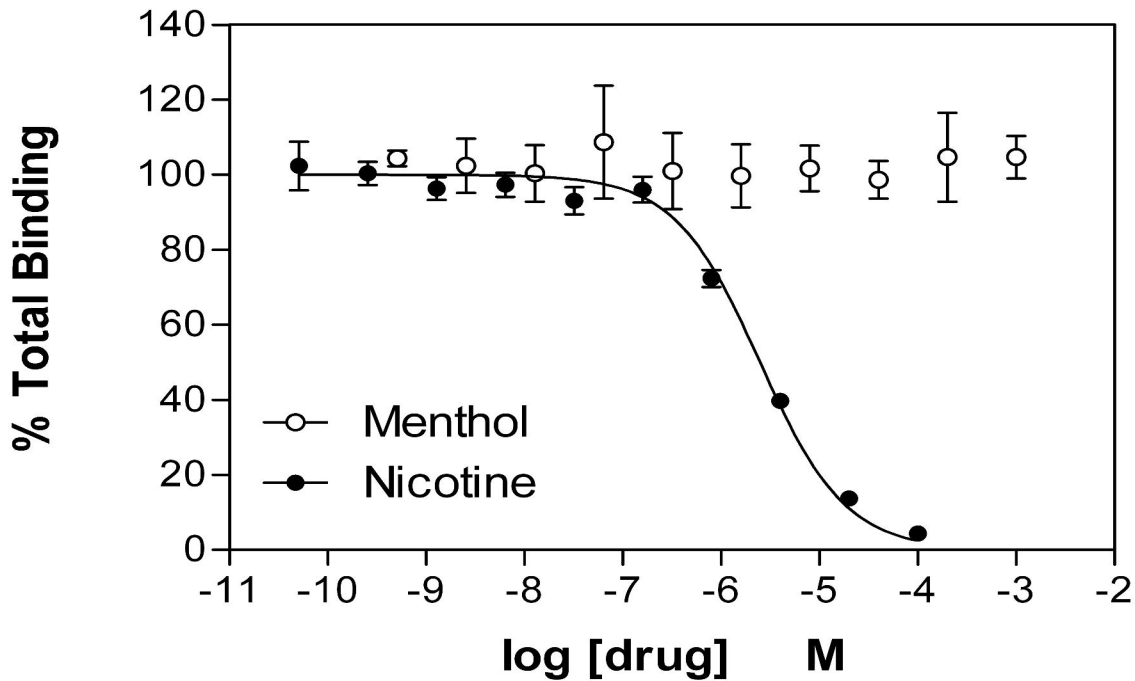


Figure 2

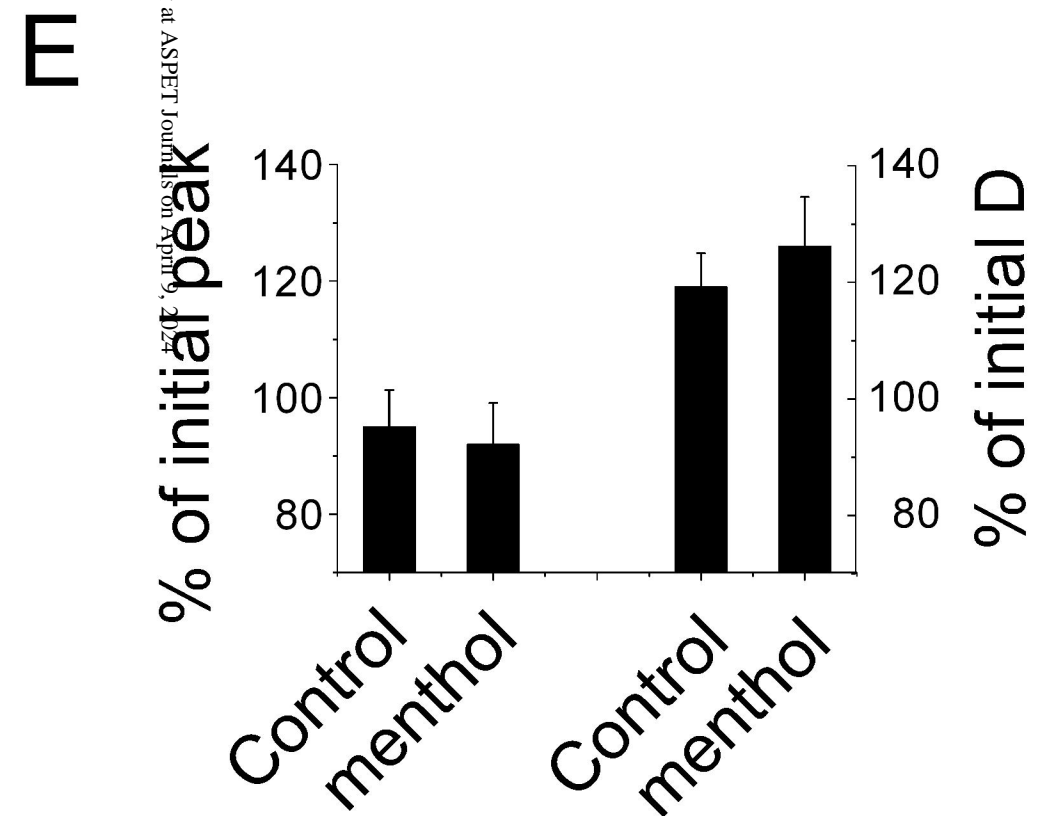
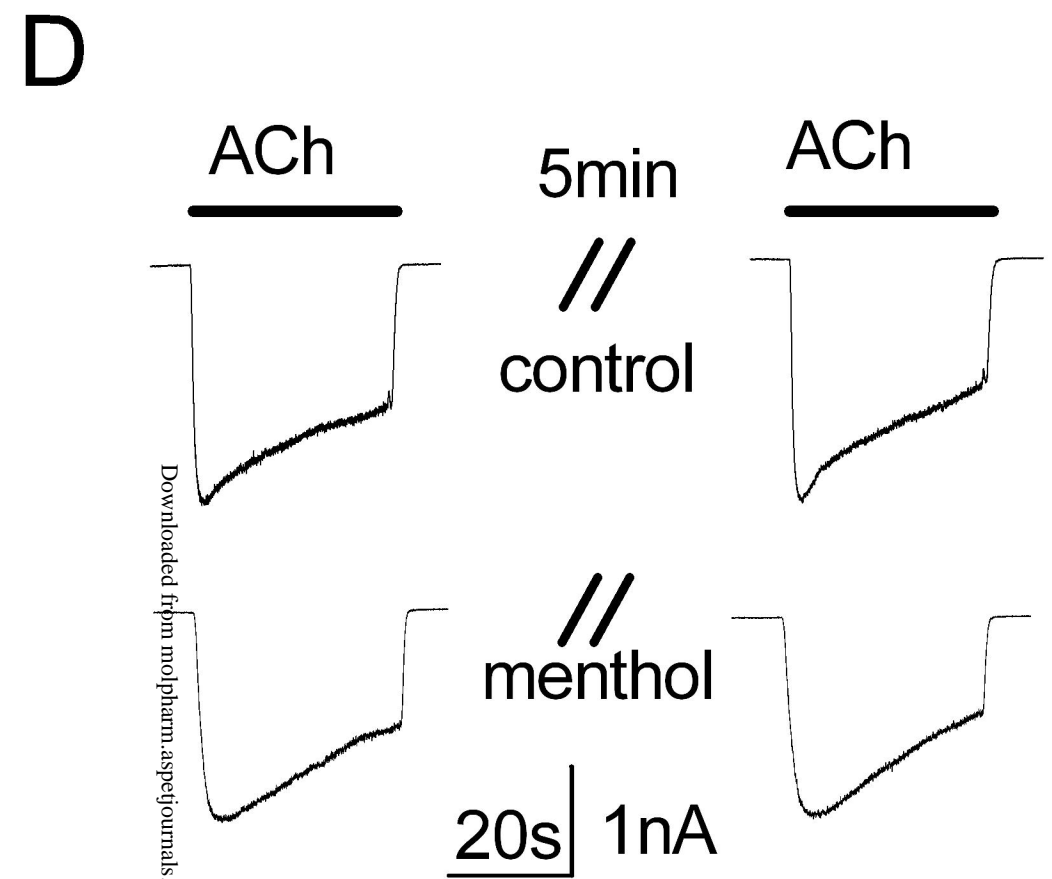
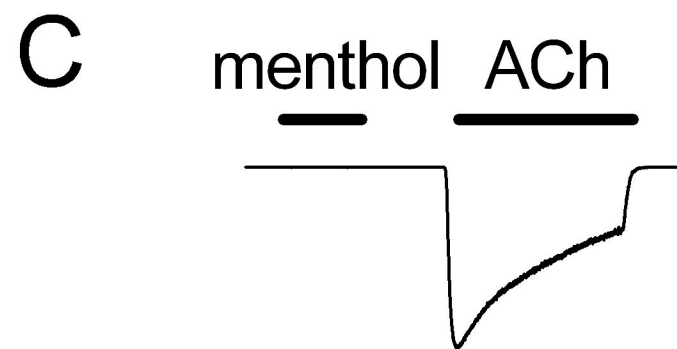
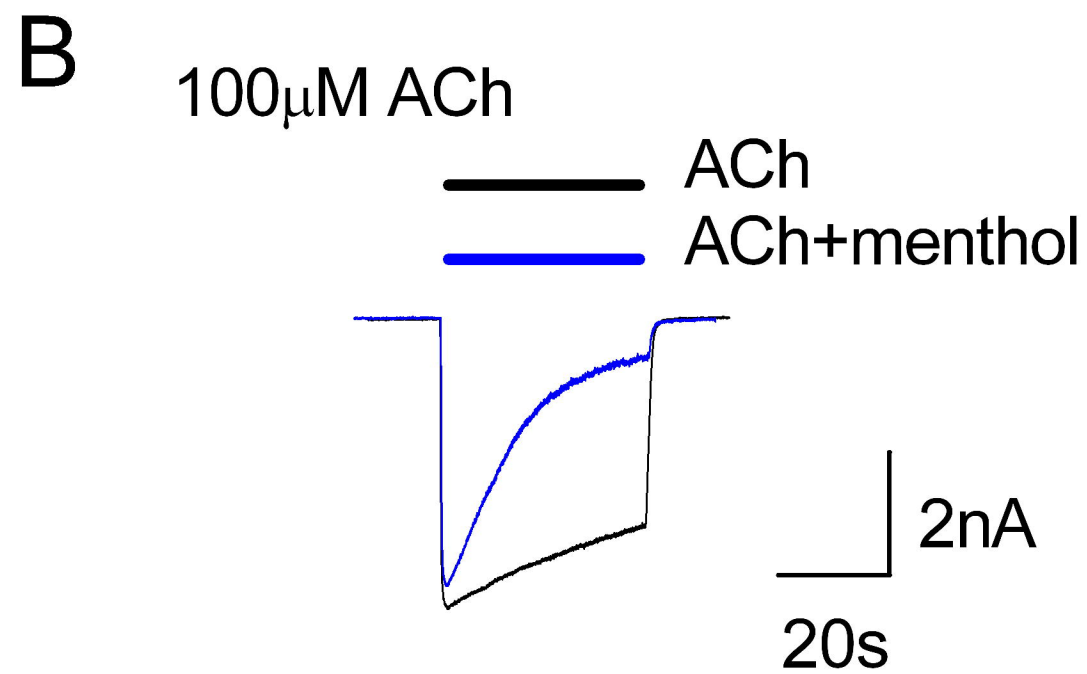
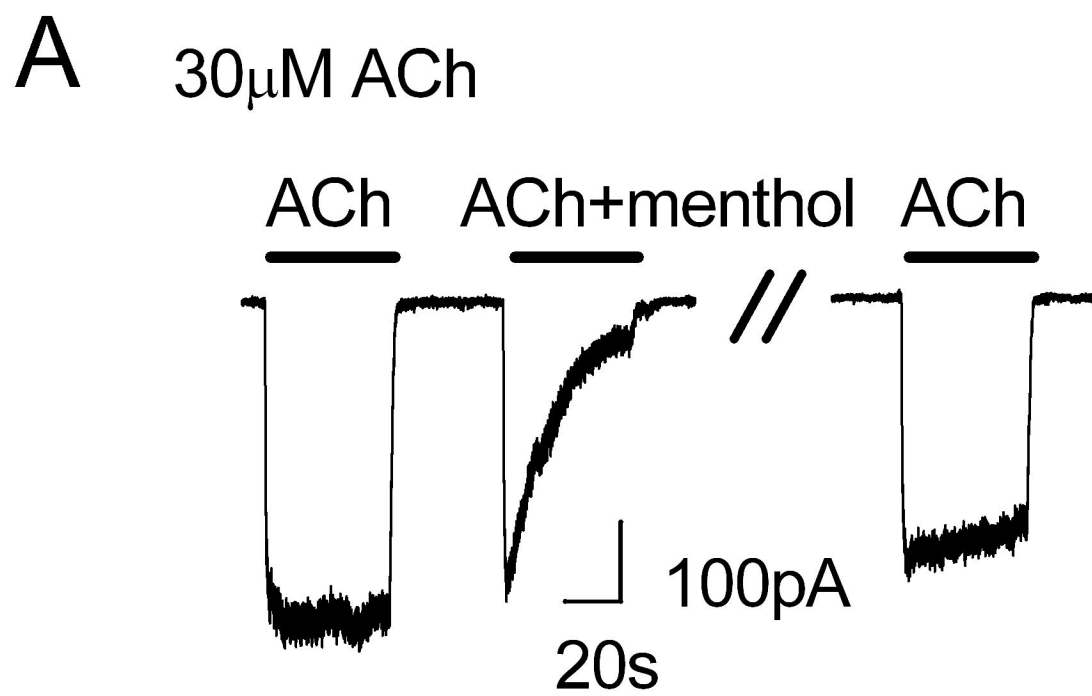
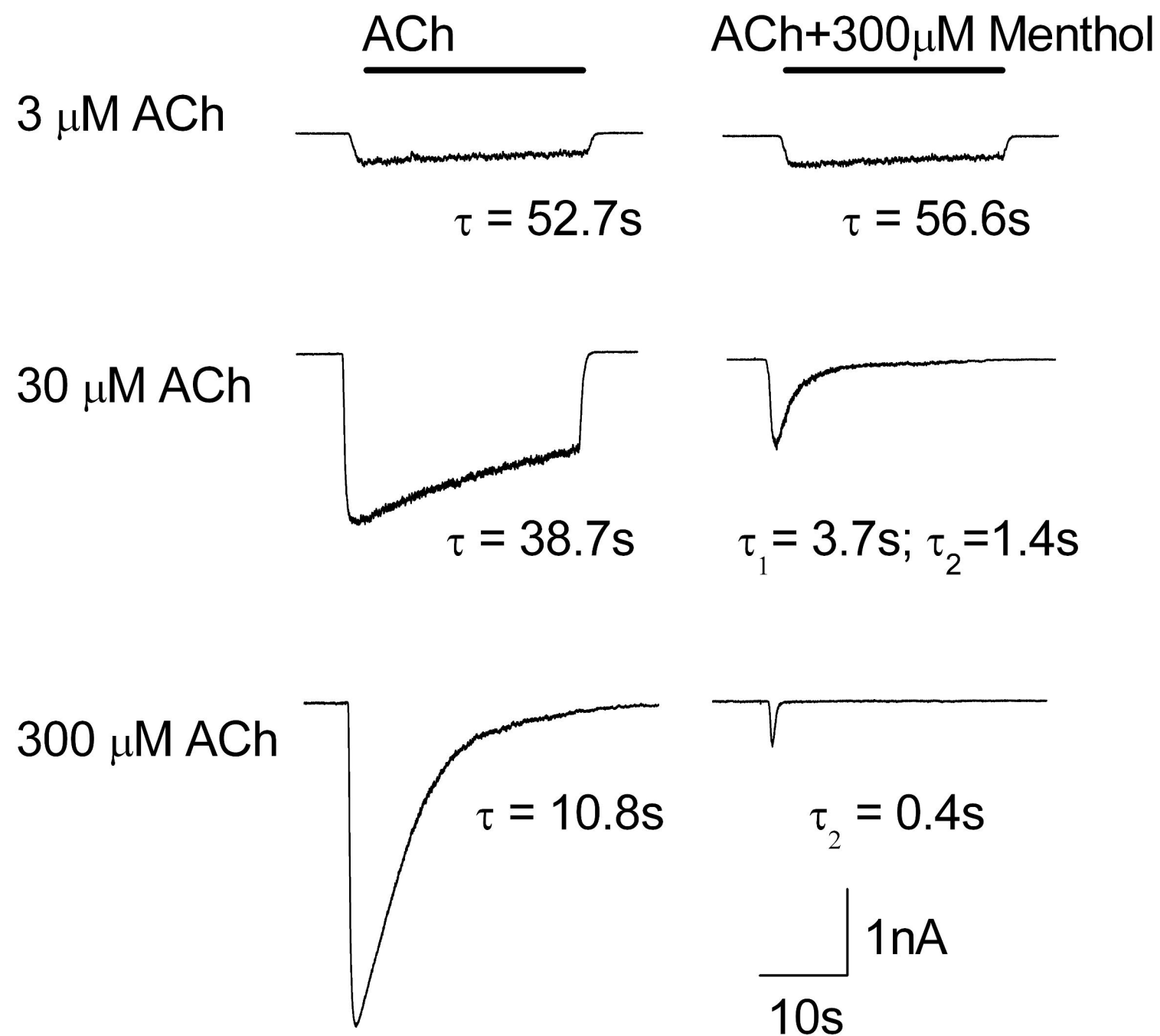


Figure 3

**A**

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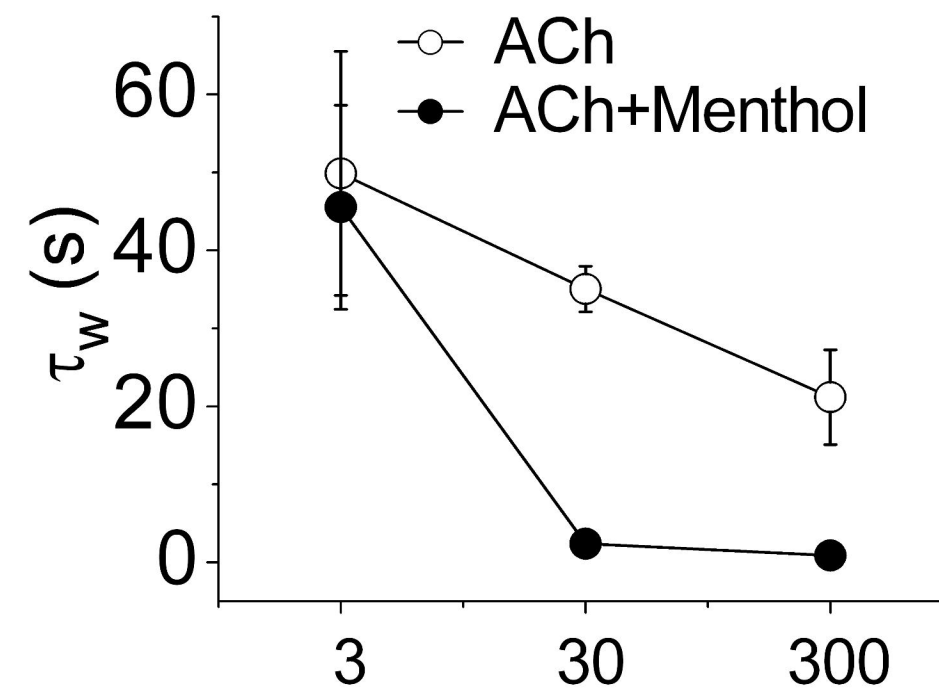
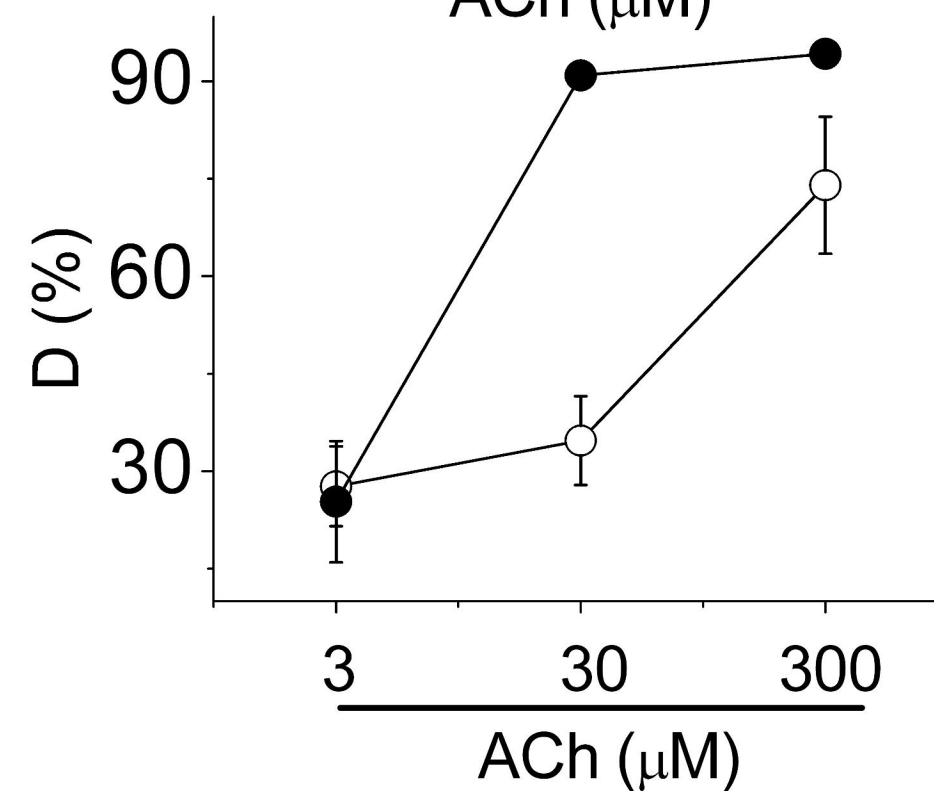
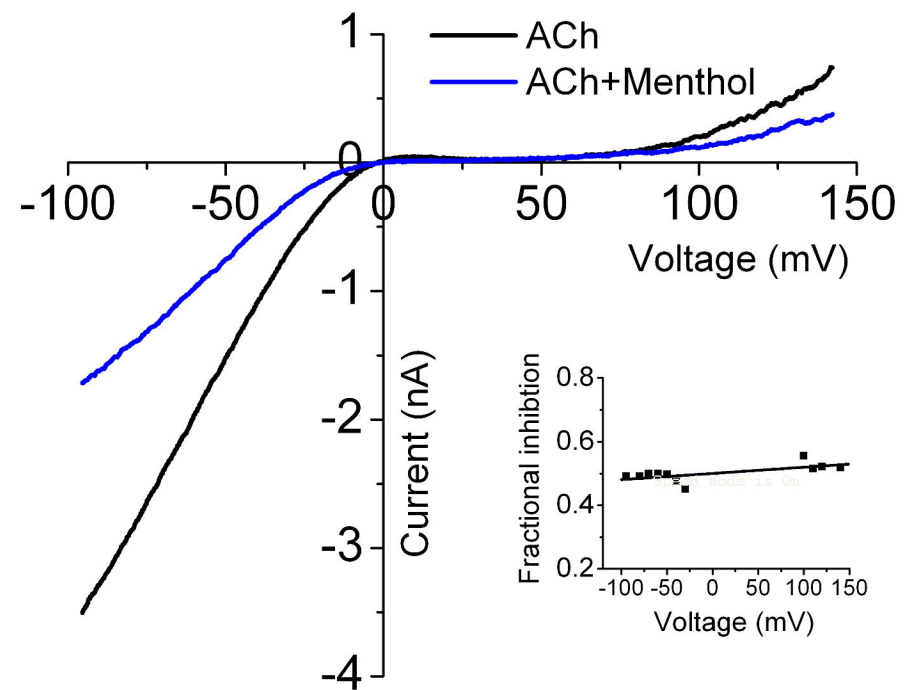
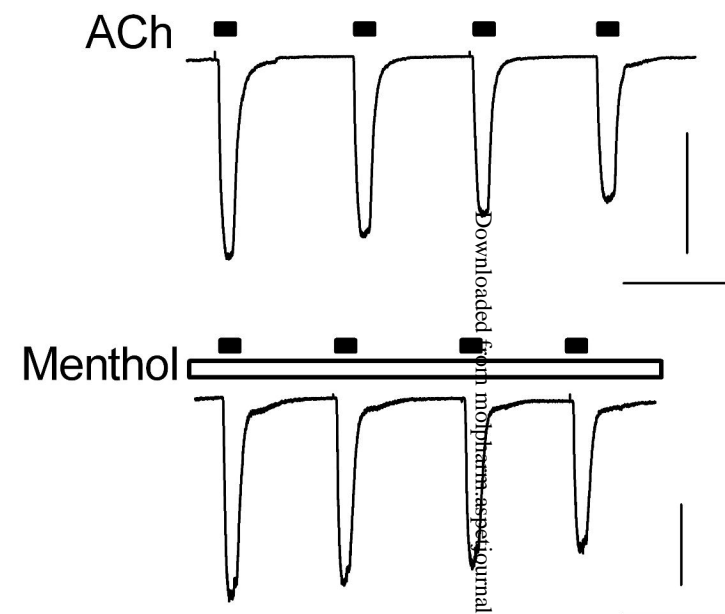
**B****C**

Figure 4

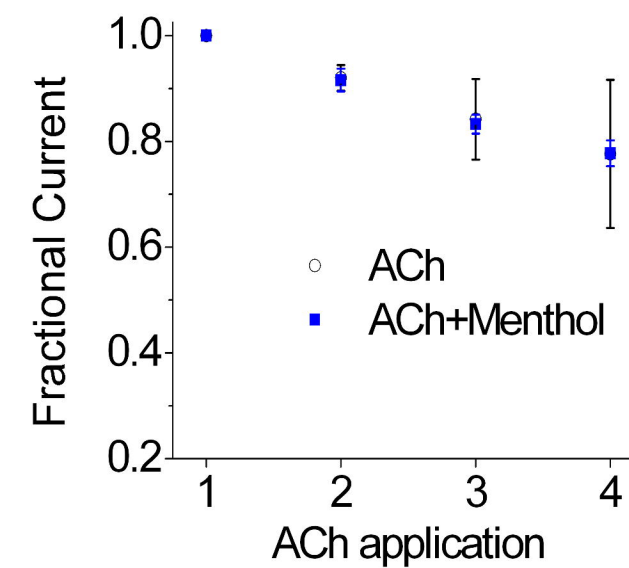
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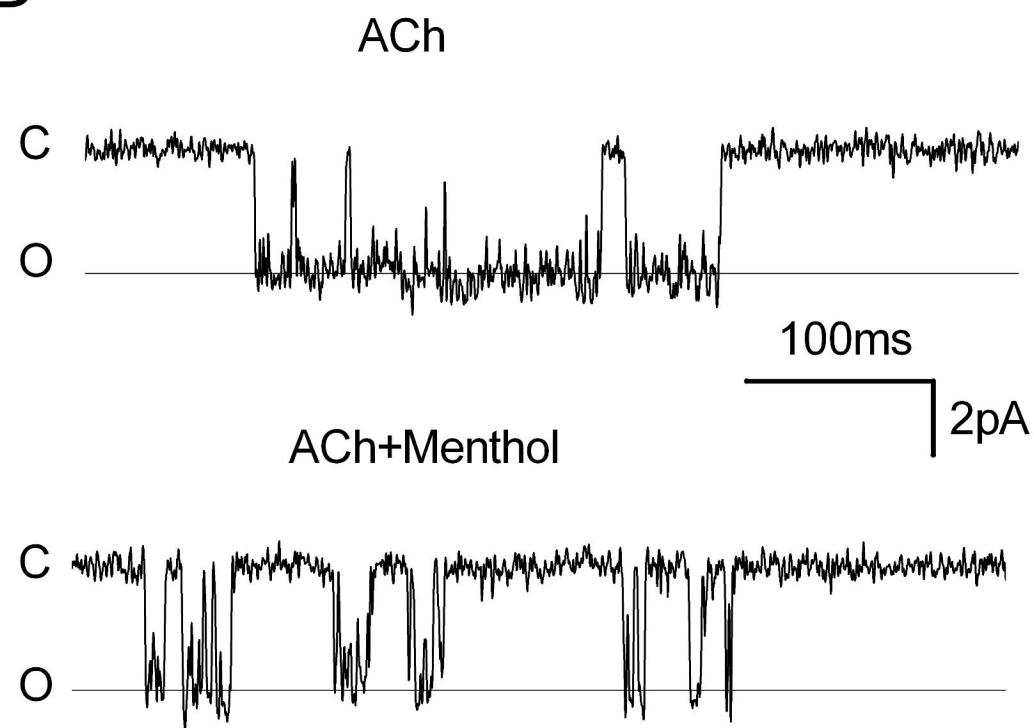
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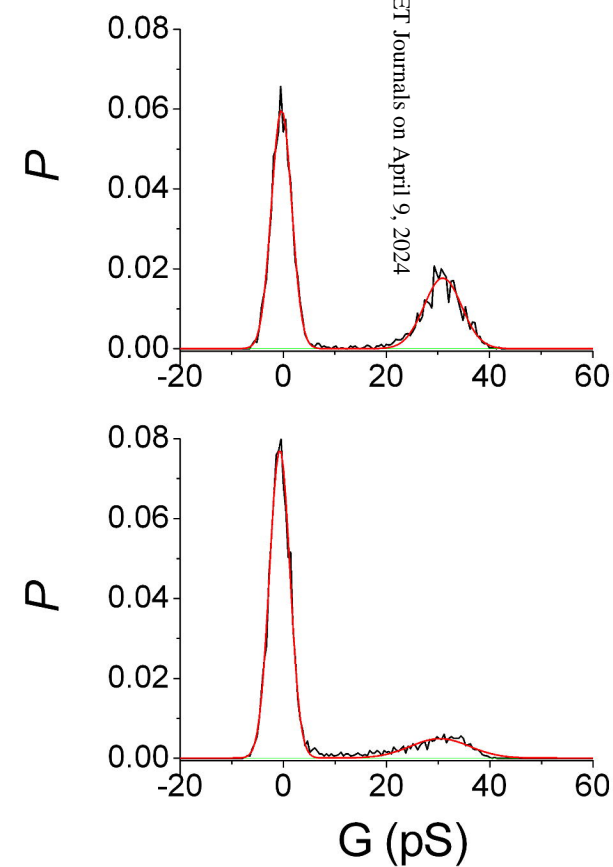
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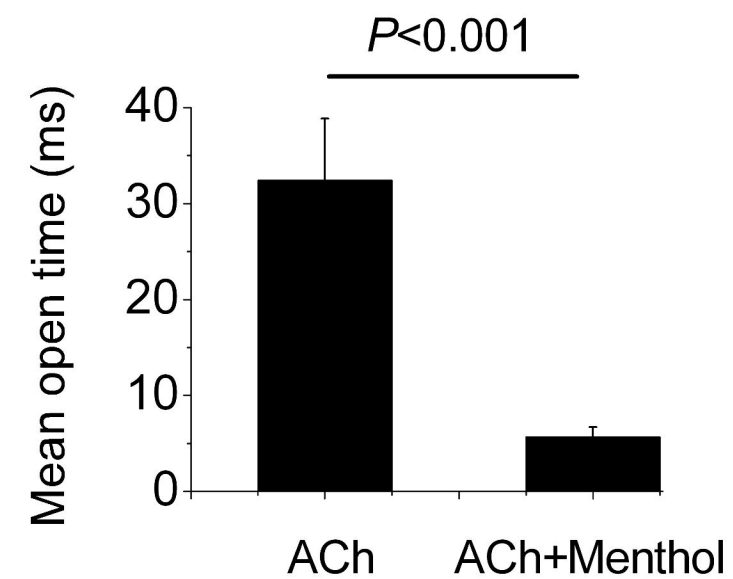
D



E



F





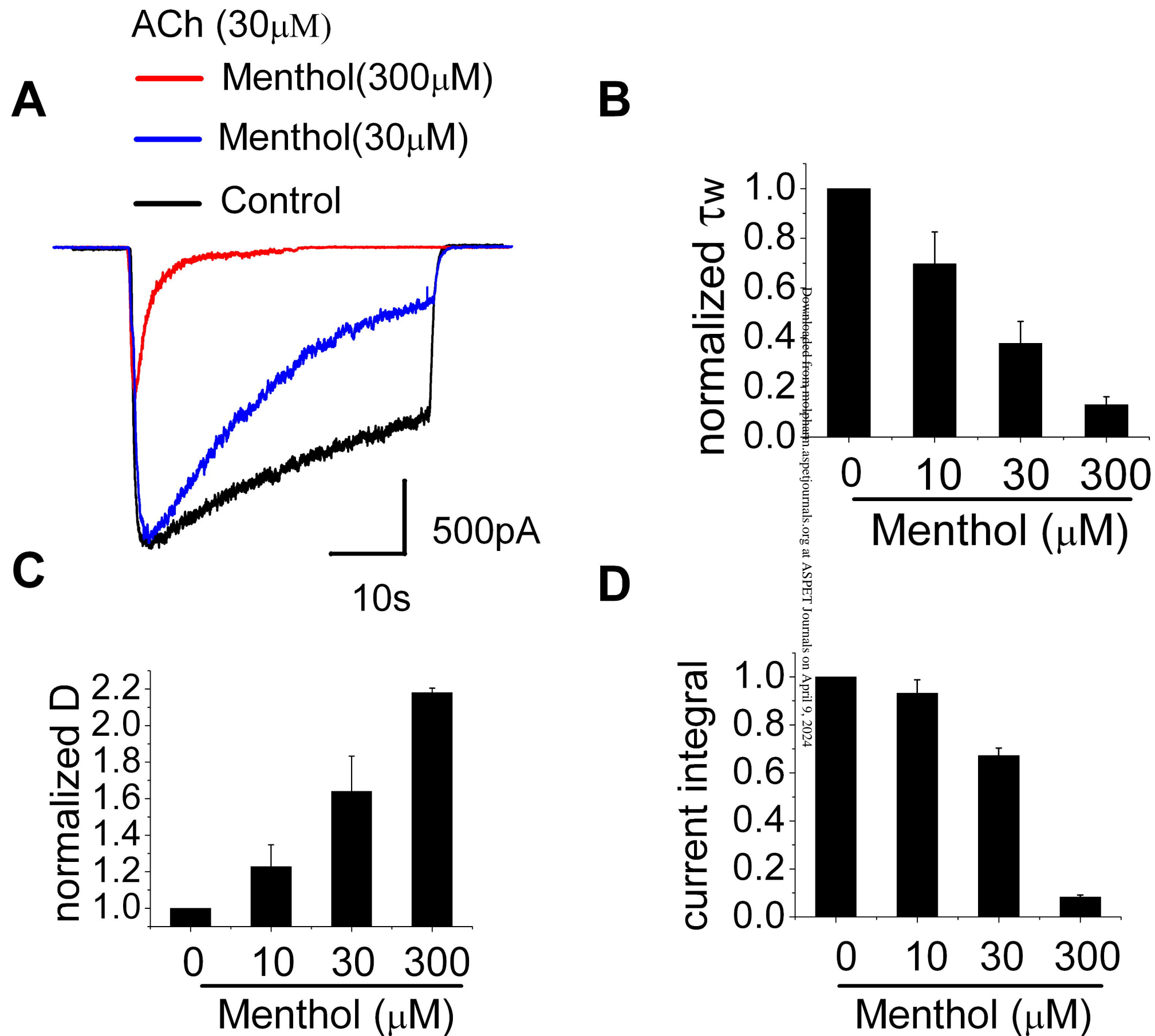


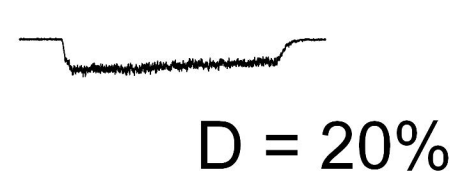
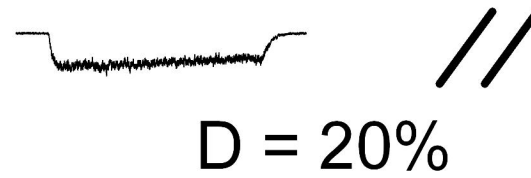
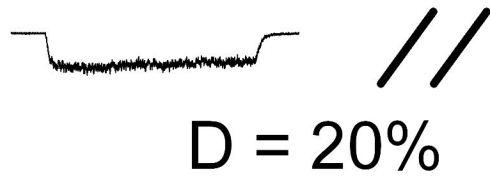
Figure 6

Nicotine

Nicotine+menthol

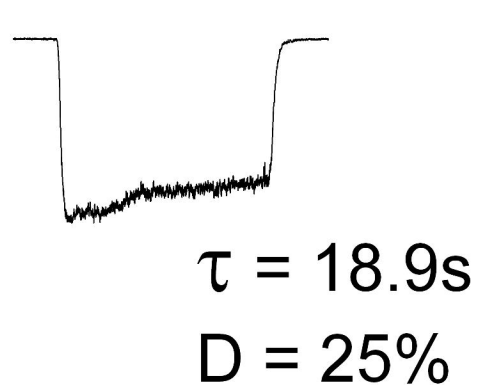
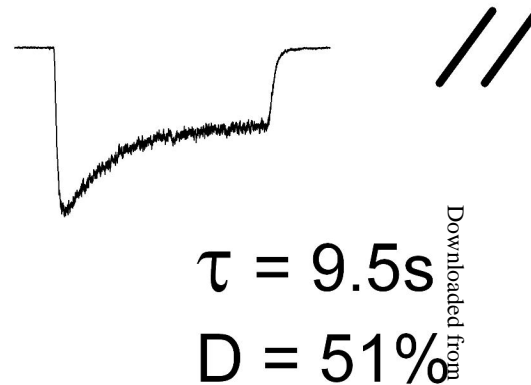
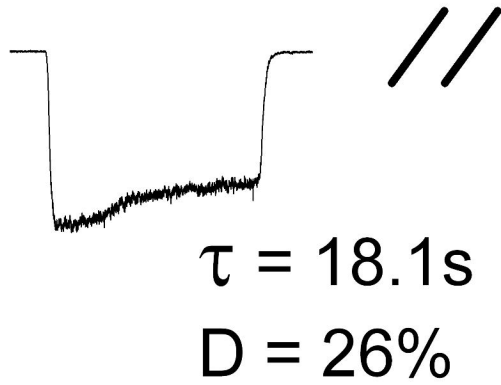
Nicotine

1 $\mu$ M Nicotine



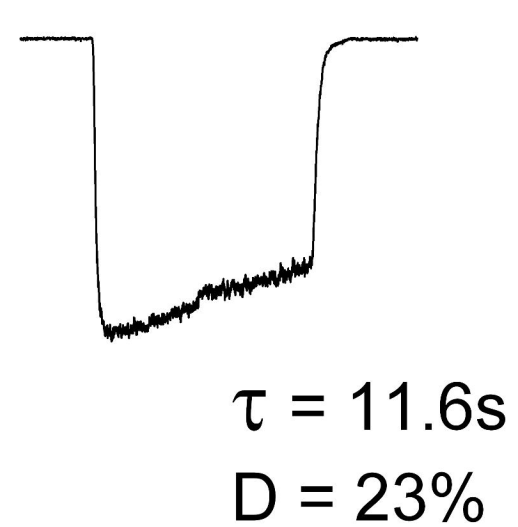
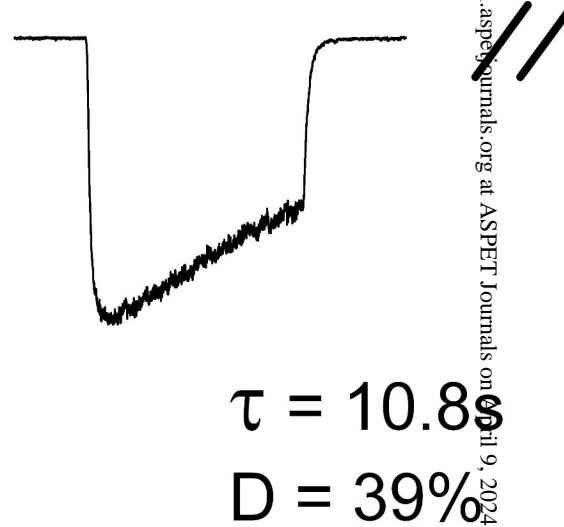
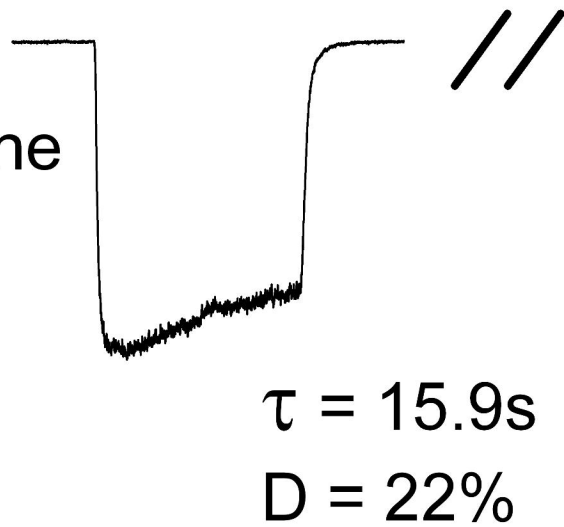
**B**

3 $\mu$ M Nicotine



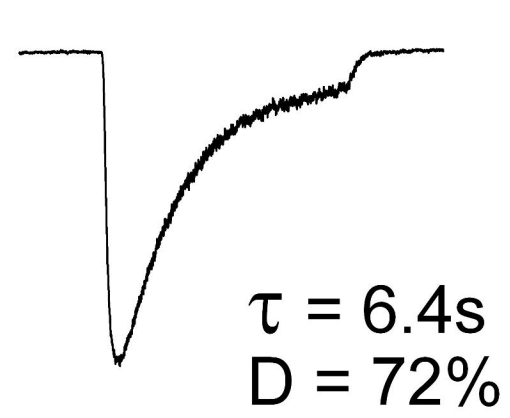
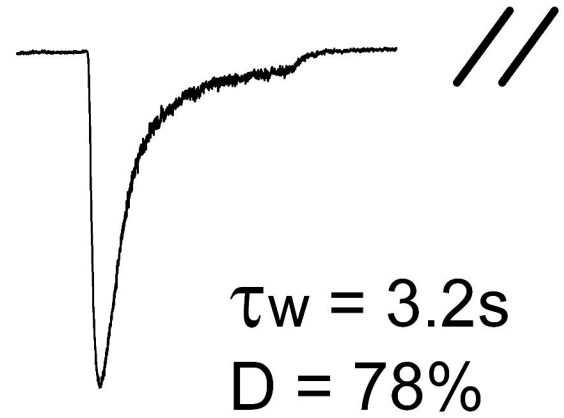
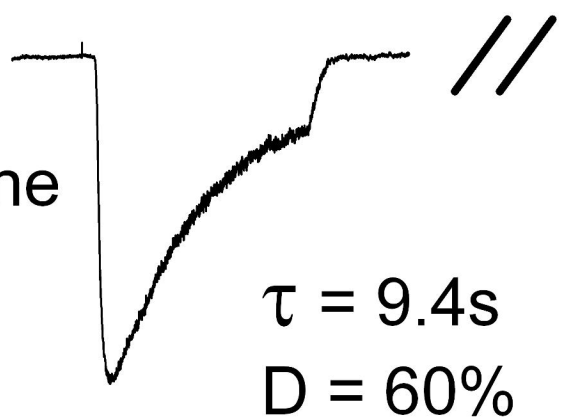
**C**

10 $\mu$ M Nicotine



**D**

100 $\mu$ M Nicotine



500pA  
10s

Figure 7

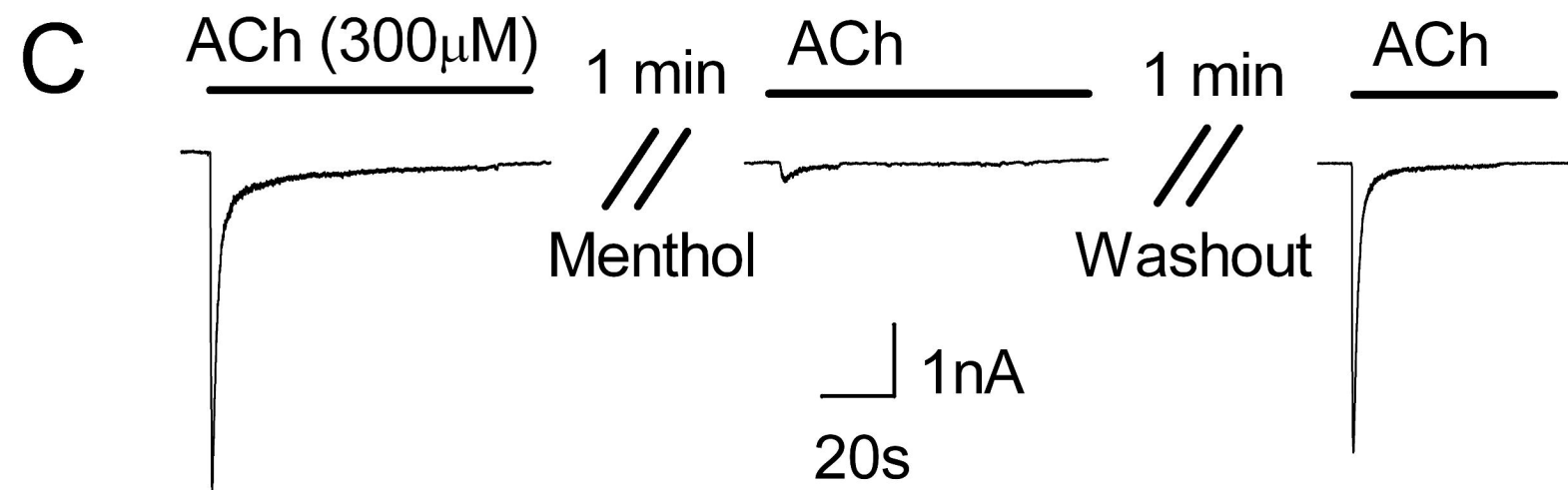
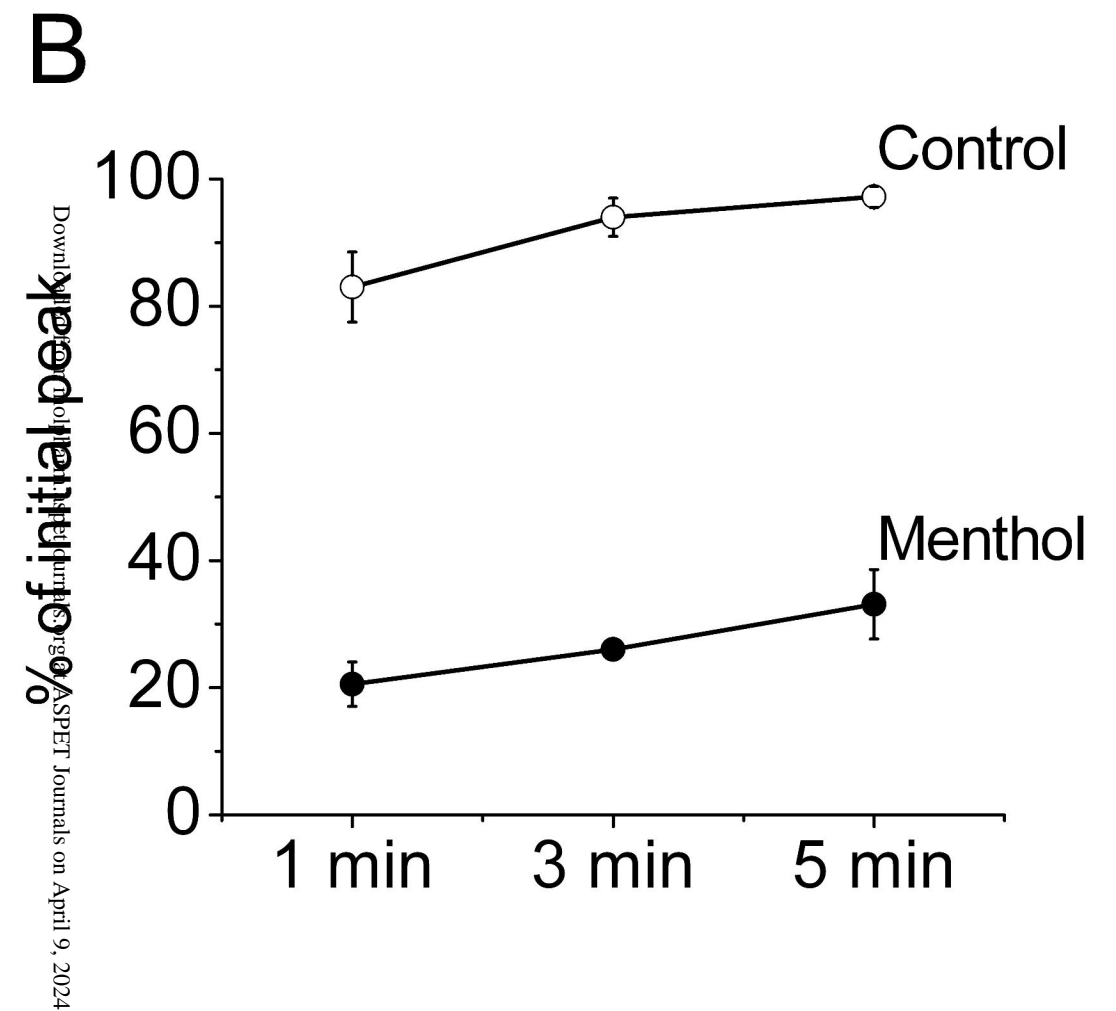
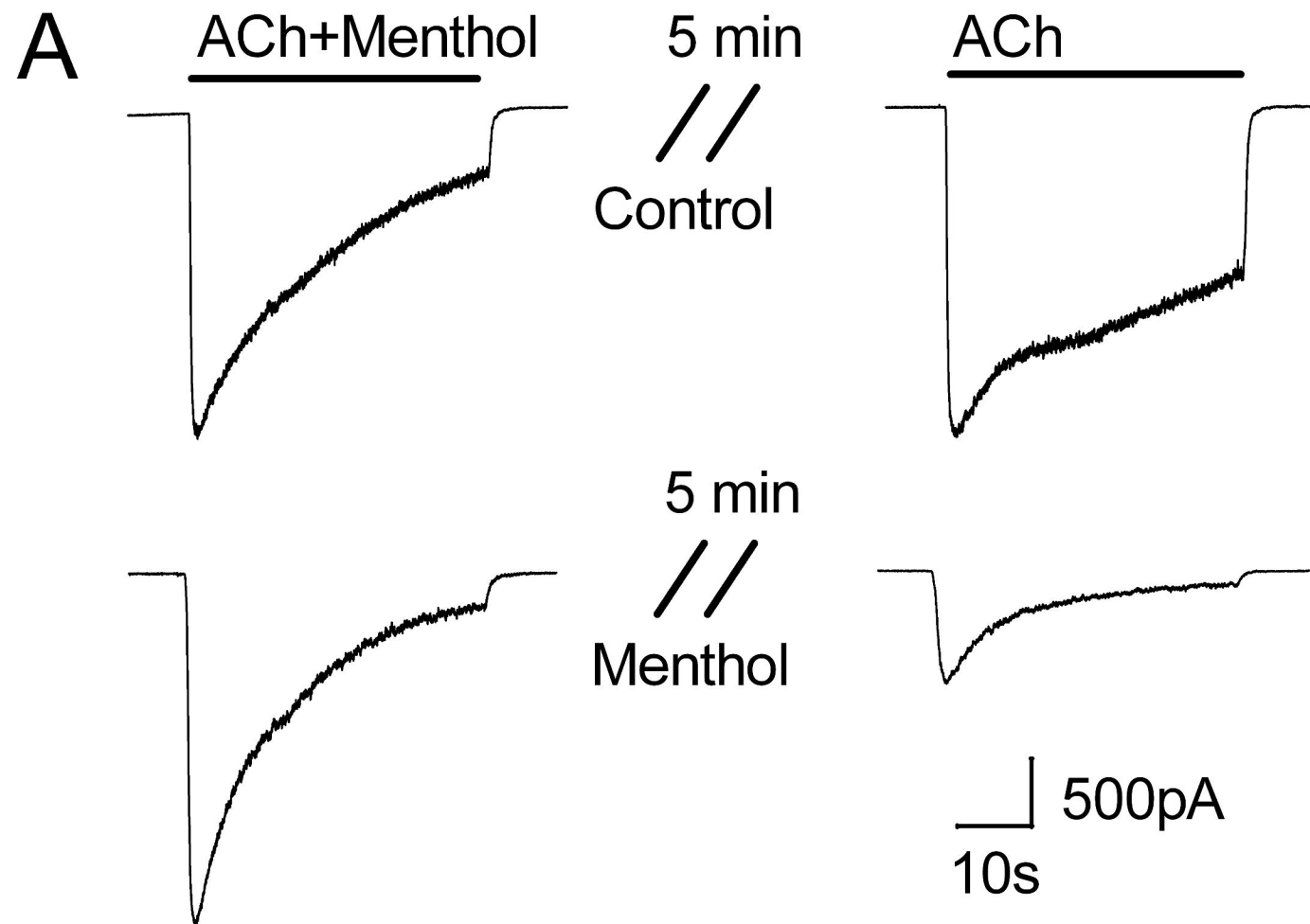


Figure 8

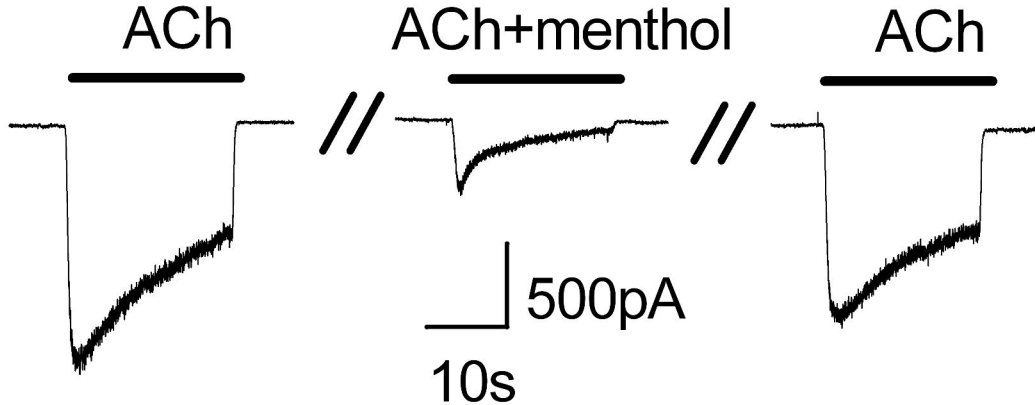


Figure 9