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Nicotine activates the chemosensory cation channel TRPA1

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Topical application of nicotine, as used in nicotine replacement therapies, causes irritation of the mucosa and skin. This reaction has been attributed to activation of nicotinic acetylcholine receptors (nAChRs) in chemosensory neurons. In contrast with this view, we found that the chemosensory cation channel transient receptor potential A1 (TRPA1) is crucially involved in nicotine-induced irritation. We found that micromolar concentrations of nicotine activated heterologously expressed mouse and human TRPA1. Nicotine acted in a membrane-delimited manner, stabilizing the open state(s) and destabilizing the closed state(s) of the channel. In the presence of the general nAChR blocker hexamethonium, nociceptive neurons showed nicotine-induced responses that were strongly reduced in TRPA1-deficient mice. Finally, TRPA1 mediated the mouse airway constriction reflex to nasal instillation of nicotine. The identification of TRPA1 as a nicotine target suggests that existing models of nicotine-induced irritation should be revised and may facilitate the development of smoking cessation therapies with less adverse effects.

Nicotine is a powerful psychoactive drug inducing an addiction that kills about five million people per year as consequence of the noxious effects of tobacco smoke[1]. In addition, nicotine elicits taste and smell sensations and, at increasing concentrations, it produces strong burning, stinging and pain[2]. Notably, all variants of smoking-cessation therapies that are based on nicotine replacement produce local irritation side effects[1,3,4], which has been suggested to reduce treatment compliance and efficacy[5]. It is currently assumed that the irritant effects of nicotine are exclusively mediated by nAChRs expressed in nerve fibers that convey painful stimuli from the skin and mucosa[6–8]. However, some observations are not completely consistent with nAChRs being the sole targets of nicotine. For example, nAChRs quickly desensitize under the high local concentrations of nicotine used in replacement therapies (up to 60 mM), but the irritating effects of nicotine are long lasting.

The TRP superfamily of cation channels is important in chemical nociception[9–11], but no TRP channel has been shown to be directly activated by nicotine. However, nicotine seems to modulate the activity of the vanilloid receptor TRPV1, sensitizing it to capsaicin stimulation[12] and desensitizing it after nicotine-induced activation of nAChRs in sensory neurons[13]. We found that nicotine actually inhibited hTRPV1 (**Supplementary Fig. 1**), indicating that TRPV1 is not directly involved in the irritation caused by nicotine.

The emerging role of the ankyrin-rich channel TRPA1 as a broadly tuned chemosensor[14] prompted us to test whether this channel contributes to the irritating effects of nicotine. TRPA1 is expressed in a subset of polymodal nociceptive neurons[15], determines behavioral responses to noxious cold[16] and to multiple irritant substances such as mustard oil (allyl isothiocyanate)[17,18] and the unsaturated aldehydes contained in cigarette smoke[19], and is involved in inflammatory pain[20,21]. We found that TRPA1 is activated by nicotine and is crucial for the airway constriction reflex caused by application of this compound to the nasal mucosa.

RESULTS

Nicotine activates TRPA1

Extracellular application of nicotine to mTRPA1-expressing Chinese hamster ovary (CHO) cells induced a reversible increase of currents (**Fig. 1a–c**), which was not observed in control cells (**Supplementary Fig. 2**). Stimulation with the nicotine analog anabasine induced a very similar mTRPA1 current activation (**Supplementary Fig. 3**). Cell-attached patch-clamp recordings (**Supplementary Fig. 4**) and Fura-2–based measurements of the intracellular Ca^{2+} concentration (**Supplementary Fig. 5**) further confirmed that nicotine activated mTRPA1. In addition, nicotine activated hTRPA1 that we transfected into CHO cells (**Supplementary Fig. 2**).

We consistently found that the magnitude of the nicotine-activated TRPA1 current declined above ~300–1,000 μM, particularly at positive potentials (**Fig. 1b–d**). Notably, the inhibitory effect reversed much faster than the activating effect, which explains the current rebound observed on washout of high doses of nicotine. This differential kinetics allowed us to dissect the activating and inhibitory effects of nicotine (**Supplementary Fig. 6**), yielding a maximal relative current increase of 4.0 ± 0.3 and 2.7 ± 0.2 at -75 and $+50$ mV,

Figure 1 Activation of TRPA1 by nicotine. (**a**–**c**) Time course of the effects of nicotine on the amplitude of mTRPA1 currents measured at −75 and +50 mV. The horizontal lines indicate the periods of extracellular application of nicotine at the indicated concentrations. The colored data points correspond to the current traces shown to the right. (**d**) Dose-dependent modulation of mTRPA1 currents by nicotine. Data points represent the relative change of the current amplitude with respect to the values in the control condition. The continuous lines represent the fit with a bimodal function comprising stimulatory and inhibitory components (see Online Methods). Error bars represent s.e.m. (**e**) EC_{50} and IC_{50} of nicotine on mTRPA1 currents at −75 mV (solid columns) and +50 mV (empty columns). Error bars represent fitting errors.

respectively, and the concentrations for half-maximal activation (EC_{50}; **Fig. 1e**) and half-maximal inhibition (IC_{50}; **Fig. 1e**).

As the response to nicotine occurred with a notable delay in whole-cell (**Fig. 1a–c**) and cell-attached (**Supplementary Fig. 4**) patch-clamp experiments, as well as in intracellular Ca^{2+}-imaging experiments (**Supplementary Fig. 5**), we predicted that nicotine acts indirectly on the channel via a mechanism involving other membrane receptors and/or intracellular signaling pathways. To investigate this possibility, we tested the action of nicotine application to the intracellular side of the membrane in cell-free inside-out patches in the absence of extra- and intracellular Ca^{2+}. Under this condition, 1 mM nicotine was still able to activate single mTRPA1 channels. These channels had a conductance of 112 ± 8 pS at −75 mV ($n = 5$) and were reversibly blocked by 1 mM menthol (**Fig. 2**), consistent with previous studies[22–26]. Notably, nicotine-induced activation was substantially faster in inside-out patches than in whole-cell recordings. This indicates that the delay in TRPA1 activation on extracellular application of nicotine reflects, at least partly, the rate of nicotine diffusion across the plasma
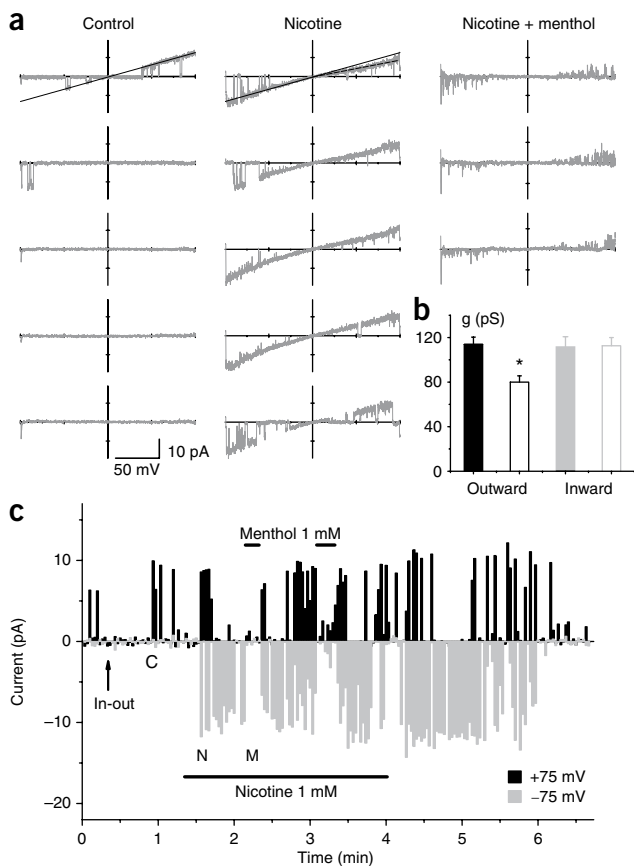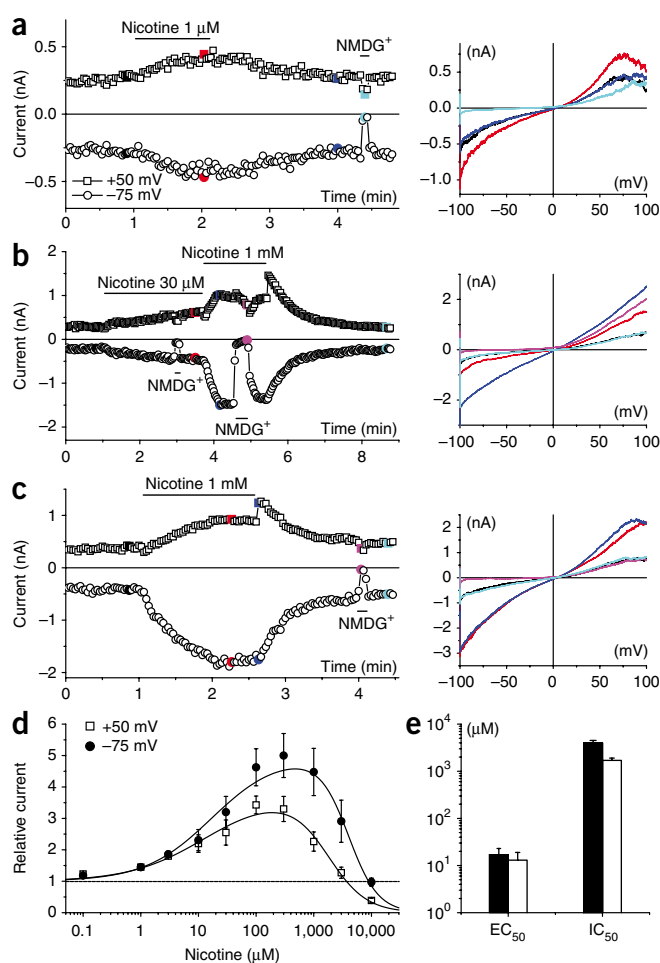
membrane. Nicotine caused a significant reduction of the single channel conductance at positive potentials, from 114 ± 7 pS in controls to 80 ± 6 pS in the presence of 1 mM nicotine ($n = 5$, $P = 0.004$). Taken together, these data indicate that nicotine interacts with TRPA1 in a membrane-delimited manner.

Activation of TRPA1 by electrophiles, such as mustard oil, cinnamaldehyde and acrolein, involves covalent modification of N-terminal cysteine residues[27,28]. Thus, we tested nicotine's effect on an mTRPA1 mutant in which the critical cysteine residue C622 is mutated to serine. Consistent with previous work[27,28], this mutant was unresponsive to mustard oil (20 μM), but was activated by 1 mM nicotine (**Supplementary Fig. 7**). These results indicate that nicotine-induced

Figure 2 Nicotine activates TRPA1 in cell-free inside-out patches. (**a**) Examples of single mTRPA1 channel currents elicited by a voltage ramp from −100 to +100 mV in the control condition (left) or with the addition of 1 mM nicotine (center) or 1 mM nicotine plus 1 mM menthol (right). Note that in the upper panels the single channel conductance at positive potentials was larger in the control condition than it was in the presence of nicotine (solid line versus dashed line). Menthol application produced strong current inhibition, showing the typical flickering pattern of block[23]. (**b**) Average single channel conductance for the inward (black) and outward (gray) currents in control (solid) and in the presence of 1 mM nicotine (empty, $n = 5$). Error bars represent s.e.m. The asterisk indicates statistically significant difference from control ($P = 0.004$). (**c**) Time course of the amplitudes of currents at −75 and +75 mV. The arrow indicates the moment of patch excision. The labels C, N and M mark the time points corresponding to the traces shown in **a**: C is the control, N is nicotine addition, and M is the addition of both nicotine and menthol.

Figure 3 Cross desensitization of TRPA1 activation by nicotine and mustard oil. (**a**,**b**) Examples of the effect of 100 µM mustard oil (MO) on the amplitude of mTRPA1 currents at −75 and +50 mV without (**a**) or with (**b**) pre-application of 100 µM nicotine. (**c**) Nicotine did not affect mTRPA1 currents after stimulation with 100 µM mustard oil. (**d**) Maximal average current amplitudes at −75 and +50 mV elicited by mustard oil without (n = 8) or with (n = 7) pre-application of nicotine and by nicotine without (n = 7) or with (n = 5) pre-application of mustard oil. Error bars represent s.e.m.

activation does not involve modification of cysteine 622, which is fully in accordance with the nonelectrophilic character of nicotine.

Activation of TRPA1 by other nonelectrophilic chemicals, such as menthol, clotrimazole and nifedipine, or by cold temperatures involves a shift of the voltage dependence of channel activation toward negative voltages[23,25,26]. To examine whether nicotine acts in a similar manner, we applied a voltage-step protocol that allowed us to assess the voltage dependence of activation from the measurement of peak tail currents. In these experiments, Ca^{2+} was omitted from the extracellular solution and 2 mM EDTA was added to avoid any interference of Ca^{2+} ions with the effect of nicotine on TRPA1 activation[29,30]. In the absence of nicotine, clear tail currents were only observed at prepulse potentials greater than +50 mV (**Supplementary Fig. 8**). In the presence of 300 µM nicotine, tail currents were already obvious at prepulse potentials above 0 mV. We fitted average tail currents in
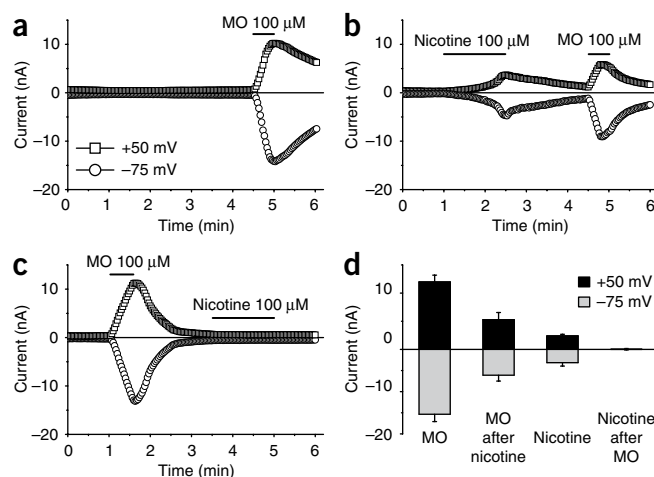
the presence of 300 µM nicotine with a Boltzmann function (see Online Methods), which yielded a voltage for half maximal activation (V_{act}) of 162 ± 4 mV and a slope factor (s_{act}) of 36.9 ± 1.5 mV. In the absence of nicotine, tail currents were far from saturation at the most depolarizing prepulse potentials (+200 mV), which precluded the accurate independent estimation of V_{act} or s_{act}. However, assuming a constant s_{act}, the nicotine-induced changes in tail currents can be accounted for by a 51-mV shift of the activation curve toward negative voltages (**Supplementary Fig. 8**). In principle, such a leftward shift of the activation curve can be a result of a stabilization of the open state, a destabilization of the closed state or both. We observed that nicotine decreased the rate of whole-cell current deactivation at negative voltages and increased the rate of activation and very positive potentials (**Supplementary Fig. 8**). This is consistent with an increased mean open time and a reduced mean close time in the presence of nicotine (**Supplementary Fig. 4**).

Oral application of mustard oil is known to prevent later stimulation with other irritant chemicals including nicotine, a phenomenon known as cross-desensitization[31]. Thus, we tested whether nicotine-induced activation of TRPA1 influences the effect of mustard oil and vice versa. Indeed, pre-activation with 100 µM nicotine significantly blunted ($P < 0.01$) a subsequent response to 100 µM mustard oil (**Fig. 3**). Conversely, pre-activation of mTRPA1 with mustard oil fully
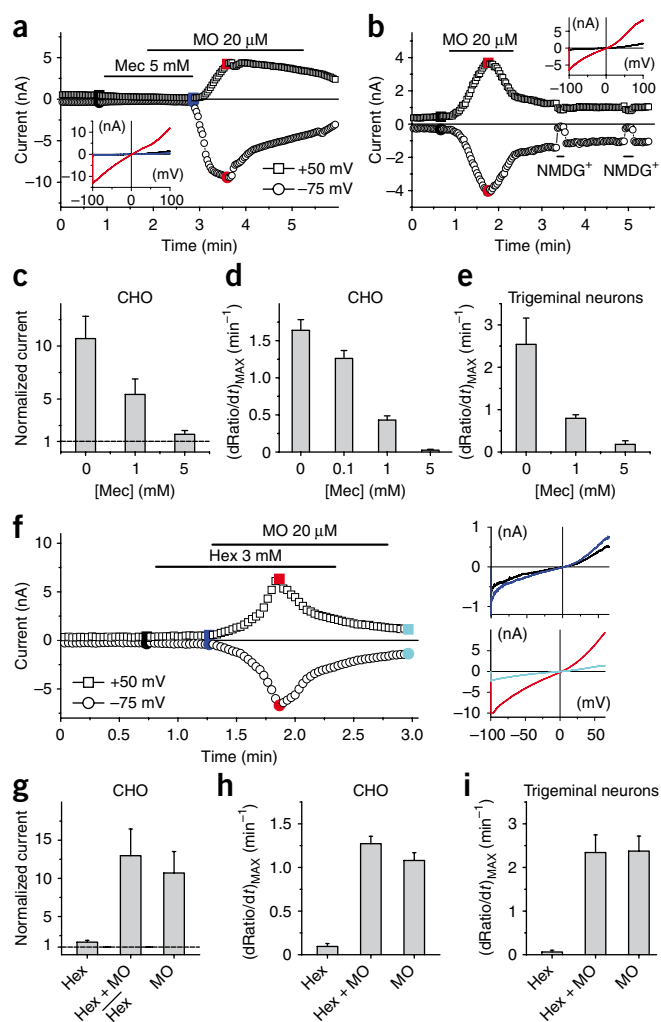

Figure 4 TRPA1 activation is prevented by the nAChR inhibitor mecamylamine, but is unaffected by hexamethonium. The colored data points correspond to the current traces shown in the inserts. (**a**,**b**) Effect of mustard oil on mTRPA1 currents, without (**a**) or with (**b**) pre-application of mecamylamine (Mec) in CHO cells. (**c**) Maximal mustard oil–induced currents (relative to the amplitude in control) at −75 mV in the absence (n = 7) and in the presence of 1 mM (n = 7) or 5 mM (n = 5) mecamylamine in CHO cells. (**d**,**e**) Maximal amplitude of the first time derivative of the intracellular Ca^{2+} signal elicited by 20 µM mustard oil in the absence and in the presence of mecamylamine in mTRPA1-expressing CHO cells (n = 22–35, **d**) or wild-type mouse trigeminal neurons (n = 12–18, **e**). (**f**) Amplitude of mTRPA1 currents at −75 and +50 mV during extracellular application of hexamethonium (Hex) and mustard oil in CHO cells. (**g**) Maximal amplitude of mTRPA1 currents (relative to the amplitude in control) at −75 mV in the presence of hexamethonium (n = 8), hexamethonium plus mustard oil (n = 8), and mustard oil (n = 7). (**h**,**i**) Maximal amplitude of the first time derivative of the intracellular Ca^{2+} signal elicited by 20 µM mustard oil in the absence or presence of 3 mM hexamethonium in mTRPA1-expressing CHO cells (n = 14 in mustard oil and n = 41 in mustard oil plus hexamethonium, **h**) or wild-type mouse trigeminal neurons (n = 28 in mustard oil and n = 35 in mustard oil plus hexamethonium, **i**). Error bars represent s.e.m.

Figure 5 Nicotine activates TRPA1 in mouse trigeminal ganglion neurons. (**a,b**) Intracellular Ca²⁺-imaging experiments showing the effects of extracellular application of 100 µM (**a**) or 1 mM (**b**) nicotine (Nic) to trigeminal ganglion neurons isolated from wild-type mice. Caps, capsaicin. (**c**) Examples of nicotine's effects on trigeminal ganglion neurons isolated from *Trpa1* knockout (KO) mice. (**d**) Examples of nicotine's effects on trigeminal ganglion neurons isolated from wild-type mice in the presence of the nAChR blocker hexamethonium. In **a**–**d**, blue represents cells sensitive to nicotine, but not to mustard oil (100 µM), dark gray represents cells that responded to both nicotine and mustard oil, orange represents mustard oil–sensitive cells that did not respond to nicotine and green represents cells that did not respond to nicotine or mustard oil. (**e**) Stack bar plot representing the proportions of neurons responding to mustard oil and nicotine (100 µM, 1 mM and 1 mM in the presence of 3 mM hexamethonium) in wild-type and *Trpa1* knockout mice. The regions are color coded as in **a**–**d**. (**f**) Cumulative probability plot of the time to the maximal response elicited by nicotine in wild-type mustard oil–sensitive cells, wild-type cells in the presence of hexamethonium, wild-type mustard oil–insensitive cells and *Trpa1* knockout cells. We took the duration of nicotine application (2 min) as the cutoff time for determining the time to peak.

abolished the response to nicotine. These data indicate that the sensory cross-desensitization between mustard oil and nicotine occurs, at least partly, at the level of the chemosensor TRPA1.

Inhibition of TRPA1 by the nAChR blocker mecamylamine

The mechanisms of nicotine-induced irritation have been extensively studied in several animal models and in humans. Notably, from the effects of mecamylamine, a general inhibitor of nAChRs, several studies have suggested that nAChRs are involved in nicotine-induced irritation[2,7,32]. However, reduction of the irritation caused by application of high concentrations of nicotine requires prolonged pre-application of mecamylamine at concentrations that are much higher than those needed to fully inhibit nAChRs *in vitro* (1–5 mM versus 30–100 µM, respectively)[7,32,33]. Because mecamylamine has structural similarities with camphor, a known inhibitor of TRPA1 (ref. 34), we hypothesized that it may also inhibit TRPA1, which would account, at least in part, for its inhibitory effect on the irritation caused by nicotine at high concentrations. We found that mecamylamine prevented the mustard oil–induced activation of mTRPA1 current with almost total inhibition at 5 mM (**Fig. 4a**–**c**). These experiments were repeated and the results confirmed in intact mTRPA1-expressing CHO cells (**Fig. 4d**) and cultured mouse trigeminal ganglion neurons (**Fig. 4e**). In addition, mecamylamine inhibited mTRPA1 currents pre-activated
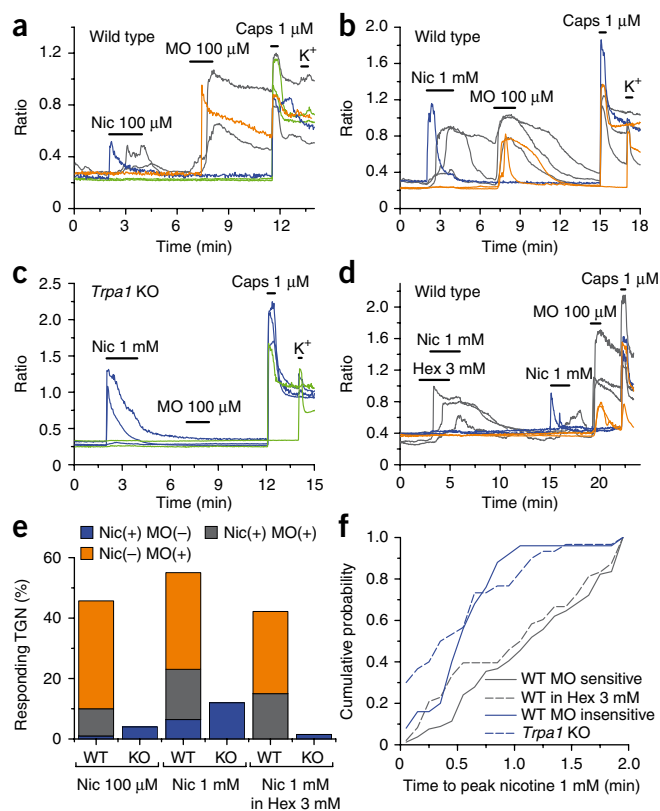
by mustard oil and nicotine (**Supplementary Fig. 9**) and the human TRPA1 ortholog (**Supplementary Fig. 10**).

Consequently, we tested whether TRPA1 is modulated by hexamethonium, a mecamylamine-unrelated nAChR inhibitor that has been widely used to assess the role of these receptors in the irritating effects of cigarette smoke[8]. In contrast with mecamylamine, hexamethonium did not substantially affect basal TRPA1 currents (**Fig. 4f,g**), nor did it prevent TRPA1 activation by mustard oil in mTRPA1-expressing CHO cells (**Fig. 4f**–**h**) and trigeminal ganglion neurons (**Fig. 4i**). Hence, hexamethonium is a better tool than mecamylamine for selectively inhibiting nAChRs without interfering with mTRPA1.

TRPA1-mediated response to the nicotine in sensory neurons

We next tested whether TRPA1 contributes to the sensory responses to nicotine in mouse trigeminal ganglion neurons. In contrast with earlier *in vitro* studies[13,35,36], we considered the relatively high EC₅₀ and slow onset of nicotine effects on TRPA1 (see above) and tested the effect of long (2 min) applications of nicotine at 100 µM and 1 mM. Exposure to 100 µM nicotine caused a significant increase ($P < 0.01$) in the intracellular Ca²⁺ concentration in ~10% (29 out of 298) of wild-type trigeminal ganglion neurons (**Fig. 5**). All of the nicotine-responsive cells were sensitive to capsaicin and 90% were
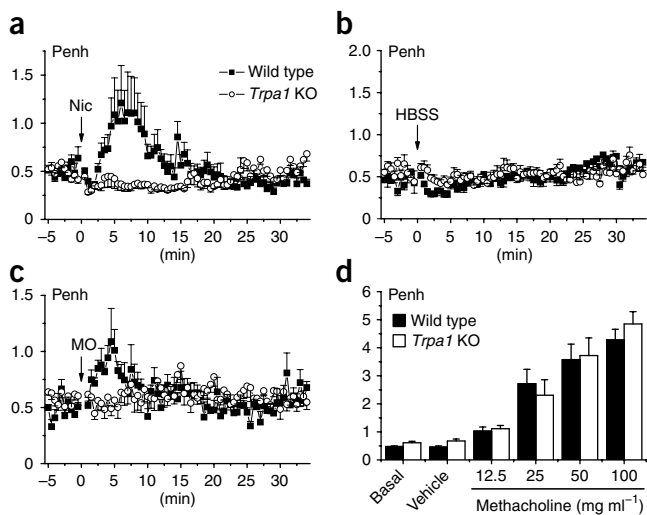

Figure 6 TRPA1 mediates the airway constriction reflex triggered by nasal instillation of nicotine and mustard oil. (**a**–**c**) The effects of nasal instillation of 10 µl of 60 mM nicotine (**a**), the vehicle (Hanks' balanced salt solution; **b**) or 50 mM mustard oil (**c**) on the Penh determined with unrestrained whole-body plethysmography in wild-type and *Trpa1* knockout mice (*n* = 4–7). The arrows mark the moment of instillation (time = 0 min). (**d**) Average Penh obtained in the basal condition and after 1 min administration of aerosols containing the vehicle alone (HBSS) or the bronchoconstrictor methacholine at various concentrations (*n* = 5 for each group). Error bars represent s.e.m.

Figure 7 Menthol inhibits nicotine-induced activation of TRPA1. (**a**) Time course of the amplitude of mTRPA1 currents at −75 and +50 mV during extracellular application of nicotine and nicotine plus menthol in CHO cells. The colored data points correspond to the current traces shown in the inset. Washout of menthol caused a strong current rebound, as has been previously reported[23,24]. (**b**) Ratiometric intracellular Ca^{2+}-imaging experiment showing that menthol inhibited the response elicited by nicotine in mTRPA1-induced CHO cells ($n = 7$). The thick trace represents the mean and the dashed traces represent the mean ± s.e.m. (**c**) Menthol inhibited nicotine-induced airway constriction reflex. The effect of nasal instillation of 10 µl of 60 mM nicotine and 10 mM menthol on wild-type mice ($n = 5$) is shown. The arrow marks the moment of instillation (time = 0 min). The data for nicotine alone is the same as that shown in **Figure 6a**. Error bars represent s.e.m.


also activated by 100 µM mustard oil, indicating that they were nociceptive neurons. In contrast, a significantly lower proportion of *Trpa1* knockout neurons (4%, 11 out of 262, $P = 0.011$) responded to 100 µM nicotine. Application of 1 mM nicotine activated 23% (88 out of 383) of wild-type trigeminal ganglion neurons; about 72% of these neurons also responded to 100 µM mustard oil (**Fig. 5b,e**). Again, the fraction of nicotine-sensitive cells was significantly reduced in *Trpa1* knockout mice (12%, 40 out of 320, $P = 0.00034$; **Fig. 5c,e**). Nicotine was able to trigger strong responses in wild-type neurons in the presence of the nAChR blocker hexamethonium (15%, 48 out of 311; **Fig. 5d**), which, as shown above, did not affect TRPA1. These responses were restricted to the mustard oil–sensitive neuronal population (**Fig. 5e**). Notably, only 4 out of 257 *Trpa1* knockout neurons responded to 1 mM nicotine in the presence of hexamethonium (<1.6%, significantly lower than in wild type, $P = 10^{-8}$; **Fig. 5e**), which indicates that the vast majority (presumably >93%) of the responses in wild-type mice are mediated by nAChRs and/or TRPA1.

Notably, the responses to nicotine in TRPA1-expressing (mustard oil sensitive) neurons were characterized by a broad distribution of the time to maximal increase (time to peak) and recovery occurred only after washout of nicotine (**Fig. 5a,b,d,f**). In contrast, in TRPA1-negative neurons, the large majority of the responses (88% in nicotine 1 mM) reached the maximum before 1 min and all of them decayed in the presence of nicotine (desensitized) within 2 min (**Fig. 5a,b,c,f**). In addition, whole-cell patch-clamp recordings in mouse trigeminal neurons revealed the presence of TRPA1-like and nAChR-like responses to extracellular nicotine application (**Supplementary Fig. 2**).

TRPA1 mediates the nasal irritation induced by nicotine

To test whether TRPA1 contributes to the known irritant effects of nicotine *in vivo*, we compared the airway constriction reflexes of wild-type and *Trpa1* knockout mice to stimulation of the nasal mucosa[37]. To monitor the respiratory function before and after nasal instillation of test solutions, we used unrestrained whole-body plethysmography and used the increase in enhanced pause (Penh) as a measure of airway constriction[38]. Penh significantly increased ($P = 0.027$) above control levels after application of nicotine in wild-type, but not in *Trpa1* knockout mice ($P = 1$; **Fig. 6a**). Instillation of vehicle alone had no effect on Penh (**Fig. 6b**), whereas instillation of mustard oil selectively increased Penh in wild-type mice (**Fig. 6c**). *Trpa1* knockout mice showed normal responses to increasing concentrations of aerosolized methacholine, a muscarinic receptor agonist causing contraction of airway smooth muscle cells[38] (**Fig. 6d**).

Menthol is popularly used as an additive in nicotine-containing products to produce cooling, soothing and analgesic effects, which are thought to counteract the irritation caused by tobacco smoke[39] and nicotine[40]. It was therefore interesting to determine the effects
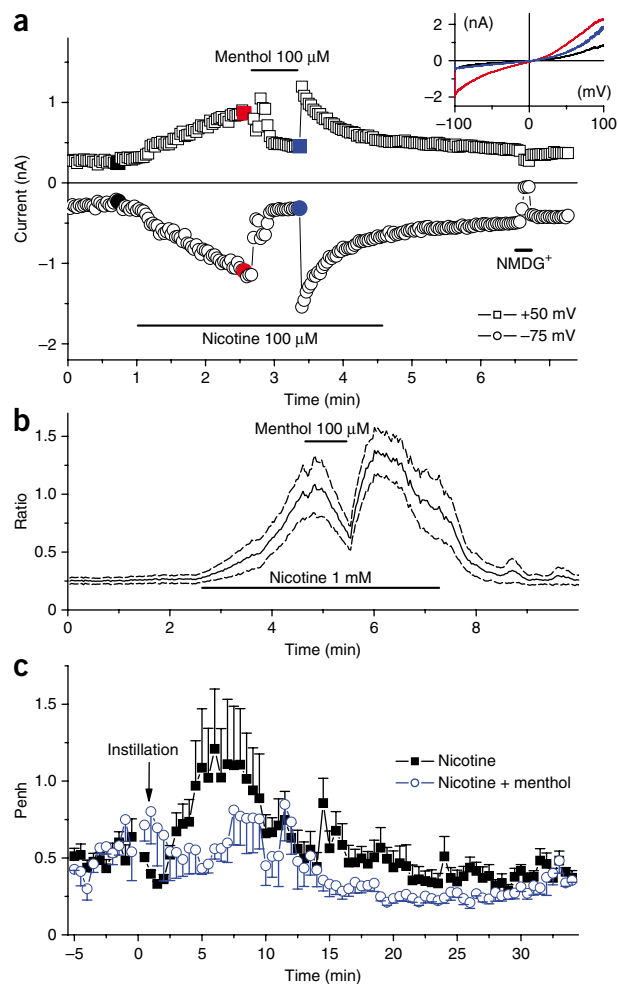
of this compound on the TRPA1-mediated responses to nicotine. Extracellular application of 100 µM menthol caused a significant reduction in mTRPA1 currents prestimulated with 100 µM nicotine (60 ± 7% at −75 mV, $P = 10^{-4}$; $n = 6$; **Fig. 7a**) and reversed the increase in intracellular Ca^{2+} concentration induced by 1 mM nicotine in mTRPA1-expressing CHO cells (**Fig. 7b**). Notably, nasal instillation of a menthol (10 mM) and nicotine (60 mM) mixture triggered a smaller Penh response than the instillation of nicotine alone (**Fig. 7c**), suggesting that menthol reduces the airway constriction reflex triggered by nicotine. The Penh response to instillation of menthol alone (10 mM) was similar to that obtained with vehicle (**Supplementary Fig. 11**). This result is consistent with a previous report that menthol causes oral trigeminal stimulation in rats only at concentrations above 32 mM (ref. 41).

DISCUSSION

It has been generally thought that the irritating effect of nicotine is exclusively mediated by nAChRs expressed in nociceptive nerves fibers[6–8,32]. In contrast, we found that nicotine caused irritation via activation of TRPA1, a cation channel involved in the transduction of noxious chemical stimuli. First, we found that nicotine stimulates heterologously expressed TRPA1, acting as a gating modifier. Second, we identified TRPA1-mediated nicotine responses in a subset of nociceptive neurons. These responses could be clearly distinguished from those mediated by nAChRs on the basis of their dose dependence, kinetics and pharmacological profile. Finally, we found that TRPA1

was necessary for the nicotine-induced airway constriction reflex in mice. To the best of our knowledge, this is the first demonstration of the *in vitro* and *in vivo* activation of a TRP channel by nicotine.

We found that nicotine actually had a bimodal action on TRPA1, with activation and inhibition occurring at low and high concentrations, respectively. As the inhibitory effect reversed much faster than the activating effect, a prominent current rebound was observed on wash-out of higher doses of nicotine. The effect of nicotine is reminiscent of that of menthol and menthol-related compounds, for which a bimodal action on the mouse TRPA1 clone has also been reported[23,24]. Using a mutagenesis approach based on the differences in menthol sensitivity of various TRPA1 orthologs, a previous study identified residues in the putative S5 segment as determinants for the stimulatory effect[24]. The study found that menthol's ability to inhibit mouse TRPA1, but not human TRPA1, is determined by specific amino acids distributed all across the putative pore domains. Our data suggest that nicotine interacts with the pore, as application of 1 mM nicotine induced a ~30% reduction of the single-channel conductance at positive potentials. Moreover, the fast relief of inhibition on nicotine washout is consistent with a binding site in the pore, which is quickly accessible from the extracellular solution. A bimodal effect has also been reported for 2-aminoethoxydiphenyl borate on TRPV3 (ref. 42), with an abrupt, but transient, increase in current on washout of high doses of the agonist.

It is well known that prior oral stimulation with the epitomic TRPA1-agonist mustard oil reduces the sensitivity to other irritant chemicals, such as nicotine[31]. Our data suggest that this cross-desensitization arises at the level of the common chemoreceptor, TRPA1. Indeed, we found that prior stimulation of TRPA1 by mustard oil fully abolished a later response to nicotine. Conversely, prior stimulation with nicotine led to a reduction of the mustard oil response. In this respect, it should be noted that nicotine and mustard oil have similar EC_{50} values for activating inward TRPA1 currents (17 and 11 μM, respectively), but that mustard oil is a much more effective activator, producing up to a 30-fold current increase, as compared with the maximal fivefold increase in inward current caused by nicotine (**Fig. 3**).

Activation of TRPA1 by electrophiles, such as mustard oil, cinnamaldehyde and acrolein, occurs through covalent modification of cysteine residues on the channel[27,28]. We were able to exclude the possibility that nicotine acts via a similar mechanism, as we found that the mustard oil–insensitive mutant C622S (ref. 28) can be readily activated by nicotine, which is consistent with the nonelectrophilic nature of nicotine. Thus, we consider it most likely that nicotine acts on TRPA1 through a noncovalent interaction with the channel, similar to the actions of other nonelectrophilic agonists, such as icilin[15], menthol[23,24], clotrimazole[25] and nifedipine[26]. Indeed, as has been reported for the latter three compounds, nicotine caused a negative shift in the voltage dependence of channel activation. In addition, we found that nicotine reduced the rate of whole-cell current deactivation and accelerated activation. These observations are fully consistent with our observations of a negative shift of the voltage dependence of channel activation and an increase in the mean open time and reduction of the mean closed time of single TRPA1 channels. Thus, nicotine causes both stabilization of the open conformation and destabilization of the closed conformation of TRPA1.

Our experiments in mouse trigeminal ganglion neurons strongly support the role of TRPA1 as mediator of nicotine-induced irritation. First, nicotine activated a subset of neurons that largely overlaps with the TRPA1-expressing (mustard oil sensitive) population. Second, the proportion of nicotine-sensitive neurons was strongly reduced in *Trpa1* knockout mice. Third, nicotine elicited robust responses in the presence of the specific nAChRs inhibitor hexamethonium. Fourth, nicotine responses were virtually abolished in *Trpa1* knockout mice neurons in the presence of hexamethonium. Finally, the responses to nicotine in TRPA1-expressing (mustard oil sensitive) neurons were kinetically different from the responses in TRPA1-negative (mustard oil insensitive) neurons. Thus, we conclude that nicotine evokes two distinct types of responses in wild-type trigeminal ganglion neurons: rapid and quickly desensitizing responses mediated by nAChRs and slower, more sustained responses mediated by TRPA1.

Our results prompt a re-evaluation of the mechanisms underlying the irritating effects of nicotine by introducing TRPA1 as a previously unknown ionotropic nicotine receptor that is distinct from nAChRs. On the one hand, the mucosal nicotine concentrations attained during tobacco smoke exposure are submicromolar[43] and are therefore lower than those necessary to activate TRPA1. Thus, the reported acute irritant effects of nicotine delivered in this way are probably mediated by nAChRs[8]. However, many studies aimed at understanding the irritating effects of nicotine on mucosal irritation *in vivo* have used millimolar concentrations of nicotine, as high as 600 mM[7,32,44]. Moreover, in many instances the inhibition of nicotine-induced responses by mecamylamine has been taken as evidence for the involvement of nAChRs. Our current data challenge this view, as we found that TRPA1 was not only activated by nicotine, but was also inhibited by mecamylamine.

To test the relevance of nicotine-induced activation of TRPA1 *in vivo*, we studied the irritating effects of nicotine on wild-type and *Trpa1* knockout mice. We simulated the application of nicotine nasal sprays, which generally contain nicotine at concentrations around 60 mM, by studying the well-known airway constriction reflex to noxious stimulation of the nasal mucosa[37,45]. Notably, this form of nicotine replacement therapy is the most effective one, but has the highest treatment dropout rate as a result of mucosal irritation[5]. Nasal instillation of nicotine provoked an increase of Penh, a surrogate measure of airway constriction, in wild-type, but not in *Trpa1* knockout, mice. The normal response of *Trpa1* knockout mice to the bronchoconstrictor methacholine indicates that the lack of nicotine- and mustard oil–induced response in these mice is not caused by an unspecific contractile dysfunction of the airway smooth muscle. Altogether, these results indicate that TRPA1 mediates the irritating effect of nasal nicotine instillation. Notably, it is apparent from our data that nAChRs did not contribute to the airway constriction reflex, which contrasts with the observation of nAChR-like responses in primary cultured trigeminal ganglion neurons. This disagreement could be explained by the transient character of nAChR activation, which may have hindered the contribution of nAChRs to the airway constriction reflex in our *in vivo* experimental model. Alternatively, the sensory neurons that respond to nicotine via activation of nAChRs may not be involved in the long-lasting airway reflex following nicotine stimulation of the nasal mucosa.

We found that menthol, a known blocker of mouse TRPA1, induced an inhibition of mouse TRPA1 currents that were pre-activated by nicotine and reduced the Penh response on intranasal instillation of nicotine in mice. Caution should be taken, however, when trying to extrapolate the effects of menthol to humans, as this compound does not inhibit human TRPA1 (ref. 24). The well-known soothing effect of menthol in tobacco-containing products[39] may well be related to activation of TRPM8, which has been shown to produce analgesia[46]. Nevertheless, our results indicate that inhibition of TRPA1 represents an interesting approach for developing smoking cessation therapies with less adverse effects.

Our findings are also relevant to the ecological role and industrial uses of nicotine and its analog anabasine, which are known to be strong repellents of herbivores. Notably, *Brassicaceae* and *Nicotiana* plants increase their production of either isothiocyanates[47] or nicotine and anabasine[48], respectively, following herbivore attack. This suggests that the promiscuous character of TRPA1 chemo-activation underlies a unified mechanism for the detection of a wide range of noxious compounds that function as botanical defensive traits.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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ONLINE METHODS

Cells and animals. We used a tetracycline-regulated system for inducible expression of mTRPA1 in CHO cells[15]. Human TRPA1 was heterologously expressed in CHO cells by transient transfection using TransIT 293 reagents (Mirus). Trigeminal ganglion neurons from adult (postnatal weeks 8–12) C57Bl/6J (wild type) and *Trpa1* knockout mice[16] were cultured as described previously[23]. Experiments were carried out in accordance with the European Union Community Council guidelines and were approved by the Animal Experiments Ethics Committee of KU Leuven.

Patch-clamp experiments. Patch-clamp recordings were performed as described previously[49]. Before current recordings, cells were rinsed with Krebs solution containing 150 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES and titrated to pH 7.4 with NaOH. Bath solutions were perfused by gravity via a multi-barreled pipette tip with a single outlet of 0.8-mm inner diameter. This system allows the full exchange of the medium bathing the recorded cell in less than 2–4 s. Currents were routinely elicited by 400-ms-long voltage ramps from −100 to +100 mV at a stimulation frequency of 0.5 Hz. The holding potential was 0 mV. The extracellular solution contained 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES and was titrated to pH 7.4 with NaOH. To monitor the quality of the recordings, we regularly tested the effect of substituting all extracellular cations with $NMDG^+$, which is largely impermeable through TRPA1 and is therefore expected to reduce the inward current to negligible background levels. The intracellular solution contained 156 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM BAPTA to minimize possible activation by intracellular Ca^{2+} (refs. 29,30) and was titrated to pH 7.2 with NaOH. The patch-clamp data were analyzed using WinASCD (G. Droogmans; ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/winascd.zip) and Origin 7.0 (OriginLab). The bimodal dose-response curves for the effects of nicotine on TRPA1 currents (**Fig. 1d**) were fit by a function of the form:

$$I_{\text{rel}} = \left(1 + \frac{Max \times [\text{Nic}]^{H_S}}{[\text{Nic}]^{H_S} + \text{EC}_{50}^{H_S}}\right) \times \frac{\text{IC}_{50}^{H_I}}{[\text{Nic}]^{H_I} + \text{IC}_{50}^{H_I}}$$

where I_{rel} is the steady-state amplitude of the current recorded in the presence of nicotine at concentration [Nic] normalized to the value in control. *Max* is the maximal relative current increase expected in the absence of inhibitory effect of nicotine, EC_{50} and IC_{50} are the effective concentrations for current stimulation and inhibition, respectively, and H_S and H_I are the corresponding Hill coefficients. *Max*, EC_{50} and H_S were fixed to the corresponding values obtained from the fit of the dose-response curves for the stimulatory effect of nicotine (see **Supplementary Fig. 6**).

Intracellular Ca^{2+}-imaging experiments. Cells were incubated with 2 µM Fura-2 acetoxymethyl ester for 30 min at 37 °C. Intracellular Ca^{2+} concentration was monitored via the ratio of fluorescence measured on alternating illumination at 357 and 380 nm using an MT-10 illumination system and cell^M software (Olympus). As a control, we used extracellular Krebs solution (see above). To identify neurons in trigeminal ganglion cultures, we applied a Krebs-based solution in which the KCl concentration was increased to 45 mM by iso-osmotic substitution of NaCl. The system for exchange of extracellular solutions was similar to that used for patch-clamp experiments (see above).

Whole-body plethysmography. The ventilatory function of mice was monitored using unrestrained whole-body plethysmography, as described previously[38]. Drugs were delivered by nasal instillation (without anesthesia) of 10 µl of test solutions to restrict the area of stimulation to the upper airways[50]. The increase in Penh was used as an indicator of bronchoconstriction. Penh is a dimensionless parameter that represents a proportion of maximal expiratory to maximal inspiratory box pressure signals in relation to the timing of expiration and is calculated as

$$\left(\frac{\text{expiratory time}}{\text{relaxation time}} - 1\right) \times \left(\frac{\text{peak expiratory flow}}{\text{peak inspiratory flow}}\right).$$

All chemicals were purchased from Sigma-Aldrich.

Statistics. Data are presented as mean ± s.e.m. Significance between groups was tested using the unpaired or paired Student's *t* tests or the χ^2 test as appropriate.

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