JPET Fast Forward. Published on August 7, 2013 as DOI: 10.1124/jpet.113.205971
JPET#205971

1. Title Page.

Nicotine's bimodal concentration-response involves nAChR, TRPV1, TRPA1 channels in mouse trachea and sensory neurons

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2. Running title page.

a) Running Title: Nicotine responses in trachea and sensory neurons

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c) The number of:

text pages (45)

tables (1)

figures (7)

references (58)

The number of words in the:

Abstract (249)

Introduction (750)

Discussion (1579).

d) Abbreviations:

CGRP - calcitonin gene-related peptide,

DRG - dorsal root ganglion;

MO – mustard oil (AITC = allyl isothiocyanate).

nAChR - nicotinic acetylcholine receptor;

NJG – nodose – jugular ganglion complex;

SP – substance P,

transient receptor potential ankyrin 1 (TRPA1);

transient receptor potential vanilloid type 1 (TRPV1);

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e) A recommended section assignment to guide the listing in the table of contents: Toxicology

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3. Abstract

High concentrations of nicotine, as in the saliva of oral tobacco consumers or in smoking cessation aids, have been shown to sensitize/activate recombinant rTRPV1 and mTRPA1 channels. Measuring stimulated CGRP release from the isolated mouse trachea we established a bimodal concentration-response relationship with a threshold below 10µM (-)-nicotine, a maximum at 100µM, an apparent nadir between 0.5 and 10mM and a renewed increase at 20mM. The first peak was unchanged in TRPV1/A1 double-null mutants as compared to wildtypes and was abolished by specific nicotinic acetylcholine receptor (nAChR) inhibitors and by camphor, discovered to act as nicotinic antagonist. The nicotine response at 20mM was strongly pH_e dependent, five-times greater at pH9 than 7.4, indicating that intracellular permeation of the (uncharged) alkaloid was required to reach the TRPV1/A1 binding sites. The response was strongly reduced in both null mutants, more so in double-null mutants. Measuring calcium transients in nodose/jugular and dorsal root ganglion neurons in response to nicotine 100µM, 48% of the vagal but only 14% of the somatic sensory neurons were activated, the latter very weakly. However, nicotine 20mM at pH9 repeatedly activated almost every single cultured neuron, partly by releasing intracellular calcium and independent of TRPV1/A1 and nAChRs. In conclusion, in mouse tracheal sensory nerves nAChRs are 200-fold more sensitive to nicotine than TRPV1/A1; they are widely co-expressed with the capsaicin receptor among vagal sensory neurons and twice as abundant as TRPA1. Nicotine is the major stimulant in tobacco, and its sensory impact through nAChRs should not be disregarded.

4. Introduction

For smokers nicotine does not only exert pharmacological effects but is an essential part of the smoking experience, providing the desirable somatosensory impact and some distinct smell (Hummel et al., 1992). In smoking cessation aids, oral tobacco products and in the "electric cigarette" nicotine is a main sensory stimulant that can cause pleasantly mild burning but eventually unpleasant, pungent, sensations (Talavera et al., 2009; Einarson & Einarson, 1997; Jimenez Ruiz et al., 2000; Bolliger et al., 2007). Such excitatory effects of nicotine derive from ubiquitous primary polymodal nerve endings, subpopulation of which is equipped with nicotinic acetylcholine receptors (nAChR; Steen & Reeh, 1993; Bernardini et al., 2001; Rau et al., 2005). The physiological role of these nAChRs is not yet clear, although it has been speculated they may subserve pain induction when acetylcholine is set free from damaged epithelial cells, e.g. in the cornea (Tanelian 1991). Many epithelia contain ACh and the machinery to produce, store, and release it (Zia et al., 2000; Wessler & Kirkpatrick, 2008 for rev.). For example, in the mouse trachea an epithelium-derived bronchoconstriction is mediated by ACh, and skin keratinocytes release graded amounts of ACh upon stimulation by noxious heat or resiniferatoxin, an activator of the capsaicin receptor TRPV1 (Moffatt et al., 2004; Nagy et al., 2006).

Nicotinic ACh receptors have not remained the only known targets of nicotine on sensory nerves: TRPV1 was shown to be directly sensitized and the almost universal chemoreceptor-channel TRPA1 to be fully activated by nicotine (Liu et al., 2004; Talavera et al., 2009*)*. Surrogate studies on cellular models even suggest an equal potency of nicotine to activate nAChR and TRPA1 (Rau et al, 2005; Talavera et al., 2009). However, a complete concentration-response relationship for nicotine on native sensory neurons, that may co-express TRPV1, TRPA1 and nAChR, has not yet been established nor have different activating components been dissected.

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Up to high millimolar nicotine concentrations are contained, for example, in nasal sprays and dissolve in human saliva when oral tobacco products are used (Benowitz et al., 2009).

Nicotine is an alkaloid (pK_a 8.02 at 37°C) and as such it is largely charged at pH 7.4, only fractions being available to permeate plasma membranes. This should not matter in the case of nAChR, because its ligand binding pockets are extracellular (Arias, 1997, for rev.). However, TRPV1 and TRPA1 have intracellular binding sites for most of their ligands and, thus, should be more sensitive to nicotine in alkaline solution (Jung et al., 1999; Talavera et al., 2009). Other means of discriminating the nicotine-activated receptor-channels are selective agonists and antagonists. However, the long approved nicotinic antagonist mecamylamine has recently been shown to block TRPA1, although in hundred times higher concentration than required for nAChR (Talavera et al., 2009). This side effect was not unexpected, as mecamylamine is structurally very similar to camphor, an established TRPA1 antagonist that also acts as weak agonist at TRPV1 (Xu et al., 2005; Talavera et al., 2009). The TRPA1 agonist acrolein has not yet been incriminated of acting through other TRP receptors; it is an endogenous product of lipid peroxidation, associated with neurodegenerative diseases, reperfusion injury and chronic inflammation (Tsirulnikov K et al., 2012). In addition, acrolein is a major constituent of smoke, in particular of the gas phase of cigarette smoke, and there it accompanies a variety of further volatile TRPA1 agonists like formaldehyde, acetaldehyde, crotonaldehyde etc. (Roemer et al., 2012).

A final aspect by which nicotine effects could be dissected is selectivity of action on particular subsets of sensory neurons. Here, we compare mouse dorsal root and nodose-jugular ganglion neurons in culture, the latter representing the cell bodies of vagal sensory nerve fibers. These neurons also provide the vast majority of

sensory nerve endings in the trachea, the isolated tissue preparation used here to measure stimulated release of calcitonin gene-related peptide (CGRP) as an index of sensory activation and neurosecretion (Kichko & Reeh, 2009). However, CGRP release is also a (patho-)physiological parameter of interest in its own right, because it goes along with substance P release, and both neuropeptides together are a hallmark of neurogenic inflammation in the trachea and elsewhere (Brain S, 1997; Myers et al., 2002). In this respect, our data show a wide segregation of specific nicotinic effects at micromolar concentrations from TRPV1/A1-dominated actions at higher millimolar levels which, in addition, become prevalent only at alkaline pH. Further, we show that vagal sensory neurons are much more sensitive to nicotine than somatic ones and that camphor is a specific, though not selective, nicotinic antagonist.

5. Materials and Methods

Animals - The experiments were carried out in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Adult C57BL/6, TRPV1-/-, TRPA1-/- and TRPA1/V1double knockout mice were used. Breeding pairs of TRPV1 and TRPA1 knock-out mice were obtained from Dr. John Davis (Davis et al., 2000) and Dr. David Corey (Kwan et al., 2006) and continuously backcrossed to C57BL/6. Double knock-out animals were generated in our animal facility by cross-mating knockouts of both strains. Mice were housed in group cages in a temperature-controlled environment on a 12 h light/dark cycle and were supplied with water and food *ad libitum*. Mice of either sex (body weight 15 – 25 g) were killed by exposure to pure CO_2 atmosphere *(approved by the Animal Protection Authority, District Government Mittelfranken, Ansbach, Germany).*

Trachea preparation - The trachea was excised together with the two main bronchi, hemisected along the saggital midline. One half of the trachea preparation was used as control and the other half for chemical treatments, taking advantage from the minor intraindividual variability of CGRP release. The preparations were placed for 30 min at 37°C in carbogen-gassed (95% O_2, 5% CO_2, obtaining pH7.4) synthetic interstitial fluid (SIF) containing (in mM) 107.8 NaCl, 3.5 KCl, 1.53 $CaCl_2$, 0.69 $MgSO_4$, 26.2 $NaHCO_3$, 1.67 NaH_2PO_4, 9.64 sodium gluconate. After the initial rest period, the isolated trachea was consecutively incubated for 5 min in each of four tubes containing 125 µl SIF or stimulated chemicals and mounted in a shaking bath at 37°C. The first two incubations were performed to determine basal CGRP release and variations at 37°C. The third tube contained the stimulating chemicals diluted in SIF (nicotine, epibatidin, acrolein, mustard oil) and, when indicated, the antagonistic compounds in addition. The final tube was for wash-out and to check for reversal.

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CGRP enzyme immunoassay (EIA). The CGRP content of the incubation fluid was measured using commercial enzyme immunoassay kits with a detection threshold of 2 pg/ml (Bertin Pharma, Montigny-le-Bretonneux, France). For this purpose 100µl sample fluid were stored on ice and mixed, immediately following the trachea incubation period, with 25µl of fivefold concentrated commercial CGRP enzyme immunoassay buffer that contained a cocktail of peptidase inhibitors. The CGRP contents were determined after the end of the experiment; the antibody reactions took place overnight. Enzyme immunoassay plates were determined photometrically using a microplate reader (Dynatech, Channel Islands, UK). All results are presented as measured by the EIA in pg CGRP/ml SIF. Reducing interindividual variability and day-to-day baseline variability, the data were normalized to the second individual baseline value (before stimulation). This value was subtracted from all four data points of a typical experiment so that only the absolute change in CGRP release (Δ pg/ml) is displayed in the figures.

Cell culture: Nodose/jugular ganglia of both sides and dorsal root ganglia of lumbar and the first two thoracic segments of the spinal column of mice were excised and transferred into Dullbecco's modified Eagle's medium (DMEM) solution containing 50µg/ml gentamicin (Sigma, Taufkirchen, Germany). The ganglia were treated with 1mg/ml collagenase and 0.1mg/ml protease for 30 min (both from Sigma) and subsequently dissociated using a fire-polished silicone-coated Pasteur pipette. The cells were plated on poly-D-lysine-coated (200µg/ml, Sigma) coverslips and cultured in TNB100 cell culture medium supplemented with TNB100 lipid-protein complex, 100 µg/ml streptomycin, penicillin (all from Biochrom, Berlin, Germany) and mouse NGF (100ng/ml, Almone Labs, Tel Aviv, Israel) at 37°C and 5% CO_2. Calcium imaging experiments were performed within 20-30 h of dissociation.

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Ratiometric [Ca2+]i measurements: Neurons of nodose/jugular or dorsal root ganglia of C57Bl/6 *and* TRPV1-TRPA1$^{-/-}$ mice were stained by 3 µM fura-2 AM and 0.02% pluronic (both from Invitrogen, Darmstadt, Germany) dissolved in TNB100 medium for about 30 min. Following a 30 min wash out period to allow fura-2-AM deesterification coverslips were mounted on an Olympus IX71 inverse microscope with a 10x objective. The ganglion cells were constantly superfused with extracellular fluid (in mM: NaCl 145, KCl 5, $CaCl_2$ 1.25, $MgCl_2$ 1, Glucose 10, Hepes 10) using a software controlled 7-channel gravity-driven common-outlet superfusion system. Fura-2 was excited at 340 and 380nm with a Polychrome V monochromator (Till Photonics, Graefelfing, Germany). Images were exposed for 200µs and acquired at a rate of 1 Hz with a 12 –bit CCD camera (Imago Sensicam QE, Till Photonics). Data were recorded and further analyzed using TILLvisION 4.0.1.3 software (Till Photonics). Background was subtracted before calculation of ratios.

All experimental protocols were pre-programmed. During experiment, the cells on coverslip were exposed to different stimulations for 20 s each (capsaicin for 10s). Between stimuli, the cells were washed with fresh extracellular buffer for 6 min for the cells to recover prior to the next stimulus. Capsaicin and mustard oil were used for cell categorization. At the end of all protocols a 30 sec stimulus of KCl 60 mM was applied to depolarize the cells as a control and as the normalization reference for comparison between different stimulations. Ratio increases reflecting calcium concentration were calculated (Poenie et al., 1986). An increase of the intracellular calcium concentration of at least 50 nM during the application period was considered as activation.

Stimulation responses were quantified as the area under the curve of the fluorescence ratio during the application period and standard error of the mean; 10 s before application was used as the reference period.

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Chemicals. Chemicals used in this study are as follows (name, abbreviation):

From Sigma (Taufkirchen, Germany): (+/-) camphor; capsaicin; mecamylamine hydrochloride; methyllycaconitine citrate hydrate; epibatidine; mustard oil (AITC = allyl isothiocyanate); acrolein; the TRPA1 blocker HC030031, *2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide*;

from Acros (Geel, Belgium) (-)-nicotine; from Biomol (Cologne, Germany) the TRPV1 inhibitor BCTC, N-(4-t-Butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide.

Initial stock solutions were made in ultrapure H_2O except for camphor, capsaicin, BCTC, mustard oil (made in 100% ethanol) and stored at – 24°C. The final solutions ready to use were freshly diluted in SIF or external solution before each experiment. The camphor 2 mM solution contained 0.2 % ethanol and other final solutions (BCTC, capsaicin, MO) contained 0.1% ethanol or less. Nicotine of 20 mM concentration overloaded the buffer capacity of the physiological solution (generating pH ~8) and required HCl titration to achieve pH 7.4 and NaOH for pH 9.

Statistical analysis. Statistical comparisons were performed using Statistica 7 software (Statsoft, Tulsa, USA). All time series of experimental values were first analyzed for the effect of stimulation (by nicotine, epibatidine, acrolein, mustard oil) as compared to baseline using the nonparametric Wilcoxon matched pairs test. The baseline-normalized, i.e. Δ, CGRP values were entered into a one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test, focusing on the peak values of stimulated CGRP release; $p < 0.05$ was considered significant and labelled with an asterisk. Data points represent means ± SEM of the given number (n) of experiments.

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6. Results

1. (-)-Nicotine-induced tracheal CGRP release shows a bimodal concentration-response. The tracheal nicotine concentration-response in wildtype (C57Bl/6) mice showed a significant increase over baseline CGRP secretion at 10 µM (-)-nicotine, a maximum at 100 µM (pH_e7.4), an apparent nadir between 0.5 and 10 mM and a second increase with 20 mM (Fig.1A). A log-linear rising flank up to 100 µM provided a half-maximal effective concentration EC_{50} of about 31 µM; significantly less stimulated CGRP release was achieved at supramaximal concentrations of 500 µM and 10 mM in comparison to 100 µM. This inversely U-shaped relationship suggested an inhibitory action of nicotine concentrations >100 µM and <20 mM. This hypothesis was tested using the ultrapotent and specific nicotinic agonist epibatidine (0.1 µM). Its effect was neither enhanced nor reduced by co-application of 1 mM nicotine (n=8, data not shown), making improbable a block of the ion channel as well as a heterologous desensitization of the nAChR. However, a homologous desensitization, typical for nAChRs, became evident when the nicotine (100 µM) exposure was extended from 5 to 10 min, and the CGRP release induced during the first 5 min returned to baseline during the second 5 min (n=4, data not shown). A further typical property of nAChRs is their absolute enantiomer-selective preference for (-) versus (+)-nicotine, the latter of which (100 µM) did not evoke any tracheal CGRP release (n=4, data not shown). Release of CGRP by vesicular exocytosis requires calcium entry into the sensory nerve fibers. Accordingly, in the presence of EGTA (10 mM) and absence of extracellular calcium the basal (constitutive) CGRP release from the trachea was significantly reduced (not shown) and no nicotine response at maximal effective concentration of 100 µM could be observed (Fig.1A) which indicates a physiological mechanism of neurosecretion.

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Double-knockout TRPA1/TRPV1=/= mice, congenic with C57Bl/6, were used to exclude the participation of these TRP channels in the assumed specific nicotinic response of the trachea. The stimulated CGRP release at 31µM and 100µM nicotine concentration was the same in knockouts as wildtypes (Fig.1B), suggesting that the lower-concentration mode of the nicotine actions is mediated by nAChRs, which will be demonstrated below.

2. Specific micromolar nicotinic actions. The pharmacology of lower-concentration nicotine-induced tracheal CGRP release was in accord with the knockout findings. The classical unselective nAChR antagonist hexamethonium abolished the nicotine response (Fig.2A). Mecamylamine, the more selective antagonist of the ganglionic, heteromeric nAChRs, showed concentration-dependent efficacy, while methyllycaconitine, preferring the CNS type of nAChR, was ineffective. This suggests an α-bungarotoxin insensitive subunit composition of the heteropentameric nAChR subtype, most likely α3ß4, in agreement with immunocytochemical findings on tracheal sensory nerves (Haberberger et al., 2004).

In contrast to the nicotinic blockers, the specific TRPV1 antagonist BCTC (Valenzano et al., 2003) and the TRPA1 antagonist HC030031 (Eid et al., 2008) did not diminish the tracheal nicotinic response (Fig.2B), while the latter was effective against the selective TRPA1 agonist acrolein, another essential constituent of tobacco smoke (Fig.2C). Mecamylamine was also tested against acrolein, because it had recently been shown to inhibit TRPA1 in much higher concentration (Talavera et al., 2009) which, however, was not the case at 100µM equimolar to nicotine (Fig.2C). To exclude any sensitizing effect of lower nicotine concentration on TRPA1 (Talavera et al., 2009), nicotine was co-applied with the TRPA1 agonists acrolein and mustard oil (MO, AITC) at low, TRPA1-selective concentrations. The CGRP-

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releasing effects were solely additive (Fig. 2D), suggesting independence of each other of nicotinic and TRPA1-mediated actions.

3. Antinicotinic effects of camphor. An interesting overlap of the antagonist profiles of TRPA1 and nAChR was discovered using camphor. This monoterpenoid, structurally similar to mecamylamine, is an established TRPA1 antagonist (Xu et al., 2005) and was not previously known to block nicotine effects on nAChR (Park et al., 2001). Although camphor is a chiral substance, both enantiomers (+ and -) at 2mM were equally effective (data not shown) abolishing the specific nicotine responses in the trachea (Fig.2B) as well as the acrolein effect (Fig.2C). This antinicotinic camphor effect was concentration-dependent in a narrow range between 0.2 and 1mM upon co-application with nicotine at half-maximal effective concentration (Fig.3A). The novel action of camphor appeared worthy to be reproduced in an independent model using cultured sensory neurons from the nodose-jugular ganglion (NJG) complex, part of which innervate the trachea and respond with calcium influx to sensory irritants such as mustard oil and capsaicin as well as to nicotine (further details see below). In this cellular model camphor also abolished the specific nicotinic response in a reversible manner and without inducing calcium influx by itself (Fig.3B). Further support for a specific antinicotinic action of camphor came again from the trachea, where it inhibited the CGRP release evoked by epibatidine, although not as effectively and by far not as potently as mecamylamine (Fig.3C). These data also confirmed the high selectivity of epibatidine for nAChR, because both TRPV1 and TRPA1 null mutants showed about the same nicotinic responsiveness as the congenic wildtypes (Fig.3C, D). A saturating concentration of epibatidine (0.1µM) evoked the same amount of tracheal CGRP release as the maximal effective nicotine concentration (100µM, Fig. 1A), confirming the 1000-fold greater potency of epibatidine over nicotine (Bradley, 1993) and indicating that all

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accessible neuronal AChRs had been activated by either agonist. Camphor showed its full inhibitory effect on epibatidine also in TRPA1 knockouts (Fig.3D), finally confirming that its antinicotinic action was not due to a TRPA1 block.

4. Multiple actions of millimolar nicotine. High concentrations (mM) of nicotine occur in the saliva of consumers of oral tobacco products and nicotine-containing chewing gums; they may as well contribute to the harshness of strong cigarettes as perceived on the laryngeal level (Talavera et al., 2009; Stanfill et al., 2011; Lee et al., 1987). TRPV1 and TRPA1 had previously been identified as mediators of high-concentration neuronal nicotine effects (Liu et al., 2004; Talavera et al., 2009). Nicotine-induced tracheal CGRP release confirmed this involvement of TRP channels by showing a deficit in nicotine (20 mM) responsiveness in TRPA1 and a complete loss in double-mutants (Fig. 4A). Although these deficits were evident at pH 7.4 of the extracellular nicotine solution, they were much more prominent at pH 9 when the alkaloid is largely uncharged (pK_a 8.02; Lu, Chen, Zhan, 2007)) and therefore well membrane permeable. This is most likely due to the fact that both TRP receptor-channels have their binding sites for most agonists at the intracellular site of the transmembrane proteins (Vriens et al., 2009). Thus, the much greater efficacy of nicotine in alkaline than neutral solution (in C57BL/6) is most likely due to a higher intracellular concentration. SIF at pH 9 (titrated by NaOH) did not stimulate any CGRP release (n=7, data not shown). The double-knockout mice for both TRPA1 and TRPV1 showed further reduced nicotine responsiveness, and hexamethonium caused an additional reduction in these animals (Fig. 4A), suggestive for a small but specific nicotinic component that was masked in wildtypes (in which 3 mM hexamethonium was ineffective against the high nicotine concentration; n=6, data not shown). Enantiomer-selectivity of the high-concentration (20 mM) alkaline nicotine effects on TRP channels was not expected but, to our surprise, was confirmed, as

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only 25% of the control CGRP release was evoked using (+)-nicotine (n=4, data not shown). Hence, both TRP channels show a clear preference for the naturally occurring (-)-nicotine but not as exclusive as nAChRs do (see above).

Even in double-knockouts and presence of hexamethonium nicotine 20 mM at pH 9 caused a small but significant increase of tracheal CGRP release over baseline, which effect appeared worth scrutinizing in the cellular model of cultured sensory neurons (Figs. 4B, C, D): NJG and DRG neurons of the TRPA1/V1 double-knockout mice were used for calcium imaging experiments. As expected, both neuron types did not respond to mustard oil at a concentration (1 mM) that would activate TRPA1 as well as TRPV1 in wildtypes (Everaerts et al. 2011). However, nicotine 20 mM at pH9 activated every single of the tested double-knockout neurons to an extent that was not significantly different from wildtype neurons (Figs. 6 & 7); extracellular solution at pH 9 did not stimulate any calcium influx by itself. Hexamethonium did not reduce this cellular nicotine response (Fig. 4D) in contrast to the residual tracheal response (Fig. 4A). However, in nominally calcium free (+ EGTA) extracellular solution the nicotine response was reversibly diminished by 75% though not abolished (Fig.4D). Thus, uncharged membrane permeable nicotine in high concentration appears to activate another calcium entry mechanism, perhaps another TRP channel besides nAChR, TRPV1 and TRPA1, and in addition, it is able to release calcium from intracellular stores. This intracellular calcium increase is obviously reversible, repeatable, pH-dependent and, thus, not likely of cytotoxic nature. The unknown calcium influx mechanism may account for the, however small, residual nicotine-induced tracheal CGRP release in double-knockouts and presence of hexamethonium (see above), because unmyelinated nerve fibers and endings require calcium influx for vesicular exocytosis in any case as they do not possess any appreciable calcium stores. The difference between nerve terminals bare of

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endoplasmic reticulum (ER) and sensory nerve cell bodies with abundant ER may account, in part at least, for the discrepancy between the small residual CGRP release from the former and the full-blown calcium transient in the latter. Accordingly, this discrepancy did not occur at pH7.4 when nicotine (20mM) is largely membrane impermeable (see below Figs. 5A, 6C).

5. PH-dependence of the cellular nicotine response. The strong pH-dependence seen with nicotine (20 mM)-induced tracheal CGRP release was scrutinized using NJG neurons of wildtype mice and calcium imaging. Alkaline pH more than doubled the prevalence of nicotine responsive cells and the magnitude of the calcium transients as compared to nicotine 20 mM at pH 7.4 (Fig. 5A). Mustard oil responses at TRPA1-specific concentration (100 µM) were clearly depressed by a preceding strong nicotine stimulus, most likely due to cross-desensitization of TRPA1 (Ruparel et al., 2007, Talavera et al., 2009). If the order of stimuli was reversed, the mustard oil response was obviously greater and excelled the nicotine response (Fig. 5B). Subsequent capsaicin responses did not appear to be much affected by the preceding stimuli. PH, whether neutral or alkaline, did not matter if the nicotine concentration was only 1 mM (Fig. 5C), suggesting that even this relatively high concentration was still predominantly acting through nAChRs which bind nicotine at the extracellular site of the receptor-channel.

6. Vagal NJG versus spinal DRG neurons. It was the specific nicotinic (100 µM) responsiveness that distinguished DRG from NJG neurons which innervate almost only internal organs including larynx and trachea. Prevalence and magnitude of the stimulated calcium transients were significantly greater among the vagal versus spinal sensory neurons (Table 1), and the pH of the nicotine solution did not matter at 100µM concentration (Figs. 6A and B). Nicotine 100 µM also did not exert appreciable heterologus desensitizing effects on the subsequent MO responses. In

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contrast, such a desensitization was obvious after nicotine 20 mM at pH 9 (Fig. 6C). High prevalence (up to 99%) as well as large magnitude of these supramaximal nicotine responses in wildtype DRG neurons were neither different from NJG cells nor from DRGs of the TRPV1/A1 double-knockouts, which again emphasizes the unspecificity of the high nicotine concentration (Table 1). Interestingly, the accentuated concentration-response relationship seen with nicotine-induced tracheal CGRP release (Fig. 1A) did not show up in NJG neurons. Nicotine 100 µM, 1 mM and 20 mM (pH 9) evoked about the same submaximal increases of intracellular calcium (compare Figs. 5D, 6A and 5A).

7. Distribution of chemosensitivities among sensory neurons. Chemosensory-induced calcium transients were used to compute Venn diagrams that illustrate the prevalences and their overlap among cultured NJG and DRG neurons at different nicotine and associated pH levels (Fig. 7). Capsaicin (0.3 µM) and MO (100 µM) sensitivities were about evenly distributed over four different random samples of neurons from both types of ganglia (n=442): Capsaicin activated 48% and MO 25% on average. Among NJG neurons, the specific nicotine sensitivity ranged with the high prevalence of capsaicin responsiveness, although in general the overlap between the chemosensory subpopulations was great but not complete; each chemical activated a number of neurons that were not sensitive to either of the other agents. Among DRG neurons, the specific nicotinic responses were rare (and small), but 20 mM nicotine (at pH 7.4) activated as many cells as capsaicin and MO together and finally all neurons, if the nicotine solution was alkalinized (pH 9). The maximized prevalence of nicotine (20 mM) responsiveness at pH9 in DRG as well as in NJG neurons comprised most of the previously chemosensitive cells but even more those units that had before appeared insensitive to all three chemicals.

This finally supports the conclusion (from Fig. 4D) that intracellular calcium release and some unknown calcium entry mechanism are activated by 20 mM nicotine at pH 9 in addition to nAChR, TRPV1 and TRPA1. The contributions of these identified receptor-channels were completely masked in the somata of sensory neurons by those unknown mechanisms of nicotine-induced intracellular calcium increase that were predominant in the cellular model. In the trachea, however, the contributions of the two TRP channels were evident, those of the nAChRs were small but discernible, and in the absence of the TRPs the CGRP release in response to 20 mM (pH 7.4) nicotine was abolished. Thus, if such high concentration of nicotine ever reached the intraepithelial nerve endings in the trachea, neurogenic inflammation would mainly be mediated through TRPV1 and TRPA1. However, a 200-times lower nicotine concentration could still exert pronounced irritancy in the airways through nAChRs.

7. Discussion

Measuring stimulated CGRP release from the isolated mouse trachea, a bimodal concentration-response relationship was established with a threshold below 10µM, an EC_{50} of 31 µM, a maximum at 100 µM (-)-nicotine, an apparent nadir between 0.5 and 10 mM, and a renewed increase at 20 mM. Using TRPV1, TRPA1 and double-null mutants as well as pharmacological tools, the micromolar activity range could be attributed to nAChRs, while the higher millimolar range was dominated by activation of TRPV1 and TRPA1 in particular at alkaline pH, when nicotine is uncharged and membrane-permeable. The (+)-nicotine enantiomer was ineffective at low and weakly effective at high concentration. The TRPA1 blocker camphor turned out to also inhibit nAChR, specifically though not selectively. Calcium imaging on cultured sensory neurons confirmed most of the results – except for the apparent nadir of the concentration-response – and revealed that the vagal NJG neurons exhibit much greater specific nicotinic sensitivity than somatic, i.e. DRG neurons. However, all neuronal cell bodies of both innervation territories showed the same large responses upon supramaximal 20 mM nicotine at unphysiological pH 9 in double-knockouts and wildtype mice, suggesting a predominant unspecific effect that was partly due to intracellular calcium release.

The nicotine EC_{50} in our trachea preparation was as low as 31µM. From recombinant rat nAChR composed of $\alpha_3\beta_4$ subunits EC_{50} values between 31 µM and 106 µM nicotine have been reported, while murine TRPA1-overexpressing Chinese hamster ovary (CHO) cells exhibited an EC_{50} of 17 µM, which suggests a complete overlap of the concentration-response relationships of nicotinic and TRPA1 receptors in heterologous expression systems (Rau et al., 2005; Talavera et al., 2009). However, in TRPA1 null mutants 4% of cultured trigeminal neurons were reported sensitive to 100µM nicotine, 12% to 1 mM nicotine, and this percentage

JPET Fast Forward. Published on August 7, 2013 as DOI: 10.1124/jpet.113.205971
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dropped to 1.6% under hexamethonium, the specific nAChR blocker (Talavera et al., 2009). This concentration-dependend increase in prevalence and decrease by application of a selective antagonist strongly indicate a specific nicotinic action in this micromolar range. In fact, 24% of our nodose-jugular ganglion neurons from TRPV1/TRPA1 double-null mutants were activated by 100 µM nicotine, while hardly any DRG neuron responded (Figs. 4B,C), suggesting that trigeminal neurons might range in between DRG and NJG with respect to nicotinic sensitivity. For rat DRG neurons much higher nicotine EC_{50} values (>200µM) have been reported (Rau et al., 2005). A high nicotinic sensitivity can be enabled by a supportive action of voltage-gated calcium channels co-expressed in sensory neurons (Fucile et al., 2005). The fractional calcium conductance of heteropentameric – as opposed to homopentameric – nAChRs (here $\alpha_3\beta_4$) is restricted but, if they depolarize the neuron by sodium influx, those calcium channels will be activated and provide cytosolic calcium increase and eventually CGRP release (Stetzer et al., 1996; Letz et al., 1997; Fucile et al., 2005).

In contrast to the cellular model, the nicotine-induced tracheal CGRP release showed this accentuated apparent nadir around 1 mM in the concentration-response curve that clearly separated a specific nicotinic, micromolar, mode of action from the TRPV1/TRPA1-dominated millimolar mode, shaping an inverted U in the lower micromolar range. Effects that decrease or revert into the contrary at supramaximal concentration or dosage are a classical hallmark of the nicotine concentration/dose-response relationship and have been demonstrated in many animal and human experimental models (Ashton et al., 1980; Nakamura et al., 1986; Clarke 1990; Perkins et al., 1994; Krebs et al., 1994; Rowell & Li, 1997; Matta et al., 2007; Picciotto 2003). The phenomenon has unanimously been attributed to the well known fast and complete homologous desensitization/tachyphylaxis of the nAChR

that is particularly evident at high agonist concentrations (Jinks & Carstens 1999; Dessirier et al., 2000; Liu & Simon 1996; Rau et al., 2005). Our results from the trachea confirmed the principle of nicotinic desensitization, but this did not seem to explain the significant loss of efficacy at supramaximal, 0.5 and 10 mM, nicotine concentration, because the (maximal) epibatidine response was not reduced by additional 1 mM nicotine. Perhaps, the low concentration (0.1 µM) of this ultrapotent agonist was still too high, creating a complete and prolonged nACh receptor occupancy so that the (1000-fold) lower affinity nicotine passed by ineffectively.

To activate recombinant TRPA1 (in CHO cells), the non-electrophilic nicotine binds to a yet unknown but definitely intracellular site of the ion channel (Talavera et al. 2009), which is consistent with the pronounced increase of nicotine effects at unphysiological, alkaline, pH in both of our models, because only deprotonated nicotine can freely pass plasma membranes. On the other hand, nicotine is also reported to block the open TRPA1 pore at higher concentrations with an IC_{50} of 2 mM (Talavera et al. 2009). This open channel block was discovered using neutral nicotine solutions, when most of the alkaloid molecules are positively charged and prone to attach to negative charges in the channel walls. Hence, the blocking side effect is most likely reduced with alkaline nicotine solution, and uncharged nicotine may even pass the TRPA1 (and TRPV1) channel that is known for its unusually wide pore, conducting even larger molecules when activated (Karashima et al., 2010). Intracellularly, nicotine would then become protonated and, thus, trapped. Thereby, 20 mM nicotine – as in our experiments – conveys a considerable buffer capacity that would alkalize the axoplasm, which is a further condition activating TRPA1 (Fujita et al., 2008). All these augmenting and interlaced mechanisms together may explain the pronounced pH dependency of millimolar nicotine actions and account for the drastic, though not simply cytotoxic, effects of alkaline nicotine. At

physiological pH these TRPA1/TRPV1-mediated and partly unspecific nicotine effects were much smaller and, actually, smaller than the specific, genuinely nicotinic micromolar effects in NJG neurons as well as in the trachea.

The TRPA1 antagonist camphor is contained in plenty of consumer products such as teas, liqueurs, spice blends, sweets, incense, snuff and in herbal cigarettes together with or without tobacco. Its role is to scent or flavor, its smell is eucalyptus-like but in higher concentration like mothballs ("camphor ball"). Also in medicinal liniments and ointments camphor is contained in high concentrations, restricted by the FDA to 11% (Federal Register 47 No. 183, p. 41716; 1982, USA). Thus, within certain dose limits camphor can be generally regarded as safe. It could therefore be added to oral nicotine-containing products such smoking cessation aids, when sensory irritation becomes a problem that reduces compliance. Camphor could reduce the pungency of these products, first by blocking TRPA1, secondly by blocking nAChRs of oral nerve endings. The suitable range of camphor concentrations appears limited to below 6 mM, because from this level on camphor evoked CGRP release by itself (from isolated mouse buccal mucosa) and augmented the nicotine (30 mM, pH 9) response, both effects mediated through TRPV1 in TRPA1 null-mutants (T. Kichko & P. Reeh, unpublished; Xu et al., 2005). Decreasing the saliva pH by acidic nicotine-based oral smoking cessation aids would be another way of preventing burning mouth sensations, according to our results. However, neutral or lower pH also reduces the nicotine absorption, if not the amount though probably the speed. Thus, the peak plasma concentrations may not be reached that are required to assuage the craving for a cigarette (Tomar & Henningfield, 1997).

When TRPA1 activation by nicotine was uncovered, the authors demonstrated the quasi physiological relevance with a whole-animal experiment: 60

mM nicotine solution was applied by nasal instillation which resulted in a mild, as compared to methacholine, bronchoconstrictory response of wildtype but not TRPA1-deleted mice. TRPV1 knockouts were not tested, but recombinant human TRPV1 was reported to be blocked rather than activated by 1 mM nicotine (Talavera et al. 2009). Previously, however, a direct sensitization, though not activation, of rat TRPV1 in CHO cells by 0.1 and 1 mM nicotine had been reported (Liu et al., 2004). Our results from the native mouse trachea are at variance with both reports: The robust effect of 0.1 mM nicotine did neither involve TRPV1 nor TRPA1 but was due to nAChRs (Fig 1B), while high millimolar nicotine engaged all three receptor-channels (Fig. 4A). The discrepancies might relate to species differences (rat/ mouse) but, first of all, they point to the principal differences between heterologous overexpression systems, cell culture models, and native tissues with intact innervation. Neither the nicotine concentrations impacting the tracheal mucosa and reaching its intraepithelial free nerve endings (Hauser-Kronberger et al., 1997), nor the local pH resulting from cigarette smoke or nicotine aerosol ("electric cigarette") inhalation are known. But, even if TRPV1/TRPA1-activating millimolar nicotine concentrations together with alkaline pH were not attained, the much more sensitive and effective nAChRs – mostly co-expressed with substance P/CGRP in sensory neurons (Rau et al., 2005) – could still mediate neurogenic inflammation in the trachea with plasma extravasation and vasodilatation, as previously demonstrated by antidromic stimulation of vagal sensory nerves (McDonald et al., 1988). In addition, nicotine largely contained in the particulate phase of cigarette smoke comes together with a multitude of volatile TRPA1 agonists in the gaseous phase such as acrolein, formaldehyde, acetaldehyde, crotonaldehyde etc. (Roemer et al., 2012). Andrè et al. (2008) have shown that the entirety of the water-soluble cigarette smoke constituents, which includes nicotine, can activate TRPA1 in the mouse

trachea. As specific nicotinic and TRPA1-activating effects are additive (Fig. 2D), the significance of the more sensitive nAChRs for sensory irritation should not be disregarded. Moreover, it is largely the same sensory neurons (Fig. 7) that mediate specific nicotinic as well as TRPV1 and TRPA1 agonistic actions and, likely, they send signals of sensory impact to the brain, differently perceived by smokers and non-smokers.

8. Acknowledgments. We thank Susanne Haux-Oertel, Iwona Izydorczyk, Annette Kuhn for excellent technical assistance and Jana Schramm for genotyping and breeding the mutant mice. Quantitative RT-PCR analysis by Andrea Link, MSc, is gratefully acknowledged.

9.Authorship Contributions.

Participated in research design: Kichko, Reeh, Kobal.

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Contributed new reagents or analytic tools: Lennerz, Neuhuber, Reeh.

Performed data analysis: Kichko, Eberhardt.

Wrote or contributed to the writing of the manuscript: Kichko, Reeh, Kobal.

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11. Footnotes

Financial support for the work was provided (in part) by Philip Morris USA and Altria Client Services Inc., Richmond (VA) USA.

12. Figure Legends.

Fig.1. A) Concentration-dependent nicotine-induced CGRP release from isolated trachea of wildtype mice; threshold concentration 10µM (significant over baseline, *****Wilcoxon test), EC_{50}~31µM. P<0.01 and smaller applies to one-way ANOVA followed by LSD Fisher test. In presence of EGTA (10mM) and absence of extracellular calcium ions no response to maximal effective nicotine concentration (100 µM). B) Specific nicotinic responses in congenic wildtype (C57Bl6) mice and TRPA1/V1 double-null mutants (Data normalized to baseline, i.e. second incubation/ sampling period).

Fig.2. Pharmacological differentiation of nicotinic and TRPA1-mediated tracheal CGRP release. P values refer to peaks of CGRP release (one-way ANOVA followed by LSD Fisher test). A) Mecamylamine concentration-dependently targeted heteropentameric nAChRs, methyllycaconitine–homopentameric ones; hexamethonium served as unselective nAChR blocker. B) The unselective TRPA1 antagonist camphor abolished half-maximal and maximal effective nicotinic concentration-responses, while the specific TRPV1 antagonist BCTC and TRPA1 antagonist HC030031 were ineffective. C) The selective TRPA1 agonist acrolein caused concentration-dependent tracheal CGRP release with a minimal effective concentration of 10µM. The TRPA1 blockers HC030031 and camphor reduced the acrolein responses, while mecamylamine was ineffective. D) The half-maximal effective nicotine concentration co-applied with low TRPA1-selective concentrations of mustard oil (AITC) and acrolein showed only additive effects.

Fig.3. Specific antinicotinic camphor effects. A) Camphor concentration-dependently decreased the half-maximal tracheal nicotinic response normalized to baseline. Insert shows % reduction of CGRP release upon co-application of nicotine and camphor. B) Camphor reversibly abolished nicotinic-induced calcium influx in cultured sensory neurons from the mouse nodose-jugular ganglion complex and did not show stimulatory effect by itself. Mustard oil (MO=AITC), capsaicin and KCl as positive controls for TRPA1, TRPV1 activation and unspecific depolarization, respectively. C) Camphor and mecamylamine reduced the tracheal response to the ultrapotent nicotinic agonist epibatidine co-applied at saturating concentrations and equally effective in WT and TRPV1-/-. D) Inhibitory camphor effect in TRPA1-/- on the tracheal epibatidine response. (P values one-way ANOVA followed by LSD Fisher test).

Fig.4. Supramaximal (20mM) nicotine effects depend on genotype and pH. A) Tracheal CGRP release at neutral and alkaline pH involving TRPA1, TRPV1, nAChR-channels; small but significant responses retained in double-knockouts (at pH9) are reduced by hexamethonium. B, C, D) Averaged calcium transients (±SEM) in sensory neurons of TRPV1/A1 double-null mutants: B) Vagal NJG neurons responding to nicotine but not MO (AITC) and pH9 (left panel); areas under the curve (AUC) showing response magnitudes and percentages (%) of neurons responding (right panel). C) Somatic DRG neurons. D) DRG neurons without extracellular calcium showed reduced percentage and AUC upon 20mM nicotine at pH9 and partial recovery upon calcium returned, hexamethonium (10mM) without effect; the columns (right panel) are ordered according to the sequence of stimuli. (P values one-way ANOVA followed by Fisher's LSD test).

Fig.5. Only supramaximal (20mM) nicotine shows pH-dependency and cross-desensitization in NJG neurons.

A) Nicotine 20mM caused greater prevalence (%) and magnitude of calcium transients at pH9 than pH7.4 and leaves cross-desensitization to mustard oil (MO=AITC). B) Different order of stimuli confirmed pH-dependency and prevented cross-desensitization. C) No difference in percentage and magnitude at 1mM nicotine pH7.4 versus pH9.

Fig.6. Prevalence and magnitude of specific nicotinic responsiveness greater in NJG than DRG neurons. A) NJG neurons, calcium transients in response to specific nicotinic concentration about equal at pH7.4 and pH9, high percentage of cells responding, no cross-desensitization of MO (AITC) responses. B) DRG neurons, low percentage of cells responding with small calcium transients, significantly smaller than NJG neurons (in A), # signifies $p<0.01$, one way ANOVA followed by LSD test. C) Almost all DRG neurons responding with large calcium transients to supramaximal nicotine at pH9, less so at pH7.4, pronounced cross-desensitization against MO (AITC).

Fig.7 Venn diagrams of nicotine (100µM vs. 20mM), mustard oil (100µM) and capsaicin (0.3µM) responsiveness of NJG versus DRG neurons at pH7.4 vs. pH9 of the nicotine solution (derived from Figs.5, 6). The total number of DRG and NJG neurons from C57Bl/6 mice was identified by their KCl responsiveness. The main difference was in the prevalence of specific nicotinic (100 µM) sensitivity, NJG>DRG, little difference between pH7.4 and pH9 (right panels). Differences shrink with 20mM nicotine, in particular at pH9, which activates most or all neurons in both types of ganglia (left panels).

JPET Fast Forward. Published on August 7, 2013 as DOI: 10.1124/jpet.113.205971
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Table 1.

Comparison of NJG versus DRG neurons of WT versus TRPV1/A1=/= mice at specific and supramaximal nicotine concentrations. The numbers are derived from data behind Figs. 3B, 4B-D, 5A and 6A-C. Number (n) of sensory neurons tested, AUC refers to magnitude of ratiomeric calcium transients, # signifies significant difference between labeled numbers ($p<0.01$, one-way ANOVA followed by LSD test).

	Nicotine 100µM pH 7.4		**Nicotine 20mM pH 9**		
	% prevalence	**AUC x 100**	**% prevalence**	**AUC x 100**	**n**
NJG Wild type	48	**25** ± 4 #	86	**24** ± 1.8	92/192
NJG TRPV1/A1=/=	24	**8.5** ± 2.8	100	**26** ± 2.6	25
DRG Wild type	14	**6** ± 1.6 #	99	**37** ± 2	110/105
DRG TRPV1/A1=/=	1	**0.7** ± 0.15	95	**41** ± 3.5	20/66

Fig.1

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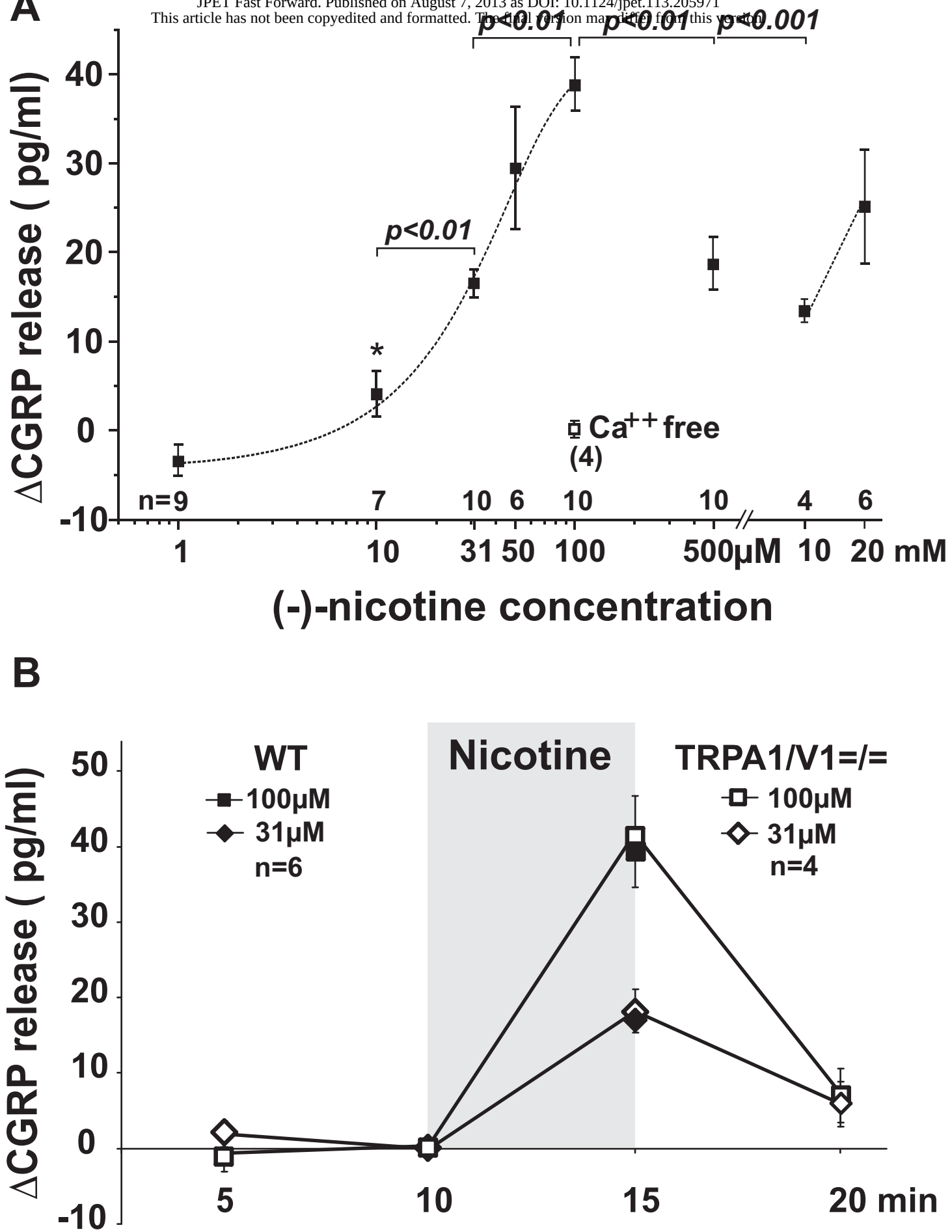

Fig.2

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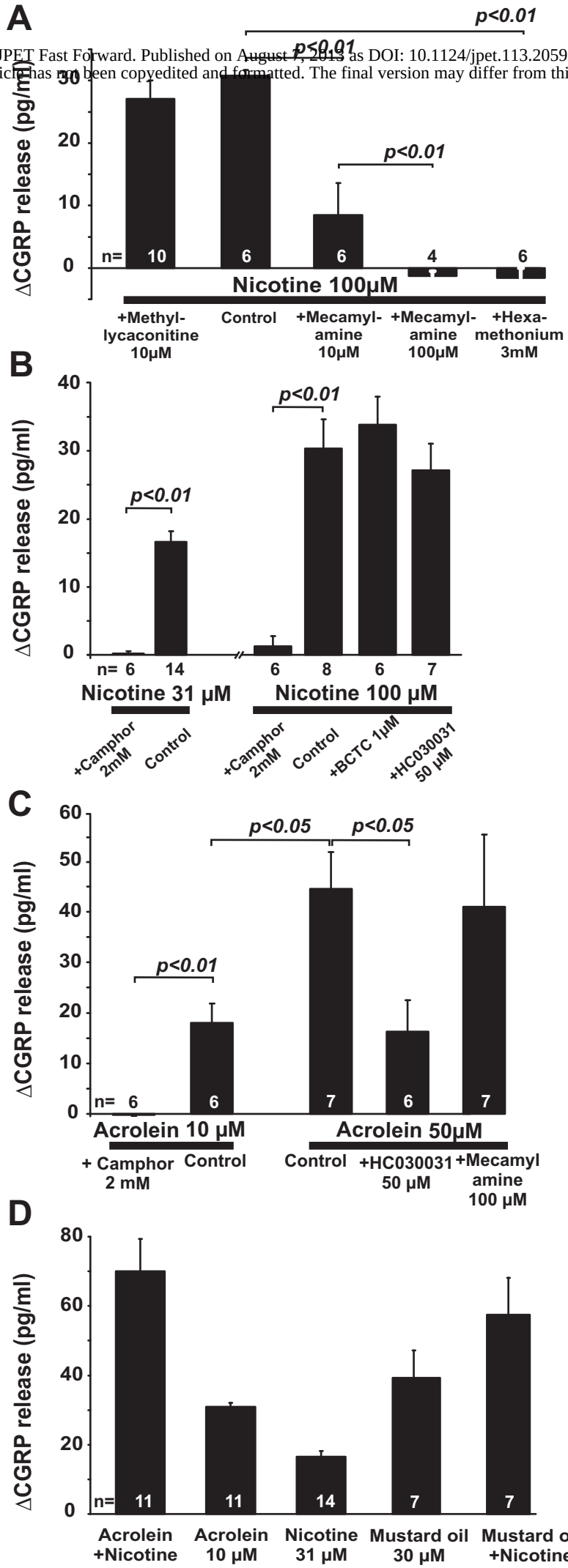

Fig.3

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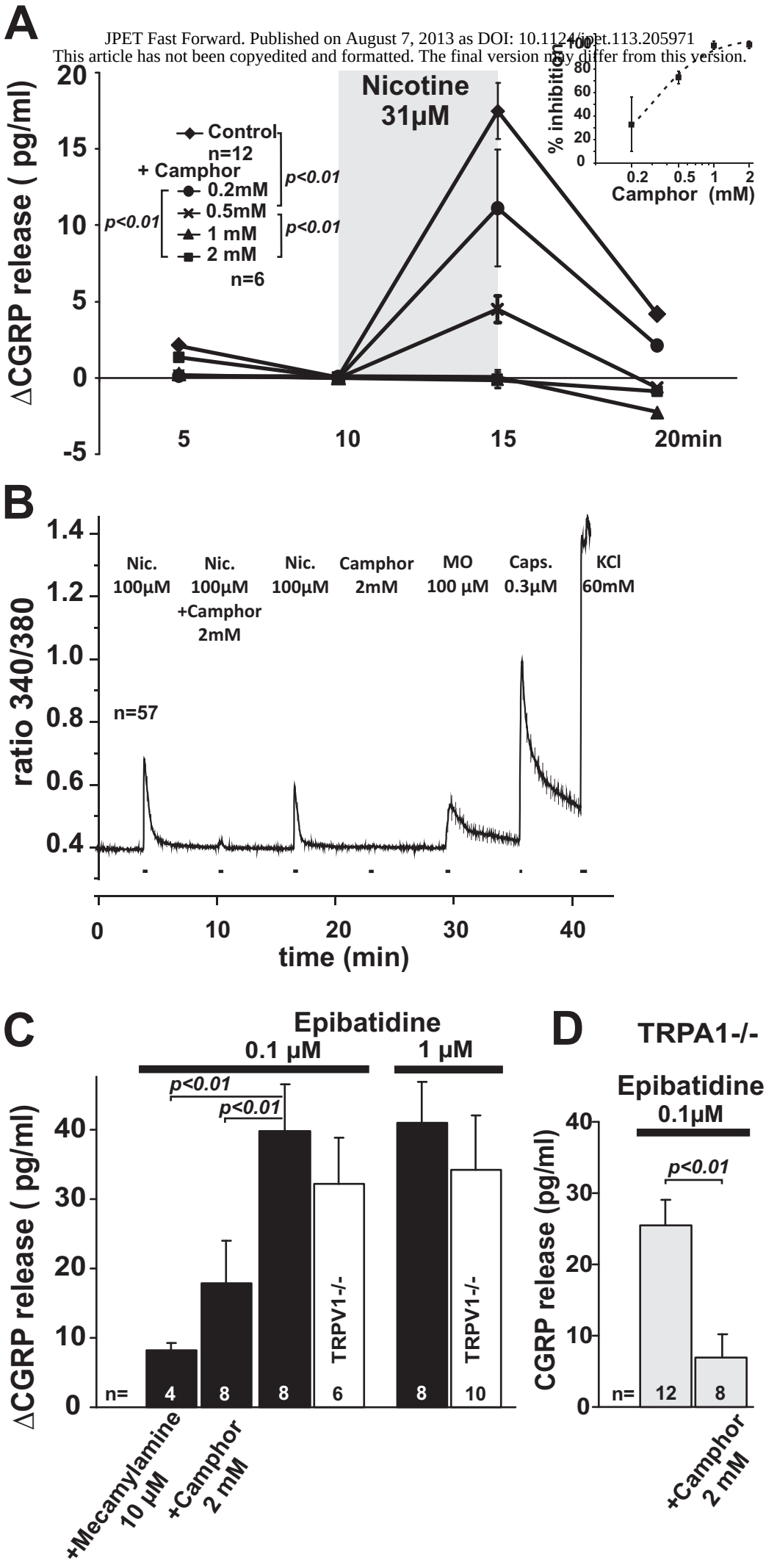

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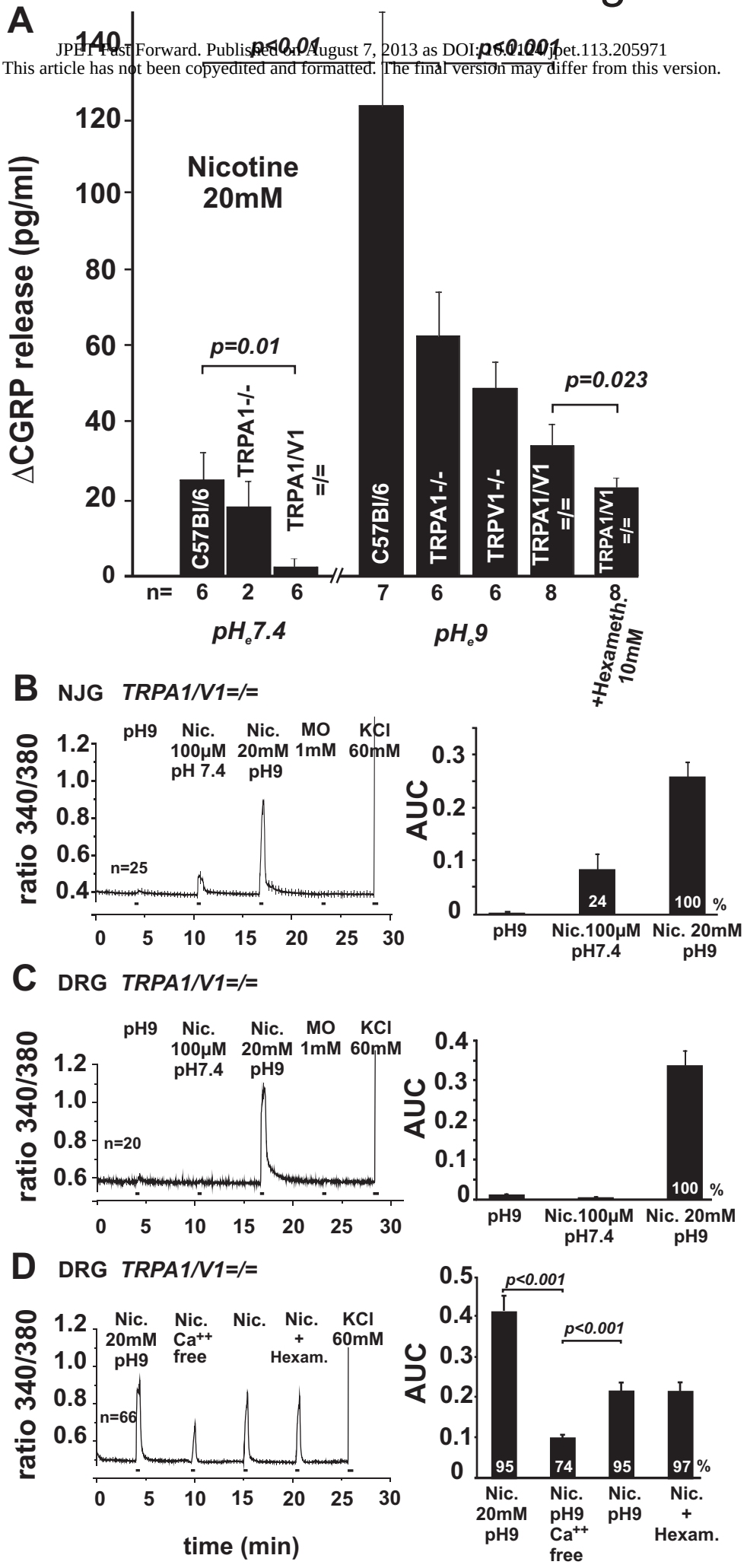

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Fig.5

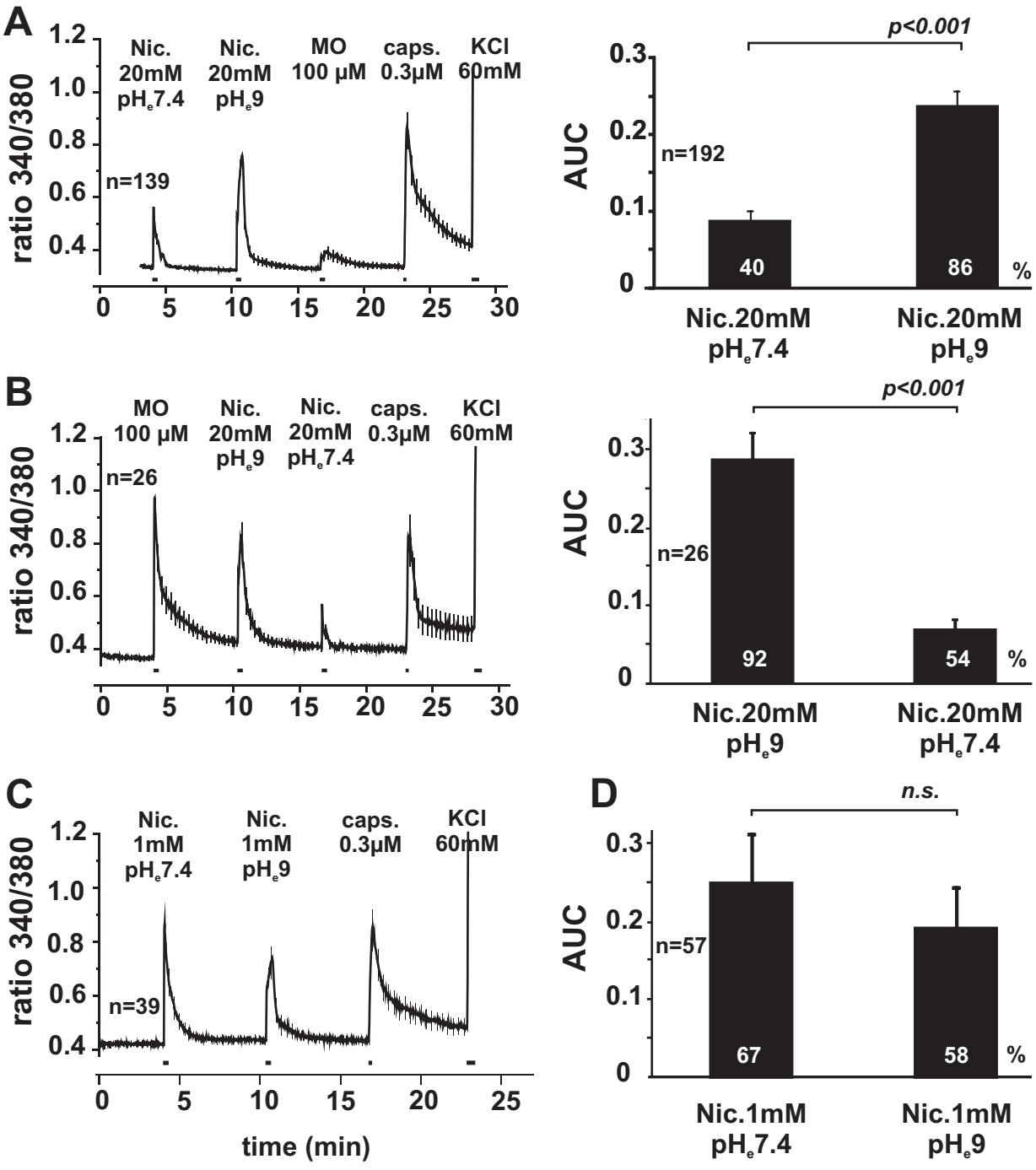

Fig.6

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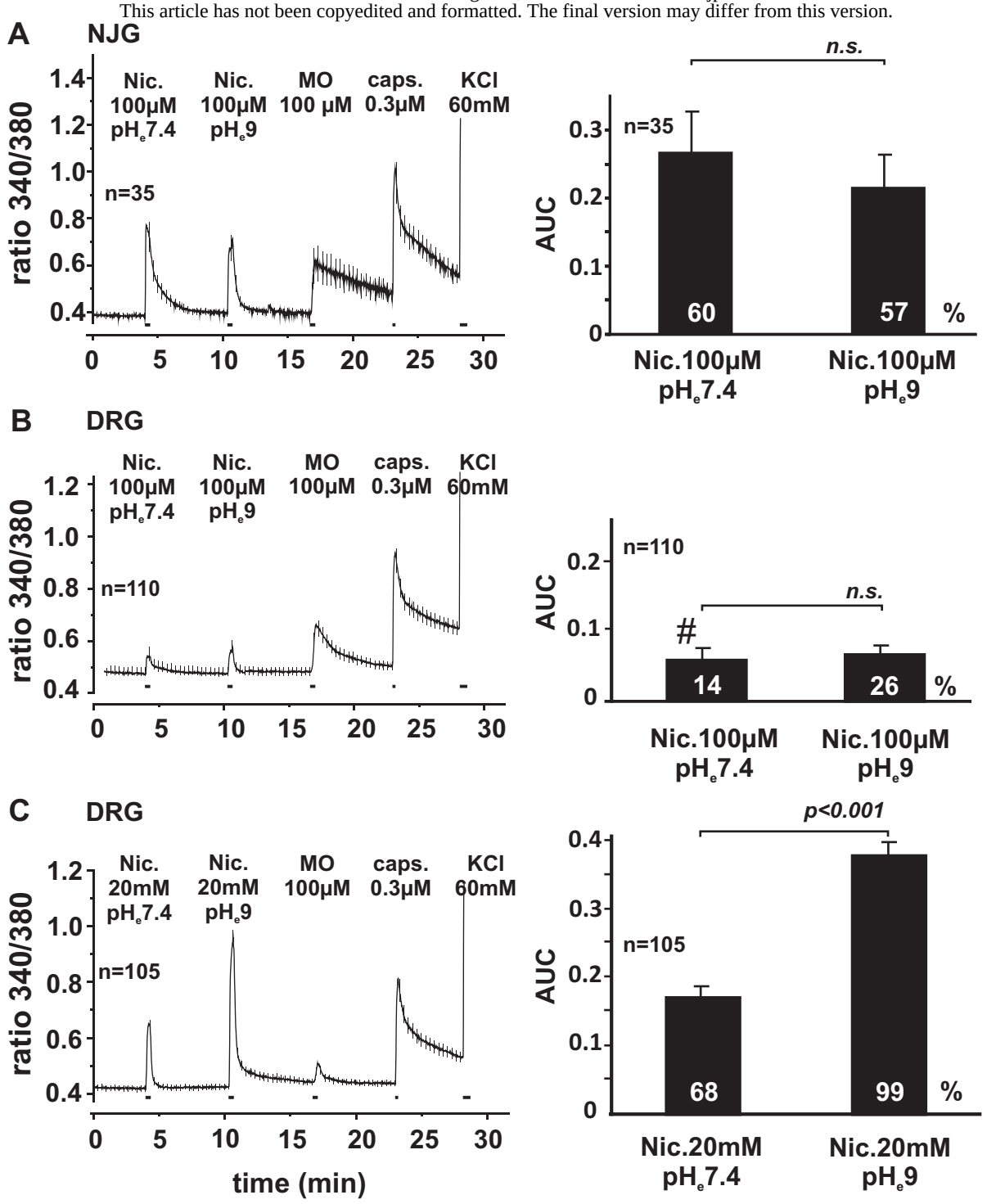

Fig.7