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ORIGINAL ARTICLE

Investigation of feeding behaviour in *C. elegans* reveals distinct pharmacological and antibacterial effects of nicotine

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Abstract
Caenorhabditis elegans is an informative model to study the neural basis of feeding. A useful paradigm is one in which adult nematodes feed on a bacterial lawn which has been pre-loaded with pharmacological agents and the effect on pharyngeal pumping rate scored. A crucial aspect of this assay is the availability of good quality bacteria to stimulate pumping to maximal levels. A potential confound is the possibility that the pharmacological agent impacts bacterial viability and indirectly influences feeding rate. Here, the actions of nicotine on pharyngeal pumping of *C. elegans* and on the *Escherichia coli* bacterial food source were investigated. Nicotine caused an immediate and concentration-dependent inhibition of *C. elegans* pharyngeal pumping, IC_{50} 4 mM (95% CI = 3.4 mM to 4.8 mM). At concentrations between 5 and 25 mM, nicotine also affected the growth and viability of *E. coli* lawns. To test whether this food depletion by nicotine caused the reduced pumping, we modified the experimental paradigm. We investigated pharyngeal pumping stimulated by 10 mM 5-HT, a food 'mimic', before testing if nicotine still inhibited this behaviour. The IC_{50} for nicotine in these assays was 2.9 mM (95% CI = 3.1 mM to 5.1 mM) indicating the depletion of food lawn does not underpin the potency of nicotine at inhibiting feeding. These studies show that the inhibitory effect of nicotine on *C. elegans* pharyngeal pumping is mediated by a direct effect rather than by its poorly reported bactericidal actions.

Keywords *Caenorhabditis elegans* · *E. coli* · Bactericide · Pharyngeal pumping · Nicotine

Introduction

Acetylcholine is the most widely used transmitter in *C. elegans* with 52 of the 118 types of neuron being cholinergic (Pereira et al. 2015). The cholinergic system is important in the regulation of many physiological functions, including locomotion, egg-laying and pharyngeal pumping (Richmond and Jorgensen 1999; Raizen et al. 1995; Duerr et al. 2001) and nicotinic receptors are involved in mediating the effect of acetylcholine on all these behaviours. The molecular pharmacology of this nicotinic cholinergic system in *C. elegans* is complex as over 40 putative nicotinic acetylcholine receptor subunits were identified in *C. elegans* (Jones and Sattelle 2004; Jones et al. 2007). In view of the functional importance of nicotinic acetylcholine receptors at *C. elegans* neuromuscular structures, the actions of the pharmacological agonist nicotine have been investigated by several groups.

The effects of nicotine on locomotion can be studied by observing worms crawling on agar plates either in the presence or absence of their food source, bacteria. In studies on crawling, worms show exploratory behaviour, forward movement accompanied by occasional turns or reversals (Zhao et al. 2003). Nicotine exhibits complex dose-dependent effects on locomotion: at 1 µM in food-free plates it reduced the mean speed of movement, increasing the concentration to 10–100 µM increased the speed (Sobkowiak et al. 2011) and at 1, 10 and 30 mM, the mean speed decreased (Sobkowiak et al. 2011). In addition, there is evidence that nicotine modulates the vulva body wall muscle that controls egg laying as concentrations between 1.5 and 6 mM caused a concentration-dependent stimulation of egg-laying (Kim et al. 2001).

The effects of nicotine on feeding may be monitored by scoring the rate of pharyngeal pumping. The stimulation of pharyngeal pumping is mainly regulated by 5-hydroxytryptamine (5-HT) and acetylcholine (Albertson and

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Thomson 1976; Franks et al. 2006) and by 2 out of 14 neuron types in the pharynx, namely M4 and MC (Avery and Horvitz 1989; Raizen et al. 1995). In the presence of bacteria, the NSM neurosecretory neurons release 5-HT (Sze et al. 2000) which activates MC and M4 neurons via volume transmission (Albertson and Thomson 1976). Depolarised MC and M4 neurons release acetylcholine and stimulate the pharynx to pump at a rate of around 250 pumps min^{-1} (Avery and Horvitz 1989).

There are two routes of scoring the effects of compounds on pumping of intact animal. The most commonly used assay involves the use of bacteria as a stimulant on a solid medium. An alternative assay, which removes the need for bacteria to stimulate pumping (Dernovici et al. 2007), is an in-liquid assay employing 5-HT. 5-HT does not only stimulate pumping, but also inhibits locomotion of worms in liquid (Horvitz et al. 1982). This paralysing effect of 5-HT enables counting of pharyngeal pumping of the intact animal in liquid. Lastly, the effects of compounds on un-stimulated pharyngeal pumping can also be investigated. It has been shown that nicotine at up to 1 mM stimulated pharyngeal pumping in intact wild-type *C. elegans* (Avery and Horvitz 1990). Using dissected *C. elegans*, nicotine at 1–100 µM had a direct stimulatory effect on the pharynx (Raizen et al. 1995). Together this suggests that there are diffusional barriers that limit the access of externally applied nicotine to intact *C. elegans* to activate internal nicotinic acetylcholine receptors.

In addition to these likely direct effects on cholinergic-dependent transmission, the worm has been shown to express several forms of nicotine-induced plasticity (Waggoner et al. 2000; Matsuura and Urushihata 2015).

The concentrations used to probe nicotinic responses in intact *C. elegans* are in the low mM range. Many observations of the alterations of *C. elegans* behaviour have been made in the presence of the bacteria food source. Interestingly, there have been a number of studies investigating the antimicrobial effects of nicotine and extracts of *Nicotiana tabacum*. Biofilm formation and the metabolic activity of *Streptococcus mutans* were enhanced at low mM concentrations of nicotine (12.5–50 mM), but inhibited at concentrations of 100 mM and above (Huang et al. 2012). Growth of planktonic *S. mutans* cells was inhibited by 12.5–50 mM nicotine. In another study, around 100–200 mM nicotine was required for the antibacterial effect against *S. mutans* (Li et al. 2012). Nicotine also inhibits the growth of a range of bacteria including *E. coli* at concentrations in the low mM range. Moreover, two novel nicotine analogues had antibacterial properties against *E. coli* when applied at 6 mM (Gandhi and Athmaram 2016; Bautista et al. 2014). Nicotine at low mM concentrations changes the energy-related metabolism and choline metabolism of *E. coli*, resulting in what the authors call nicotine stress (Ding et al. 2014).

During the course of experimental investigations designed to probe the cholinergic pharmacology of pharyngeal function we observed that *E. coli* (OP50 strain) grew less well on plates containing nicotine. This presents an alternative interpretation of earlier reports on the effect of nicotine on *C. elegans* behaviour. That is, rather the response to nicotine being a direct consequence of its pharmacological actions on nicotinic acetylcholine receptors within the circuits of the worm that regulate behaviour, the effect could be explained by an indirect effect of nicotine on the quality of the worm's food source. Indeed the literature is replete with evidence of the intricate and discrete effect of changes in the quantity and quality of the bacterial food source on *C. elegans* behaviour, including feeding (Shtonda and Avery 2006). Therefore, here we have conducted parallel studies in which we investigate the effects of nicotine on *C. elegans* pharyngeal pumping and *E. coli* growth to determine whether any direct effects of nicotine on *E. coli* could indirectly affect its potency on pumping. This reveals that the nicotine-dependent modulation of a food activated pharyngeal circuit is underpinned by a direct effect on cholinergic transmission.

Materials and methods

Maintenance of *C. elegans*

Caenorhabditis elegans were maintained on 5-cm Petri Dishes containing 5 ml of nematode growth medium (NGM) and 0.05 ml of seeded *E. coli*, strain OP50 ($OD_{600nm} = 0.6$). To maintain viable *C. elegans*, each week 2 adult hermaphrodites were picked and placed onto a fresh seeded plate and left to propagate. Worms were handled using a platinum rod. Developmentally synchronised hermaphrodites, that is, L4 + 1 (young adults), were used in all assays. This was ensured by picking L4 worms, identified by a crescent shape around the vulva the day before the assay. All experiments were carried out at 20 °C and pharyngeal pumping was scored under a binocular microscope. Wild-type N2 Bristol strain hermaphrodites were used in the current investigation.

Drug solutions

All compounds were purchased from Sigma-Aldrich and dissolved in ddH_2O. The stock solutions of 250 mM (−)-nicotine hydrogen tartrate, 100 mM 5-HT creatinine sulphate monohydrate and 14 mM (50 µg/mL) ampicillin sodium salt were prepared in ddH_20. These stock solutions were aliquoted and stored at −20 °C. Once defrosted, they were stored at 4 °C and used within 2 weeks or discarded.

Optical density measurements

The OD_{600nm} of all *E. coli* OP50 culture samples were measured in a spectrophotometer, after being diluted 1 in 10 with ddH_2O.

NGM plates

The standard procedure for making NGM plates was followed (Brenner 1974). 3 ml of the NGM was pipetted into each well of a six well plate and left to dry overnight. 500 µl nicotine was added to 4.5 ml of NGM; 3 mL of nicotine-NGM mix was pipetted into each well of a 6 well plate. This was repeated pro-rata for the concentrations of nicotine used in this study, that is, 0, 0.1, 1, 5, 10 and 25 mM.

The effects of nicotine on pharyngeal pumping on an agar plate arena

Preparation of OP50 food patch on agar plates

A single colony of *E. coli* was taken from an OP50 stock plate using a pipette tip and placed into 5 ml of Lysogeny Broth (LB). The OP50 culture was incubated at 37 °C overnight in a shaking incubator. The following day, the overnight culture was diluted 100 times in 5 mL LB and grown as previously until it reached an optical density (OD_{600nm}) of 0.6. 50 µl of the OD = 0.6 OP50 culture was pipetted into each well of a six well plate and the maintenance plates. The plates were left overnight to allow the OP50 lawn to grow.

Pharyngeal pumping measurements

L4 hermaphrodites were picked from the culture onto a fresh NGM plate. 24-h later, their pumping rate on food was recorded. Following this, 6 individuals were placed in each of the 6-well plate filled with *E. coli* seeded nicotine/vehicle infused NGM. 10 min after transfer to the drug/vehicle induced arena, pharyngeal pumping of worms present on food was recorded at this 0 time point [delaying the initial count ensures that worms had recovered from the pick-mediated inhibition of pharyngeal pumping (Chalfie et al. 1985)]. Pharyngeal pumping was also counted at six and twenty-four hours post-exposure.

Worms were viewed under the optical microscope and pumping rates made by visual observations. One pharyngeal pump was defined as a complete forward and backward movement of the grinder in the pharynx. The rate was counted for a period of 1 min and expressed in Hz.

The effects of nicotine on pharyngeal pumping in the liquid assay

M9 was used as the vehicle for the liquid medium assays with the addition of 0.1% BSA (bovine serum albumin) (w/v). The assay was performed in a 24-well plate in a total volume of 500 µl. 50 µL of 5-HT was added to achieve a final concentration of 10 mM. This paralysed the worms (Gurel et al. 2012) and induced pumping in the absence of food. After 30 min, 50 µl of nicotine was pipetted into the wells containing the 5-HT stimulated worms to provide final concentrations in the wells of 0.1, 1, 5, 10 and 25 mM. The effect of nicotine on 5-HT induced pumping was scored (as described previously) at time zero and after 10, 20, 30, 40, 50 and 60 min. Data are presented in Hz.

OP50 growth assay

An overnight culture was made as described above. This was then diluted 100 times in 10 mL LB and grown until OD reached ~ 0.6. Following, 4.5 mL of 0.6 culture was added to 0.5 mL of nicotine to give final concentrations. In parallel, a distinct incubation was supplemented with 140 µM of the bactericidal ampicillin. This ampicillin incubation was used as a positive control with known bactericidal effects. The OD of these incubations was measured 0.5, 1, 1.5, 2, 2.5, 3 and 24 h after the indicated drug addition.

To visually represent the effects of nicotine or ampicillin on OP50 growth, images of OP50 lawn grown on drug-containing NGM were taken. Plates were prepared as described above. 24 h after seeding, the images of OP50 lawns were taken using USB camera to capture the entire food lawn. Plates were also viewed under Nikon eclipse 800 microscope at 10 × magnification, and images captured with Hamamatsu camera. The scale was set using a graticule and scale bars added using ImageJ software.

Viable cell count

OP50 overnight cultures with the indicated concentrations of nicotine or ampicillin were made as described previously. 24 h after OP50 growth in the presence of the indicated nicotine or ampicillin, the cultures were diluted 10^6 times. 30 µL of each dilution was spread on a quadrant of an LB-agar plate. Plates were placed in the 37 °C incubator to allow bacterial growth. The following day, the number of colonies in each quadrant was counted and this number multiplied by the dilution to give the viable cell count, for example, Viable Cell Count = 30 × 1,000,000 equals 30,000,000.

Statistical analysis

Statistical analysis was carried out using both one- and two-way analysis of variance [ANOVA] where a result was recorded as significant if $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****). Post hoc analysis using Sidak with the levels of significance and P values being the same as two-way ANOVA. Error bars show the standard error of the mean [SEM]. All performed with GraphPad Prism 7.

Results

An established approach for assessing the effects of compounds on feeding of *C. elegans* is to place worms on agar plates seeded with *E.coli* (OP50 strain) and treated with a drug pre-equilibrated into the solid medium. In the presence of food, *C. elegans* exhibits rhythmic and sustained pharyngeal pumping (Fig. 1a) which enables them to ingest food. This behaviour was reduced by nicotine concentrations ≥ 1 mM (Fig. 1a, b). The inhibition was visible 10 min post-exposure and sustained throughout the duration of the experiment (24 h), (Fig. 1a). The IC_{50} of nicotine on pharyngeal pumping was 4 mM (95% CI = 3.4 to 4.8 mM) (Fig. 1b). During the experiments, it was observed that the OP50 lawn appeared reduced as the nicotine concentration increased (Fig. 2). In particular, exposure of OP50 to nicotine concentrations ≥ 5 mM led to a visually depleted food lawn. This suggested that a reduction in food source might contribute to the reduction in pumping observed at these concentrations.

To investigate how the nicotine was affecting bacterial growth, it was added to the exponentially growing OP50 cultures. Optical density (OD) was analysed as a measure of bacterial growth, and the results are shown in Fig. 3. Over the course of 24 h, the bacteria incubated in control conditions grew continuously and steadily, reaching a final OD of approximately 4. Addition of nicotine at 0.1–25 mM led to the concentration-dependent inhibition of bacterial growth. This effect could be observed after 1-h incubation and became increasingly obvious with time. The growth was partially inhibited by 5 mM nicotine and fully inhibited by 10–25 mM nicotine (Fig. 3a). A normalised OD graph gave an IC_{50} value of 3.2 mM (95% CI = 2.4 to 6.0 mM), (Fig. 3b). The effect of nicotine was compared to that of the positive control ampicillin, a known bactericide (Kohanski et al. 2007). The number of colonies of *E. coli* is shown in Fig. 4a, from which it can be seen that 5 mM nicotine greatly reduces the number of colonies while 10 mM nicotine is bactericidal. 0.14 mM (50 µg/ml) ampicillin also showed bactericidal effects (Fig. 4a). The IC_{50} of nicotine on colony growth was 2.2 mM (95% CI = 0.5 to 6.9 mM) (Fig. 4b).

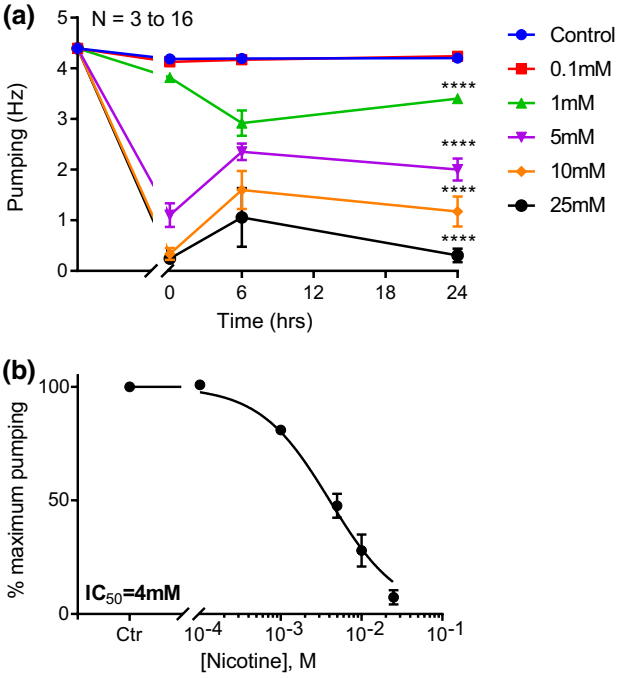

Fig. 1 Effects of nicotine on on-food pharyngeal pumping of *C. elegans*. **a** The concentration dependence and the time course for the effects of nicotine on pharyngeal pumping of *C. elegans*. Pumping rate of worms immediately prior transfer to the experimental arena and at 0 (10 min post transfer), 6, and 24 h post- exposure to 0.1–25 mM nicotine. Pumping was counted for a period of 1 min and expressed as pumps/s (Hz). Each experiment was repeated three times and the total number of individual worms tested is given on the graph. **b** Concentration-response curve for the effect of nicotine on pharyngeal pumping. Graph was generated by taking 24-h time points and expressed as a % of control pharyngeal pumping. IC_{50} value (drug concentrations generating 50% inhibition of the response) is given. Data points and error bars are mean ± SEM, respectively. **** $P < 0.0001$ by two-way ANOVA with post hoc analysis using Sidak

This suggests that the bacterial lawns in the plates used to measure pumping may be compromised by exposure to ≥ 5 mM nicotine. To test this we measured the effect of nicotine in a pharyngeal pumping assay that does not require the presence of bacteria. In this assay, exogenously applied 5-HT acts as a food mimic to stimulate pharyngeal pumping. This alternative assay to score the action of nicotine on pumping removes the need for the presence of bacteria (Dernovici et al. 2007) and is conducted in liquid. Worms were treated with 10 mM 5-HT. This had a dual effect: paralysing (Gurel et al. 2012) and a stimulatory effect on pharyngeal pumping (Fig. 5a). The pharyngeal pumping rate elicited by 5-HT was comparable to that seen in the presence of food (Figs. 1a and 5a). Addition of 0.1–25 mM nicotine resulted in a concentration-dependent inhibition of pumping (Fig. 5a), with an IC_{50} of 2.9 mM (95% CI = 3.1 to 5.1 mM) (Fig. 5b).

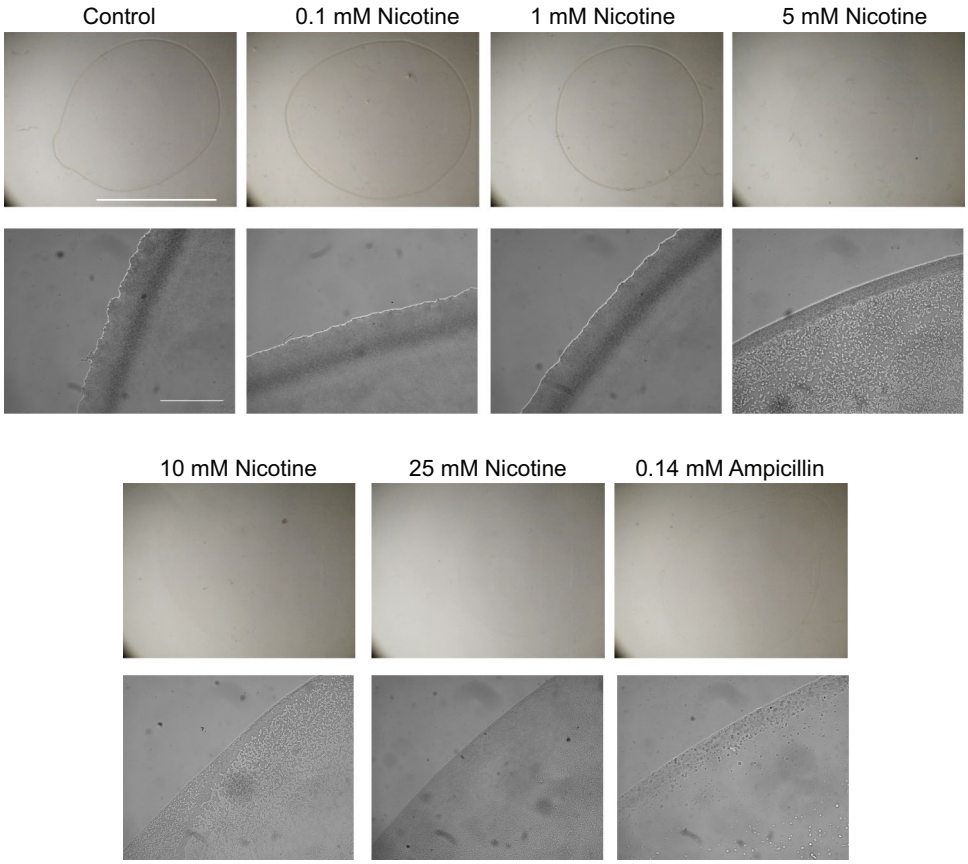

Fig. 2 Growth of OP50 food lawns on drug-treated NGM plates showing food lawn depletion at high nicotine concentrations or 140 µM ampicillin. An image of the entire and 10× magnified edge of OP50 lawns grown in the presence of increasing concentrations of nicotine or 0.14 mM (50 µg/mL) of ampicillin. Scale bar of upper image = 0.5 cm, whereas that in the lower image = 0.5 mm

Since the IC_{50} of nicotine on pumping in the agar plate assay which requires the presence of *E. coli*, and in the liquid assay in which 5-HT is used to stimulate pumping is comparable (4 and 2.9 mM, respectively, Figs. 1b and 5b), the antimicrobial effect of nicotine is most likely not responsible for the reduced pharyngeal pumping on plate-assays.

Discussion

The present investigation demonstrates that high concentrations of nicotine have a direct inhibitory effect on *E. coli* growth and that the IC_{50} values for the effect of nicotine on pharyngeal pumping and OP50 growth are 4 mM and 3.2 mM, respectively. This raises the possibility that the effects of nicotine on *C. elegans* behaviour may not be due to a direct pharmacological action on nicotinic acetylcholine receptors but rather might reflect an indirect consequence of the negative impact of nicotine on the quality and abundance of *C. elegans* food source. However, the finding that the IC_{50} value for nicotine on pharyngeal pumping in liquid in the presence of 5-HT but the absence of bacteria was 2.9 mM would suggest the bactericidal action of nicotine does not influence the potency of nicotine on pumping. One explanation is that the residual bacterial lawn is likely of sufficient density to cue pumping. Indeed, bacterial lawns made from highly diluted cultures are still capable of achieving maximal pumping (Chiang et al. 2006; our unpublished observations).

Since high concentrations of nicotine have similar effects on the OP50 as ampicillin, it is likely that nicotine is also bactericidal (Fig. 5a). This conclusion is supported by observing that the high concentrations of nicotine almost entirely killed the bacterial colony population in viable cell count assay and depleted food lawns (Figs. 2, 4a). Nicotine, 12.34 mM or higher, alters energy-related metabolism of *E. coli*, including changes to glycolysis and inhibition of the tricarboxylic acid cycle (Ding et al. 2014). As discussed in the introduction, nicotine can both stimulate and inhibit bacterial growth, depending on the concentration applied (Huang et al. 2012; Zaidi et al. 2012). In the current study a nicotine concentration of around 5 mM and higher was required to affect growth of OP50 *E. coli.* Our data suggest care should be taken in the interpretation of food-dependent *C. elegans* experiments where agents affecting bacterial biology are used (Cabreiro et al. 2013).

Nicotine has been shown to modify a number of *C. elegans* behaviours. It is likely that its action on the nervous system is complex and likely to influence more than one circuit. This suggestion is reinforced by the literature where the variable effects of nicotine are very concentration-dependent

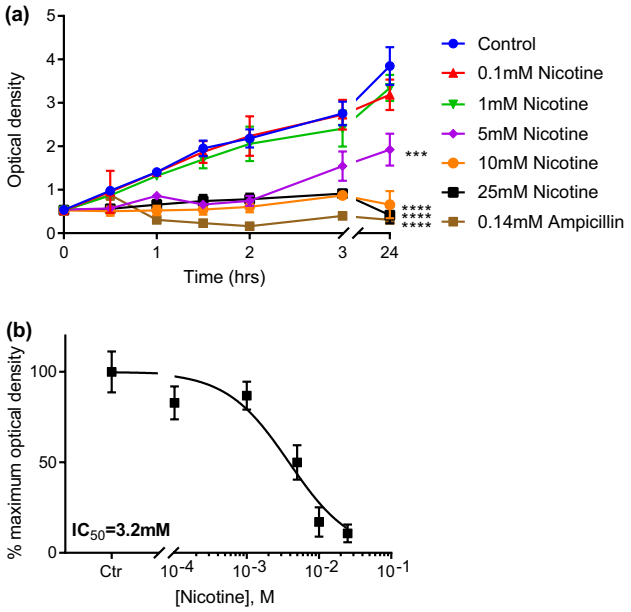

Fig. 3 Effects of nicotine on *E. coli* growth. **a** The concentration dependence and time course for the effects of nicotine on growth of *E. coli*. The effect of increasing concentration of nicotine or 0.14 mM ampicillin on the optical density of a culture of *E. coli* OP50. 3–7 independent repeats were performed. **b** Concentration-response curve for the effects of nicotine on *E. coli* growth. 24-h time points were taken and expressed as a % control optical density. IC_{50} value is given on the graph. Data points and error bars are mean ± SEM, respectively. *** $p < 0.001$, **** $P < 0.0001$ by two-way ANOVA with post hoc analysis using Sidak

(Sobkowiak et al. 2011; Urushihata et al. 2016). Nicotine effects also vary depending on whether it is applied acutely or chronically and at what stage in the development of the worm. Apart from having an effect on *E. coli* growth and in turn, an effect on *C. elegans* development and behaviour, nicotine has effects on worm gene expression. Chronic, µM nicotine exposure for 24-h resulted in the up regulation of eleven genes and these worms showed significantly increased locomotion (Polli et al. 2015). The effect of nicotine is both time-dependent and concentration-dependent (Sobkowiak et al. 2011). Nicotine can also affect *C. elegans*' response to touch (Smith et al. 2013). These authors also found that the expression of ten protein-coding genes was affected by nicotine when applied at 6.17–194.5 µM. Interestingly, lower concentrations of nicotine had greater effect than higher concentrations.

One striking observation associated with nicotine-dependent effects that are reported in worms exposed to external drug is the wide concentration response range: 10 µM to 50 mM. Although the bioaccumulation over time may underlie the requirement for mM dosing it is striking that there is evidence for rapid behavioural responses to nicotine. In the current study, with the higher concentrations we see pronounced behavioural inhibition within 10 min of the worms making contact with the drug laced plate (Fig. 1a). The initial delay in measuring pumping to allow recovery from pick-mediated inhibition precludes a more precise definition of the onset of inhibition. However, this time course might suggest an additional consideration that some nicotine sensitive receptors are more directly exposed to external drug. Can such sites at which nicotine

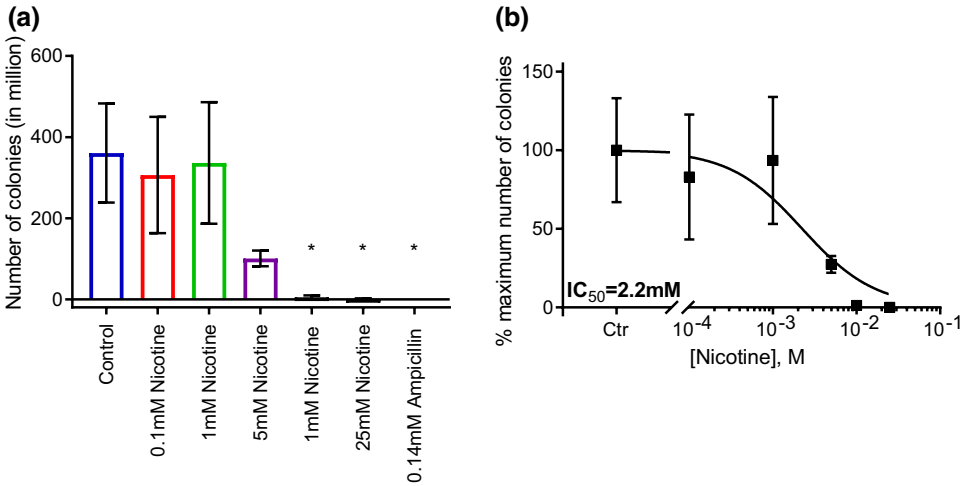

Fig. 4 Effect of nicotine on E. *coli* viability. **a** Concentration dependence for the effects of nicotine or ampicillin on viability of OP50. Number of OP50 colonies formed overnight on the LB-agar plate from the sample of cultures grown for 24 h either in the presence of 0.1–25 mM nicotine or 0.14 mM ampicillin. 3–6 independent repeats were performed. **b** Concentration response curve for the effect of nicotine on OP50 viability. Data expressed as a % of number of colonies formed from control culture. IC_{50} is given on the graph. Data points and error bars are mean ± SEM, respectively. * $p < 0.05$, by one-way ANOVA with post hoc analysis using Sidak

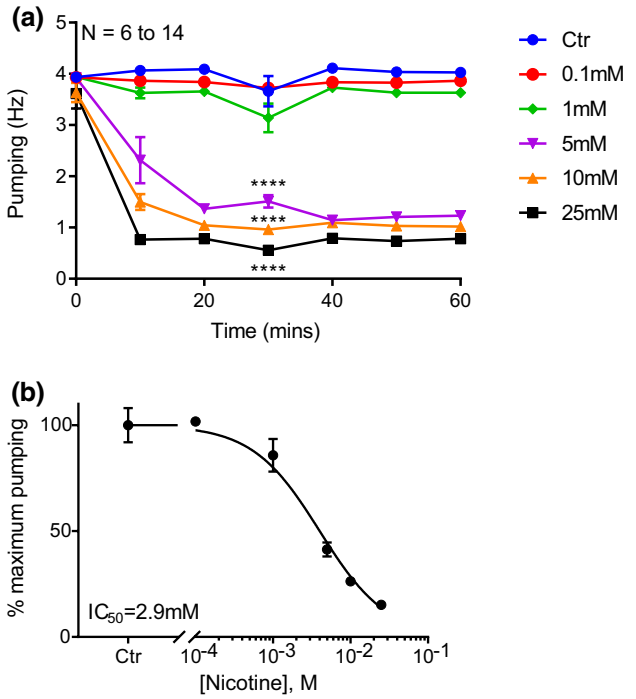

Fig. 5 Effect of nicotine on 5-HT induced pharyngeal pumping in liquid medium. **a** Concentration dependence and time course for the effects of nicotine on 5-HT induced pumping. Nicotine was added following 30 min 10 mM 5-HT exposure. Pharyngeal pumping was counted for a time period of 30 s and expressed as number of pumps/s (Hz). Each experiment was repeated three times and the total number of individual worms tested is given on the graph. **b** Concentration response curve for the effect of nicotine on pumping. Graph was generated by taking 30-min time points and expressed as % control pumping rate. IC_{50} is given on the graph. Data points and error bars are mean ± SEM, $^{****}P < 0.0001$ by way of two-way ANOVA with post hoc analysis using Sidak

acts to influence *C. elegans* behaviour be identified? One site could be the DES-2 subunit containing receptors located on inner labial sensory neurons, IL2 s (Treinin et al. 1998). This would allow a rapid and low external concentration response, as nicotine does not have to penetrate the cuticle barrier. IL2 s synapse onto other sensory neurons, including RIH, interneurons, including RIP, and motoneurons, including RME, URA and RMD (Durbin 1987). All these synapses are cholinergic since IL2 s synthesise acetylcholine (Pereira et al. 2015). It is interesting that RIPs are the only neurons connecting the somatic nervous system to the pharyngeal nervous system of *C. elegans* where it synapses with I1 (Albertson and Thomson 1976) and is well placed to mediated an extrapharyngeal modulation of pharyngeal pumping. The glutamatergic inner labial polymodal neurons, IL1 s, which respond to mechanosensory stimulation, also have the cholinergic subunit DEG3 that could be activated by nicotine (Serrano-Saiz et al. 2013; Treinin and Chalfie 1995). IL1 s also have strong synapses onto RIP. Thus, there are two sensory neuron systems which could be activated by nicotine upstream before mediating a downstream inhibition of pharyngeal neurons.

In conclusion, this study has shown that mM nicotine has bactericidal properties and reduces bacterial cell lawns but this should not affect the behaviour of *C. elegans* when grown on seeded agar plates. These data reinforce the profound modulation of the activated pharynx by nicotine (Raizen et al. 1995). Our experiments would support a direct impact on the core cholinergic drive of the pump but also highlight wider routes with the pharyngeal nervous system or upstream sensory pathways that have a more direct or open access to drug in the external environment. Teasing apart nicotine concentrations and time dependence of various modulated behaviours provides a powerful approach to understand the distinct ways in which cholinergic transmission organises invertebrate behaviour. This is only made more intriguing by the fact that nicotine is a natural secondary metabolite of plants utilised to protect them from animals and as might be intimated here, bacterial disruption.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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