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Concentration- and time-dependent behavioral changes in *Caenorhabditis elegans* after exposure to nicotine

Robert Sobkowiak [a,*], Mateusz Kowalski [b], Andrzej Lesicki [a]

[a] *Department of Cell Biology, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland*
[b] *Poznan University of Technology, Faculty of Electrical Engineering, Institute of Control and Information Engineering, Piotrowo 3a, 60-965 Poznań, Poland*

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ABSTRACT

Nicotine induces profound behavioral responses in the model organism *Caenorhabditis elegans*. We tested the effect of a broad range of concentrations of nicotine (from 0.001 mM to 30 mM in nematode growth medium) on *C. elegans* locomotor behavior. We also followed the time-course influence on the sensitivity of *C. elegans* to nicotine (from 0 min to 300 min). A low concentration (0.001 mM) of this alkaloid causes a reduction of the speed of movement. By contrast, moderate concentrations (0.01 and 0.1 mM) induced acceleration of the mean speed of locomotion of *C. elegans*. High doses of nicotine (above 1 mM) induced slowing down of the movements and, finally, paralysis. Time-dependent analysis revealed that the stimulating effect of nicotine abolished the slowing down of *C. elegans* in control experiments after 30 min in the presence of 0.001, 0.1 and 10 mM nicotine. In the presence of 0.1 mM nicotine, the stimulation phase lasted up to 70 min. The evidence indicates that nicotine can have dual effects on the speed of locomotion, which is dependent on differences in its dosage and treatment time.

1. Introduction

The common soil nematode *Caenorhabditis elegans* is widely used to test various toxicants, including metals, pesticides, and numerous other agents (see reviews: Helmcke et al., 2010; Leung et al., 2008). The worm has been a model organism in studies on a number of drugs, including alcohol (Lee et al., 2009; Mitchell et al., 2007; Thompson and de Pomerai, 2005), cocaine (Ward et al., 2009), amphetamine (Carvelli et al., 2010), opioids (Nieto-Fernandez et al., 2009), and nicotine (Feng et al., 2006; Gottschalk et al., 2005; Sobkowiak and Lesicki, 2009). These drugs have been investigated by using neurological-related parameters, such as behavioral (locomotion, chemotaxis, feeding, etc.), molecular (DNA damage), and morphological (body bending) endpoints.

One of important issues in performing behavioral studies is the identification of a specific endpoint. Many tests performed with *C. elegans*, to determine behavioral effects after exposures to toxicants, were focused on mortality, head thrash, body bend, forward turn, backward turn, and Omega/U turn, feeding alterations, chemotaxis and altering behavior to avoid a toxicant, and learning (ability to associate a particular temperature with food and return to that temperature under starvation conditions) (see review: Leung et al., 2008 and articles cited therein). Among them, locomotion was revealed to be one of the most sensitive indicators of nicotine effects. Although changes in behavior may be an indicator of general toxic stress, some endpoints, including locomotion, also may be more sensitive to neural toxicants (like nicotine) than to toxicants that do not act directly on neuromuscular targets.

Estimation of locomotion rate as an endpoint was used in research on nicotine by Feng et al. (2006). They developed a *C. elegans* model of nicotine-dependent behavior. Their results indicated that *C. elegans* displays several types of behavioral responses to nicotine, which parallel those observed in vertebrates. In addition, those authors showed that nicotine responses in *C. elegans* require nicotinic acetylcholine receptors (AChRs), the molecular targets of nicotine that are known to mediate nicotine dependence in mammals. Feng et al. (2006) focused on behavioral changes in worms treated with nicotine concentrations of 0–0.005 mM.

In the present study, we tested the effect of a broader range of concentrations of nicotine on *C. elegans* locomotor behavior. We also described time course of the speed of movement during the acute response of *C. elegans* to nicotine (i.e. 0–300 min after treatment). To determine the locomotor behavior in *C. elegans*, we employed an automated multi-worm tracking system, which has been designed to observe multiple animals at a low magnification and track the position of each animal over time. (It is necessary to record their movements at a low magnification to keep all tracked animals within the field of view of the microscope.) The Parallel Worm Tracker from the laboratory of Miriam Goodman (Stanford University) extracts the positions of the worm centroids (i.e. their visual "centers of gravity") and determines the tracks of locomotion of individual worms (Ramot et al., 2008). This system has the advantage over many similar ones in

* Corresponding author. Tel.: +48 618295825; fax: +48 618295822.
E-mail address: robsob@amu.edu.pl (R. Sobkowiak).

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that several worms can be tracked simultaneously. This approach is computationally efficient and has proved useful in measuring the time-dependent changes of movement evoked by nicotine.

2. Materials and methods

2.1. Strain maintenance

All tests were performed on the wild-type Bristol N2 strain of *C. elegans* obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (Duluth, Minnesota, USA). Standard methods were used for the maintenance and manipulation of strains (Stiernagle, 2006). Nematodes were maintained at 22 °C on 5-cm NGM agar plates seeded with *Escherichia coli* (OP50).

All the experimental procedures presented in this paper were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Medium

The nematode growth medium (NGM, 1.25× standard concentration) was autoclaved, next cooled to ~50 °C, followed by addition of appropriate amounts of $CaCl_2$, $MgSO_4$, and KPO_4 buffer pH 6.0 prior to pouring plates (Stiernagle, 2006). (−)-Nicotine (free base; Sigma-Aldrich) was diluted in water. Before reaching the final concentrations of nicotine (0.001 mM, 0.01 mM, 0.1 mM, 1 mM, 10 mM and 30 mM,) we prepared in separate tubes 5× nicotine stock solutions. The pH of the nicotine solutions was adjusted to 6.0 (to match NGM without nicotine) with concentrated HCl. One mL of nicotine stock solution was next placed in 5-cm sterile Petri plates as a few drops. To prepare the assay plates, 4 mL of cooled 1.25× NGM were added — then the final concentrations of nicotine were reached. In the control variant we added 1 mL of water instead of 1 mL of nicotine stock solution. Before medium solidification, all Petri dishes were gently swirled to mix NGM with nicotine well. The plates were stored inverted at 4 °C. Pre- and post-exposure pH measurements were made by using pH-indicator strips (pH 5.2–7.2, Merck, Darmstadt, Germany).

3. *C. elegans* synchronization

Large quantities of *C. elegans* were grown in liquid 100 mL S medium, using concentrated *E. coli* OP50 as a food source (Stiernagle, 2006). L1 larvae were prepared by egg synchronization: hermaphrodites were lysed in 20% bleach (Clorox), 0.5 M NaOH, until fragmented, and eggs were incubated in M9 buffer without food overnight (14–18 h) at 20 °C with agitation (150 rpm), to allow larvae to hatch and arrest the development because of starvation. The L1 larvae were next spun down at 450 g (5 min), and about 600 worms (in 1.5 µL total volume of M9 buffer) were placed in several drops on each assay plate (5 cm across) with enriched nematode growth medium (ENG), seeded with *E. coli* OP50. The number of worms could not be strictly controlled. We found, however, that the worms survived 3 days at 20 °C on the seeded plate, and by then became adult and capable of egg laying.

3.1. Behavioral assays

Assays were performed on adults aged 70–74 h. Each experiment lasted 450 min and was carried out at room temperature in the absence of food. Plates were placed on a video apparatus for recording. The automated tracking system comprises a stereomicroscope (Olympus SZ11), a modified (with unscrew lens) web camera (Logitech QuickCam Pro 9000) with 640×480 resolution to acquire worm videos, and a desktop PC (Compaq dc7800, HP) running under Windows XP. The transmitted light sources were custom built and the illumination did not exceed 1 $\mu mol \times m^{-2} \times s^{-1}$ (measurement made by Quantum Meter Photosynthetic Photon Flux, Apogee Instruments Inc.). The light covered the whole plate and was applied for 1 min every 10 min (light started 15 s before recordings). The stereomicroscope was fixed to its lowest magnification (1.8×) during the whole experiment.

Recordings were made using the WormCapture (Ramot et al., 2008) at a rate of 10 frames/s; video recordings (30 s long each) were automatically saved every 10 min. The resolution was fixed at 77.7 pixel/mm (calibration was made by using an empty hemocytometer grid at the working magnification of 1.8×). Video recordings were carried out at room temperature (21–22 °C).The captured movies were transformed into a black silhouette of the animal on a white background by using Adobe After Effects CS3. The Parallel Worm Tracker platform, developed by the laboratory of Miriam Goodman (Stanford University, Stanford, CA), was used to characterize adult movement. This is an automated tracking system developed to quantify the locomotion of multiple individual worms in parallel. Captured movies were analyzed using MATLAB (MathWorks, Natick, MA) scripts (Ramot et al., 2008; http://med.stanford.edu/wormsense/tracker/). The Parallel Worm Tracker consists of three basic modules. To obtain detailed data including the mean speed of individual worms, we developed this system and created a forth module (called WormExtractor). The individual worm movements were tracked by WormAnalyzer (Ramot et al., 2008) and recorded to an Excel spreadsheet by WormExtractor. The WormExtractor source code for the Parallel Worm Tracker and a User Manual can be downloaded from http://www.staff.amu.edu.pl/~robsob/WormExtractor/.

Adult animals were first observed in covered plates for 60 min on native ENG plates, on which they grew up for 3 days from the L1 stage to adulthood. The 30-s videos were taken every 10 min for 60 min (naïve, untouched state, first plate). After 68 min, the animals were placed onto fresh, unseeded NGM plates free of nicotine (naïve, touched state, second plate) using the “chunking” method (Stiernagle, 2006). Instead of a scalpel or spatula, we used a thin-walled metal tube (4.4 cm across) to cut a chunk of agar from a 3-day-old ENG Petri plate (5 cm across) containing only adult worms. The chunk (with worms on top) was moved on a broad round spatula and inverted onto the fresh assay plate. There were usually hundreds of worms on the chunk of agar and they stuck to the new plates. After quick removal of air bubbles between the sticking agars, by very gentle pressing, the old agar was discarded. This method results in the transfer of over 70% of worms. Thus it is an effective method for rapidly transferring a large number of animals without scratching the agar surface (important for obtaining high-contrast videos) and with minimized mechanical stimulation of worms.

Adult animals were next observed on the foodless plate (second plate) without nicotine for 70 min. Recording began no more than 2 min after transfer.

To quantify nicotine resistance, adults were again transferred, in the same way as described above, to a plate containing nicotine (third plate) at the concentrations indicated. Again recording began no more than 2 min after transfer.

To analyze worm locomotion data, we analyzed 1350 video films. A total of 20 558 individual worm tracks were analyzed. Average locomotion speed was calculated by tracking 3416 worms in the control experiment, 1461 worms in the presence of 0.001 mM nicotine, 2252 worms in the presence of 0.01 mM nicotine, 2077 worms in the presence of 0.1 mM nicotine, 4113 worms in the presence of 1 mM nicotine, 3136 worms in the presence of 10 mM nicotine, 4103 worms in the presence of 30 mM nicotine.

3.2. Statistical analysis

The measurements from 5 experiments were pooled and the mean values (± standard error) were calculated. The data were not normally distributed, as determined by the Shapiro-Wilk's *W*-test and Kolmogorov–Smirnov and Lilliefors method. Due to this, the Kruskal–Wallis test followed by Dunn's multiple comparison *post-hoc* test were performed. In cases where two groups were compared, a Mann–Whitney *U*-test was used. The Friedman test was used to compare dependent samples. Statistical significance was considered at $P<0.05$. The calculations and graphs were done by using the computer program Statistica (StatSoft, Inc., Tulsa, Oklahoma, USA).

4. Results

4.1. Average speed of naïve worms

Data obtained in our lab using the tracking system has demonstrated that naïve worms in the first ENG medium plate moved at a mean baseline speed (Fig. 1, time from −140 to −80 min) equal to 0.051 mm/s, and their speed subsequently remained more or less constant, representing baseline movement.

After transferring to a new NGM plate without bacteria (second plate), the worms displayed an increase in locomotion rate, with an average speed of 0.055 mm/s (Fig. 1, time from −70 to −10 min). The increase (by 0.004 mm/s), compared to speed movement on the first plate, is statistically significant (Friedman test, $P<0.001$).

We noticed that right after the transfer ($t=-70$ min), more animals moved, and at a faster speed. After 20–30 min, however, their speed decreased to the baseline level. We considered the initial accelerated movement as a response to a mechanical stimulus, whereas we reasoned that the stable level of locomotion represented baseline movement. Anyway, after $t=-40$ min, the mean speed gradually increased (Fig. 1).

4.2. Nicotine dose-dependent response of C. elegans

Upon exposure to nicotine, adult *C. elegans* demonstrated dose-dependent behavioral responses (Fig. 2). Low doses (0.001 mM

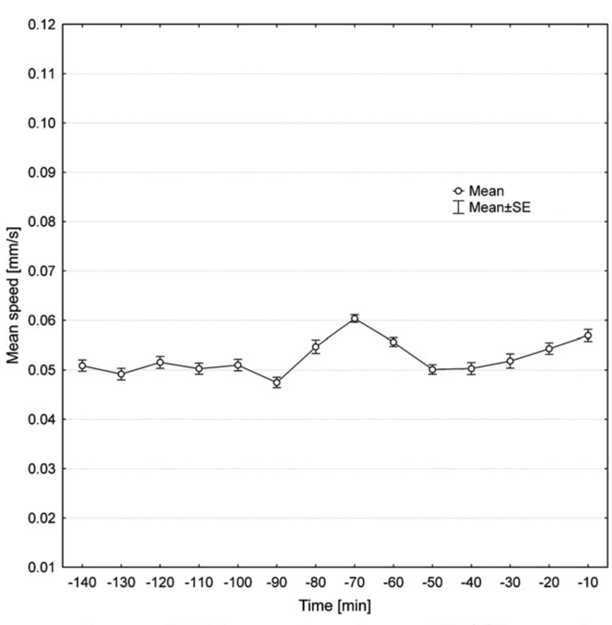

Fig. 1. The mean locomotor activity (centroid speed) of *Caenorhabditis elegans* versus time on nicotine-free plates. Lack of significant locomotor stimulation in animals on the first plate; mean baseline speed between −140 and −80 min was 0.051 mm/s. After transfer to the second NGM nicotine-free plate, the mean speed between −70 and −10 min increased to 0.055 mm/s (Friedman test at $P<0.001$), which seems to be a locomotor response to a mechanical stimulus. First plate $n=10987$; second plate $n=7884$, worm transfer efficiency 71.7%.

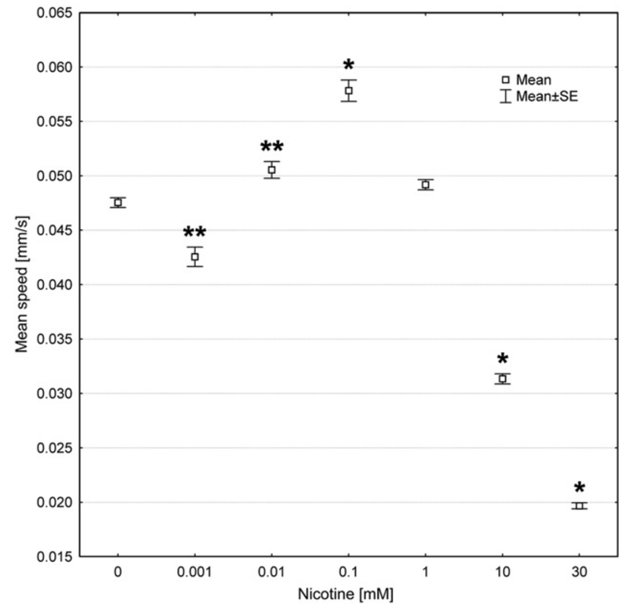

Fig. 2. The nicotine-induced locomotor response of *Caenorhabditis elegans*. Worms were tracked on plates with 0, 0.001, 0.01, 0.1, 1, 10, and 30 mM nicotine. In each experiment, worms were tracked for 30 s every 10 min. The treatment lasted 300 min. The mean speed was calculated from all collected data. Significance of differences from the control: *$P<0.01$ and **$P<0.05$ (Kruskal–Wallis test). $n\geq1461$.

nicotine) led to hypokinesis, leading to slowing down of average movement by about 10.5% (Kruskal–Wallis test, $P<0.05$), compared to the control (no nicotine). Moderate doses (0.01 mM and 0.1 mM) induce locomotor hyperactivity (hyperactive locomotion), elevating the mean speed by about 6.3% (Kruskal–Wallis test, $P<0.05$) in the presence of 0.01 mM nicotine and by about 22% (Kruskal–Wallis test, $P<0.01$) in the presence of 0.1 mM nicotine (Fig. 2, see also Supplemental Data Table S1). We did not observe any statistical difference among the mean speed of worms in the presence of 1 mM nicotine and control conditions. Doses higher than 1 mM nicotine lead to slowing the movement of *C. elegans*. In the presence of 10 mM nicotine, the decrease in the mean speed, compared to the control, reached 34.1% (Kruskal–Wallis test, $P<0.01$). Nicotine at a concentration of 30 mM evoked average slowing down of the speed by about 58.6% (Kruskal–Wallis test, $P<0.01$).

4.3. Nicotine time-dependent response of C. elegans

In the control variant, the mean speed throughout the experiment was not constant (Figs. 1 and 3). A strong slowing down of movements was observed 30 min after the last transfer i.e. to the third plate (0 mM nicotine, Fig. 3, see also Supplemental Data Table S2). Beside this, the average speed of worms in control conditions slowly decreased and was the lowest at the end of the experiment ($t=300$ min) (Fig. 3). The nematodes showed a remarkable decline in locomotion in the presence of 30 mM nicotine (Fig. 3F). The slowing down was also observed after 90 min in the presence of 0.001, 0.1, and 10 mM nicotine (Fig. 3A, C, E).

At $t=30$ min, when the worms reached a lowest speed, the average speed of control animals was 0.041 mm/s, right after mechanical stimulation linked with the transfer onto the third plate, whereas at time 0, the control animals moved at a rate of 0.056 mm/s (Fig. 3), so the decrease in speed was about 27%.

Statistically significant locomotor stimulation was also observed in the presence of 0.001 mM ($t=30$ min), 0.01 mM ($t=0$ min), 0.1 mM ($t=0$, 10, 30, 50, 60, 70, 130, 200, 230 min), 1 mM ($t=0$, 10, 170, 180, 200, 220, 300 min), and 10 mM ($t=30$ min). In the concentration

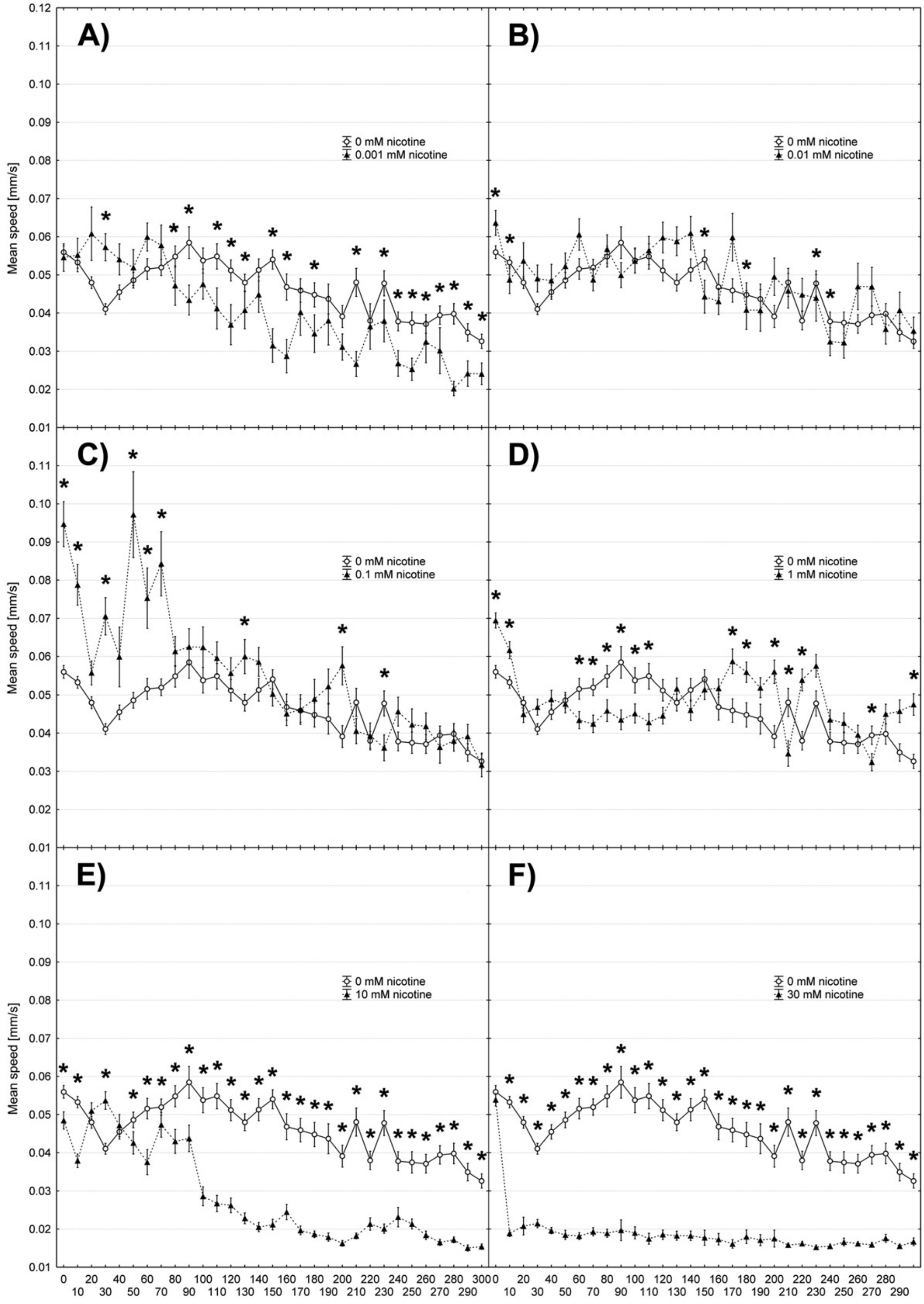

Fig. 3. The locomotor activity (centroid speed) of *Caenorhabditis elegans* versus time at various concentrations of nicotine on bacteria-free plates. The data are means and standard error (SE) of 5 experiments, each done with hundreds of worms per plate. Asterisks denote significant differences from the control (Mann–Whitney *U* test, $P<0.05$).

range of 0.001 to 10 mM nicotine, after $t=30$ min, in all cases the speed of movement was higher than in the control. Statistically significant locomotor stimulation at $P<0.05$ (Mann–Whitney *U* test) was confirmed at 0.001, 0.1 and 10 mM nicotine (Fig. 3A, C, E). The most pronounced stimulating effect of nicotine is visible at 0.1 mM nicotine, where the stimulation phase lasted up to 70 min (Fig. 3C).

We also observed switching between the stimulating and inhibiting effects of nicotine at concentrations ranging from 0.001 to 1 mM. Before 110 min, the worms in nicotine moved slower than in the control, but after another 50 min they started to move faster (Fig. 3A, D). After 10 min in the presence of 30 mM nicotine, most of the animals almost completely stopped moving.

5. Discussion

In our study we used nicotine, which is an agonist of nicotinic receptor with the natural ligand acetylcholine. Nicotine can stimulate or depress movement. This process is dependent on concentration and time. We estimated the nicotine-induced behavioral response of *C. elegans* in a broad range of concentration of this alkaloid (0.001 mM to 30 mM). To our knowledge, the lowest concentration of nicotine was used by Feng et al. (2006) — 0.0005 mM to 0.005 mM. The highest concentration (30–31 mM) of nicotine was applied by Gottschalk et al. (2005) and Waggoner et al. (2000) for nicotine paralysis and adaptation experiments.

Our study shows that nicotine, probably by the acetylcholine signal pathway, can greatly induce faster movements (Fig. 3C: at 0.1 mM nicotine, after 50 min, the worms moved 2-fold faster than in the control). We also show that nicotine can abolish the slowing down after 30 min (Fig. 3A, C, E; Mann–Whitney *U* test, $P<0.05$). A similar increase (data read from the graph) was observed by Feng et al. (2006) during 16 min of incubation of worms with nicotine. Previous studies have shown that nicotine modulates the locomotion of *C. elegans* (Feng et al., 2006; Gottschalk et al., 2005). Feng et al. (2006) noticed that when worms were assayed on a plate containing nicotine, the animals displayed a distinct locomotor behavior, compared to the control: the animals began to gradually speed up their locomotion. They named it a "locomotion–stimulation phase". They found that 0.0015 mM nicotine gradually speeds up locomotion rate of worms at the time range of 3 to 16 min. Our data confirm information on the stimulating role of nicotine. At the nearest concentration of nicotine (0.001 mM) we verified that worms after 30 min moved about 27% faster (Mann–Whitney *U* test, $P<0.05$) than in the control. The acceleration is most pronounced in our experiment in the presence of 0.1 mM nicotine after 50 min, where the speed in nicotine was 100% higher than in the control conditions (Fig. 3C).

To quantify the effects of nicotine on worm locomotion, we used the wild-type Bristol N2 strain of *C. elegans*. According to literature data, the wild-type hermaphrodites from the laboratory strain N2 are solitary in plentiful food; they aggregate into swarms when food is limited but disperse when food is absent (de Bono and Bargmann, 1998). To obtain the solitary and active strains for our experiments, animals were assayed on food-free plates. During 3 days of *C. elegans* incubation, the bacteria from an ENG plate were completely eaten and the worms became adult. Such conditions enable computer analysis of single worms, because WormTracker can analyze the speed of individual worms then.

Many studies have shown that the absence or presence of food markedly influences the average speed of wild-type worms (de Bono and Bargmann, 1998; Karbowski et al., 2006; Ramot et al., 2008). Our results on the mean speed of worms on plates without bacteria (0.051 mm/s on the first plate, 0.055 mm/s on the second plate, and 0.047 mm/s on the third plate) are closest to the value obtained by Feng et al. (2006). We minimized the number of steps in the pre-experimental phase to avoid some potential artifacts. Collecting and washing steps were omitted, as the animals were transferred directly from the culture plates (first plate) to the assay plates (second and third plates).

Sawin et al. (2000) reported that the feeding status (well-fed or starved) as well as the presence or absence of food (bacteria) affects the rate of locomotion of *C. elegans*. Also the transfer of *C. elegans* onto another plate affects locomotion rate of worms as a result of mechanostimuli (Sawin et al., 2000; Zhao et al., 2003). In our control experiment, 30 min after transferring the animals onto the third plate, we observed 27% slowing of movements (Fig. 3, comparison of mean speed at 0 min and 30 min). A similar slowing down (27%), triggered by mechanosensory neurons, was obtained by Sawin et al. (2000) in an experiment with well-fed *C. elegans* after transferring the nematode to food.

By observing worms in liquid over longer periods, Ghosh and Emmons (2008) found that after transfer from a solid surface into liquid, a worm initially swam continuously under assay conditions for a period of 92.6 ± 2.5 min, after which it stopped and lay quiescent. In our experiment, if we sum up the time from the first transfer (Fig. 1, $t=-70$ min) to slowing down 30 min after the second transfer, we obtain in total 100 min, which roughly corresponds to the time of 92.6 min estimated by Ghosh and Emmons (2008). We suppose that this might be a result of mechanical stimulation when the animal is moved to the assay plate, whereas the slowing down is likely to be triggered by a mechanosensory stimulus to the surface of the animal. In our experiments, the worms were exposed to tactile stimuli during transfer. Over longer periods (>4 h), the time of movements gradually declined and time of quiescence became longer in Ghosh and Emmons' (2008) study. In our research, similar extended periods of hypokinesia were seen for adult worms crawling on an agar surface. They postulated that quiescence is evoked by increased cholinergic activity, i.e. that acetylcholine is involved in this process. To test further the role of acetylcholine in swimming–quiescence cycling, Ghosh and Emmons (2008) examined the swimming behavior of animals with increased acetylcholine signaling. They found that the slowing response involves a mechanosensory stimulus mediated by acetylcholine (nicotine).

The physiologic effects of nicotine on *C. elegans* are multiple, complex and dose-dependent. At a higher concentration of nicotine (e.g. 30 mM), the stimulating phase at the neuromuscular junction is obscured by a rapidly developing paralysis, partly due to receptor desensitization (Fig. 3F). We found that nicotine significantly decreased average locomotion rate in wild-type worms above 1 mM in a dose-dependent manner, indicating that nicotine can evoke a hypolocomotor response in *C. elegans* (Fig. 1).

High doses (10 and 30 mM nicotine) induced muscle hypercontraction paralysis (akinesia). These behaviors are qualitatively very similar to those described in the literature. Exposure to relatively high nicotine concentrations, ranging from 20 to 30 mM in liquid culture for wild-type young adults, causes rapid paralysis of body wall muscles within 10–15 min (Matta et al., 2007).

The results of our experiments indicated that behavioral responses of worms to nicotine are parallel to those observed in mammals. The effects of nicotine on locomotion vary according to dose and over time. In other animal models, acute administration of nicotine evokes dual changes in locomotor activity (Matta et al., 2007). The effects of nicotine on spontaneous locomotor activity in rats are complex and include both stimulant and depressant actions. In rodent models, nicotine at low doses stimulates locomotor activity in naïve animals, though it initially induces transient hypoactivity. At higher doses, a biphasic depressant/activating effect becomes apparent, culminating in ataxia and catalepsy at very high doses and followed by increased locomotion (Dwoskin et al., 1999; Frenk and Dar, 2000).

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Competing interests

The authors have declared that no competing interests exist.

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