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Nicotine-motivated behavior in *Caenorhabditis elegans* requires the nicotinic acetylcholine receptor subunits *acr-5* and *acr-15*

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Abstract

Signaling at nicotinic acetylcholine receptors in *Caenorhabditis elegans* controls many behaviors, including egg-laying and locomotor activity. Here, we show that *C. elegans* approaches a point source of nicotine in a time-, concentration- and age-dependent manner. Additionally, nicotine paired with butanone under starvation conditions prevented the reduced approach to butanone that is observed when butanone is paired with starvation alone and pairing with nicotine generates a preference for the tastes of either sodium or chloride over baseline. These results suggest nicotine acts as a rewarding substance in *C. elegans*. Furthermore, the nicotinic receptor antagonist mecamylamine, the smoking cessation pharmacotherapy varenicline, mutation of the *dop-1* and *dop-2* dopamine receptors, and mutations of either *acr-5* or *acr-15*, two nicotinic receptor subunit genes with sequence homology to the mammalian α7 subunit, all reduced the nicotine approach behavior. These two mutants also were defective at associating the presence of nicotine with butanone under starvation conditions and *acr-5* mutation could obviate the effect of pairing nicotine with salts. Furthermore, the approach deficit in *acr-15* mutants was rescued by selective re-expression in a subset of neurons, but not in muscle. *Caenorhabditis elegans* may therefore serve as a useful model organism for nicotine-motivated behaviors that could aid in the identification of novel nicotine motivational molecular pathways and consequently the development of novel cessation aids.

Introduction

In response to the public health threat posed by tobacco smoking, the neurobiological mechanisms of nicotine have been examined widely (Laviolette & van der Kooy, 2004; Dani & Harris, 2005). Nicotine is the main psychoactive component of tobacco, and is necessary to maintain smoking (Donny *et al.*, 2007). Although the three smoking cessation pharmacotherapies in common use – nicotine replacement, bupropion and varenicline – double the quit rate compared with untreated smokers, this results in only 20% of quit attempts ending in long-term abstinence (Jorenby *et al.*, 2006). These low quit rates highlight the need to identify novel targets for drugs of higher efficacy than those on the market today. To achieve this end, it is imperative to understand the mechanisms by which nicotine guides motivated behavior.

Rodents have often been used to model nicotine-motivated behaviors. Unfortunately, genetic studies in rodents are hindered by their relatively small brood size, their long gestation period, the expense of generating and maintaining knockout or transgenic animals, and the virtual impossibility of performing forward genetic screens. These limitations are overcome in the nematode *Caenorhabditis elegans*, which represents an excellent model organism in which to perform genetic studies due to the availability of many well-defined knockout animals, the short generation time and their well-characterized nervous system. *Caenorhabditis elegans* expresses at least 27 nicotinic acetylcholine receptor subunits, several of which share a high degree of sequence homology with mammalian subunits (Jones & Sattelle, 2004; Rand, 2007). At least a subset of these combine to form nicotine-responsive receptors, as nicotine can modulate several behaviors in the worm, including egg-laying (Waggoner *et al.*, 2000; Kim *et al.*, 2001), spicule ejection in the male (Matta *et al.*, 2007) and neuromuscular transmission (Lewis *et al.*, 1980). Importantly, some nicotine-dependent effects including locomotor stimulation, withdrawal and sensitization were shown to be neurally mediated (Feng *et al.*, 2006), which parallels the case in rodent models (Balfour *et al.*, 1998; Di Chiara, 2000; Picciotto *et al.*, 2000). Whereas the mechanisms underlying nicotine-induced locomotor stimulation in *C. elegans* have been explored (Feng *et al.*, 2006), these may not reflect the mechanisms underlying motivated

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behavior. As the motivational and locomotor activating effects of drugs, including nicotine, have been attributed to related but distinct mechanisms in rodents (Boye *et al.*, 2001; Sellings *et al.*, 2008; Shabat-Simon *et al.*, 2008), it is premature to assume that the same mechanisms controlling psychomotor activation directly underlie motivated behavior.

In the present study, we aimed to develop a model of nicotine-motivated behavior in *C. elegans*. We examined both approach to a point source of nicotine as well as how nicotine pairing modified the response to olfactory or taste stimuli. These results suggest that nicotine motivation can be modeled in *C. elegans*, and that such a model may prove useful in the identification of novel genes implicated in the control of nicotine-motivated behaviors.

Methods

Subjects

Nematodes were maintained at 20 °C on nematode growth medium [NGM: 50 mM NaCl, 20 g/L bactoagar, 2.5 g/L bactopeptone, 13 μM cholesterol, 1 mM $Ca(CH_3COO)_2$, 1 mM $MgSO_4$, 25 mM KH_2PO_4, pH 7.0] seeded with *Escherichia coli* (OP50) (Brenner, 1974). The acr-15;Ex(Pglr-1::acr-15) and acr-15;Ex(Pmyo-3::acr-15) transgenic lines were provided by Dr X. S. Shaun Xu (Feng *et al.*, 2006). All other *C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). The mutant alleles used were as follows: *acr-2(ok1887)*, *acr-3(ok2049)*, *acr-5(ok180)*, *acr-5(ok182)*, *acr-5(ok205)*, *acr-7 (tn863)*, *acr-8(ok1240)*, *acr-9(ok933)*, *acr-11(ok1345)*, *acr-12 (ok367)*, *acr-14(ok1155)*, *acr-15(ok1214)*, *acr-16(ok789)*, *acr-18 (ok1285)*, *acr-19(ad1674)*, *acr-21(ok1314)*, *cat-1(e1111)*, *cat-2 (e1112)*, *dat-1(ok157)*, *deg-3(tu1851)*, *dop-1(vs101)*, *dop-1(vs101)*, *dop-2(vs105)*, *dop-2(vs105)*, *dop-3(ok295)*, *dop-4(tm1392)*, *eat-2 (ad465)*, *lev-1(e211)*, *lev-8(x15)*, *unc-29(e193)*, *unc-38(e264)* and *unc-11(ic9);unc-63(e384).* All animals were age synchronized at the L1 larval stage and tested 52–54 h post plating as young adults unless otherwise specified, as previously described. (Pereira & van der Kooy, 2012).

Behavioral assays

All nicotine approach assays were performed in triplicate with 2–4 plates per replicate at room temperature (22–23 °C), with the exception of the varenicline and bupropion experiments, which were performed in duplicate. All butanone/nicotine pairing assays were performed with at least $n = 4$ at 20 °C. Worms were synchronized at the L1 stage and tested 52–54 h after plating on NGM plates seeded with *E. coli* OP50 at 20 °C unless otherwise indicated.

Chemotaxis assay

Chemotaxis (CTX) assays were performed with standard 100-mm Petri dishes containing 6 mL of chemotaxis agar [10 mM MOPS, pH 7.2, 0.25% (v/v) Tween 20, 15 g/L agar]. Where necessary, odorants (benzaldehyde or 2-butanone) were diluted in ethanol and reported as percentages by volume. Plates were sealed with a strip of parafilm around the edge during all odorant exposures. Standard 1-h CTX assays were performed as described previously (Colbert & Bargmann, 1995; Barron *et al.*, 2005). Briefly, 10 min before the assay, 1 μL of 1 M NaN_3 was applied to the centers of two test spots that were 6 cm apart. This acted to immobilize worms that reached the spot during the assay. Individuals (50–200) were then placed at the center of the plate between the two spots, 1 μL of the test odorant was placed at one spot and 1 μL of ethanol was applied to the control spot. After 1 h, worms within a 2-cm radius of either spot were counted, and a chemotaxis index (CI), defined as the number of animals at the test spot minus the number of animals at the control spot and divided by the total number of animals on the plate outside the rectangle where worms were initially plated, was calculated. A positive CI indicates an attraction to the odor, and a negative CI indicates an aversion.

Nicotine approach assay

Nicotine approach assays were performed with standard 100-mm Petri dishes containing 6 mL of chemotaxis agar [10 mM MOPS, pH 7.2, 0.25% (v/v) Tween 20, 15 g/L agar]. The nicotine gradient was formed by adding 10 μL of a given concentration of nicotine tartrate salt solution in water with the pH adjusted to 7.3 ± 0.1 at a distance of 1 cm from one side of the plate. The nicotine was left to

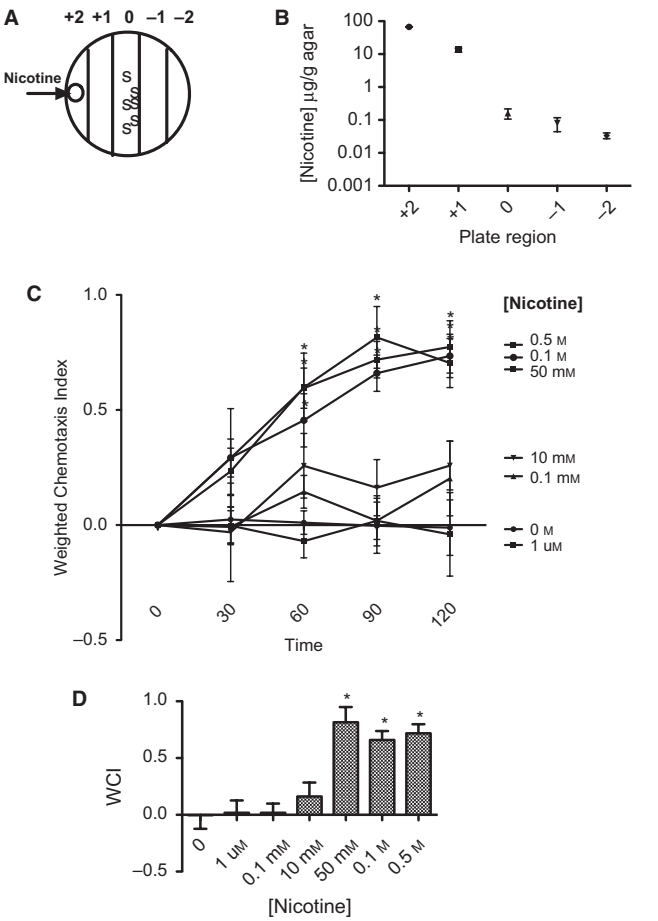

FIG. 1. *Caenorhabditis elegans* climbs a nicotine gradient in a manner consistent with unconditioned reward. (A) Assay for nicotine approach. Worms were plated in the central quintile of a standard 10-cm Petri dish containing 6 mL chemotaxis agar onto which 10 μL of nicotine had been spotted 3 h prior to worm placement. (B) A concentration gradient of nicotine is formed when 10 μL of 50 mM nicotine was spotted at one end of a plate. (C) Worms approached nicotine in a time- and concentration-dependent manner. Nicotine concentrations are listed on the right beside their corresponding value at 120 min. A significant increase over baseline was seen for nicotine concentrations 50 mM and higher at all time points beginning at 60 min (*$P < 0.05$, Bonferroni). (D) Further analysis of the nicotine approach observed at 90 min revealed a significant approach to 50 mM, 0.1 M and 0.5 M nicotine (*$P < 0.05$, Dunnett's test).

diffuse for 3 h, at which point worms were placed in the central compartment of the weighted chemotaxis assay plate (Fig. 1A). Worms were examined at a defined time point after plating. Unless otherwise mentioned, the concentration of nicotine solution used was 50 mM and the time point examined after plating onto nicotine plates was 90 min.

Locomotor spreading assay

Worms were spotted in the center circle of a standard 100-mm Petri dish containing 6 mL of chemotaxis agar and their position relative to the center spot was recorded after a defined time period (90 min later unless otherwise stated). A weighted average, assigning a value of 3 to the outer ring, 2 to the next ring, 1 to the ring adjacent to the center circle and 0 to the center circle, was calculated.

Quantification of body bend frequency and omega turns

Worm tracking to calculate body bend frequency was performed using a system consisting of an EM-CCD camera (QuantEM:512SC; Photometrics, Tucson, AZ, USA) mounted onto a microscope (MVX10; Olympus, Richmond Hill, ON, CAN), and a motorized stage (BioPrescision; Ludl, Hawthorne, NY, USA). Custom worm tracker software was used to automatically move the stage to re-center the worm under the field of view during recording (H. Suzuki, pers. comm.). Worms were manually picked off maintenance plates and allowed to acclimate to the testing plate for at least 2 min. Injured worms were discounted. Images were acquired at 5 frames/s and 512 × 512 pixels for 1 min. The body bend frequency data were then extracted using a slightly modified version of the software previously described (Cronin *et al.*, 2005). Analysis of omega turns was done manually using a standard 4× light microscope. Omega turns were counted immediately after transfer for a duration of 10 min or at the 14-16 interval. All worms were age synchronized at the L1 larval stage as above. Plates containing uniform levels of nicotine and pharmacological agents were made by dissolving 30 μL of a 1000× concentrated stock (in double distilled H20) into 30 mL of liquid agar, mixed by rigorous pipetting and plated on to large 10-cm Petri dishes. For control plates, an equivalent amount of water was used.

Age-dependence assay

Worms were synchronized at the L1 larval stage and plated on NGM plates seeded with *E. coli* OP50 bacteria. Worms were plated 216, 144, 96, 72, 52 or 40 h prior to examination in the nicotine approach assay, benzotaxis assay or locomotor assay. Starting at 52 h after placement on food plates, worms were washed daily from food plates with M9 buffer three times to remove eggs and larvae from the adult population and were re-plated onto new food plates.

Butanone conditioning assay

The butanone conditioning assay was based on the standard assay for adaptation (Colbert & Bargmann, 1995) and modified from the butanone enhancement assay (Torayama *et al.*, 2007). N2 worms (approximately 500 per plate) were washed twice with M9 buffer and once with distilled water, and were placed on a 10-cm Petri dish containing 6 mL of NGM agar in the absence or presence of nicotine (50 nM–50 mM) for conditioning. 2-Butanone (4 μL) was added to each of six pieces of Parafilm affixed to the Petri dish lid and the plate was sealed with Parafilm. After a 60-min conditioning trial, worms were gently washed once from the conditioning plates using distilled water, and were tested for their chemotaxis to 0.5% 2-butanone as described above.

Nicotine-salt reward assay

CTX plates with a concentration of 75 μM salt (either sodium acetate or ammonium chloride) were used to condition worms. For nicotine pairing, 3 h prior to conditioning, half of the conditioning plates were spotted with 3 5-μL drops of 50 mM nicotine tartrate solution at 25-mm intervals along the diameter of the plate. Worms were transferred to CTX-only plates, without salt or nicotine, for 30 min prior to conditioning to acclimate. Worms were then transferred to the center of the conditioning plates and left for 1 h. Conditioning plates without nicotine were used as controls for the assay. After conditioning, worms were washed and transferred to the center of the testing plates. For testing plates, 3.5 h prior to use, 5 μL of sodium acetate (2 M) and ammonium chloride (2.5 M) were spotted on opposite ends of large CTX plates, 2 cm from the edge to form two opposing diffusion gradients. Ninety minutes after transferring worms to the testing plate, the CI was calculated using the number of worms within 2 cm of either salt according to the formula [(Conditioning Salt approach – Control Salt Approach)/Total]. All experiments were performed using young adult hermaphrodites (52 h after plating L1 larvae).

Pharmacology

For experiments involving mecamylamine (Sigma, St Louis, MO, USA), the drug was dissolved directly in chemotaxis agar to final concentrations ranging from 100 nM to 10 mM, and the plates were left uncovered after pouring at 22 °C for 30 min. After preparation, plates were used in the assays described above. For experiments involving varenicline, pills were crushed using a mortar and pestle, and then were placed in water. As varenicline is highly water-soluble (DrugBank), the supernatant was removed from the insoluble components, which were washed three times with double distilled H_2O. Drug loss of 5% during this process was assumed for all molarity calculations.

Determination of nicotine concentration gradient in CTX agar

Three nicotine diffusion plates per replicate were prepared as in the nicotine approach assay (i.e. 3 h of diffusion after addition of 10 μL of a 50 mM solution of nicotine tartrate). At the end of the 3-h diffusion period, samples were separated manually and placed into tubes representing each of the five sections on the WCI plate (see Fig. 1A).

One gram of CTX agar was added to 0.1 mL of 10 M NaOH, 50 μL of 1.3 μg/mL 5-methylcotinine as the internal standard and 20 μL of distilled water. To create the calibration curve, 1 g of nicotine-free CTX agar was added to 0.1 mL of 10 M NaOH, 50 μL of 5-methylcotinine (IS, 1.3 μg/mL) and 20 μL nicotine bitartrate in water to obtain final concentrations of 0.01–80.0 μg nicotine base/g agar. The samples and standards were homogenized and then extracted using liquid-liquid extraction with 5 mL of dichloromethane by shaking for 10 min as previously described (Kowalski *et al.*, 2007). After centrifugation at 1400 ***g*** for 10 min, 25 μL of 6 M HCl was added to the organic fraction and this was evaporated to dryness under a nitrogen stream at 37 °C. The residue was re-dissolved in 105 μL of Milli-Q water and 90 μL was subjected to HPLC analysis for nicotine concentration assessment (Siu *et al.*, 2006). All chromatography was performed at room temperature.

Separation of nicotine and IS was achieved using a ZORBAX Bonus-RP column (5 mm, 150 × 4.6 mm; Agilent Technologies Inc., Mississauga, ON, Canada) and a mobile phase consisting of acetonitrile/potassium phosphate buffer (10 : 90, v/v, pH 5.07) containing 3.3 mM heptane sulfonic acid and 0.5% triethylamine. The separation was performed with isocratic elution at a flow rate of 0.9 mL/min. Nicotine sample concentrations were determined from the calibration curve. A calibration graph was constructed by plotting the measured nicotine/IS peak-area ratio vs. the concentration of the nicotine standards. Linear regression analysis was used to calculate the slope and intercept, which was then used to determine the nicotine concentrations in the study samples. The limit of quantification was 10 ng nicotine per gram of agar.

Statistical analysis

All CTX, weighted CTX and locomotor indices were plotted as the mean ± standard error of the means, and were calculated from at least eight (approach assay) or four (butanone conditioning assay) test plates. Multiple group comparisons were performed with ANOVAS followed by Bonferroni corrected *t*-tests, Tukey's test or Dunnett's test as appropriate. Significance was set at $P < 0.05$ (two-tailed).

Results

Wild-type worms climb the nicotine gradient

In the first set of experiments, we quantified the behavior of young adult *C. elegans* on an agar plate with an established nicotine gradient. Fifty to 200 young adult hermaphrodite N2 worms (52 h after hatching) were placed in the middle of a chemotaxis agar plate on which 10 μL of a nicotine tartrate salt solution (pH 7.3 ± 0.1) of varying concentrations had been spotted at one end and allowed to diffuse for 3 h (Fig. 1A). Under these conditions, a defined concentration gradient of nicotine was established in the CTX agar (50 mM nicotine spot; one-way ANOVA F (4, 34) = 930.6 $P < 0.05$; +2 vs. all and +1 vs. all $P < 0.05$; $n = 7$; Tukey's; Fig. 1B). Although the initial conentration of nicotine in the 10-μL spot was quite high (50 mM), the nicotine diffused rapidly throughout the plate, where the volume of agar was by far greater than the volume of the nicotine spot. The region containing the origin of the 10-μL spot of 50 mM nicotine tartrate solution was found to have a nicotine concentration of 67.26 ± 1.06 μg/mL of agar, which translates to 4.15 μM – an order of magnitude below that of the concentrated spot. For the next compartment, these figures were 13.38 ± 1.85 μg/mL (0.82 μM), the center compartment was 0.16 ± 0.05 μg/mL (10 nM), the next compartment was 0.08 ± 0.04 μg/mL (4 nM), and the region farthest from the nicotine spot was 0.03 ± 0.01 μg/mL (2 nM).

Worms approached the nicotine spot in a time- and concentration-dependent manner (two-way ANOVA: concentration $F_{6,196} = 7.36$, $P < 0.05$; time $F_{4,196} = 6.03$, $P < 0.05$; $n = 8$; Fig. 1C); after 90 min, worms on test plates containing 50 mM, 0.1 or 0.5 M nicotine climbed the nicotine gradient (one-way ANOVA: $F_{6,49} = 12.36$, $P < 0.05$; $P < 0.05$ for 0 vs. 50 mM, 0.1 or 0.5 M, $n = 8$; Dunnett's test; Fig. 1D). That worms seek higher concentrations of nicotine is consistent with a rewarding effect of nicotine.

Worms do not leave the nicotine spot

Fifty to 200 young adult Bristol N2 worms (52 h after hatching) were placed in the center circle of a chemotaxis agar plate where nicotine or vehicle had been spotted in the center (Fig. 2A). Worms were allowed to move freely for 30–120 min. Worms on a vehicle spotted plate left the center, yet at higher nicotine concentrations, worms stayed near the point source (two-way ANOVA: interaction $F_{12,80} = 25.07$, $P < 0.05$; 0 vs. 50 mM, 0.1 and 0.5 M all $P < 0.05$, *t*-test with Bonferroni correction; $n = 6$; Fig. 2B).

To verify that remaining at the nicotine spot was not a result of nicotine-induced akinesis, worms were placed directly on the nicotine spot and filmed 0, 15, 30, 60, 90 and 120 min later. Worms on vehicle spots quickly left the center, and at 90 min, few remained in the center. After 30 min, most worms on 0.5 M nicotine were akinetic (Fig. 2C). Worms on the 0.1 M nicotine spot became either extremely ataxic or akinetic by 90 min. Conversely, all of the worms on the 50 mM spot were moving at 90 min, although they exhibited more omega bends than did worms on vehicle spots (two-way ANOVA: interaction $F_{15,187} = 8.31$, $P < 0.05$; $P < 0.05$ for 0 vs. 50 mM at 15, 30, 60 and 90 min; $n = 8$–9; *t*-test with Bonferroni correction; Fig. 2I), suggesting that the worms were backtracking to stay on the 50 mM nicotine spot. The fraction of worms moving was reduced only between 0 and 0.1 or 0.5 M plates (two-way ANOVA interaction $F_{15,160} = 45.41$, $P < 0.05$, $P < 0.05$ for 0 vs. 0.1 at 90 and 120 min and for 0 vs. 0.5 M at 15, 30, 60, 90 and 120 min), whereas the number of worms moving on 50 mM plates vs. 0 M plates was not significantly different (Fig. 2C). These results suggest that worms plated onto spots of 0.1 or 0.5 M nicotine became akinetic, whereas those on 50 mM nicotine spots did not.

When a spot of 1% benzaldehyde, an odorant that elicits a strong naïve approach in *C. elegans*, was placed at one side of the plate 90 min after the worms were placed in the center (Fig. 2D), worms on plates without nicotine approached benzaldehyde more than those on nicotine plates, and worms on 50 mM plates approached benzaldehyde more than did those on plates containing 0.1 or 0.5 M nicotine (one-way ANOVA: $F_{3,32} = 175.1$, $P < 0.05$; 0 vs. 50 mM, 0.1 and 0.5 M all $P < 0.05$; 50 mM vs. 0.1 and 0.5 M all $P < 0.05$; $n = 9$; Tukey's test; Fig. 2E). These data suggest that worms on plates with 50 mM nicotine spots allowed to diffuse for 3 h can move if given another reward nearby, although most worms stay on the nicotine spot. When worms were removed from nicotine-containing plates after 90 min and tested for benzaldehyde approach on fresh, nicotine-free plates, only worms initially exposed to 0 or 50 mM nicotine showed significant benzaldehyde approach (one-way ANOVA: $F_{3,20} = 48.91$; 0 vs. 0.1 and 0.5 M all $P < 0.05$; 50 mM vs. 0.1 and 0.5 M all $P < 0.05$; $n = 6$; Tukey's test; Fig. 2F). This suggested that worms exposed to 50 mM nicotine can approach appetitive stimuli once removed from the nicotine source.

To fully address the question of whether worms on plates with 0.1 or 0.5 M nicotine spots did not migrate towards 1% benzaldehyde because of locomotor deficits (rather than, for example, an indifference to appetitive stimuli), the proportion of worms leaving the central rectangle where worms were initially placed on the benzaldehyde approach plate after nicotine exposure (Fig. 2D) was compared with worms never exposed to nicotine. Most worms initially plated on 0.1 and 0.5 M nicotine stayed at the center rectangle whereas those initially plated on the no nicotine and 50 mM plates left (one-way ANOVA: $F_{3,20} = 40.96$, $P < 0.05$; 0 vs. 0.1 and 0.5 M, $P < 0.05$; $n = 6$; Dunnett's test; Fig. 2G). The significantly lower proportion of worms leaving the rectangle in the 0.1 and 0.5 M relative to the 50 mM or control plates suggests the lack of approach can be attributed to locomotor deficits. Taken together, these results suggest that worms on plates containing a 50 mM nicotine spot allowed to diffuse for 3 h approach and remain at the spot not because of akinesis, but because of a rewarding effect. To determine

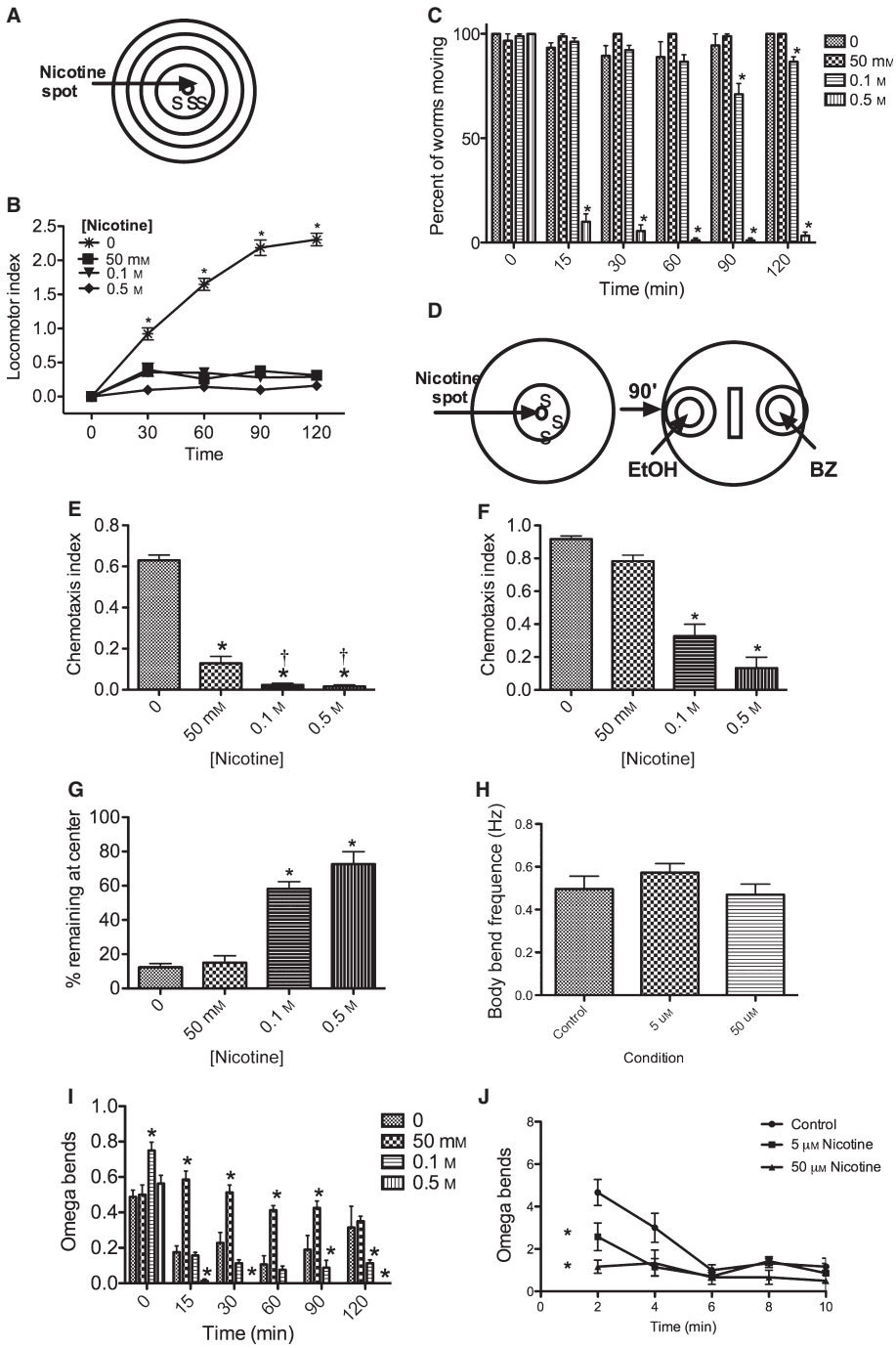

FIG. 2. Nicotine-induced changes in locomotor activity do not account for nicotine approach. (A) Assay for nicotine effects on locomotor activity. A 10 μL spot of nicotine was placed at the center of an assay plate and allowed to diffuse for 3 h. (B) Worms plated onto plates containing nicotine-free spots moved away from the center in a time-dependent fashion, but not those plated on nicotine spots (*$P < 0.05$, *t*-tests with Bonferroni correction). (C) Further analysis of the locomotor activity of worms on the central nicotine spot revealed that significant numbers of worms placed directly on a 0.5 M nicotine spot were paralysed after 15 min on the plate, 0.1 M nicotine rendered worms ataxic with some paralysed at 90 min, while those on 50 mM plates were still moving 120 min after plating (*$P < 0.05$, unpaired *t*-tests with Bonferroni correction). (D) Assay for subsequent benzaldehyde approach. Worms exposed to nicotine were given a choice between dilute benzaldehyde counterbalanced by ethanol solvent. (E) Fewer worms left the central spot when nicotine was present to approach 1% benzaldehyde (*$P < 0.05$ vs. 0 and †$P < 0.05$ vs. 50 mM, Tukey's test). (F) Worms plated on 50 mM nicotine approached 1% benzaldehyde when first washed off the nicotine plate (*$P < 0.05$, Dunnett's test). (G) The fraction of worms that stayed at the central starting point did not differ between worms plated on 0 vs. 50 mM nicotine, but increased significantly for worms plated on 0.1 and 0.5 M nicotine (*$P < 0.05$, Dunnett's test). (H) An automated worm tracker system revealed no difference in body bend frequency with change in nicotine concentration. (I) Worms on the 50 mM nicotine spot exhibit an enhancement in omega bends. Worms were placed on the center of the agar plate where nicotine (or water vehicle) had been spotted 3 h previously, dried and then examined immediately ($t = 0$) and at various times afterwards. Worms on 50 mM nicotine plates exhibited more omega bends than those on water plates. Worms on 0.5 M nicotine plates were akinetic 15 min after plating. Worms on 0.1 M nicotine plates initially exhibited more omega bends, and became progressively more ataxic and akinetic. (*$P < 0.05$, Bonferroni) (J) Worms picked on to plates with nicotine, although without a gradient, exhibit lower, rather than higher, numbers of omega turns relative to control during the first 2 min for both 5 and 50 μM concentrations ($P < 0.05$, Bonferroni).

whether nicotine concentrations the worms experience affect locomotor function, we employed an automated measure of body bends of worms on plates with homogeneous levels of nicotine in the range they would experience in the highest section of the gradient (Fig. 1B). Worms analysed at the 5 or 50 μM nicotine concentrations displayed no changes in locomotor function (one-way ANOVA: $F_{2,26} = 1.123$, NS $P > 0.05$; $n = 9$; Fig. 2H). Even after 90 min on nicotine, we found no evidence of decreased locomotor ability (0 μM nicotine 46.19 ± 8.75 bends/min, $n = 16$ vs. 5 μM nicotine 58.00 ± 7.27 bends/min, $n = 17$ worms; mean ± SEM, *t*-test $P > 0.05$). Consistent with a rewarding effect, worms on 50 mM spot plates exhibited an increase in omega turn frequency over control and high nicotine concentration plates (two-way ANOVA: $F_{15,187} = 8.31$, $P < 0.05$; $n = 8$–9; with Bonferroni correction; Fig. 2I). Yet it remained possible that worms remained near the 50 mM nicotine not because it was an attractive stimulus, but merely because nicotine illicited an unconditioned increase in omega turns. We therefore counted the number of omega turns performed by worms picked on to CTX plates containing homogenous levels of nicotine (Fig. 2J). The results indicate that in the initial stages both nicotine concentrations showed a decrease in omega turns over baseline, while after 4 min omega turn levels were indistinguishable from controls (two-way ANOVA $F_{8,80} = 2.633$, $P < 0.05$; $n = 6$–7; Bonferroni; Fig. 2J). When we analysed worms around the 15-min mark (14–16 min post transfer), we found no significant effect of nicotine treatment (0 μM nicotine 3.00 ± 0.67 turns, $n = 9$ vs. 5 μM nicotine 2.23 ± 2.14 turns, $n = 7$ vs. 50 μM nicotine 4.43 ± 2.99 bends, $n = 7$; mean ± SEM; two-way ANOVA $F_{2,22} = 0.7598$ $P > 0.05$; Bonferroni; Fig. 2I). This suggests that worms initiate increased omega turns to stay near a high concentration of nicotine rather than as a general unconditioned response to uniform concentrations of nicotine.

Worms approach nicotine more strongly during a narrow developmental window

In both animal models and humans, studies suggest that adolescence represents a time of heightened vulnerability to nicotine (Slotkin, 2002; Barron *et al.*, 2005). Adolescence is the period of development between the onset and reaching of sexual maturity, and in *C. elegans* it is analogous to the period between the L4–adult transition and the onset of egg-laying, or 46–59 h after hatching at 20 °C (Epstein & Shakes, 1995).

We tested different ages of N2 worms for nicotine approach, which was absent in worms 40 h after hatching (L4), peaked in worms 52 h after hatching (young adult), started to decline 72 h post hatching, and was virtually absent by 96 h after hatching (one-way ANOVA: $F_{5,48} = 16.83$; $P < 0.05$ for 52 h vs. all but 3 days, and 3 days vs. all but 52 h; $n = 9$; Tukey's test; Fig. 3A). To verify that this approach was not confounded by differences in locomotion at the various stages, worms were placed in the middle of a CTX agar plate, and their position 90 min later was recorded and a weighted average taken (Fig. 3B). Only 40 h (L4) worms differed significantly in gross locomotion from other stages (one-way ANOVA: $F_{5,48} = 88.27$, $P < 0.05$; L4 vs. all other ages $P < 0.05$; $n = 9$; Tukey's test; Fig. 3B). To ensure that this was not the result of reduced attention and approach to appetitive stimuli, approach to a dilute concentration of benzaldehyde was examined (Fig. 3C). No age group exhibited reduced approach to 0.01% benzaldehyde (one-way ANOVA: $F_{5,48} = 0.95$; $n = 9$; NS; Fig. 3C). We also employed an automated system to examine body bend frequency to look for an interaction between age and nicotine treatment (Fig. 3D). No such interaction was found, suggesting the results were not due to differences in nicotine's locomotor effects with age, although body bend frequency did eventually decline with age (two-way ANOVA: $F_{5,96} = 0.4243$ $P < 0.05$; 9-day vs. all $P < 0.05$; $n = 9$; Bonferroni; Fig. 3D). Taken together, these results suggest that nicotine approach peaks in the developmental period prior to sexual fertility and quickly tapers off afterwards.

Nicotine approach is dose-dependently blocked by mecamylamine and varenicline

Nicotine approach was examined in the presence of mecamylamine, a nicotinic acetylcholine receptor antagonist, in CTX agar. Mecamylamine reduced nicotine approach at concentrations of 1 and 10 mM (one way ANOVA: $F_{6,51} = 11.11$, $P < 0.05$; $P < 0.05$ for 0 vs. 1 and 10 mM; $n = 6$–9; Dunnett's test; Fig. 4A left). Mecamylamine at 10 mM, but not at 1 mM, significantly reduced basal locomotor activity (one-way ANOVA: $F_{2,23} = 9.89$, $P < 0.05$; $P < 0.05$ for 0 vs. 10 mM; $n = 8$–9; Dunnett's test; Fig. 4B left), but neither of these concentrations significantly reduced approach to 0.01% benzaldehyde (one-way ANOVA: $F_{2,23} = 1.305$; $n = 9$; NS; Fig. 4C left). To test whether the combination of nicotine and mecamylamine resulted in reduced locmotor effects, we analysed body bend frequency in worms transferred to CTX plates lacking drug or with either homogenous nicotine, mecamylamine or both (Fig. 4D left). We found no effect of either treatment, alone or together, on body bend frequency (one-way ANOVA: $F_{3,35} = 0.0401$; $n = 9$; NS; Fig. 4D left). These results support the conclusion that climbing the nicotine gradient is a nicotine-specific effect mediated by nicotinic receptors.

To determine if these motivational processes could be affected by pharmacotherapies already on the market for smoking cessation, we tested the effect of varenicline, a partial agonist of the α4β2 receptor, in the agar on the approach to nicotine. Varenicline treatment did not result in a reduction of locomotor activity (one-way ANOVA: $F_{5,48} = 1.347$; $n = 9$; NS; Fig. 4B right), and had no effect on 0.01% benzaldehyde approach at any concentration (one-way ANOVA: $F_{5,48} = 0.6457$; $n = 9$; NS; Fig. 4C right). However, there was a dose-dependent decrease in nicotine approach with increasing concentrations of varenicline (one-way ANOVA: $F_{5,48} = 5.368$, $P < 0.05$ for 10 μM and greater; $n = 9$; Fig. 4A right). Again we analysed body bend frequency with treatment with nicotine or varenicline alone or simultaneously (Fig. 4D right). Similarly, we found no effect from any treatment on this parameter (one-way ANOVA: $F_{3,35} = 1.577$; $n = 9$; NS; Fig. 4D). Thus, the currently prescribed pharmacotherapy varenicline can specifically inhibit approach to nicotine, while leaving general chemotactic and locomotor behavior unperturbed.

Mutations in two α7 orthologs, acr-5 and acr-15, significantly reduced nicotine approach

Twenty-one nicotinic acetylcholine receptor subunit mutants were screened for basal locomotor activity deficits. Six of these exhibited significantly reduced general locomotor activity (one-way ANOVA: $F_{21,193} = 41.67$, $P < 0.05$; N2 vs. *lev-1*, *unc-29*, *unc-38*, *unc-63*, *deg-3*, *eat-2*, all $P < 0.05$; $n = 26$; Dunnett's test; Fig. 5A). Of the remaining 16 mutants, all were examined for approach to 50 mM nicotine after 90 min diffusion. Worms with mutations in either *acr-5* or *acr-15* exhibited reduced nicotine approach (one-way ANOVA: $F_{15,148} = 3.688$, $P < 0.05$; N2 vs. *acr-5* and *acr-15*, both $P < 0.05$; $n = 29$; Dunnett's test; Fig. 5B). Subsequent examination of their approach to dilute benzaldehyde revealed that mutant worms exhibited normal approach to this stimulus (0.01%: one-way ANOVA:

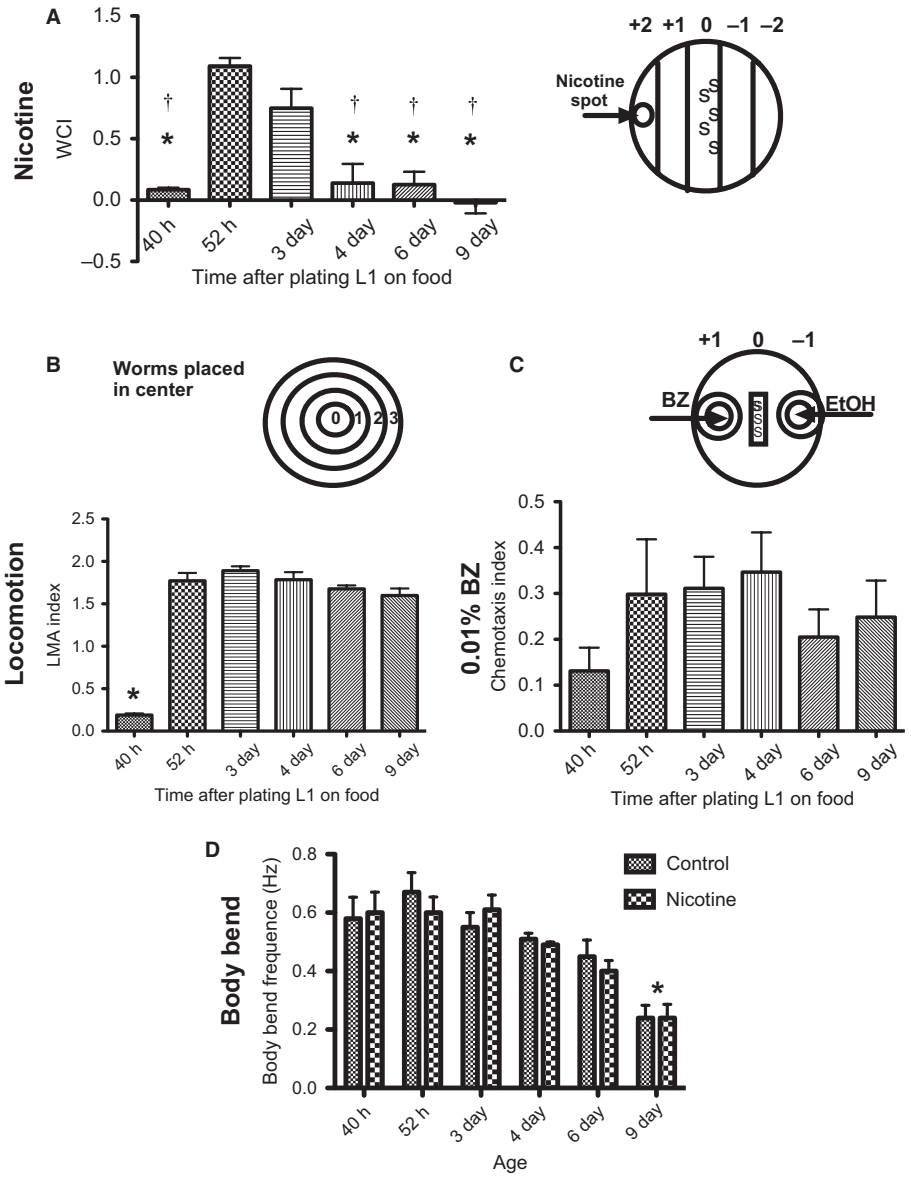

FIG. 3. The magnitude of nicotine approach peaks in young adulthood. For (A)-(B) a 50 mM nicotine drop was employed. (A) Worms placed on food at 20 °C as synchronized L1s 52 h prior to testing for nicotine approach exhibited enhanced approach to nicotine compared with other ages (*$P < 0.05$ vs. 52 h worms; †$P < 0.05$ all vs. 3-day, Tukey's test). Schematic of assay used for approach to nicotine top right. (B) Only 40 h (L4) worms exhibited reduced locomotor activity (*$P < 0.05$ compared with all other ages, Tukey's test). Schematic of locomotor assay above graph (middle left). (C) Approach to 0.01% benzaldehyde was not significantly different in any age of worms examined. Schematic of assay above graph (middle right). (D) An automated measure of body bends found no interaction between age and 5 μM homogenous nicotine treatment, although body bends did decline with age (*$P < 0.05$, Bonferroni).

$F_{15,145} = 1.63$; $n = 9$; NS; Fig. 5C). Examination of all available *acr-5* mutants showed that none of them approached nicotine (data not shown). Furthermore, examination of an interaction between gene and nicotine treatment on locomotor activity also found no effect (see below). Thus, the *acr-5* and *acr-15* nicotinic receptors specifically blocked nicotine but not benzaldehyde approach, suggesting that these receptor subunits are implicated in nicotine approach rather than in appetitive behavior generally.

The function of the acr-15 acetylcholine receptor is required specifically in glutamatergic sensitive neurons to mediate the effects of nicotine reward

As neuronally expressed *acr-15* was previously found to be important for nicotine-induced locomotor behaviors (Feng *et al.*, 2006), we further examined its involvement in motivated behaviors. Re-expression of *acr-15* under the myo-1 promoter (muscles) did not rescue the nicotine approach behavior. However, re-expression under the glr-1 promoter (mainly command interneurons) rescued the phenotype (one-way ANOVA: $F_{3,44} = 21.35$, $P < 0.05$; N2 vs. *acr-15* and myo-3 rescue $P < 0.05$; glr-1 rescue vs. *acr-15* and myo-3 rescue $P < 0.05$; $n = 12$; Tukey's test; Fig. 6A). This strongly suggests that nicotine is acting on *acr-15*-containing nicotinic receptors located in neurons to mediate nicotine approach.

Worms are able to undergo adaptation to various odorants, including the odorant butanone, sensed by the AWC olfactory sensory neuron pair, which results in a decreased chemotatic response to the initially attractive odor (Toryama *et al.*, 2007). However, this adaptated response can be obviated, and indeed the attraction even increased (termed 'butanone enchancment') by the presence of food

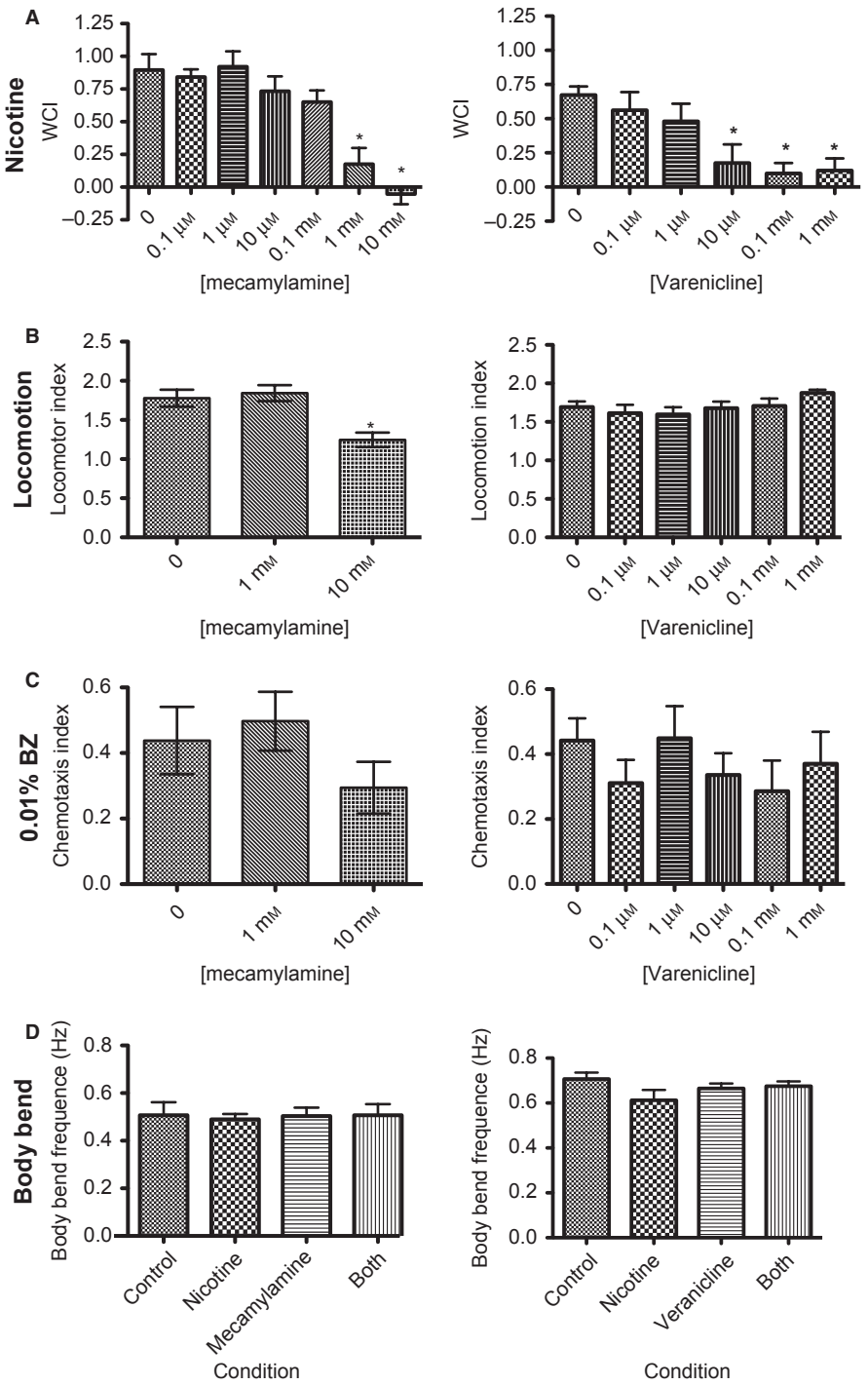

FIG. 4. Mecamylamine and varenicline inhibit nicotine approach in a dose-dependent manner. (A) The addition of mecamylamine (left) and varenicline (right) to nicotine approach assay plates significantly reduced approach (*$P < 0.05$, Dunnett's test). (B) The addition of 10 mM, but not 1 mM, mecamylamine to assay agar reduced locomotor activity (*$P < 0.05$, Dunnett's test) (left), while no effect was seen from any dose of varenicline in the locomotor assay. (C) The addition of mecamylamine (left) or varenicline (right) to assay agar did not reduce approach to 0.01% benzaldehyde. (D) An automated measure of body bends did not reveal any effect of 5 μM homogenous nicotine treatment or 1 mM mecamylamine when worms were exposed to either or both (left). Similarly, no effect was seen when worms were exposed to either 5 μM homogenous nicotine treatment, 1 mM varenicline or both (right).

during odorant conditioning. This perturbation of the adapted response is taken to be a result of pairing the odor with a rewarding stimulus. We therefore investigated whether nicotine could similarly substitute as a rewarding stimulus. The results demonstrated that worms previously exposed to 50 mM nicotine in the presence of 100% butanone approached 0.5% butanone, whereas worms exposed to plates lacking nicotine in the presence of 100% butanone later avoided 0.5% butanone (two-way ANOVA: $F_{4,63} = 3.791$, $P < 0.05$; Tukey's test: $P < 0.05$ for naïve vs. no nicotine and 50 nM nicotine vs. no nicotine; Fig. 6B), suggesting that climbing the nicotine gradient represented an unconditioned rewarding effect of nicotine. Furthermore, the *acr-5* and *acr-15* mutants, which blocked approach to

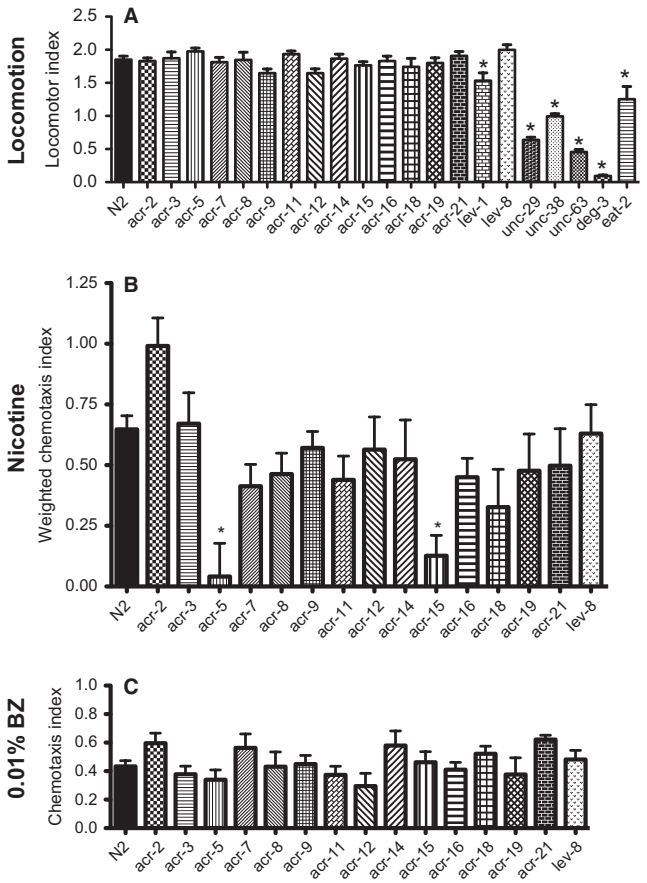

FIG. 5. Mutations in *acr-5* and *acr-15* reduced nicotine approach. (A) Twenty-one known mutants in nicotinic acetylcholine receptor subunits were examined for changes in locomotor activity. Six of these exhibited reduced basal locomotion (*$P < 0.05$, Dunnett's test), and the remaining 15 were examined further for nicotine approach. (B) Mutants in the *acr-5* and *acr-15* genes, both of which are alpha subunits and have sequence similarity to the α7 subunit in mammals, significantly reduced nicotine approach (*$P < 0.05$, Dunnett's test). (C) In a subsequent examination of approach to 0.01% benzaldehyde, none of the 15 mutants exhibited a significant change in approach compared with N2 worms.

nicotine, also eliminated the ability of nicotine to preserve approach to butanone by mutants in butanone (two-way ANOVA: F(4, 63) = 3.791 naïve vs. butanone conditioned for both *acr-5* and *acr-15* $P < 0.01$; mutants in butanone conditioned vs. nicotine paired for both *acr-5* and *acr-15* $P > 0.05$; Bonferroni; Fig. 6C). In addition, a rewarding effect of nicotine pairing could be seen with other sensory modalities. Worms can sense the ions sodium and chloride in agar through the ASE amphid sensory neuron pair (Bargmann & Horvitz, 1991) and behavior to salts can be modified by experience and is genetically tractable (Wen *et al.*, 1997). We therefore investigated whether such behavior could be modified by pairing with nicotine (Fig. 6D). When worms were exposed to either sodium or chloride ions in agar and then tested to a weak stimulus of either, no attraction was seen (two-way ANOVA: $F_{3,16} = 0.3485$, $P < 0.05$; $n = 3$; Bonferroni; Fig. 6D). If, however, either ion was first paired with nicotine prior to testing, a significant increase over baseline could be seen. Most importantly, this rewarding effect could be blocked by *acr-5* mutation. These results strongly argue for a rewarding, rather than locomotor, effect of nicotine, which again is dependent specifically on *acr-5* and *acr-15* function.

Worms lacking the dop-1 and dop-2 dopamine receptors do not approach nicotine

Considering the extensive research that suggests that dopamine (DA) transmission is important in mediating drug-motivated behaviors, we examined the effects of mutations in DA receptors, transporters and biosynthetic enzymes on nicotine approach, locomotor activity and benzaldehyde approach. A first examination of worms with mutations in genes involved in DA synthesis, reuptake and signaling suggested that the mutant *cat-2*, which encodes a worm ortholog of tyrosine hydroxylase, exhibited reduced nicotine approach (one-way ANOVA: $F_{7,65} = 2.88$, $P < 0.05$, N2 vs. *cat-2* $P < 0.05$; $n = 11$; Dunnett's test; Fig. 7A). However, this mutant also exhibited a significant reduction in general locomotor activity (one-way ANOVA: $F_{7,57} = 5.327$, N2 vs. *cat-1*, *cat-2* and *dop-1* $P < 0.05$; $n = 6$; Dunnett's test; Fig. 7B), although approach to 0.01% benzaldehyde was unaffected (one-way ANOVA: $F_{7,62} = 5.19$, $P < 0.05$, N2 vs. *cat-1* $P < 0.05$, Dunnett's test; Fig. 7D). We observed reductions in approach to nicotine in both *dop-1* and *dop-2* mutants, although these did not reach statistical significance, we further investigated these mutants in a separate series of experiments.

When *dop-1*, *dop-2* and *dop-1;dop-2* double mutant worms were further examined (Fig. 7D–F), both *dop-2* and *dop-1;dop-2* worms exhibited reduced nicotine approach (one-way ANOVA: $F_{3,20} = 5.54$, $P < 0.05$, N2 vs. *dop-2* and *dop-1;dop-2* $P < 0.05$, Dunnett's test, Fig. 7D) with no impact on locomotor activity (one-way ANOVA: $F_{3,20} = 2.02$, NS, Fig. 7E), or approach to 0.01% benzaldehyde (one-way ANOVA: $F_{3,20} = 0.79$, NS; Fig. 7F). Again we employed an automated analysis of body bend frequency to look for an interaction between strain and nicotine treatment. We analysed *dop-1*, *dop-2*, *dop-1;dop-2*, as well as the previously identified *acr-5* and *acr-15* (two-way ANOVA: $F_{5,96} = 0.2972$; $n = 8$; NS; Fig. 7G). The results indicate no interaction or effect of strain on body bend frequency. These results suggested that DA transmission at both DOP-1 and DOP-2 impacted nicotine-motivated behavior, with the latter playing a more significant role.

Discussion

Research into pharmacotherapies targeting maladaptive motivational, reward and learning processes in drug dependence is hindered by the absence of high-throughput models examining relevant aspects of drug actions. In the present study, we have demonstrated that the nematode *C. elegans* exhibits motivational behavior patterns towards nicotine similar to those observed in mammalian models. These results provide strong evidence for the existence of nicotine-dependent motivation in *C. elegans* that is genetically tractable.

Motivation can be viewed as a condition under which a stimulus elicits goal-directed behavior. Utilizing this definition, unconditioned nicotine approach in *C. elegans* falls under the umbrella of motivated behavior – worms respond in a manner that preferentially gains access to higher concentrations of nicotine. Although invertebrates exhibit behavioral effects in response to several drugs that are misused in humans, such as nicotine- or cocaine-induced locomotion (Bainton *et al.*, 2000; Feng *et al.*, 2006; Ward *et al.*, 2009) or locomotor sensitization (McClung & Hirsh, 1999; Feng *et al.*, 2006), as well as ethanol approach (Cadieu *et al.*, 1999; Wolf & Heberlein, 2003; Lee *et al.*, 2009), the present study represents the first, to our knowledge, in which nicotine serves as a primary motivating stimulus in invertebrates. Nicotine approach was observed in *C. elegans* that were previously nicotine-naïve, and this stands in contrast to

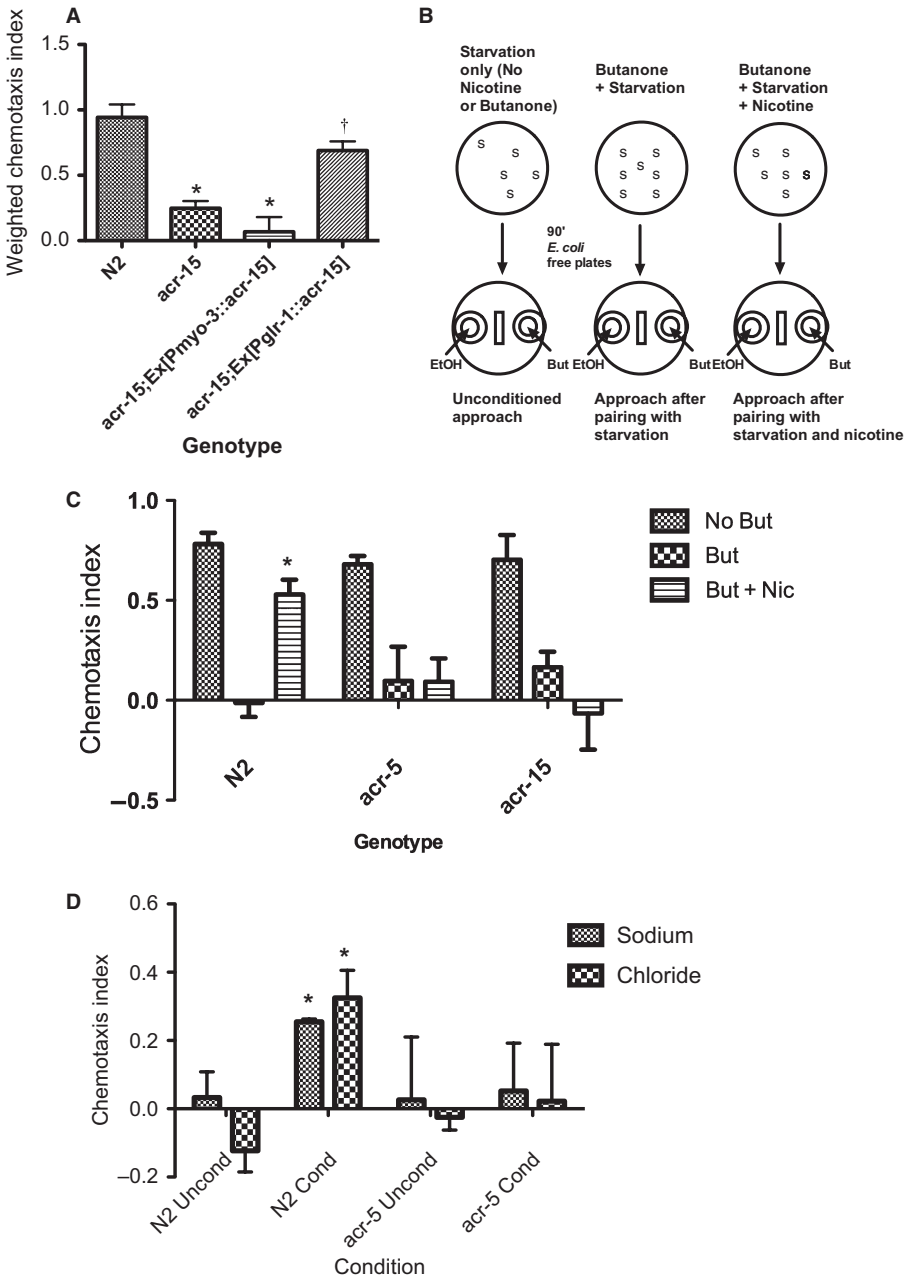

FIG. 6. *acr-15* is required in command interneurons and *acr-5* and *acr-15* are required for mediating rewarding effects of nicotine in butanone conditioning. *acr-5* is also required in mediating a preference for salt taste paired with nicotine. (A) N2 worms approached nicotine whereas *acr-15* mutants did not. Worms on an *acr-15* background expressing *acr-15* under the myo-3 promoter [*acr-15*;Ex(Pmyo-3::acr-15)] did not approach nicotine, whereas those expressing *acr-15* under the glr-1 promoter [*acr-15*;Ex(Pglr-1::*acr-15*)] exhibited approach to nicotine [$^{*}P < 0.05$ vs. N2; $^{\dagger}P < 0.05$ vs. *acr-15* and *acr-15*;Ex(Pmyo-3::*acr-15*)] ($^{*}P < 0.05$, Tukey's). (B) Diagram laying out the paradigm of butanone adaptation. Manipulations are listed at the top while conceptual explanations of the condition are listed at the bottom. Worms were placed on an agar plate lacking food for 1 h with no butanone or nicotine (Starvation only), with butanone conditioning (Butanone + Starvation) or pairing butanone and nicotine (Butanone + Starvation + Nicotine). After this period worms were transferred to a test plate and given a choice between 0.5% butanone and ethanol solvent. (C) Chemotaxis index results from the paradigm laid out in (b). N2, *acr-5* and *acr-15* worms were tested for approach to 0.5% butanone in either the naïve state ('No But') or after conditioning to butanone in the absence ('But') or presence of nicotine ('But + Nic'). All genotypes demonstrated significant reduction in approach to butanone between naïve and conditioned, while N2 demonstrated a significant rescue of the approach by the presence of nicotine during conditioning (* indicates significant difference in N2 'But' vs. 'But+Nic', $P < 0.001$) ($^{*}P < 0.001$, Bonferonni); this effect of nicotine was not seen with either *acr-5* or *acr-15* (*acr-5* 'butanone' vs. 'paired', $P > 0.05$; *acr-15* 'butanone' vs. 'paired', $P > 0.05$). (D) Nicotine's rewarding effect can also modulate behavior to ions. N2 (left) worms show no attraction to dilute Na^{+} or Cl^{-} when unpaired ('N2 Uncond') ($^{*}P < 0.05$, Bonferroni). However, pairing of either ion with nicotine (as done with butanone in (b)) results in a large increase in attraction to either ion ('N2 Cond'). This rewarding effect of pairing with nicotine was eliminated in the *acr-5* mutant (right), which revealed levels of approach indistinguishable from unconditioned ($P > 0.05$).

ethanol approach behavior in *C. elegans*, which was observed only after chronic ethanol treatment (Lee *et al.*, 2009). In *Drosophila melanogaster*, ethanol approach probably represents a secondary reinforcing effect resulting from the presence of ethanol in decaying organic matter (Cadieu *et al.*, 1999; Wolf & Heberlein, 2003). Furthermore, our study shows the presence of nicotine with butanone

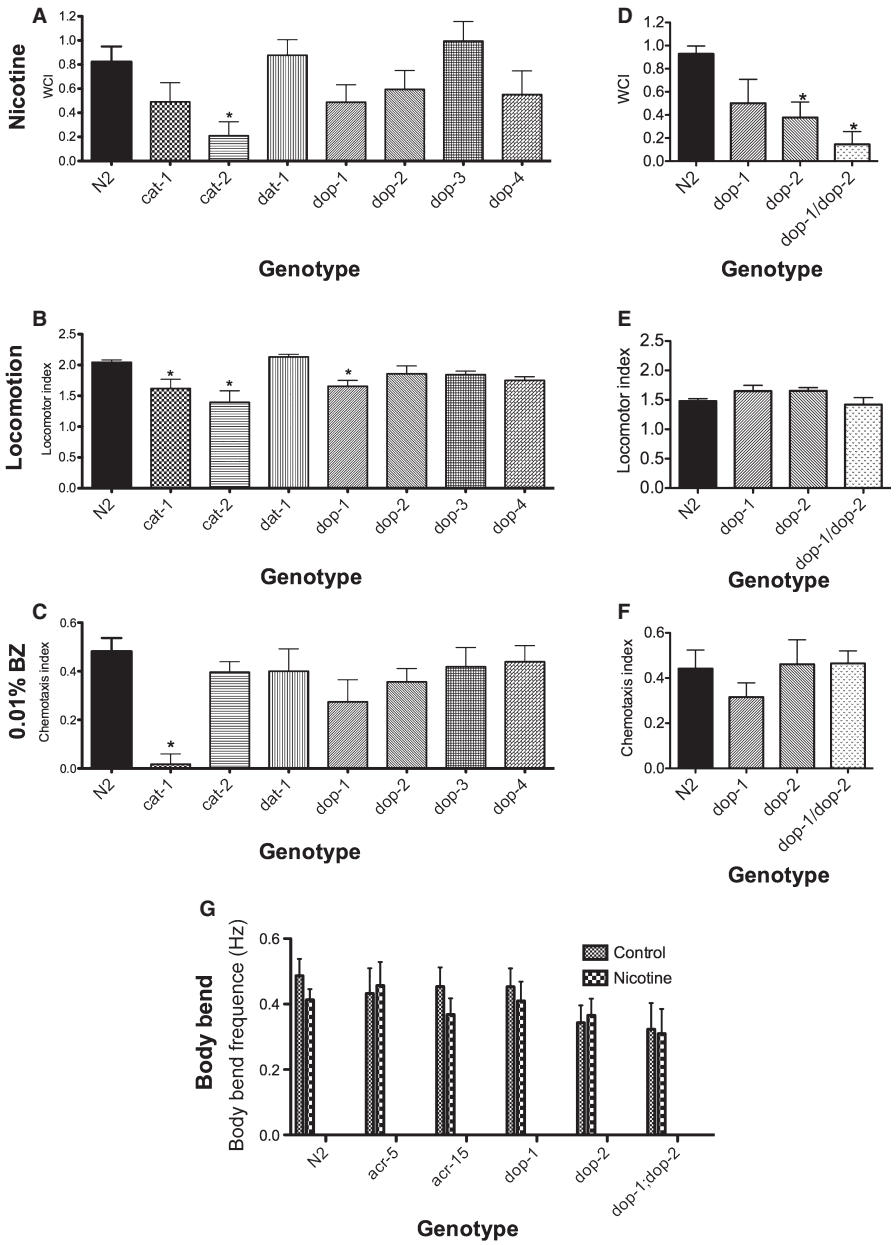

FIG. 7. The double mutant *dop-1;dop-2* exhibits reduced nicotine approach. (A) Several known mutants in DA synthesis and receptors were examined in the nicotine approach assay. *cat-2* worms exhibited a significantly reduced approach to nicotine (*$P < 0.05$, Dunnett's test). (B) Three mutants, *cat-1*, *cat-2* and *dop-1*, exhibited a significant reduction in locomotor activity (*$P < 0.05$, Dunnett's test). (C) Only *cat-1* mutants exhibited a reduction in approach to 0.01% benzaldehyde (*$P < 0.05$, Dunnett's test). (D) When re-examined in a separate experiment, *dop-2* and the double mutant *dop-1;dop-2* exhibited reduced nicotine approach (*$P < 0.05$, Dunnett's test). (E, F) Neither *dop-1*, *dop-2* nor *dop-1;dop-2* had reductions in locomotor activity or approach to 0.01% benzaldehyde. (G) An automated analysis of body bend frequency of *acr-5*, *acr-15* and the DA receptor mutants found no interaction between strain and 5 μM homogenous nicotine treatment.

under starvation conditions blocked the negative effect of pairing the odor with starvation, which we suggest occurs because nicotine counters the negative pairing by pairing the odor with the rewarding effect of nicotine. In addition, nicotine pairing greatly enhanced the attractive response of worms to ions paired with nicotine over their baseline unpaired responses. These behaviors exhibited by *C. elegans* in the current study are consistent with nicotine acting as a primary reward, rather than as a secondary reinforcer or as a locomotor stimulant.

The effect of nicotine on worm locomotion previously was investigated by Sobkowiak *et al.* (2011), which makes for an interesting comparison, although this is hindered by the distinct assays employed. However, a number of congruences can be highlight. While at 1 μM their work suggests a mild reduction in locomotion, at 10 μM a stimulatory effect on locomotion is seen. Our analysis of nicotine concentrations in the gradient generated from the 50 mM drop (Fig. 1B) revealed that in the highest section (the quarter of the plate with the drop) the concentration was approximately 5 μM. Most interesting is that this concentration is approximately in the middle of the Sobkowiak points for inhibitory and stimulatory effects on locomotion, thus suggesting their net effects may be cancelled in our assay. Yet as our body bend analysis did not reveal these subtle effects of nicotine reported by Sobkowiak *et al.*, this suggests that any such effects are small enough as to not present an alternative explaination

of the data presented in our manuscript. Finally, the Sobkowiak study reports paralysis at doses above 1 mM. This is higher than any dose tested in our manuscript, yet we were able to see significant levels of paralysis on 0.5 and 0.1 mM spotted plates, where the actual concentration worms are exposed to is probably orders of magnitude lower. This suggests that our assay is far more sensitive in accounting for the confounding effects of paralysis and our use of a myriad of controls (not limited to body bend frequency and general chemotactic ability) rigorously ensures that our data are not an artifact of its effects.

In mammals, the α4β2-containing nicotinic receptor is important for nicotine-motivated behaviors (Picciotto *et al.*, 1998; Watkins *et al.*, 1999; Laviolette & van der Kooy, 2003; Walters *et al.*, 2006; Sagara *et al.*, 2008; Xi *et al.*, 2009). Pharmacological blockade of such receptors prevents nicotine place preference and place aversion (Laviolette & van der Kooy, 2003; Walters *et al.*, 2006), as well as nicotine self-administration (Watkins *et al.*, 1999). Additionally, animals lacking a functional β2 subunit do not self-administer nicotine (Picciotto *et al.*, 1998). In the present study, the α4β2 receptor partial agonist varenicline attenuated nicotine approach in a dose-dependent manner (Fig. 4A right). Unfortunately, strains carrying mutations in the worm ortholog of β2 (*unc-38*) as well as one of the α4 orthologs (*unc-29*) exhibited abnormal locomotor activity and it was therefore not possible to further clarify the role of these subunits in nicotine approach (Fig. 5). The α7 subunit has also been implicated in nicotine motivation, although its function remains ambiguous (Nomikos *et al.*, 2000; Laviolette & van der Kooy, 2003; Walters *et al.*, 2006; Sagara *et al.*, 2008; Xi *et al.*, 2009). Pharmacological blockade of these receptors does not consistently block nicotine place preference (Laviolette & van der Kooy, 2003; Walters *et al.*, 2006), nor does it prevent nicotine-mediated enhancement of intracranial self-stimulation (Sagara *et al.*, 2008). In the current study, worms lacking functional *acr-15* and *acr-5* subunits of the nicotinic acetylcholine receptor did not approach nicotine, nor did they exhibit conditioned nicotine behavior (Fig. 6C, D), and both of these genes encode α subunits that bear sequence similarity to the α7 subunit in mammals (Mongan *et al.*, 2002; Rand, 2007). However, deficits in nicotine-induced locomotor stimulation exhibited by the *acr-16* mutant (another α7 ortholog) were rescued by re-expressing mouse α4β2 but not mouse α7 under the acr-16 promoter (Feng *et al.*, 2006), suggesting that α7 orthologs may act functionally in a manner analogous to α4β2-containing receptors in mammals to elicit nicotine's behavioral effects.

The present study also showed that *C. elegans* approached nicotine within a narrow developmental window. Although approach to several volatile odorants decreases as a function of age (Glenn *et al.*, 2004), such a mechanism is unlikely to fully explain the reduction in nicotine approach, as worms plated 4, 6 or 9 days prior to testing, none of which approached nicotine, did not show significant reductions in approach to 0.01% benzaldehyde (Fig. 3C) and body bend frequency only significantly declined by 9 days (Fig. 3D). Evidence in humans and rodents has suggested that adolescents may be more susceptible to the behavioral effects of nicotine than are adults (Slotkin, 2002; Barron *et al.*, 2005), and this phenomena may be replicated in *C. elegans.*

Dopamine signaling in the worm is important for several behaviors, including the basal slowing response (Sawin *et al.*, 2000), area restricted searching (Hills *et al.*, 2004), T-maze learning (Qin &

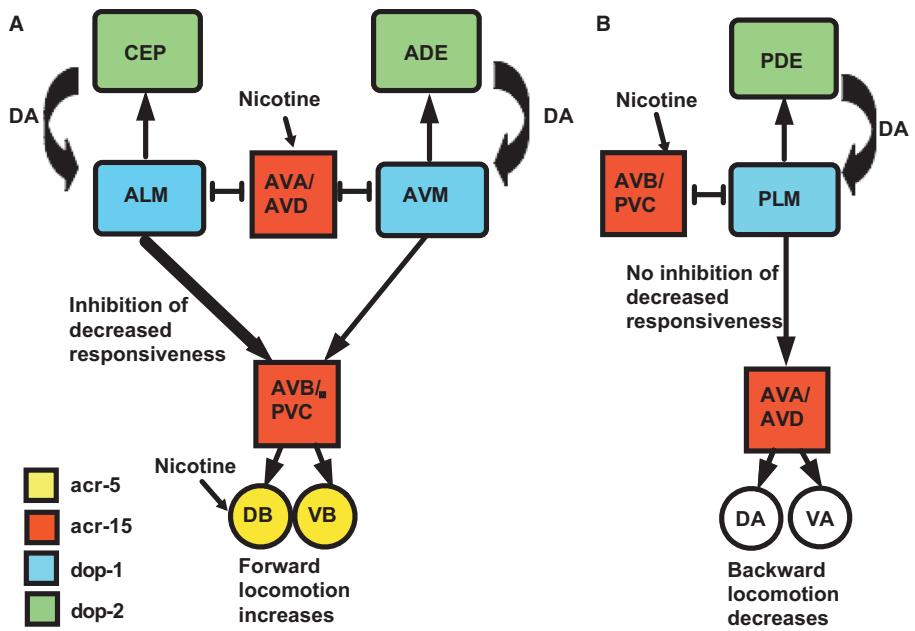

FIG. 8. Proposed neural circuit for nicotine-motivated behavior. Nicotine acts upon ACR-15-containing nicotinic acetylcholine receptors in the command interneurons (red), which then activate the touch mechanosensory neurons (blue) via gap junctions (flat arrowheads). The touch neurons then activate DOP-2-expressing dopaminergic neurons (green) via chemical synapses (arrows). DA released from these neurons acts extrasynaptically on DOP-1 receptors on the touch mechanosensory neurons (blue). (A) In the CEP–ALM forward locomotion circuit, DOP-1-dependent inhibition of reduced responsiveness of ALM to repeated stimulation occurs, thereby providing sustained input to the AVB/PVC command interneurons (Kindt *et al.*, 2007). These neurons then project to the B-class motor neurons, where ACR-5 is expressed. As a result of both the sustained input from ALM to AVB/PVC, and activation of ACR-5-containing nicotinic receptors on B-class motor neurons, forward locomotion up the nicotine gradient increases. (B) In the PDE–PLM backwards locomotion circuit, no DOP-1-dependent inhibition of reduced responsiveness occurs (Kindt *et al.*, 2007). The PLM neuron therefore exhibits decreased responsiveness with time, and the A-type motor neurons are not activated by the input from the AVA/AVD command interneurons. Backwards locomotion is inhibited.

Wheeler, 2007) and habituation to mechanical stimuli (Sanyal *et al.*, 2004; Kindt *et al.*, 2007). Tap habituation is mediated by the touch circuitry, consisting of the touch mechanosensory neurons (ALM, AVM and PLM), which project to the command interneurons (AVA, AVB, PVC and AVD), which further project to the A- and B-type motor neurons to elicit locomotion (Sanyal *et al.*, 2004; Kindt *et al.*, 2007; Giles and Rankin, 2009). We propose that nicotine may be acting on specific targets in the touch circuit to elicit approach (Fig. 8). Note that the dop-1 and dop-2 receptors act at two distinct levels of mechano-sensory neurons. The mechanosensory neurons (ALM, AVM and PLM) are connected to the command interneurons (in which acr-15 rescue restored approach), by both chemical synapses and by gap junctions (Chen *et al.*, 2006) (Fig. 8). It is plausible that nicotine's primary target is ACR-15-containing nicotinic receptors expressed on command interneurons, which excite the mechanosensory neurons via gap junctions. The ALM mechanosensory neurons, which project to the CEP cephalic dopaminergic neurons, elicit dopamine release from the CEP neurons that acts extrasynaptically on DOP-1 receptors located on the ALM neuron to prevent the reduced responsiveness of ALM that occurs with repeated stimulation (Kindt *et al.*, 2007) (Fig. 8A). This mechanism could serve to enhance nicotine approach by selectively maintaining neuronal responsiveness to nicotine in the ALM neuron that modulates forward locomotion. DOP-1 signaling in the PLM–PDE circuitry was found not to prevent reduced PLM responsiveness to stimuli with repeated exposures (Kindt *et al.*, 2007), suggesting that backwards locomotion could become inhibited (Fig. 8B). The effect of *acr-5* mutation on nicotine approach and its expression downstream of the CEP/ALM pathway (Fig. 8A) suggest that nicotine is acting to enhance forward locomotion towards higher nicotine concentrations. However, we cannot rule out a role for either the ADE–AVM or PLM–PDE projections on nicotine approach. In both mammals and *C.* elegans, dopamine modulates motivational output, which manifests itself in *C.* elegans as a motor program that elicits approach or reversal behavior from a relevant stimulus. A previous study in which re-expressing *acr-15* under the *glr-1* promoter rescued the locomotor deficit in *acr-15* mutant worms also showed that ablation of the AVA had the same effect, therefore specifically implicating the command interneurons in their effect (Feng *et al.*, 2006). It is therefore plausible, but not certain, that the command interneurons are also responsible for our observed motivated behaviour. However, and significantly, nicotine motivation and locomotor stimulation are dissociable in the worm. More specifically, *acr-15* mutants exhibited reductions in both nicotine-induced psychomotor activation and nicotine-motivated behavior (current study), whereas *acr-16* mutants exhibited reductions in only psychomotor activation but not in motivation and *acr-5* mutants showed reduced nicotine motivation but not reduced psychomotor activation (current study and Feng *et al.*, 2006). These results suggest that a behavioral double dissociation exists with respect to nicotine-induced locomotion vs. motivation, even if both behaviors are mediated through the same neuronal circuit.

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Abbreviations

CI, chemotaxis index; CTX, chemotaxis; DA, dopamine; IS, internal standard; NGM, nematode growth medium.

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