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A *C. elegans* Model of Nicotine-Dependent Behavior: Regulation by TRP-Family Channels

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SUMMARY

Nicotine, the primary addictive substance in tobacco, induces profound behavioral responses in mammals, but the underlying genetic mechanisms are not well understood. Here we develop a *C. elegans* model of nicotine-dependent behavior. We show that worms exhibit behavioral responses to nicotine that parallel those observed in mammals, including acute response, tolerance, withdrawal, and sensitization. These nicotine responses require nicotinic acetylcholine receptor (nAChR) family genes that are known to mediate nicotine dependence in mammals, suggesting functional conservation of nAChRs in nicotine responses. Importantly, we find that mutant worms lacking TRPC (transient receptor potential canonical) channels are defective in their response to nicotine and that such a defect can be rescued by a human TRPC channel, revealing an unexpected role for TRPC channels in regulating nicotine-dependent behavior. Thus, *C. elegans* can be used to characterize known genes as well as to identify new genes regulating nicotine responses.

INTRODUCTION

Nicotine dependence is a worldwide health problem and represents the leading preventable cause of death in industrialized countries (Champtiaux and Changeux, 2004; Laviolette and van der Kooy, 2004). The primary molecular target of nicotine is nicotinic acetylcholine receptors (nAChRs), a family of pentameric calcium-permeable cation channels (Champtiaux and Changeux, 2004). In mammals, nicotine binds to nAChRs in the ventral tegmental area, leading to stimulation of the mesolimbic dopamine system (Laviolette and van der Kooy, 2004). While much is known about the role of nicotine in directly modulating the function of nAChRs, the genetic mechanisms by which such modulation leads to prolonged behavioral and neurological changes are poorly understood.

Due to their simple nervous system and amenability to genetic manipulation, invertebrate organisms such as *C. elegans* and *Drosophila* have been widely utilized as genetic models to study various phenomena in neurobiology, including substance dependence. For example, recent studies in *Drosophila* have provided new insights into the mechanisms of alcohol tolerance/intoxication and cocaine sensitivity, and work in *C. elegans* has identified novel players involved in alcohol intoxication (Andretic et al., 1999; Bainton et al., 2005; Davies et al., 2003; Scholz et al., 2005). The *C. elegans* genome encodes 28 nAChR genes, many of which have been shown to form functional nAChRs in heterologous systems (Jones and Sattelle, 2004). While the role of nicotine in regulating the activity of muscle cells (e.g., egg-laying and body-wall muscles) has been well characterized (Gottschalk et al., 2005; Lewis et al., 1980; Waggoner et al., 2000), the effects of nicotine on the function of the nervous system, which mediates nicotine dependence in vertebrates, have not been evaluated.

TRP (transient receptor potential) channels represent a superfamily of cation channels conserved from worms to humans and comprise seven subfamilies (TRPC, TRPV, TRPM, TRPN, TRPA, TRPP, and TRPML) (Montell, 2005; Ramsey et al., 2006). The founding members of the TRP superfamily are the TRPC (TRP canonical) channels, which can be activated following the stimulation of phospholipase C and/or depletion of internal calcium stores (Montell, 2005). However, the precise mechanisms leading to TRPC activation remain unclear and somewhat controversial. Studies in cell culture systems have implicated TRPCs in a wide variety of physiological processes in mammals ranging from muscle relaxation/contraction, fluid secretion, and growth-cone guidance and morphology to acrosome reaction (Montell, 2005; Ramsey et al., 2006). Nevertheless, the genetic evidence supporting such functions is limited.

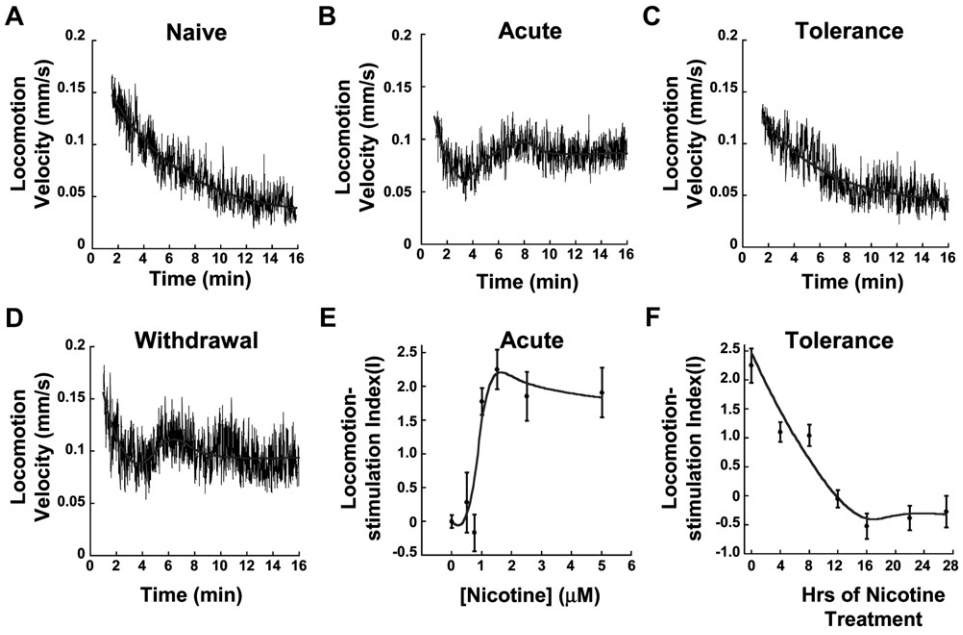

Figure 1. *C. elegans* Responds to Acute Nicotine Treatment, Develops Tolerance to Chronic Nicotine Exposure, and Exhibits Withdrawal Symptoms upon Nicotine Cessation

(A) The locomotion activity (centroid speed) of naive animals (N2) versus time on nicotine-free plates. Naive wild-type animals picked to a fresh plate moved at high speed initially because of the mechanical stimuli resulting from picking. Speed during the first 1.5 min is not shown due to high variations. See Figure S2D for longer time points. Shown is a sample trace averaged from 10 animals.

(B) Acute nicotine response. Assays were performed as in (A) except on plates containing 1.5 μM nicotine.

(C) Worms become adapted to nicotine after chronic nicotine treatment. Worms treated with 1.5 μM nicotine overnight were analyzed for their response to nicotine on nicotine plates.

(D) Nicotine cessation induces withdrawal responses. Worms treated with 1.5 μM nicotine for 16 hr were moved to nicotine-free plates.

(E) Nicotine responses in naive animals are dose dependent. The locomotion-stimulation index was used to quantify nicotine effects as described in the Supplemental Data. In this and all other figures, unless otherwise noted, error bars represent SD.

(F) The time course of nicotine tolerance (adaptation). Worms were treated with 1.5 μM nicotine for various lengths of time, and their response to nicotine was subsequently analyzed on plates containing the same concentration of nicotine. $n \geq 10$.

In the current study, we developed a *C. elegans* model of nicotine-dependent behavior. We show that worms display acute and chronic behavioral responses to nicotine that parallel those observed in mammals. These responses require nAChRs that are known to be critical for nicotine dependence in mammals. Significantly, we have found that TRPC channels regulate nicotine-dependent behavior by functionally modulating nicotine-induced cellular responses in the locomotion circuitry. Our results suggest that *C. elegans* can be used as a genetic model for identifying and characterizing neuronal genes regulating nicotine responses.

RESULTS

Worms Respond to Acute Nicotine Exposure, Develop Tolerance to Chronic Nicotine Treatment, and Exhibit Withdrawal Responses upon Nicotine Cessation

In rodent models, nicotine stimulates locomotor activity in naive animals, though it initially induces transient hypoactivity (Dwoskin et al., 1999). Chronic nicotine treatment adapts animals to nicotine (Dwoskin et al., 1999; Laviolette and van der Kooy, 2004). In addition, nicotine cessation evokes withdrawal symptoms (Kenny and Markou, 2001). To quantify the effects of nicotine on worm locomotion, we used a worm tracking system that records worm locomotion and reports its activity, such as locomotion velocity, in real time (Li et al., 2006).

We first examined the locomotion behavior of naive animals. After being transferred to a new environment (i.e., a fresh plate with bacteria), the animals exhibited a continuous decay in locomotion speed until reaching a relatively steady state (Figure 1A; see also Figure S2D in the Supplemental Data available with this article online), consistent with previous observations (Zhao et al., 2003). However, when assayed on plates containing nicotine, the animals displayed a distinct locomotion behavior: After a brief rapid decay in locomotion speed, the animals began to gradually speed up their locomotion (Figure 1B). We named this phenomenon "locomotion-stimulation." The locomotion-stimulation phase, which was not seen in naive animals, became evident starting at ~4 min after the acute nicotine incubation and perdured until the speed

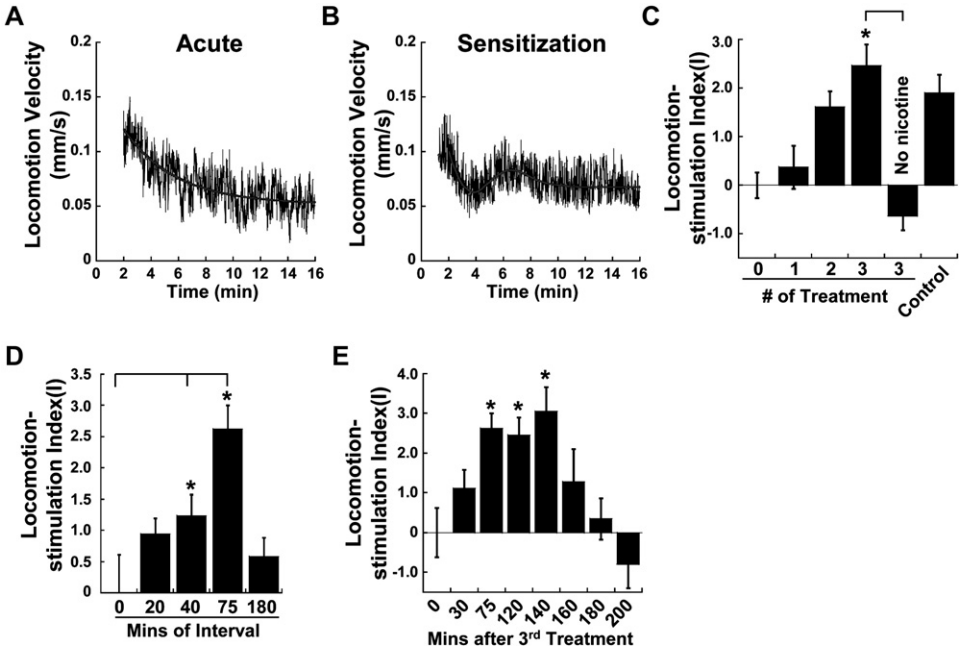

Figure 2. Repeated Intermittent Nicotine Treatments Sensitize Worms' Response to Nicotine

(A) Lack of significant locomotion-stimulation in presensitized animals. Naïve wild-type animals were assayed on plates containing 500 nM nicotine. Shown is a sample trace averaged from ten animals.

(B) Behavioral sensitization by repeated intermittent nicotine treatments. Wild-type worms were treated with 500 nM nicotine for 20 min up to three times. Between each treatment, worms were left on nicotine-free plates for 75 min. Two hours after the third nicotine treatment, their response to nicotine was assayed on plates containing 500 nM nicotine.

(C) Behavioral sensitization by repeated intermittent nicotine treatments. If no nicotine was included during the treatment, no behavioral sensitization was observed. Control represents data from naive worms in response to a higher concentration of nicotine (5 μM). *$p < 0.05$ (ANOVA with Kruskal-Wallis H test).

(D) Continuous nicotine treatment fails to induce behavioral sensitization. Worms were treated with nicotine as in (B), except that the duration of the interval between each treatment varied as indicated. *$p < 0.03$ (ANOVA with Kruskal-Wallis H test).

(E) The time course of nicotine sensitization. The protocol was the same as in (B), except that after the last treatment, worms were incubated on nicotine-free plates for various lengths of time as indicated. Subsequently, they were analyzed for response to 500 nM nicotine. *$p < 0.03$ (ANOVA with Kruskal-Wallis H test). $n \geq 10$.

plateaued (Figure 1B). Such a response to nicotine was dose dependent and peaked at around 1.5 μM (Figure 1E). Interestingly, the nicotine concentration in human blood peaks at ~500 nM after consumption of one cigarette (Pidoplichko et al., 1997).

We next tested the long-term effects of nicotine on locomotion and found that, after chronic nicotine treatment, worms developed tolerance to nicotine in a time-dependent manner (Figures 1C and 1F; Figure S2E). As a result, these nicotine-adapted animals behaved similarly to naive animals on nicotine-free plates, demonstrating that chronic nicotine treatment elicits tolerance (adaptation) in *C. elegans* (Figures 1C and 1F).

To test the effects of nicotine withdrawal, we moved the animals that were chronically treated (>16 hr) with nicotine to nicotine-free plates. In response to such nicotine cessation, these nicotine-adapted animals began to increase their locomotion speed at ~4 min after nicotine withdrawal and displayed a locomotion-stimulation phase similar to that observed in naive animals responding to acute nicotine exposure (Figure 1D). This suggests that these nicotine-adapted animals became dependent on nicotine for their naive-like behavior on nicotine plates (Figure 1D). These results indicate that nicotine cessation can induce withdrawal responses in *C. elegans*.

Worms Can Be Behaviorally Sensitized to Nicotine by Repeated Intermittent Nicotine Challenges

In vertebrates, repeated intermittent administrations of nicotine can sensitize an animal's response to nicotine, a process believed to be critical for the development of nicotine dependence (Dwoskin et al., 1999; Laviolette and van der Kooy, 2004). To test whether worms can be sensitized to nicotine, we treated naive animals for 20 min with a lower concentration of nicotine that did not induce significant locomotion-stimulation in naive animals (Figure 2A). Subsequently, we moved these nicotine-treated animals to nicotine-free plates for a recovery period before subjecting them to another round of treatment. After three doses of such treatment, the same low concentration of nicotine then evoked robust

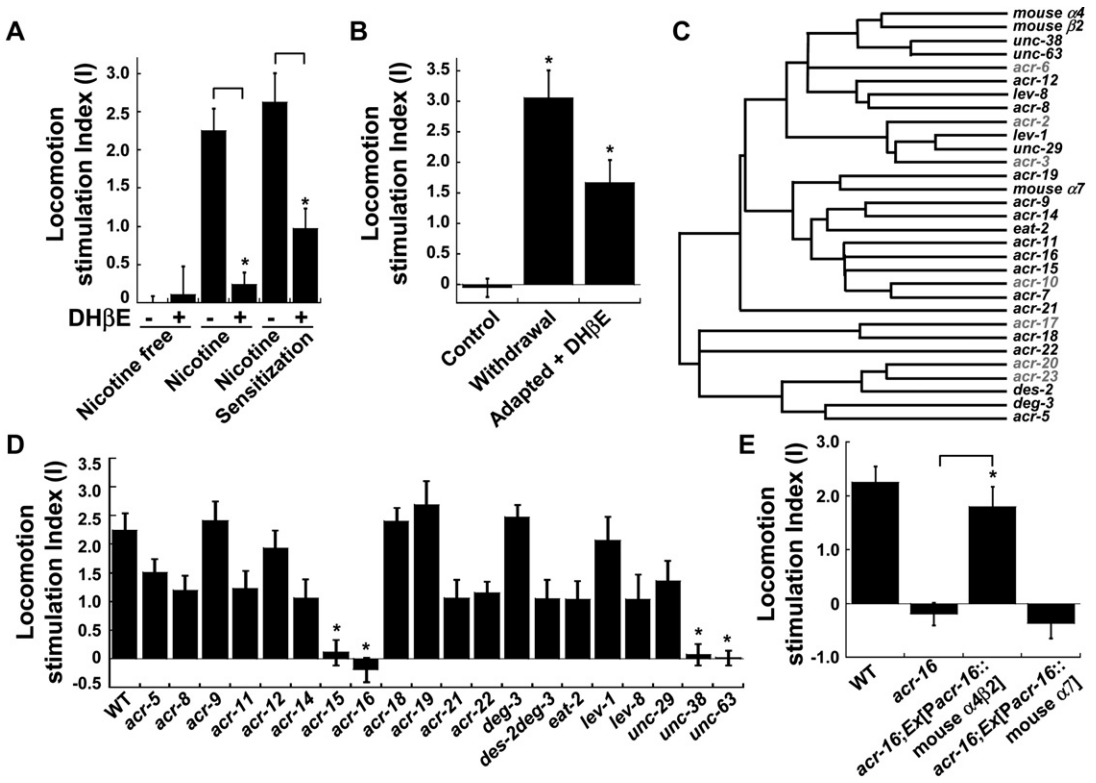

Figure 3. Nicotine-Dependent Behavior Requires Nicotinic Receptors

(A) Suppression of behavioral responses to nicotine by DHβE (20 μM). DHβE did not significantly reduce the basal locomotion rate (Figure S2C). *$p < 0.005$ (Mann-Whitney U test).

(B) DHβE induces withdrawal symptoms in nicotine-adapted animals. Nicotine-adapted animals (16 hr treatment) were moved to plates containing both DHβE and nicotine. Control represents data from nicotine-adapted animals assayed on nicotine-containing plates (no withdrawal treatment). *$p < 0.03$ (ANOVA with Kruskal-Wallis H test).

(C) Dendrogram of *C. elegans* nAChRs. Mouse α4, β2, and α7 were included. The nAChR genes that do not have mutants available are in light gray.

(D) No significant acute response to nicotine is detected in four nAChR mutants. See Experimental Procedures for allele names. *$p < 0.001$ (ANOVA with Kruskal-Wallis H test).

(E) Mouse α4β2 nAChR can restore the acute response to nicotine in *acr-16(ok789)* mutant animals. *$p < 0.02$ (ANOVA with Kruskal-Wallis H test). $n \geq 10$.

locomotion-stimulation in these animals, indicating that worms can be sensitized to nicotine (Figures 2B and 2C).

It could be argued that the observed nicotine sensitization might be caused by the accumulation of nicotine in worms. Two observations argue against this possibility. First, nicotine sensitization can only be seen with intermittent nicotine treatments (Figure 2D), whereas continuous nicotine treatments, which would presumably result in the accumulation of more nicotine, failed to induce sensitization (Figure 2D). Second, the sensitization effect did not peak until the treated animals rested on nicotine-free plates for 1 hr after the last dose of nicotine treatment (Figure 2E), providing further evidence that the observed sensitization effect was not simply due to the accumulation of nicotine.

Taken together, our data show that worms exhibit nicotine-dependent behavior: They respond to acute nicotine treatment, develop tolerance to chronic nicotine exposure, display withdrawal symptoms upon nicotine cessation, and become sensitized to nicotine after repetitive nicotine challenges. These behavioral responses to nicotine seem to parallel those observed in vertebrates.

Nicotine-Dependent Behavior Requires nAChRs

Having developed a model for characterizing nicotine-dependent behavior, we then asked what genes might underlie this behavior in *C. elegans*. In vertebrates, the psychostimulatory effects of nicotine require nAChRs, the molecular target of nicotine (Champtiaux and Changeux, 2004). As a first step to test whether these receptors are also required for nicotine responses in *C. elegans*, we challenged worms with DHβE, a nAChR competitive antagonist (Champtiaux and Changeux, 2004). This antagonist suppressed the acute nicotine response as well as nicotine sensitization in wild-type animals (Figure 3A). In vertebrates, nAChR antagonists can mimic the effects of nicotine cessation by inducing withdrawal-like symptoms in nicotine-treated animals in the presence of nicotine

(Kenny and Markou, 2001). We also observed a similar DHβE-induced phenomenon in *C. elegans* (Figure 3B). These results provide strong pharmacological evidence that nicotine-dependent behavior in *C. elegans* requires nAChRs.

We next sought to provide genetic evidence for the requirement of nAChRs for nicotine responses in *C. elegans*. The *C. elegans* genome encodes 28 nAChRs (Jones and Sattelle, 2004), most of which have mutants available (Figure 3C). We screened these mutants and found that *acr-15* and *acr-16* mutant worms lacked response to acute nicotine treatment (Figure 3D), though both mutants were otherwise superficially wild-type (Francis et al., 2005; Touroutine et al., 2005; Figure S1). As a result of this defect, the mutant worms were also defective in nicotine withdrawal and sensitization (Figures S2A and S2B). Thus, ACR-15 and ACR-16 are required for nicotine-dependent behavior. *unc-63* and *unc-38* mutants were also defective in response to nicotine (Figure 3D); however, it remains possible that such a deficit might result from a nonspecific defect in locomotion because both mutants, particularly *unc-63*, are severely uncoordinated (Culetto et al., 2004; Fleming et al., 1997). Nonetheless, our results demonstrate that nicotine-dependent behavior in *C. elegans* requires nAChRs.

The Mouse nAChR α4β2 Can Functionally Substitute for Worm nAChRs in Nicotine-Dependent Behavior

The essential role of nAChRs in nicotine-dependent behavior prompted us to explore the possibility that mammalian nAChRs may functionally substitute for *C. elegans* nAChRs in this behavior. As the mouse α4β2 heteromeric channel is the only nAChR that has thus far been found to be essential and sufficient for mediating nicotine dependence in mice (Champtiaux and Changeux, 2004; Maskos et al., 2005; Tapper et al., 2004), we expressed this mouse nAChR as a transgene in the *acr-16* mutant background under the *acr-16* promoter. We found that mouse α4β2 rescued the mutant phenotype in acute response, withdrawal, and sensitization, though the transgene did not significantly affect locomotion of wild-type worms (Figure 3E; Figures S2A and S2B; data not shown). Another mouse nAChR, α7, failed to rescue the mutant phenotype (Figure 3E). While it is possible that such a negative result might simply stem from aberrant expression of mouse α7 in worm cells, it is nonetheless noteworthy that knockout studies in mice do not support an essential role for α7 in nicotine dependence (Orr-Urtreger et al., 1997). The observation that mammalian nAChRs can functionally substitute for their *C. elegans* homolog in response to nicotine strongly suggests that at least some of the genes regulating nicotine responses in mammals are functionally conserved in *C. elegans*.

nAChRs Primarily Act in Neurons to Mediate Nicotine-Dependent Behavior

Both neuron- and muscle-type nAChRs are present in mammals; however, it is the neuronal, but not the muscular, nAChRs that primarily mediate the psychostimulatory effects of nicotine (Champtiaux and Changeux, 2004). We therefore wondered whether this is also the case with *C. elegans* nAChRs. However, *acr-16* is not neuron specific, has strong expression in muscle cells, and has been reported to function as a nAChR in the neuromuscular junction (Ballivet et al., 1996; Francis et al., 2005; Touroutine et al., 2005; Figure S3B). Similarly, *acr-15* is also expressed in both neurons and muscles (Figure S3A). In the nervous system, *acr-15* can be found in interneurons (including command interneurons), motor neurons, and pharyngeal neurons (Figure S3A). We generated transgenic animals expressing ACR-15 specifically in neurons or muscles in the *acr-15* mutant background under a neuron- or muscle-specific promoter, respectively. The neuronal, but not the muscular, expression of ACR-15 rescued the nicotine defects in *acr-15* mutant animals including acute response, withdrawal, and sensitization (Figure 4A and Figures S2A and S2B). Similar results were also obtained with *acr-16* (Figure 4A and Figures S2A and S2B). It should be noted that nicotine can affect muscle function under certain conditions. For example, high concentrations of nicotine paralyze worms by acting in the muscle (Gottschalk et al., 2005). Nevertheless, under our conditions, nAChRs seem to primarily act in neurons to mediate their function in nicotine responses.

Command Interneurons Are Important for Mediating Nicotine Responses

Interestingly, expression of *acr-15* under the *glr-1* promoter rescued the nicotine phenotype in *acr-15* mutants (Figure 4A). This promoter drives expression primarily in command interneurons, the central players in the locomotion circuitry, and a few other neurons (Hart et al., 1995; Maricq et al., 1995), suggesting that *acr-15* may act in some of these neurons to mediate its function in nicotine-dependent behavior. The same promoter, however, did not significantly rescue the *acr-16* mutant phenotype (Figure 4A), indicating the involvement of additional or different sets of neurons for *acr-16* function in nicotine-dependent behavior. Thus, we chose to focus on *acr-15* for further characterization.

The observation that the *glr-1* promoter-driven *acr-15* rescued the mutant phenotype suggests that command interneurons may be important for mediating nicotine-dependent behavior. Therefore, we killed these neurons (AVA, AVB, AVD, and PVC) with a laser. Worms lacking PVC or AVD did not exhibit a significant defect in response to nicotine incubation (Figure 4B). In contrast, ablation of AVA rendered worms unresponsive to nicotine, demonstrating an essential role for this neuron in nicotine-dependent behavior (Figure 4B). Ablation of AVB also impaired worms' response to nicotine, though the ablated worms retained residual nicotine response (Figure 4B). However, unlike other command interneurons, AVB ablation makes worms severely uncoordinated (Chalfie et al., 1985; data not shown); thus, it remains possible that the observed nicotine defects in these worms may result from

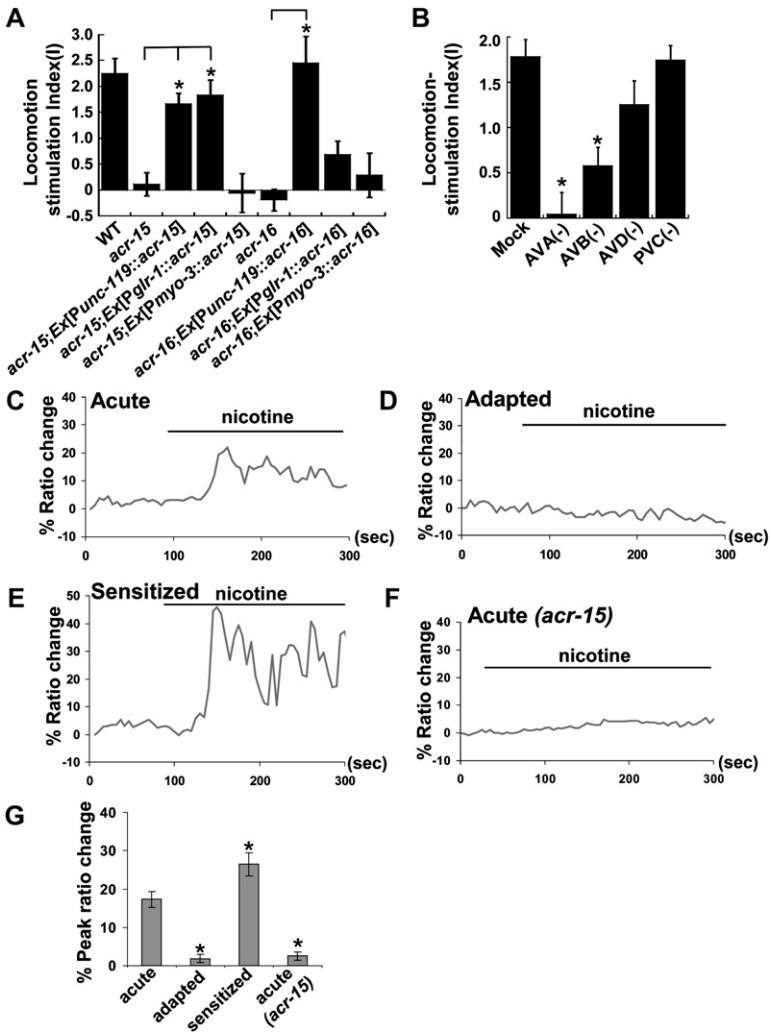

Figure 4. Nicotine Induces Calcium Responses in Command Interneurons that Are Important for Nicotine-Dependent Behavior

(A) Neuronal, but not muscular, expression of *acr-15* and *acr-16* restored the acute response to nicotine in naive *acr-15(ok1214)* and *acr-16(ok789)* animals, respectively. The *unc-119* and *myo-3* promoter was used to drive expression of *acr-15* and *acr-16* in neurons and muscles, respectively. n $\geq$ 10. *p < 0.04 (ANOVA with Kruskal-Wallis H test).

(B) Worms lacking the command interneuron AVA do not respond to nicotine incubation. AVA, AVB, AVD, or PVC neurons were ablated by laser microbeam. *p < 0.006 (ANOVA with Kruskal-Wallis H test).

(C) Nicotine induces robust calcium responses in the command interneuron AVA of live worms. G-CaMP and DsRed2 were coexpressed as a transgene under the *nmr-1* promoter. AVA was selected for imaging. Nicotine (100 μM) was perfused toward the animal immobilized on an agarose pad. The percentage change of the ratio of G-CaMP/DsRed2 fluorescence was plotted versus time. DsRed2 is insensitive to calcium changes (Li et al., 2006). Shown is a representative trace.

(D) Worms that were behaviorally adapted to nicotine show little if any response to nicotine in AVA. Worms that were treated with nicotine overnight as described in Figure 1C were subjected to imaging for nicotine-induced calcium responses as described in (C).

(E) Nicotine-induced calcium responses are potentiated in worms that were behaviorally sensitized to nicotine. Worms that received three doses of intermittent nicotine treatment as described in Figure 2 were imaged for nicotine-induced calcium responses as described in (C).

(F) ACR-15 is critical for nicotine-induced calcium responses. The same G-CaMP transgene was crossed into the *acr-15* mutant background.

(G) Histogram of calcium imaging experiments. The nicotine-induced calcium responses were greatly reduced in nicotine-adapted wild-type worms and naive *acr-15* worms (*p < 0.001, ANOVA with Dunnett test) but were potentiated in nicotine-sensitized wild-type worms (*p < 0.05, ANOVA with Dunnett test). n $\geq$ 5. Error bars represent SEM.

a nonspecific deficit in locomotion. As such, we decided to focus on AVA for further characterization.

To provide physiological evidence that command interneurons are important for nicotine responses, we recorded the activity of these neurons by calcium imaging of live animals expressing G-CaMP, a genetically encoded calcium sensor (Nakai et al., 2001). G-CaMP and cameleon have been successfully used in *C. elegans* to monitor neuronal activity (Kahn-Kirby et al., 2004; Li et al., 2006; Suzuki et al., 2003). DsRed was coinjected as a reference marker, allowing for ratiometric imaging. We focused on AVA because of its essential role in nicotine-dependent behavior and its expression of ACR-15 (Figure 4A and Figure S3A). In naive animals, acute nicotine perfusion elicited robust calcium responses in AVA (Figures 4C and 4G). In contrast, such responses were greatly reduced in the animals that were behaviorally adapted to nicotine by chronic nicotine treatment (Figures 4D and 4G), though ACR-15 was upregulated in these animals (Figures S3C and S3D). Chronic nicotine treatment also upregulates nAChRs in mammals, the mechanism of which is not fully understood (Marks et al., 1986). Notably, in the animals that were behaviorally sensitized to nicotine, the nicotine-induced calcium responses were significantly potentiated (Figures 4E and 4G). Importantly, very little response to nicotine perfusion was observed in *acr-15* mutant animals (Figures 4F and 4G), suggesting that ACR-15 is important for mediating the observed calcium responses.

In support of this in vivo observation, we found that, when expressed in HEK293T cells, ACR-15 was capable of forming a functional nAChR that can be activated by nicotine (Figure S4). Taken together, these results provide a cellular and molecular mechanism for nicotine regulation of locomotion behavior and reveal a correlation between behavioral responses and cellular physiology.

TRPC Channels Are Required for Nicotine-Dependent Behavior

Having examined some genes known to regulate nicotine responses, we then sought to identify novel genes involved in the process. In an effort to characterize *C. elegans* TRPC channels, we became intrigued by the possibility that these channels might regulate nicotine-dependent behavior. As a first step, we challenged wild-type animals with 2-APB, a TRPC-channel inhibitor (Montell, 2005; Ramsey et al., 2006), and found that this drug abolished the response to acute nicotine treatment as well as to nicotine withdrawal in wild-type animals, suggesting that TRPC channels are required for nicotine-dependent behavior (Figure 5A and Figure S5A). However, 2-APB impinges on several other targets in addition to TRPC channels (Montell, 2005; Ramsey et al., 2006). Therefore, we decided to examine TRPC mutants.

The *C. elegans* genome encodes three TRPC-channel homologs, TRP-1, TRP-2, and TRP-3 (Xu and Sternberg, 2003), all of which share the same domain structure with human and fly TRPCs (Figure 5B). These include three or four ankyrin repeats and a coiled-coil domain in the N terminus followed by six putative transmembrane domains and a TRP domain in the C-terminal cytoplasmic tail (Figure 5B). We isolated one *trp-1* allele that deleted the promoter region as well as the majority of the N terminus and one *trp-2* allele that ablated half of the transmembrane domains (Figures 5C and 5D). Both alleles are likely to be null.

Both *trp-1* and *trp-2* mutant animals were superficially wild-type (Figure S1 and data not shown), though some moderate locomotion abnormalities can be detected with our tracking system (Z.F. and X.Z.S.X., unpublished data). Importantly, both *trp* mutants lacked response to acute nicotine treatment, consistent with our pharmacological results (Figure 5E). As a result of this defect, these mutants were also defective in nicotine withdrawal and sensitization (Figures S5A and S5B). As a control, mutants lacking the sperm-specific TRPC channel TRP-3, the TRPV channel OSM-9, or the TRPM channel GTL-1 all responded normally to nicotine incubation (Colbert et al., 1997; Teramoto et al., 2005; Xu and Sternberg, 2003) (Figure 5E). Transgenic expression of wild-type copies of the *trp-2* gene in the *trp-2* mutant background restored mutant animals' responses to nicotine including acute response, withdrawal, and sensitization (Figure 5E and Figures S5A and S5B). Similar results were obtained with *trp-1* rescue (Figure 5E and Figures S5A and S5B). Therefore, TRPC channels are required for nicotine-dependent behavior in *C. elegans*.

TRP-1 has been reported to be expressed in multiple classes of neurons, such as interneurons (including command interneurons), motor neurons, pharyngeal neurons, and sensory neurons (Colbert et al., 1997). TRP-2 is also expressed in these types of neurons (Figures S5D and S5E). Expression of TRP-2 under the *glr-1* promoter can rescue the *trp-2* mutant phenotype, including acute response, withdrawal, and sensitization (Figure 5E and Figures S5A and S5B). Similar results were obtained for *trp-1* (Figure 5E). This promoter was also able to rescue the *acr-15* mutant phenotype (Figure 4A), suggesting that TRPC channels and ACR-15 might act in the same groups of neurons or circuits to regulate nicotine-dependent behavior.

TRP-2 Promotes Receptor-Operated Calcium Entry In Vitro

We then asked whether worm TRPCs can function, like their mammalian counterparts, as receptor-operated channels (Montell, 2005; Ramsey et al., 2006). Although the precise mechanisms leading to activation of mammalian TRPCs remain uncertain, they all can be activated following the stimulation of phospholipase Cβ (PLCβ) when expressed in heterologous systems (Montell, 2005; Ramsey et al., 2006). We used TRP-2 as an example and isolated its cDNA by RT-PCR. Expression of TRP-2 in HEK293T cells promoted receptor-operated calcium entry elicited by perfusion of carbachol (Figures 6A, 6B, and 6G). Carbachol is known to induce such calcium entry by stimulating PLCβ via its endogenous receptors in HEK293 cells that are coupled to heterotrimeric G proteins (Montell, 2005; Ramsey et al., 2006). Further evidence came from the observation that the TRP-2-dependent activity in HEK293T cells can be blocked by U73122, a PLC inhibitor (Figure 6H) (Montell, 2005; Ramsey et al., 2006). As is the case with mammalian TRPCs, the TRP-2-dependent activity in HEK293T cells was sensitive to 2-APB (Figure 6H). In addition, TRP-2 appeared to be permeable to Ba^{2+} and Sr^{2+}, a feature shared by several mammalian TRPCs (Figures 6C–6G) (Montell, 2005; Ramsey et al., 2006). These observations provide strong evidence that TRP-2 can function as a receptor-operated channel either on its own or by interacting with endogenous TRP proteins.

PLCβ/EGL-8 Is Required for Nicotine-Dependent Behavior

The requirement of PLCβ for TRP-2 activation in vitro raises the possibility that PLCβ may play a role in nicotine-dependent behavior in vivo. *egl-8* encodes the worm PLCβ homolog that is ubiquitously expressed in the nervous system (Lackner et al., 1999; Miller et al., 1999). *egl-8* mutant animals did not exhibit significant response to acute nicotine incubation or withdrawal (Figure 7A and Figure S6A). We also examined the role of Gq/11 because this type of protein is known to function upstream of PLCβ (Montell, 2005). A reduction-of-function Gq/*egl-30* mutant lacked response to nicotine incubation; however,

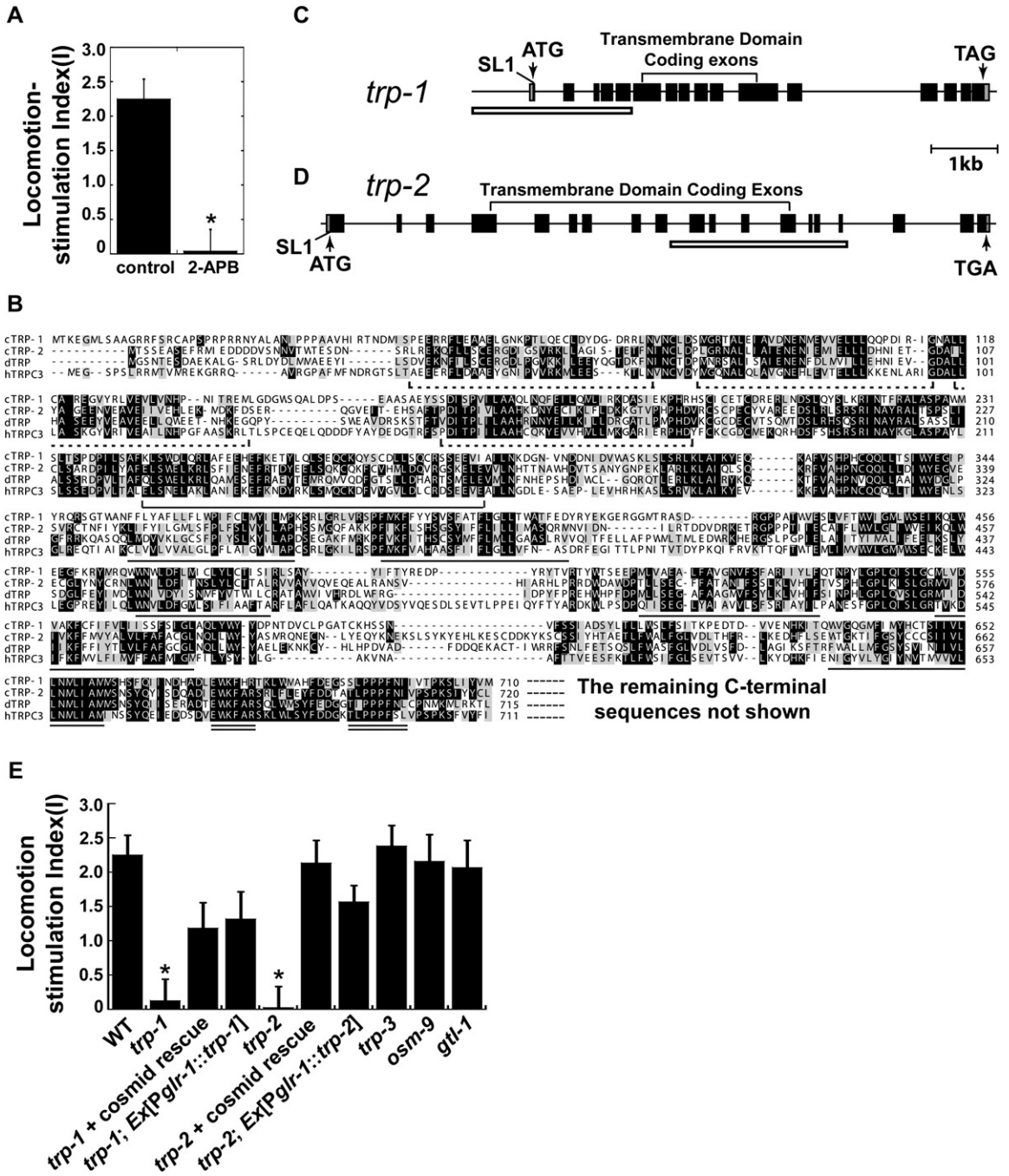

Figure 5. TRPC Channels Are Required for Nicotine-Dependent Behavior

(A) 2-APB blocks wild-type animals' acute response to nicotine. Naive wild-type animals were assayed on nicotine plates with or without (control) 50 μM 2-APB. *$p < 0.03$ (Mann-Whitney U test).

(B) Sequence alignment of worm TRP-1 (cTRP-1), worm TRP-2 (cTRP-2), fly TRP (dTRP), and human TRPC3 (hTRPC3). The ankyrin repeats, coiled-coil domain, six putative transmembrane domains, and TRP domain are indicated by the underlying dashed brackets, solid brackets, solid lines, and double lines, respectively.

(C and D) Gene structure and mutations of *trp-1* (C) and *trp-2* (D). The positions of the *trans*-spliced leader sequence (SL1), the first Met, and the stop codon are indicated. The underlying hollow rectangle indicates the fragment deleted in the *trp-1(sy690)* (C) and *trp-2(sy691)* (D) mutants.

(E) *trp-1(sy690)* and *trp-2(sy691)* mutant animals do not respond to acute nicotine treatment. *Ex*[P*glr-1::trp-2*] and *Ex*[P*glr-1::trp-1*] denote transgenes expressing TRP-2 and TRP-1 under the *glr-1* promoter, respectively. See Experimental Procedures for allele names. *$p < 0.02$ (ANOVA with Kruskal-Wallis H test). $n \geq 10$. Error bars represent SD.

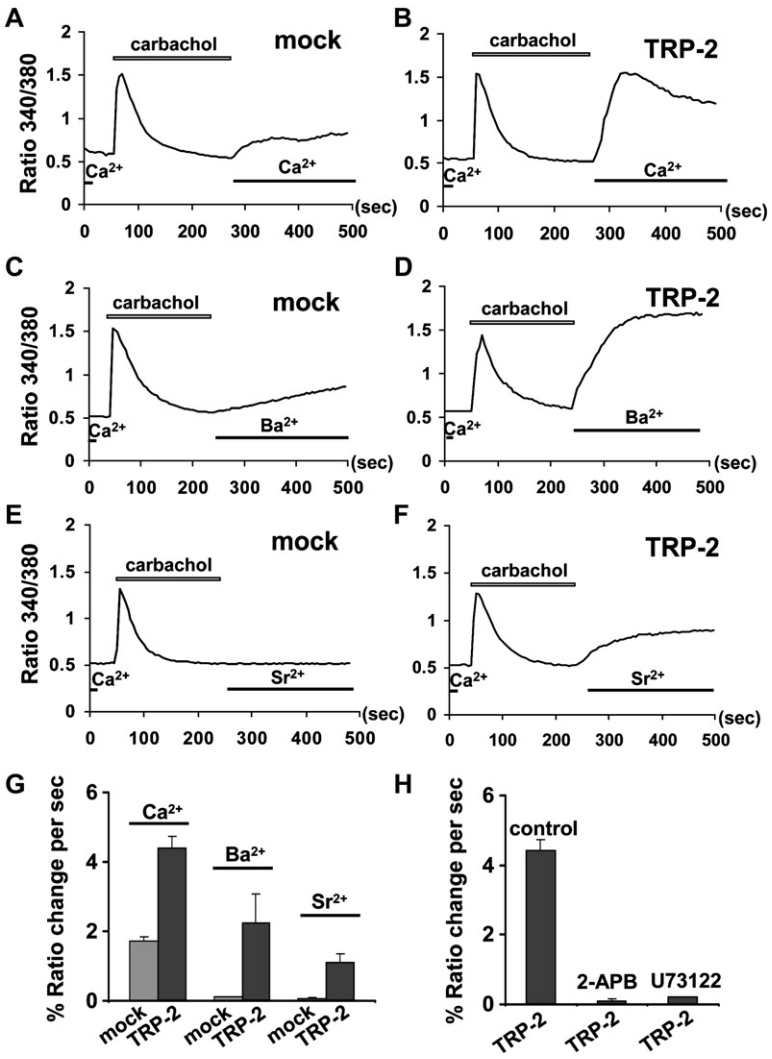

Figure 6. TRP-2 Promotes Receptor-Operated Calcium Entry In Vitro

Shown are representative traces of mock-transfected (A, C, and E) and TRP-2-transfected (B, D, and F) cells.

(A and B) Expression of TRP-2 cDNA in human HEK293T cells promotes receptor-operated calcium entry. The initial calcium peak represents calcium release from internal calcium stores. Subsequently, calcium was added to the bath to reveal the activity of receptor-operated channels.

(C and D) TRP-2 is permeable to Ba^{2+}.

(E and F) TRP-2 is permeable to Sr^{2+}.

(G) Histogram of TRP-2-dependent activities in HEK293T cells. The percentage ratio change (Δ340/380 nm) per second during the first 30 s, but not the percentage peak ratio change, was used to quantify receptor-operated channel activity because Ba^{2+} and Sr^{2+} responses did not desensitize. Error bars represent SEM.

(H) TRP-2-dependent calcium activity in 293 cells is sensitive to 2-APB and U73122. The control data (no drug) are duplicated from (G). 2-APB (100 μM) was applied right before calcium reapplication, while U73122 (10 μM) was added 10 min prior to carbachol perfusion. $n \geq 6$. Error bars represent SEM.

we cannot exclude the possibility that such a defect might result from a nonspecific deficit in locomotion because this mutant is severely uncoordinated (Brundage et al., 1996; Miller et al., 1999). Nonetheless, our results demonstrate an in vivo role for PLCβ in regulating nicotine-dependent behavior in *C. elegans* and are also consistent with the role of TRPC channels in this behavior.

Human TRPC3 Can Functionally Substitute for *C. elegans* TRP-2 in Nicotine-Dependent Behavior

In light of the functional similarity between TRP-2 and mammalian TRPCs in heterologous systems, we reasoned that mammalian TRPCs might be able to substitute for the function of TRP-2 in nicotine-dependent behavior in *C. elegans*. To explore this possibility, we generated transgenic animals expressing human *TRPC* genes in the *trp-2* mutant background under the *trp-2* promoter. Three human *TRPC* genes (hTRPC1, 3, and 4) were tested, with each representing a subgroup of the human TRPC subfamily (Montell, 2005; Ramsey et al., 2006). While negative results were obtained with hTRPC1 and 4, expression of hTRPC3 restored the responses of *trp-2* mutant animals to nicotine, including acute response, withdrawal, and sensitization (Figure 7A and Figure S6). These results reveal functional conservation of TRPC channels in regulating nicotine-dependent behavior.

TRPC Channels Are Important for Nicotine-Induced Calcium Responses in Command Interneurons

The question arises as to how TRPC channels regulate nicotine-dependent behavior. One possibility is that nAChRs could depend on TRPC channels for their expression. However, this does not seem to be the case. For example, TRP-2 was not required for ACR-15 expression (Figure S3E and data not shown). As TRPCs and ACR-15 may act in the same groups of neurons or circuits, we tested whether TRPC channels can functionally regulate nAChR activity by imaging nicotine-induced calcium responses in live animals expressing the genetically encoded calcium sensor G-CaMP. We found that

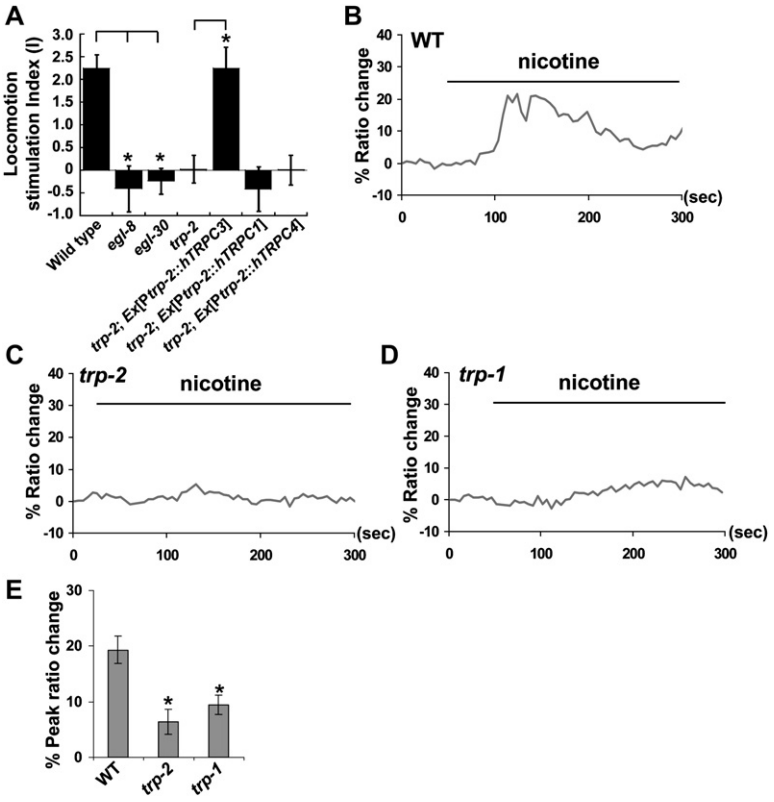

Figure 7. TRPC Channels Are Important for Nicotine-Induced Calcium Responses in Command Interneurons

(A) Expression of human TRPC3 restores the acute response to nicotine in *trp-2(sy691)* mutant animals. hTRPC1, hTRPC3, and hTRPC4 cDNAs were expressed as transgenes (i.e., *Ex*[P*trp-2::hTRPC1*], *Ex*[P*trp-2::hTRPC3*], and *Ex*[P*trp-2::hTRPC4*]) under the *trp-2* promoter in *trp-2(sy691)* mutant animals. n $\geq$ 10. Error bars represent SD. *p < 0.02 (ANOVA with Kruskal-Wallis H test).

(B) Nicotine induces robust calcium responses in the command interneuron AVA of live worms. Nicotine (100 μM) was perfused toward the animal immobilized on an agarose pad. The percentage change of the ratio of G-CaMP/DsRed2 fluorescence was plotted versus time. A representative trace is shown.

(C and D) Nicotine-induced calcium responses are greatly reduced in *trp-2(sy691)* (C) and *trp-1(sy690)* (D) mutant worms. The same G-CaMP transgene was crossed into the *trp-2(sy691)* or *trp-1(sy690)* mutant background.

(E) Histogram of calcium imaging experiments. The nicotine-induced calcium responses were greatly reduced in *trp-2(sy691)* (*p < 0.003, ANOVA with Dunnett test) and *trp-1(sy690)* mutant worms (*p < 0.03, ANOVA with Dunnett test). n $\geq$ 8. Error bars represent SEM.

nicotine-induced calcium responses in the command interneuron AVA were greatly reduced in *trp-2* and *trp-1* mutant worms (Figures 7B–7E). Thus, it appears that TRPC channels can functionally regulate nicotine-induced neuronal activity in the locomotion circuitry, providing a cellular mechanism for TRPC-channel regulation of nicotine-dependent behavior.

DISCUSSION

C. elegans Can Be Used as a Genetic Model to Characterize Genes Regulating Nicotine Responses

In the current study, we developed a *C. elegans* model of nicotine-dependent behavior. Our results indicate that *C. elegans* displays several types of behavioral responses to nicotine that parallel those observed in vertebrates. These include acute response, tolerance, withdrawal, and sensitization. In addition, we show that nicotine responses in *C. elegans* require nAChRs, the molecular targets of nicotine that are known to mediate nicotine dependence in mammals (Champtiaux and Changeux, 2004; Laviolette and van der Kooy, 2004). These results demonstrate that at least some of the genes regulating nicotine responses in mammals are functionally conserved in *C. elegans* and suggest that *C. elegans* is a valuable system for characterizing genes regulating nicotine responses.

While only 17 nAChR genes are present in mammals, the *C. elegans* genome encodes 28 such genes. Among the worm nAChR genes that have been examined for expression patterns, all have been found to be expressed in neurons (Jones and Sattelle, 2004). Although much is known about the role of these nAChRs in regulating muscle activity (Francis et al., 2005; Gottschalk et al., 2005; Richmond and Jorgensen, 1999; Touroutine et al., 2005; Waggoner et al., 2000), their functions in the nervous system are not well understood. We have shown that at least two nAChR genes, *acr-15* and *acr-16*, are required for nicotine-dependent behavior. Both nAChRs can be activated by nicotine when expressed in heterologous systems (Figure S4; Ballivet et al., 1996). Although these two nAChRs are expressed in both neurons and muscles, as is the case with their mammalian counterparts, we have found that under our conditions, they primarily act in neurons to regulate nicotine-dependent behavior. One site of action for ACR-15 seems to be in the command interneurons. Nevertheless, it remains unclear in which neurons ACR-16 acts to mediate its function in nicotine responses. Nicotine dependence in mammals is a highly complex phenomenon entailing the function and coordination of multiple nAChR genes and brain regions. In worms, it may also involve the action of multiple nAChRs expressed in different classes of neurons that are directly or indirectly connected to the locomotion circuitry.

Our laser ablation and calcium imaging experiments suggest that command interneurons are important for nicotine-dependent behavior. This is consistent with the

critical role of these neurons in locomotion (Chalfie and White, 1988). Nevertheless, our results do not exclude the involvement of other types of neurons in regulating nicotine-dependent behavior. Among the four major pairs of command interneurons, AVA neurons are essential for nicotine-dependent behavior; AVB neurons may also play an important role. One function for AVA neurons is to regulate spontaneous reversal frequency during locomotion (Chalfie et al., 1985; Zheng et al., 1999). However, these neurons also receive synaptic input from, as well as synapse onto, other command interneurons including AVB and PVC, both of which are known to regulate forward movement (Chalfie et al., 1985; White et al., 1986). Upon nicotine stimulation, AVA and AVB may tune the activity of the locomotion circuitry, leading to the observed behavioral effects.

TRPC Channels Are Important for Nicotine-Dependent Behavior

We have also begun to identify proteins that were not previously known to regulate nicotine-dependent behavior and have found that TRPC channels are required for this behavior in *C. elegans*. Specifically, *trp* mutant worms are defective in the acute nicotine response, which might lead to defects of these mutants in other nicotine responses such as nicotine withdrawal and sensitization. The role of TRPC channels in nicotine responses is also supported by the observation that PLCβ, a protein important for TRPC-channel activation, is critical for nicotine-dependent behavior in *C. elegans*.

How do TRPC channels regulate nicotine-dependent behavior? Our rescue experiments suggest that TRPC channels and the nAChR ACR-15 may act in the same groups of neurons or circuits to mediate their function in nicotine responses. Indeed, worms lacking TRPC channels are defective in nicotine-induced, ACR-15-dependent calcium responses in some command interneurons. Thus, while other mechanisms may also contribute, one mechanism by which TRPC channels regulate nicotine-dependent behavior appears to be through functionally modulating nicotine-induced command interneuron activity in the locomotion circuitry. TRPC channels may do so by acting directly in command interneurons and/or indirectly via network activity.

All six human TRPC channels are expressed in the central nervous system (CNS) (Montell, 2005; Ramsey et al., 2006). Despite extensive in vitro studies in cell culture systems implicating mammalian TRPC channels in various neuronal activities in the CNS, such as synaptic transmission, growth-cone guidance and morphology, and neurite extension (reviewed in Ramsey et al., 2006), the genetic evidence supporting such roles for these channels is still lacking. Our studies identify an unexpected role for TRPCs in nervous-system function and reveal a functional link between TRP-family channels and nicotinic signaling. The observation that a human TRPC homolog can functionally substitute for a *C. elegans* TRPC channel also raises the possibility that these channels might regulate nicotine dependence and perhaps other types of substance dependence in mammals.

EXPERIMENTAL PROCEDURES

Genetics and Molecular Biology

The following mutant alleles were used in the study: *acr-5(ok180)*, *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(ok967)*, *acr-21(ok1314)*, *deg-2(u695)deg-3(u662)*, *deg-3(tu1851)*, *eat-2(ad465)*, *eat-4(ky5)*, *egl-8(n488)*, *egl-30(md186)*, *gtl-1(ok375)*, *lev-1(e211)*, *lev-8(x15)*, *osm-9(ky10)*, *trp-1(sy690)*, *trp-2(sy691)*, *trp-3(sy693)*, *unc-29(x29)*, *unc-38 (x20)*, and *unc-63(x13)*.

To generate the *unc-119* promoter-driven transgenic lines, the *unc-119* promoter was amplified by PCR from a plasmid (PBY103), fused by PCR with a PCR fragment (amplified from genomic DNA) encoding the coding region *of acr-15* and *acr-16*, and injected into the *acr-15(ok1214)* and *acr-16(ok789)* mutant background, respectively. Transgenic lines expressing the *myo-3* and *glr-1* promoter-driven transgenes were generated with the same strategy. The *myo-3* promoter and *glr-1* promoter were amplified by PCR from pPD136.64 (a gift from A. Fire) and genomic DNA, respectively (Hart et al., 1995; Maricq et al., 1995). To make transgenic worms expressing the transgene P*acr-16::mouseα4β2*, the *acr-16* promoter (~6 kb) was amplified by PCR from genomic DNA; fused by PCR with a PCR fragment of the mouse α4 cDNA or β2 cDNA coding region linked to the *unc-54* 3′UTR, respectively; and coinjected at a ratio of 2:3 into the *acr-16(ok789)* mutant background. To generate transgenic lines expressing P*trp-2::hTRPC3*, the *trp-2* promoter (~3.5 kb) was amplified from the cosmid R06B10, fused by PCR with a PCR fragment of human TRPC3 cDNA coding region linked to the *unc-54* 3′UTR, and directly injected into the *trp-2(sy691)* mutant background. The P*trp-2::hTRPC1* and P*trp-2::hTRPC4* transgenes were constructed with the same strategy. The cosmids ZC21 and R06B10 were used to rescue the *trp-1(sy690)* and *trp-2(sy691)* mutants, respectively. At least two independent lines were analyzed for each transgene. Both *trp* alleles were backcrossed to N2 seven times before behavioral analysis.

Cell Culture and Calcium Imaging

Cell culture and calcium imaging were performed as previously described (Li et al., 2006; Xu and Sternberg, 2003). See details in Supplemental Data.

Behavioral Analysis

L4 hermaphrodites were picked 16 hr before behavioral analysis. The NGM plates used for tracking were freshly spread with a thin layer of *E. coli* OP50 5 min prior to tracking. Tracking was performed at 20°C–21°C and a relative humidity of ~40% with the lid off. The tracking system consists of a stereomicroscope (Zeiss Stemi 2000C) mounted with a digital camera (Cohu 7800), a digital motion system (Parker Automation) that follows worm movement, and a home-developed software package. To record locomotion, animals' images were grabbed at a rate of 2 Hz for 16 min. The locomotion velocity of the animal at each time point, computed as centroid displacement (mm) per second, was plotted and displayed in real time during tracking. The vision/motion data were also compressed, integrated, and stored as a commonly used multimedia file format (AVI). Nicotine was included in media right before plates were poured. DHβE and 2-APB were directly spread on the surface of NGM plates and allowed to diffuse for >16 hr prior to use. Animals were preincubated with DHβE for 1 hr prior to analyzing their response to nicotine.

Behavioral Quantification

Methods for quantifying locomotion-stimulation are described in the Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and six figures can be found with this article online at http://www.cell.com/cgi/content/full/127/3/621/DC1/.

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Z.F. and X.Z.S.X. conceived and designed the experiments. Z.F. and W.L. performed the experiments and analyzed the data. A.W., B.J.P., and E.R.L. helped perform some experiments and paper writing. P.W.S. contributed critical reagents, intellectual input, and help with paper writing. X.Z.S.X. and Z.F. wrote the paper.

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