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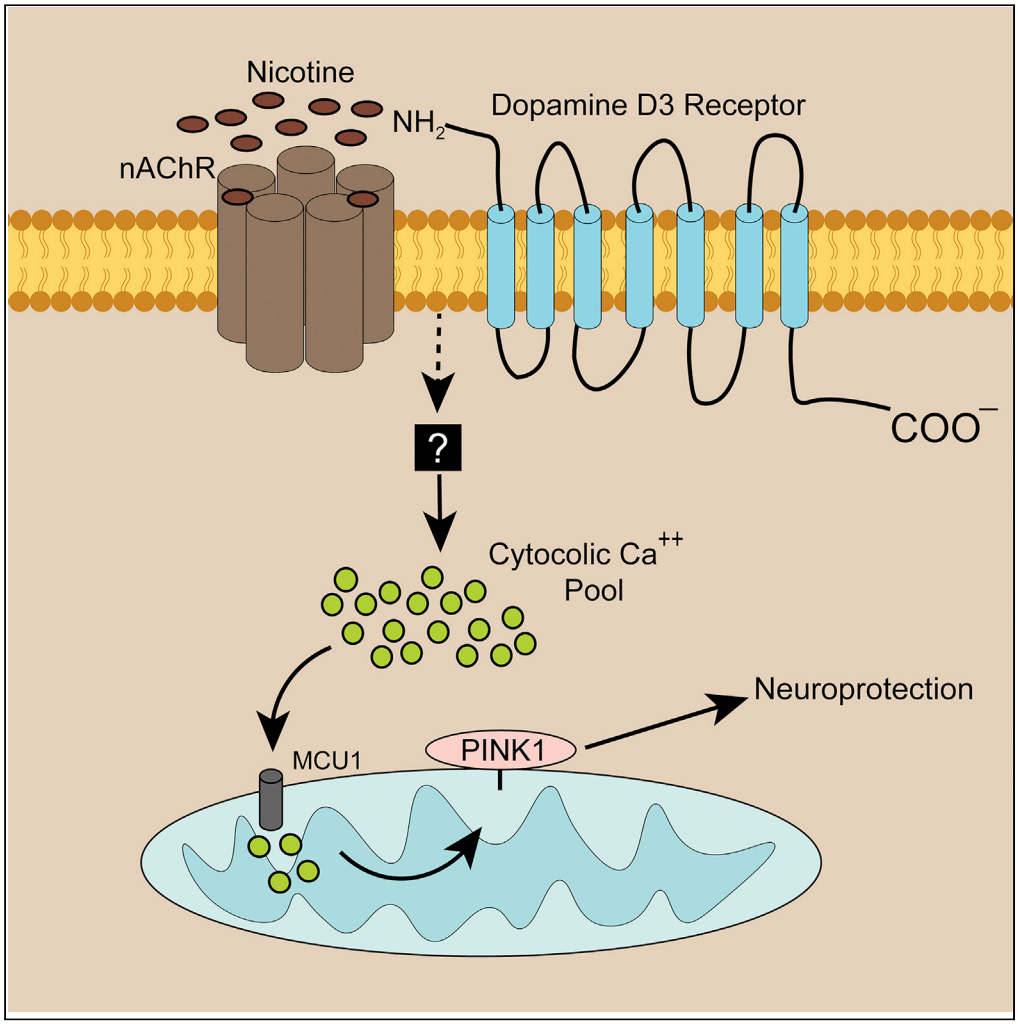

J. Brucker Nourse, Jr., Gilad Harshefi, Adi Marom, ..., Kim A. Caldwell, Guy A. Caldwell, Millet Treinin

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HIGHLIGHTS

Establishment of a *C. elegans* model for nicotine-induced neuroprotection

Dopaminergic neuron-expressed nAChRs exhibit conserved functions

Nicotine-induced nAChR- and D3R-dependent signaling is neuroprotective *in vivo*

Nicotine-induced protection involves mediators of mitochondrial quality control

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Conserved nicotine-activated neuroprotective pathways involve mitochondrial stress

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SUMMARY

Tobacco smoking is a risk factor for several human diseases. Conversely, smoking also reduces the prevalence of Parkinson's disease, whose hallmark is degeneration of *substantia nigra* dopaminergic neurons (DNs). We use *C. elegans* as a model to investigate whether tobacco-derived nicotine activates nicotinic acetylcholine receptors (nAChRs) to selectively protect DNs. Using this model, we demonstrate conserved functions of DN-expressed nAChRs. We find that DOP-2, a D3-receptor homolog; MCU-1, a mitochondrial calcium uniporter; PINK-1 (PTEN-induced kinase 1); and PDR-1 (Parkin) are required for nicotine-mediated protection of DNs. Together, our results support involvement of a calcium-modulated, mitochondrial stress-activated PINK1/Parkin-dependent pathway in nicotine-induced neuroprotection. This suggests that nicotine-selective protection of *substantia nigra* DNs is due to the confluence of two factors: first, their unique vulnerability to mitochondrial stress, which is mitigated by increased mitochondrial quality control due to PINK1 activation, and second, their specific expression of D3-receptors.

INTRODUCTION

Epidemiological studies show that tobacco smoking reduces the prevalence of Parkinson's disease (PD) (Li et al., 2015). A hallmark of PD is degeneration of *substantia nigra* dopaminergic neurons (DNs). Effects of tobacco smoking on degeneration of these neurons may depend on tobacco-derived nicotine and on nicotinic acetylcholine receptors (nAChRs). Support for this hypothesis comes from several lines of evidence. These include (1) expression studies showing that *substantia nigra* DNs express multiple nAChR subtypes having high affinity for nicotine and thus likely to be affected by the low nicotine concentrations present in the blood of tobacco smokers (Jones et al., 2001; Zoli et al., 2002); (2) analysis of PD models showing protective effects of nicotine on *substantia nigra* DNs (Huang et al., 2009; Liu et al., 2012; Quik et al., 2007); (3) genetic studies implicating RIC3, a chaperone of nAChRs, in PD (Sudhaman et al., 2016); and (4) a recent study that identified *CHRNA6* (encoding a neuronal nAChR subunit selectively affecting DN activity [Quik et al., 2011]) as a genetic modifier of infantile parkinsonism (Martinelli et al., 2020).

Several nAChR-dependent processes may explain the neuroprotective effects of nicotine. Nicotine acting through nAChRs was shown to activate protective signaling pathways within neurons (Dineley et al., 2001; Stevens et al., 2003; Toborek et al., 2007; Yu et al., 2011). Nicotine-mediated neuroprotection may also be a by-product of altered neuronal activity following chronic nicotine exposure, as reported for smokers (Dani, 2015). Additionally, nicotine may protect neurons indirectly through established neuroinflammation-reducing effects resulting from activation of astrocyte-expressed α7 nAChRs (Jurado-Coronel et al., 2016). However, despite such neuroprotective effects, smoking is in fact a known risk factor for cancer, stroke, heart disease, and other prevalent human disorders, including neurodegenerative diseases such as multiple sclerosis (MS) and Alzheimer's disease. Mechanisms suggested to reduce prevalence of PD afforded by tobacco smoking should, therefore, also provide plausible explanations for selectivity of these effects (Chang et al., 2014; Jiang et al., 2020; Li et al., 2015; Otuyama et al., 2019; Rosso and Chitnis, 2019). To date, mechanisms conferring the selective protection to *substantia nigra* DNs, afforded by tobacco smoking, remain undefined.

The nematode *Caenorhabditis elegans* provides a simple and well-characterized model for the study of nicotine-mediated protection of DNs. The well-described nervous system of *C. elegans* includes eight well-characterized DNs (Sawin et al., 2000), which provide an advantageous model for the analysis of

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PD-modifying mechanisms. Exposure to 6-OHDA or MPTP (toxins specifically affecting DNs) or overexpression of human α-synuclein (α-syn; a cause of PD) have similar degeneration-causing effects on *C. elegans* and human DNs (Lakso et al., 2003; Nass et al., 2002, 2005). Research using this model has led to the identification and characterization of conserved genes, pathways, and small molecules that function to modulate neurodegeneration (Gaeta et al., 2019).

Here, we have undertaken a systematic functional analysis of nAChRs in *C. elegans* DNs. We show that nAChRs alter a dopamine-dependent behavioral response, basal slowing response (BSR), via DN-expressed nAChRs. We also describe nicotine-dependent protection against 6-OHDA toxicity by DN-expressed nAChRs. These results establish *C. elegans* as a conserved model to study the functional effects of nAChRs on DNs, *in vivo*. Furthermore, we demonstrate the mechanistic utility of this nematode model by identifying nicotine-activated neuroprotective signaling pathways functioning in DNs. Results of this analysis suggest involvement of the mitochondrial stress response, mitochondrial calcium accumulation, PINK-1, and PDR-1 (whose human homologs have been implicated in PD) in nicotine-mediated protection of DNs (Mouton-Liger et al., 2017). The extensive axons, dense synapses, and high basal activity of *substantia nigra* DNs were suggested to increase the metabolic load on these neurons, leading to their increased vulnerability to perturbations in mitochondrial function (Ge et al., 2020). Our findings, revealing that nicotine-induced protection is dependent on PINK-1 and PDR-1, both known to promote mitophagy, may explain selective protection afforded by tobacco smoking to these neurons. Moreover, we show that *C. elegans* DOP-2, a D3-receptor (D3R) homolog, is required for nicotine-mediated protection. D3Rs are selectively expressed in nigrostriatal DNs where they function as auto-receptors (Joyce and Millan, 2007; Yang et al., 2020). The involvement of D3Rs in nicotine-mediated protection may further explain the selective protection afforded to DNs by tobacco smoking, as a source of chronic nicotine exposure.

RESULTS

RIC-3 and nAChRs function within DNs of *C. elegans* to enhance dopaminergic signaling

C. elegans is a simple genetic model, with a well-characterized nervous system that can be used to study various cellular phenomena, such as functions of nAChRs in DNs. To determine if nAChRs possess a conserved function in *C. elegans* DNs, as shown for mammalian nAChRs, we examined their effects on the BSR (reduced locomotion on food), a behavior dependent on dopamine release from DNs (Sawin et al., 2000). RIC-3, a chaperone of nAChRs, is required for maturation of all known nAChRs in *C. elegans* (Halevi et al., 2002; Jospin et al., 2009). Thus, a loss-of-function mutation in *ric-3* (*ric-3(lf)*) can be used as proxy for effects of loss-of-nAChR function on DN-dependent behaviors. BSR analysis of *ric-3(lf)* animals shows a significant reduction in basal slowing (Figures 1A and 1B). Rescue experiments in which *ric-3* was expressed selectively in DNs restored basal slowing for *ric-3(lf)* animals (Figures 1C and 1D). Both *ric-3(lf)* and DN-specific expression of *ric-3* specifically affect locomotion on food but not off food, a result consistent with RIC-3 being required within DNs to increase dopaminergic signaling in response to the presence of food. These results suggest that nAChRs of *C. elegans*, like mammalian nAChRs, affect DN function.

In *C. elegans,* RIC-3 functions to enhance the maturation of nAChRs, but not of other ligand-gated ion channels that were examined (Halevi et al., 2002). Thus, effects of RIC-3 on dopaminergic signaling seen in Figure 1 are likely to be mediated by nAChRs expressed in DNs that require RIC-3 for their functional expression. Furthermore, transcriptomic analysis suggests that 17 nAChR subunits are expressed in *C. elegans* DNs (Cao et al., 2017; Spencer et al., 2011). To examine whether the effects of RIC-3 on dopaminergic signaling are dependent on nAChR activity in DNs, we assayed loss-of-function mutants for various nAChR subunits using the BSR assay (Table S1). Results of this analysis revealed that 4 of 13 subunits examined (ligand-binding α subunits, UNC-38 and UNC-63, and non-α subunits ACR-2 and LEV-1) are required for proper BSR (Figure 2).

All four subunit mutants affecting BSR were also examined for their sensitivity to exogenous dopamine. In *C. elegans*, the application of exogenous dopamine causes paralysis of wild-type animals (Chase et al., 2004). None of the mutations examined significantly reduced the paralyzing effects of exogenous dopamine (Figure 2). In fact, *lev-1(lf)* even showed enhanced responsiveness to exogenous dopamine (Figure 2H). Considering these data, reduced BSR in these mutants is likely to be a result of decreased dopamine release from DNs and not of reduced responsiveness to dopamine. This together with the results on RIC-3 functioning in DNs to affect BSR strongly support a conserved role for nAChRs in modulating DN activity and dopamine release from these neurons.

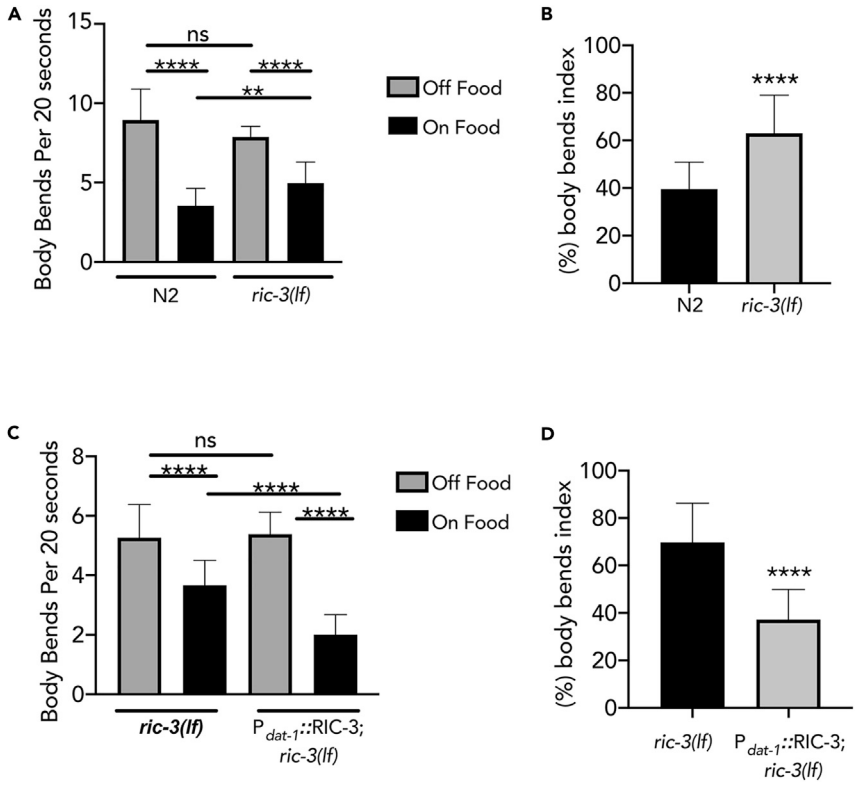

Figure 1. RIC-3 functions in DNs to enhance basal slowing response

(A and B) *ric-3(lf)* exhibit reduced BSR when compared with wild-type (N2) animals. N = 2, n = 20 animals on and off food.

(C and D) Selective expression of RIC-3 in DNs enhances BSR when compared with *ric-3(lf)* animals. N = 2, n = 13–15 animals on and off food.

(A and C) Locomotion speed was measured by number of body bends per 20 s on food (black bars) or off food (gray bars).

(B and D) BSR index (% body bends per 20 s on food per animal relative to average body bends per 20 s off food in the same strain and experiment) for data in (A or C).

Data presented as average ± SD; significance was examined using, one-way ANOVA with Tukey's post hoc multiple comparisons (A and C) or two-tailed unpaired Student's t test (B and D); ns, $p > 0.05$; **$p = 0.0063$, ****$p < 0.0001$.

Nicotine activation of nAChRs protects *C. elegans* DNs from 6-OHDA toxicity

Nicotine was shown to protect mammalian DNs from 6-OHDA, a DN-specific neurotoxin, in a nAChR-dependent manner (Ryan et al., 2001). To determine if the neuroprotective effects of nicotine on DNs are conserved in *C. elegans*, we treated animals chronically for 96 h with nicotine and examined the effects of this treatment on 6-OHDA-induced neurodegeneration (Figure 3A). The nicotine concentration selected (62 μM) was previously shown to significantly affect gene expression but not fertility or viability of *C. elegans* (Polli et al., 2015; Smith et al., 2013). Our results demonstrate that animals treated only with nicotine have no change in DN morphology (Figures 3B and 3D, relative to Figure 3C), signifying that nicotine has no deleterious effect at the tested concentration. 6-OHDA-only treatments (Figures 3B and 3E, relative to Figure 3C) caused significant neurodegeneration, whereas chronic exposure to nicotine provided robust neuroprotection from 6-OHDA (Figures 3B and 3F). These data demonstrate that nicotine significantly protects DNs from the selective neurodegeneration induced by 6-OHDA.

We explored additional nicotine exposure paradigms, including a 1-h acute exposure that was co-administered with 6-OHDA, and a 1-h pre-exposure to nicotine, which was administered 24 h before the 6-OHDA treatment and that was combined with acute exposure (Figure 4A). As shown in Figures 4B and 4C, acute nicotine treatment did not provide significant protection to DNs, whereas adding pre-exposure to acute exposure was protective, albeit at a significance level lower than that observed with chronic nicotine exposure ($p = 0.0178$ versus $p = 0.0002$, respectively) (Figure 4C versus 3B). Therefore, all subsequent experiments utilized chronic treatment with nicotine to maximize its protective effects.

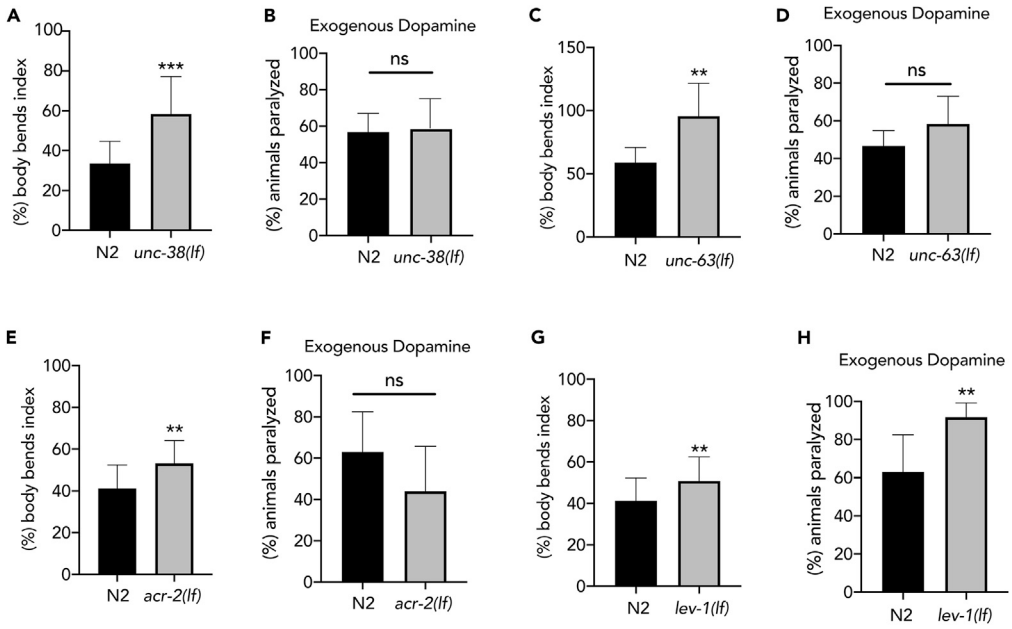

Figure 2. Mutations in nAChR subunits affecting basal slowing

(A–H) Loss-of-function mutations in *unc-38* (A), *unc-63* (C), *acr-2* (E), and *lev-1* (G) lead to a reduced slowing on food, increased BSR index (% body bends per 20 s on food per animal relative to average body bends per 20 s off food in the same experiment and strain) when compared with wild-type (N2) animals. Owing to day-to-day variability in the BSR index control N2 animals are examined on the same experiment as the subunit mutant (N2, N = 2, n = 9–30 each and nAChR subunit mutants, N = 2, n = 9–15 each). The same mutations in *unc-38* (B), *unc-63* (D), *acr-2* (F), and *lev-1* (H) did not reduce sensitivity to the paralyzing effects of exogenous dopamine. Results depicted as % animals paralyzed after 20 min in the presence of exogenous dopamine, 6–10 plates each, 10 animals per plate. Data represented as average ± SD; significance was examined using two-tailed unpaired Student's t test; ns, $p > 0.05$; **$p < 0.0097$, ***$p = 0.0004$. See also Table S1 and Figure S2.

Notably, the two paradigms of nicotine-induced neuroprotection in *C. elegans* (chronic and pre-exposure) have also been observed to be neuroprotective in mammalian experimental models (Huang et al., 2009; Janson et al., 1992). The long-lasting (24 h) effects of nicotine pre-exposure (Figure 4C relative to 4B) are consistent with nicotine-dependent gene expression changes enabling or enhancing nicotine-induced protection of DNs. The concentrations of nicotine used in our study were previously shown to affect the expression of multiple genes (Polli et al., 2015; Smith et al., 2013). Moreover, these effects raised the possibility that chronic nicotine exposure may protect the DNs against 6-OHDA exposure by reducing expression of *dat-1*, encoding the transporter responsible for 6-OHDA influx into DNs (Nass et al., 2005). Using quantitative RT-PCR, our results show that *dat-1* expression levels are not significantly altered following chronic nicotine exposure, and thus are unlikely to explain the nicotine-induced protection from 6-OHDA (Figure S1).

To determine if the expression of nAChRs in DNs is required for nicotine-induced neuroprotection in *C. elegans*, we knocked down *ric-3* selectively in DNs. RNAi silencing in DNs was accomplished by the RNAi feeding method using a *sid-1(lf)* mutant strain that only expresses SID-1, a dsRNA transporter, in DNs (Harrington et al., 2012). As depicted in Figure 3G, DN-specific knockdown of *ric-3* in animals not treated with nicotine does not affect 6-OHDA-induced neurodegeneration when compared with the empty vector (EV) RNAi control. However, *ric-3* silencing does abolish the neuroprotective effects of nicotine on animals exposed to 6-OHDA when compared with the nicotine-treated EV control (Figure 3G). Silencing of *ric-3* in DNs not treated with 6-OHDA did not impact DN morphology (not shown). Elimination of nicotine-induced neuroprotection following selective *ric-3* silencing in DNs implies a vital role for DN-expressed nAChRs to convey the protective effects of nicotine.

To further investigate whether nAChRs are required for protective effects of nicotine on DNs against 6-OHDA toxicity, we individually tested seven nAChR subunits. These subunits were examined by either DN-specific RNAi knockdown or loss-of-function mutant alleles. The seven subunits examined included

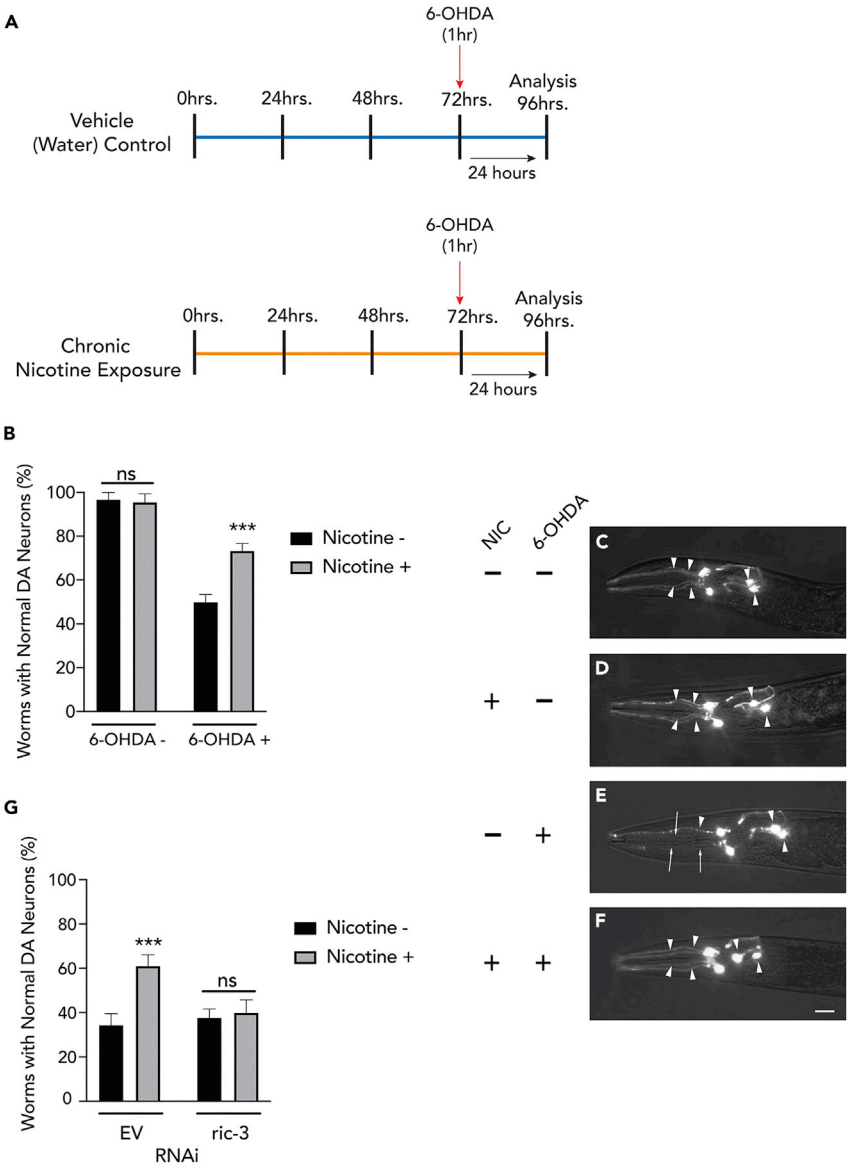

Figure 3. Chronic nicotine treatment protects DNs in a RIC-3-dependent manner

(A) Timeline of experiments. (A, top) Control experiments without nicotine exposure. (A, bottom) Animals in nicotine group exposed to 62 μM nicotine from hatching and during and after 6-OHDA exposure. Degeneration of DNs was scored 24 h after 10 mM 6-OHDA/2 mM ascorbic acid exposure or 2 mM ascorbic acid alone.

(B) Chronic nicotine treatment attenuates 6-OHDA-induced DN degeneration in BY250 worms expressing only GFP in their DNs [$P_{dat\text{-}1}$::GFP (*vtIs7*)].

(C–F) Representative images matching the conditions of (B). (C) Solvent (ddH_2O/2 mM ascorbic acid) control with all six anterior DNs intact (arrowheads). (D) Nicotine-only treatment with all six anterior DNs intact (arrowheads). (E) 6-OHDA-induced DN degeneration, with three degenerated (arrows) and three intact (arrowheads) DNs depicted. (F) Nicotine treatment of 6-OHDA-treated animals with all six anterior DNs intact and protected from 6-OHDA toxicity (arrowheads). Scale bar, 10 μM.

(G) DN-specific RNAi knockdown of *ric-3* (using UA202 worms) abolishes nicotine-induced protection against 6-OHDA toxicity compared with empty vector (EV) control.

Data are represented as average ± SD (N = 3, n = 30 each). Significance was examined using two-way ANOVA with Tukey's post hoc multiple comparison test (A, F); ns, $p > 0.05$; ***$p < 0.0008$.

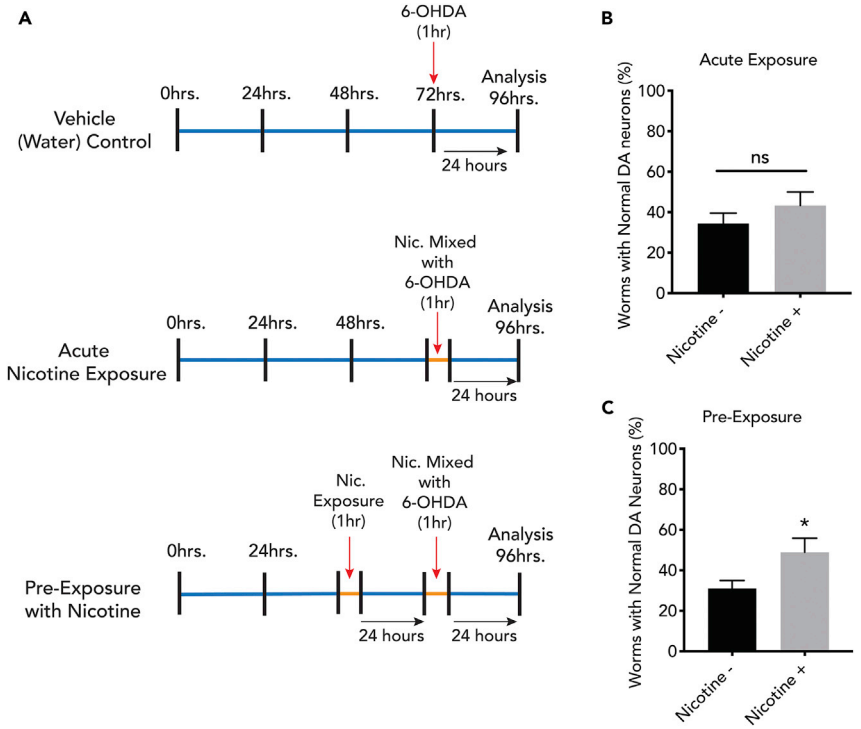

Figure 4. Pre-exposure to nicotine protects DNs from 6-OHDA

(A) Timeline depicting protocols of nicotine treatments. (A, top) Control protocol. (A, middle) Acute exposure: addition of 62 μM to the assay mix only during 6-OHDA treatment. (A, bottom) Pre-exposure: 1-h pre-exposure to 62 μM nicotine the day before 6-OHDA + acute exposure to 62 μM nicotine during the 6-OHDA treatment. Examined in animals expressing GFP only in DNs.

(B) Acute nicotine is not neuroprotective.

(C) A nicotine pre-exposure + acute exposure is protective against 6-OHDA toxicity.

Data represented as average ± SD (N = 3, n = 30 worms each). Significance was examined using two-tailed unpaired Student's t test; ns, $p > 0.05$; *$p = 0.0178$. See also Figure S1.

the four subunits shown to be needed for proper BSR—subunits likely to function in DNs to affect dopamine release—and three additional subunits shown to be expressed in DNs through transcriptomics data (Table S1 (Cao et al., 2017; Spencer et al., 2011). This approach identified four subunits (α subunits ACR-12 and ACR-20, and non-α subunits LEV-1 and UNC-29) that are necessary for nicotine-induced neuroprotection (Figure 5). Notably, *lev-1(lf)* was the only mutant found to impact both dopamine signaling (Figure 2G) and nicotine-induced DN protection (Figure 5A).

Together, these results demonstrate that, similar to mammals, nicotine elicits a neuroprotective effect on *C. elegans* that is dependent on nAChR expression in DNs.

Collectively, our results suggest that *C. elegans* DNs express at least two distinct nAChRs: (1) one affecting DN activity and dopaminergic signaling and (2) the other mediating the protective effects of nicotine. Evidence for this hypothesis comes from the following considerations: nAChRs are pentamers (heteromers or homomers); however, we identified seven subunits involved in either dopamine signaling or neuroprotection. Moreover, only one subunit, LEV-1, was shown to affect both DN activity and DN protection; ACR-2, UNC-38, and UNC-63 only affect BSR, whereas ACR-12 and UNC-29 only affect nicotine-mediated protection of DNs. No viable mutant is available for *acr-20*, thus this subunit was only examined for its effects on nicotine-induced protection of DNs using DN-selective RNAi silencing. Interestingly, this subunit was shown to function as a homomeric receptor and thus may form a third DN-expressed nAChR (Baur et al., 2015).

Notably, the nicotine concentration used here was shown to affect expression of multiple nAChR subunits. Specifically, chronic exposure to 62 μM nicotine was shown to increase expression of three of the four

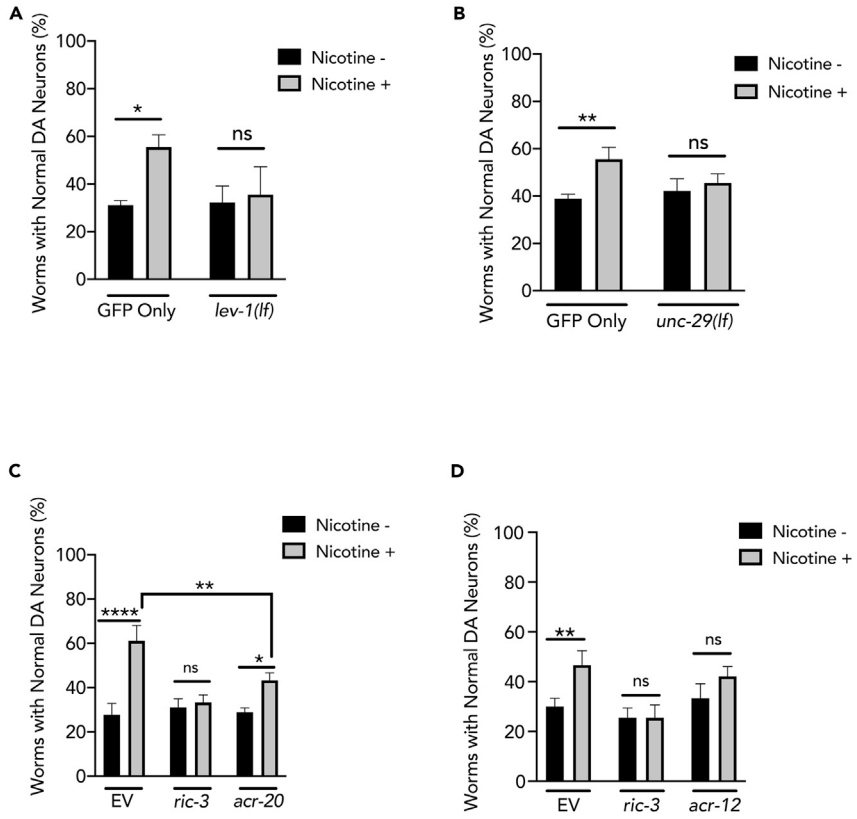

Figure 5. nAChR subunits needed for the protective effects of chronic nicotine

(A–D) Loss-of-function mutations in (A) *lev-1* and (B) *unc-29*, and DN-specific knockdown (using UA202 worms) of (C) *acr-20* or (D) *acr-12* eliminated or significantly reduced (*acr-20*) the protective effects of chronic nicotine exposure. Animals were grown in the presence (gray bars) or absence (black bars) of 62 μM nicotine and exposed to 10 mM 6-OHDA/2 mM ascorbic acid (N = 3, n = 30 each).

Data are represented as average ± SD. Significance was examined using two-way ANOVA with Tukey's post hoc multiple comparison test; ns, $p > 0.05$; *$p < 0.0158$, **$p < 0.0098$, ****$p < 0.0001$. See also Table S1 and Figure S2.

protective subunits (LEV-1, UNC-29, and ACR-20; Figure 5) and to reduce expression of two of the three subunits affecting DN activity but not DN neuroprotection (ACR-2 and UNC-63; Figure 2) (Polli et al., 2015; Smith et al., 2013). In accordance with findings showing that chronic nicotine exposure reduces expression of nAChR subunits needed for enhanced dopaminergic signaling, this treatment also reduced the BSR (Figure S2).

Conserved mechanisms enable the protective effects of nicotine

Studies involving mammalian cell cultures have identified several genes and pathways mediating nicotine-induced neuroprotection. One such study showed that the neuroprotective effects of nicotine involve increased cytosolic calcium, voltage-gated calcium channels, and the calcium- and calmodulin-dependent phosphatase, calcineurin (Stevens et al., 2003). To determine whether these mechanisms are conserved, we examined the effects of nicotine-induced neuroprotection following DN-specific knockdown of *egl-19*, encoding a voltage-gated calcium channel, and of *tax-6*, encoding the *C. elegans* homolog of calcineurin. These experiments were performed using a strain enabling selective RNAi in DNs (Harrington et al., 2012). Animals were raised on RNAi bacteria for two generations (P0 and F1), with only the F1 generation receiving chronic treatment of nicotine (Figure 6A). In this and the following RNAi experiments, *ric-3* RNAi was used as an internal control. Previous studies demonstrated that the individual knockdown of *egl-19* or *tax-6* caused DN protection from neurodegeneration induced by α-synuclein, a protein implicated in human PD, thus implying degeneration-enhancing activities of these two genes in DNs (Caraveo et al., 2014). Similarly, when *egl-19* or *tax-6* are knocked down selectively in DNs, this results in reduced 6-OHDA-dependent neurodegeneration, even without nicotine treatment (Figures 6B and 6C). These

data support a role for these two genes in enhancing degeneration of DNs. This protective effect is not additive when combined with nicotine, suggesting that voltage-gate calcium channels (EGL-19; Figure 6B) and calcineurin (TAX-6; Figure 6C) function downstream of nAChR activation. However, lack of additivity is insufficient evidence to definitively implicate this pathway, as we cannot rule out a ceiling effect masking additivity.

Effects of knocking down *egl-19* on DN survival suggest a role for cytosolic calcium dysregulation in vulnerability of these neurons to 6-OHDA and in nicotine-induced protection. The endoplasmic reticulum-resident calcium pump (SERCA) is an important regulator of cytosolic calcium; *sca-1* is the only *C. elegans* ortholog of this gene (Cho et al., 2000). The inhibition of this pump has been shown to protect DNs against α-synuclein-dependent neurodegeneration (Betzer et al., 2018). However, knockdown of *sca-1* in DNs does not protect against 6-OHDA-induced toxicity either in the presence or in the absence of nicotine (Figure 6D). Thus, SERCA activity does not affect DN survival following 6-OHDA exposure and is not needed for nicotine-induced protection of these neurons.

Another signaling protein shown to enable nicotine-induced protection of mammalian DNs is the dopamine D3 receptor, D3R (Bono et al., 2019), an effect requiring its interaction with the β2 nAChR subunit (Bontempi et al., 2017). The *C. elegans* dopamine receptor, DOP-2, shares many similarities with D3R including being expressed in DNs, functioning via G_i /G_o to inhibit adenylyl cyclase, and acting as an auto-receptor to inhibit dopamine release (Formisano et al., 2020; Suo et al., 2003). To examine whether DOP-2 functions as part of the protective signaling pathway activated by nicotine, we knocked down *dop-2* specifically in DNs. We found that, like *ric-3*, *dop-2* knockdown does not enhance neurodegeneration, but its expression in DNs is required for nicotine-mediated neuroprotection (Figure 6E). This result suggests conservation of the interaction between nAChRs and the D3R/DOP-2 in nicotine-induced protection.

Our results show that, in *C. elegans*, DOP-2 function is required for nicotine-induced protection of DNs, a result consistent with studies involving human D3R signaling in DNs (Bontempi et al., 2017). Our data also support involvement of calcium-dependent signaling in nicotine-induced neuroprotection similarly to what was shown in mammals. Taken together, we demonstrate the conservation of mechanisms enabling nicotine-induced neuroprotection between *C. elegans* and mammals (Stevens et al., 2003).

Mitochondria and nicotine-induced neuroprotection

By stimulating nAChRs, nicotine was shown to affect mitochondrial function and induce a mitochondrial stress response (Gergalova et al., 2012; Lykhmus et al., 2014; Zahedi et al., 2019). To examine whether chronic nicotine exposure impacts mitochondrial stress in *C. elegans*, we used an established transcriptional reporter for mitochondrial stress, *hsp-6*::GFP (Yoneda et al., 2004). Our results depicted significantly higher *hsp-6*::GFP expression in nicotine-treated animals. These results show a systemic increase in the mitochondrial stress response resulting from chronic nicotine exposure on days 4 and 7 post-hatching, when compared with worms not exposed to nicotine (Figure 7A).

To further examine the effects of chronic nicotine exposure on the mitochondrial stress response, we quantified its impact on endogenous RNA transcript levels of *hsp-6* and *timm-23* (encoding an inner mitochondrial membrane transporter), both of which exhibit increased expression in response to mitochondrial stress (Tian et al., 2016). RNA was extracted from nicotine-treated and -untreated young adults (3 days after hatching) followed by quantitative RT-PCR. Results of this analysis revealed that chronic nicotine exposure in wild-type animals leads to increased expression of both *hsp-6* and *timm-23*, a result supporting chronic nicotine exposure-dependent activation of mitochondrial stress (Figure 7B). Using this assay, we examined the role of nAChRs and DOP-2 in mediating nicotine-induced activation of mitochondrial stress. Results of this analysis (Figures 7C and 7D) show that RIC-3, the conserved nAChR chaperone used as proxy for nAChRs, and DOP-2 are both needed for nicotine-dependent upregulation of these genes.

Surprisingly, in *ric-3(lf)* mutants chronic nicotine exposure downregulates *hsp-6* and *timm-23* expression (Figure 7C). This result suggests an nAChR-independent effect of nicotine on mitochondrial stress. Interestingly, nicotine was shown to directly bind and inhibit mitochondrial complex I (Malińska et al., 2019). Thus, nicotine appears to exert two opposite effects on mitochondria, only one of which is nAChR dependent. Importantly, in wild-type animals the nAChR-dependent activation of mitochondrial stress is higher

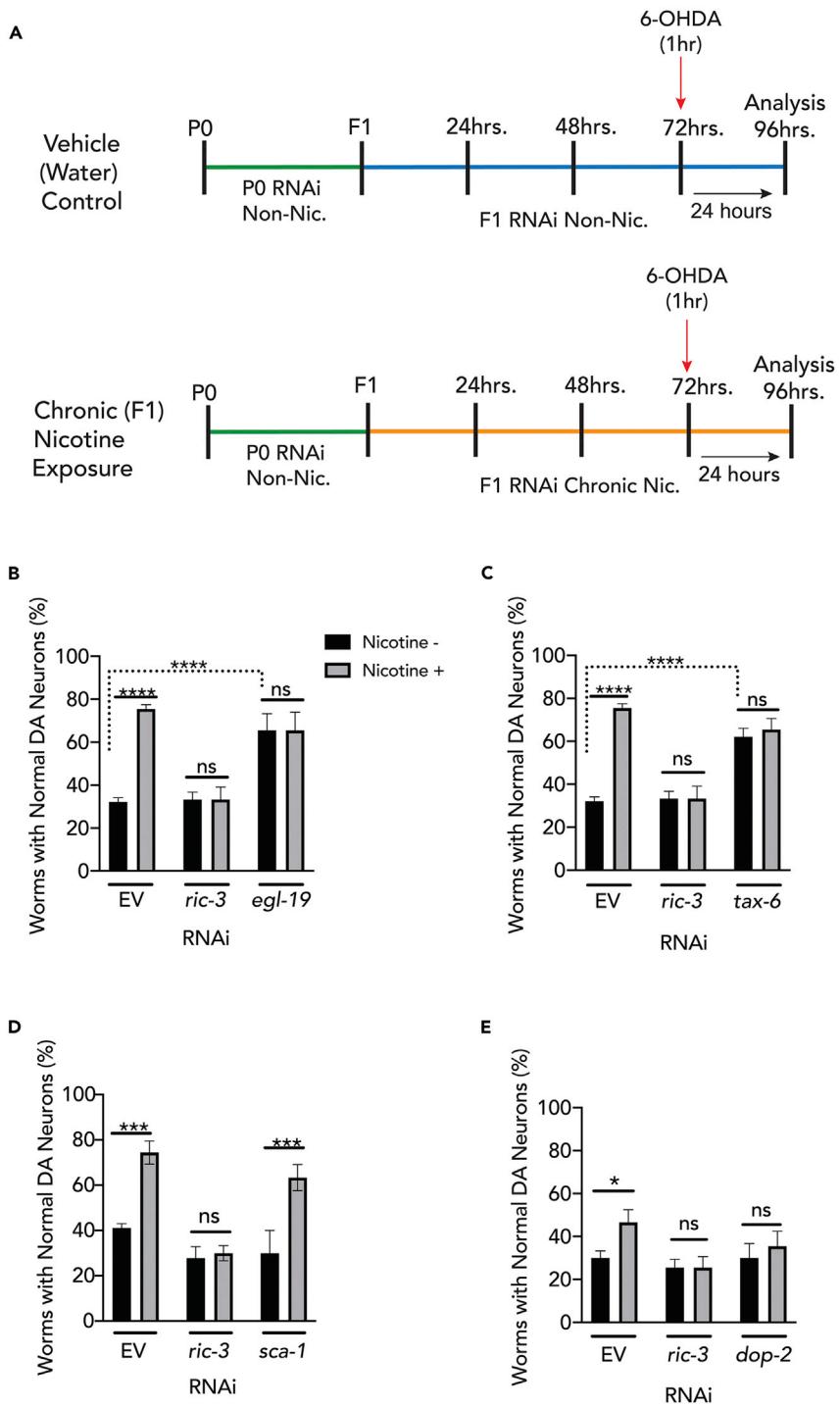

Figure 6. Conserved mechanisms mediate protective effects of nicotine

(A) Timeline depicting RNAi and chronic nicotine treatments. Animals were raised on RNAi bacteria for two generations (P0 and F1). Only the F1 generation was exposed to nicotine.

(B–E) RNAi experiments involving the knockdown of *ric-3*, *egl-19* (B), *tax-6* (C), *sca-1* (D), or *dop-2* (E) in the DN-specific RNAi-sensitive strain, UA202, when animals were grown in the presence (gray bars) or absence (black bars) of 62 μM nicotine and exposed to 10 mM 6-OHDA/2 mM ascorbic acid. (B) *egl-19* and (C) *tax-6* knockdown alone protected DNs from 6-OHDA toxicity and nicotine had no additional effects. (D) *sca-1* knockdown did not affect nicotine-induced

Figure 6. *Continued*
protection. (E) *dop-2* knockdown abolished nicotine-induced protection from 6-OHDA. For internal experimental consistency, 6-OHDA and nicotine controls (EV and *ric-3* RNAi-treated UA202 animals) were conducted concurrently. Data shown as average ± SD (N = 3, n = 30 animals each). Significance was examined using two-way ANOVA with Tukey's post hoc multiple comparison test; ns, $p > 0.05$; ****$p < 0.0001$, ***$p = 0.0001$, *$p = 0.0259$.

than the opposite nAChR-independent effect; thus, in wild-type animals chronic exposure to nicotine leads to a net increase in mitochondrial stress. The different effects seen in *dop-2(lf)* relative to *ric-3(lf)* mutants (Figures 7D versus 7C) suggest that DOP-2 facilitates nAChR-induced activation of mitochondrial stress in some but not all cells. This hypothesis is consistent with the limited expression of *dop-2* (a small subset of neurons) relative to the wide expression of *ric-3* (most neurons and muscles [Halevi et al., 2002]).

Our results demonstrate that the relationship between nicotine and mitochondrial stress response is conserved between *C. elegans* and mammals (Figures 7A and 7B). This relationship, however, has not been previously linked to nicotine-induced neuroprotection. To investigate whether pathways known to mediate responses to mitochondrial stress also modulate the neuroprotective effects of nicotine, we examined five genes (*mcu-1*, *micu-1*, *atfs-1*, *pink-1*, and *pdr-1*) shown to influence the mitochondrial stress response and to function in *C. elegans* DNs, as demonstrated by their effects on degeneration of dopaminergic neurons (Martinez et al., 2015, 2017).

MCU-1 is calcium uniporter involved in mitochondrial calcium import and homeostasis. MICU-1 is a calcium sensor responsible for coupling increased cytosolic calcium to increased activity of the MCU-1 (Pendin et al., 2014). The silencing of *mcu-1* in animals not exposed to nicotine did not enhance neurodegeneration when compared with the EV control, but *mcu-1* silencing neutralized the neuroprotective effects of nicotine against 6-OHDA, when compared with the nicotine-treated EV control (Figure 8A). Knockdown of *micu-1* did not enhance neurodegeneration of animals not treated with nicotine and did not reduce nicotine-mediated neuroprotection (Figure 8B). Thus, whereas MCU-1 is required for nicotine-induced protection of DNs, MICU-1 is not. This suggests that nicotine induces calcium influx into mitochondria through MCU-1 in a manner independent of MICU-1 function. Interestingly, MICU −/− knockout mice, which exhibit severe mitochondrial defects in calcium homeostasis and developmental abnormalities, show greatly improved mitochondrial calcium parameters as they age, suggestive of MCU1-associated functional remodeling in the absence of MICU1 (Liu et al., 2016). It is also clear that differential expression of uniporter regulatory proteins, like MICU1, may account for functional tuning of calcium uptake in a tissue-specific manner (Paillard et al., 2017). It is intriguing to speculate that, in a disease of aging like PD, the neuroprotective selectivity of DNs in response to nicotine may reflect a functional change in uniporter dynamics that can both respond to and influence mitochondrial stress.

To better discern the molecular mechanism enabling nicotine-induced protection and its association with mitochondrial stress, we examined the dependency of this response to nicotine on ATFS-1 activity. ATFS-1 is a transcription factor that shuttles to the nucleus as part of the mitochondrial unfolded protein response (UPR^{mt}) to activate protective gene expression (Nargund et al., 2012). Constitutive activation of ATFS-1 in DNs was previously shown to paradoxically enhance α-synuclein-dependent neurodegeneration, suggesting that chronic activation of ATFS-1 signaling may have deleterious effects (Martinez et al., 2017). Consistent with these earlier findings, we show that *atfs-1* knockdown in animals not treated with nicotine results in dopaminergic neuroprotection from 6-OHDA toxicity (Figure 8C). Interestingly, these effects are not additive to the neuroprotection conferred by nicotine. Instead, the combined effects of *atfs-1* knockdown and chronic nicotine exposure resulted in a significant decrease, but not a complete loss, of neuroprotection (Figure 8C).

The effects of ATFS-1 knockdown on nicotine-induced protection cannot be simply explained by genetic epistasis. To further explore the role of ATFS-1 in nicotine-induced protection we also examined the effects of chronic nicotine exposure on an *atfs-1(et15)* gain-of-function (*gf*) allele. Our results show that the constitutive activation of UPR^{mt} by *atfs-1(gf)* does not significantly affect 6-OHDA toxicity while eliminating nicotine-induced protection Figure 8D. Taken together with the outcomes of ATFS-1 depeletion by RNAi, these results suggest that the protective effects of chronic nicotine exposure are at least partly mediated through ATFS-1 and UPR^{mt}. However, the benefits of nicotine exposure are negated when UPR^{mt} is constitutively activated, a cellular state reflecting chronic and severe proteostatic imbalance resulting from dysregulated transcription of hundreds of mitochondrial-targeted proteins (Martinez et al., 2017; Nargund et al., 2012).

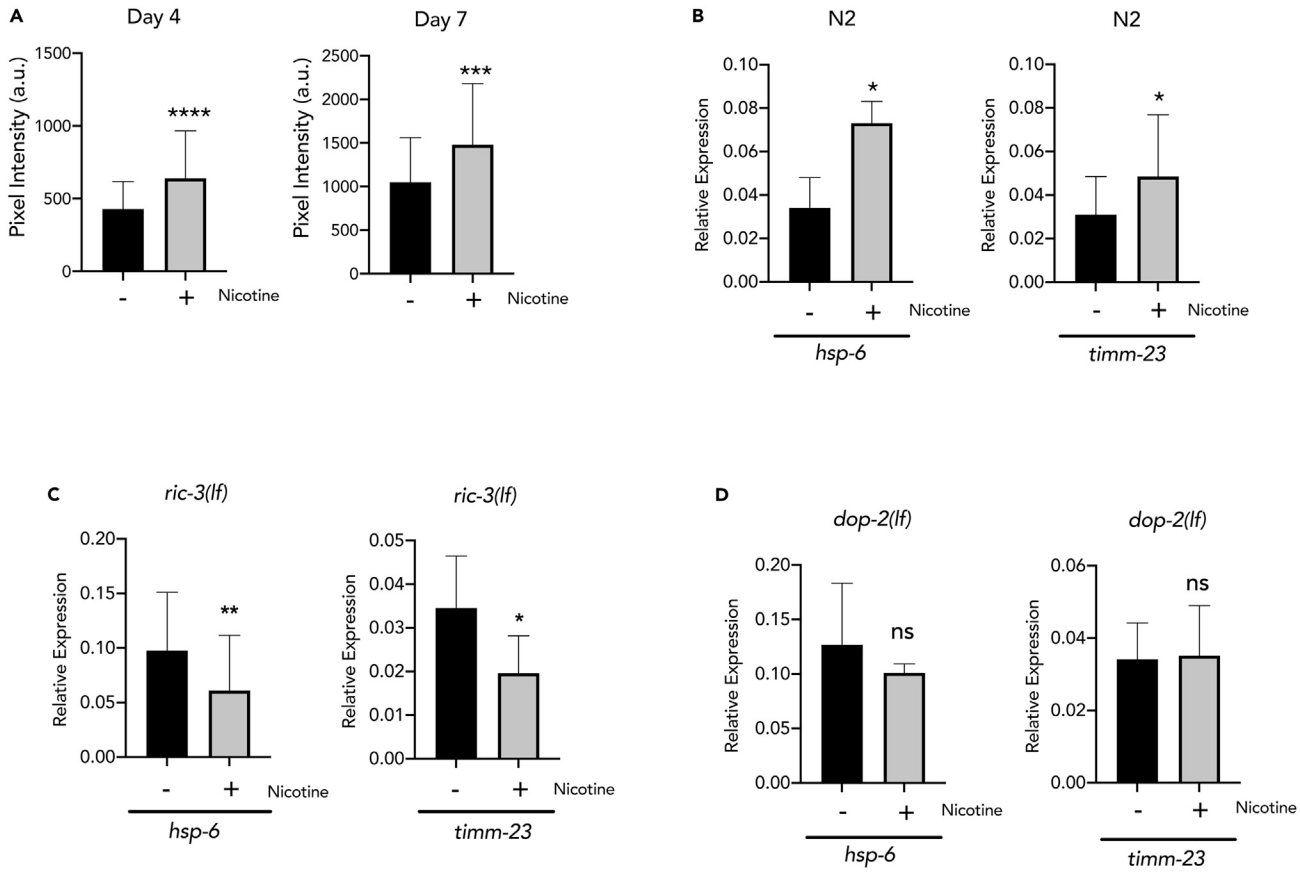

Figure 7. Chronic nicotine exposure induces mitochondrial stress

(A) A *hsp-6*::GFP reporter strain chronically treated with 62 μM nicotine showed increased mitochondrial stress (N = 2-3, n = 30 each).

(B) Effects of chronic nicotine on *timm-23* and *hsp-6* RNA levels in wild-type (N2), (C) *ric-3(lf)*, or (D) *dop-2(lf)* animals (N = 2, n = 4; two independent experiments, each analyzed twice and in triplicates).

Data shown as average ± SD, and significance was examined using two-tailed unpaired Student's t test (A) or paired Student's t test (B–D); ns, $p>0.05$; ****$p<0.0001$; ***$p=0.0002$; **$p=0.0013$; *$p<0.04915$.

Last, we examined the interaction of nicotine with PINK-1, a kinase that is stimulated by mitochondrial stress to activate mitophagy and additional protective processes mostly involving mitochondrial quality control (Ge et al., 2020; Mouton-Liger et al., 2017). RNAi silencing of *pink-1*, without nicotine, does not enhance the degeneration of dopaminergic neurons when compared with the EV control but it has been shown to do so in the presence of other neuronal stressors, such as α-synuclein overexpression in DNs (Martinez et al., 2015). Here, notably, *pink-1* silencing neutralizes the neuroprotective effects of nicotine against 6-OHDA toxicity when compared with the nicotine-treated EV control (Figure 8E). Thus, PINK-1 is likely to function downstream of nAChR activation in DNs to enable the neuroprotective effects of chronic nicotine exposure. PDR-1 functions downstream of PINK-1 to promote mitophagy (Mouton-Liger et al., 2017). Using *pdr-1(lf)* animals we show that this gene, like *pink-1*, is necessary for nicotine-induced protection (Figure 8F). Together our results describe a previously unreported mechanism of neuroprotection whereby nicotine modulates survival of DNs *in vivo* through activation of a PINK-1/PDR-1-dependent pathway, an established pathway of mitochondrial quality control where mutations in these genes have been directly implicated in familial PD.

DISCUSSION

Epidemiological studies show that tobacco smoking reduces the prevalence of PD by 40%–50% (Ma et al., 2017). This strikingly protective effect of smoking is surprising considering the multiple epidemiological studies indicating smoking to be a risk factor for many diseases, including neurodegenerative diseases such as MS and Alzheimer disease (Chang et al., 2014; Jiang et al., 2020; Rosso and Chitnis, 2019).

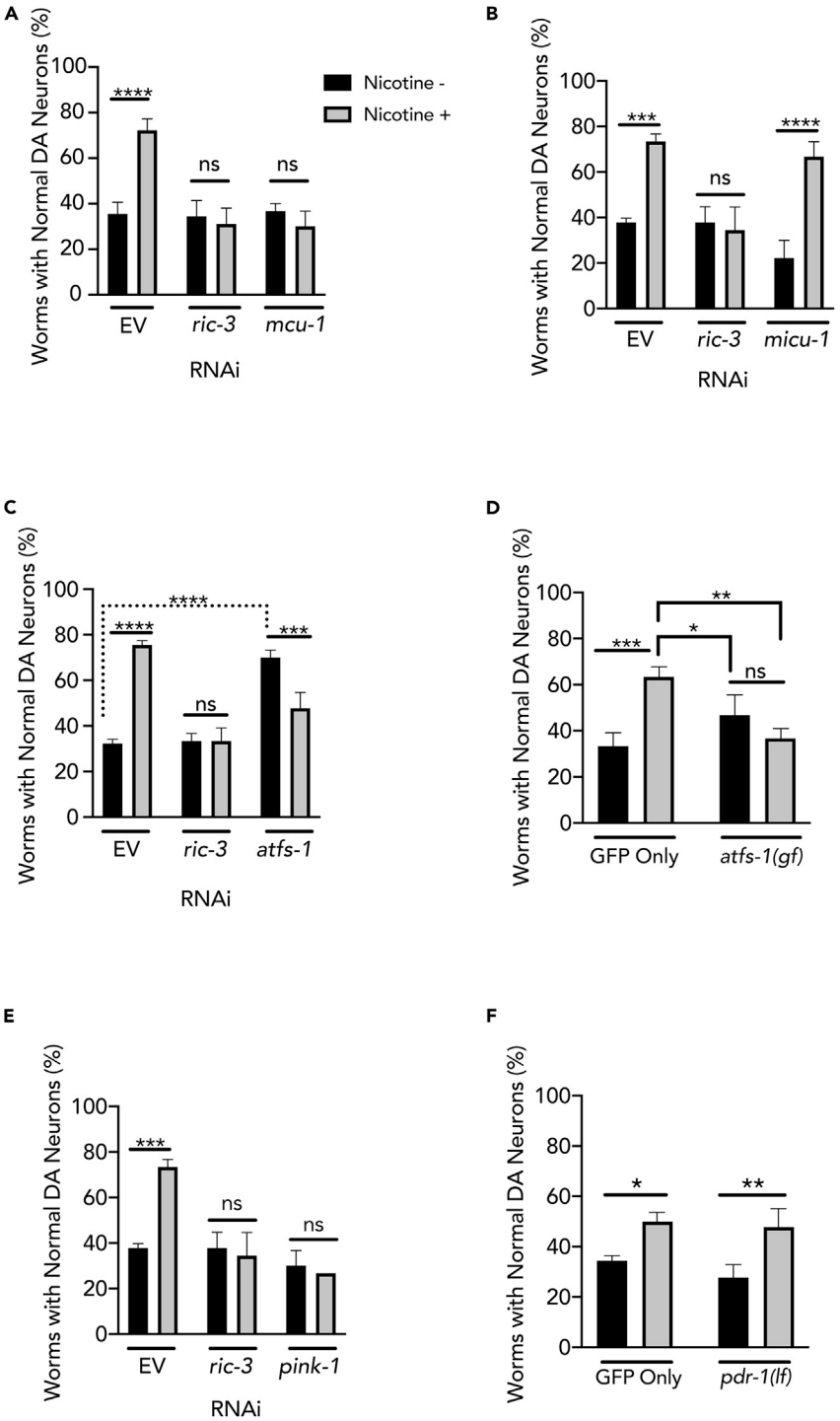

Figure 8. Mitochondria-associated genes and nicotine-induced protection

(A–D) RNAi knockdown of gene targets in the DNs of selectively RNAi-sensitive UA202 animals grown in the presence (gray bars) or absence (black bars) of 62 μM nicotine and exposed to 10 mM 6-OHDA/2 mM ascorbic acid (N = 3, n = 30 each). (A) *mcu-1* knockdown abolished nicotine-induced protection. (B) *micu-1* knockdown had no effect on nicotine-induced protection. (C) *atfs-1* knockdown alone protects against 6-OHDA toxicity, whereas the addition of nicotine exposure decreased its protective effects. (D) Nicotine-induced protection is lost in *atfs-1(gf)* animals (UA406). (E) *pink-1* knockdown abolishes nicotine-induced protection.

Figure 8. *Continued*

(F) *pdr-1(lf)* similarly eliminates nicotine-induced protection. As *pdr-1(lf)* is in a UA202 background, the "GFP Only" control for (E) is UA202 for consistency.

Data shown as average ± SD and significance was examined using two-way ANOVA with Tukey's post hoc multiple comparison test; ns, $p > 0.05$; *$p < 0.03$, **$p < 0.007$, ***$p < 0.0004$, ****$p < 0.0001$.

Protective mechanisms suggested to reduce the prevalence of PD in smokers should, therefore, explain the selective protection of *substantia nigra* DNs documented among smokers. In addition, such mechanisms should further our understanding of molecular factors that impact the survival of DNs as a selectively vulnerable neuronal subclass in PD. In this study, we identify molecular mechanisms enabling the effects of tobacco-derived nicotine on DNs, mechanisms that may explain the selective protection of *substantia nigra* DNs afforded by this activity.

Neuroprotective effects of tobacco-derived nicotine and nAChRs was suggested as an explanation for the reduced prevalence of PD in smokers. Indeed, previous work has shown that nicotine and nAChRs protect neurons from various insults (Picciotto and Zoli, 2008). Here, we demonstrated that nicotine-induced protection of *C. elegans* DNs is dependent on DN-expressed nAChRs, thus revealing the conservation of nicotine-induced neuroprotection between mammals and *C. elegans*. Our results show that *C. elegans* nAChRs are needed both for DN activity, and thus for dopaminergic signaling, and for nicotine-induced neuroprotection of DNs. We identified seven nAChR subunits that are needed for either DN activity or nicotine-induced neuroprotection against 6-OHDA toxicity. Only one of these subunits affects both nAChR-dependent activities. Thus, our results suggest distinct DN-expressed nAChRs affecting DN activity or DN survival.

One explanation for the protective effects of chronic nicotine exposure is reduced DN activity leading to reduced excitotoxicity (Choi, 1992). Chronic exposure to the same nicotine concentration used in our study has been shown to reduce the expression of two nAChR subunits, ACR-2 and UNC-63 (Polli et al., 2015), which are suggested by our results to function within DNs to promote dopaminergic signaling. Moreover, we show that chronic nicotine exposure reduced the BSR consistent with reduced dopaminergic signaling. However, our results also show that the knockout or knockdown of *acr-2* or *unc-63,* respectively, does not protect the DNs against 6-OHDA-induced toxicity. Thus, we conclude that reduced dopaminergic signaling is not sufficient for DN protection.

Prior studies suggested that interactions between nAChRs and D3Rs are required for nicotine-mediated protection of human DNs (Bono et al., 2019). D3Rs are selectively expressed in DNs, and agonists for D3Rs were shown to protect and even restore neuronal functions disrupted in PD (Joyce and Millan, 2007). Here, we show that nicotine-mediated protection following knockdown of *dop-2*, a *C. elegans* D3R homolog (Formisano et al., 2020), is completely abolished. In addition, a *dop-2*(*lf*) mutation reduced chronic nicotine-induced and nAChR-dependent activation of mitochondrial stress. Thus, we suggest that nicotine activates a protective nAChR- and D3R-dependent signaling pathway to selectively protect DNs. These results are consistent with previously published results showing that in mammalian cells nAChRs physically interact with D3Rs to enable nicotine-dependent activation of D3R signaling (Bontempi et al., 2017). This interaction presents a plausible explanation for DN-selective nicotine-mediated protection.

Together, our results establish *C. elegans* DNs as a conserved model enabling investigations of nicotine-induced neuroprotective pathways. Mammalian studies show that nicotine, through nAChR-activation, affects several signaling pathways. These pathways include calcium signaling and the mitochondrial stress response. In the present study, we examined the role of these pathways in nicotine-induced protection of DNs using a *C. elegans* model.

Based on our results with *egl-19* and *tax-6* knockdown, we suggest that voltage-gated calcium channels (EGL-19) and the calcium/calmodulin-dependent phosphatase (TAX-6), calcineurin, function downstream of nAChR activation. Studies in mammalian cell cultures suggested two distinct functions for voltage-gated calcium channels in nicotine- and nAChR-induced neuroprotection. First, they are activated by nicotine-induced and nAChR-dependent depolarization to increase cytosolic calcium. Second, they are the target of calcineurin, which is activated by increased cytosolic calcium to dephosphorylate and inactivate them,

thus reducing toxic calcium influx into neurons (Stevens et al., 2003). Future work should examine whether this second mechanism is conserved in *C. elegans*. We note that knockdown of both TAX-6 and EGL-19 protects against 6-OHDA toxicity without nicotine exposure. Furthermore, previous work showed that knockdown of these genes also protects against neurodegeneration induced by α-synuclein (Caraveo et al., 2014). Thus, these two proteins contribute both to 6-OHDA and to α-synuclein-induced neurodegeneration. Of special interest is calcineurin whose complex and tunable effects on neurodegeneration of DNs have been documented in *C. elegans* (Caraveo et al., 2014). Therefore, nicotine-induced regulation of calcineurin activity may reflect an evolutionarily conserved mechanism that can be targeted to reduce neurodegeneration.

Additional analysis supports the involvement of the calcium uniporter (MCU-1), enabling calcium influx into mitochondria, in nicotine-mediated protection. We show that nicotine activates mitochondrial stress in *C. elegans* and that the neuroprotective effects of nicotine require PINK-1, a protein known to be activated by mitochondrial membrane depolarization, and PDR-1 (Parkin), a cytosolic E3 ubiquitin ligase that ubiquitinates mitochondrial proteins. The PINK1/PDR-1 pathway is an established mechanism for activating mitophagy to remove damaged mitochondria (Mouton-Liger et al., 2017; Rolland et al., 2019).

Mitochondrial stress, membrane depolarization, and accumulation of unfolded proteins activate two distinct processes: (1) ATFS-1-dependent upregulation of gene expression promoting enhanced mitochondrial repair; two known ATFS-1 targets are HSP-6 and TIMM-23, whose expression is upregulated by nicotine; (2) PINK1/Parkin-dependent mitochondrial quality control, best known for elimination of damaged mitochondria via mitophagy. Our results suggest that nicotine-induced neuroprotection is a consequence of both these processes. In the context of previously reported evidence of the role of these genes in modulating neurodegeneration, it is clear that the response to mitochondrial damage is likely to require a delicate balance between repair and elimination of damaged mitochondria. We speculate that the complex interactions between mitochondria and nicotine-induced neuroprotection, exemplified by its effects following reduction or chronic upregulation of ATFS-1-mediated transcription, reflect this fine balance. Moreover, this type of response duality is an established factor in mitochondrial dynamics; mitohormesis, defined as the beneficial effect of transient induction of a cytoprotective response to low-level exposure or production of typically toxic stressors (i.e., reactive oxygen species), precisely mirrors the nicotine-evoked outcomes (Gohel and Singh, 2021).

Involvement of protective mitochondrial-dependent pathways in nicotine-mediated protection is consistent with recent analysis of a *CHRNA6* mutation. This mutation was identified as a genetic modifier that enhanced the severity of infantile parkinsonism and is likely to reduce activity of a DN-expressed nAChR. Importantly, this mutation was also identified as an enhancer of the disease-causing effects of a mutation in *WARS2*, mitochondrial tryptophanyl tRNA synthase, which was shown to reduce mitochondrial energy production (Martinelli et al., 2020).

The results of our investigation demonstrate that *C. elegans* represents a simple and well-characterized model system that facilitates mechanistic understanding of nicotine-induced neuroprotection. Our results suggest that an interaction between nAChRs and a D3R homolog mediates a nicotine-dependent increase in cytosolic calcium. This, in turn, leads to calcium influx into mitochondria through the mitochondrial calcium uniporter, MCU-1, to produce mild mitochondrial stress, sufficient for the activation of PINK-1-dependent protective processes within DNs.

Two findings from this research may explain the selective protection of *substantia nigra* DNs by tobacco smoking. First, the effects of nicotine require DOP-2, a D3R homolog, which is selectively expressed in DNs. Second, activation of PINK1-dependent mitochondrial quality control may selectively protect *substantia nigra* DNs, as these neurons were suggested to be uniquely susceptible to mitochondrial stress. Furthermore, many of the environmental and genetic risk factors for PD affect mitochondrial function (Ge et al., 2020).

In all, this research reveals a functional intersection of nicotinic receptor activity, dopaminergic neurotransmission, and mitochondrial dynamics with a capacity to selectively modulate DN survival, and its clinical manifestations in PD. Although tobacco smoking itself will certainly never be a panacea for PD, the molecular underpinnings of how it mysteriously confers protection provide for a unique and potentially valuable translational path into unexplored therapeutic strategies to combat degeneration of DNs.

Limitations of this study

Differences between *C. elegans* nAChRs and DNs and mammalian nAChRs and DNs preclude direct implications from our studies to mammals. Future studies should, therefore, validate our results concerning the roles of mitochondrial stress and mitochondrial quality control in nicotine-induced protection of mammalian DNs.

Our results implicate increased mitochondrial quality control in nicotine-induced protection. Future work should directly examine the effects of chronic nicotine exposure on mitochondrial dynamics and specifically on mitophagy.

Resource availability

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Materials availability

Please contact us for further information on methods or for request of strains.

Data availability

Data will be available upon request.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2021.102140.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.B.N., G.A.C., K.A.C., and M.T.; Investigation, J.B.N., G.H., A.M., A.K., and H.C.B.-A.; Visualization, J.B.N. and H.C.B.-A.; Supervision, J.B.N., G.A.C., K.A.C., and M.T.; Funding acquisition, G.A.C., K.A.C., and M.T.; Writing, J.B.N., G.A.C., K.A.C., and M.T.

DECLARATION OF INTERESTS

All authors declare that they have no competing interests.

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Supplemental Information

Conserved nicotine-activated neuroprotective pathways involve mitochondrial stress

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Supplemental information

nAChR subunit	BSR reduced	Nicotine-mediated neuroprotection	Expressed in DNs[1]
unc-38	+	-	+/*
unc-63	+	-	+/*
acr-2	+	-	+/-
lev-1	+	+	+/*
acr-12	-	+	+/*
acr-20	n.d.	+	+/*
unc-29	-	+	+/-
acr-5	-	n.d.	+/*
acr-8	-	n.d.	+/-
acr-15	-	n.d.	+/-
acr-18	-	n.d.	+/-
acr-21	-	n.d.	+/-
deg-3	-	n.d.	+/*
des-2	-	n.d.	+/*

Table S1. *C. elegans* nAChR subunits examined in this study. Related to Figures 2 and 5.

Indicated for each subunit: analysis of whether a loss-of-function mutant in the gene displayed reduced BSR (+), no effect (-), or was not determined (n.d.). Additionally, whether the loss-of-function mutants or RNAi knockdown (used for *acr-12*, *acr-20*) demonstrated a loss-of-nicotine-mediated neuroprotection (+) or not (-) following exposure to 6-OHDA. [1]Expression information for these subunits in DNs was compiled from reports of Spencer et al., 2011 (+) and Cao et al., 2017 (*). It should be noted that occasional discrepancies between these two expression studies exist and might have resulted from the use of different developmentally staged animals or from the sensitivity of profiling method used and/or signal-to-noise ratio.

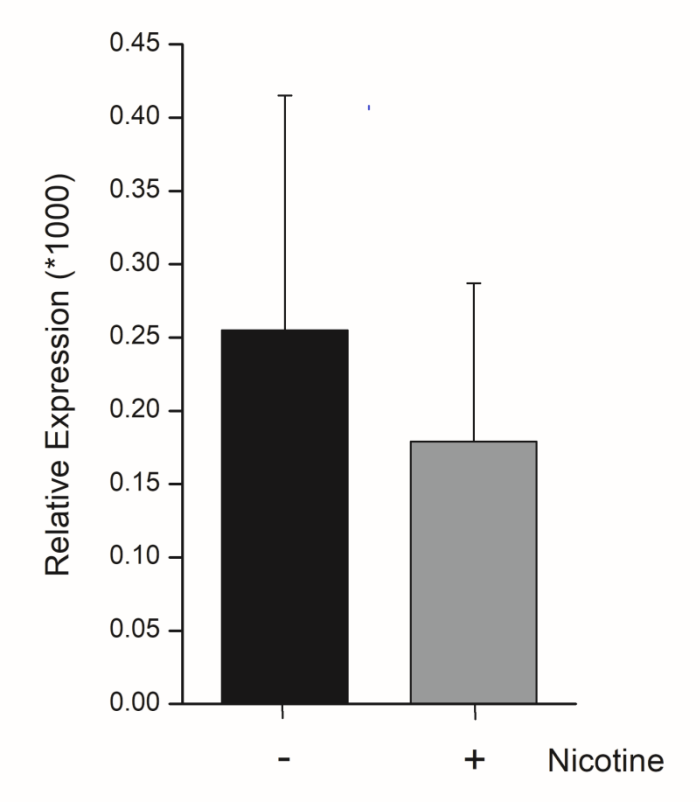

Figure S1. Effects of chronic nicotine exposure on *dat-1* expression. Related to Figure 4.

Wild-type (N2) animals were chronically treated with 62µM nicotine and expression of *dat-1* was examined using quantitative RT-PCR (N=2, n=4; two independent experiments, each analyzed twice and performed in triplicate). Data shown as average ± SD and significance was examined using paired Student's *t*-test; p >0.05.

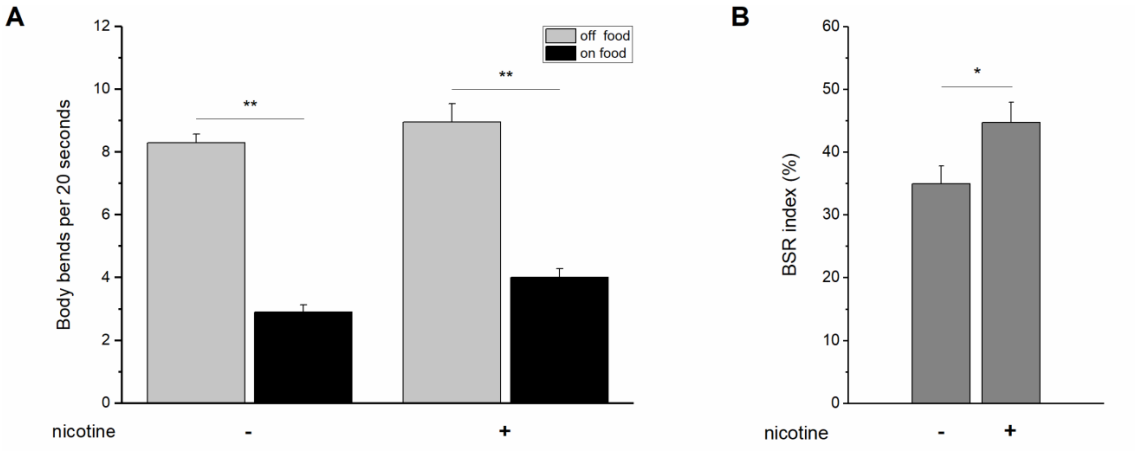

Figure S2. Chronic nicotine exposure reduces the basal slowing response. Related to figures 2 and 5.

Wild-type (N2) animals exposed to chronic nicotine (62µM nicotine before and during the assay) exhibit reduced BSR when compared to animals grown without nicotine. N=2, n=10 animals on and off food. (A) Locomotion speed was measured by the number of body bends per 20 seconds on food (black bars) or off food (grey bars). (B) BSR index (% body bends per 20 seconds on food per animal relative to average body bends per 20 seconds off food in the same growth conditions and experiment, this normalization reduces noise due to day-to-day variation in experimental conditions). Data is presented as average $\pm$ SE; significance was examined using ANOVA with Tukey's correction (A) or two-tailed unpaired Student's *t*-test (B), *-P<0.05; ** - p <0.01. Note: nicotine exposure increases locomotion speed both on and off food, but neither effect is significant.

Transparent Methods

Strains

Nematodes were maintained using standard procedures (Brenner, 1974). The wild-type is Bristol N2. Additional *C. elegans* strains examined are *dop-2(vs105), ric-3(md1181)* and nAChR subunit mutants: *unc-38(e264), unc-63(x37), acr-12(ok367), acr-8(ok1240), acr-2(ok1887), lev-1(e211), unc-29(e1072), acr-15(ok1214), acr-21(ok1314), acr-18(ok1285), deg-3des-2(u773)* (a deletion affecting both subunits), and *acr-5(ok180).* All mutants examined in this study are considered *loss-of-function (lf)* mutants.

Analysis of DN survival following 6-OHDA exposure was examined using BY250 P_{dat-1}*::*GFP (*vtIs7),* a kind gift from Randy Blakely (Florida Atlantic University). For the analysis of effects of nAChR mutations on DN survival, the mutations in the following nAChR subunit mutations were crossed into BY250: *unc-38(e264), unc-29(e1072), acr-2(ok1887) and lev-1(e211).* For analysis of *pdr-1(lf),* strain UA316 [*pdr-1(gk448)*; *sid-1(pk3321);* P_{dat-1}*::sid-1,* P_{myo-2}::mCherry #15.1[*baIn36*]; P_{dat-1}*::*GFP(*vtIs7*)] was used (Kim et al., 2018). For analysis of the effects of chronic upregulation of ATFS-1, strain UA406 [*atfs-1(et15)*; P_{dat-1}*::*GFP(*vtIs7*)] was generated by crossing BY250 into QC115 (Rauthan et al., 2013) as described in Martinez et al., 2017.

For DN-specific RNAi knockdown of genes, we used a *sid-1* mutant strain expressing *sid-1* only in DNs; this strain is UA202 [*sid-1(pk3321);* P_{dat-1}*::sid-1,* P_{myo-2}::mCherry #15.1[*baIn36*]; P_{dat-1}*::*GFP(*vtIs7*) (Harrington et al., 2012). RNAi clones were obtained from the Ahringer library (Kamath et al., 2003) except *atfs-1*, which was generated separately (Martinez et al., 2017).

For mitochondrial stress assays, we used a chromosomally-integrated transcriptional reporter strain that expresses GFP under control of the *hsp-6* promoter (SJ4100 [*hsp-6::*GFP (*zcIs13*)]) (Yoneda et al., 2004).

Basal slowing assay (BSR)

Basal slowing experiments were conducted as described by (Chase et al., 2004; Sawin et al., 2000). In these experiments movement of animals on food is compared to their movement off food. Experiments were done on fresh NGM plates. For food containing plates we added

100µl of a ten-fold concentrated freshly grown late logarithmic stage HB101 bacteria. Bacteria were gently spread on the plates, air dried and then transferred to 37°C for overnight growth. Empty plates were also maintained overnight at 37°C. Animals for experiments were picked at the fourth larval stage to fresh plates for overnight growth at 20°C. Prior to the experiment plates were equilibrated at 20°C. Animals were transferred to plates with or without food using a glass capillary containing M9. Each animal was gently washed of bacteria before transfer to the new plate. 90 seconds after transfer to the experimental plate each animal was examined for appearance of body bends at the head. For each animal the number of sinusoidal bends initiating at the head and propagating backwards was counted for two consecutive 20 second periods, and the number given for each animal is the average of these two measurements. For experiments on food animals were placed in the middle of the bacterial lawn, and animals that moved to the edge of this lawn or that exited the food lawn were not analyzed. For BSR assays following chronic nicotine exposure, nicotine (62 µM) was added both to plates in which the animals were grown and to assay plates.

Exogenous dopamine response assay

Experiments were performed as described by Chase et al. (2004). Dopamine containing plates were prepared the day prior to assay. These plates contained 100 ml double distilled water, 1.7gr agar, 11.4µl acetic acid and 0.379 gr dopamine hydrochloride (Abcam, ab1210565-5-B) to a final concentration of 20mM. Dopamine was added after the agar cooled down to 50°C. Prior to the experiment plates were air dried for one-hour. For experiments fourth larval stage animals were picked to fresh plates for overnight growth at 20°C. For analysis of the dopamine response 10 animals were placed on each dopamine plate and inspected for movement every 15 minutes for one-hour. Temperature for experiments was maintained at approximately 20°C. Animals were considered to be paralyzed if for 15 seconds no sinusoidal wave was seen in the head region. Animals showing only small head movements were considered paralyzed. In each experiment N2 controls were examined in parallel to mutant animals.

RIC-3 rescue of the basal slowing response

For expression of *ric-3* in DNs only, we PCR amplified a 0.8 Kb fragment containing the *dat-1* promoter using the following primers (5’ to 3’):

Dat1draIIIF, GATCACGTAGTGCTTCCATGAAATGG

and Dat1sphIR, CAGCTTGCATGCACAAACTTGTATC.

These primers include a *draIII* or a *SpHI* sites to enable cloning of the resulting PCR product upstream to a RIC-3::GFP fusion lacking the non-conserved coiled coil domain (Biala et al., 2009). The resulting plasmid was injected at 5ng/µl concentration into *ric-3(md1181)* animals together with a coelomocyte marker, CC::RFP at 50ng/µl and supplemented with empty Bluescript vector as carrier 100 ng/µl. For the basal slowing experiments transgenic L4 animals expressing RFP in their coelomocytes were picked the day prior to the experiment for overnight growth at 20°C.

6-OHDA treatment and nicotine exposures

Animals were analyzed for degeneration of DNs using established procedures and criteria for *C. elegans* (Cao et al., 2005; Ray et al., 2014). Briefly, animals were synchronized and grown at 20°C on NGM plates seeded with OP-50 bacteria. On day-3 post-hatching, animals were exposed to 10mM 6-OHDA/2mM ascorbic acid (Tocris Bioscience) for one-hour at 20°C. After the one-hour incubation, 6-OHDA was oxidized by adding 0.5X M9 and washed three times with ddH_2O then plated back onto fresh, appropriate plates matching their respective conditions. Twenty-four hours after exposure, three replicates of 30 worms were scored for dopaminergic neurodegeneration of the six anterior DNs, totaling to 90 worms per treatment. Worms were scored as normal if no degenerative phenotypes (dendritic blebbing, swollen soma, broken projections, or a missing neuron) were observed in the six anterior [four CEPs (cephalic) and two ADEs (anterior deirid)] DNs.

Animals were treated with nicotine (Sigma N3876) using three different exposure paradigms – chronic, acute, and pre-exposure. These were performed as follows: 1) for chronic nicotine exposure, animals were raised on NGM plates with nicotine added to the agar to the final concentration of 62µM and nicotine was added to the 6-OHDA assay mix. Animals were placed back onto nicotine containing plates post-6-OHDA treatments; 2) for acute nicotine tests, animals were only exposed to nicotine while in the 6-OHDA assay mix; 3) for pre-exposure, a one-hour nicotine treatment was done 24-four hours prior to 6-OHDA, in addition to nicotine being present in the 6-OHDA assay mix. Animals in the control groups were never exposed to nicotine. The solvent used for nicotine was ddH_2O.

DN-specific knockdown of genes

RNAi knockdown of genes expressed in the DNs was accomplished using established procedures for RNAi by bacterial feeding (Harrington et al., 2012; Ray et al., 2014). Briefly, bacterial strains harboring constructs used for the production of dsRNA were obtained from the Ahringer *C. elegans* library (Kamath et al., 2003). Bacteria were isolated and grown overnight in Luria broth (LB) media containing 100µg/ml ampicillin. Nematode growth media (NGM) plates containing 50µg/ml ampicillin, 1mM IPTG, with or without 62µM nicotine, were seeded with 300µl of RNAi bacterial culture, allowed to dry and grown overnight. UA202 (DN-specific RNAi sensitive) animals were grown on RNAi conditions for two generations (Ray et al., 2014). The first generation was raised on RNAi plates without nicotine. Their embryos were collected and synchronized by hypochlorite treatment (Lewis and Fleming, 1995) and then placed onto agar plates corresponding to their respective RNAi conditions. Experimental plates had 62µM nicotine added to the agar for a chronic exposure (see above methods) while control treatments were kept off of nicotine for the entirety of the experiment. This method allows for stronger silencing of targeted genes without the nicotine-induced alteration of gene expression in the first generation. The second generation was exposed to 10mM 6-OHDA/2mM ascorbic acid at day-3 post-hatching for one-hour and then plated back onto RNAi-nicotine plates of their respective condition. They were then scored 24-hours later for DN neurodegeneration.

Mitochondrial stress assay

Experiments testing the influence of nicotine on mitochondrial stress used the reporter strain SJ4100 (*hsp-6*::GFP (*zcIs13*)) (Yoneda et al., 2004). Animals were raised and kept on NGM plates seeded with OP-50 bacteria that had nicotine added to the agar to a final concentration of 62µM; control plates had no nicotine. GFP fluorescent images of 2-3 replicates, with 30 animals per replicate, were taken on days four and seven post-hatching. Focusing on an anatomically consistent region measuring 275 x 275 pixels comprising the same defined area in each animal (muscular region directly posterior to the pharynx), images were taken and pixel intensity was measured using MetaMorph software.

Quantitative RT-PCR

For RNA extraction, synchronized animals were collected 3 days post hatching. Animals were collected into epi tubes and washed 3 times with M9 by centrifugation at 500g for 2 minutes. Finally, the animal containing pellet was resuspended in 250 µl of Trizol to which was added one spoonful of zirconium beads (cat. No. ZrOB05, Next Advance). This mixture was frozen in -80°C overnight or until needed. On the day of isolation, the tubes were wrapped in parafilm and placed, still frozen, in a Bullet blender (Next Advance) at speed setting 8 for 3 minutes and at speed setting 9 for 4 minutes in a 4°C room. To each tube 200 µl of cold chloroform was added, and the mixture was gently mixed for 15 seconds followed by 3 minutes of incubation at room temperature. This was followed by centrifugation at 17,000g for 15 minutes at 4°C and the aqueous phase was transferred into new microfuge tubes. To this 500 µl of EtoH were added, the tubes were placed in liquid nitrogen for 5 minutes, then centrifuged at 17000g for 10 minutes at 4°C. The resulting pellet was washed 2 times with 75% EtOH, air dried and resuspended in 40 µl of double-deionized water followed by purification using a Qiagen RNeasy mini kit (cat. No. 74104) with DNAse treatment (cat. No. 79254, Qiagen).

Single-stranded cDNA was synthesized from 1µg total RNA using the High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). Gene expression was quantified using the StepOne Plus Real Time PCR System (Applied Biosystems). For each 20µl qPCR reaction volume, 10µl Fast SYBR Green master mix (Applied Biosystems), primers at concentrations chosen in calibration experiments (all from Integrated DNA Technologies), 2µl cDNA (20ng) and double-deionized water were mixed together. qPCR was performed at 95° for 20 seconds followed by 40 cycles of 95° for 3 seconds and then 60° for 30 seconds, followed by the melt curve stage. All reactions were run in triplicate. All raw data cycle thresholds were well within the range of reliable detection. Primers were as listed below, at final concentrations of 300 nM each; signal threshold was set at 0.8. For statistical analysis, raw CT at threshold was recorded, normalized to *ubq-2* (whose expression is unchanged following chronic nicotine exposure) and final expression was calculated as $2^{-\Delta CT}$. Primers used:

ubq-2

Forward – 5'-GGAATCCCACCAGATCAGC -3'

Reverse – 5'-GGAGAGATGGCTCGATGATTC -3'

hsp-6

Forward – 5'-CAAACTCCTGTGTCAGTATCATGGAAGG-3'

Reverse – 5'-GCTGGCTTTGACAATCTTGTATGGAACG-3'

timm-23

Forward – 5'-AGTGCCGGAATGAACTTCTC -3'

Reverse – 5'-GTTGATCCAAGGCGAGGAC -3'

dat-1

Forward – 5'-ACCAGGACTACACATACCCAAC -3'

Reverse – 5'-CTCGTTGCCATTTCTCAGATATCG -3

Statistical Analysis

Data are represented as SD for all figures, "n" is number of animals examined and "N" is number of independent experiments or plates in dopamine experiments. Significance in BSR experiments were examined by two different methods: 1) one-way ANOVA with Tukey's *post hoc* multiple comparisons test was used to compare the number of body bends; 2) unpaired two-tailed Student's *t*-test was used to compare BSR index (number of bend bends on food relative to off food). Unpaired two-tailed Student's *t*-test was used to compare paralyzing effects of exogenous dopamine. Due to variability in gene expression between experiments unpaired Student's *t*-test was used to examine effects of chronic nicotine exposure. Pixel intensity comparisons used unpaired two-tailed Student's *t*-test with 3 replicates of 30 worms per replicate (day seven used 2 replicates). All neurodegeneration experiments included 3 replicates of 30 worms per replicate and comparisons done by two-way ANOVA with Tukey's *post hoc* multiple comparisons test. $p < 0.05$ was used the minimum threshold to determine statistical significance. Statistical comparisons were generated using GraphPad Prism.

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