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Insulin signaling genes modulate nicotine-induced behavioral responses in *C. elegans*

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Abstract

Insulin signaling has been suggested to modulate nicotine dependence, but the underlying genetic evidence has been lacking. Here, we used the nematode, *C. elegans*, to investigate whether genetic alterations in the insulin signaling pathway affect behavioral responses to nicotine. To do so, we challenged drug-naïve *C. elegans* with an acute dose of nicotine [100 µM] while recording changes in their locomotion speed. While nicotine treatment stimulated locomotion speed in wild-type *C. elegans*, the same treatment reduced locomotion speed in mutants defective in insulin signaling. This phenotype could be suppressed by mutations in *daf-16*, a gene encoding a FOXO transcription factor that acts downstream of insulin signaling. Our data suggest that insulin signaling genes, *daf-2*, *age-1*, *pdh-1*, *akt-1*, and *akt-2* modulate behavioral responses to nicotine in *C. elegans*, revealing a genetic link between nicotine behavior and insulin signaling.

Keywords

nicotine; insulin; behavior; neuron; *daf-2*; *C. elegans*; *daf-16*; FOXO; drugs of abuse; tobacco; addiction; smoking; diabetes

Introduction

Tobacco use is the leading cause of preventable deaths in the developed world, with tobacco-related deaths reaching hundreds of thousands annually. Despite this, the one-year success rate of tobacco cessation is less than five percent (USPHS 2008), which means for every 100 tobacco users who attempt to abstain, more than 95 individuals will have relapsed within a year of their quit date. A plurality of these addicts will cite weight gain as a major component in their relapse, and the majority of current tobacco users will identify their fear of weight gain as the largest impediment to attempting a tobacco cessation program (Shen *et al.* 2014). Even newly abstinent tobacco users who do not increase caloric intake appear to gain weight, particularly as body fat, upon tobacco cessation (Bergman *et al.* 2012), and these abstinent smokers have a dramatically increased risk of developing type II diabetes

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Conflicts of Interest

None.

within the first 24 months of nicotine abstinence (Bajaj 2012). Equally disconcerting, however, is that prolonged nicotine use increases body fat, particularly intra-abdominal body fat (Bergman *et al.* 2012), thereby increasing waist-to-height ratio, which is the metric that most reliably predicts risk for type II diabetes, metabolic syndrome, non-alcoholic fatty liver disease, coronary artery disease, and cerebrovascular disease (Kahn *et al.* 2014). As dysfunction in insulin signaling is a major cause of obesity and diabetes, it has been suggested that the insulin pathway may play a role in modulating tobacco dependence (O'Dell *et al.* 2014; Richardson *et al.* 2014). However, the genetic evidence supporting such a role for insulin signaling in nicotine dependence remains elusive.

C. elegans represents a powerful genetic model for studying various questions in neurobiology, including drug dependence. For example, it has been reported that *C. elegans* responds to nicotine in both a concentration-dependent and time-dependent manner and display a wide range of behavioral responses to nicotine (Feng *et al.* 2006; Sobkowiak *et al.* 2011). *C. elegans* is capable of navigating up a nicotine gradient (Sellings *et al.* 2013). Nicotine has also been paired with salts to probe CS-US associations in nematodes (Musselman *et al.* 2012). In addition to nicotine, *C. elegans* has been used as a model to characterize other types of drugs of abuse, such as cocaine and ethanol (Davies and McIntire 2004; Davies *et al.* 2003; Ward *et al.* 2009). Interestingly, the genes mediating drug-dependent behaviors in *C. elegans* are found to be evolutionarily conserved. For instance, *C. elegans* depends on nAChRs for nicotine-induced behaviors and require BK channels for ethanol-induced responses (Crowder 2004; Davies and McIntire 2004; Feng *et al.* 2006).

In *C. elegans*, the gene *daf-2* encodes an ortholog of the receptor tyrosine kinases of the insulin and IGF-1 receptor family (Fig 1A). While only one single insulin receptor ortholog exists in *C. elegans*, *daf-2* has splice variants for at least three distinct isoforms, each with its own putative expression pattern and specialized functions (Kwon *et al.* 2010; Ohno *et al.* 2014). In mammals, and likely in nematodes, insulin and IGF-1 receptors are an $\alpha 2/\beta 2$ heterotetramer with α -subunit ligand-binding domains and β -subunit kinase domains (Ohno *et al.* 2014). The insulin and IGF signaling (IIS) pathway plays a crucial role in a host of physiological processes in *C. elegans*, including lifespan and aging (Kenyon 2010); lipid metabolism and fat regulation (Ashrafi 2007); energy homeostasis and body weight management (Daws *et al.* 2011); as well as a role in learning and memory (Labouebe *et al.* 2013; Tomioka *et al.* 2006). However, a role for IIS in drug-induced behaviors has not been explored in this organism. Here, we present studies that seek to investigate a genetic link between a behavioral response to nicotine and the IIS pathway.

Methods

Subjects and Strains

We maintained the *C. elegans* strains on nematode growth medium (NGM) [50 mM NaCl, 32 g/L BBL Agar, 2.5 g/L peptone, 13 μ M cholesterol, 1 mM Ca(CH₃COO)₂, 1mM MgSO₄, 25 mM KH₂PO₄, pH 7.0] seeded with *Escherichia coli* (OP50) at 20°C (Avery and You 2012). For any strain not already in our possession, we purchased the line from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). For the studies presented here, we used strains carrying mutations in *C. elegans* insulin signaling

genes. Each strain is named for the genetic mutation it carries. The strains used are as follows: Bristol N2 (as wild-type), *daf-2(e1368)*, *daf-2(e1370)*, *age-1(hx546)*, *pdk-1(sa709)*, *akt-1(mg306)*, *akt-2(ok393)*, *daf-16(mgDF46)*, *daf-16(mu86)*, *daf-18(e1375)*, and the double mutant *daf-2(e1368);daf-16(mu86)*.

Acute Nicotine-Induced Locomotion Assay

For the acute nicotine locomotion assay, we grew *C. elegans* on nicotine-free NGM with a 200 μ L lawn of *E. coli* (OP50) and picked L4 hermaphrodites onto fresh plates 16–20 hours before the behavioral test, depending on genetic background. At test, we transferred a single animal per plate containing a thinly-spread lawn of 40 μ L OP50 and the appropriate nicotine concentration. As described previously (Hsu *et al.* 2009; Liu *et al.* 2013), an automated system tracked the animal as it crawled freely on the plate for 16 minutes. We discarded the first 11 minutes as the animal acclimated to the novel environment and absorbed the nicotine through the cuticle. We then calculated the average crawl speed for the last five minutes of the recording. For the wortmannin studies, the plates also contained the appropriate concentrations of the PI3K inhibitor. We excluded from analysis any animal that crawled up the side of the plate at any point. Each group consisted of at least 20 animals.

Drugs

We used double distilled water to dilute molecular nicotine (10 μ M, 50 μ M, 100 μ M, 500 μ M, and 1000 μ M) and wortmannin (10 nM, 100 nM, 1000 nM). We applied drugs directly to the NGM and allowed them to diffuse throughout the medium for 24–48 h to ensure uniform concentration.

Data Analysis

We used a one-way ANOVA with Dunnett's Multiple Comparison Test to test dose-response curves and Student's *t*-Test for all other statistical analyses.

Results

Acute nicotine treatment stimulates locomotion in wild-type but depresses it in *daf-2* mutants

Acute nicotine treatment induces increased locomotion in naïve worms, and this increased locomotor response depends upon nAChRs (Feng *et al.* 2006). This increased locomotor response depends upon nAChRs (Feng *et al.* 2006). As reported previously, here we also found that wild-type *C. elegans* increased their locomotion speed in response to an acute nicotine challenge. This acute nicotine response was dose-dependent, with an optimal response to 100 μ M nicotine and nicotine-induced paralysis at 1000 μ M nicotine (Fig 1B).

Interestingly, we observed that, unlike wild-type animals, when exposed to nicotine, *daf-2* mutants (both *e1368* and *e1370*) showed a dose-dependent decrease in locomotor response (Fig 1C). As these two alleles did not differ from one another, we present them here collectively. This is not merely a defective acute nicotine response; rather, *daf-2* mutants show a nicotine phenotype that appears to be a nearly complete inversion of the wild-type response.

Mutants deficient in AGE-1 show inverted nicotine phenotype

We next tested whether other players in the IIS pathway also regulate nicotine-induced locomotor response. DAF-2 signals to the PI3K homolog, AGE-1, which catalyzes the conversion of PIP₂ to PIP₃. We thus tested how *age-1* mutants respond to nicotine challenge, and found that these animals showed the same inverted nicotine phenotype as *daf-2* mutants (Fig 2A). We also applied a pharmacological approach by testing how the PI3K inhibitor, wortmannin, affects nicotine responses. While wild-type *C. elegans* exposed to nicotine and a low (10 nM) dose of wortmannin responded to nicotine with an increased locomotion speed (Fig 2B), those animals exposed to nicotine and a moderate dose of wortmannin (100 nM) recapitulated the decreased locomotor response seen from *age-1* mutants (Fig 2C). Higher concentrations of wortmannin (1000 nM), however, are toxic to *C. elegans*. Thus, wortmannin treatment can mimic the inverted nicotine phenotype in a dose-dependent manner. These data together demonstrate that *age-1* regulates nicotine-induced behavioral responses.

C. elegans defective in genes downstream of AGE-1 show depressed nicotine phenotype

Once AGE-1 converts PIP₂ to PIP₃, PIP₃ activates the PDK ortholog PDK-1. As such, we next turned our attention to this kinase and its downstream targets. Fig 3A shows that *pdk-1* mutant animals exhibit the same decreased locomotor response to nicotine as *daf-2* and *age-1* mutants. We next chose to investigate the targets of PDK, namely AKT/PKB. The *C. elegans* genome encodes two AKT homologs in the IIS pathway, *akt-1* and *akt-2*. Loss of function in either *akt-1* (Fig 3B) or in *akt-2* (Fig 3C) also results in the decreased locomotor phenotype in response to an acute nicotine challenge. Therefore, mutants deficient in genes acting downstream of *age-1*, including *pdk-1*, *akt-1*, and *akt-2*, all show the same inverted behavioral phenotype as *daf-2* in response to acute nicotine challenge.

DAF-16/FOXO is required for nicotine-induced locomotor responses

The FOXO family of transcription factors is the primary target of IIS. In *C. elegans*, *daf-16* encodes the sole FOXO transcription factor. AKT-1 and AKT-2 signal to DAF-16 through phosphorylation. As *daf-16* acts downstream of *daf-2*, mutations in *daf-16* can also suppress *daf-2* mutant phenotypes in dauer formation and longevity (Kenyon 2010). As the IIS pathway acts to suppress DAF-16 function, unlike other genes in the IIS pathway, *daf-16* mutants usually show a phenotype distinct from those in the IIS pathway. Indeed, we found that strains carrying either of the null alleles, *daf-16(mu86)* or *daf-16(mgDF46)*, showed no notable responses to acute nicotine treatment (Fig 4A). As these two alleles did not differ from one another, we present them here collectively. This indicates that *daf-16* is required for behavioral responses to nicotine. With the disparity in the *daf-2* and *daf-16* phenotypes, we next tested whether these two genes are, in fact, functioning in the same pathway to mediate an acute nicotine response. If the *daf-2* and *daf-16* genes act in the same pathway in this acute nicotine behavior, the *daf-2;daf-16* double mutant should perform similarly to the *daf-16* or *daf-2* mutant alone. Indeed, *daf-2;daf-16* double mutants showed the same nicotine defect as observed with the single *daf-16* mutants, suggesting that these two genes, *daf-2* and *daf-16*, are functioning in the same pathway (Fig 4B). Therefore, *daf-16* acts downstream of the IIS pathway to modulate the acute response to nicotine challenge.

Discussion

In the current study, we present the first genetic evidence supporting a role for IIS in modulating behavioral responses to nicotine. We show that wild-type *C. elegans* dose-dependently increases locomotor speed in response to acute nicotine treatment. Interestingly, the highest doses of nicotine elicited a paralytic effect. Such a biphasic effect of nicotine has also been observed in rodents (Jerome and Sanberg 1987). Rather than increasing their locomotion speed as does wild-type *C. elegans*, *daf-2* mutants move slower when acutely challenged with nicotine. In these animals, nicotine appears to act as a depressant rather than as a stimulant. This effect does not appear to result from a simple shift of the dose curve, as no stimulatory effect of nicotine was observed at low concentrations of nicotine in *daf-2* mutants. In addition, though nicotine suppresses locomotion speed in *daf-2* mutants, these animals do not become paralyzed at low doses. In fact, both wild-type and *daf-2* strains first show nicotine-induced paralysis at the same concentration (1000 μ M). Thus, the inverted nicotine phenotype observed in *daf-2* mutants is not caused by a hypersensitive paralysis effect caused by nicotine.

This inverted nicotine phenotype persists throughout the kinase cascade downstream of DAF-2. *C. elegans* deficient in the PI3K gene, *age-1*, respond to nicotine with a reduced locomotor speed, and this genetic effect is recapitulated dose-dependently when wild-type *C. elegans* experience nicotine in the presence of the PI3K inhibitor wortmannin. As such, both genetic and pharmacologic manipulations seem to support this unique effect of an insulin signaling gene in the acute locomotor response to nicotine. Moreover, loss of function in the PDK ortholog *pdh-1*, or in either of the AKT genes *akt-1* or *akt-2*, leads to a similar depressed response to nicotine. All these observations support a role for IIS genes in modulating nicotine-induced behavioral responses.

The *C. elegans* genome encodes a single FOXO transcription factor, DAF-16. This FOXO transcription factor is necessary for extending lifespan via decreased insulin signaling in nematodes (Kenyon 2010), and is crucial in a number of other phenomena that require insulin receptor activity (Kenyon 2010; Murphy and Hu 2013; Ohno *et al.* 2014). Accordingly, we tested two separate null alleles for *daf-16* in this acute nicotine paradigm. In both cases, *daf-16* mutants showed no notable changes in locomotion speed in response to nicotine and could also suppress the inverted nicotine phenotype of *daf-2* mutants, indicating that DAF-16 is required for mediating acute nicotine responses and acts downstream of IIS. IIS is known to suppress the function of DAF-16. However, *daf-16* mutants do not exhibit a phenotype opposite to that observed in *daf-2* mutants, but are instead insensitive to nicotine. This is not surprising as DAF-16 is a transcription factor and regulates the expression of hundreds of genes, some of which could be required for mediating normal nicotine responses (Larsen 2003). Future studies are needed to identify specific DAF-16 target genes that mediate nicotine responses and explore a potential role of insulin and dopamine signaling in the rewarding or reinforcing effects of nicotine in *C. elegans*.

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Figure 1. Locomotor Responses of Wild-Type and *daf-2* Worms

A. The insulin signaling pathway in nematodes consists of the insulin and insulin-like growth factor receptor homolog, *daf-2*, and the kinase homologs downstream of this receptor, *age-1*, *pdk-1*, *akt-1*, and *akt-2*. The pathway culminates with *daf-16*, which is the only nematode FOXO transcription factor homolog in *C. elegans*. **B.** Wild-type, nicotine-naïve nematodes show a dose-dependent increase in their crawl speed subsequent to an acute nicotine challenge. When compared to the nicotine-free ('0 μ M') group, the '100 μ M' group crawls significantly faster, while the '1000 μ M' group crawls significantly slower, most

likely due to nicotine-induced paralysis at this concentration. The '10 μ M' group is not significantly different from control animals [one-way ANOVA $p < 0.001$; *post hoc* Dunnett's MCT]. **C.** The locomotor response of nematode strains carrying alleles hypomorphic for the IIS ortholog, DAF-2 show a dose-dependent decrease crawl speed in response to nicotine. As the two strains tested, *daf-2(e1368)* and *daf-2(e1370)*, were not different from one another, their data are presented here collectively. Neither the '10 μ M' nor the '50 μ M' groups were significantly different from the '0 μ M' group, although the '50 μ M' did show a trend towards significance. The '100 μ M', '500 μ M', and '1000 μ M' groups were all crawled significantly slower than the nicotine-free control group when faced with an acute nicotine challenge [one-way ANOVA $p < 0.001$; *post hoc* Dunnett's MCT]. Animals in the '1000 μ M' showed signs of nicotine-induced paralysis comparable to what was seen with wild-type animals, while the '100 μ M' and '500 μ M' worms show normal, non-paralytic body curvature.

Figure 2. *age-1* Function is Crucial for Normal Nicotine Response

A. Strains carrying an allele hypomorphic for *age-1* decrease their crawl speed in response to an acute 100 μ M dose of nicotine. *age-1* than do nicotine-naïve *age-1* [one-tailed *t*-Test, $p < 0.001$]. **B.** shows the effects of Wortmannin, a PI3K inhibitor on the locomotor response of wild-type nematodes challenged with a 100 μ M dose of nicotine. Wortmannin dose-dependently affects the nicotine-responsive behaviors of nematodes. 10 nM Wortmannin has no effect on the wild-type acute nicotine response as worms receiving both nicotine and wortmannin have an increased crawl speed than worms receiving wortmannin alone [one-tailed *t*-Test, $p < 0.01$]. **C.** Wild-type worms experiencing a 100 μ M nicotine challenge in the presence of 100 nM Wortmannin have a decreased crawl speed when compared to the Wortmannin alone group [one-tailed *t*-Test, $p < 0.01$]. This phenocopies worm strains hypomorphic for *age-1*. Note, the wortmannin-free data are duplicated in Fig 2B and 2C for ease of visual comparison. These data were not included in the analysis

Figure 3. Downstream Kinases Yield the Inverted Nicotine Phenotype

A. *pdk-1* mutants phenocopy *daf-2* mutants in response to an acute nicotine challenge. *pdk-1* mutants crawl at slower speed in response to a 100 μM nicotine challenge than do *pdk-1* mutants not exposed to nicotine [one-tailed *t*-Test, $p < 0.001$]. **B.** The two *C. elegans* Akt/PKB homologs behave similarly in response to an acute 100 μM nicotine challenge. *akt-1* mutants decrease their crawl speed in response to a 100 μM challenge when compared to nicotine-naïve mutants [one-tailed *t*-Test, $p < 0.001$].

C. This decrease crawl speed persists in *akt-2* mutants exposed to 100 μM nicotine when compared to their nicotine-naïve controls [one-tailed *t*-Test, $p < 0.01$].

Figure 4. *daf-16* Is Necessary for a Normal Nicotine Response

A. As the two strains tested, *daf-16(mgDF46)* and *daf-16(mu86)*, were not different from one another, their data are presented here collectively. *daf-16* mutants exposed to 100 μ M nicotine do not crawl at a different speed than nicotine-naïve *daf-16*/FOXO mutants [one-tailed *t*-Test].

B. *daf-2;daf-16* double mutants exposed to 100 μ M nicotine crawl at a speed that is not significantly different from double mutants exposed to no nicotine [one-tailed *t*-Test]. As the double mutant phenocopies the *daf-16* single mutant instead of presenting a phenotype intermediate to the *daf-2* and *daf-16* single mutants, it is likely that these two genes are working in the same pathway in response to nicotine and *daf-16* is downstream of *daf-2*.