Genetic Regulation of Nicotine-Responsive Behaviors in the Nematode, *C. elegans*

by

Seth A. Wescott

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in The University of Michigan
2015

Doctoral Committee:
Professor X.Z. Shawn Xu, Chair
Assistant Professor Asim A. Beg
Professor Margaret E. Gnegy
Associate Professor Michael M. A. Sutton
Assistant Professor Bing Ye

DEDICATON

This work is dedicated to my 22 shipmates who ended their lives yesterday,

to my 22 shipmates who end their lives today,

and to my 22 shipmates who will end their lives tomorrow.

ACKNOWLEDGMENTS

The work presented here represents the culmination of nearly a decade of my life. While I collected, analyzed, and interpreted all of the data present in this manuscript, this feat would not have been possible if I had been acting in isolation. At the very least, all of the work I present here was completed in collaboration with my dissertation advisor, Shawn Xu, who graciously offered me an intellectual haven in his lab and the financial support to conduct these studies. Similarly, when I came to the lab, I had no background in nematodes, genetics, or molecular biology. Without the patience and understanding of my colleagues in lab, I may never have learned the skills necessary to complete these studies. I would especially like to thank Manish Rauthan, Beverly Piggott, and Jingwei Gao who were instrumental in guiding me through initial learning process within the lab. Furthermore, science is not an internal process but a process that requires discussion among individuals who can offer unique perspectives in possible data interpretation, in trouble-shooting, and presentation preparation. For their roles in these aspects of this dissertation, I would like to thank Elizabeth Ronan, Jianke Gong, Guang Li, Adam Iliff, Lei Chun, and Bi Zhang.

Outside of the lab, an entire second, although in many ways more important, tier of a support system is necessary. These are the individuals who recognize when it is time to take break, who help you up off the mat when haywire experiments knock you down, and who build you up to throw yourself back into what can be an incredibly frustrating process. Among this tier

of support is my entire cohort in the Neuroscience Graduate Program, particularly: Ali Althaus, Ashley Ingiosi, Blair Sutton, Graham Atkin, Trace Henry, and Travis Dickendesher. Also among this tier of support are the friends from endeavors unrelated to science, such as Aneesha Badrinarayan, Steve Kreps, Diane Trahanas, Danny Rivera, and the entire community of CrossFit734, especially: Aaron Brewer, Wayne Gilbert, Julie Jahn, Sheila Reeves, Lisa O'Donnell, Eric Santure, Lubna Grewal, Jason May, Lauren May, Jake Blackmer, and Rose Moya. Without these people buttressing me outside of lab, I'm not certain that I could have finished this degree. And, finally, none of this would have been possible without Mitch and Jaime Roitman who were the first to encourage me in science and to give me the opportunity to begin. Humility dictates that, as a tribute to and acknowledgment of everyone named here, to everyone who has helped me through the years but went unnamed, and to my family who have always been supportive of me, throughout the remainder of this manuscript, I use first-person plural pronouns (we, our).

A National Research Service Award from the National Institute of Deafness and Communication Disorders [5 F31 DC012241] funded the work I present here. I would like to thank the National Institutes of Health for their gracious support.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

At its core, addiction is a pathological state of motivation. While motivation itself is an adaptive psychological tool, when the motivation to seek out and engage a specific pharmacological agent, such as nicotine, or a biologically relevant stimulus, such as highly palatable food, exerts undue control over the behavior of an animal, the adaptive tool of motivation is transformed into the maladaptive state of addiction. The insulin signaling system plays a vital role in motivated behaviors, particularly in food-seeking behavior. The transient receptor potential (TRP) channels, meanwhile, are deeply involved in the sensory systems necessary for an organism to monitor its environment in order to locate biologically relevant stimuli. Here, we explore a role for the insulin signaling system in the locomotor response of nematodes to an acute nicotine challenge as well as a role for TRP channels in mediating nicotine-approach behavior in these animals. The studies presented here suggest novel models for how drugs of abuse can usurp adaptive motivational processes. We demonstrate that nematodes with a compromised insulin signaling system respond to an acute nicotine challenge as though it were a depressant rather than a stimulant. This effect persists in both genetic and pharmacologic manipulations of the insulin signaling system. We also demonstrate that *C. elegans* will chemotax to a source of nicotine and that vanilloid TRP channels are necessary for this behavior. The findings from the studies presented here may serve to identify novel therapeutic targets for the treatment of nicotine abuse, which is a leading public health concern throughout the developed world.

Chapter I

A Brief Overview of Nicotine-Responsive Behavior

'Addiction' is a term without a strict definition. In a recent review, Wise and Koob lament this very fact and its implications in research related to drugs of abuse (Wise and Koob, 2014). As they point out, clinical experts tasked with establishing diagnostic criteria – such as the American Psychological Association, the American Psychiatric Association, and the World Health Organization – all fail to offer a scientific definition for 'addiction'. In fact, between and within these august scholarly bodies, heated arguments rage about how an appropriate, methodical definition of the term 'addiction' should read (Edwards, 2012; Schuckit, 2012). Furthermore, the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) – the metric by which clinical diagnoses of psychological and psychiatric disorders are made – largely avoids the use of the word 'addiction' altogether (Wise and Koob, 2014). In addition to all of this, researchers who are largely held to be leaders in the study of drug-related behaviors and the physiological effects of drugs of abuse often give wildly different definitions for the term. Robinson, for example, each consider most-specifically the shift from volitional to compulsory drug-taking behavior to be commensurate with 'addiction' (Edwards, 2012). Everitt prefers to focus on the impulsivity of drug-taking behavior as a hallmark of 'addiction' (Edwards, 2012). Koob suggests that 'addiction' is best-defined as the relapse to drug-taking in an abstinent animal (Belin et al., 2013). Volkow, meanwhile, classifies 'addiction' as the effect of adaptations of

motivational neural circuits in response to chronic drug exposure (Belin et al., 2013). Similarly, Nestler focuses on epigenetic responses of cells to drugs of abuse while still comfortably using the term 'addiction' (Belin et al., 2013). This lack of strict definition for the term 'addiction' has lead both Wise and White (White, 1989; Wise and Koob, 2014), independently, to recall the character Humpty Dumpty in Lewis Carroll's *Through the Looking Glass* who said: "When I use a word, it means just what I choose it to mean – neither more nor less".

Perhaps the most versatile definition of 'addiction' is that it is a state of pathological motivation. Motivation is an adaptive process that allows an organism to garner biologically necessary stimuli and avoid potential threats. Food-gathering, mate-seeking, and denning are all examples of appetitive motivated behaviors (Roitman et al., 2004), while the avoidance of predators and withdrawal from noxious stimuli are examples of aversive motivated behaviors (Badrinarayan et al., 2012). In a healthy psychological state, the motivation for each of these behaviors is tempered with the internal state of the animal — hunger, thirst, exhaustion — and the current environment — proximity to a predator, exposure to elements, availability of palatable food or acceptable mate. In addiction-related states, a natural reward — like food or sex — or a drug of abuse — such as nicotine, cocaine, or opioids — arrogates the balance among these various motivations and foists itself to the forefront at the expense of biological imperatives and despite adverse consequences (Koob, 2009b; Parylak et al., 2011; Robinson, 2004; Robinson and Berridge, 1993; Schulteis and Koob, 1994). It is this persistent, compulsive drug-taking in the face of adverse consequences that separates addiction from recreational drug use in humans and a number of animal models seeks to capture or explain this aspect of addiction (Koob, 2009b; Koob and Le Moal, 2005; Robinson, 2004). Neural pathways mediating motivated behaviors are an especially vulnerable point of entry for drugs of abuse in mammals,

and chief among these weak spots is the mesolimbic dopamine (DA) system. This system extends from the ventral tegmental area (VTA) of the midbrain to forebrain targets such as the nucleus accumbens (NAc), the prefrontal cortex (PFC), and the amygdala. (Everitt et al., 1999; Fields et al., 2007; Kauer, 2004; Kauer and Malenka, 2007; Koob, 2009a; Margolis et al., 2006; Mihov and Hurlemann, 2012; Niehaus et al., 2009; Stamatakis et al., 2014; Yap and Miczek, 2008).

Introduction to Insulin Signaling in Nicotine-Responsive Behaviors

The Ventral Tegmentum

Often, the VTA and the A10 DA cells are considered synonymously. However, while the VTA includes the entirety of the A10 DA region, the VTA is a complicated and heterogeneous collection of nuclei (Ikemoto, 2007; Ikemoto and Bonci, 2014; Wise and Koob, 2014). The tegmental cellular heterogeneity is laid out in a rostrocaudal gradient and the forebrain targets appear to follow this gradient in a lateromedial manner (Ikemoto, 2007; Lammel et al., 2008). That is, cells in the medial and caudal part of the VTA send their axons preferentially to medial forebrain structures: the medial PFC (mPFC) and, in particular, the medial portion of the NAc shell subregion. Meanwhile, laterally and anteriorly situated VTA neurons target more lateral forebrain structures: the amygdala and the NAc core and the lateral portion of the NAcs (Lammel et al., 2008). In addition, DA transporter mRNA and the dopamine D2 autoreceptor expression appears to decrease along this lateromedial gradient, with DA neurons targeting the mPFC and the NAcs expressing the lowest levels of DA transporters and D2 autoreceptors. Thus, medial DA neurons express the lowest levels of both somatodendritic DA transporters and D2 receptors as well as the lowest levels of these proteins in the distal axon terminal fields of these

neurons (Lammel et al., 2008). This suggests that the DA neurons projecting to the mPFC and the NAcs see the least self-regulation.

While the DA neurons are arranged in this anteroposterior gradient, the A10 dopamine region is largely contained within the posterior aspect of the VTA while the anterior VTA has a larger population of GABA and glutamate cells In fact, immunostaining for tyrosine hydroxylase (TH) – the rate limiting step in DA biosynthesis – shows enrichment near the parabrachial pigmented and paranigral nuclei of the VTA that diminishes in density laterally and caudally (Faure et al., 2014; Ikemoto, 2007; Lammel et al., 2008). A similar gradient exists for the GABA and glutamate neurons of the VTA. GABA neurons are most prevalent in the anteriolateral regions of the ventral tegmentum while glutamate neurons are localized closer to the midline and with greater prevalence rostral to the DA neurons (Faure et al., 2014; Ikemoto, 2007; Sanchez-Catalan et al., 2014).

In discussing GABAergic nuclei within the VTA, it is important to note the GABA cells of the rostromedial tegmental nucleus (RMTg), lie caudal to, and modulate the firing rate of, A10 dopamine neurons (Bourdy and Barrot, 2012). These RMTg GABA cells express high levels of μ-opioid receptors (Jalabert et al., 2011), are subject to endocannabinoid control (Zangen et al., 2006) and one of their major targets outside the VTA is the lateral hypothalamus (Richardson et al., 2014) – a major center for control of feeding behaviors. Both the μ-opioid and the cannabinoid receptors act to decrease output from RMTg neurons, which disinhibits the DA neurons under their control, while reciprocal glutamate afferents from the lateral hypothalamus drive GABA output onto these DA neurons, increasing inhibitory control over them (Bourdy and Barrot, 2012; Bourdy et al., 2014; Jalabert et al., 2011; Kaufling et al., 2010). This lateral

hypothalamus-ventral tegmentum axis is likely an important circuit, at least in part, for indicating post-prandial satiety. This seems especially likely given the role insulin plays within the VTA.

Nicotine, Insulin, and the Ventral Tegmentum

Insulin receptors are expressed throughout the VTA (Bruijnzeel et al., 2011; Davis et al., 2010; Daws et al., 2011). On DA neurons, activation of the intracellular insulin signaling pathway, through intracerebroventricular (ICV) infusion of the peptide hormone, increases cell-surface expression of both the DA transporter and the D2 autoreceptor (Figlewicz et al., 1994; Schoffelmeer et al., 2011). Moreover, a nine peptide fragment of the insulin β-chain appears to increase binding of DA to its transporter (Liu et al., 2001), thus decreasing the activity of extracellular dopamine. Furthermore, insulin induces an AMPAR trafficking-independent form of long-term depression (LTD) in VTA DA neurons (Grueter et al., 2010; Labouebe et al., 2013). That is to say, insulin decreases the output from tegmental DA neurons. VTA μ-opioid receptor activity, meanwhile, is dependent upon insulin response substrate (IRS) and Akt/PKB, which are canonical elements of the insulin signaling pathway (Russo et al., 2007). Furthermore, the phosphoinositide 3-kinase (PI3K) – another canonical insulin signaling element – is necessary for somatodendritic dopamine release within the tegmentum (Daws et al., 2011).

Insulin is the major hormone responsible of maintaining energy homeostasis (Anthony et al., 2006; Boghossian et al., 2009; Bruijnzeel et al., 2011; Davis et al., 2010; Daws et al., 2011; Figlewicz et al., 2008) so it is unsurprising this peptide would exert some modicum of regulatory control over the mesolimbic DA system that is so crucial in effecting motivated behaviors. Nicotine – the exogenous ligand derived from tobacco for which nicotinic acetylcholine receptors (nAChR) are named – is also an anorectic agent and these nAChR are enriched

throughout the VTA (Figlewicz et al., 2008; Konner et al., 2011; Labouebe et al., 2013). In mammals, nAChR are pentameric proteins consisting of at least one of nine α-subunits (α2-α10) and sometimes one or more β-subunits (β2-β4) (Faure et al., 2014). The composition and stoichiometry of the subunits can determine a number of functional and pharmacological properties of the nAChR. For example, a heteropentamer containing an α4 and β2 subunit, with or without other subunits (henceforth, α4β2*), are heavily up-regulated in response to chronic nicotine exposure and have a high affinity for a number of nicotinic agonists (Changeux et al., 1998; Picciotto et al., 1998), while the homopentameric α7 nAChR has a relatively low affinity for ACh but rapidly activates and desensitizes once the ligand successfully binds is highly permissive of calcium entry (Changeux and Edelstein, 2005). The expression pattern of these various nAChR can greatly affect the role of ACh, and the ACh-mimetic nicotine, in influencing both DA neuron and GABA neuron activity within the VTA. DA neuron somata in the ventral tegmentum, for example, mostly express receptors containing α4, α5, α6, α7, β2, and β3 subunits while the axons of these neurons in the forebrain only express receptors containing α4, α6, and β2 subunits. GABA neurons in the VTA, on the other hand, mainly express α7 and α4β2 receptors but the axon terminals of glutamatergic cortical afferents largely express the α7 receptor alone, which has a higher affinity for nicotine than it does for ACh ($EC50_{nicotine}$: 111.3 μM; $EC50_{ACh}$: 179.6 μM). This preference for nicotine over the endogenous ligand persists in the α4β2 receptors, as well ($EC50_{nicotine}$: 5.5 μM; $EC50_{ACh}$: 68.1 μM) (Changeux and Edelstein, 2005; Di Chiara, 2000; Faure et al., 2014; Kaufling et al., 2010; Picciotto et al., 1998), which partially explains how nicotine is such a potently addictive drug of abuse.

Spontaneous tegmental DA neuron activity shifts between a basal, tonic activity with relatively low frequency of action potential firing (generally not more than 10 Hz) that is

dependent upon a pacemaker mechanism (Exley et al., 2008; Exley and Cragg, 2008; Exley et al., 2011; Exley et al., 2012) and brief, phasic activity with high frequency bursts of action potentials. These phasic bursts are, at least partially, dependent upon acetylcholine, most likely from cholinergic afferents from the laterodorsal tegmental nucleus (LDTg), as inactivation of this nucleus prevents DA neuron bursting even in the presence of glutamate (Lodge and Grace, 2006) and the posterior pedunculopontine tegmental nucleus (PPTg), which is necessary for cue-induced DA release in freely behaving rodents and can induce DA neuron bursting directly (Floresco et al., 2003; Pan and Hyland, 2005; Pan et al., 2005). The β2 subunit is necessary for setting the permissive state that allows spontaneous DA neuron activity to switch from tonic to phasic, as *β2-/-* mice show only the tonic, low-level pacemaker firing rate dissociated A10 neurons demonstrate (Maskos et al., 2005). Moreover, GABA neurons within the VTA appear to be a necessary regulator of this tonic-to-phasic activity as β2 expression in DA neurons alone does not rescue the defect of *β2-/-* mice but expression of this subunit in GABA neurons alone can rescue the phenotype (Avale et al., 2008). Additionally, the α4 subunit appears to be crucial for determining the architecture of spontaneous bursting patterns in the A10 neurons. This is putatively due to an imbalance between ACh activity directly at the DA neurons and indirectly through the GABA neurons (Exley et al., 2011), and the up-regulation of α4* nAChR following chronic nicotine exposure (Nashmi et al., 2007) supports this explanation, as one would expect prolonged nicotine exposure to require increased inhibitory control from GABAergic neurons to dampen the aberrant cholinergic drive on the DA neurons. In addition to cholinergic control of spontaneous DA neuron activity, it is likely that nAChR play a vital role in coordinating the activity of VTA neuron ensembles (Floresco et al., 2003; Lodge and Grace, 2006; Pan and Hyland, 2005; Pan et al., 2005) as subpopulations of these neurons respond differentially to the

stimuli with different motivational valence (Lammel et al., 2011; Lammel et al., 2014; Lammel et al., 2012; Ungless et al., 2004). That is, some VTA neurons respond preferentially to appetitive stimuli while other VTA neurons prefer stimuli with a more aversive character.

The Nucleus Accumbens

The NAc, or ventral striatum, is a major limbic target of tegmental dopamine neurons. This subcortical structure is divided, anatomically, into the NAc core and the NAc shell (Aragona et al., 2008; Aragona et al., 2009; Badrinarayan et al., 2012; Carlezon and Thomas, 2009; Ikemoto, 2007; Lammel et al., 2008). The shell is a comet-shaped structure with the head of the 'comet' lying medial to the NAc core and the horn, or lateral aspect, of the shell (tail of the 'comet') wrapping under the inferior aspect of the core (Baldo, 2001). While the core versus shell divisions were originally anatomically-derived, there are some functional differences between the two subregions. The NAc shell receives DA input from the more medial VTA neurons, which are not as enriched in DA transporter and D2 receptors on their axons (Ikemoto, 2007; Lammel et al., 2008). Consequently, the shell tends to have a higher frequency of spontaneous DA release events ('transients') than the core subregion (Aragona et al., 2008; Aragona et al., 2009; Badrinarayan et al., 2012; Twining et al., 2014; Wheeler et al., 2011). Moreover, stimulus-evoked DA events differ between the core and the shell. The dopamine in the NAc shell responds preferentially to an unconditioned stimulus, while the NAc core DA responses are typically phase-locked to a CS with training (Aragona et al., 2008; Aragona et al., 2009; Badrinarayan et al., 2012; Day et al., 2007; Day et al., 2006; Roitman et al., 2004; Roitman et al., 2008; Twining et al., 2014; Wheeler et al., 2011). Furthermore, the extracellular DA of the NAc shell shows a rostrocaudal gradient in the motivational valence of stimuli to

which it responds, while NAc core DA does not (Aragona et al., 2008; Badrinarayan et al., 2012; Phillips et al., 2003; Robinson et al., 2003). This is likely due to what the DA responses 'tells' the accumbens about the stimulus. The shell DA seems to encode information about the motivational salience of the stimulus, while DA in the NAc core appears to carry information about the predictive nature of the stimulus (Aragona et al., 2008; Badrinarayan et al., 2012; Loriaux et al., 2011). That is, the NAc core DA levels do not respond to the US because the unconditioned properties have no predictive value as they are what the CS is predicting.

What the core and shell subregions have in common is their cytoarchitecture. Regardless of subregion, the major projection neuron of the NAc are the GABAergic medium spiny neurons (MSNs), which are enmeshed in a matrix of GABAergic fast-spiking interneurons (FSIs) and cholinergic tonically active neurons (TANs) (Atallah et al., 2014). These TANs are of particular interest as they are synaptic targets of GABAergic afferents from the VTA (Chuhma et al., 2014) that show firing patterns reminiscent of tegmental extracellular electrophysiology during reward-learning trials. Namely, the firing pattern of both tegmental neurons and TANs appear to track differences in the expected outcome versus the actual outcome of an event or action (Atallah et al., 2014). While the tegmental neurons track reward expectation (Mirenowicz and Schultz, 1996), however, the TANs track performance, becoming increasingly quieter as the animal becomes increasingly more proficient at a task, but rebounding when reward contingency and, therefore, performance suddenly changes (Atallah et al., 2014). Interestingly, there is evidence to suggest that TANs can drive GABA release from midbrain DAergic terminals as a rapid braking system for the MSN projections (Pitman et al., 2014). Reciprocally, DAergic terminals induce a pause in the tonic activity of the TANs (Atallah et al., 2014).

Nicotine, Insulin, and the Nucleus Accumbens

While the nAChR α4 and β2 subunits are of particular importance in the VTA, the α6 and α7 subunits are major players within the NAc. As in the VTA, in the NAc, homomeric α7 nAChRs are localized largely to the axon terminals of pyramidal cell afferents from the cortex (Faure et al., 2014). However, in the NAc α7 receptors seem to be of greater influence due to greater expression levels of these proteins (Laviolette et al., 2004; Laviolette and van der Kooy, 2004) and the activity of both the cholinergic TANs as well as cholinergic afferents from the LDTg and PPTg (Laviolette et al., 2002). The α6* nAChR, especially the α6β2*, is necessary for mediating the rewarding properties of both nicotine and cocaine (Sanjakdar et al., 2014), although it is unclear whether these receptors are expressed on the MSN or FSI cell-types.

Again, it is unsurprising that feeding-related peptides would have activity within a structure like the NAc that is concerned with predicting when and where food, and other motivationally salient stimuli, will be available (Baldo, 2001; Roitman et al., 2004). While many of the effects insulin has on the NAc are mediated through alterations in tegmental DA signaling, insulin does have activity within the NAc, particularly on DA terminals where it can regulate DA transporter action through the IRS/Akt pathway discussed previously (Russo et al., 2007). Furthermore, both insulin receptors and dopamine receptors – particularly the D2 receptor – converge on Akt/PKB (Sugano et al., 2006), which suggests the possibility of crosstalk between these two intracellular signaling pathways in NAc MSNs. Concurrently, amylin – a gut peptide released with insulin – has a specific action in the NAc. Namely, intra-NAc amylin infusions shut down the motivation to feed (Baldo, 2001). Furthermore, while μ-opioid receptor activation in the NAc can induce gluttonous feeding behaviors, amylin receptor activity blocks this pharmacologically-driven feeding behavior (Baisley and Baldo, 2014).

Nicotine, Insulin, and Nematodes

While the VTA and NAc are entry points for drugs of abuse to usurp mammalian neural circuits, a command interneuron, known as AVA, is a point of vulnerability for the soil nematode, *Caenorhabditis elegans*, when it is exposed to nicotine concentrations high enough to diffuse through the cuticle. Nicotine-exposed worms increase their crawl-speed acutely; they adapt to prolonged nicotine treatment; they show withdrawal behaviors when removed from a nicotine-rich environment; and, they show sensitization to repeated nicotine administration. These early studies provided evidence of a clear role for the AVA command interneuron, and its nAChR and TRPC Channels in these behaviors (Feng et al., 2006). Another research group recently probed whether *C. elegans* nicotine chemotaxis can be used as a nematode model of motivated behaviors (Sellings et al., 2013). Their studies showed worms will chemotax up a nicotine concentration gradient towards a point-source of the drug placed directly onto the medium and worms will show a place preference for a non-paralyzing concentration of nicotine (50 μM) versus vehicle as well as conditioning a preference for butanone [a normally repellant odorant (Bargmann, 2006)] after it was paired with nicotine. This van der Kooy group also identified two nACh receptors (ACR-5, ACR-15) and two DOPamine receptors (DOP-1, DOP-2) as critical for nematode nicotine approach behavior in this paradigm (Sellings et al., 2013). These researchers posit a complicated model to explain this nicotine approach behavior in which nicotine diffuses through the cuticle to act as ligand at ACR-15 receptors expressed on the AVA command interneuron. Through gap junctions, this AVA depolarization activates the mechanosensory neural circuits that feed back onto a second command interneuron, AVB, which then releases ACh onto the B-type, cholinergic motor neurons, which express ACR-5 receptors.

This pathway, they suggest, then induces locomotion towards the point-source of nicotine (Sellings et al., 2013). Consequently, the AVA command interneuron may serve as a nematode analog to the VTA and NAc in mammals as a point of vulnerability in nicotine-responsive behaviors.

Nicotine has numerous effects on systems regulating mammalian energy homeostasis. For example, chronic nicotine users are more susceptible to insulin insensitivity and the adipocytes of these users may be insulin resistant long before other signs or symptoms of insulin insensitivity present clinically (Xu et al., 2012). Nicotine down-regulates some feeding-related peptides, such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), but up-regulates others, like cocaine-and-amphetamine-response transcript (CART) and α-melanocyte stimulating hormone (α-MSH) (Dandekar et al., 2011; Fornari et al., 2007; Kramer et al., 2007a; Kramer et al., 2007b). In fact, α-MSH acting at the melanocortin 4 receptor (MC4R) — defects in which cause an autosomal-dominant form of obesity (Pardini et al., 2006) — appears to mediate, at least in part, the orexigenic effects of chronic nicotine withdrawal in abstinent users (Bellinger et al., 2010). In addition to these feeding peptides, nicotine also stimulates the cytokines tumor necrotic factor-α (TNFα) and interleukin-6 (IL-6), which affect appetite and lipid metabolism (Wang et al., 2011). IL-6, acting independently of TNF, serves as a myokine that may influence energy homeostasis and metabolism of muscle cells and brown adipocytes (Pedersen, 2006; Pedersen and Fischer, 2007), although it is unclear whether nicotine works through this myocyte-derived IL-6. It is clear, however, that nicotine can suppress energy release from brown adipocytes (Gao et al., 2008; Gochberg-Sarver et al., 2012; Yoshida et al., 1999). Furthermore, nicotine diminishes the levels of circulating adiponectin, a peptide hormone that may suppress a number of metabolic disorders including type II diabetes, NFALD, obesity, and a host of others

(Inoue et al., 2011; Won et al., 2014). Importantly, these metabolic effects of nicotine, especially its contributions to glucose intolerance and insulin resistance, occur regardless of whether the nicotine exposure is through an active (smoking a cigarette) or passive (second-hand smoke) route (USPHS, 2008).

In *C. elegans*, the gene *daf-2* (abnormal DAuer Formation) encodes an ortholog of the receptor tyrosine kinases of the insulin receptor (IIS) family. The mammalian insulin receptor, insulin-like growth factor receptors (IGF1R and IGF2R), and insulin receptor-related receptor (INSRR) share close to 30% sequence homology with the DAF-2 receptor (Murphy and Hu, 2013). While only a single IIS ortholog exists in *C. elegans*, *daf-2* has splice variants for three distinct isoforms, each with its own putative expression pattern and specialized functions (Ohno et al., 2014). Unfortunately, we know very little about the DAF-2b and DAF-2c isoforms to date. However, DAF-2c seems to be most similar to DAF-2a, appears to be solely expressed in neurons, and is orthologous to the B isoform of the mammalian insulin receptor. The DAF-2b isoform, on the other hand, is most similar to IGFR but is also enriched in neurons (Murphy and Hu, 2013). In mammals, and likely in nematodes, IIS are an α2/β2 heterotetramer with α-subunit ligand-binding domains and β-subunit kinase domains (Ohno et al., 2014). Upon ligand binding, the kinase domains recruits an IRS protein, IST-1 (Insulin response SubsTrate homolog) that, through an adaptor protein, activates PI3K - the nematode homolog of which is AGE-1 (AGEing alteration) (Murphy and Hu, 2013). AGE-1/PI3K phosphorylates the 3' position on the inositol ring of phosphoinositide (PIP_3), thereby increasing PIP_3-dependent signaling complex activity. Thus, AGE-1/PI3K works in direct opposition to DAF-18, a phosphatase and tensin (PTEN) homolog, that dephosphorylates PIP_3 (Murphy and Hu, 2013).

A result of PIP_3 activity is the recruitment and phosphorylation of phosphoinositide-dependent kinase (PDK) - whose *C. elegans* homolog is the conveniently named, PDK-1 (Murphy and Hu, 2013). In turn, PDK-1 phosphorylates the nematode homologs of Akt/PKB, AKT-1 and AKT-2 (Murphy and Hu, 2013). These Akt homologs, however, are most similar to mammalian AKT2 than AKT1 (Murphy and Hu, 2013). Together, they act semi-redundantly in the regulation, through phosphorylation, of several target proteins or protein complexes. Among these are the Ras cell survival pathway, rictor/mTORC2 complex, and the FoxO family of forkhead box transcription factors, the only ortholog of which in *C. elegans* is DAF-16 (Murphy and Hu, 2013). Phosphorylation of DAF-16/FoxO prevents the translocation of this transcription factor to the nucleus, sequestering it to the cytosol (Murphy and Hu, 2013). Importantly, loss of function mutations in *daf-16* suppress the phenotypes seen when the function of any kinase upstream of the transcription factor is disrupted (Murphy and Hu, 2013), which implies DAF-16/FoxO activity is required for these phenotypes dependent upon insulin signaling. **Fig 1.1** summarizes this kinase cascade.

Transient Receptor Potential Channels in Drug-Related Behaviors

Transient Receptor Potential Channels in Drug Dependence

In humans, drugs of abuse target different neurotransmitter systems, but they all converge on midbrain DA neurons in the VTA or in the projections of these neurons to forebrain structures, such as the amygdala, striatum, especially the nucleus accumbens, and prefrontal cortex (Lammel et al., 2008). Some drugs have a straightforward action on DA signaling, such as cocaine and amphetamine, which act as indirect monoamine agonists by blocking the clearance of DA from the parenchyma, thereby prolonging the activity of the transmitter at its cognate

receptors (Porter-Stransky et al., 2011; Stuber et al., 2005). The action of other drugs, such as nicotine and ethanol, seems to be more complex. These drugs mainly interact with GPCRs, monoamine transporters, or alter the function of ion channels to modulate DA levels in appetitive motivation (Luscher and Ungless, 2006), learning (Jones et al., 2010), and executive control circuits in the brain (Koob, 2010). An increasing number of studies suggest that transient receptor potential (TRP) channels are important targets of second messengers in these mammalian neural circuits that become compromised in addiction.

TRP channels are perhaps best known for their role as one of the prominent protein superfamilies modulating sensory signaling pathways (Montell, 2001, 2005; Nilius and Owsianik, 2011). The members of the TRP channel superfamily have six transmembrane domains that form homo- or heterotetrameric cation channels, with strong homology to its founding member, the *Drosophila* protein, TRP. The TRP superfamily includes seven subfamilies: canonical (TRPC), vanilloid (TRPV), ankyrin (TRPA), melastatin (TRPM), polycystin (TRPP), MucoLupin (TRPML) and NompC-like (TRPN). These functionally divergent, non-selective cation channels are conserved from nematodes to vertebrates and are considered to be coincidence detectors and convergent signal integrators (Kang et al., 2010; Xiao and Xu, 2009) (for summary, see **Fig 1.2**). The diverse activation mechanisms and biophysical properties of different TRP family members allow these proteins to modulate complex behaviors, especially behaviors related to drug-seeking and drug-taking. (Cavalie, 2007; Gulbransen et al., 2008; Oliveira-Maia et al., 2009). Here, we outline the emerging role for TRP channels in drug dependence.

Canonical TRP (TRPC) channels in drug dependence

Of the TRP channel superfamily, TRPC channels are most closely homologous to the *Drosophila* TRP, the founding member of the TRP channel superfamily (Montell and Rubin, 1989). These channels are mainly activated in a phospholipase C (PLC)-dependent manner (Venkatachalam and Montell, 2007). In humans, there are six TRPC channels that form homo- and heterotetramers (Venkatachalam and Montell, 2007). These are multi-functional channels implicated in the regulation of diverse physiological functions, such as kidney filtration, acrosomal reaction, vascular tone and pheromone recognition (Nilius and Owsianik, 2011). Specific to drug dependence, genome-wide association (GWA) studies between smoker and non-smoker cohorts implicate TRPC channels in nicotine addiction. These studies particularly identify the TRPC7 channel among other novel genes that were previously not associated with addiction (Bierut et al., 2007; Lessov-Schlaggar et al., 2008). TRPC7 is enriched in brain tissue, especially in striatal regions where it impinges on neurons imperative for behavioral responses to drugs of abuse (Numaga et al., 2007). Interestingly, another GWA study implicates TRPC4 in drug dependence, based on comparisons between European-American and African-American polysubstance abusers or non-abusing controls (Uhl et al., 2008). TRPC4 is important for the vasorelaxation of arteries and neurotransmitter release from thalamic dendrites (Cavalie, 2007).

While direct evidence demonstrating a role for mammalian TRPC channels in drug addiction is still lacking, rodent fear-learning studies reveal a clear role for TRPC5 in forming associations between an unconditioned stimulus (US) and a conditioned stimulus (CS) in the amygdala (Riccio et al., 2009). The amygdala is critical for learning associations between the CS and US (Schafe et al., 2005), and human drug users show event-related potentials (ERP) viewing drug-related paraphernalia similar to the ERPs they show when viewing positive emotional

stimuli (Dunning et al., 2011). In a functional MRI study, the amygdala showed decreased focal signal in response to an unpredicted cocaine administration (Breiter et al., 1997).

Cocaine modulates intrinsic plasticity of accumbens neurons (Kourrich et al., 2007) and affects metabotropic glutamate receptor (mGluR)-dependent synaptic plasticity in the nucleus accumbens (Huang et al., 2011) and prefrontal cortex (Huang et al., 2007). TRPC1 is an mGluR target in cerebellar Purkinjie cells (Kim et al., 2003), while both TRPC3 and TRPC7 are known targets of mGluR activity in striatal cholinergic interneurons (Berg et al., 2007). Moreover, TRPC5 mRNA is located within the shell subregion of the nucleus accumbens (Fowler et al., 2007), which is preferentially activated by cocaine (Aragona et al., 2008) and is particularly responsive to the unconditioned aspects of stimuli (Wheeler et al., 2011). It will be interesting to test whether TRPC channels have a role in the motivational, learning and executive control circuits drugs of abuse undermine when recreational drug users succumb to addiction.

The most direct evidence supporting a role for TRPC channels in drug-related behaviors comes from the nematode *Caenorhabditis elegans*. *C. elegans* requires the TRPC homologues TRP-1 and TRP-2 for nicotine-dependent behaviors (Feng et al., 2006). The *C. elegans* genome encodes members of all the seven TRP channel subfamilies (Xiao and Xu, 2009). Most of these members are involved in various chemosensory or mechanosensory pathways, either as primary sensors or as signal transducers or amplifiers (Xiao and Xu, 2009). There are three TRPC subfamily members in *C. elegans*: TRP-1, TRP-2 and TRP-3. While TRP-3 is enriched in sperm, the neuronally-expressed TRP-1 and TRP-2 modulate nicotine-dependent behavior in *C. elegans* (Feng et al., 2006; Xu and Sternberg, 2003).

C. elegans exhibits a variety of behavioral responses to nicotine, including acute response, adaptation, withdrawal and sensitization. Specifically, acute nicotine treatment

stimulates locomotion (Feng et al., 2006), an innate behavior that forms the foundation of most, if not all behaviors (Piggott et al., 2011). Repeated intermittent administration of nicotine sensitizes *C. elegans* to nicotine, and long-term nicotine treatment elicits tolerance to the drug (Feng et al., 2006). Nicotine-adapted worms exhibit hyperlocomotion when placed in a nicotine-free environment, a withdrawal response to nicotine (Feng et al., 2006). These nicotine dependent behaviors require the *C. elegans* nicotinic acetylcholine receptor (nAChR) genes *acr-15* and *acr-16* (Feng et al., 2006). Both genes function in neurons to modulate nicotine responses in worms. Notably, *trp-1* and *trp-2* mutant animals are severely defective in nicotine dependent behaviors (Feng et al., 2006). Interestingly, TRP-1 and TRP-2 appear to act downstream of the nAChRs ACR-15 and ACR-16 in a PLC-dependent manner (Feng et al., 2006). This work further demonstrates that neuronal expression of ACR-15 and ACR-16 as well as TRP-1 and TRP-2 are required for nicotine-induced behaviors in *C. elegans* (Feng et al., 2006). Moreover, neuronal Ca^{2+} influx is greatly diminished in response to nicotine exposure in *trp-1* or *trp-2* null mutant worms, suggesting that these TRPC channels functionally regulate neuronal nicotine responses (Benowitz, 2008; Feng et al., 2006). Interestingly, the mouse $\alpha4\beta2$ nAChR, which is known to be essential for nicotine-associated behaviors, can rescue nicotine behavioral defects in *acr-15* null mutant animals; similarly, the human TRPC3 channel functionally substitutes for worm TRP-2 in nicotine responses (Feng et al., 2006), suggesting that the role of TRPC channels and nAChRs in nicotine responses may be evolutionarily conserved.

In addition to this functional interaction with nAChR, TRPC channels interact with both CREB and Homer proteins, which are important for gene transcription related to drug dependence and drug-related changes in neural plasticity (Pandey et al., 2005; Ron and Jurd, 2005; Talavera et al., 2008). Both TRPC3 and TRPC6 overexpression potentiate phosphorylation

of CREB which stimulates both early and late CREB-dependent gene transcription (Jia et al., 2007). The role of this CREB-dependent transcription in drug-induced neural plasticity is well documented (Kumar et al., 2011; Philpot et al., 2012). Homer proteins are a group of EVH1 domain-containing scaffolding proteins involved in coupling metabotropic glutamate receptors (mGluR1) and inositol-1,4,5-triphosphate receptors (IP_3R) with TRPC channels (Mast et al., 2010; Yuan et al., 2003). Homer-IP_3R interactions regulate trafficking of TRPC3 to the plasma membrane, while coupling of mGluR and IP_3R with TRPC channels results in mGluR-mediated neuronal conductance, which may have a role in drug-related behavioral plasticity (Kim et al., 2006). Together, these data make a case for more in-depth studies of mammalian TRPC channels in relation to drugs of abuse.

Vanilloid TRP (TRPV) channels in drug dependence

TRPV channels share homology with the founding member of the subfamily, TRPV1, which was identified through its response to the vanilloid capsaicin. These channels respond to a range of stimuli, such as heat, mechanical stimulation, and pro-inflammatory agents as well as other chemical stimuli (Kauer and Gibson, 2009; Venkatachalam and Montell, 2007). Mammalian neurons expressing TRPV1 show a decrease in the amplitude of capsaicin-induced action potentials after acute nicotine treatment. Moreover, repeated and intermittent nicotine treatment sensitizes capsaicin-induced currents in these cells (Liu et al., 2004). Moreover, TRPV1 is also known to interact with many nAChRs and is associated with anxiogenic behavioral responses, indicating that this channel might be responsible for the anxiety and 'nervousness' associated with nicotine withdrawal responses (Casarotto et al., 2012). Besides, the TRPV1 activity is potentiated by ethanol, and *Trpv1* null mutants show higher preference to

ethanol and higher consumption in two-bottle choice assays as compared to wild-type mice (Blednov and Harris, 2009). These findings suggest the role for TRPV1 channels in specific behaviors associated to ethanol dependence.

In invertebrates, the *Drosophila* TRPV homologue *inactive* (*iav*) mediates behavioral sensitization to cocaine (McClung and Hirsh, 1998). In this model, stereotypical behavioral responses to cocaine include intense grooming at low doses, with moderate doses affecting rapid rotations and sideways or backward movements. High doses, in turn, result in tremors and paralysis. With repeated cocaine administration, these behaviors become more vigorous in response to decreased cocaine concentrations. This behavioral sensitization, however, is not present in *iav* null mutants despite a wild-type response to acute cocaine exposure (McClung and Hirsh, 1998). This sensitization deficit appears to result from decreased levels of the monoamines tyramine and octopamine, implicating TRPV proteins in the regulation of monoamine neurotransmitter systems (McClung and Hirsh, 1999). Thus, both invertebrate studies and the findings in rodents suggest TRPV proteins as targets for understanding the action that drugs of abuse have on the brain.

In mammals, the endocannabinoid anandamide (AEA) activates not only the CB1 and CB2 GPCRs but also TRPV1. A recent study demonstrates that TRPV1 is critical for long-term depression (LTD) of medium spiny neurons (MSN) in the rodent nucleus accumbens and cocaine administration disrupts this phenomenon (Grueter et al., 2010). TRPV1 channels are also critical for coupling ACh signals with the endocannabinoid 2-archidonylglycerol (2AG) in the striatum. This coupling is vital for both LTD and long-term potentiation (LTP) at corticostriatal synapses (Musella et al., 2010). Furthermore, the TRPV1 agonist capsaicin induces LTP in the amygdala (Zschenderlein et al., 2011). In addition, repeated methamphetamine exposure increases TRPV1

mRNA within the prefrontal cortex (Tian et al., 2010), a brain region responsible for inhibiting unwanted actions whose dysfunction can lead to hyperactivity and compulsive behaviors such as drug-taking (Koob, 2009b). Collectively, these studies suggest that TRPV1 channels may play a role in usurping natural motivational, learning and executive control circuits to effect addiction.

Other TRP channel subfamilies in drug dependence

Besides TRPC and TRPV subfamilies, other TRPs (mainly TRPA and TRPM) are involved either in primary sensing of addictive drugs or in their long-term effects. In vertebrates, nicotine activates both TRPM5-dependent and independent gustatory pathways. The TRPM5-dependent mechanism affects a general taste pathway and is required for nicotine-specific behavioral and gustatory cortex circuit responses. It has also been shown to be involved in peripheral sensing of nicotine in the nasal cavity (Gulbransen et al., 2008; Oliveira-Maia et al., 2009).

TRPA1, meanwhile, is involved in nicotine-induced irritation and facilitates the mouse airway constriction reflex to nasal administration of nicotine (Talavera et al., 2008). This channel is also known to be responsible for the airway neurological inflammation caused by α,β-unsaturated aldehydes, one of the main caustic agents in cigarette smoke (Andre et al., 2008). These facts make TRPA1 a potential nicotine target for developing smoking cessation therapeutics with milder side effects. While TRPA1 acts as an irritant-sensing channel in cigarette smoke, the menthol receptor TRPM8 acts as a counterirritant channel in menthol-flavored cigarettes (Willis et al., 2011). Activation of TRPM8 by menthol suppresses the irritant sensation caused by TRPA1 during smoking, thus masking the caustic irritants and promoting smoking behavior. These differential actions of TRP channels in the periphery might be

important in the preliminary stages of nicotine dependence. In addition, ethanol inhibits TRPM8, while potentiating the activity of TRPV1 (Benedikt et al., 2007). Further evidence of the complicated role TRP channels play in drug use is seen with 'hangover pain', a pathological symptom after ethanol consumption, which is mediated by TRPA1 (Bang et al., 2007).

The effects of addictive drugs on primary targets, such as their cognate receptors, and secondary targets, such as kinases and lipases that those receptors modulate, are well known. However, the role of those gene families with less obvious involvement in drug addiction, such as TRP family channels, remains unclear. Interestingly, there is growing evidence implicating TRP channels in drug dependence. TRPC channels, in particular TRPC4/7 were identified in two GWA studies. Similarly, two *C. elegans* TRPC homologues (TRP-1 and TRP-2) are essential for nicotine dependent behaviors, and their mammalian counterparts can functionally substitute for them, suggesting a functional conservation among species.

On the other hand, TRPV channels are implicated in the control of extracellular monoamine levels, as well as in anxiety-related behaviors, suggesting that these channels might be responsible for the neural changes that lead to the adverse effects of withdrawal and behavioral sensitization following repeated drug use. This TRP channel subfamily is not only involved in behavioral responses to several drugs of abuse, but also performs conserved roles in motivational, learning and executive control circuits usurped by drugs of abuse to elicit addiction. It is important to note that many more members of the TRP superfamily are implicated in responses to drugs of abuse both at the primary sensing level (TRPA1 and TRPM8) and in maintaining long-term neural changes (TRPM5). These properties, with the ever-growing evidence related to their association with drugs of abuse, support a role for TRP channels in the

development of drug dependence, and further investigation is needed to fully understand their functional significance in addiction.

C. elegans as a Model for Nicotine-Responsive Behaviors

Here, we have touched briefly upon the fundamental psychological processes believed to effect addictive behaviors in mammals. We have also given a superficial description of only two of the brain regions dysregulated when an individual moves from recreational drug use into compulsive, addictive behaviors. While rodent and primate models will always be vital to understanding these processes, the complexity of their physiology and their psychology present certain challenges. To sidestep these obstacles, we elected to use the soil nematode, *C. elegans*, to evaluate simple models of drug-related behaviors – namely, the stimulation of locomotor response to acute nicotine challenge and a native olfactory approach behavior. *C. elegans* is an hermaphroditic species that is capable of self-fertilization, so has no real motivation to find a mate and it obtains water from the medium and bacterial lawn on which it grows (Avery and You, 2012; Fitch, 2005; Schafer, 2005), so its motivated behaviors – its psychology, in a manner of speaking – is limited to maintaining a readily accessible supply of food and avoiding noxious stimuli.

The nematode physiology is equally simple (Coghlan, 2005), which is not to say they are any less elegant in their evolutionary niche than any other species. *C. elegans* grows to approximately 1 mm in length and has an egg-to-egg generation time of roughly 4 days (Fitch, 2005), making genetic manipulations much faster than in primates, rodents, or even flies. The hermaphrodite nervous system consists of 302 neurons (Hobert, 2010) and the translucent cuticle makes the neurons readily accessible for inspection. Furthermore, thanks to sectioning with serial

electron microscopy (White et al., 1986), the complete *C. elegans* connectome is available. Moreover, these nematodes respond similarly to many drugs of abuse in a manner commensurate with their mammalian counterparts (Feng et al., 2006; Sellings et al., 2013; Sobkowiak et al., 2011; Ward et al., 2009). For example, in response to nicotine, *C. elegans* exhibits acute locomotor stimulation, shows tolerance and adaptation to prolonged exposure, and demonstrates a withdrawal response once the drug is removed (Feng et al., 2006). Furthermore, worms will approach a source of nicotine by following the increasing concentration gradient (Sellings et al., 2013). Accordingly, we delved more deeply into the nematode acute nicotine response, identifying an effect of defective insulin signaling mutants and we identified TRPV family protein, with no previously characterized phenotype, that plays a crucial role in a nicotine olfactory response and chemotaxis.

Insulin Family Receptor Signaling in *C. elegans*

Fig 1.1

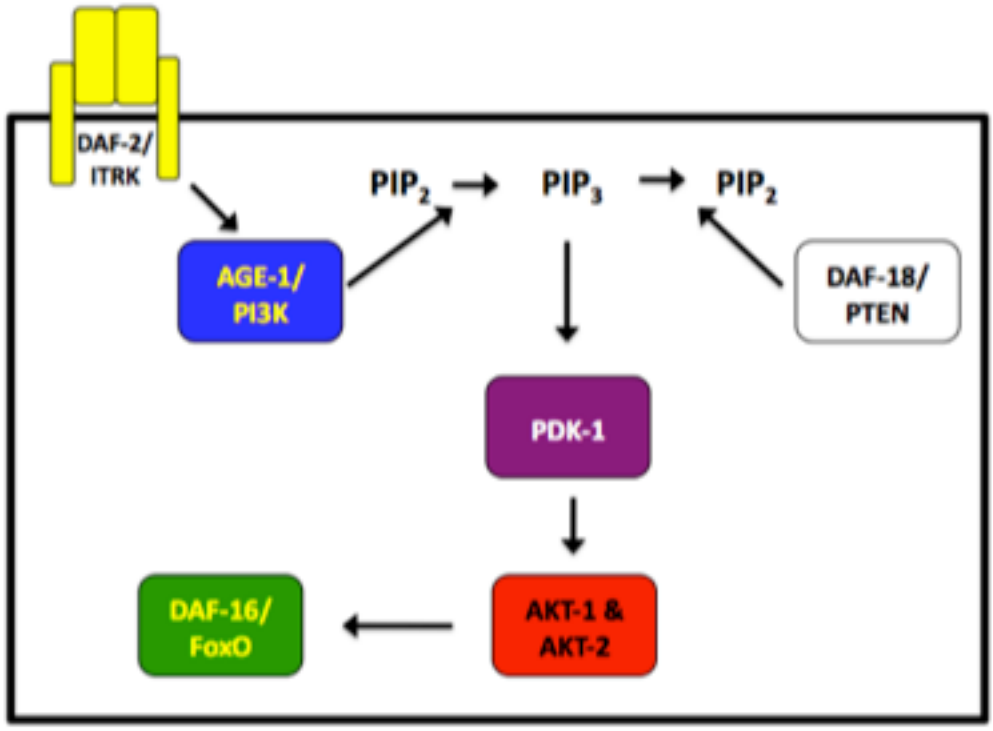

Fig 1.1 illustrates the intracellular signaling cascade following ligand activation of the insulin family receptor, DAF-2, in the nematode, *C. elegans*. Once a peptide hormone binds to the IIS, accessory proteins recruit and activate AGE-1/PI3K. In converting PIP_2 to PIP_3, AGE-1/PI3K activates PDK-1, which targets the PKB homologs, AKT-1 and AKT-2. The Akt homologs, in turn, phosphorylate DAF-16/FoxO, preventing its translocation to the nucleus.

TRP Channel Phylogenetic Tree

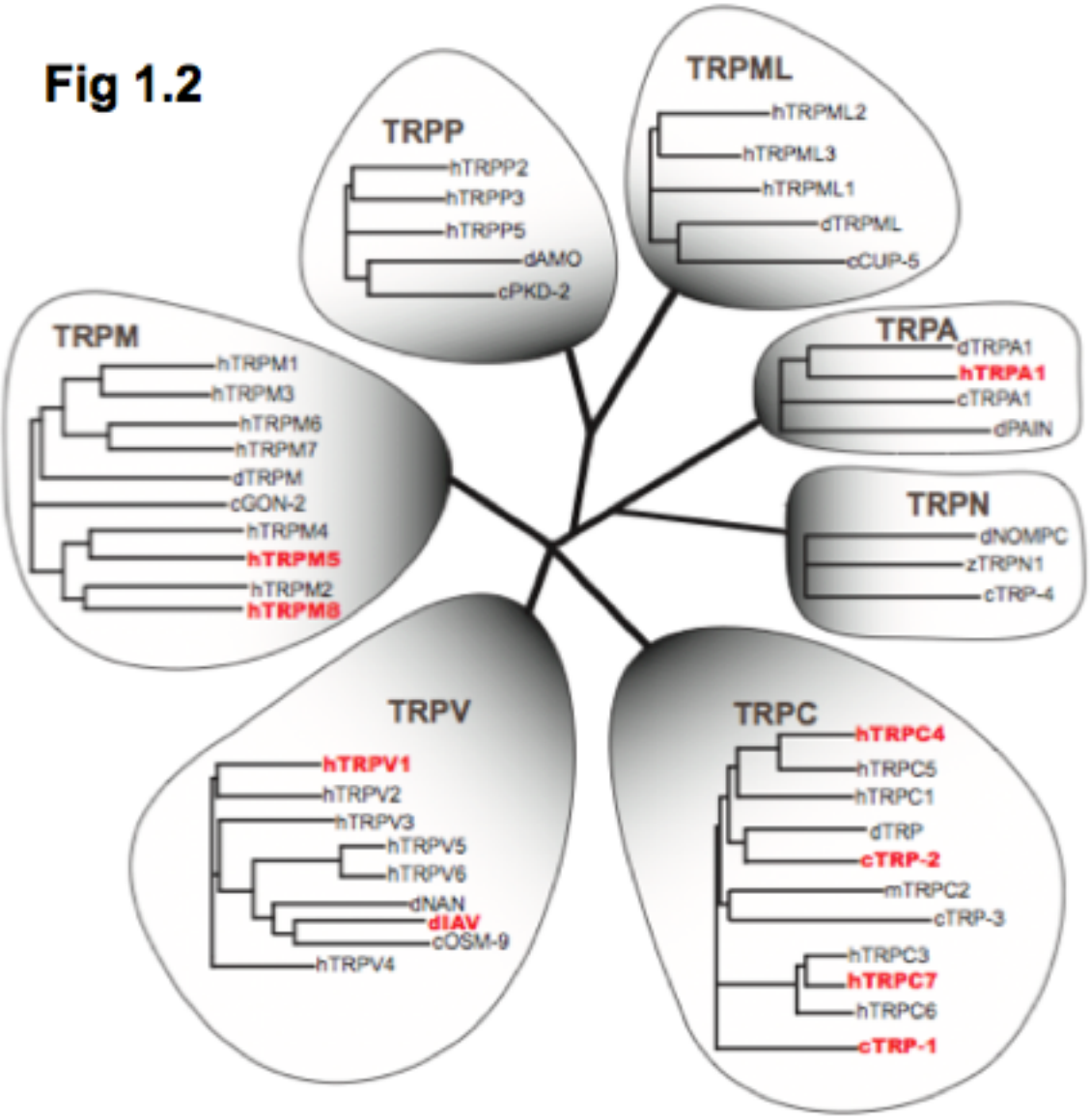

Fig 1.2 shows a TRP channel phylogenetic tree with all human TRP channels and representatives from other species. Proteins in red are implicated in addiction biology. The letter before the protein name indicates the species — c: *C. elegans*; d: *Drosophila melanogaster*; h: *Homo sapiens*; m: *Mus musculus*; z: *Danio rerio*.

Chapter II

Insulin Signaling Genes Modulate Nicotine-Responsive Behaviors in *C. elegans*

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 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 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 reliable predicts risk for type II diabetes, metabolic syndrome, non-alcoholic fatty liver disease (NFALD), coronary artery disease, and cerebrovascular disease (Kahn et al., 2014).

As described previously, in *C. elegans*, the gene *daf-2* encodes an ortholog of the receptor tyrosine kinases of the insulin receptor (IIS) family. While only a single IIS ortholog

exists in *C. elegans*, *daf-2* has splice variants for three distinct isoforms, each with its own putative expression pattern and specialized functions (Ohno et al., 2014). Unfortunately, we know very little about the DAF-2b and DAF-2c isoforms to date. However, DAF-2c seems to be most similar to DAF-2a, appears to be solely expressed in neurons, and is orthologous to the B isoform of the mammalian insulin receptor. The DAF-2b isoform, on the other hand, is most similar to IGFR but is also enriched in neurons (Murphy and Hu, 2013). In mammals, and likely in nematodes, IIS are an α2/β2 heterotetramer with α-subunit ligand-binding domains and β-subunit kinase domains (Ohno et al., 2014).

The insulin signaling pathway plays a crucial role in a host of physiological processes, including lifespan and aging (Murphy and Hu, 2013); 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). However, despite the interaction of many, or even most, of these processes with nicotine use in humans, very little work exists exploring these connections. Therefore, we present studies here that seek to investigate a link between a behavioral response to an acute nicotine challenge and the insulin signaling pathway.

Methods

Acute Nicotine-Induced Locomotion Assay

For the acute nicotine locomotion assay, we grew worms on nicotine-free NGM with a 200 µL 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 worm per plate containing a thinly-spread lawn of 40 µL OP50 and the appropriate nicotine

concentration. An automated system tracked the worm as it crawled freely on the plate for 16 minutes. As described previously (Feng et al., 2006), we discarded the first 11 minutes as the worm 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 AGE-1/PI3K inhibitor. We excluded from analysis any worm that crawled up the side of the plate at any point. Each group consists of 20 animals.

Nicotine Locomotor Sensitization Assay

For the locomotor sensitization assay, we grew and picked worms as in the acute locomotor assay. On the day of test, however, we transferred worms to a clean plate containing a lawn of OP50 and either 100 μM nicotine or vehicle for 1 hour before transferring them to a second clean, nicotine-free plate for an additional hour. Finally, the worms crawled freely on a plate covered in 40 μL OP50 and a 10 μM Nicotine concentration. We then collected and analyzed the data as in the acute locomotor assay.

Subjects and Strains Used

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 μM cholesterol, 1 mM Ca(CH3COO)2, 1mM MgSO4, 25 mM KH2PO4, pH 7.0] seeded with *Escherichia coli* (OP50) at 20ºC (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 the following strains: 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).

Results

Wild-Type Nematodes Show Nicotine-Induced Locomotor Stimulation

Previously, our lab demonstrated that an acute nicotine challenge induces increased locomotion in naïve nematodes. This increased locomotor response depends upon the nAChR, ACR-15, and the TRPC homologs, TRP-1 and TRP-2 within the AVA command interneuron (Feng et al., 2006). Here, we began by replicating these data. As seen in **Fig 2.1**, naive, wild-type nematodes increase their crawl speed in response to an acute nicotine challenge. This acute nicotine response is dose-dependent, with an optimal response to 100 μM nicotine and nicotine-induced paralysis at 1000 μM nicotine.

Human drug users often experiment with one or more drugs of abuse but, for many of these individuals, drug use will remain recreational without devolving into addiction. However, drug-naïve individuals who experience a high initial dose of drug succeeded by a period of abstinence are more likely to slide into use of the drug compulsively and to exhibit more destructive addiction-related behaviors (Koob and Le Moal, 2005; Robinson, 2004). We, therefore, attempted to model this single-dose sensitization in *C. elegans*. To do so, we pre-exposed worms to 100 μM nicotine for one hour followed by a one hour period of nicotine abstinence. Following this second hour, we then tracked the locomotor response of the worms to 10 μM nicotine – a concentration that does not stimulate locomotion in naïve animals. **Fig 2.2** shows that animals pre-exposed to a single, high dose of nicotine before the nicotine challenge

respond to a 10 μM nicotine dose as though it were the 100 μM nicotine dose. This suggests that nematodes, like mammals, are also sensitive to a single-dose regimen for nicotine sensitization.

DAF-2/IIS Hypomorphs Reduce Crawl Speed in Response to Acute Nicotine

The insulin signaling pathway affects neural circuits that underlie motivated and addiction-related behaviors in mammals, especially the mesolimbic DA system (Daws et al., 2011). Accordingly, we sought to determine whether strains carrying hypomorphic alleles for the nematode IIS homolog, *daf-2*, had abnormal acute nicotine responses. In **Fig 2.3** we see that, unlike wild-type animals, when worms carrying alleles hypomorphic for the IIS gene, *daf-2(e1368)* or *daf-2(e1370)*, show a dose-dependent decrease in locomotor response, which does not differ between the two alleles (data not shown). This is not merely a defective acute nicotine response, rather *daf-2*/IIS mutants show a nicotine phenotype that appears to be a nearly complete inversion of the wild-type nicotine phenotype. Moreover, this inverted nicotine phenotype sensitizes in *daf-2*/IIS mutants with previous high (100 μM) concentration nicotine exposure (**Fig 2.4**). As such, not only do DAF-2/IIS mutants show an inverted nicotine phenotype, but this behavioral inversion is also susceptible to single-dose sensitization.

AGE-1/PI3K Underperformance Yields Inverted Nicotine Phenotype

Upon ligand-binding at DAF-2/IIS, the nematode IRS homolog, IST-1, recruits and phosphorylates AGE-1/PI3K, which catalyzes the conversion of PIP_2 to PIP_3. As the first major kinase target of the IIS, we next tested whether a line carrying a hypomorphic allele of *age-1* would decrease crawl speed in response to an acute 100 μM nicotine challenge. Nematodes with an under-functioning AGE-1/PI3K show the same inverted nicotine phenotype as the *daf-2*/IIS

mutants (**Fig 2.5**). As the genetically under-performing AGE-1/PI3K results in nematodes crawling slower in response to nicotine, we asked whether we could effect a similar outcome pharmacologically. To do so, we grew wild-type worms overnight on the AGE-1/PI3K inhibitor, wortmannin, and then tested the response of these worms to 100 μM nicotine. As shown in **Fig 2.6**, while wild-type worms exposed to nicotine and a low (10 nM) dose of wortmannin responded to nicotine with an increased crawl speed, wild-type animals exposed to nicotine and a moderate dose of wortmannin (100 nM) recapitulated the decreased locomotor response seen from *age-1*/PI3K mutants. A high (1000 nM) dose of wortmannin appeared to sicken the worms, which resulted in no locomotor changes between worms in the nicotine or control conditions. Meanwhile, as an AGE-1/PI3K hypomorph, which converts PIP_2 to PIP_3, shows a decreased crawl speed in response to nicotine, we reasoned that loss of function in the PTEN homolog, DAF-18, which works antagonistically to AGE-1/PI3K converting PIP_3 back to PIP_2, would induce an exaggerated nicotine response. That is, it would increase crawl speed in response to nicotine above wild-type levels. However, as **Fig 2.7** shows, we found instead that *daf-18*/PTEN mutants show a blunted, but not inverted, nicotine phenotype. Therefore, a nematode with underperforming AGE-1/PI3K reduces its crawl speed in response to an acute nicotine challenge regardless of whether the source of underperformance derives from a genetic or pharmacologic source. However, hyperactivity of AGE-1/PI3K, through loss of the antagonizing phosphatase, does not reverse the polarity of this inversion.

Kinases Downstream of AGE-1/PI3K Yield the Inverted Nicotine Phenotype

Once AGE-1/PI3K converts PIP_2 to PIP_3, PIP_3 activates the hypomorphic PDK ortholog, PDK-1, As such, we next turned our attention to this kinase and its downstream targets. **Fig 2.8**

shows that PDK-1 hypomorphic animals exhibit the same decreased locomotor response to nicotine as *daf-2*/IIS and *age-1*/PI3K mutants. It was logical, then, to investigate targets of PDK, which include Akt/PKB. The *C. elegans* genome encodes two Akt homologs involved in the IIS signaling pathway, *akt-1* and *akt-2*. Loss of function in either *akt-1* or in *akt-2* also shows the decreased locomotor phenotype in response to an acute nicotine challenge (**Fig 2.9**). Therefore, the kinases downstream of AGE-1/PI3K, *pdk-1*, *akt-1*, and *akt-2* all show the same inverted behavioral phenotype in response to acute nicotine challenge as *daf-2*/IIS.

DAF-16/FoxO Nuclear Activity is Critical for a Normal Nicotine Phenotype

The ultimate target of the IIS signaling pathway is the FoxO family of transcription factors. In *C. elegans*, *daf-16* encodes the sole FoxO transcription factor. In lifespan and dauer formation assays, *daf-16*/FoxO mutations rescue the phenotypes of *daf-2*/IIS mutants (Murphy and Hu, 2013). We, therefore, expected worms carrying one of the loss of function *daf*-16/FoxO mutations to show the reduced crawl speed in response to acute nicotine challenge. However, while strains carrying either of the null alleles, *daf-16(mu86)* or *daf-16(mgDF46)*, showed defects in their acute nicotine response (**Fig 2.10**), these *daf-16*/FoxO mutants did not show the decreased locomotor response observed from DAF-2/IIS mutants. As these two alleles did not differ from one another, we present them here collectively. With the disparity in the *daf-2*/IIS and *daf-16*/FoxO 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*/IIS and *daf-16*/FoxO genes act in the same pathway in this nicotine-responsive behavior, we expect the *daf-2;daf-16* double mutant should perform similarly to the *daf-16*/FoxO mutant alone. If, however, the two genes are functioning in distinct pathways for this nicotine-responsive behavior, we expect a double

mutant phenotype that is intermediate to the individual single mutants. As **Fig 2.11** demonstrates, *daf-2;daf-16* double mutants show the same nicotine defect we see with the *daf-16*/FoxO mutants suggesting these two genes, *daf-2*/IIS and *daf-16*/FoxO, are functioning in the same pathway here, as well. Therefore, while *daf-16*/FoxO is downstream of *daf-2*/IIS in the signaling pathway mediating the acute response to nicotine challenge, *daf-16*/FoxO shows a defective, but not inverted, phenotype in this nicotine responsive-behavior.

Discussion

In *C. elegans*, the insulin receptor and the insulin-like growth factor have not yet diverged, and the product of the *daf-2*/IIS gene functions as a single receptor tyrosine kinase that is responsive to the approximately 40 insulins encoded in the genome of these animals (Murphy and Hu, 2013). In mammals, all of the known insulin family tyrosine receptor kinases activate the PI3K/PDK/Akt kinase cascade (Sugano et al., 2006), which is the major kinase target of DAF-2, as well (Murphy and Hu, 2013). Interestingly, there is evidence to suggest that nAChR activation of this same kinase cascade might mediate the neuroprotective effects of nicotine in aging neurons (Davis et al., 2010; Daws et al., 2011). Moreover, we know that weight-gain is the reason cited most often for relapse following tobacco cessation (Bergman et al., 2012), which implicates an interaction between cholinergic signaling pathways and the IIS signaling pathways.

The studies presented here replicate the wild-type locomotor stimulation in response to nicotine our lab has described previously (Feng et al., 2006). We show that wild-type *C. elegans* dose-dependently increases crawl speed in response to an acute nicotine challenge, with the highest doses of nicotine having a paralytic effect on the worms. Additionally, while we have yet

to explore it in greater detail, we show here that this acute locomotor nicotine phenotype is prone to single-dose sensitization. This brief, high-dose exposure to nicotine, preceding an abstinent period before a low-dose challenge is commensurate with human addiction-prone, drug-taking behaviors. That is, individuals who cycle through periods of drug binge and abstinence may be more likely to devolve into destructive addiction-related behaviors than lighter, if more consistent, recreational users (Koob and Le Moal, 2005; Robinson, 2004).

When we turned our attention to worms carrying reduction-of-function alleles for the IIS ortholog gene, *daf-2*, we found that nicotine-naive nematodes yield an inverted phenotype when acutely challenged with nicotine. Rather than increasing their crawl speed as wild-type worms do, or simply ignoring the nicotine as *acr-15*/α7nAChR null mutants do, the *daf-2*/IIS hypomorphic mutants crawl slower when acutely challenged with nicotine. In these worms, nicotine appears to act as a depressant rather than as a stimulant. This effect is not present at low doses of nicotine and becomes more apparent with increasing nicotine concentration. However, the worms do not become paralyzed, despite this decreased locomotor speed. In fact, both wild-type and *daf*-2/IIS worms first show nicotine-induced paralysis at the same concentration (1000 μM). Moreover, this effect sensitizes just as the wild-type nicotine response does. When naive *daf-2*/IIS worms experience a high dose of nicotine followed by a brief period of abstinence, they respond robustly to a low dose of nicotine that elicits no response in naive animals. This nicotine response is, again, a decrease in crawl speed when challenged with the low nicotine dose.

This inverted nicotine phenotype persists throughout the kinase cascade downstream of the IIS, as well. Worms hypomorphic for the PI3K homolog, *age-1*, respond to nicotine with a slowed crawl speed and this genetic effect is dose-dependently recapitulated when wild-type worms experience nicotine in the presence of the AGE-1/PI3K inhibitor, wortmannin. As such,

both genetic and pharmacologic manipulations seem to corroborate this unique effect of an insulin signaling pathway that is under-performing when exposed to nicotine. Moreover, loss of function in the PDK ortholog, *pdk-1*, or in either of the Akt2 homologs, *akt-1* or *akt-2*, show a similar response to nicotine. For ease of inspection, **Fig 2.12** collates all of these genotypes.

The *C. elegans* genome encodes a single FoxO transcription factor, *daf-16*. This FoxO transcription is necessary for extending life-span via decreased IIS signaling in nematodes (Murphy and Hu, 2013) and is crucial in a number of other phenomena that require IIS activity as well. Accordingly, we tested two separate null alleles for *daf-16*/FoxO in this acute nicotine paradigm. In both cases, *daf-16*/FoxO mutants showed no decreased crawl speed in response to nicotine. However, strains carrying either of the null alleles were also defective in the wild-type nicotine response. As *daf-2*/IIS and *daf-16*/FoxO mutants differed phenotypically from one another, we next sought to determine whether these two genes act in the same pathway or in separate pathways in response to an acute nicotine challenge. To probe this, we used a *daf-2;daf-16* double mutant. If the two genes are acting in the same pathway, the double mutant should phenocopy the downstream gene, in this case *daf-16*/FoxO. If, however, the two genes are acting in separate pathways, the double mutant should show some form of an intermediate phenotype. As it happens, the double mutants phenocopy *daf-16*/FoxO mutants, which suggests that *daf-16*/FoxO is acting downstream of *daf-2*/IIS here as well. It is likely that the divergent phenotypes seen between these two genotypes is due to a secondary loss of activity from one of the gene products the FoxO transcription regulates.

As all of the kinases known to be between *daf-2*/IIS and *daf-16*/FoxO resulted in a decreased crawl speed, we hypothesized that a loss-of-function mutation in the PTEN homolog, *daf-18*, would yield an exaggerated increase in crawl speed to levels greater than those seen with

wild-type worms in response to nicotine. Alas, this was not the case. Rather, we found that *daf-18*/PTEN mutants neither increased nor decreased their crawl speed in response to nicotine, which phenocopies the *acr-15*/α7nAChR mutants (Feng et al., 2006) and our *daf-16*/FoxO findings.

It would seem that the most likely explanation of these findings involves a precarious balance of kinase and phosphatase activity within the cell to elicit a normal acute nicotine phenotype. Under normal conditions, the phosphorylation of DAF-16/FoxO is tightly regulated. In its unphosphorylated state, DAF-16/FoxO is free to translocate to the nucleus and induce gene transcription. Phosphorylation, however, locks DAF-16/FoxO into the cytosol. Accordingly, a loss of DAF-16/FoxO activity, either through null mutation in the *daf-16*/FoxO gene or increased phosphorylation due to decreased DAF-18/PTEN activity results in an inability to respond to acute nicotine as one or more FoxO-regulated genes are not transcribed or are transcribed ineffectively. Meanwhile, when the IIS or any of its kinases are under-performing, DAF-16/FoxO is less likely to be phosphorylated and becomes overly permissive in gene transcription, and some constellation of its gene targets interact to produce the decreased crawl speed in response to an acute nicotine challenge. This is summarized in **Fig 2.13**.

It is unlikely that nicotine is acting directly at the IIS. Rather, we expect that nicotine is acting first at a nAChR (probably ACR-15) and is inducing the release of one or more insulins. These ligands then are likely acting at the IIS on some target cell capable of regulating crawl speed. In fact, our lab recently identified a neuron that may serve to control speed generally (Li et al., 2014), and it is an attractive candidate to examine in this nicotine paradigm. With roughly 40 insulins working redundantly with each other (Murphy and Hu, 2013), however, it seems

improbable to identify a single insulin whose loss would be sufficient to cause the inverted nicotine phenotype of *daf-2*/IIS mutants.

Locomotor Response of Wild-Type Nematodes to Acute Nicotine Challenge

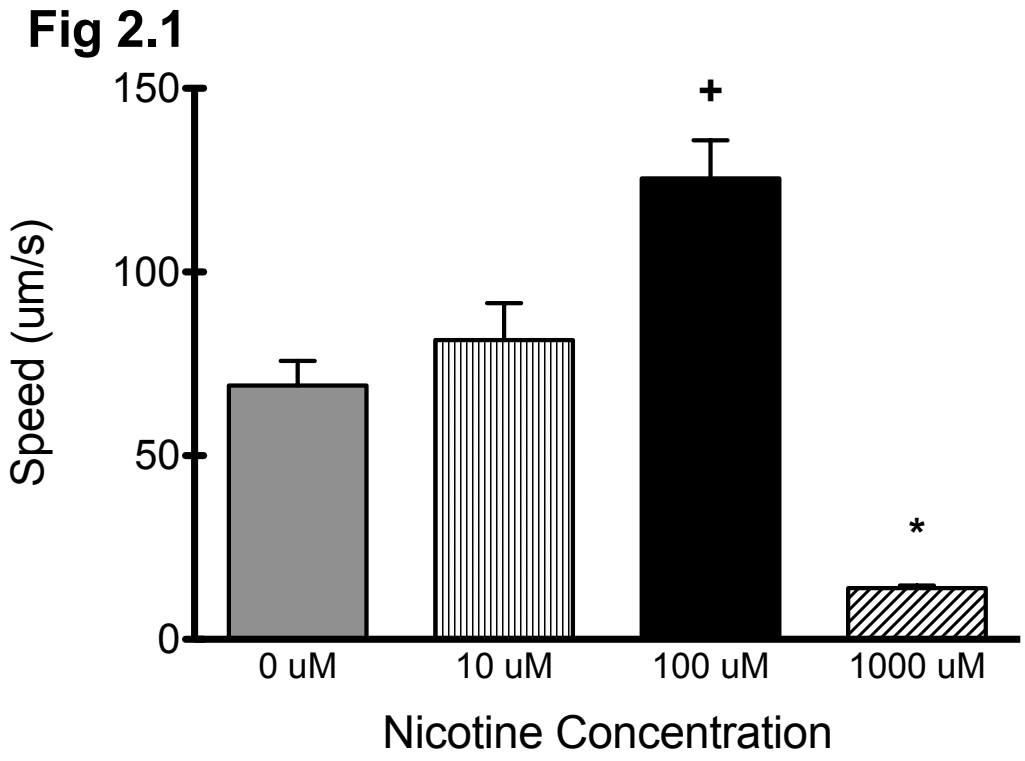

Fig 2.1 shows the locomotor response of nicotine-naive nematodes at a range of concentrations. When compared to the nicotine-free ('0 μM') group [Mean: 69.1 μm/s ± 6.88], the '100 μM' group [Mean: 125.5 μm/s ± 10.69] crawls significantly faster, while the '1000 μM' group [Mean: 13.9 μm/s ± 0.78] crawls significantly slower, most likely due to nicotine-induced paralysis at this concentration. The '10 μM' group [Mean: 81.5 μm/s ± 10.29] is not significantly different from control animals [one-way ANOVA $p < 0.0001$; *post hoc* Dunnett's MCT].

Single-Dose Sensitization of Wild-Type Acute Nicotine Locomotor Response

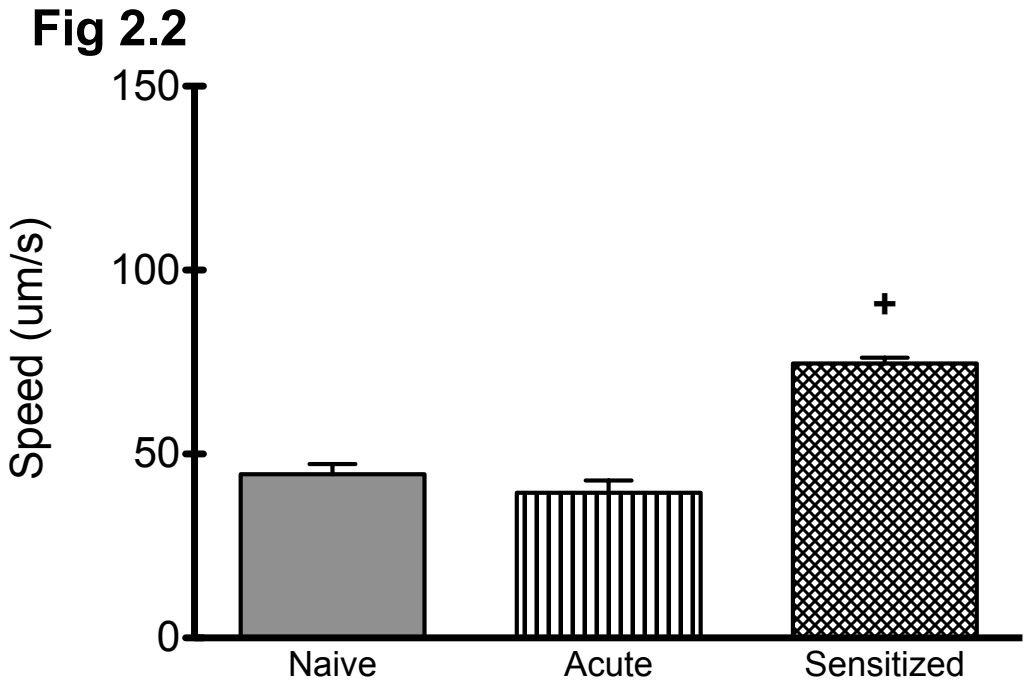

Fig 2.2 shows the locomotor response of wild-type nematodes in an acute or sensitizing nicotine paradigm. Animals in both the 'Acute' group [Mean: 39.5 μm/s ± 3.37] and 'Sensitized' group [Mean: 74.6 μm/s ± 1.62] received a 10 μM nicotine challenge. However, 'Sensitized' received a one hour pretreatment with 100 μM nicotine followed by an hour of nicotine withdrawal before the 10 μM nicotine challenge. 'Acute' animals experienced fresh, nicotine-free plates for each of these transfers. When compared to the 'Naive' group [Mean: 44.5 μm/s ± 2.87] that never experienced nicotine, the 'Sensitized' animals crawled significantly faster in response to a 10 μM nicotine challenge, while 'Acute' worms behaved no differently than the 'Naive' controls [one-way ANOVA $p < 0.0001$; *post hoc* Dunnett's MCT].

Locomotor Response of *daf-2*/IIS Mutants to Acute Nicotine Challenge

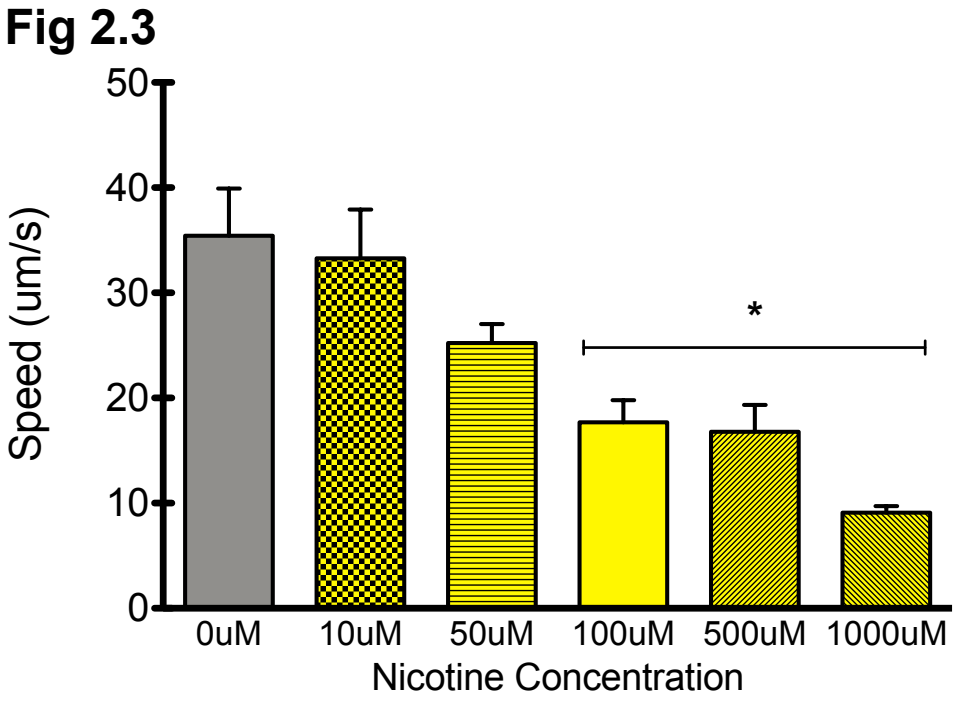

Fig 2.3 shows the locomotor response of nematode strains carrying alleles hypomorphic for the IIS ortholog, DAF-2. As the two strains tested, *daf-2(e1368)* and *daf-2(e1370),* were not different from one another, their data are presented here collectively. Neither the '10 μM' [Mean: 33.3 μm/s ± 4.73] nor the '50 μM' [Mean: 25.2 μm/s ± 1.85] groups were significantly different from the '0 μM' [Mean: 35.4 μm/s ± 4.58] group, although the '50 μM' did show a trend towards significance. The '100 μM' [Mean: 17.7 μm/s ±2.11], '500 μM' [Mean: 16.8 μm/s ±2.56], and '1000 μM' [Mean: 9.1 μm/s ± 0.63] groups were all crawled significantly slower than the nicotine-free control group when faced with an acute nicotine challenge [one-way ANOVA $p < 0.0001$; *post hoc* Dunnett's MCT]. Animals in the '1000 μM' showed signs of nicotine-induced paralysis comparable to what was seen with wild-type animals, while the '100 μM' and '500 μM' worms show normal, non-paralytic body curvature.

Single-Dose Sensitization of *daf-2*/IIS Acute Nicotine Locomotor Response

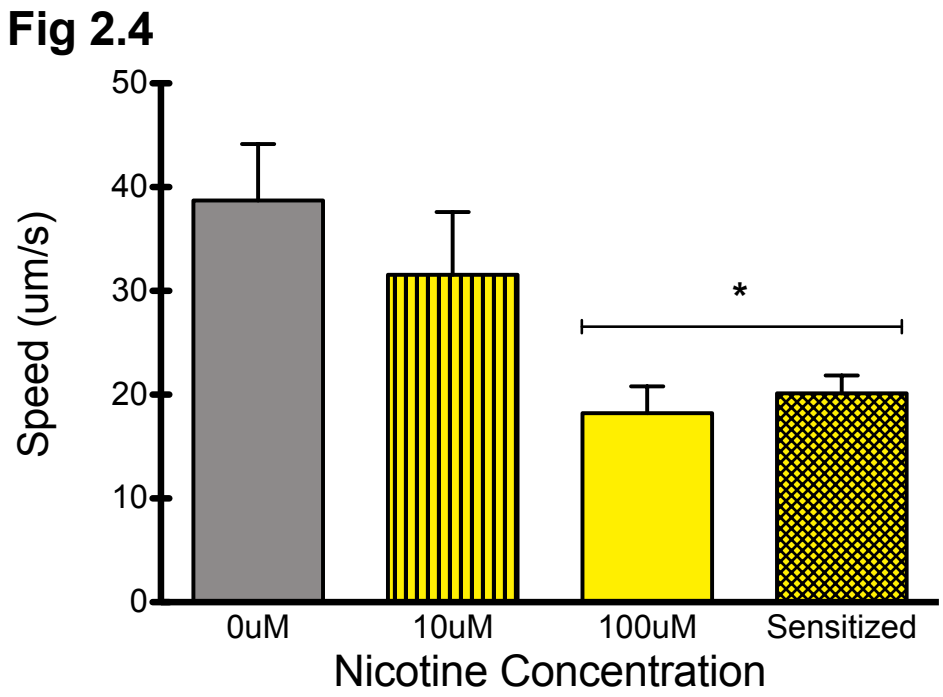

Fig 2.4 shows that the decreased crawl speed of *daf-2* mutants is subject to single-dose sensitization in a manner commensurate with wild-type animals. Again, animals in both the '10 μM' group [Mean: 31.5 μm/s ± 6.21] and 'Sensitized' group [Mean: 20.1 μm/s ±1.77] received a 10 μM nicotine challenge. However, 'Sensitized' received a one hour pretreatment with 100 μM nicotine followed by an hour of nicotine withdrawal before the 10 μM nicotine challenge. '0 μM' and '100 μM' [Mean: 18.2 μm/s ± 2.65] animals experienced fresh, nicotine-free plates for each of these transfers. When compared to the '0 μM' group [Mean: 38.8 μm/s ± 5.57] or the '10 μM' group [Mean: 31.5 μm/s ± 6.21], the 'Sensitized' animals crawled significantly slower in response to a 10 μM nicotine challenge and were no different from naive animals receiving a 100 μM, while '10 μM' worms behaved no differently than the '0 μM' controls [one-way ANOVA $p < 0.01$; Dunnett's MCT].

Locomotor Response of *age-1*/PI3K Mutants to Acute Nicotine Challenge

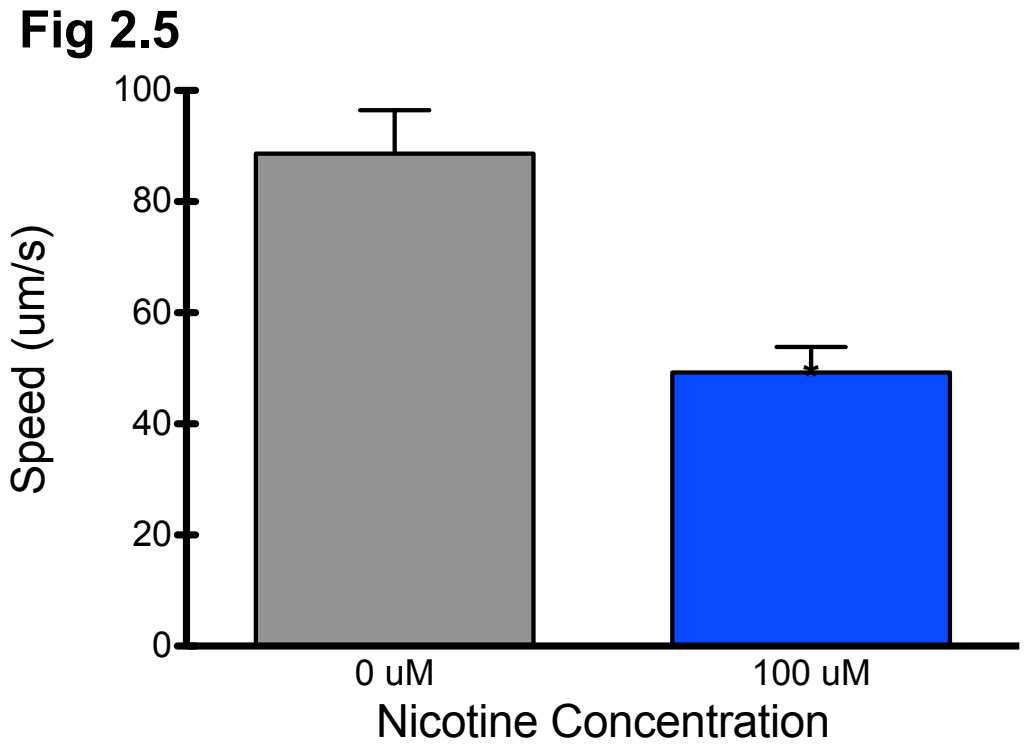

Fig 2.5 shows the locomotor response of *age-1*/PI3K mutants in response to an acute 100 μM dose of nicotine. *age-1*/PI3K mutants crawl slower in response to a 100 μM nicotine challenge [Mean: 49.3 μm/s ± 4.70] than do nicotine-naive *age-1*/PI3K mutants [Mean: 88.6 μm/s ± 8.03] [one-tailed *t-Test*, $p < 0.0001$].

Effects of *age-1*/PI3K Inhibitor Wortmannin on Acute Nicotine Response

Fig 2.6a

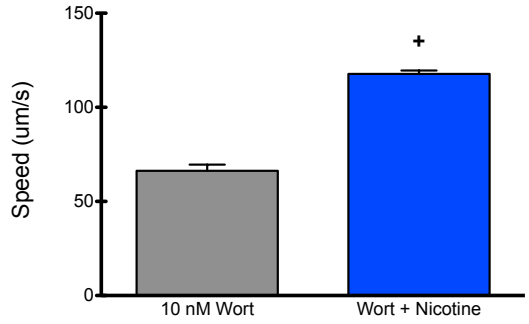

Fig 2.6b

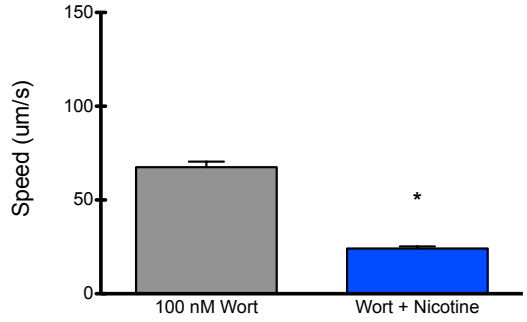

Fig 2.6c

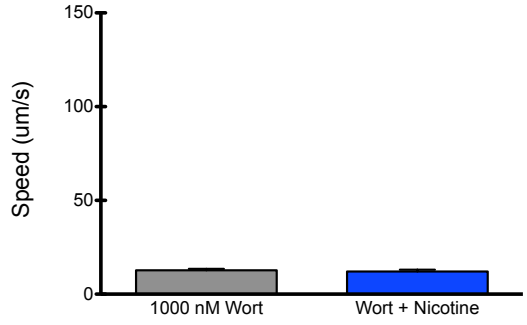

Fig 2.6 shows the effects of Wortmannin, a PI3K inhibitor on the locomotor response of wild-type nematodes challenged with a 100 µM dose of nicotine. **Fig 2.6a** shows that 10 nM Wortmannin has no effect on the wild-type acute nicotine response as worms receiving both nicotine and wortmannin [Mean: 117.7 µm/s ± 1.84] have an increased crawl speed than worms receiving wortmannin alone [Mean: 66.3 µm/s ± 3.38] [one-tailed *t-Test*, $p < 0.01$]. **Fig 2.6b** shows wild-type worms experiencing a 100 µM nicotine challenge in the presence of 100 nM Wortmannin [Mean: 24.1 µm/s ± 1.10] have a decreased crawl speed when compared to the Wortmannin alone group [Mean: 67.5 µm/s ± 3.03] [one-tailed *t-Test*, $p < 0.01$]. This phenocopies worm strains hypomorphic for AGE-1/PI3K. **Fig 2.6c** demonstrates the high, 1000 nM dose of Wortmannin sickens the animals resulting in minimal movement whether nicotine is present [Mean: 12.07 µm/s ± 1.00] or absent [Mean: 12.7 µm/s ± 0.81] [one-tailed *t-Test*, $p = 0.2842$].

Locomotor Response of *daf-18*/PTEN Mutants to Acute Nicotine Challenge

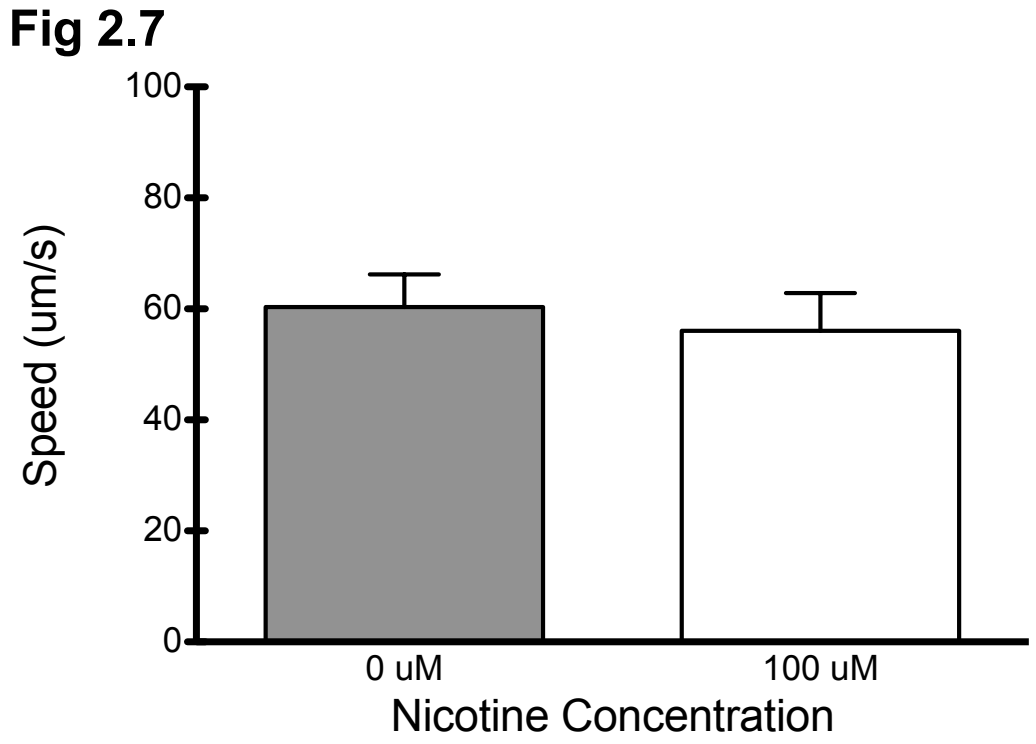

Fig 2.7 shows the locomotor response of *daf-18*/PTEN mutants in response to an acute 100 μM nicotine challenge. *daf-18*/PTEN mutants crawl at the same speed whether nicotine is present [Mean: 56.0 μm/s ± 6.98] or absent [Mean: 60.3 μm/s ± 6.05] [one-tailed *t-Test*, p = 0.3199].

Locomotor Response of *pdk-1* Mutants to Acute Nicotine Challenge

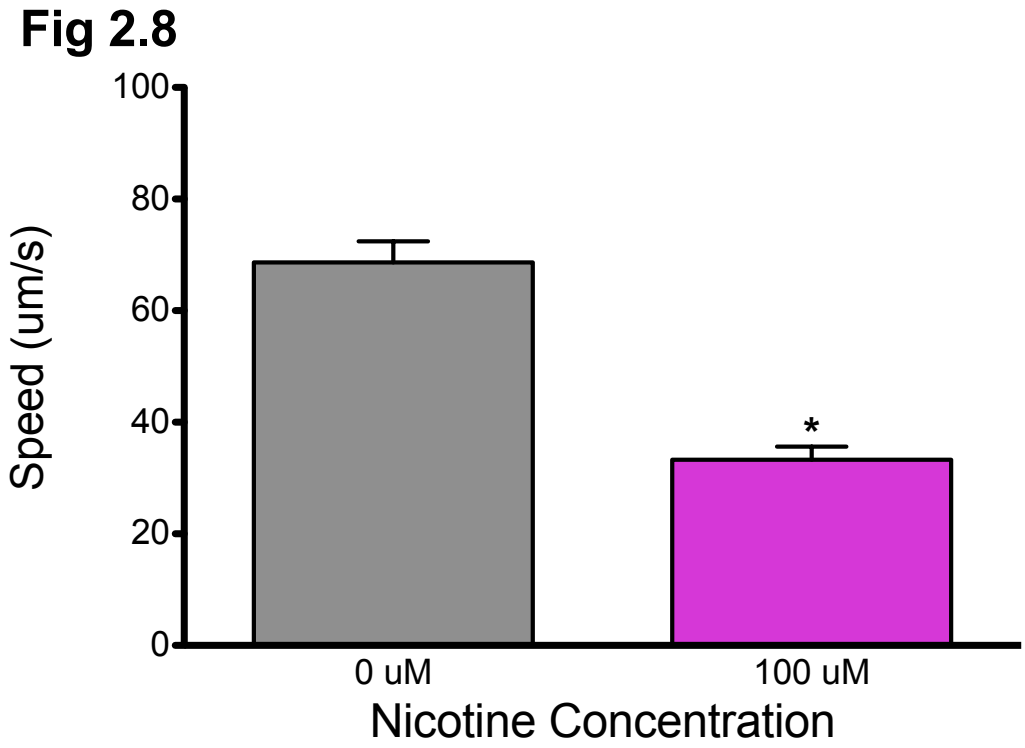

Fig 2.8 shows the change in locomotor output of *pdk-1* mutants in response to an acute nicotine challenge. *pdk-1* mutants crawl at slower speed in response to a 100 μM nicotine challenge [Mean: 33.3 μm/s ± 2.40] than do *pdk-1* mutants not exposed to nicotine [Mean: 68.6 μm/s ± 3.88] [one-tailed *t-Test*, $p < 0.0001$].

Locomotor Response of AKT Mutants to Acute Nicotine Challenge

Fig 2.9a

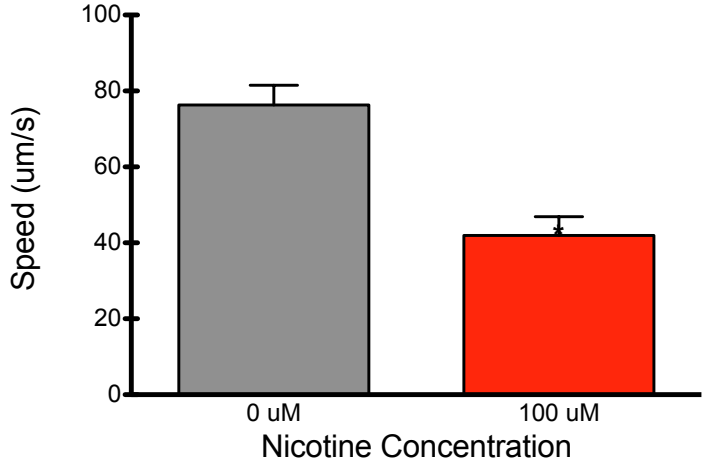

Fig 2.9b

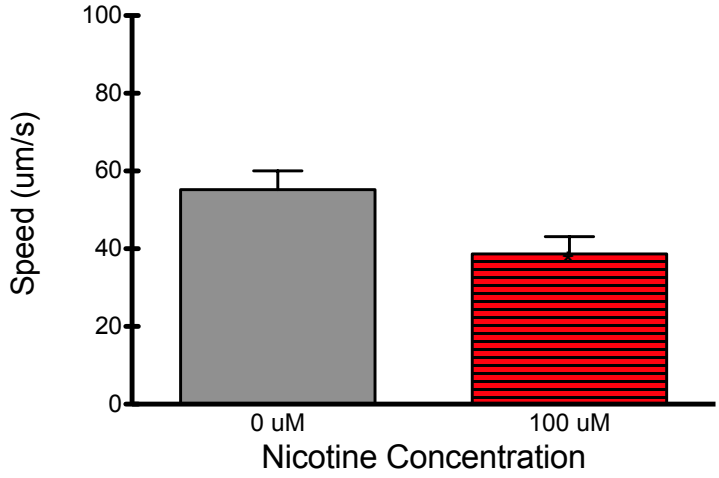

Fig 2.9 shows the change in crawl speed of the two *C. elegans* Akt/PKB homologs in response to an acute 100 µM nicotine challenge. **Fig 2.9a** demonstrates the decrease in locomotion of *akt-1* mutants in response to a 100 µM challenge [Mean: 42.0 µm/s ± 5.04] when compared to nicotine-naive mutants [Mean: 76.3 µm/s ± 5.35] [one-tailed *t-Test*, $p < 0.0001$]. **Fig 2.9b** shows *akt-2* mutants exposed to 100 µM nicotine [Mean: 38.6 µm/s ± 4.58] crawl slower than their nicotine-naive controls [Mean: 55.2 µm/s ± 4.99] [one-tailed *t-Test*, $p < 0.01$].

Locomotor Response of *daf-16*/FoxO Mutants to Acute Nicotine Challenge

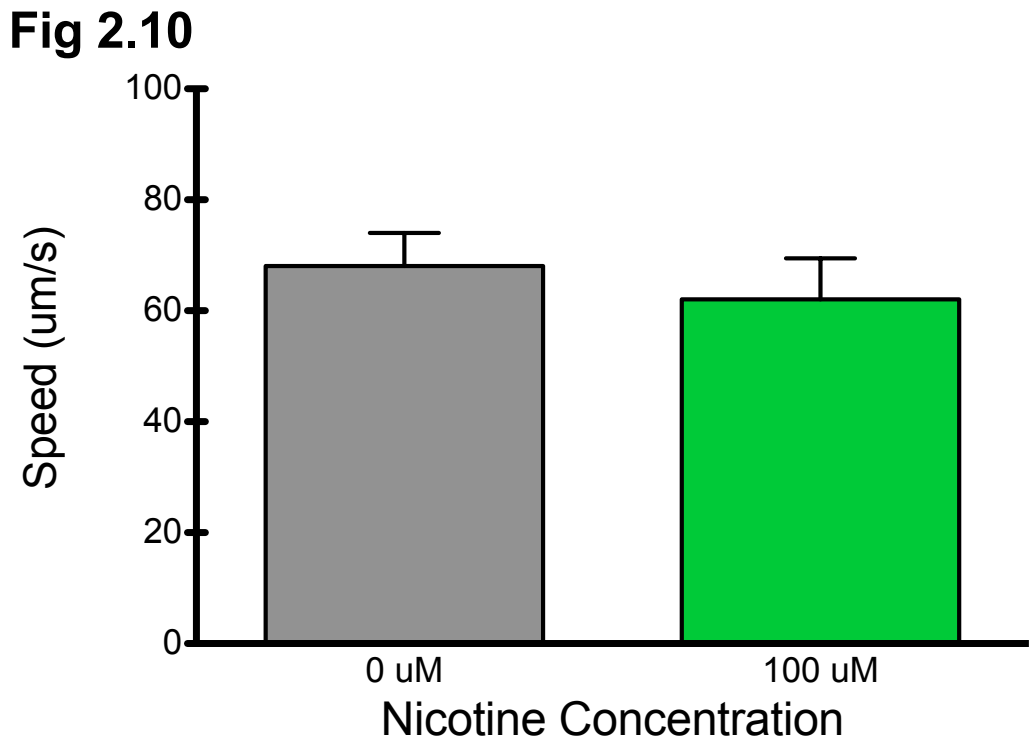

Fig 2.10 shows the locomotor response of *daf-16*/FoxO mutants. As the two strains tested, *daf-16(mgDF46)* and *daf-16(mu86),* were not different from one another, their data are presented here collectively. *daf-16*/FoxO mutants exposed to 100 μM nicotine [Mean: 62.1 μm/s ± 7.55] do not crawl at a different speed than nicotine-naive *daf-16*/FoxO mutants [Mean: 68.1 μm/s ± 6.13] [one-tailed *t-Test*, p = 0.2657].

Locomotor Response of *daf-2:daf-16* Mutants to Acute Nicotine Challenge

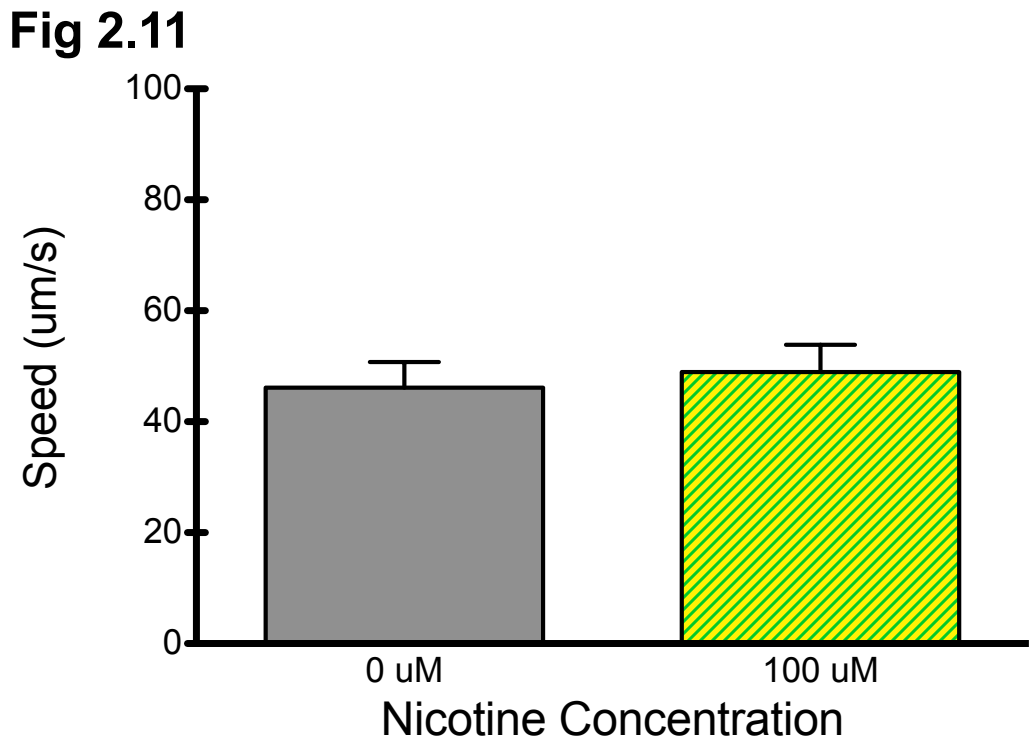

Fig 2.11 shows the locomotor response of *daf-2;daf-16* double mutants in response to an acute 100 µM nicotine challenge. Double mutants exposed to 100 µM nicotine [Mean: 48.9 µm/s ± 5.07] crawl at a speed that is not significantly different from double mutants exposed to no nicotine [Mean: 46.2 µm/s ± 4.74] [one-tailed *t-Test*, $p < 0.3418$]. As the double mutant phenocopies the *daf-16*/FoxO single mutant instead of presenting a phenotype intermediate to the *daf-2*/IIS and *daf-16*/FoxO single mutants, it is likely that these two genes are working in the same pathway in response to nicotine and *daf-16*/FoxO is downstream of *daf-2*/IIS.

Comparison of Insulin Signaling Mutants in Acute Nicotine Response

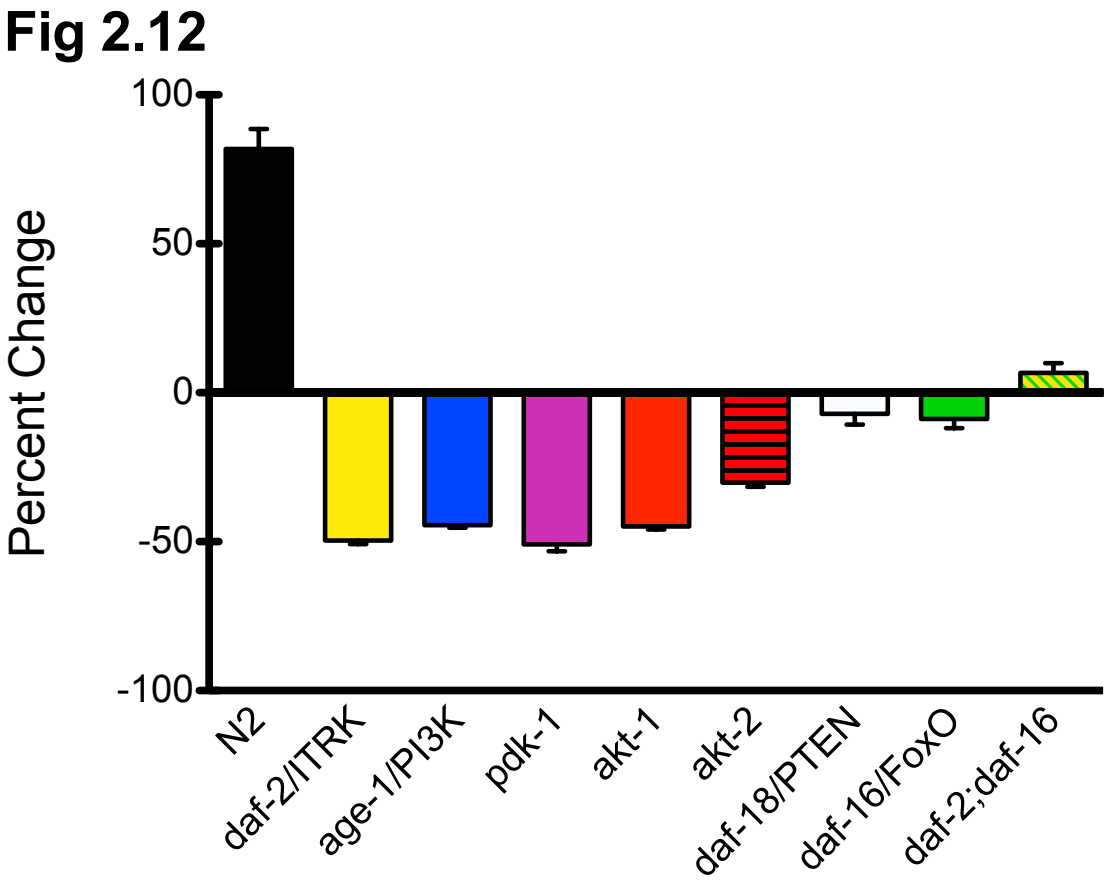

Fig 2.12 shows the percent change in response of insulin signaling mutants to an acute challenge of 100 μM nicotine relative to their controls on nicotine-free plates. The DAF-2/IIS mutants and the mutants for each of the downstream kinases — AGE-1/PI3K, PDK-1, AKT-1, and AKT-2 — show the inverted nicotine phenotype, the depressant response to nicotine. Loss of either DAF-18/PTEN or DAF-16/FoxO, however, results in a blunted, defective nicotine phenotype.

Model of Insulin Signaling Mutants Response to Acute Nicotine Challenge

Fig 2.13

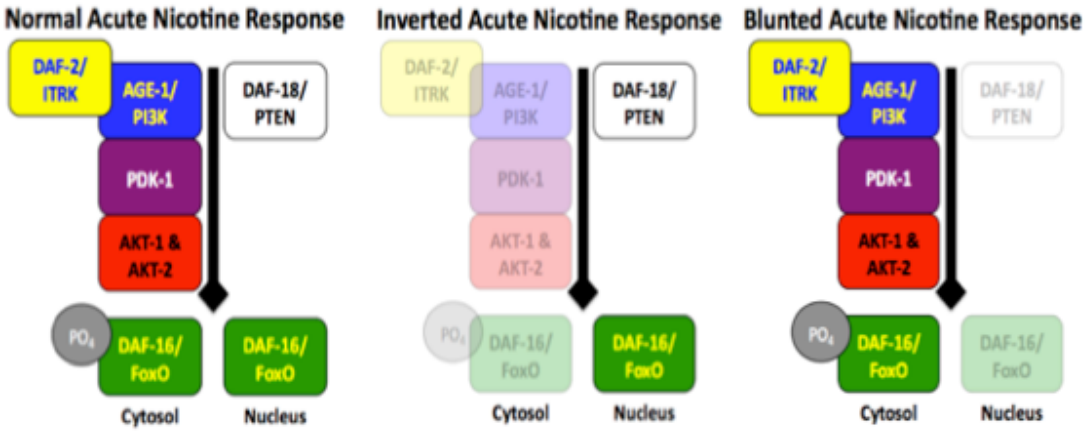

Fig 2.13 illustrates a model that may explain the role of insulin signaling in these aberrant nicotine responses. A normal, stimulating nicotine response depends upon a proper balance between the cytosolic and nuclear activity of DAF-16/FoxO. In its phosphorylated state, DAF-16/FoxO is sequestered in the cytosol while the unphosphorylated state allows DAF-16/FoxO to translocate to the nucleus. When the transcription factor is in physiologic balance between these two states, nicotine induces a stimulatory response. However, when DAF-16/FoxO is overly phosphorylated, as in the case of the *daf-18*/PTEN mutants, or missing, as in the case of the *daf-16*/FoxO mutants, the loss of nuclear activity results in the blunted response and defective nicotine phenotype. In contrast, reduced phosphorylation, as in the case of the *daf-2*/IIS and downstream kinase mutants is overly permissive of DAF-16/FoxO activity in the nucleus, which results in the inverted, depressant response phenotype when the animal experiences nicotine challenge.

Chapter III

TRPV Channels Regulate Nicotine Chemotaxis in the Nematode, *C. elegans*

As explained previously, drug addiction is a psychiatric disease state in which the rewarding effects of a drug of abuse come to usurp adaptive neural mechanisms mediating motivated behaviors, resulting in the compulsive and impulsive self-administration of a chemical substance, despite negative consequences for these actions (Koob and Le Moal, 2005; Robinson, 2004; Robinson and Berridge, 1993). While direct cognate receptors for most drugs of abuse are known, we have only just begun to plumb the complicated depths of the synaptic plasticity drugs of abuse induce in neurons (Kauer, 2004; Kauer and Malenka, 2007). In fact, an increasing body of literature suggests transient receptor potential (TRP) channels may be important secondary targets for drugs of abuse in mammalian and invertebrate models of addiction-related behaviors (Wescott et al., 2013).

In order to survive, an organism must be able to monitor its environment. *C. elegans* employs a number of sensory systems - osmosensation, chemosensation, mechanosensation - to achieve this end. Pertinent to the discussion at hand, chemosensation can be further divided into gustation — the ability to "taste" or detect salts and water-soluble molecules — and olfaction — the ability to "smell" or detect volatile, airborne chemicals (Bargmann, 2006). Typically, these nematodes can detect volatile chemicals at much lower concentrations than they detect water-soluble chemicals. Generally, nematode olfaction detects in nano- to low micormolar range

while gustation is more often in the micro- to low millimolar range (Bargmann, 2006). The ciliated chemosensory neurons necessary for olfaction and gustation are enclosed in the amphid, which is a nematode-specific sensory structure comprised of a series of invaginations near the pharynx at the anterior end of the worm. Within the amphid, there are three distinct pairs of neurons known to mediate olfaction (Bargmann, 2006). The AWA and AWC neurons are responsible for detecting appetitive or attractive odorants (Bargmann et al., 1993), while the AWB neurons respond to non-noxious, aversive or repulsive odorants (Troemel et al., 1997). A separate, polymodal neuron pair, the ASH neurons, are thought to be responsible for detecting most noxious stimuli regardless of sensory modality (Tobin et al., 2002).

Among the appetitive odorants to which *C. elegans* chemotaxes benzaldehyde, butanone, isoamyl alcohol, and 2,3-pentanedione all activate the AWC neurons, while AWA detects diacetyl and pyrazine. Both neuron classes are capable of detecting 2,4,5-trimethylthiazole, however (Bargmann, 2006; Bargmann et al., 1993). Curiously, the AWC neurons demonstrate a random lateralization across individual animals such that one AWC neuron detects benzaldehyde and the other detects butanone but neither neurons detects both these chemicals, but with no predictable sequence to whether the right or left AWC will be receptive to benzaldehyde (Troemel et al., 1999). To date, no studies have identified this sort of lateralization in the AWA neurons. In turning attention to drugs of abuse, evidence exists demonstrating that nematodes can chemotax up a nicotine concentration gradient (Sellings et al., 2013) but the study in question was unable to identify a chemosensory neuron responsible for detecting nicotine.

As one might expect, in addition to neurons, a number of genes are imperative for appropriate chemosensory behaviors in *C. elegans*. Prime among the genes known to have a role in nematode chemosensation are several G proteins, especially G_i-like genes, including *odr-3*

(ODorant Response abnormal), *gpa-2* (G Protein Alpha subunit), *gpa-3*, *gpa-5*, *gpa-6*, and *gpa-13* (Bargmann, 2006). The ODR-3 G protein, which is localized to sensory cilia, is necessary for full chemosensory responses to every odorant characterized to date (Jansen et al., 1999; Roayaie et al., 1998).

In AWA, for example, ODR-3 serves a G protein downstream of the diacetyl-sensitive GPCR encoded by *odr-10* (Roayaie et al., 1998). Diacetyl acts as a ligand at ODR-10 receptors. This increases ODR-3 activity, which eventually leads to signal transduction through a heteromeric ion channel encoded by the TRPV genes *osm-9* (OSMotic avoidance abnormal) and *ocr-2* (Osm-9 and Capsaicin receptor-Related) (Colbert, 1997; Tobin et al., 2002). A similar process appears to be at work for all known olfactory stimuli as well as for most other chemosensory and noxious stimuli (Bargmann, 2006). In fact, in the nociceptive ASH neuron, ODR-3 and the OSM-9/OCR-2 complex appear to have clear roles in response to noxious stimuli (Kahn-Kirby et al., 2004). Interestingly, not only are both OSM-9 and OCR-2 proteins localized to the sensory cilia in both AWA and ASH, but they each appear to be necessary for the appropriate trafficking of the other. Mutants lacking either *ocr-2* or *osm-9* are defective in olfactory chemotactic responses for every volatile chemical tested in *C. elegans* (Colbert and Bargmann, 1995), while, at present, no one has identified a clear behavioral phenotype for nematodes lacking a functional copy of *ocr-1*, another TRPV gene expressed in the AWA and ADL neurons.

Oddly, there are precious few studies to examine nicotine responses in freely behaving nematodes and most of the studies that exist are relatively recent and none have investigated a role for TRPV Channels in these behaviors. As noted previously, work from our lab shows that *C. elegans* exhibits a number of behavioral response when exposed acutely to nicotine (Feng et

al., 2006). Nicotine-exposed worms increase their crawl-speed acutely; they adapt to prolonged nicotine treatment; they show withdrawal behaviors when removed from a nicotine-rich environment; and, they show sensitization to repeated nicotine administration. These early studies provided evidence of a clear role for the AVA command interneuron, and its nAChR and TRPC Channels in these behaviors (Feng et al., 2006). For the behavioral paradigm in question, worms crawled freely on plates with a uniform nicotine concentration and it was assumed the nicotine effects were due to the diffusion of the drug through the cuticle until it reached its cognate receptor on the command interneuron.

More recently, a group consisting of Sellings, Pereira and their colleagues probed whether *C. elegans* nicotine chemotaxis as a nematode model of motivated behaviors (Sellings et al., 2013). Their studies showed worms will chemotax up a nicotine concentration gradient of the drug allowed to diffuse through the medium and worms will show a place preference for a non-paralyzing concentration of nicotine (50 μM) versus vehicle as well as conditioning a preference for butanone [a normally repellant odorant (Bargmann, 2006)] after it was paired with nicotine. The Sellings-Pereira group also identified both two nACh receptors (ACR-5, ACR-15) and two DOPamine receptors (DOP-1, DOP-2) as critical for nematode nicotine approach behavior in this paradigm (Sellings et al., 2013). Sellings and Pereira posit a complicated model to explain this nicotine approach behavior in which nicotine diffuses through the cuticle to act as ligand at ACR-15 receptors expressed on the AVA command interneuron. Through gap junctions, this AVA depolarization activates the mechanosensory neural circuits that feed back onto a second command interneuron, AVB, which then releases ACh onto the B-type, cholinergic motor neurons, which express ACR-5 receptors. This pathway, they suggest, then induces locomotion towards the point-source of nicotine (Sellings et al., 2013).

Methods

Chemotaxis Assays

For chemotaxis assays, we used standard 10 cm Petri dishes containing 15 mL of an optimized nematode chemotaxis medium [1.6% BBL agar; 1 mM $MgSO_4$; 1 mM CaCl; 5 mM KPO_4]. We poured chemotaxis plates fresh within the week of testing, allowed the plates to settle overnight on the bench, before storing them at 4ºC until used. On test day, we allowed the plates to warm on the bench to 20ºC, before partitioning the outside of the dish with felt-tip pen into three sections - an "attractant" (A), a "control" (R), and a "middle" (M) section. We then set the plates with the lids removed on the bench for an additional hour to allow for the evaporation of any excess moisture remaining from the time stored in the cooler. At the end of this hour, we washed each strain of worms from the plate on which it was grown into a 1.5 mL microcentrifuge tube with 1.5 mL S Basal [100 mM NaCl; 6 mM K_2HPO_4; 44 mM KH_2PO_4; 13 µM Cholesterol in H_20] and allowed them to settle for 3 min before drawing off the supernatant and rinsing them twice more with S Basal before a final rinse with ddH_20. We then transferred a population of 50-150 L4 and early Day 1 worms to the "start box" on each chemotaxis plate, adding the attractant (nicotine or diacetyl diluted in ethanol) and the control (ethanol diluent alone). We then placed the dishes upside down and allowed the to sit undisturbed for one hour. At the end of the hour, we calculated a chemotaxis index (CI) by counting the number of worms within the "A" partition less the worms in the "C" partition and dividing that number by the total number of worms to leave the "start box": (A-C) ÷ (A+C+M) = CI. This yielded a CI between +1.00 (absolute attraction) and -1.00 (absolute repulsion) with a score of 0.00 being chance. **Fig 3.1** illustrates this. We replicated each chemotaxis assay in triplicate.

Subjects and Strains Used

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 μM cholesterol, 1 mM Ca(CH3COO)2, 1mM MgSO4, 25 mM KH2PO4, pH 7.0] seeded with *Escherichia coli* (OP50) at 20ºC. 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 the following strains: Bristol N2 (as wild-type), *acr-2(ok1887)*, *acr-5(ok180)*, *acr-8(ok340)*, *acr-9(ok933)*, *acr-11(ok1345)*, *acr-12(ok367)*, *acr-14(ok455)*, *acr-15(ok1214)*, *acr-16(ok789)*, *acr-18(ok1285)*, *acr-19(ok967)*, *deg-3(u701)*, *eat-2(ad465)*, *lev-8(x15)*, *lgc-40(n4545)*, *ocr-1(ak46)*, *ocr-1(ok132)*, *ocr-2(ak47)*, *ocr-3(ok1559)*, *ocr-4(vs137)*, *odr-3(n2150)*, *odr-10(ky32)*, *osm-9(ky10)*, *trpa-1(ok999)*, the double mutant *ocr-1(ok132);ocr-2(ak47)*, the quadruple mutant *ocr-1(ok132);ocr-2(ak47);osm-9(ky32);trpa-1(ok999)*, and the rescue line: *ocr-1(ok132);xuEx[Podr-10::OCR1::SL2::mCherry]*.

Results

Worms Can Chemotax to a Point-Source of Nicotine

As previously discussed, we decided to investigate whether *C. elegans* is capable of crawling to a point-source of nicotine that is not diffusing through the NGM or through the cuticle of the animal. To this end, we tested a range of nicotine dilutions (in ethanol) by placing a 2 μL drop of the nicotine:ethanol mixture on the lid of the plate while the worms crawled on the surface of the NGM. To prevent the nicotine from dripping onto the medium, the plates sat upside-down during the test. This paradigm has proven reliable in diacetyl olfactory chemotaxis

assays (Bargmann et al., 1993) and, as such, we used diacetyl diluted in ethanol (1:1000) as a positive control (**Fig 3.2a**). As **Fig 3.2b** shows, worms chemotax reliably to nicotine (1:50) when compared to ethanol alone [two-tailed *t*-Test, $p < 0.0001$], although their chemotaxis to diacetyl (1:1000) is significantly greater than the nicotine chemotaxis (**Fig 3.2c**). While we did not explore it further here, the difference in chemotaxis index between nicotine and diacetyl is likely due to the ability of nematodes, like mammals, to select among various stimuli in their environment. To do so, different appetitive stimuli should have different motivational salience or weighting.

While worms reliably chemotax to a 1:50 dilution of nicotine, we next explored their chemotaxis to a range of nicotine dilutions. As seen in **Fig 3.3**, wild-type worms are able to chemotax to a host of nicotine dilutions successfully when compared to the No Nicotine control. Worms responded with robust chemotaxis to the 1:100, 1:50, 1:25, 1:5, and Pure Nicotine dilutions, with animals in each of these conditions significantly better than those in the No Nicotine condition at finding the "attractant" spot. As the 1:50 dilution seemed to be elicit a particularly optimal response in wild-type worms, we selected this 1:50 nicotine dilution for the candidate screens to follow. In the 1:500 condition, worms performed no better than animals in the No Nicotine group.

Generally, especially with drugs of abuse, there is some lower threshold at which the drug is ineffective in eliciting a response as well as an upper threshold at which the amount of drug present has catastrophic effects on the ability of the animal to perform. We expected to see this typical "inverted **U**" for a dose-response curve across the various dilutions in this assay. What we see, however, is less of an analog, dose-response curve and much more a digital, responsive-unresponsive threshold. There is a dilution below which worms begin to respond in

this olfactory assay, but once the threshold is reached worms respond similarly to most dilutions above that point. This is reminiscent of the 'digital cliff' in the telecommunications industry where a digital signal is perceived to have perfect reception or not to exist at the receiving end where as analog signals gradually decrease in strength and clarity until they are too degraded for reception. Additionally, this could be compared to the 'all-or-nothing' nature of action potential – once threshold is reached, the depolarization of the neuron results in an action potential firing down the axon whether more depolarization is present or not but sub-threshold excitations do not. Regardless of the imagery we choose to describe it, wild-type nematodes are capable of chemotaxing to a source of nicotine.

Acetylcholine Receptors Have No Apparent Role in Nicotine Chemotaxis

Nicotine is an exogenous ligand at nAChR, a family of receptors for which the *C. elegans* genome encodes at least 27 genes with approximately 20 of these encoding putative α-subunits. As these proteins were the most likely candidate to serve as nicotine receptor, we next screened all of the available nAChR α-subunit mutants without any noticeable phenotypically abnormal locomotor behaviors. Despite our expectations to the contrary, none of the strains carrying null mutations in a nAChR α-subunit we tested were defective in their ability to locate a point-source of nicotine (**Fig 3.4**). This suggests that something else must act as the nicotine chemoreceptor.

TRPV Genes Regulate Nicotine Chemotaxis

Previous work from our lab demonstrates a role for TRP channels in an acute nicotine response (Feng et al., 2006) and TRPV channels play a significant role in nematode sensory behaviors (Bargmann et al., 1993; Colbert and Bargmann, 1995; Colbert, 1997; Tobin et al.,

2002; Troemel et al., 1997; Troemel et al., 1999). Meanwhile, nAChR appear to have no role in our paradigm for chemotaxis to nicotine. Accordingly, we next screened worm lines with null mutations in TRPV homologs (**Fig 3.5**). Despite the fact that *ocr-1* has never shown a significant role in chemosensation previously, two separate null alleles for *ocr-1* show deficits in nicotine chemotaxis. Moreover, the quadruple mutant *ocr-1;ocr-2;osm-9;trpa-1*, has the same deficit in chemotaxis as the *ocr-1* mutants alone (data not shown). The nicotine chemosensory deficits of *ocr-1* mutants persisted at all dilutions tested (**Fig 3.6**). Together, these data suggest that OCR-1 is necessary for nicotine chemotaxis.

In nearly every chemosensory behavior tested in *C. elegans*, OSM-9 has a vital, if not necessary, role in transducing the chemical signal (Colbert, 1997). In our nicotine chemotaxis assay, however, we see that worms lacking OSM-9 are still capable of locating the nicotine source. In fact, *osm-9* mutants show improved chemotaxis when compared to wild-type worms at the 1:50 dilution and this exaggerated nicotine chemotaxis of *osm-9* mutants was present at all dilutions (**Fig 3.7**). This would seem to suggest that not only is OSM-9 not necessary for nicotine olfaction, but it may actually be detrimental to this behavior.

In diacetyl olfaction, both OSM-9 and OCR-2 are necessary and their proper localization to the sensory cilia depend on one another (Colbert and Bargmann, 1995). Curiously, however, *ocr-2* mutants have normal nicotine chemotaxis at most dilutions, but show a deficit in chemotaxis to the 1:5 and pure nicotine conditions (**Fig 3.8**). The *ocr-1;ocr-2* double mutant, however, showed no nicotine chemotaxis at any dilution (**Fig 3.9**) further demonstrating the necessity of *ocr-1* in this nicotine chemotaxis.

Meanwhile, at the 1:50 nicotine dilution, *ocr-3*, *ocr-4*, *trpa-1* all show a normal nicotine chemotaxis phenotype. As *ocr-3* appears to be expressed only in rectal glands, we did not

explore this gene further. OCR-4 is expressed in the mechanosensory OLQ neurons. On the chance the expression pattern for this protein is incomplete and since it does have some neuronal expression, we elected to test *ocr-4* mutants on a range nicotine dilutions. Similarly, as the mammalian TRPA1 is expressed in the nasal airway and can bind nicotine, we decided to test these mutants on a full range of nicotine doses. However, we saw no effect on nicotine chemotaxis in either *trpa-1* or *ocr-4* null mutants any of the tested dilutions (**Fig 3.10**).

We next attempted to rescue nicotine chemotaxis by expressing OCR-1 cDNA solely in neurons. In the *ocr-1* mutant background, a transgenic line expressing OCR-1 cDNA behind a neuron-specific promoter rescued the nicotine chemotaxis defects of these animals (**Fig 3.11**). Not only is OCR-1 necessary for nicotine olfaction, but its presence in neurons alone restores this behavior in *ocr-1* null mutants.

Discussion

Chemosensation in *C. elegans* is divisible into gustation and olfaction. While worm gustation typically detects chemicals in the high micromolar to millimolar concentrations, worm olfaction tends to detect chemicals at much lower concentrations, down to the low nanomolar concentrations (Bargmann, 2006). As olfaction also detects primarily volatile chemicals as opposed to solutes, it is possible that worms use olfaction to locate potential sources of food from a distance, and switch to a more gustatory strategy to maximize time spent near an abundant food source. In either case, TRP channels — particularly TRPV channels — play a critical role in nematode chemosensation (Bargmann, 2006; Bargmann et al., 1993; Colbert and Bargmann, 1995; Colbert, 1997; Tobin et al., 2002; Troemel et al., 1997; Troemel et al., 1999).

In the studies presented here, we see wild-type worms can chemotax to a point-source of nicotine over a range concentrations. Interestingly, this chemotaxis behavior does not show the classic bell-shaped curve one typically expects to see in drug-induced behaviors over a range of doses. Rather, the behavioral response is more similar to the phenomenon of a 'digital cliff'. The worm responds until it reaches some lower detection threshold at which point the nicotine elicits no response, analogous to the initiation of an action potential in higher-order animals.

Typically, one would expect to see a faltering of the behavioral response at higher drug concentrations for a number of reasons — a drug make become paralytic or even toxic at high concentrations, for example. In the case of psychomotor stimulants in mammals, high drug concentrations paradoxically decrease locomotor activity, particularly when measuring movement by breaks in an infrared beam-grid as the animal ceases to pace around the testing environment but falls to stereotypical stimulant-induced behaviors such as jaw-grinding, excessive grooming, paw-treading, or scratching behaviors (Bonasera et al., 2008). In nematodes, specifically, high concentrations of nicotine will result in paralysis, as shown elsewhere in this manuscript. However, in our olfactory paradigm, even in the 'pure nicotine' condition, the nicotine was not allowed enough time to diffuse fully through the medium thereby preventing worms from absorbing or accumulating a large enough internal concentration of nicotine to experience this paralytic effect. Thus, the wild-type worms do not show the expected faltering of behavioral response at these higher doses.

The most clear-cut finding we see in these nicotine chemotaxis studies is the necessity of *ocr-1* gene expression with the neurons for normal chemotaxis to nicotine. The normal nicotine olfactory phenotype of *odr-3*, however, is more remarkable. The *odr-3* gene product is necessary in a plethora sensory neurons and sensory responses, including gustation, mechanosensation,

osmosensation, and nociception as well as olfaction (Bargmann, 2006). It is, therefore, striking that the studies we present here suggest no necessary role for *odr-3* in nicotine olfaction (data not shown). There are two possible, though not mutually exclusive, explanations for this. First, these data would seem to suggest that normal nematode nicotine chemotaxis utilizes some other G-protein signaling pathway. The second possibility is that this nicotine chemotaxis is not a normal nematode behavior outside of the laboratory, but at the bench we are able to control the environment of the animal in such a way as to unmask a latent effect nicotine can have at these TRP channel proteins. In either case, these studies and those that may follow, will shine a light on novel therapeutic approaches for nicotine use, because we have potentially identified a non-canonical nicotine target. This is, perhaps, especially likely as none of the nAChR alpha subunits we tested seem to have a role in this behavior. We should note that nAChR alpha subunit mutants with an 'uncoordinated' phenotype that we did not test appear to be expressed in body wall muscles or ventral cord motor neurons. No one has reported expression of these subunits in sensory neurons or interneurons.

While we were unable to detect an involvement for any nAChR alpha subunits, it is important to remember that the Sellings-Pereira group identified both *acr-5* and *acr-15* as necessary genes for nicotine chemotaxis in their paradigm (Sellings et al., 2013). It seems that the most likely explanation of this discrepancy between the two studies is the placement of the nicotine itself. The Sellings-Pereira group placed the nicotine directly on the NGM and allowed it diffuse through the medium to construct their concentration gradient. Consequently, the worm was able to absorb nicotine through the cuticle, which permitted the drug to work throughout the body of the worm. Our lab has already shown that, when worms absorb nicotine through the cuticle, the ACR-15 receptors on AVA neurons play a critical role in the nematode locomotor

response (Feng et al., 2006). Additionally, the ACR-5 receptor is expressed on cholinergic ventral cord motor neurons (Winnier et al., 1999). It is likely that in worms lacking this receptor, these motor neurons would be less active and, consequently, their post-synaptic partners would likely up-regulate the surface expression of their own nAChR. This confluence of events would result in a stronger paralytic effect at lower nicotine concentrations. Therefore, worms with loss-of-function *acr-5* mutations would have increased difficulty crawling to a point-source of nicotine. In our chemotaxis paradigm, however, it is unlikely nicotine had adequate time to diffuse through the cuticle fully. As such, our assay did not return a chemotaxis defect for these two nAChR mutants.

Moving forward, we have developed a working model we will use to continue investigating this phenomenon. In this model, G-proteins activated through some unknown receptor act at TRPV channels to mediate nicotine chemotaxis. Chemotaxis to the full range of nicotine is mediated largely through OCR-1 with some small contribution from OCR-2 at higher nicotine levels, while OSM-9 contributes to a 'no-go' signal at all concentrations. Consequently, in the *ocr-1* mutants that lack the necessary OCR-1, we see no nicotine chemotaxis. However, in the *ocr-2* mutants we still see chemotaxis at the lower nicotine levels mediated through OCR-1 despite the lack of OCR-2 in these animals. Finally, in animals lacking OSM-9, we see an exaggerated nicotine response as they lose the 'no-go' signal. **Fig 3.12** summarizes this working model.

Nicotine Chemotaxis Paradigm

Fig 3.1

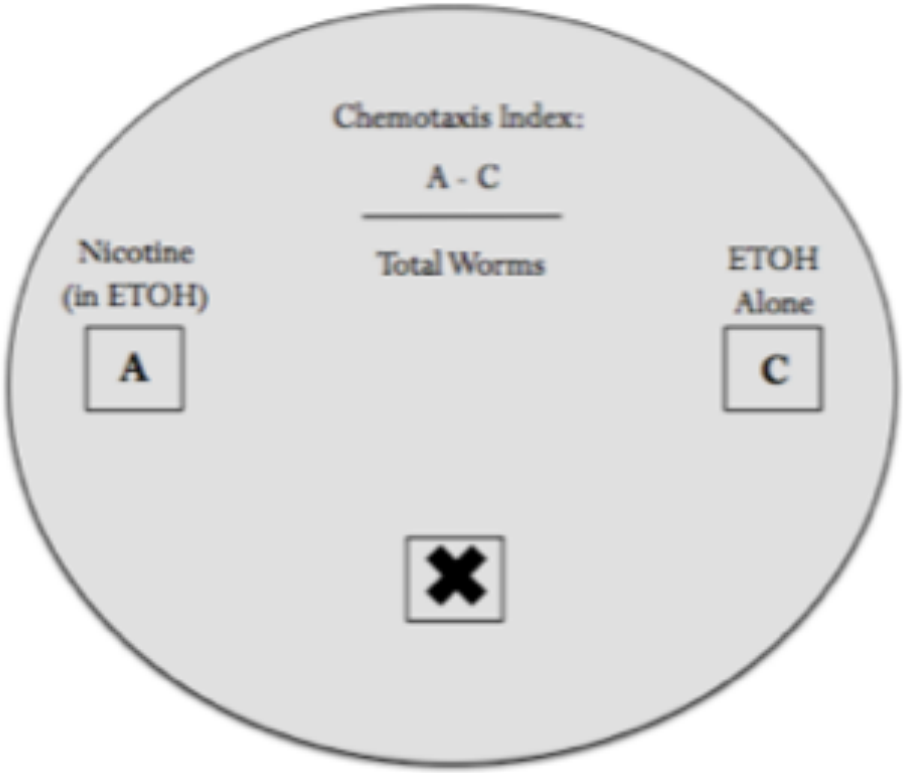

Fig 3.1 illustrates the nicotine chemotaxis paradigm. Worms crawled freely for one hour. At the of the hour, we calculated a chemotaxis index (CI) by counting the number of worms within the "A" partition less the worms in the "R" partition and dividing that number by the total number of worms to leave the start box, marked with the '**x**': (A-C) ÷ (A+C+M) = CI. This yielded a CI between +1.00 (absolute attraction) and -1.00 (absolute repulsion) with a score of 0.00 being chance.

Nicotine Chemotaxis of Wild-Type Nematodes

Fig 3.2a

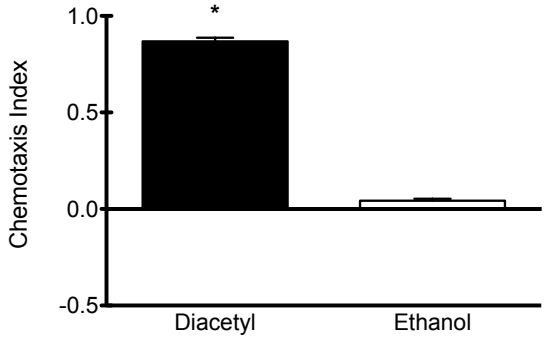

Fig 3.2b

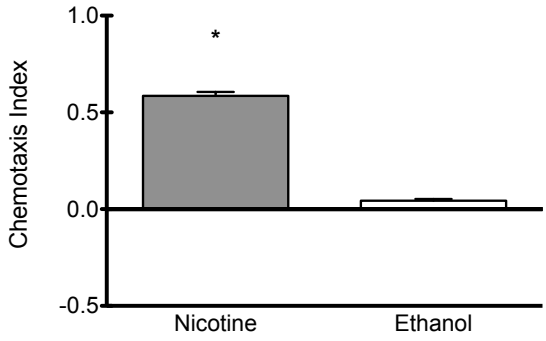

Fig 3.2c

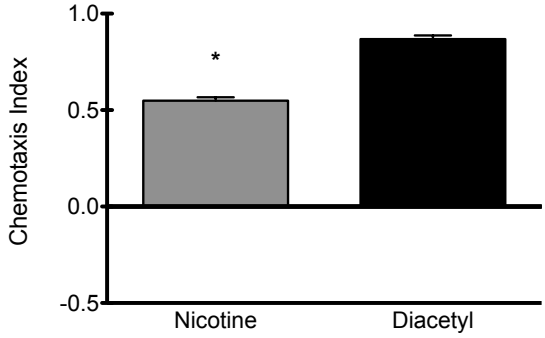

Fig 3.2 shows olfactory chemotaxis to volatile chemicals. **Fig 3.2a** shows nematodes crawl toward a 1:1000 dilution of the volatile diacetyl [Mean: 0.868 ± 0.0200] significantly more than they do to the ethanol vehicle [Mean: 0.043 ± 0.0109] [two-tailed *t*-Test, $p < 0.0001$]. **Fig 3.2b**, likewise shows the nematode preference for a 1:50 dilution of nicotine [Mean: 0.585 ± 0.0435] over the ethanol vehicle [Mean: 0.043 ± 0.0090] [two-tailed *t*-Test, $p < 0.0001$]. **Fig 3.2c**, however shows that, while nematodes will chemotax to both nicotine and diacetyl, they have a significant preference for diacetyl [Mean: 0.853 ± 0.0210] over nicotine [Mean: 0.552 ± 0.0179] [two-tailed *t*-Test, $p < 0.0001$].

Wild-Type Nicotine Chemotaxis

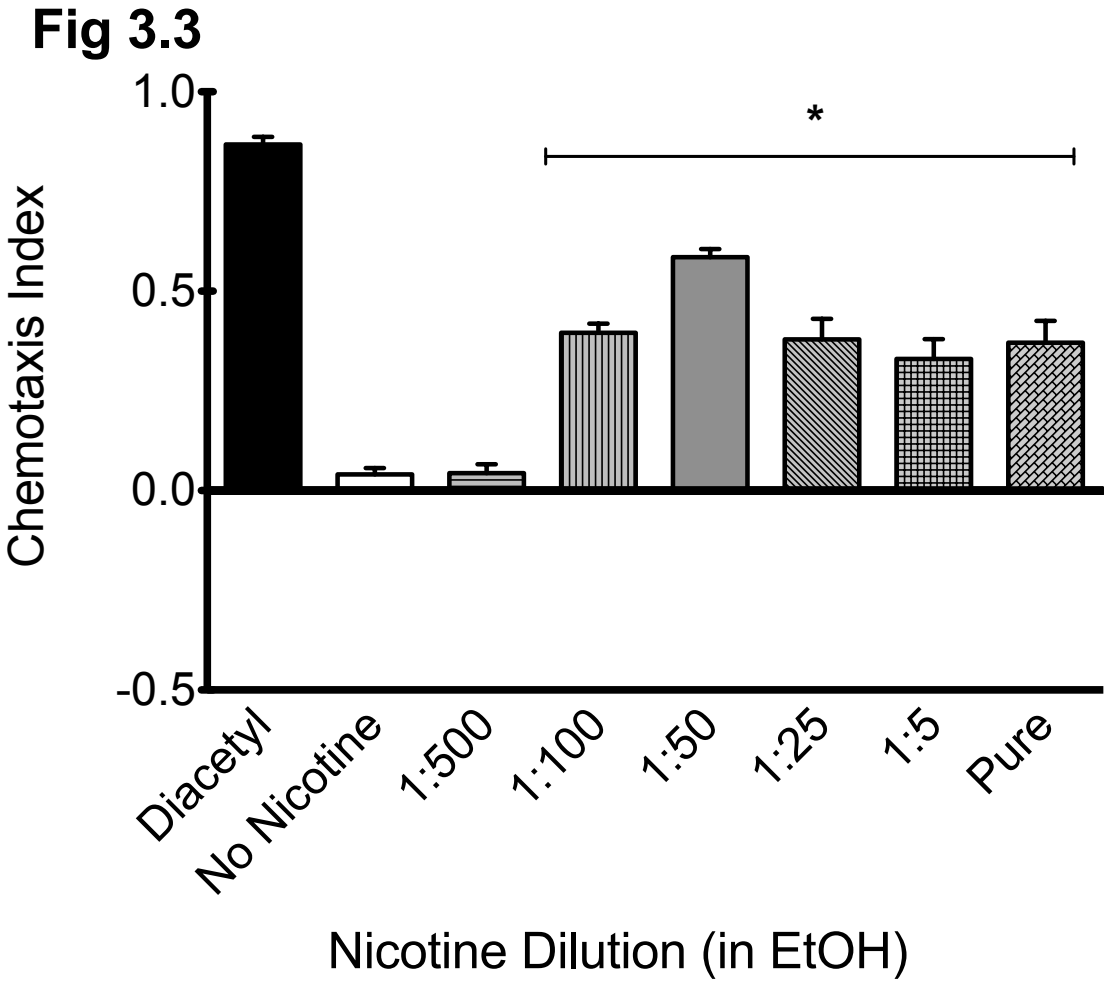

Fig 3.3 shows the olfactory chemotaxis to nicotine of wild-type nematodes. While worms are unable to chemotax to a 1:500 [Mean: 0.044 ± 0.0263] dilution of nicotine in ethanol, worms in the '1:100' [Mean: 0.396 ± 0.0257], '1:50' [Mean: 0.585 ± 0.0355], '1:25' [Mean: 0.379 ± 0.0563], '1:5' [Mean: 0.330 ± 0.0806], and 'Pure' [Mean: 0.371 ± 0.0599] groups all show reliable to chemotaxis in comparison to the 'No Nicotine' [Mean: 0.041 ± 0.0177] group [one-way ANOVA $p < 0.0001$, Bonferroni's MCT].

Screen of Acetylcholine Receptor Mutants in Nicotine Chemotaxis

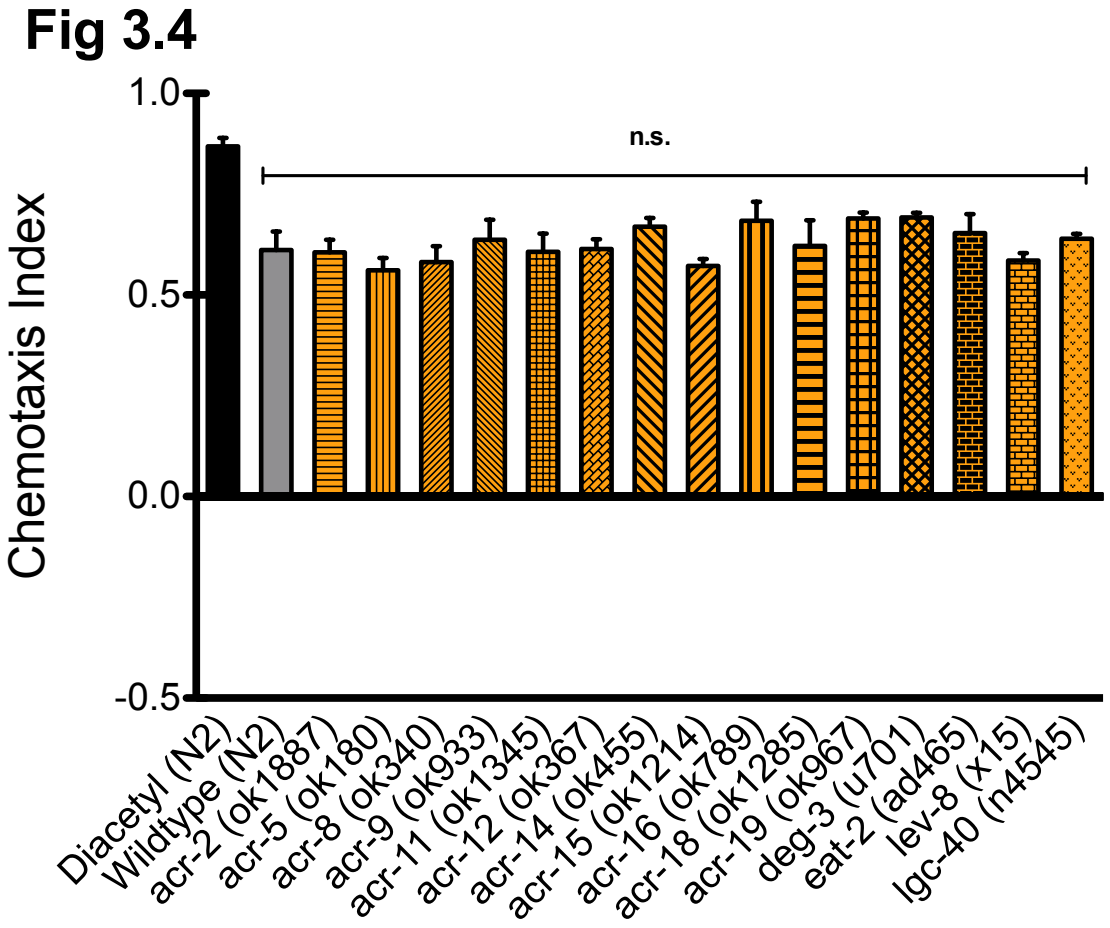

Fig 3.4 shows a screen of strains carrying null mutations in a putative α-subunit of nicotinic acetylcholine receptors in the nicotine chemotaxis paradigm. None of the nAChR mutants tested showed a deficit in nicotine olfactory chemotaxis [one-way ANOVA $p = 0.1630$].

Screen of TRP Channel Mutants in Nicotine Chemotaxis

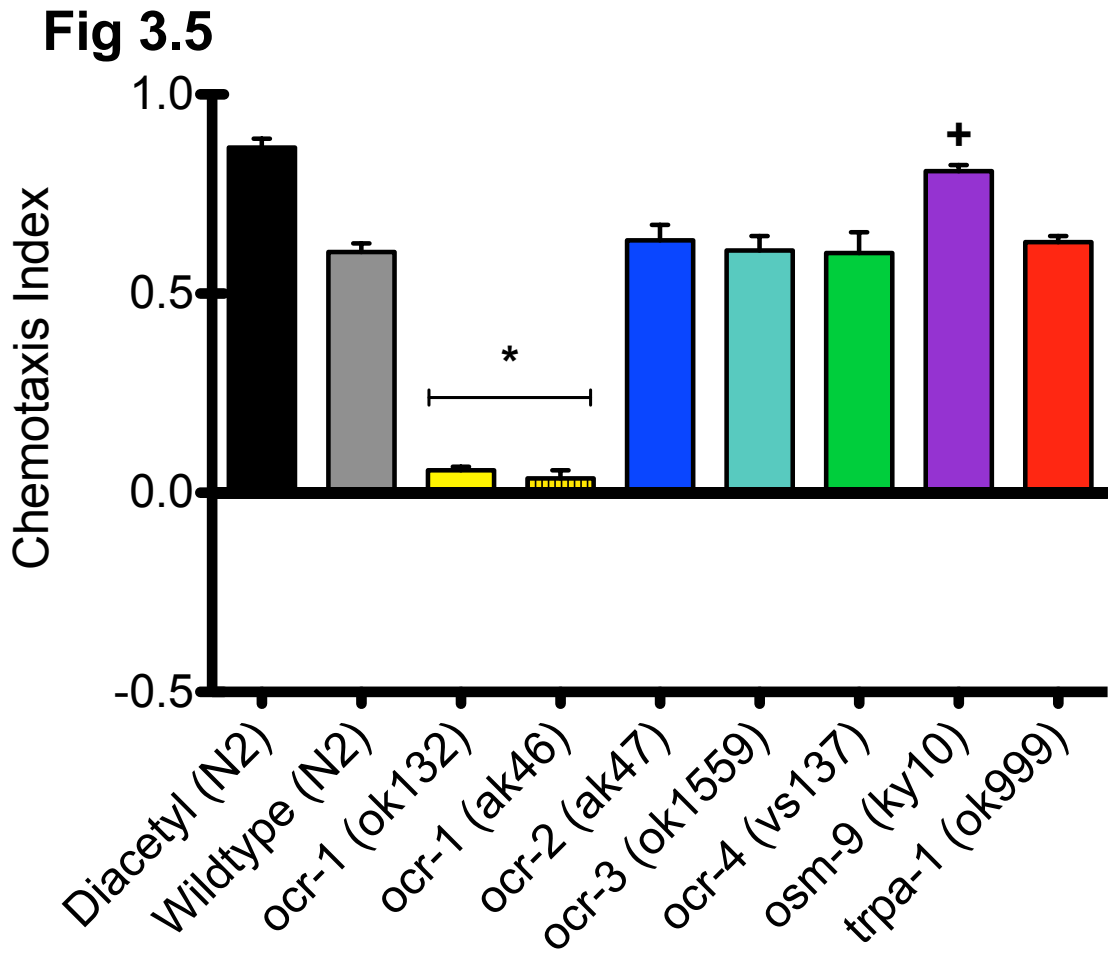

Fig 3.5 shows a screen of TRP channel mutants in the nicotine olfaction paradigm. When compared to wild-type [Mean: 0.605 ± 0.0249] animals, *ocr-2* [Mean: 0.634 ± 0.0474], *ocr-3* [Mean: 0.608 ± 0.0422], *ocr-4* [Mean: 0.602 ± 0.0642], and *trpa-1* [Mean: 0.629 ± 0.0177] showed no defect in nicotine chemotaxis at the 1:50 dilution of nicotine. The *osm-9* [Mean: 0.807 ± 0.0178] mutants showed a significant increase nicotine chemotaxis, while both strains carrying either the ok132 [Mean: 0.056 ± 0.0114] or ak46 [Mean: 0.036 ± 0.0249] allele of *ocr-1* showed a significant defect in olfactory nicotine chemotaxis [one-way ANOVA $p < 0.0001$, Dunnett's MCT].

ocr-1 Mutants Nicotine Chemotaxis

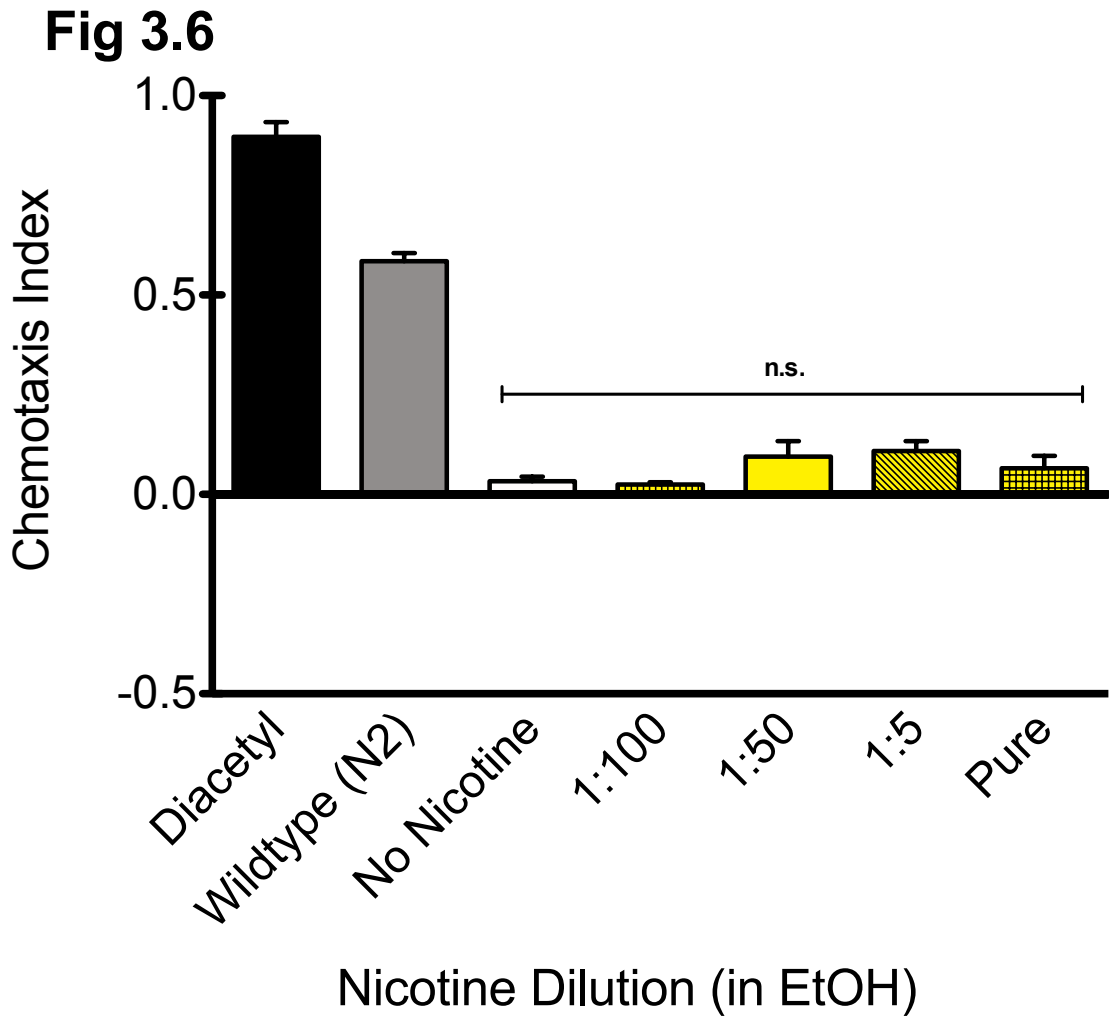

Fig 3.6 shows the nicotine chemotaxis response of *ocr-1* mutants. Mutants on plates with a 1:100 [Mean: 0.025 ± 0.0068], 1:50 [Mean: 0.095 ± 0.0450], 1:5 [Mean: 0.109 ± 0.0291], or Pure [Mean: 0.066 ± 0.0361] nicotine dilutions showed no difference when compared to animals on a plate with no nicotine [Mean: 0.033 ± 0.0136] [one-way ANOVA $p = 0.1377$].

***osm-9* Mutants Nicotine Chemotaxis**

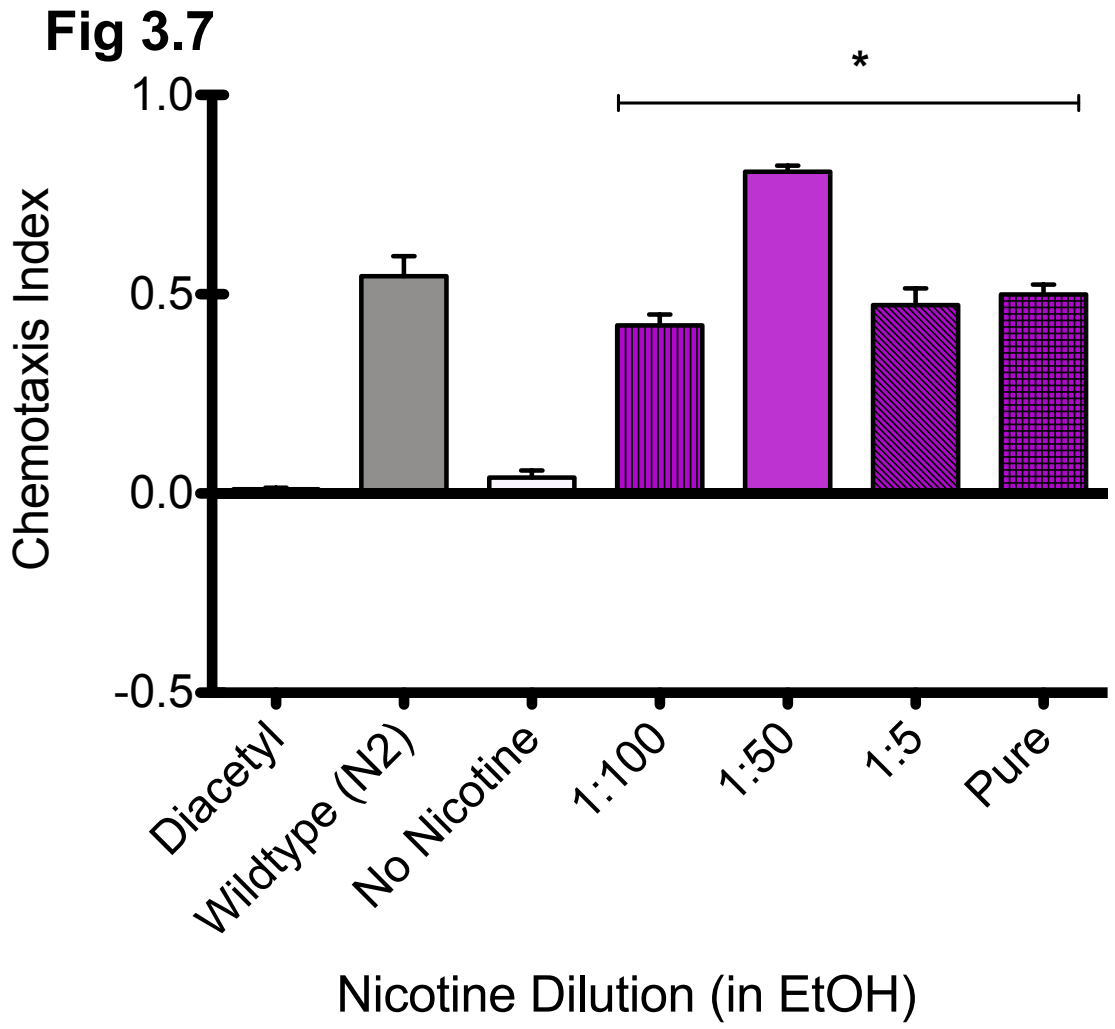

Fig 3.7 shows the nicotine chemotaxis response of *osm-9* mutants. Mutants on plates with a 1:100 [Mean: 0.422 ± 0.0317], 1:50 [Mean: 0.807 ± 0.0178], 1:5 [Mean: 0.473 ± 0.0486], or Pure [Mean: 0.500 ± 0.0289] nicotine dilutions showed significant nicotine chemotaxis when compared to animals on a plate with no nicotine [Mean: 0.040 ± 0.0207] [one-way ANOVA $p < 0.0001$, Dunnett's MCT]. While not tested directly, the chemotaxis index at each dilution of these mutants appears to be qualitatively more robust than wild-type animals at the same dilution.

***ocr-2* Mutants Nicotine Chemotaxis**

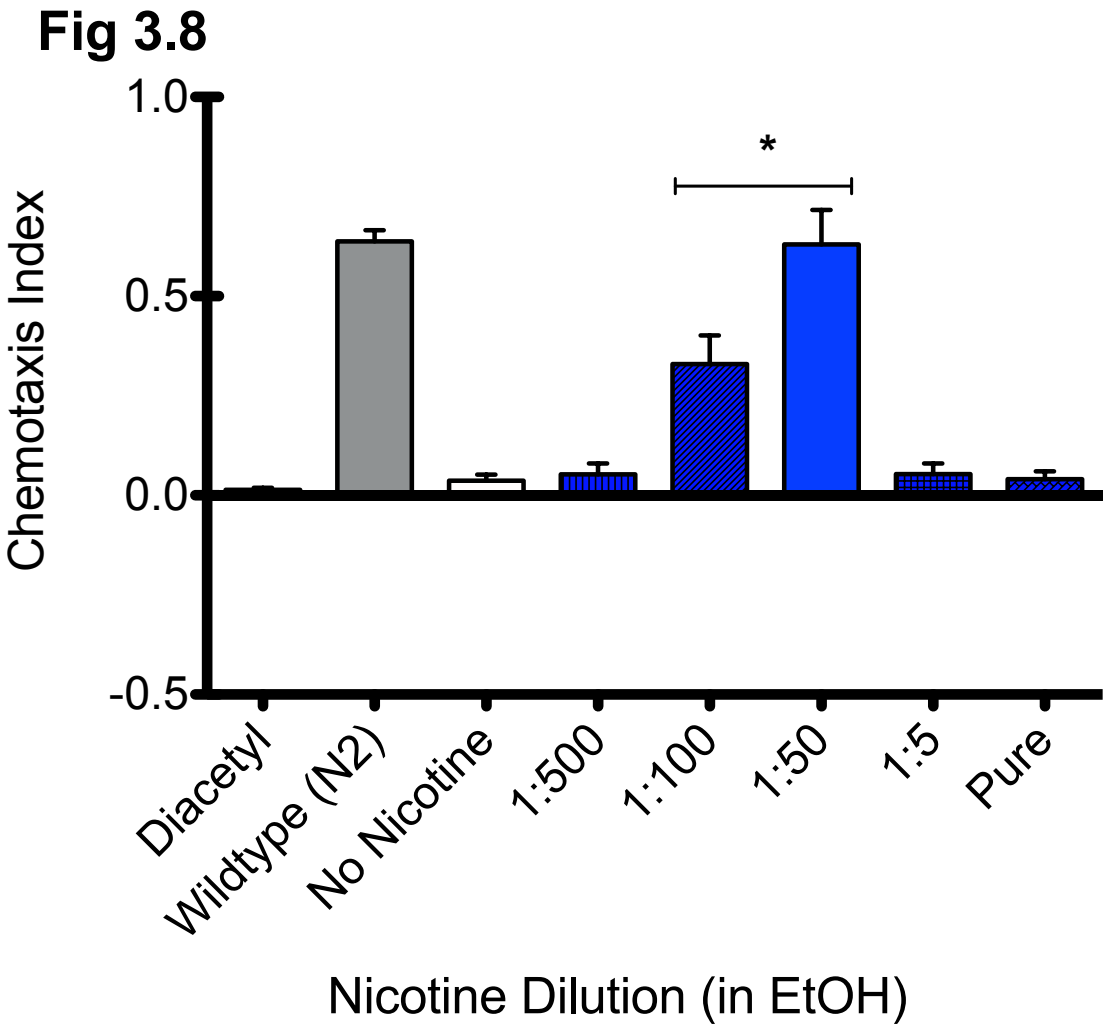

Fig 3.8 shows the nicotine chemotaxis response of *ocr-2* mutants. When compared to the 'no nicotine' group [Mean: 0.037 ± 0.0173], the '1:500' [Mean: 0.053 ± 0.0313], '1:5' [Mean: 0.054 ± 0.0308], and 'Pure' [Mean: 0.041 ± 0.0231] groups show no significant difference, while the '1:100' [Mean: 0.330 ± 0.0830] and '1:50' [Mean: 0.630 ± 0.1001] groups each show significant chemotaxis to the nicotine source [one-way ANOVA $p < 0.0001$, Dunnett's MCT].

ocr-1;ocr-2 Double Mutants Nicotine Chemotaxis

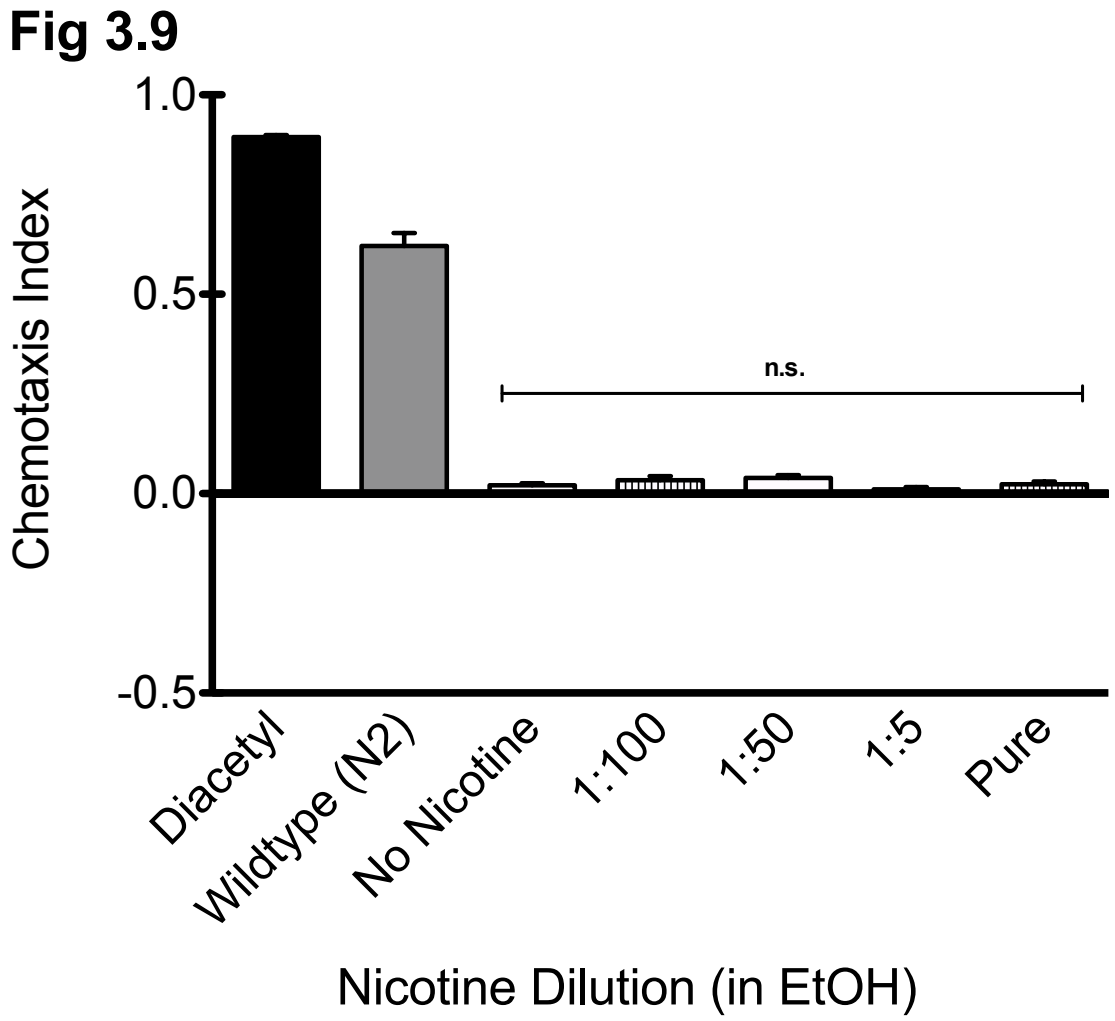

Fig 3.9 shows the nicotine chemotaxis response of *ocr-1;ocr-2* double mutants. Double Mutants on plates with a 1:100 [Mean: 0.033 ± 0.0127], 1:50 [Mean: 0.040 ± 0.0082], 1:5 [Mean: 0.011 ± 0.0067], or Pure [Mean: 0.023 ± 0.0087] nicotine dilutions showed no difference when compared to animals on a plate with no nicotine [Mean: 0.021 ± 0.0066] [one-way ANOVA p = 0.1148].

Other TRP Channel Mutants Nicotine Chemotaxis

Fig 3.10a

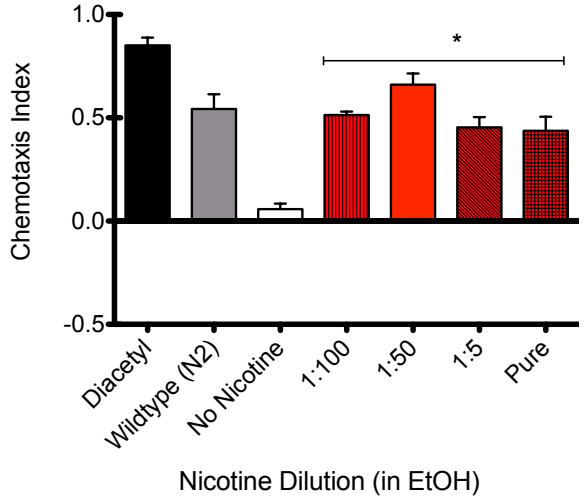

Fig 3.10b

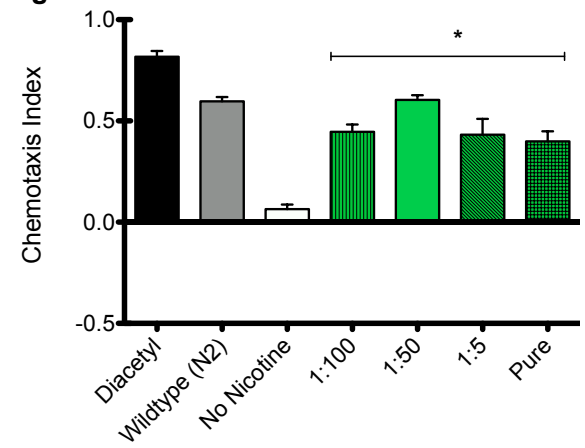

Fig 3.10 shows the dose ranges of *trpa-1* and *ocr-4* mutants. **Fig 3.10a** shows the nicotine chemotaxis response of *trpa-1* mutants. Mutants on plates with a 1:100 [Mean: 0.514 ± 0.0201], 1:50 [Mean: 0.660 ± 0.0668], 1:5 [Mean: 0.454 ± 0.0606], or Pure [Mean: 0.437 ± 0.0834] nicotine dilutions showed significant nicotine chemotaxis when compared to animals on a plate with no nicotine [Mean: 0.058 ± 0.0326] [one-way ANOVA $p < 0.0001$, Dunnett's MCT]. **Fig 3.10b** shows the nicotine chemotaxis response of *ocr-4* mutants. Mutants on plates with a 1:100 [Mean: 0.446 ± 0.0437], 1:50 [Mean: 0.604 ± 0.0282], 1:5 [Mean: 0.432 ± 0.0958], or Pure [Mean: 0.399 ± 0.0613] nicotine dilutions showed significant nicotine chemotaxis when compared to animals on a plate with no nicotine [Mean: 0.064 ± 0.0280] [one-way ANOVA $p < 0.001$, Dunnett's MCT].

Rescue of OCR-1 in *ocr-1* Mutant Background

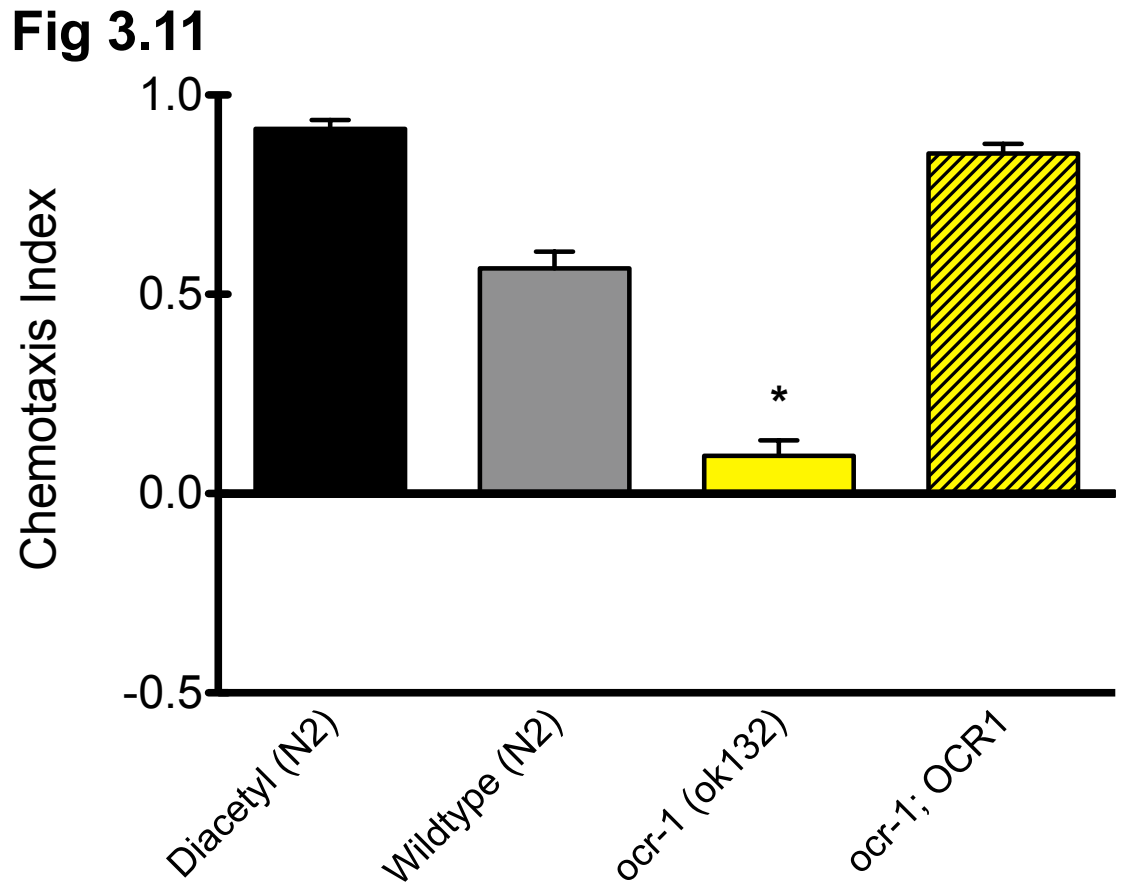

Fig 3.11 shows the nicotine chemotaxis of transgenic animals rescuing OCR-1 specifically within neurons in an *ocr-1* null background. At the 1:50 nicotine dilution, animals carrying a transgene to express OCR-1 within neurons [Mean: 0.853 ± 0.0277] show nicotine chemotaxis at levels that exceed wild-type animals [Mean: 0.565 ± 0.0482], rescuing the defect in this behavior seen in the *ocr-1* mutants [Mean: 0.095 ± 0.0450] [one-way ANOVA $p < 0.0001$, Bonferroni's MCT].

Working Model of TRPV-Mediated Nicotine Chemotaxis

Fig 3.12

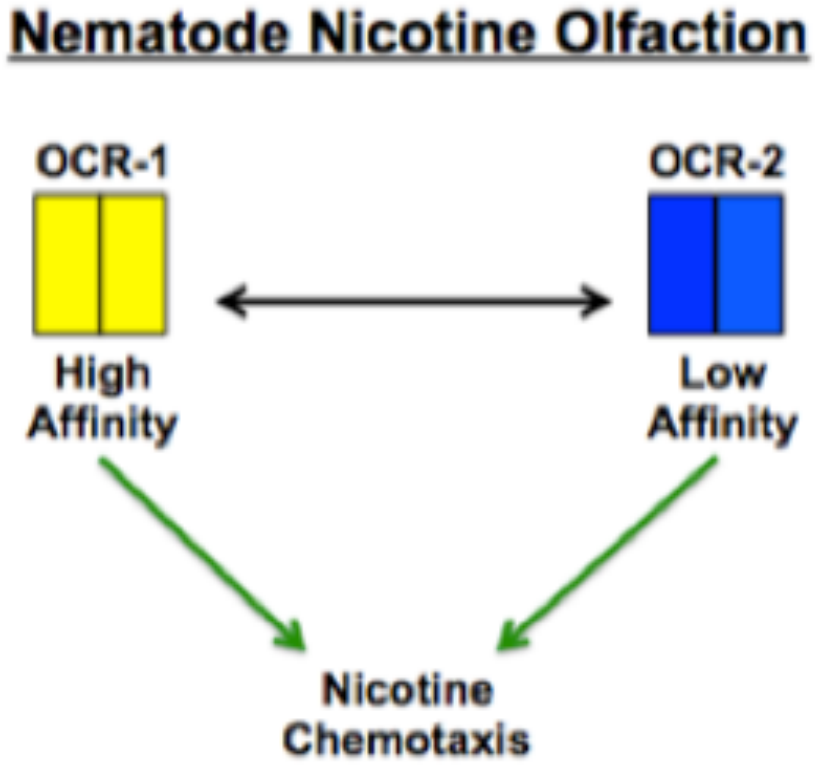

Fig 3.12 shows a working model to explain the role of TRPV proteins in nicotine olfaction. In this model, nicotine targets OCR-1, either directly or indirectly. At low levels of nicotine, OCR-1 alone mediates the nicotine chemotaxis response. As nicotine levels rise, the much lower affinity and closely homologous, OCR-2, comes online to mediate part of this nicotine chemotaxis at the more pure nicotine levels.

Chapter IV

Concluding Remarks

C. elegans is a simple metazoan organism, but in its simplicity lies its strength as a model organism. As an hermaphroditic species capable of self-fertilization that has no need for denning, the motivated behaviors of these animals are largely limited to food-seeking and to the avoidance of noxious stimuli. This, in conjunction with the tractability of nematode genome and simplicity of its nervous system, makes *C. elegans* a powerful, if unlikely, tool for investigating drug-responsive behaviors at the molecular and cellular level.

A number of research groups, among them our own, have demonstrated the feasibility of *C. elegans* as a model for studying drugs of abuse. Sobkowiak and colleagues, for example, demonstrated that nematodes respond to nicotine in both a concentration-dependent and time-dependent manner (Sobkowiak et al., 2011). As discussed previously, the Sellings-Pereira group showed that worms are capable of navigating up a nicotine gradient (Sellings et al., 2013). Musselman and colleagues, furthermore, have used nicotine paired with salts to probe CS-US associations in nematodes (Musselman et al., 2012). Our lab, meanwhile, has shown that worms respond to an acute nicotine challenge with an increase in crawl speed that is dependent upon TRP channels (Feng et al., 2006). Additionally, our lab has also shown that acute cocaine can also elicit a hypolocomotive behavioral response in *C. elegans* (Ward et al., 2009). Moreover,

Davies and colleagues have done exciting work in identifying genes involved in the nematode response to ethanol (Davies and McIntire, 2004).

Herein, we present two additional lines of research adding to the growing literature supporting *C. elegans* as model for understanding drugs of abuse more completely. In probing the insulin signaling pathway mutants, we sought to explore more deeply the acute nicotine response of nematodes and we turned to olfactory chemotaxis identify a novel role for TRP channels in a second nicotine response paradigm. We are the first to demonstrate an olfactory component to nicotine chemotaxis in *C. elegans* and no one has yet to report a direct effect of the insulin pathway on nicotine-related behaviors.

While the field is still evolving, a role for insulin signaling in addiction biology is becoming increasingly more obvious. Insulin receptors are enriched within mammalian brain regions susceptible to pharmacological effects from drugs of abuse (Daws et al., 2011; Konner et al., 2011; Labouebe et al., 2013). Insulin regulates DA transporter activity through an Akt-dependent pathway (Pardini et al., 2006; Russo et al., 2007; Sugano et al., 2006). This same IIS-Akt pathway is what we discovered to be crucial in the normal nematode acute nicotine response. Moreover, acetylcholine receptors are vital regulators of DA neuron activity in the mammalian midbrain (Exley et al., 2008; Exley and Cragg, 2008; Exley et al., 2011; Exley et al., 2012; Faure et al., 2014; Picciotto et al., 1998; You et al., 2008). It is clear to see how simultaneous disruptions in both these regulators of extracellular DA concentrations could evoke pathology. In fact, the various interactions among the insulin signaling pathway, acetylcholine receptors, midbrain DA neurons could readily explain the high incidence of co-morbidity among addiction moieties and energy homeostasis disorders. At the very least, the continuously increasing literature expounding upon these interactions should illustrate the importance of monitoring

caloric input/output of individuals undergoing a tobacco cessation or other drug rehabilitation program.

While TRP channels are best known for their role in sensory systems (Kauer and Gibson, 2009; Montell, 2001, 2005; Venkatachalam and Montell, 2007; Wescott et al., 2013), they are gaining recognition as important players in modulating central responses to drugs of abuse (Kauer and Gibson, 2009; Wescott et al., 2013). Of particular interest are the TRPV family channels, which are targets of endocannabinoids in mammals (Grueter et al., 2010). Incidentally, endocannabinoids appear to mediate the insulin-dependent plasticity of tegmental DA neurons in mammals (Labouebe et al., 2013). Despite all of the circumstantial evidence within the literature to support interactions among the acetylcholine, insulin, and endovanilloids pathways, it remains possible that the inverted, depressant-like response our insulin signaling mutants show in response to nicotine could be due to their increased susceptibility to the toxic effects of nicotine, despite the fact that they do not appear to succumb to nicotine-induced paralysis any earlier than wild-type nematodes. Regardless, even if this interpretation were to prove true, it would still support a clinical relevance for monitoring the insulin signaling pathway in current and recovering addicts, especially nicotine addicts.

Here, however, we described a clearly novel sensory role for the TRPV channel OCR-1 in *C. elegans* olfaction. For all previously known volatile chemicals to which nematodes respond, one or more G-proteins are necessary to activate a TRP channel (Bargmann, 2006; Micale et al., 2010). Nicotine, surprisingly, appears to act directly at OCR-1, bypassing *odr-3* and the G-protein pathway. If nicotine can act directly at TRPV channels, one might be tempted to suggest that the smell of vaporized nicotine from smoking tobacco may mediate some of the addicting properties of nicotine. However, as roughly 1,000 different chemicals compose

cigarette smoke making the smoke a highly complex odorant, this seems unlikely. A number of recent studies have suggested that the unconditioned properties of cocaine in the periphery could, over time, act as a conditioned stimulus predicting the unconditioned euphoria the drug induces (Porter-Stransky et al., 2011; Wise et al., 2008). This seems to be a more plausible effect of nicotine activating TRPV channels in the pharynx and nasal mucosa — peripheral, physiological effects of nicotine could come to serve as a CS that predicts the central US properties of the drug once it reaches the brain.

More likely still, is a role for TRPV channels acting within the central nervous system to modulate or gate the window for associative learning, particularly within the mesolimbic dopamine system. As some evidence already implicates the endovanilloids in neural mechanisms thought to underlie learning and memory (Grueter et al., 2010; Kauer, 2004; Kauer and Gibson, 2009; Labouebe et al., 2013), plumbing the role of TRPV channels in mammalian motivated behaviors and associative learning promises exciting results in the coming years. For example, the typically retrograde nature of the endocannabinoids suggests that TPRV channels could play a crucial role in terminal control of neurotransmitter release within the nucleus accumbens, prefrontal cortex, and amygdala, fine-tuning midbrain dopamine signals or coordinating among various neurotransmitter systems to improve the signal-to-noise ratio in the chemical communication of the brain. In addition, overwhelming evidence already demonstrates that various TRP channels pair with metabotropic glutamate receptors (Bengtson et al., 2004; Berg et al., 2007; Fowler et al., 2007; Grueter et al., 2010; Huang et al., 2007; Huang et al., 2011; Kim et al., 2003; Musella et al., 2010; Riccio et al., 2009). It could be most exciting to learn that some monoamine GPCRs can physically or functionally couple with TRP channels to stabilize the window of time necessary for coincidence detection and synaptic plasticity. Furthermore, as yet,

very little work has examined the role of TRP channels in these underdogs of synaptic physiology and addiction. In any case, the effects of TRP channels already identified in the mesolimbic dopamine system make them a compelling target for an adjunct pharmacotherapy in tobacco cessation programs.

As we continue to explore *C. elegans* as a model for addiction-related behaviors, it will be interesting to see how hard they will work to reach a point-source of nicotine. For example, nematodes avoid crossing copper-containing regions of their environment. Perhaps worms previously exposed to nicotine would be more willing to cross a copper barrier to reach a nicotine source than their drug-naïve controls. This would be commensurate with a human, or rodent model organism, who continues drug-seeking despite negative consequences. More interesting still, would be if TRPV channel mutants then failed to show this behavior. With regard to the insulin findings presented here, a number of questions still remain. It would be useful to identify the exact neuron or neurons in which *daf-2*/IIS signaling modulates changes in locomotion following nicotine exposure. Additionally, identifying which gene or genes that *daf-16*/FoxO regulate in response to nicotine could help to discover novel targets in human nicotine dependence treatment. Furthermore, as Akt activity also regulates the mTOR pathway, there exists an entire avenue of study yet to pursue in nematodes. Both of these lines of research – the interactions between insulin signaling and drugs of abuse as well as the role of TRP channels in addiction biology – promise to yield exciting results in the years to come.

References

Andre, E., Campi, B., Materazzi, S., Trevisani, M., Amadesi, S., Massi, D., Creminon, C., Vaksman, N., Nassini, R., Civelli, M., *et al.* (2008). Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents. The Journal of clinical investigation *118*, 2574-2582.

Anthony, K., Reed, L.J., Dunn, J.T., Bingham, E., Hopkins, D., Marsden, P.K., and Amiel, S.A. (2006). Attenuation of insulin-evoked responses in brain networks controlling appetite and reward in insulin resistance: the cerebral basis for impaired control of food intake in metabolic syndrome? Diabetes *55*, 2986-2992.

Aragona, B.J., Cleaveland, N.A., Stuber, G.D., Day, J.J., Carelli, R.M., and Wightman, R.M. (2008). Preferential enhancement of dopamine transmission within the nucleus accumbens shell by cocaine is attributable to a direct increase in phasic dopamine release events. J Neurosci *28*, 8821-8831.

Aragona, B.J., Day, J.J., Roitman, M.F., Cleaveland, N.A., Wightman, R.M., and Carelli, R.M. (2009). Regional specificity in the real-time development of phasic dopamine transmission patterns during acquisition of a cue-cocaine association in rats. The European journal of neuroscience *30*, 1889-1899.

Ashrafi, K. (2007). Obesity and the regulation of fat metabolism. WormBook : the online review of C elegans biology, 1-20.

Atallah, H.E., McCool, A.D., Howe, M.W., and Graybiel, A.M. (2014). Neurons in the ventral striatum exhibit cell-type-specific representations of outcome during learning. Neuron *82*, 1145-1156.

Avale, M.E., Faure, P., Pons, S., Robledo, P., Deltheil, T., David, D.J., Gardier, A.M., Maldonado, R., Granon, S., Changeux, J.P., *et al.* (2008). Interplay of beta2* nicotinic receptors and dopamine pathways in the control of spontaneous locomotion. Proceedings of the National Academy of Sciences of the United States of America *105*, 15991-15996.

Avery, L., and You, Y.J. (2012). C. elegans feeding. WormBook : the online review of C elegans biology, 1-23.

Badrinarayan, A., Wescott, S.A., Vander Weele, C.M., Saunders, B.T., Couturier, B.E., Maren, S., and Aragona, B.J. (2012). Aversive stimuli differentially modulate real-time dopamine transmission dynamics within the nucleus accumbens core and shell. J Neurosci *32*, 15779-15790.

Baisley, S.K., and Baldo, B.A. (2014). Amylin Receptor Signaling in the Nucleus Accumbens Negatively Modulates mu-opioid-Driven Feeding. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *39*, 3009-3017.

Bajaj, M. (2012). Nicotine and insulin resistance: when the smoke clears. Diabetes *61*, 3078-3080.

Baldo, B.A.K., A. E. (2001). Amylin infusion into rat nucleus accumbens potently depresses motor activity and ingestive behavior. Am J Physiol Regulatory Integrative Comp Physiol *281*, R1232–R1242.

Bang, S., Kim, K.Y., Yoo, S., Kim, Y.G., and Hwang, S.W. (2007). Transient receptor potential A1 mediates acetaldehyde-evoked pain sensation. The European journal of neuroscience *26*, 2516-2523.
Bargmann, C.I. (2006). Chemosensation in C. elegans. WormBook : the online review of C elegans biology, 1-29.
Bargmann, C.I., Hartwieg, E., and Horvitz, H.R. (1993). Odorant-selective genes and neurons mediate olfaction in C. elegans. Cell *74*, 515-527.
Belin, D., Belin-Rauscent, A., Murray, J.E., and Everitt, B.J. (2013). Addiction: failure of control over maladaptive incentive habits. Current opinion in neurobiology *23*, 564-572.
Bellinger, L.L., Wellman, P.J., Harris, R.B., Kelso, E.W., and Kramer, P.R. (2010). The effects of chronic nicotine on meal patterns, food intake, metabolism and body weight of male rats. Pharmacology, biochemistry, and behavior *95*, 92-99.
Benedikt, J., Teisinger, J., Vyklicky, L., and Vlachova, V. (2007). Ethanol inhibits cold-menthol receptor TRPM8 by modulating its interaction with membrane phosphatidylinositol 4,5-bisphosphate. Journal of neurochemistry *100*, 211-224.
Bengtson, C.P., Tozzi, A., Bernardi, G., and Mercuri, N.B. (2004). Transient receptor potential-like channels mediate metabotropic glutamate receptor EPSCs in rat dopamine neurones. The Journal of physiology *555*, 323-330.
Benowitz, N.L. (2008). Neurobiology of nicotine addiction: implications for smoking cessation treatment. The American journal of medicine *121*, S3-10.
Berg, A.P., Sen, N., and Bayliss, D.A. (2007). TrpC3/C7 and Slo2.1 are molecular targets for metabotropic glutamate receptor signaling in rat striatal cholinergic interneurons. J Neurosci *27*, 8845-8856.
Bergman, B.C., Perreault, L., Hunerdosse, D., Kerege, A., Playdon, M., Samek, A.M., and Eckel, R.H. (2012). Novel and reversible mechanisms of smoking-induced insulin resistance in humans. Diabetes *61*, 3156-3166.
Bierut, L.J., Madden, P.A., Breslau, N., Johnson, E.O., Hatsukami, D., Pomerleau, O.F., Swan, G.E., Rutter, J., Bertelsen, S., Fox, L.*, et al.* (2007). Novel genes identified in a high-density genome wide association study for nicotine dependence. Human molecular genetics *16*, 24-35.
Blednov, Y.A., and Harris, R.A. (2009). Deletion of vanilloid receptor (TRPV1) in mice alters behavioral effects of ethanol. Neuropharmacology *56*, 814-820.
Boghossian, S., Lemmon, K., Park, M., and York, D.A. (2009). High-fat diets induce a rapid loss of the insulin anorectic response in the amygdala. American journal of physiology Regulatory, integrative and comparative physiology *297*, R1302-1311.
Bonasera, S.J., Schenk, A.K., Luxenberg, E.J., and Tecott, L.H. (2008). A novel method for automatic quantification of psychostimulant-evoked route-tracing stereotypy: application to Mus musculus. Psychopharmacology *196*, 591-602.
Bourdy, R., and Barrot, M. (2012). A new control center for dopaminergic systems: pulling the VTA by the tail. Trends in neurosciences *35*, 681-690.
Bourdy, R., Sanchez-Catalan, M.J., Kaufling, J., Balcita-Pedicino, J.J., Freund-Mercier, M.J., Veinante, P., Sesack, S.R., Georges, F., and Barrot, M. (2014). Control of the nigrostriatal dopamine neuron activity and motor function by the tail of the ventral tegmental area. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *39*, 2788-2798.

Breiter, H.C., Gollub, R.L., Weisskoff, R.M., Kennedy, D.N., Makris, N., Berke, J.D., Goodman, J.M., Kantor, H.L., Gastfriend, D.R., Riorden, J.P.*, et al.* (1997). Acute effects of cocaine on human brain activity and emotion. Neuron *19*, 591-611.
Bruijnzeel, A.W., Corrie, L.W., Rogers, J.A., and Yamada, H. (2011). Effects of insulin and leptin in the ventral tegmental area and arcuate hypothalamic nucleus on food intake and brain reward function in female rats. Behavioural brain research *219*, 254-264.
Carlezon, W.A., Jr., and Thomas, M.J. (2009). Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. Neuropharmacology *56 Suppl 1*, 122-132.
Casarotto, P.C., Terzian, A.L., Aguiar, D.C., Zangrossi, H., Guimaraes, F.S., Wotjak, C.T., and Moreira, F.A. (2012). Opposing roles for cannabinoid receptor type-1 (CB(1)) and transient receptor potential vanilloid type-1 channel (TRPV1) on the modulation of panic-like responses in rats. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *37*, 478-486.
Cavalie, A. (2007). Ionic channels formed by TRPC4. Handbook of experimental pharmacology, 93-108.
Changeux, J.P., Bertrand, D., Corringer, P.J., Dehaene, S., Edelstein, S., Lena, C., Le Novere, N., Marubio, L., Picciotto, M., and Zoli, M. (1998). Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. Brain research Brain research reviews *26*, 198-216.
Changeux, J.P., and Edelstein, S.J. (2005). Allosteric mechanisms of signal transduction. Science *308*, 1424-1428.
Chuhma, N., Mingote, S., Moore, H., and Rayport, S. (2014). Dopamine neurons control striatal cholinergic neurons via regionally heterogeneous dopamine and glutamate signaling. Neuron *81*, 901-912.
Coghlan, A. (2005). Nematode genome evolution. WormBook : the online review of C elegans biology, 1-15.
Colbert, H.A., and Bargmann, C.I. (1995). Odorant-specific adaptation pathways generate olfactory plasticity in C. elegans. Neuron *14*, 803-812.
Colbert, H.A.S., T. L.; Bargmann, C. I. (1997). OSM-9, A Novel Protein with Structural Similarity to Channels, Is Required for Olfaction, Mechanosensation, and Olfactory Adaptation in Caenorhabditis elegans. J Neurosci *17*, 8259–8269.
Dandekar, M.P., Nakhate, K.T., Kokare, D.M., and Subhedar, N.K. (2011). Effect of nicotine on feeding and body weight in rats: involvement of cocaine- and amphetamine-regulated transcript peptide. Behavioural brain research *219*, 31-38.
Davies, A.G., and McIntire, S.L. (2004). Using C. elegans to screen for targets of ethanol and behavior-altering drugs. Biological procedures online *6*, 113-119.
Davis, J.F., Choi, D.L., and Benoit, S.C. (2010). Insulin, leptin and reward. Trends in endocrinology and metabolism: TEM *21*, 68-74.
Daws, L.C., Avison, M.J., Robertson, S.D., Niswender, K.D., Galli, A., and Saunders, C. (2011). Insulin signaling and addiction. Neuropharmacology *61*, 1123-1128.
Day, J.J., Roitman, M.F., Wightman, R.M., and Carelli, R.M. (2007). Associative learning mediates dynamic shifts in dopamine signaling in the nucleus accumbens. Nature neuroscience *10*, 1020-1028.
Day, J.J., Wheeler, R.A., Roitman, M.F., and Carelli, R.M. (2006). Nucleus accumbens neurons encode Pavlovian approach behaviors: evidence from an autoshaping paradigm. The European journal of neuroscience *23*, 1341-1351.

Di Chiara, G. (2000). Role of dopamine in the behavioural actions of nicotine related to addiction. European journal of pharmacology *393*, 295-314.
Dunning, J.P., Parvaz, M.A., Hajcak, G., Maloney, T., Alia-Klein, N., Woicik, P.A., Telang, F., Wang, G.J., Volkow, N.D., and Goldstein, R.Z. (2011). Motivated attention to cocaine and emotional cues in abstinent and current cocaine users--an ERP study. The European journal of neuroscience *33*, 1716-1723.
Edwards, G. (2012). "The evil genius of the habit": DSM-5 seen in historical context. Journal of studies on alcohol and drugs *73*, 699-701.
Everitt, B.J., Parkinson, J.A., Olmstead, M.C., Arroyo, M., Robledo, P., and Robbins, T.W. (1999). Associative processes in addiction and reward. The role of amygdala-ventral striatal subsystems. Annals of the New York Academy of Sciences *877*, 412-438.
Exley, R., Clements, M.A., Hartung, H., McIntosh, J.M., and Cragg, S.J. (2008). Alpha6-containing nicotinic acetylcholine receptors dominate the nicotine control of dopamine neurotransmission in nucleus accumbens. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *33*, 2158-2166.
Exley, R., and Cragg, S.J. (2008). Presynaptic nicotinic receptors: a dynamic and diverse cholinergic filter of striatal dopamine neurotransmission. British journal of pharmacology *153 Suppl 1*, S283-297.
Exley, R., Maubourguet, N., David, V., Eddine, R., Evrard, A., Pons, S., Marti, F., Threlfell, S., Cazala, P., McIntosh, J.M., *et al.* (2011). Distinct contributions of nicotinic acetylcholine receptor subunit alpha4 and subunit alpha6 to the reinforcing effects of nicotine. Proceedings of the National Academy of Sciences of the United States of America *108*, 7577-7582.
Exley, R., McIntosh, J.M., Marks, M.J., Maskos, U., and Cragg, S.J. (2012). Striatal alpha5 nicotinic receptor subunit regulates dopamine transmission in dorsal striatum. J Neurosci *32*, 2352-2356.
Faure, P., Tolu, S., Valverde, S., and Naude, J. (2014). Role of nicotinic acetylcholine receptors in regulating dopamine neuron activity. Neuroscience *282C*, 86-100.
Feng, Z., Li, W., Ward, A., Piggott, B.J., Larkspur, E.R., Sternberg, P.W., and Xu, X.Z. (2006). A C. elegans model of nicotine-dependent behavior: regulation by TRP-family channels. Cell *127*, 621-633.
Fields, H.L., Hjelmstad, G.O., Margolis, E.B., and Nicola, S.M. (2007). Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. Annual review of neuroscience *30*, 289-316.
Figlewicz, D.P., Bennett, J.L., Aliakbari, S., Zavosh, A., and Sipols, A.J. (2008). Insulin acts at different CNS sites to decrease acute sucrose intake and sucrose self-administration in rats. American journal of physiology Regulatory, integrative and comparative physiology *295*, R388-394.
Figlewicz, D.P., Szot, P., Chavez, M., Woods, S.C., and Veith, R.C. (1994). Intraventricular insulin increases dopamine transporter mRNA in rat VTA/substantia nigra. Brain research *644*, 331-334.
Fitch, D.H. (2005). Introduction to nematode evolution and ecology. WormBook : the online review of C elegans biology, 1-8.
Floresco, S.B., West, A.R., Ash, B., Moore, H., and Grace, A.A. (2003). Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nature neuroscience *6*, 968-973.

Fornari, A., Pedrazzi, P., Lippi, G., Picciotto, M.R., Zoli, M., and Zini, I. (2007). Nicotine withdrawal increases body weight, neuropeptide Y and Agouti-related protein expression in the hypothalamus and decreases uncoupling protein-3 expression in the brown adipose tissue in high-fat fed mice. Neuroscience letters *411*, 72-76.
Fowler, M.A., Sidiropoulou, K., Ozkan, E.D., Phillips, C.W., and Cooper, D.C. (2007). Corticolimbic expression of TRPC4 and TRPC5 channels in the rodent brain. PloS one *2*, e573.
Gao, Y.J., Holloway, A.C., Su, L.Y., Takemori, K., Lu, C., and Lee, R.M. (2008). Effects of fetal and neonatal exposure to nicotine on blood pressure and perivascular adipose tissue function in adult life. European journal of pharmacology *590*, 264-268.
Gochberg-Sarver, A., Kedmi, M., Gana-Weisz, M., Bar-Shira, A., and Orr-Urtreger, A. (2012). Tnfalpha, Cox2 and AdipoQ adipokine gene expression levels are modulated in murine adipose tissues by both nicotine and nACh receptors containing the beta2 subunit. Molecular genetics and metabolism *107*, 561-570.
Grueter, B.A., Brasnjo, G., and Malenka, R.C. (2010). Postsynaptic TRPV1 triggers cell type-specific long-term depression in the nucleus accumbens. Nature neuroscience *13*, 1519-1525.
Gulbransen, B.D., Clapp, T.R., Finger, T.E., and Kinnamon, S.C. (2008). Nasal solitary chemoreceptor cell responses to bitter and trigeminal stimulants in vitro. Journal of neurophysiology *99*, 2929-2937.
Hobert, O. (2010). Neurogenesis in the nematode Caenorhabditis elegans. WormBook : the online review of C elegans biology, 1-24.
Huang, C.C., Yang, P.C., Lin, H.J., and Hsu, K.S. (2007). Repeated cocaine administration impairs group II metabotropic glutamate receptor-mediated long-term depression in rat medial prefrontal cortex. J Neurosci *27*, 2958-2968.
Huang, C.C., Yeh, C.M., Wu, M.Y., Chang, A.Y., Chan, J.Y., Chan, S.H., and Hsu, K.S. (2011). Cocaine withdrawal impairs metabotropic glutamate receptor-dependent long-term depression in the nucleus accumbens. J Neurosci *31*, 4194-4203.
Ikemoto, S. (2007). Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. Brain research reviews *56*, 27-78.
Ikemoto, S., and Bonci, A. (2014). Neurocircuitry of drug reward. Neuropharmacology *76 Pt B*, 329-341.
Inoue, K., Takeshima, F., Kadota, K., Yoda, A., Tatsuta, Y., Nagaura, Y., Yoshioka, S., Nakamichi, S., Nakao, K., and Ozono, Y. (2011). Early Effects of Smoking Cessation and Weight Gain on Plasma Adiponectin Levels and Insulin Resistance. Internal Medicine *50*, 707-712.
Jalabert, M., Bourdy, R., Courtin, J., Veinante, P., Manzoni, O.J., Barrot, M., and Georges, F. (2011). Neuronal circuits underlying acute morphine action on dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America *108*, 16446-16450.
Jansen, G., Thijssen, K.L., Werner, P., van der Horst, M., Hazendonk, E., and Plasterk, R.H. (1999). The complete family of genes encoding G proteins of Caenorhabditis elegans. Nature genetics *21*, 414-419.
Jia, Y., Zhou, J., Tai, Y., and Wang, Y. (2007). TRPC channels promote cerebellar granule neuron survival. Nature neuroscience *10*, 559-567.

Jones, J.L., Day, J.J., Aragona, B.J., Wheeler, R.A., Wightman, R.M., and Carelli, R.M. (2010). Basolateral amygdala modulates terminal dopamine release in the nucleus accumbens and conditioned responding. Biological psychiatry *67*, 737-744.
Kahn, H.S., El ghormli, L., Jago, R., Foster, G.D., McMurray, R.G., Buse, J.B., Stadler, D.D., Trevino, R.P., Baranowski, T., and Group, H.S. (2014). Cardiometabolic risk assessments by body mass index z-score or waist-to-height ratio in a multiethnic sample of sixth-graders. Journal of obesity *2014*, 421658.
Kahn-Kirby, A.H., Dantzker, J.L., Apicella, A.J., Schafer, W.R., Browse, J., Bargmann, C.I., and Watts, J.L. (2004). Specific polyunsaturated fatty acids drive TRPV-dependent sensory signaling in vivo. Cell *119*, 889-900.
Kang, L., Gao, J., Schafer, W.R., Xie, Z., and Xu, X.Z. (2010). C. elegans TRP family protein TRP-4 is a pore-forming subunit of a native mechanotransduction channel. Neuron *67*, 381-391.
Kauer, J.A. (2004). Learning mechanisms in addiction: synaptic plasticity in the ventral tegmental area as a result of exposure to drugs of abuse. Annual review of physiology *66*, 447-475.
Kauer, J.A., and Gibson, H.E. (2009). Hot flash: TRPV channels in the brain. Trends in neurosciences *32*, 215-224.
Kauer, J.A., and Malenka, R.C. (2007). Synaptic plasticity and addiction. Nature reviews Neuroscience *8*, 844-858.
Kaufling, J., Waltisperger, E., Bourdy, R., Valera, A., Veinante, P., Freund-Mercier, M.J., and Barrot, M. (2010). Pharmacological recruitment of the GABAergic tail of the ventral tegmental area by acute drug exposure. British journal of pharmacology *161*, 1677-1691.
Kim, J.Y., Zeng, W., Kiselyov, K., Yuan, J.P., Dehoff, M.H., Mikoshiba, K., Worley, P.F., and Muallem, S. (2006). Homer 1 mediates store- and inositol 1,4,5-trisphosphate receptor-dependent translocation and retrieval of TRPC3 to the plasma membrane. The Journal of biological chemistry *281*, 32540-32549.
Kim, S.J., Kim, Y.S., Yuan, J.P., Petralia, R.S., Worley, P.F., and Linden, D.J. (2003). Activation of the TRPC1 cation channel by metabotropic glutamate receptor mGluR1. Nature *426*, 285-291.
Konner, A.C., Hess, S., Tovar, S., Mesaros, A., Sanchez-Lasheras, C., Evers, N., Verhagen, L.A., Bronneke, H.S., Kleinridders, A., Hampel, B*., et al.* (2011). Role for insulin signaling in catecholaminergic neurons in control of energy homeostasis. Cell metabolism *13*, 720-728.
Koob, G.F. (2009a). Brain stress systems in the amygdala and addiction. Brain research *1293*, 61-75.
Koob, G.F. (2009b). Neurobiological substrates for the dark side of compulsivity in addiction. Neuropharmacology *56 Suppl 1*, 18-31.
Koob, G.F. (2010). The role of CRF and CRF-related peptides in the dark side of addiction. Brain research *1314*, 3-14.
Koob, G.F., and Le Moal, M. (2005). Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. Nature neuroscience *8*, 1442-1444.
Kourrich, S., Rothwell, P.E., Klug, J.R., and Thomas, M.J. (2007). Cocaine experience controls bidirectional synaptic plasticity in the nucleus accumbens. J Neurosci *27*, 7921-7928.
Kramer, P.R., Guan, G., Wellman, P.J., and Bellinger, L.L. (2007a). Nicotine's attenuation of body weight involves the perifornical hypothalamus. Life sciences *81*, 500-508.

Kramer, P.R., Kramer, S.F., Marr, K., Guan, G., Wellman, P.J., and Bellinger, L.L. (2007b). Nicotine administration effects on feeding and cocaine-amphetamine-regulated transcript (CART) expression in the hypothalamus. Regulatory peptides *138*, 66-73.
Kumar, D., Deb, I., Chakraborty, J., Mukhopadhyay, S., and Das, S. (2011). A polymorphism of the CREB binding protein (CREBBP) gene is a risk factor for addiction. Brain research *1406*, 59-64.
Labouebe, G., Liu, S., Dias, C., Zou, H., Wong, J.C., Karunakaran, S., Clee, S.M., Phillips, A.G., Boutrel, B., and Borgland, S.L. (2013). Insulin induces long-term depression of ventral tegmental area dopamine neurons via endocannabinoids. Nature neuroscience *16*, 300-308.
Lammel, S., Hetzel, A., Hackel, O., Jones, I., Liss, B., and Roeper, J. (2008). Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. Neuron *57*, 760-773.
Lammel, S., Ion, D.I., Roeper, J., and Malenka, R.C. (2011). Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. Neuron *70*, 855-862.
Lammel, S., Lim, B.K., and Malenka, R.C. (2014). Reward and aversion in a heterogeneous midbrain dopamine system. Neuropharmacology *76 Pt B*, 351-359.
Lammel, S., Lim, B.K., Ran, C., Huang, K.W., Betley, M.J., Tye, K.M., Deisseroth, K., and Malenka, R.C. (2012). Input-specific control of reward and aversion in the ventral tegmental area. Nature *491*, 212-217.
Laviolette, S.R., Alexson, T.O., and van der Kooy, D. (2002). Lesions of the tegmental pedunculopontine nucleus block the rewarding effects and reveal the aversive effects of nicotine in the ventral tegmental area. J Neurosci *22*, 8653-8660.
Laviolette, S.R., Gallegos, R.A., Henriksen, S.J., and van der Kooy, D. (2004). Opiate state controls bi-directional reward signaling via GABAA receptors in the ventral tegmental area. Nature neuroscience *7*, 160-169.
Laviolette, S.R., and van der Kooy, D. (2004). The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. Nature reviews Neuroscience *5*, 55-65.
Lessov-Schlaggar, C.N., Pergadia, M.L., Khroyan, T.V., and Swan, G.E. (2008). Genetics of nicotine dependence and pharmacotherapy. Biochemical pharmacology *75*, 178-195.
Li, Z., Liu, J., Zheng, M., and Xu, X.Z. (2014). Encoding of Both Analog- and Digital-like Behavioral Outputs by One C. elegans Interneuron. Cell *159*, 751-765.
Liu, L., Zhu, W., Zhang, Z.S., Yang, T., Grant, A., Oxford, G., and Simon, S.A. (2004). Nicotine inhibits voltage-dependent sodium channels and sensitizes vanilloid receptors. Journal of neurophysiology *91*, 1482-1491.
Liu, Z., Wang, Y., Zhao, W., Ding, J., Mei, Z., Guo, L., Cui, D., and Fei, J. (2001). Peptide derived from insulin with regulatory activity of dopamine transporter. Neuropharmacology *41*, 464-471.
Lodge, D.J., and Grace, A.A. (2006). The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America *103*, 5167-5172.
Loriaux, A.L., Roitman, J.D., and Roitman, M.F. (2011). Nucleus accumbens shell, but not core, tracks motivational value of salt. Journal of neurophysiology *106*, 1537-1544.
Luscher, C., and Ungless, M.A. (2006). The mechanistic classification of addictive drugs. PLoS medicine *3*, e437.

Margolis, E.B., Lock, H., Hjelmstad, G.O., and Fields, H.L. (2006). The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? The Journal of physiology *577*, 907-924.
Maskos, U., Molles, B.E., Pons, S., Besson, M., Guiard, B.P., Guilloux, J.P., Evrard, A., Cazala, P., Cormier, A., Mameli-Engvall, M., *et al.* (2005). Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature *436*, 103-107.
Mast, T.G., Brann, J.H., and Fadool, D.A. (2010). The TRPC2 channel forms protein-protein interactions with Homer and RTP in the rat vomeronasal organ. BMC neuroscience *11*, 61.
McClung, C., and Hirsh, J. (1998). Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in Drosophila. Current biology : CB *8*, 109-112.
McClung, C., and Hirsh, J. (1999). The trace amine tyramine is essential for sensitization to cocaine in Drosophila. Current biology : CB *9*, 853-860.
Micale, V., Cristino, L., Tamburella, A., Petrosino, S., Leggio, G.M., Di Marzo, V., and Drago, F. (2010). Enhanced cognitive performance of dopamine D3 receptor "knock-out" mice in the step-through passive-avoidance test: assessing the role of the endocannabinoid/endovanilloid systems. Pharmacological research : the official journal of the Italian Pharmacological Society *61*, 531-536.
Mihov, Y., and Hurlemann, R. (2012). Altered amygdala function in nicotine addiction: insights from human neuroimaging studies. Neuropsychologia *50*, 1719-1729.
Mirenowicz, J., and Schultz, W. (1996). Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. Nature *379*, 449-451.
Montell, C. (2001). Physiology, phylogeny, and functions of the TRP superfamily of cation channels. Science's STKE : signal transduction knowledge environment *2001*, re1.
Montell, C. (2005). The TRP superfamily of cation channels. Science's STKE : signal transduction knowledge environment *2005*, re3.
Montell, C., and Rubin, G.M. (1989). Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. Neuron *2*, 1313-1323.
Murphy, C.T., and Hu, P.J. (2013). Insulin/insulin-like growth factor signaling in C. elegans. WormBook : the online review of C elegans biology, 1-43.
Musella, A., De Chiara, V., Rossi, S., Cavasinni, F., Castelli, M., Cantarella, C., Mataluni, G., Bernardi, G., and Centonze, D. (2010). Transient receptor potential vanilloid 1 channels control acetylcholine/2-arachidonoylglicerol coupling in the striatum. Neuroscience *167*, 864-871.
Musselman, H.N., Neal-Beliveau, B., Nass, R., and Engleman, E.A. (2012). Chemosensory cue conditioning with stimulants in a Caenorhabditis elegans animal model of addiction. Behavioral neuroscience *126*, 445-456.
Nashmi, R., Xiao, C., Deshpande, P., McKinney, S., Grady, S.R., Whiteaker, P., Huang, Q., McClure-Begley, T., Lindstrom, J.M., Labarca, C., *et al.* (2007). Chronic nicotine cell specifically upregulates functional alpha 4* nicotinic receptors: basis for both tolerance in midbrain and enhanced long-term potentiation in perforant path. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 8202-8218.
Niehaus, J.L., Cruz-Bermudez, N.D., and Kauer, J.A. (2009). Plasticity of addiction: a mesolimbic dopamine short-circuit? The American journal on addictions / American Academy of Psychiatrists in Alcoholism and Addictions *18*, 259-271.

Nilius, B., and Owsianik, G. (2011). The transient receptor potential family of ion channels. Genome biology *12*, 218.
Numaga, T., Wakamori, M., and Mori, Y. (2007). Trpc7. Handbook of experimental pharmacology, 143-151.
Ohno, H., Kato, S., Naito, Y., Kunitomo, H., Tomioka, M., and Iino, Y. (2014). Role of synaptic phosphatidylinositol 3-kinase in a behavioral learning response in C. elegans. Science *345*, 313-317.
Oliveira-Maia, A.J., Stapleton-Kotloski, J.R., Lyall, V., Phan, T.H., Mummalaneni, S., Melone, P., Desimone, J.A., Nicolelis, M.A., and Simon, S.A. (2009). Nicotine activates TRPM5-dependent and independent taste pathways. Proceedings of the National Academy of Sciences of the United States of America *106*, 1596-1601.
Pan, W.X., and Hyland, B.I. (2005). Pedunculopontine tegmental nucleus controls conditioned responses of midbrain dopamine neurons in behaving rats. J Neurosci *25*, 4725-4732.
Pan, W.X., Schmidt, R., Wickens, J.R., and Hyland, B.I. (2005). Dopamine cells respond to predicted events during classical conditioning: evidence for eligibility traces in the reward-learning network. J Neurosci *25*, 6235-6242.
Pandey, S.C., Chartoff, E.H., Carlezon, W.A., Jr., Zou, J., Zhang, H., Kreibich, A.S., Blendy, J.A., and Crews, F.T. (2005). CREB gene transcription factors: role in molecular mechanisms of alcohol and drug addiction. Alcoholism, clinical and experimental research *29*, 176-184.
Pardini, A.W., Nguyen, H.T., Figlewicz, D.P., Baskin, D.G., Williams, D.L., Kim, F., and Schwartz, M.W. (2006). Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis. Brain research *1112*, 169-178.
Parylak, S.L., Koob, G.F., and Zorrilla, E.P. (2011). The dark side of food addiction. Physiology & behavior *104*, 149-156.
Pedersen, B.K. (2006). The anti-inflammatory effect of exercise: its role in diabetes and cardiovascular disease control. Essays in biochemistry *42*, 105-117.
Pedersen, B.K., and Fischer, C.P. (2007). Beneficial health effects of exercise--the role of IL-6 as a myokine. Trends in pharmacological sciences *28*, 152-156.
Phillips, P.E., Robinson, D.L., Stuber, G.D., Carelli, R.M., and Wightman, R.M. (2003). Real-time measurements of phasic changes in extracellular dopamine concentration in freely moving rats by fast-scan cyclic voltammetry. Methods in molecular medicine *79*, 443-464.
Philpot, R.M., Engberg, M.E., and Wecker, L. (2012). Effects of nicotine exposure on locomotor activity and pCREB levels in the ventral striatum of adolescent rats. Behavioural brain research *230*, 62-68.
Picciotto, M.R., Zoli, M., Rimondini, R., Lena, C., Marubio, L.M., Pich, E.M., Fuxe, K., and Changeux, J.P. (1998). Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature *391*, 173-177.
Piggott, B.J., Liu, J., Feng, Z., Wescott, S.A., and Xu, X.Z. (2011). The neural circuits and synaptic mechanisms underlying motor initiation in C. elegans. Cell *147*, 922-933.
Pitman, K.A., Puil, E., and Borgland, S.L. (2014). GABAB modulation of dopamine release in the nucleus accumbens core. The European journal of neuroscience *40*, 3472-3480.
Porter-Stransky, K.A., Wescott, S.A., Hershman, M., Badrinarayan, A., Vander Weele, C.M., Lovic, V., and Aragona, B.J. (2011). Cocaine must enter the brain to evoke unconditioned dopamine release within the nucleus accumbens shell. Neuroscience letters *504*, 13-17.

Riccio, A., Li, Y., Moon, J., Kim, K.S., Smith, K.S., Rudolph, U., Gapon, S., Yao, G.L., Tsvetkov, E., Rodig, S.J.*, et al.* (2009). Essential role for TRPC5 in amygdala function and fear-related behavior. Cell *137*, 761-772.
Richardson, J.R., Pipkin, J.A., O'Dell, L.E., and Nazarian, A. (2014). Insulin resistant rats display enhanced rewarding effects of nicotine. Drug and alcohol dependence *140*, 205-207.
Roayaie, K., Crump, J.G., Sagasti, A., and Bargmann, C.I. (1998). The G alpha protein ODR-3 mediates olfactory and nociceptive function and controls cilium morphogenesis in C. elegans olfactory neurons. Neuron *20*, 55-67.
Robinson, D.L., Venton, B.J., Heien, M.L., and Wightman, R.M. (2003). Detecting subsecond dopamine release with fast-scan cyclic voltammetry in vivo. Clinical chemistry *49*, 1763-1773.
Robinson, T.E. (2004). Neuroscience. Addicted rats. Science *305*, 951-953.
Robinson, T.E., and Berridge, K.C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain research Brain research reviews *18*, 247-291.
Roitman, M.F., Stuber, G.D., Phillips, P.E., Wightman, R.M., and Carelli, R.M. (2004). Dopamine operates as a subsecond modulator of food seeking. J Neurosci *24*, 1265-1271.
Roitman, M.F., Wheeler, R.A., Wightman, R.M., and Carelli, R.M. (2008). Real-time chemical responses in the nucleus accumbens differentiate rewarding and aversive stimuli. Nature neuroscience *11*, 1376-1377.
Ron, D., and Jurd, R. (2005). The "ups and downs" of signaling cascades in addiction. Science's STKE : signal transduction knowledge environment *2005*, re14.
Russo, S.J., Bolanos, C.A., Theobald, D.E., DeCarolis, N.A., Renthal, W., Kumar, A., Winstanley, C.A., Renthal, N.E., Wiley, M.D., Self, D.W.*, et al.* (2007). IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates. Nature neuroscience *10*, 93-99.
Sanchez-Catalan, M.J., Kaufling, J., Georges, F., Veinante, P., and Barrot, M. (2014). The antero-posterior heterogeneity of the ventral tegmental area. Neuroscience *282C*, 198-216.
Sanjakdar, S.S., Maldoon, P.P., Marks, M.J., Brunzell, D.H., Maskos, U., McIntosh, J.M., Bowers, M.S., and Damaj, M.I. (2014). Differential Roles of alpha6beta2* and alpha4beta2* Neuronal Nicotinic Receptors in Nicotine- and Cocaine-Conditioned Reward in Mice. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology.
Schafe, G.E., Doyere, V., and LeDoux, J.E. (2005). Tracking the fear engram: the lateral amygdala is an essential locus of fear memory storage. J Neurosci *25*, 10010-10014.
Schafer, W.R. (2005). Egg-laying. WormBook : the online review of C elegans biology, 1-7.
Schoffelmeer, A.N., Drukarch, B., De Vries, T.J., Hogenboom, F., Schetters, D., and Pattij, T. (2011). Insulin modulates cocaine-sensitive monoamine transporter function and impulsive behavior. J Neurosci *31*, 1284-1291.
Schuckit, M.A. (2012). Editor's corner: editorial in reply to the comments of Griffith Edwards. Journal of studies on alcohol and drugs *73*, 521-522.
Schulteis, G., and Koob, G. (1994). Neuropharmacology. Dark side of drug dependence. Nature *371*, 108-109.
Sellings, L., Pereira, S., Qian, C., Dixon-McDougall, T., Nowak, C., Zhao, B., Tyndale, R.F., and van der Kooy, D. (2013). Nicotine-motivated behavior in Caenorhabditis elegans requires

the nicotinic acetylcholine receptor subunits acr-5 and acr-15. The European journal of neuroscience *37*, 743-756.
Shen, X., Bachyrycz, A., Anderson, J.R., Tinker, D., and Raisch, D.W. (2014). Quitting patterns and predictors of success among participants in a tobacco cessation program provided by pharmacists in New Mexico. Journal of managed care pharmacy : JMCP *20*, 579-587.
Sobkowiak, R., Kowalski, M., and Lesicki, A. (2011). Concentration- and time-dependent behavioral changes in Caenorhabditis elegans after exposure to nicotine. Pharmacology, biochemistry, and behavior *99*, 365-370.
Stamatakis, A.M., Sparta, D.R., Jennings, J.H., McElligott, Z.A., Decot, H., and Stuber, G.D. (2014). Amygdala and bed nucleus of the stria terminalis circuitry: Implications for addiction-related behaviors. Neuropharmacology *76 Pt B*, 320-328.
Stuber, G.D., Roitman, M.F., Phillips, P.E., Carelli, R.M., and Wightman, R.M. (2005). Rapid dopamine signaling in the nucleus accumbens during contingent and noncontingent cocaine administration. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *30*, 853-863.
Sugano, T., Yanagita, T., Yokoo, H., Satoh, S., Kobayashi, H., and Wada, A. (2006). Enhancement of insulin-induced PI3K/Akt/GSK-3beta and ERK signaling by neuronal nicotinic receptor/PKC-alpha/ERK pathway: up-regulation of IRS-1/-2 mRNA and protein in adrenal chromaffin cells. Journal of neurochemistry *98*, 20-33.
Talavera, K., Nilius, B., and Voets, T. (2008). Neuronal TRP channels: thermometers, pathfinders and life-savers. Trends in neurosciences *31*, 287-295.
Tian, Y.H., Lee, S.Y., Kim, H.C., and Jang, C.G. (2010). Repeated methamphetamine treatment increases expression of TRPV1 mRNA in the frontal cortex but not in the striatum or hippocampus of mice. Neuroscience letters *472*, 61-64.
Tobin, D.M.D.M.M., Amanda Kahn-Kirby, E.L.P., Gary Moulder, R.B., and Andres V. Maricq, a.C.I.B. (2002). Combinatorial Expression of TRPV Channel Proteins Defines Their Sensory Functions
and Subcellular Localization in C. elegans Neurons. Neuron *35* 307–318.
Troemel, E.R., Kimmel, B.E., and Bargmann, C.I. (1997). Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in C. elegans. Cell *91*, 161-169.
Troemel, E.R., Sagasti, A., and Bargmann, C.I. (1999). Lateral signaling mediated by axon contact and calcium entry regulates asymmetric odorant receptor expression in C. elegans. Cell *99*, 387-398.
Twining, R.C., Wheeler, D.S., Ebben, A.L., Jacobsen, A.J., Robble, M.A., Mantsch, J.R., and Wheeler, R.A. (2014). Aversive Stimuli Drive Drug Seeking in a State of Low Dopamine Tone. Biological psychiatry.
Uhl, G.R., Drgon, T., Johnson, C., Fatusin, O.O., Liu, Q.R., Contoreggi, C., Li, C.Y., Buck, K., and Crabbe, J. (2008). "Higher order" addiction molecular genetics: convergent data from genome-wide association in humans and mice. Biochemical pharmacology *75*, 98-111.
Ungless, M.A., Magill, P.J., and Bolam, J.P. (2004). Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. Science *303*, 2040-2042.
USPHS (2008). A clinical practice guideline for treating tobacco use and dependence: 2008 update. A U.S. Public Health Service report. American journal of preventive medicine *35*, 158-176.
Venkatachalam, K., and Montell, C. (2007). TRP channels. Annual review of biochemistry *76*, 387-417.

Wang, X., Yang, Z., Xue, B., and Shi, H. (2011). Activation of the cholinergic antiinflammatory pathway ameliorates obesity-induced inflammation and insulin resistance. Endocrinology *152*, 836-846.
Ward, A., Walker, V.J., Feng, Z., and Xu, X.Z. (2009). Cocaine modulates locomotion behavior in C. elegans. PloS one *4*, e5946.
Wescott, S.A., Rauthan, M., and Xu, X.Z. (2013). When a TRP goes bad: transient receptor potential channels in addiction. Life sciences *92*, 410-414.
Wheeler, R.A., Aragona, B.J., Fuhrmann, K.A., Jones, J.L., Day, J.J., Cacciapaglia, F., Wightman, R.M., and Carelli, R.M. (2011). Cocaine cues drive opposing context-dependent shifts in reward processing and emotional state. Biological psychiatry *69*, 1067-1074.
White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans. Philosophical transactions of the Royal Society of London Series B, Biological sciences *314*, 1-340.
White, N.M. (1989). Reward or reinforcement: what's the difference? Neuroscience and biobehavioral reviews *13*, 181-186.
Willis, D.N., Liu, B., Ha, M.A., Jordt, S.E., and Morris, J.B. (2011). Menthol attenuates respiratory irritation responses to multiple cigarette smoke irritants. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *25*, 4434-4444.
Winnier, A.R., Meir, J.Y., Ross, J.M., Tavernarakis, N., Driscoll, M., Ishihara, T., Katsura, I., and Miller, D.M., 3rd (1999). UNC-4/UNC-37-dependent repression of motor neuron-specific genes controls synaptic choice in Caenorhabditis elegans. Genes & development *13*, 2774-2786.
Wise, R.A., and Koob, G.F. (2014). The development and maintenance of drug addiction. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *39*, 254-262.
Wise, R.A., Wang, B., and You, Z.B. (2008). Cocaine serves as a peripheral interoceptive conditioned stimulus for central glutamate and dopamine release. PloS one *3*, e2846.
Won, W.Y., Lee, C.U., Chae, J.H., Kim, J.J., Lee, C., and Kim, D.J. (2014). Changes of plasma adiponectin levels after smoking cessation. Psychiatry investigation *11*, 173-178.
Xiao, R., and Xu, X.Z. (2009). Function and regulation of TRP family channels in C. elegans. Pflugers Archiv : European journal of physiology *458*, 851-860.
Xu, T.Y., Guo, L.L., Wang, P., Song, J., Le, Y.Y., Viollet, B., and Miao, C.Y. (2012). Chronic exposure to nicotine enhances insulin sensitivity through alpha7 nicotinic acetylcholine receptor-STAT3 pathway. PloS one *7*, e51217.
Xu, X.Z., and Sternberg, P.W. (2003). A C. elegans sperm TRP protein required for sperm-egg interactions during fertilization. Cell *114*, 285-297.
Yap, J.J., and Miczek, K.A. (2008). Stress and Rodent Models of Drug Addiction: Role of VTA-Accumbens-PFC-Amygdala Circuit. Drug discovery today Disease models *5*, 259-270.
Yoshida, T., Sakane, N., Umekawa, T., Kogure, A., Kondo, M., Kumamoto, K., Kawada, T., Nagase, I., and Saito, M. (1999). Nicotine induces uncoupling protein 1 in white adipose tissue of obese mice. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity *23*, 570-575.
You, Z.B., Wang, B., Zitzman, D., and Wise, R.A. (2008). Acetylcholine release in the mesocorticolimbic dopamine system during cocaine seeking: conditioned and unconditioned contributions to reward and motivation. J Neurosci *28*, 9021-9029.

Yuan, J.P., Kiselyov, K., Shin, D.M., Chen, J., Shcheynikov, N., Kang, S.H., Dehoff, M.H., Schwarz, M.K., Seeburg, P.H., Muallem, S.*, et al.* (2003). Homer binds TRPC family channels and is required for gating of TRPC1 by IP3 receptors. Cell *114*, 777-789.
Zangen, A., Solinas, M., Ikemoto, S., Goldberg, S.R., and Wise, R.A. (2006). Two brain sites for cannabinoid reward. J Neurosci *26*, 4901-4907.
Zschenderlein, C., Gebhardt, C., von Bohlen Und Halbach, O., Kulisch, C., and Albrecht, D. (2011). Capsaicin-induced changes in LTP in the lateral amygdala are mediated by TRPV1. PloS one *6*, e16116.