BEHAVIORAL CHARCTERIZATION OF *C. ELEGANS* HOMOLOGS OF PARKINSON'S DISEASE ASSOCIATED GENES

by

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder of central nervous system affecting more than 10 million people globally. This disease has a strong genetic component with 14% of individuals with PD reporting a first-degree relative with the disease. While genes like PARK2 and PINK1 have been associated with familial early onset PD, LRRK2, DNAJC13, and VPS35 have been linked to idopathic PD. These genes are largely involved in the maintenance of mitochondria or in the quality control via the cellular machinery that degrades unneeded proteins. Mutations in these genes affect the function and survival of particular neurons in parts of the brain that regulate normal movement, balance, and coordination. One endophenotype of PD is abnormal habituation; habituation is a simple form of learning in which repeated stimulation causes a decrement in responding over time. By investigating how these PD associated genes are involved in habituation, we hope to better understand the underlying pathophysiology induced by these genetic differences. In this study mechanosensory habituation was examined in *Caenorhabditis elegans* (*C.elegans*; microscopic transparent roundworms) in both wild-type worms and in worms with mutations in homologues of genes implicated in PD. Each strain of mutant worms showed a unique combination of basal characteristics and habituation phenotypes that distinguished them from wild-type worms. The integrity of dopamine neurotransmission was also investigated using the SWIP assay and ON/OFF Food Habituation assay. The *C. elegans* homologue of the PD gene VPS35, *vps-35*, an essential component of the retromer complex, was found to display the characteristics of abnormal dopamine signaling and was chosen for additional testing. Experiments using channelrhodopsin confirmed a lower dopamine signaling phenotype, with overexpression and rescue strains of this mutant being created to further explore how this protein affects behavior. These experiments set

the groundwork for using *C. elegans* as a genetic model of PD that can help us better understand this complicated neurological disorder.

Preface

The work I describe in this thesis was performed in the lab of Dr. Catharine Rankin, my graduate supervisor who provided the direction for this research project. This thesis was written by me, with feedback given by Dr. Rankin and my committee members (Dr. Matt Farrer, Dr. Kurt Haas, and Dr. Stan Floresco) used to make revisions. The work from Chapter 1 was primarily conducted by me, with two undergraduate students, Donya Fozoonmayeh and Omid Ebrahimzadeh, assisting me with strain maintenance and running replications of habituation experiments at 10s ISI for *lrk-1*, *pdr-1*, and *pink-1*. All experiments presented in Chapter 2 were performed by me. For Chapter 3, the majority of the experiments I conducted on my own. Andrea McEwan assisted me with injections of the *vps-35* cosmid into N2 and Jeanne Valencia ran replications of the 10s ISI habituation data for *mig-14* and *dpy-23*. I performed all statistical analysis of the data, with Dr. Andrew Giles writing the data analysis software that made these calculations possible.

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List of Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
BF	basal forebrain
CGC	Caenorhabditis Genetic Center
COM	center of mass
COMT	catechol-O-methyltransferase
DBS	deep brain stimulation
DNA	deoxyribonucleic acid
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
HL	final level habituation
HR	habituation rate
ISI	interstimulus interval
L-dopa	levodopa
MSN	medium spiny neurons
MWT	Multi-Worm Tracker
NGM	nematode growth medium
PCR	polymerase chain reaction
PD	Parkinson's Disease
SEM	standard error of the mean
SNCA	alpha synuclein
SWIP	Swimming Induced Paralysis

UBC	The University of British Columbia
YFP	yellow fluorescent protein

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To my parents, who have always told me to follow my dreams and have supported me always!

Chapter 1: Introduction

In the modern world, one of our greatest priorities is to gain a better understanding of medicine and the diseases that affect us as we age. About 1% of the population is affected by Parkinson's Disease (PD) by the age of 65, with that number increasing to 4% by the age of 85 (de Lau et al., 2006; Schrag & Schoot, 2006). As the population continues to age and life expectancy continues to increase, finding treatments and cures for neurological disorders becomes more of a priority than ever before. But as we learn more about these conditions, it also has become obvious that they are more complicated than was originally thought. The use of model organisms has become increasingly important in furthering research about the underlying causes of diseases such as PD. By looking at homologs to human proteins and using animals with simpler and more understood neural system, we can begin to piece together the complex nature of PD.

1.1 Parkinson's disease symptoms and pathology

Like many other neurological disorders, PD is diagnosed by the presence of both physical and psychological symptoms. Most noticeable and well recognized is the resting tremor that often manifests unilaterally in one of the limbs and can spread bilaterally as the disease progresses. The tremor is often accompanied by bradykinesia, rigidity, and a shuffling gait. These physical symptoms are accompanied by more subtle disturbances to normal life, including problems sleeping and fatigue. Neuropsychological issues such as depression and anxiety can also develop due to the disorder. In addition, patients experience cognitive problems, including issues with memory and learning. This includes a deficit in the ability to habituate to repeated stimuli, an important and evolutionarily conserved behavior (Rankin et al., 2009). In PD, these

deficits can be found in a variety of behaviours, which alludes to large-scale changes in the brain and how it processes information.

PD can be characterized on a whole brain level by several noticeable changes. The first of these is a large neuronal loss of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNc) (Fearnley &Lees, 1991). While the loss is most pronounced in the SNc, there is also significant depletion at the cholinergic inputs to the basal forebrain (BF) (Ziegler et al., 2013)**.** The other main pathological feature is the appearance of Lewy bodies. These abnormal masses appear in nerve cells and are largely an accumulation of alphasynuclein (SNCA) (Spillantini et al., 1997). Normally, SNCA is localized at the membrane of presynaptic terminals, but its overall function is still not fully understood. These nigrostriatal dopamine deficient cells are a sign of something going wrong on a cellular level, with the death of these neurons having a very large impact on brain function. Striatal dopamine depletion leads to enhanced medium spiny neurons (MSN) pathway activity, eventually leaving to overactivity of the indirect pathway. It is this lack of inhibition that causes much of the unwanted movement (Kreitzer and Malenka, 2008). This abnormal signaling in the basal ganglia causes movement to become slow and uncoordinated, with tremors further impeding everyday tasks.

One of the earliest and most successful treatments for PD was levodopa (L-dopa) therapy, developed by Arvid Carlson in the 1950s; L-dopa is the biochemical precursor for all catecholamines including dopamine (Seiden and Carlsson, 1963). L-dopa allows the depleted striatal circuit to continue to participate in dopamine signaling by producing more dopamine than the neurons would naturally be able to produce on their own. This delays any further degradation and abnormal signaling of these important circuits. Initially, patients can see large improvements with this therapy; however often they become unresponsive to the treatment as the disease

progresses. In addition to L-dopa, there have been several other class of drugs used to treat the symptoms of PD. This includes the use of dopamine agonists such as ropinirole and pramipexole or the catechol-O-methyltransferase (COMT) inhibitors such as entacapone and tolcapone (Fox et al., 2011). These COMT inhibitors work in tandem with L-dopa, making dopamine more readily available while the dopamine agonists stimulate D2 and D3 receptors directly. Other classes of drugs such as anticholinergics, amantadine, clozapine, etc. have been used as possible treatments, however, while they can help with non-motor symptoms, they have not been shown to have a meaningful positive effect on the PD motor symptoms (Fox et al., 2011).

A more invasive treatment, deep brain stimulation (DBS), has also been shown to be effective in treating PD. DBS involves the surgical implantation of an implantable pulse generator which can deliver current to regions of the brain in order to relieve motor symptoms (Wichmann & Delong, 2006; DeLong & Benabid, 2014). Regions such as the subthalamic nucleus and the globus pallidus have proven effective for stimulation in order to relieve physical symptoms (Fox et al., 2011). Less invasive methods such as physical therapy and exercise have also shown to have some efficacy in alleviating symptoms.

1.2 Habituation and Parkinson's disease

Habituation is a simple form of non-associative learning in which there is a progressive reduction of behavioral response probability and magnitude with repetition of a stimulus. An animal will initially respond to a stimulus but if there is no harm or benefit predicted by the stimulus, the animal learns that the stimulus is irrelevant and will no longer respond to it (Rankin et al., 2009). This innate form of learning is adaptive and allows organisms to properly respond to their environment.

Habituation has been found to be abnormal in patients with many different neurological disorders, including PD. Experiments that were performed as early as the 1950s established that the human blink reflect (which is controlled by the orbicularis oculi muscle) can be stimulated in multiple different ways (Kugelberg, 1952; Rushworth, 1962; Shahani, 1970). With repeated stimulation, the late portion of the response (R2) habituates over time (Esteban & Giménez-Roldán, 1975). However, in individuals diagnosed with PD, this eye blink reflex is shorter in latency, has higher amplitude, and decreased habituation (Rushworth, 1962; Kimura, 1973). Treatment with L-dopa or amantadine saw a reversal in these characteristics, with the eye blink behavior returning to the baseline of healthy individuals (Penders & Delwuide 1971, Messina et al. 1972). In a more recent study, Teo et al. (1997) studied habituation of the cortical auditory evoked potential (P1) in patients with PD as compared to age-matched controls. Patients with PD showed a decrease in habituation at a 250-ms and 500-ms ISI as compared to their healthy counterparts. In addition, the decrease was proportional to the severity of PD disease progression. These two stimuli have also been studied together, with studies finding that untreated PD patients took longer to habituate to auditory stimuli that induced a blink reflex (Rey at l., 1996). However subsequent studies have led some mixed results as to whether there are habituation deficits in muscle reflexes in patients with PD. A recent study by Zoetmulder et al., (2014) found in their study that there was no significant difference between PD patients and controls for eye blink habituation. However, it is important to note that their PD patients were treated with L-DOPA, while patients in previous studies were not. This is a clear difference in methods, with L-DOPA being known to treat both motor and psychological symptoms in PD. Differences in habituation results can be influenced by medication or changes in methodology, making these very important differences to control for.

Habituation has also been studied in the context of auditory startle, with the event related responses habituating more slowly in PD patients than in controls. This is thought to reflect frontal lobe and hippocampal dysfunction (Jiang et al., 2000). The possible involvement of the frontal lobe has been further implicated by deficits found in habituation to novel stimuli (Tsuchiya et al., 2000). Another aspect of habituation that has been measured in PD is the response to pain, with a lack of habituation when repetitive pain stimuli are used to stimulate the sympathetic sudomotor system (Schestatsky et al., 2007).

One of the physical symptoms of PD includes difficulty maintaining balance. One way to measure a patient's ability to balance is to use dynamic posturography in which a platform causes the toes to be stretched upwards. The center of mass (COM) is measured to note shifting in posture and ability to regain balance. With serial stimuli, healthy controls are able to habituate to this perturbation by the second trial, while PD patients did not habituate until the fifth trial (Nanhoe-Mahabier et al., 2012). Other balances measures, such as the pull-test, that are traditionally used in clinical PD diagnosis, still need to be studied in the context of habituation.

Together, these studies connect the basic sensory and motor deficits to learning and the ability of the brain to store relevant information. These studies highlight the underlying dysregulation of sensory processing that affects those with PD. It also suggests using movement perturbations as an important tool for looking at disruptions to learning and memory. Therefore, habituation becomes a tool that can be used to understand PD in a new and unique way. By gaining a better understanding of how habituation is effected by genes that are also implicated in PD, we hope to gain knowledge about this complicated interaction.

1.3 Parkinson's disease genetics

When PD was first discovered in 1917 by James Parkinson, the underlying mechanisms of the disorder were completely unknown (Eyles et al., 1955). But as technology has continued to improve, scientists have been able to identify some of the genes that make an individual more susceptible to develop PD.

It has long been theorized that PD would have a genetic component as a result of observations made that the disease would sometimes run in families. This knowledge has led researchers to create large pedigrees in families around the world in order to track disease inheritance. With this approach, genes have become linked to PD, with those found in numerous and/or larger families around the globe being the simplest ones to link. As more knowledge was added, it became clear that there is a unifying function between the proteins in that they are somehow involved the basic recycling systems within the cell.

1.3.1 Alpha-synuclein

In the mid-1990s, the composition of Lewy Bodies found in the post mortem brains of PD patients was analyzed and found to be composed mainly of alpha-synuclein (Spillantini et al. 1997). Of particular interest was an Ala to Thr substitution (A53T) that was found in several pedigrees in which autosomal-dominant familial PD was found (Polymeropoulos et al., 1997). Although the function of SNCA is not fully understood, it has been shown to interact with CSPα and SNAREs at the presynaptic membranes (Chandra et al., 2005). Additionally, SNCA has been shown to modulate the dopamine transporter, with mutations contributing to neurotoxicity (Sidhu et al., 2004). Locus duplication of this gene has been linked to early-onset forms of PD, while substitutions such as Ala30Pro, Glu46Lys, His50Gln, Gly51Asp and Ala53Thr have been implicated in late-onset PD (Trinh & Farrer, 2013).

1.3.2 Leucine rich repeat kinase 2

Leucine Rich Repeat Kinase (LRRK) has GTPase and kinase functions, however much of its role is not well understood (Esteves et al., 2014). This large 2527 amino acid protein has many cellular functions, though its primary role is endosomal (Greggio & Cookson, 2009; Cookson 2010). This could allow LRRK2 to have an active role in mitochondrial function, autophagy, and many other pathways (Cookson 2010). Asn1437His, Arg1441Cys/Gly/His, Tyr1699Cys, Gly2019Ser and Ile2020Thr are autosomal dominant mutations that have been found to segregate in families with idiopathic PD, while other mutations can simply increase chances of developing sporadic PD (Zimprich et al., 2004; Kachergus et al., 2005; Aasly et al., 2010; Farrer & Trinh, 2013; Mata et al., 2012).

1.3.3 Receptor mediated endocytosis 8

Receptor Mediated Endocytosis 8 (also known as RME8 or location DNAJC13) functions in endosomal trafficking and is necessary for receptor mediated endocytosis. RME8 contains a DNAJ-domain that binds to endosomal membranes and interacts with HSP70, a clathrin uncoating ATPase (Girard et al., 2005). In addition, DNAJC13 can interact with sorting nexin 1 of the retromer complex, which in turn creates clathrin carriers that move cargo from the endosomes to the trans-golgi network (Popoff et al., 2009). Therefore, DNAJC13 seems to act as a coordinator of the retromer complex, with mutations in this gene leading to abnormalities in cargo trafficking (Popoff et al., 2009). Familial late onset parkinsonism has been linked with the Asn855Ser mutation using genome sequences of Canadian Mennonite families (Vilarino-Guell et al., 2014).

1.3.4 Vacuolar protein sorting factor 35

Vacuolar Protein Sorting Factor 35 (VPS35) is a part of the retromer complex, which is involved in the retrograde transport of proteins from the endosomes to the trans-Golgi network (Bonifacion & Rojas, 2006; Bonifacio &Hurley, 2008). This retromer complex includes VPS35, VPS29, and VPS26, and a pair of nexins. VPS35 directly attaches to specific cargos, with its main cargo outside of neurons being the cation-independent mannose 6-phosphate (M6PR) receptor (Hierro at al., 2007). It is believe that mutations in VPS35 disrupt normal binding to these cargos, leading to abnormal recycling of M6PR and the Vps10p, along with other cargos that have yet to be identified (Zimprich et al., 2013). In addition, VPS35 has also been shown to be crucial for recruiting the WASH complex, which is crucial for recycling some membrane proteins, to the endosomes. Specifically, FAM21 is necessary for this interaction (Zavodszky et al., 2014). The only definitive mutation in VPS35 that has been shown to segregate with late-onset PD is Asp620Asn (Vilarino-Guell et al., 2011; Follett et al., 2014).

1.3.5 Parkin E3 ubiquitin protein ligase

Parkin E3 Ubiquitin Protein Ligase (known as PARKIN or PARK2) has the job of targeting proteins for protoeasomal degradation , mitochondrial fission, vesicle trafficking, and general survival pathways (Bingol et al., 2014; Walden & Martinez, 2012; Greene et al., 2003). Recently, the crystal structure of parkin has been isolated, confirming that this ubiquitin ligase has a complicated RING structure that is regulated by phosphorylation, cysteine modifications and ubiquitin (Trempe et al., 2013). Mutations in *parkin* are typically recessive and cause early-onset PD which often shows different symptoms than idiopathic PD (Kitada et al., 1998; Bonifati, 2012). Loss of function in *parkin* accounts for a large number of sporadic early-onset cases (Abbas et al., 1999).

1.3.6 PTEN-induced kinase-1

PTEN-induced kinase-1 (PINK1) works upstream of PARKIN to protect cells from stress-induced mitochondrial dysfunction (Clark et al., 2004). PINK1 works by phosphorylating the ubiquitin necessary for activating *parkin* (Koyano et al., 2014). Like *parkin*, mutations in PINK1 are typically autosomal recessive and are responsible for early-onset PD (Valente et al., 2004; Bonifati, 2012).

1.3.7 Parkinson's protein 7 DJ-1

DJ-1, or Parkinson's protein 7, was the third major PD locus (PARK7) to be associated with early-onset autosomal recessive PD (Bonifati et al., 2003; van Duijn et al., 2001). DJ-1 is also found in the mitochondria, however it does not have enzymatic activity (Kahle et al., 2009). Instead, DJ-1 modulates the antioxidative, antiapoptotic, and transcriptional pathways, having an overall cytoprotective function (Kahle et al., 2009).

1.4 *C. elegans* as a model system

The tiny roundworm *C. elegans* has become a powerful model organism for scientific research since it was first used in 1965 by Sydney Brenner (Wood, 1988). Their small size and rapid growth makes them easy and inexpensive to maintain in a laboratory environment. In 1998, the *C. elegans* genome was sequenced and mapped as part of the Human Genome Project, making all of their genetic information readily available (Hodgkin et al., 1998). This has allowed the worm community to create a comprehensive database of all known *C. elegans* genes, their functions, interactions, and whether mutations in a specific gene are available in a strain (www.wormbase.org). In addition, the nervous system of an adult hermaphrodite worm was fully reconstructed in 1986 by White and colleagues. This makes *C. elegans* an incredibly useful model for studying the nervous system. With this simple system being so well understood, it

allows us to study complex circuits and processes that would be impossible to study in mammalian systems.

1.5 *C. elegans* habituation and the role of dopamine

Like humans, *C. elegans* are able to learn to ignore irrelevant stimuli, a non-associative form of learning called habituation. In *C. elegans*, this plasticity has been best characterized using a paradigm called tap habituation, which was first described by Rankin et al. in 1990 and described in more detail in by Rankin and Broster (1992). Worms are able to sense mechanical stimuli such as vibration through the agar that they live on. When the petri plate is tapped on the side, the vibrations cause the worm to make a reversal response, in which the worm stops moving forward and instead moves backward. Taps were delivered at a 10s interstimulus interval (ISI), with a total of 30 taps being distributed. The reversal response to tap decreases in frequency and response magnitude with repeated stimulation. In order to determine that the response was in fact learned and not simply due to fatigue, the worms were also dishabituated using shock. Since the habituated worms reverse to tap after this shock stimulus, it was concluded that the decremented response to taps was in fact habituation. This study showed that *C. elegans* were able to learn, something that had not been previously thought possible. Other ISIs for short-term habituation have since been tested, with genes showing differential effects on habituation depending on the ISI. This idea was originally proposed by Broster and Rankin in 1992, with supporting evidence coming from mutations in genes such as *cmk-1*. Worms with mutations in *cmk-1* show a wild-type habituation phenotype at a 10s ISI but show slower habituation at a 60s ISI (Timbers et al., submitted). This has led to the working theory that different genes are involved in storing information in response to specific patterns of stimulation.

Recent analysis of these responses in a large cohort of mutant strains of worms has shown that response magnitude can be broken down into three genetically dissociable components: reversal probability, reversal duration and reversal speed (Giles et al., in preparation). As a result of this analysis these are the three measures used to analyze and understand habituation in C. *elegans*. Since the first study of tap habituation (Rankin et al 1990), the genes and mechanisms responsible for short-term habituation in *C. elegans* have been studied in great detail. The complexity of the circuits and gene networks involved is astonishing and continues to grow more complicated.

C. elegans express many of the classical well characterized neurotransmitters including dopamine, serotonin, GABA, and glutamate (Horvitz *et al.*, 1982; Loer, 1993; Hart *et al.*, 1995). Because of the importance of dopamine to PD it is interesting to note that dopamine has been shown to effect tap habituation by making it faster, with the D1-like dopamine receptor *dop-1* expressed on the touch sensory neurons thought to mediate this behavior (Sanyal et al., 2004). This seems to be due to the modulation of excitability of the anterior touch sensory neurons (Kindt et al., 2007).

Another important finding in regards to dopamine is that its involvement in habituation is dependent on the presence of food. Wild-type worms that are tested for habituation on food will respond at a higher frequency than worms off of food (Kindt et al., 2007). Because this difference is dependent on dopamine, mutants that are deficient in dopamine, such as *dop-1*, do not habituate more rapidly when worms are off of food. This due to the fact that without dopamine, they are not able to sense whether or not they are on food. This makes an on/off food testing paradigm for habituation an easy way to test whether mutant strains are dopamine deficient.

1.6 *C. elegans* homologs of Parkinson's disease genes

C. elegans share about 35% percent of their genome with humans, with many proteins and cellular processes having remarkably similar roles (wormbase.org). Many of these conserved genes are playing a key role in general cellular function. Of the genes that are currently hypothesized to play a key role in PD, five are found in *C. elegans*. These are PARK2, PINK1, LRRK2, VPS35, and RME8 (See Table 1.6).

The *C. elegans* homolog of human PARK2 is encoded by the gene *pdr-1*, which is expressed in muscles and in neurons (wormbase.org). It has been shown that has the same conserved function as part of the degradation machinery that mediates ubiquitin conjugation (Springer et al., 2005). When modeling PD dysfunction in *C elegans, pdr-1* has been shown to be involved in mitochondrial stability and aggregating in a similar way as what is seen in some juvenile autosomal recessive forms of PD (Ved et al., 2005; Springer et al., 2005).

The homolog of PINK1 is encoded by the *C. elegans* gene *pink-1*¸ which is expressed in neurons, pharyngeal muscles, and the vulva (wormbase.org). In the worm this gene has the same conserved function of acting as a serine/threonine kinase, working upstream of Parkin in mitochondrial homeostasis (Park et al., 2006; Samann et al., 2008).

While humans have a LRRK1 and LRRK2, *C. elegans* only have one gene, *lrk-1*, which shares homology to both of these proteins. This gene in *C. elegans* has been shown to be involved in the localization of synaptic vesicle proteins, working antagonistically to *pink-1* to regulate oxidative and endoplasmic reticulum stress response (Samann et al., 2008; Sakaguchi et al., 2007; Springer et al., 2005). This large protein can be found in neurons, the pharynx, and the vulva (wormbase.org).

The DNAJ13AC locus, which encodes RME8, has a homologue in C, elegans, *rme-8*. In *C. elegans, rme-8* regulates retromer-mediated endosome to the Golgi retrograde transport (Zhang et al., 2001). Specifically, it associates with SNX and clathrin chaperone Hsc70 to regulate clathrin and sort MIG-14, playing a large role in the retrograde recycling of cargo (Shi et al., 2009). This protein is expressed widely, especially in muscles, neurons, hypodermis, and the intestines (wormbase.org).

The homolog of VPS35, a major component of the retromer complex, is encoded by the genes *vps-35* in *C. elegans*. Importantly, this gene is necessary for recycling of an AMPA-type glutamate receptor subunit, *glr-1* (Zhang et al., 2012)**.** In *C. elegans* VPS35 is also expressed in nearly every cell type, with significant expression in the head neurons, epidermis, body wall muscles, gonad, and pharynx (wormbase.org).

Gene (allele	Strain	Mutation	Function	Human Homolog
pdr-1 (gk448)	**VC1024**	355 bp deletion (loss of function)	Ubiquitination Mitochondria	PARK2/PARKIN
pink-1 (ok3538)	**RB2547**	~500bp deletion unknown location	Oxidative stress Mitochondria	PINK1
lrk-1 (km17)	**KU17**	1917 bp deletion, exon 16, end of protein/catalyitc domains		LRRK2
lrk-1 (tm1898)	**TM1898**	377 bp deletion + 9bp (triple Arg) insertion		LRRK2
rme-8 (b1023)	**DH1206**	A/T substition, exon 10, unknown functional significance	Receptor mediated endocytosis	DNAJC13
vps-35 (ok1880)	**VC1390**	1590 bp deletion, exon 4	Retromer complex	VPS35
vps-35 (hu68)	**KN555**	Deletion 3' of exon 2 to the 7th intron	Retromer complex	VPS35
dpy-23 (e840)	**CB840**	1000 kb deletion, ORFs	Adaptin, receptor mediated endocytosis	Mu2, subunit of AP2
mig-14 (mu71)	**CF367**	Unknown	Wntless	Wntless
mig-14 (ga62)	**EW12**	C→T exon 7	Wntless	Wntless

Table 1: Parkinson's Disease Homolog's *in C. elegans* Tested in this Study

Chapter 2: Behavioral Characterization of Parkinson's Disease Associated Genes

2.1 Introduction

By using a model system such as *C. elegans*, complex genetic questions about interactions and about how a gene affects the system on a larger level can begin to be answered. The easiest way to do this is to first characterize behavior. How does a mutation in a particular gene change the behavioral output of a small worm? Is there anything fundamentally different about their natural state or how they are able to learn? These are all important questions, that when answered, can help expand our knowledge of a complex genetic condition such as Parkinson's Disease.

2.2 Methods

2.2.1 *C. elegans* strain maintenance

C. elegans of various genetic backgrounds were obtained or generated for behavioral testing. Four strains, VC1024(*gk4478*), RB2547(*ok3538)*, VC1390(*ok1880*), KN555(hu68) and DH1206 (*b1023*) were requested from the from Caenorhabditis Genetic Center (CGC) at the University of Minnesota. KU17(*km17*) was requested from the Mori Lab and TM1898 was obtained from the National Bioresource Project of Japan. All worms were maintained on petri plates containing Nematode Growth Medium (NGM) and were fed the OP50 strain of *E.coli* as a bacterial lawn. Strains were also frozen using a standard protocol in order to preserve them. Testing was performed using age synchronized adult worms grown at either the standard 20C or 15C (depending on the strain). Subsequent colonies were maintained via chunking or bleaching.

Worm Maintenance: Petri plates were filled with hot NGM agar and left to dry for up to two days. Plates there then seeded with OP50 E. coli on a disinfected bench space under a Bunsen burner. If plates were to be used for strain maintenance, 4-5 drops were put unto the plate. If the plates were for testing, 50 ul of OP50 was put on the surface with a pipette and

spread with a sterilized glass spreader to generate an even, thin lawn of bacteria. Plates were then left to dry overnight.

Worms were synchronized by picking 8 gravid hermaphrodites and placing them into a 10ul drop of 1:1 NAOH and bleach. This combination of chemicals disintegrates the worms, leaving the eggs that were present in the gonad intact on the surface of the agar. These eggs will then grow up to be the same age, within the margin of several hours (wormbook.org).

2.2.2 Behavioral characterization

The Multi-Worm Tracker is a real-time computer system that is used to capture and systematically analyze the movement and behavior of *C. elegans*. This tool can be used to record spontaneous movements and induced behaviors such as habituation of responses to a tap stimulus. This MWT program can capture the behavior of dozens of *C.elegans* on a Petri plate at a single moment, allowing for up to 120 animals to be tracked reliably at the same time (Swierczek & Giles et al., 2011). This software includes real-time analysis of things such as speed, body position, and outline.

Strains were tested at two different interstimulus intervals (ISIs) for habituation. Four-day old age synchronized worms were placed on the MWT and tracked for 5 minutes to establish baseline behavior. The habituation protocol was then performed, with 30 taps being administered to the side of the petri plate by a computer controlled mechanical solenoid, meaning that each tap is of the same magnitude and duration. This was done at both a 10s ISI and a 60s ISI.

2.2.3 Data analysis and statistics

A software program named “Beethoven” was used to analyze the habituation data for proportion reversing, reversal duration, and speed over the course of the experiments.

Data collected during the 10ISI habituation experiment was further analyzed using the program "Maestro". Body size, speed, and spontaneous reversals were found to be of special interest. Data files for these variables were further analyzed using the program R. For basal characteristics, significance was found using a one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) test.

In order to ascertain significance between habituation curves, a more complicated approach had to be taken. A java based analysis program called "Habituation Analysis" was used to graph and determine significance between habituation curves. This program, written by Dr. Andrew Giles, first calls on 'Choreography' which is able to process the raw tracker data. The analysis then uses the program Octave to perform all mathematical calculations and draw graphs. This was done for initial response, final level habituation (HL), and habituation rate (HR) for reversal probability, reversal duration, and reversal speed. HL refers to the final asymptotic level of the habituation curve, while HR is the slope of the straight line that best fits the habituation curve. Either an asymptotic or fitted value was assigned to curves based on whether the half-life of the curve was greater than 1/3 of the taps. The curve of each plate was compared to the median of all plates in order to find the standard error for HL and HR. These standard errors were then used to perform t-tests to determine significance. In the case that a habituation measure did not fit a curve, a linear regression was used in R to calculate a value for the slope of the curve. These values were then compared using a t-test to determine significance.

2.3 Results

2.3.1 Basal measures of behavioral characterization

Since the target genes are all involved in basic cellular function, it was import to ascertain whether any of the mutant worms' natural characteristics or behaviors were altered due to

expression of a non-wild-type allele. Body size of control wild-type N2 worms was 1.39 mm^2 (See Table 2)**.** For *lrk-1*, allele *km17* had a body size of 1.53 mm^2 which is significantly larger than N2 ($p<.001$), while *tm1898* had a body size of 1.37 mm^2 and was not different from wild-type. Both *pdr-1(gk448)* and *pink-1*(ok3538) were significantly different with body sizes of 1.56 mm^2 ($p<.001$) and 1.075 mm^2 ($p<.001$) respectively. Both alleles of *vps-35* were also significantly smaller than wild-type at .92 ($p<.001$) for *ok1880* and at 1.11 mm^2 ($p<.001$) for *hu68*. Finally, *rme-8(b1023)* was also significantly smaller with a body size of 1.07 mm^2 ($p<.001$).

The next basal measure that was analyzed was speed. Wild-type N2 worms moved at an average rate of .066 mm/s during a 100s timeframe (see Table 2). For the *lrk-1* strains, *km17* was found to move at a speed of .111 mm/s ($p<.001$), while *tm1898* moved at a rate .063 mms/. *Pdr-1(gk448)* and *pink-1(ok3538)*, had basal speeds of .081 mm/s ($p<.05$) and .049 mm/s ($p<.01$) as compared to N2. *Rme-8(b1023)* also had a significantly different speed as compared to wild-type, moving at .152 mm/s ($p<.0001$). Alleles for *vps-35* did not show a significant speed different from N2, moving at rates of .057 mm/s and .077 mm/s for *ok1880* and *hu68* respectively.

The reversal responses that worms make are crucial to understanding their underlying circuitry. Because reversals are the responses measured for habituation to the tap stimulus, it is also important to measure the spontaneous reversals that are taking place during the 5 min baseline period before taps. Wild-type worms performed an average of 1.72 spontaneous reversals per minute (see Table 2). *lrk-1* worms performed significantly more reversals per minute, with a rate of 2.66 per minute for *lrk-1(km17)* ($p<.01$) and a rate of 4.53 for *lrk-1(tm1898)* ($p<.001$). Allele *hu68* for the gene *vps-35* was found to perform 4.68 reversals per

minute (p<.001), however allele *ok1880* only performed 1.96 reversals per minute (p=.97). *rme-8*(b1023) was also found to perform significantly more reversals per minute at compared to N2 at a rate of 2.73 (p<.01). *Pdr-1(gk448)* and *pink-1(ok3538)* were not significantly different from wild-type, performing 2.45 and 1.88 reversals respectively.

For the spontaneous reversal distance (mm), only the alleles of *vps-35* were found to have phenotypes that were significantly different from N2 (see Table 2). The average reversal distance for N2 was found to be .188 mm, while *ok1880* had a distance of .119 mm (p<.05) and *hu68* had a reversal distance of .101 mm (p<.01). For *lrk-1(km17*), reversal distance was found to be .230 mm, while for *lrk-1(tm1898),* it was found to be .155 mm. *Pdr-1(gk448)* reversed an average distance of .191 mm and *pink-1(ok3538)* .143 mm. Finally *rme-8(b1023)* reversed an average of .238 mm.

2.3.2 Initial response to tap

For tap habituation, the first measure that is important to characterize is the initial response to tap. This helps us understand how animals differ in their baseline response to a mechanosensory stimulus. Initial response was characterized for reversal probability, reversal duration, and for reversal speed, with each measure compared to the wild-type initial response. *lrk-1(km17)* worms were found to have lower initial response to tap for reversal duration (p<.001) and reversal speed (p<.05), while *lrk-1(tm1898)* had a lower initial response for reversal speed (p<.01;Figure 1; Figure 2). For *pdr-1*(gk448) initial response was not significantly different from wild-type for any measure (Figure 3). *pink-1(ok3538)* was found to have a higher initial reversal duration (p<.001) and a lower initial reversal speed (p<.001) as compared to N2 (Figure4). The first allele of *vps-35, ok1880*, had an initial response lower for reversal probability (p<.01), higher for reversal duration (p<.01), and lower for reversal speed (p<.001)

when compared to wild-type (Figure 5). *vps-35(hu68)* largely phenocopies *ok1180*, with a lower initial response for reversal probability ($p<.01$), duration ($p<.001$), and speed ($p<.01$; Figure 6). And finally, *rme-8(b1023)* had a higher initial response for reversal duration ($p<.01$) as compared to N2 (Figure 7). A summary of this data can be found in Table 3.

When looking at initial response for 60s ISI, some similar response patterns emerge as was observed at a 10s ISI. For *lrk-1(km17)*, initial response was also found to be lower for reversal duration ($p<.01$), and *tm1898* also displayed lower initial responses speed ($p<.05$) when compared to wild-type (Figure 7; Figure 8). For *pdr-1(gk448)*, no significant differences were found at a 60s ISI (Figure 10). *pink-1(ok3538)* was found to have a lower initial response for speed ($p<.05$) and a higher initial response for reversal duration ($p<.01$) as compared to N2 (Figure 11). For *vps-35*, the allele *ok1880* showed a significantly lower initial responses for probability ($p<.01$), duration ($p<.01$), and speed ($p<.001$) as compared to wild-type (Figure 12). In the case of *vps-35(hu68)* lower initial response for reversal speed ($p<.001$) was recorded (Figure 13). Finally, for *rme-8(b1023)* initial response to tap was higher for reversal speed ($p<.05$; Figure14). A summary can be found in Table 4.

2.3.3 10s ISI tap habituation

For each strain the final habituated level and habituation rate were analyzed for reversal probability, duration and speed and compared to N2 control worms. *lrk-1(km17)* worms were found to have a lower final habituation level for reversal probability ($p<.01$) compared to N2 (Figure 1). The other allele of *lrk-1*, *tm1898*, also had a faster rate of habituation for reversal probability ($p<.01$) and a lower final habituation level for reversal probability ($p<.001$; Figure 2). For *pdr-1*(gk448) the only significantly different phenotype from N2 was a higher final habituation level for reversal speed ($p<.01$; Figure 3). *pink-1(ok3538)* was found a faster rate of

habituation for reversal duration (p<.01) and for speed (<.001). In addition, this allele had a higher final level habituation for duration (p<.01) and lower final habituation level for speed (p<.01; Figure 4). The first allele of *vps-35*, *ok1880*, had a faster rate of habituation for reversal duration and for reversal speed (p<.01; Figure 5). *vps-35(hu68)* showed a lower final habituation level for reversal probability (p<.05) and a higher final habituation level for reversal speed (p<.05) (Figure 6). *rme-8(b1023)* did not show any significant differences for the habituation measures (Figure 7).

Strain	Gene Mutation	Allele	Body Size (mm2)	Basal Speed (mm/s)	Spontaneous Reversals (#/m)	Spontaneous Reversal Distance (mm)
N2	**n/a**	**n/a**	**1.39**	**0.066**	**1.72**	**0.188**
KU17	*lrk-1*	*km17*	1.533*	0.111*	2.66*	0.230
TM1898	*lrk-1*	*tm1898*	1.37	0.063	4.53*	0.155
VC1024	*pdr-1*	*gk448*	1.566*	0.081*	2.46	0.191
RB2547	*pink-1*	*ok3538*	1.075*	0.049*	1.88	0.143
VC1390	*vps-35*	*ok1180*	0.916*	0.057	1.98	0.119*
KN555	*vps-35*	*hu68*	1.110*	0.077	4.68*	0.101*
DH1206	*rme-8*	*b1023*	1.070*	0.152*	2.73*	0.238

Table 2: **Basal Behavioral Characteristics of Mutant Worms**.
(*) denotes (p<.05) as compared to N2.

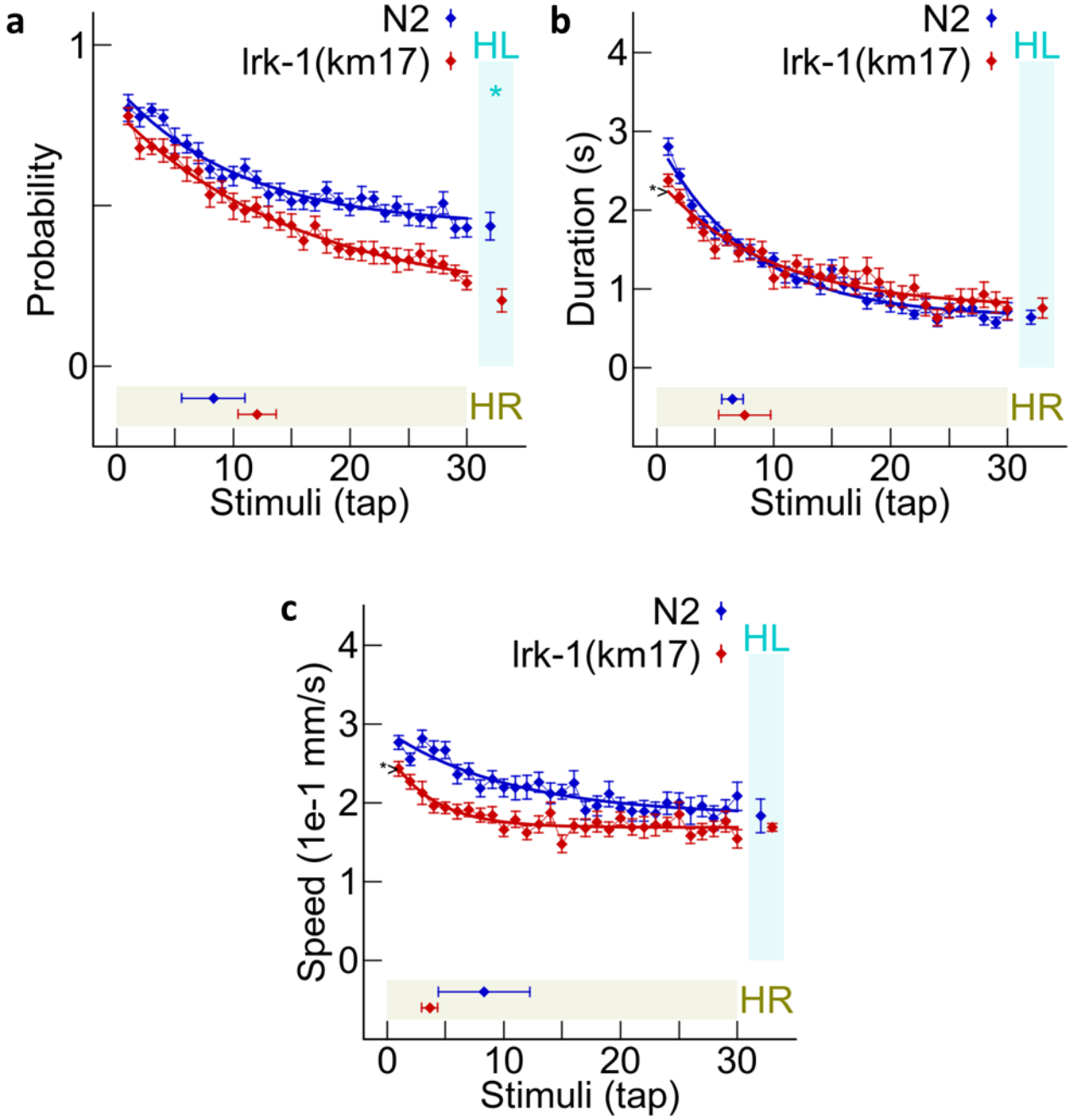

Figure 1: 10s ISI Tap Habituation for *lrk-1(km17)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

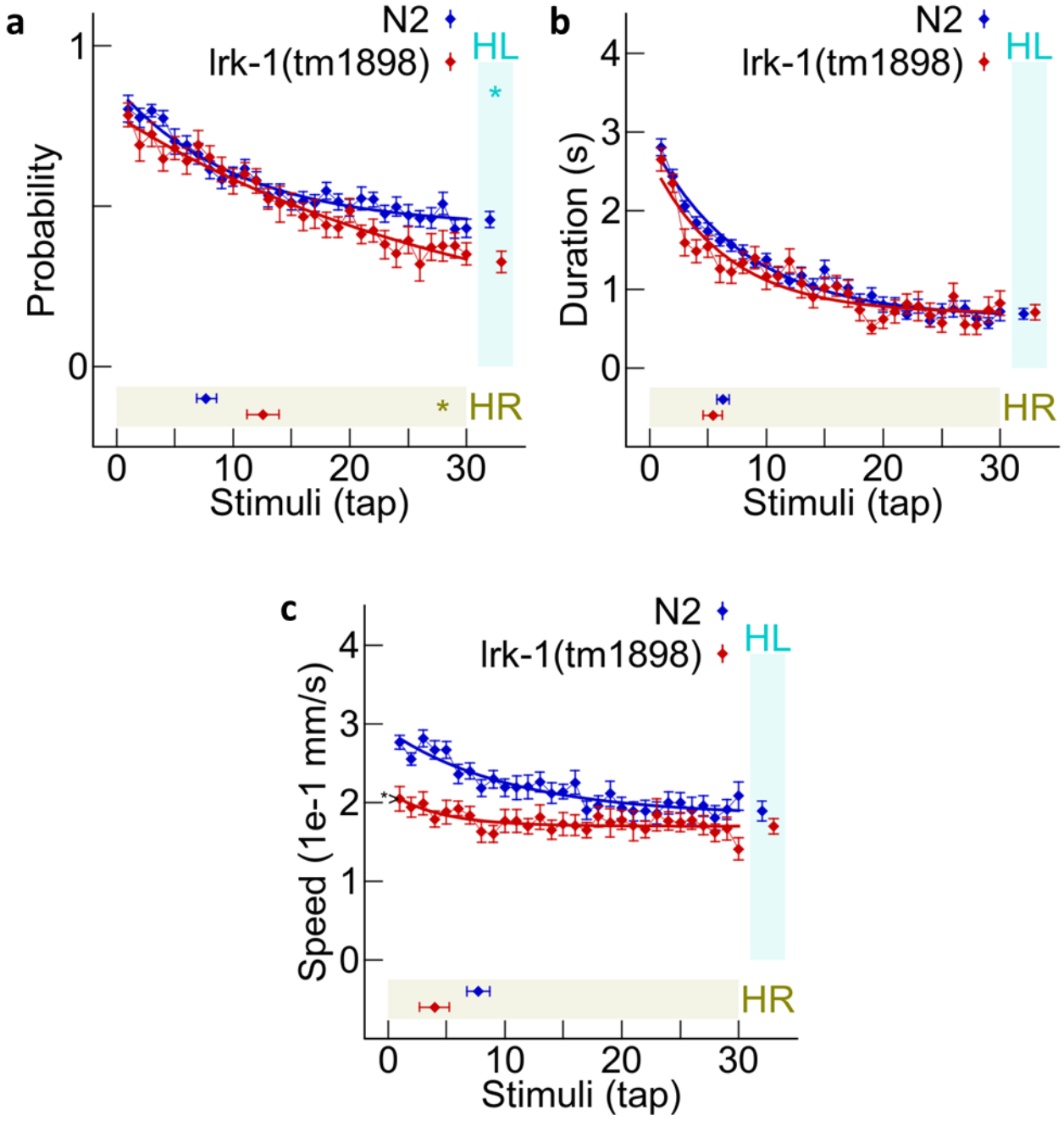

Figure 2:10s ISI Tap Habituation for lrk-1(tm1898)
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

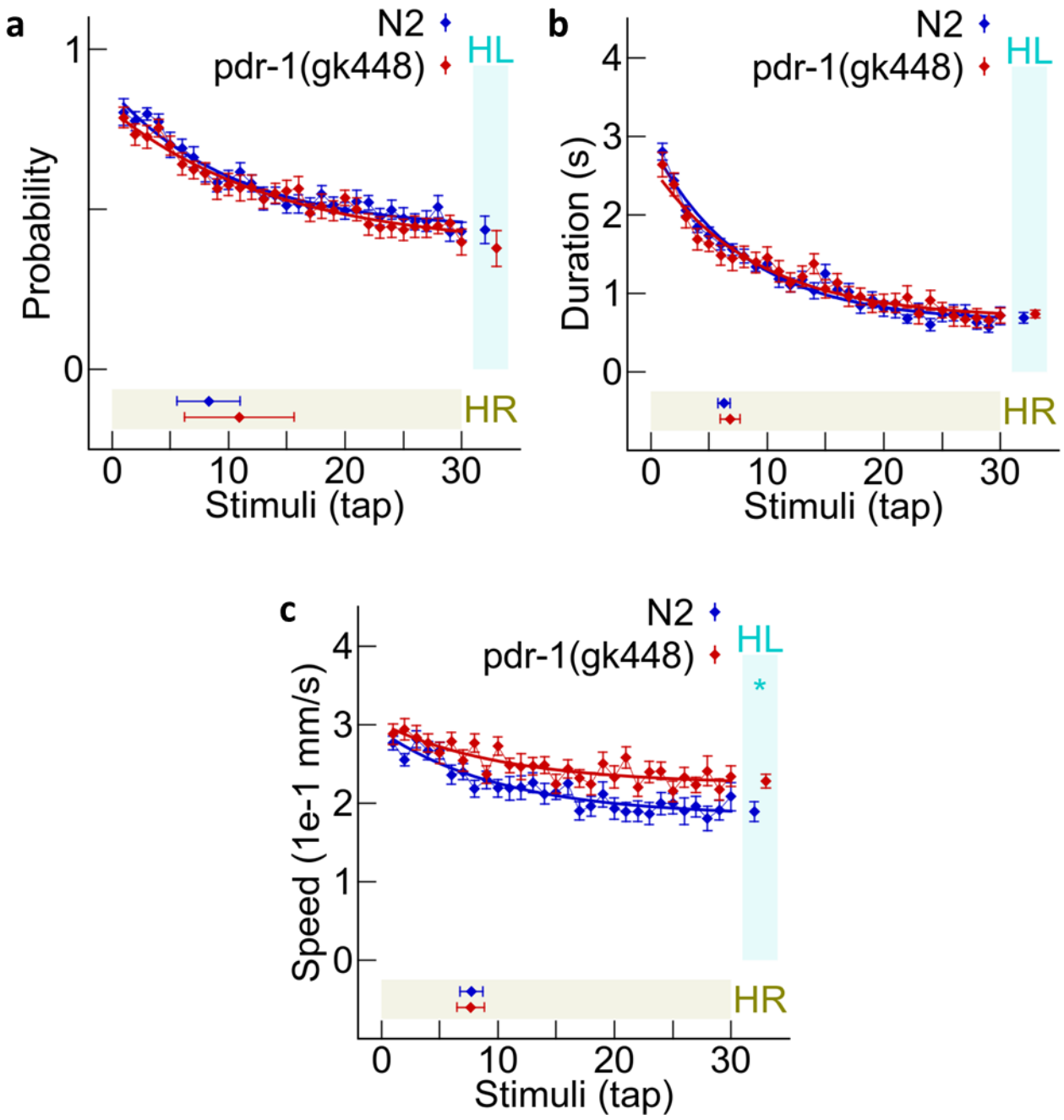

Figure 3: 10s ISI Tap Habituation for *pdr-1(gk448)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

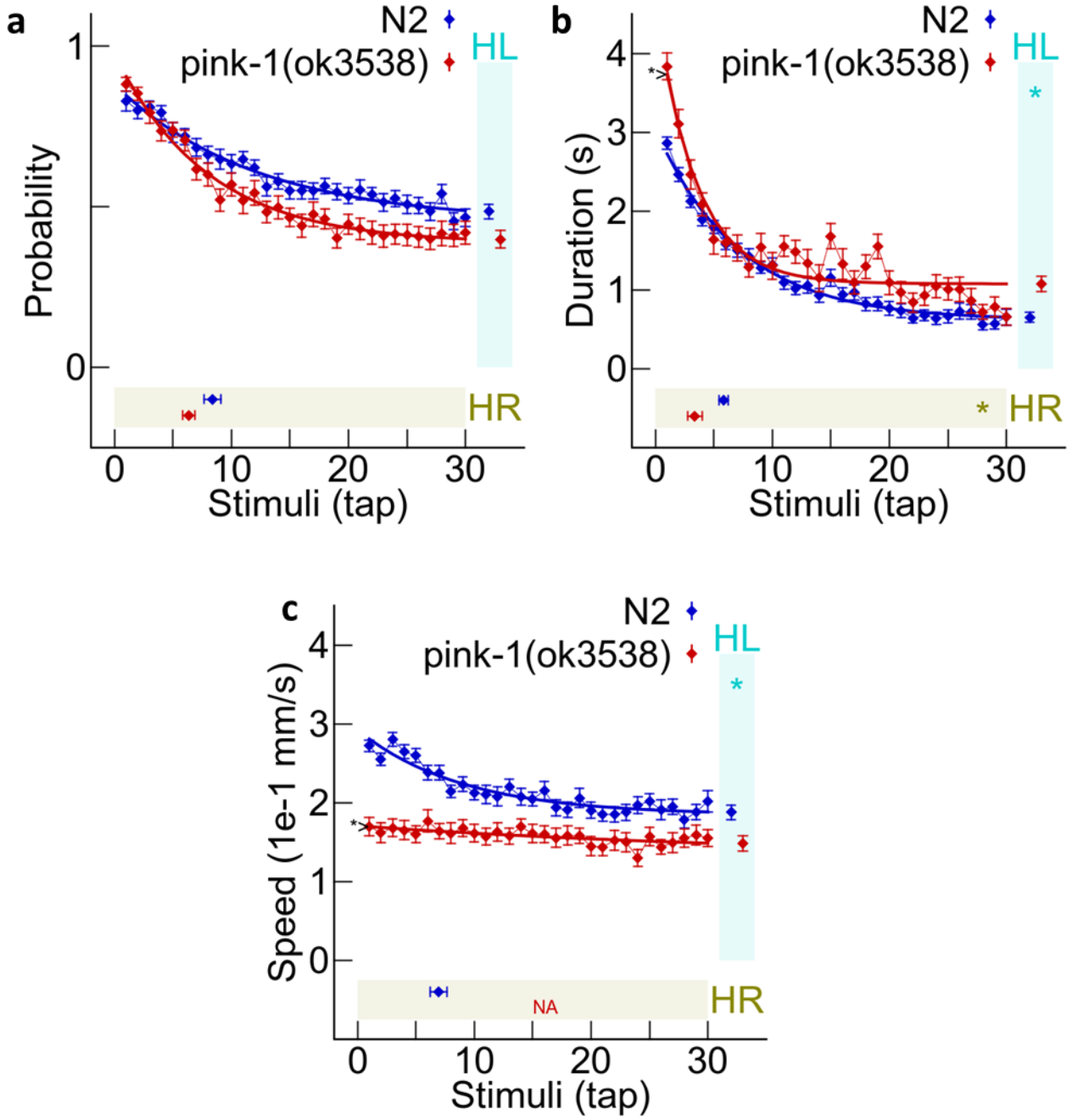

Figure 4: 10s ISI Tap Habituation for *pink-1(ok3538)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

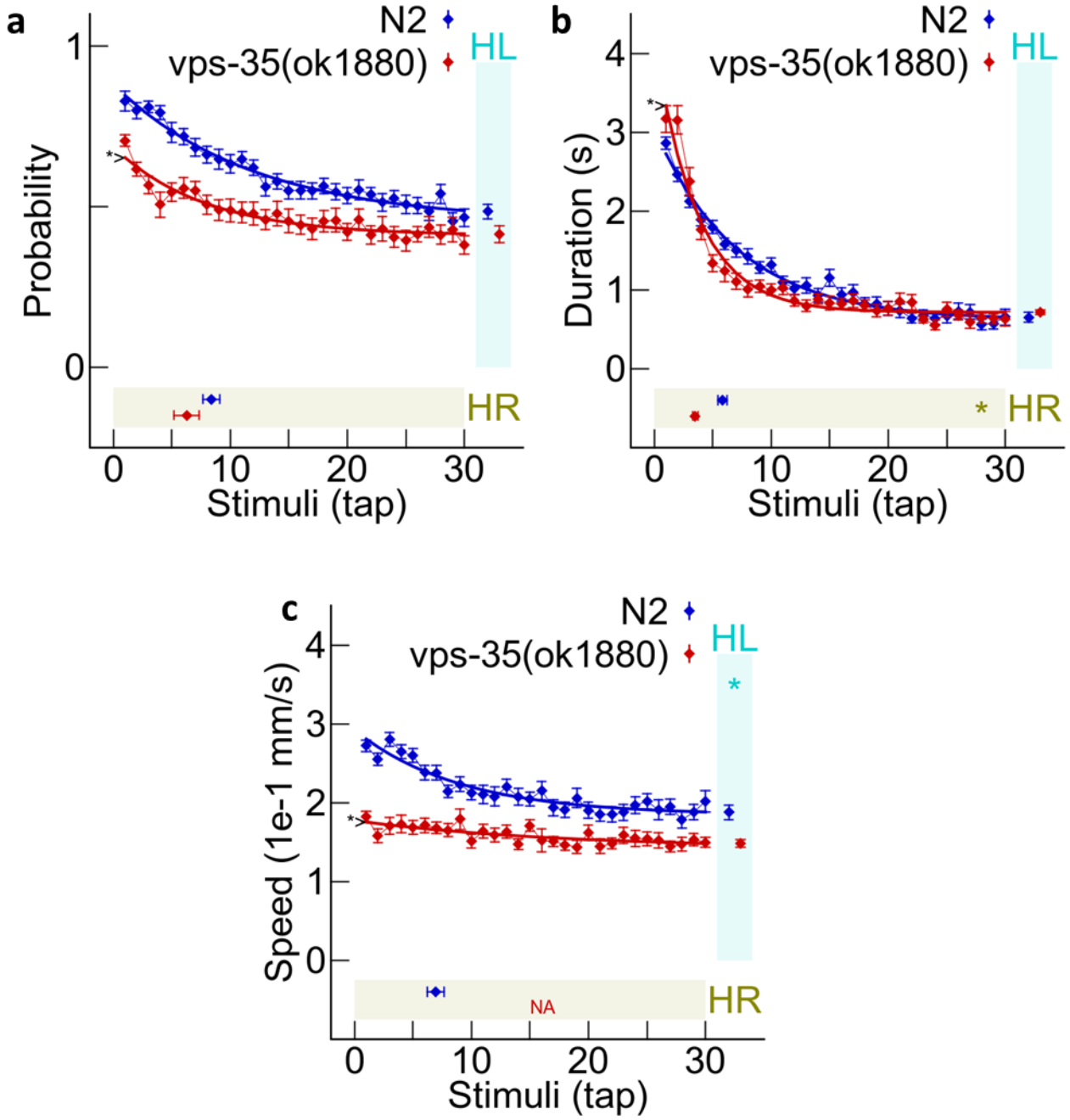

Figure 5: 10ISI Tap Habituation vps-35(ok1880)
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

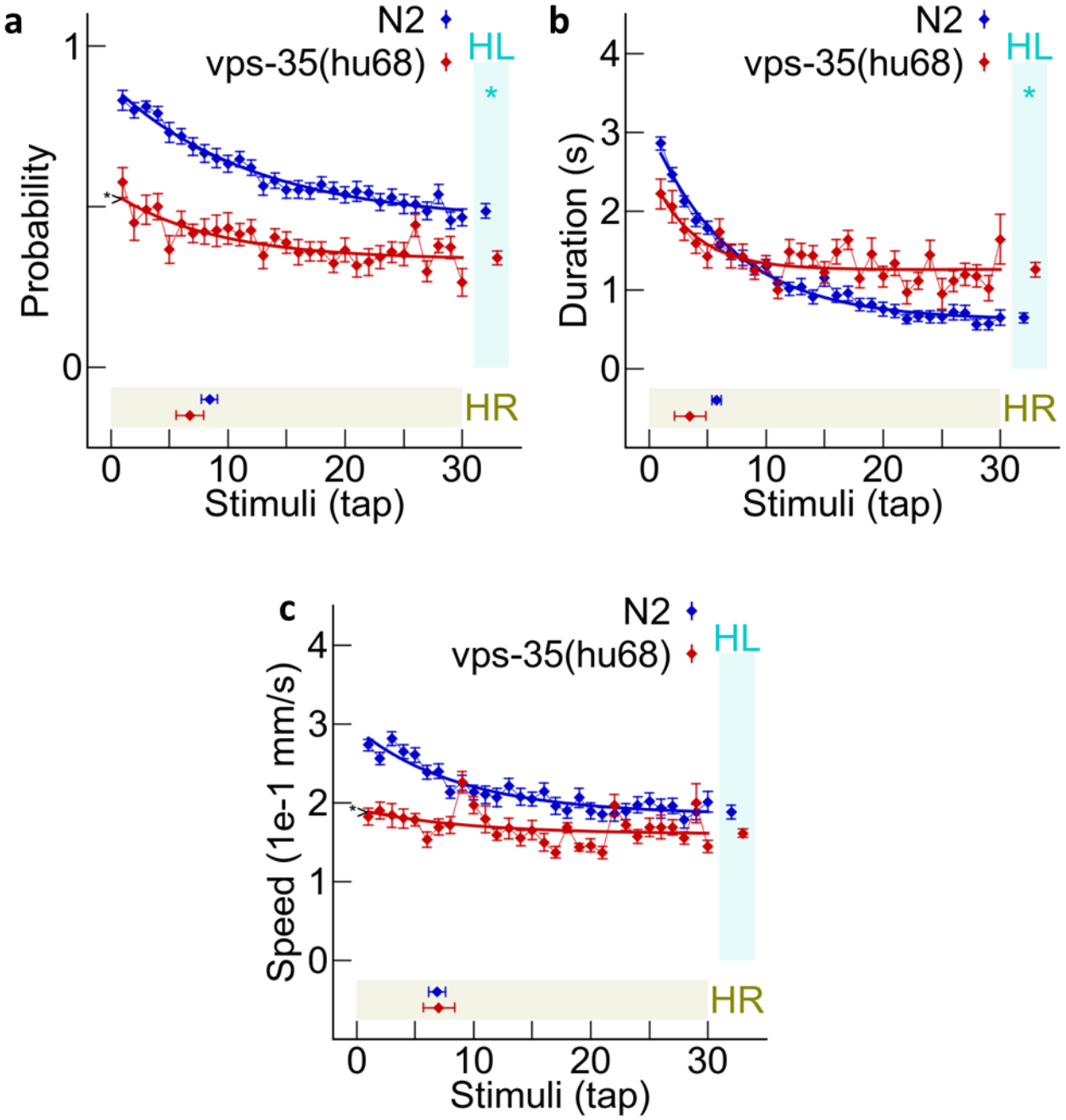

Figure 6: 10ISI Tap Habituation vps-35(hu68)
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

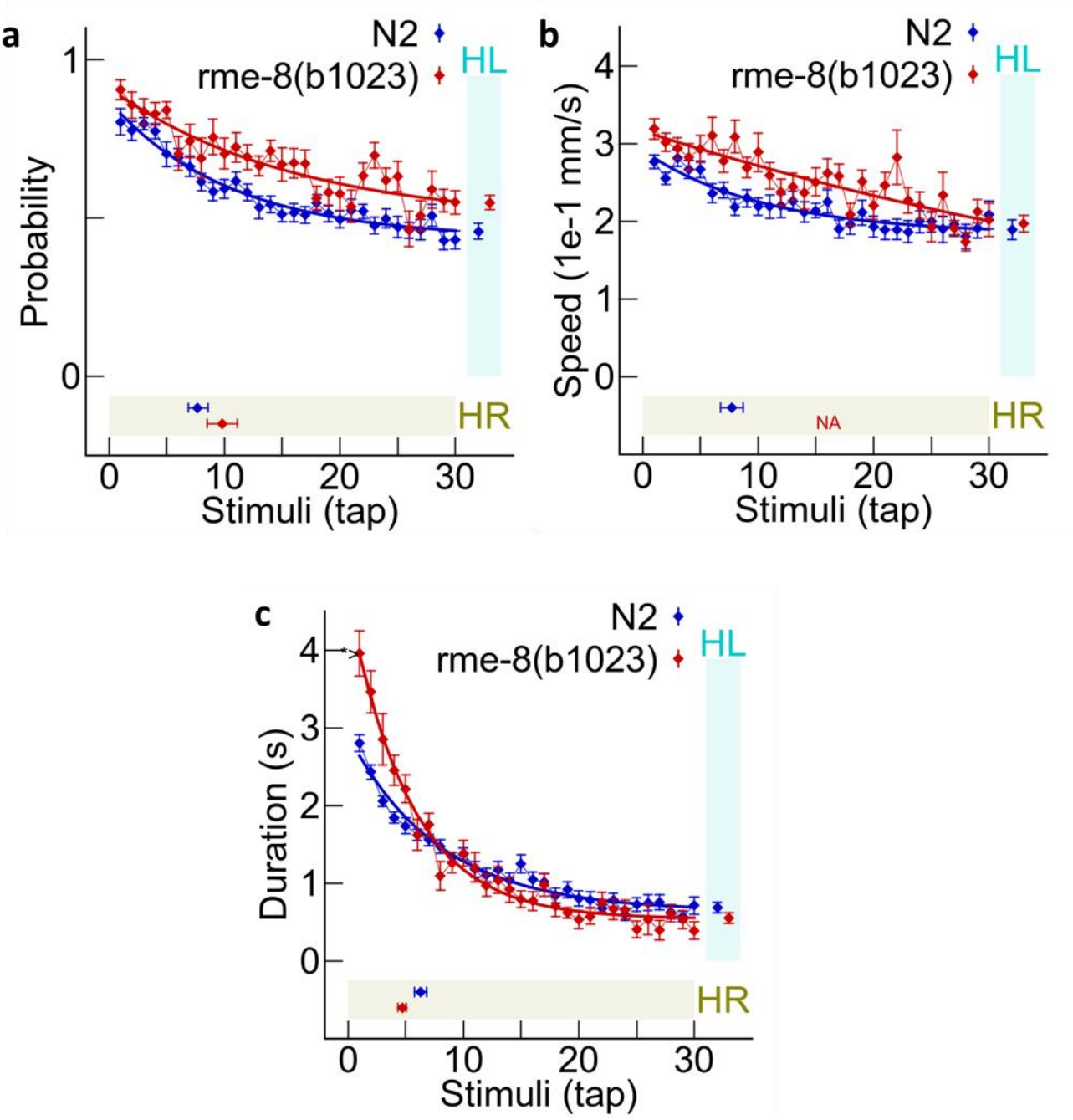

Figure 7: 10ISI Tap Habituation *rme-8(b1023)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

Strain	Initial Response Probability	Initial Response Duration	Initial Response Speed	Probability Rate	Duration Rate	Speed Rate	Probability Final Level	Duration Final Level	Speed Final Level
lrk-1(km17)		-	-				-		
lrk-1(tm1898)			-				-		
pdr-1(gk448)									-
pink-1(ok3538)		+	-		+	-			
vps-35(ok1180)	-	+	-		+	-			-
vps-35(hu68)	+	-	-			-	-	+	
rme-8(b1023)		+							

Table 3: Tap Habituation Summary for 10s ISI
Differences noted are as compared to N2, with all noted differences being significant, with (-) indicates lower than and (+) higher than N2. Specific p values can be found in text.

2.3.4 60s ISI tap habituation

For *lrk-1(km17)*, reversal final habituation level was found to be lower for both probability and duration ($p<.05$; $p<.001$; Figure 8). For the second allele of *lrk-1*, *tm1898*, final habituation level for reversal duration was also found to be lower than N2 ($p<.05$; Figure 9). For *pdr-1(gk448)*, no significant differences were found at a 60s ISI (Figure 10). *pink-1(ok3538)* was found to have a lower final habituation level for reversal speed as compared to N2 ($p<.05$; Figure 11). For the gene *vps-35*, allele *ok1880* showed a significant decrease in the final habituation level for reversal probability ($p<.01$; Figure 12). In the case of *vps-35(hu68)*, the only significantly different characteristics were a lower final habituation level for reversal probability ($p<.01$; Figure 13). Finally, for *rme-8(b1023)* final level habituation was significantly higher compared to N2 for reversal probability ($p<.05$; Figure 14).

2.4 Discussion

For each of the genes tested there was a significant change in the worms' response to tap stimulation. These differences could be subtle and only be seen in the initial response to tap or they could be more dramatic and be reflected in multiple measures. This information can help us understand which genes to follow-up on order to better understand learning and its relationship to PD. Strains such as *pdr-1(gk448)* displayed a singular role in habituation, effecting only the final level of reversal speed for 10s ISI. Since this *parkin* has mainly been associated with early onset PD, it appears as though *pdr-1* is not the most promising target for PD related research in *C. elegans* given the results from the assay used in these experiments. The same can be said for *rme-8*, which mainly has phenotypes in regards to basal characteristics, but not for habituation. However, alleles of *vps-35* show many differences in habituation and for basal characteristics. pink-1 and lrk-1 also appear to be good candidates for follow-up in regards to habituation,

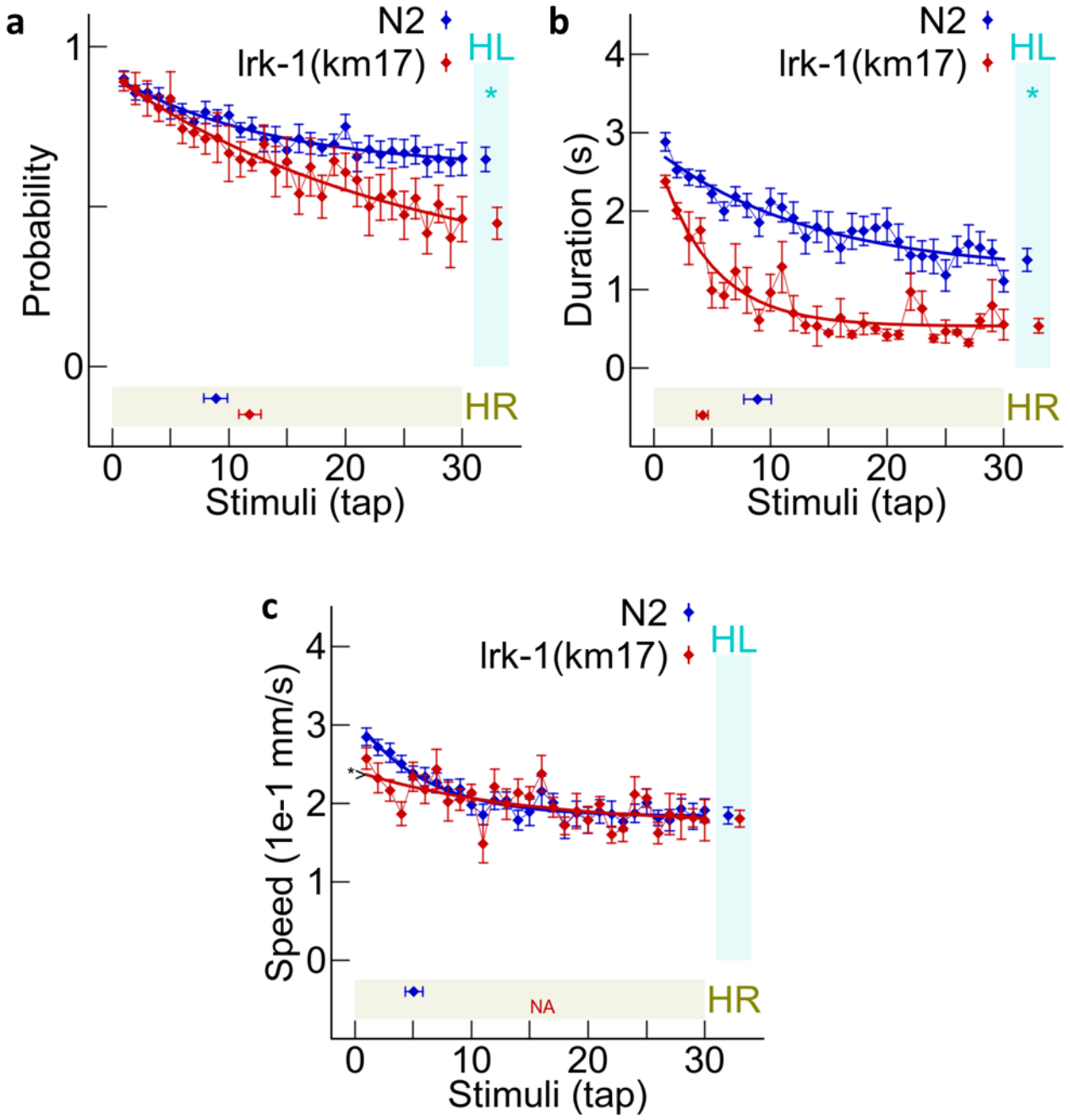

Figure 8: 60s ISI Tap Habituation for *lrk-1(km17)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

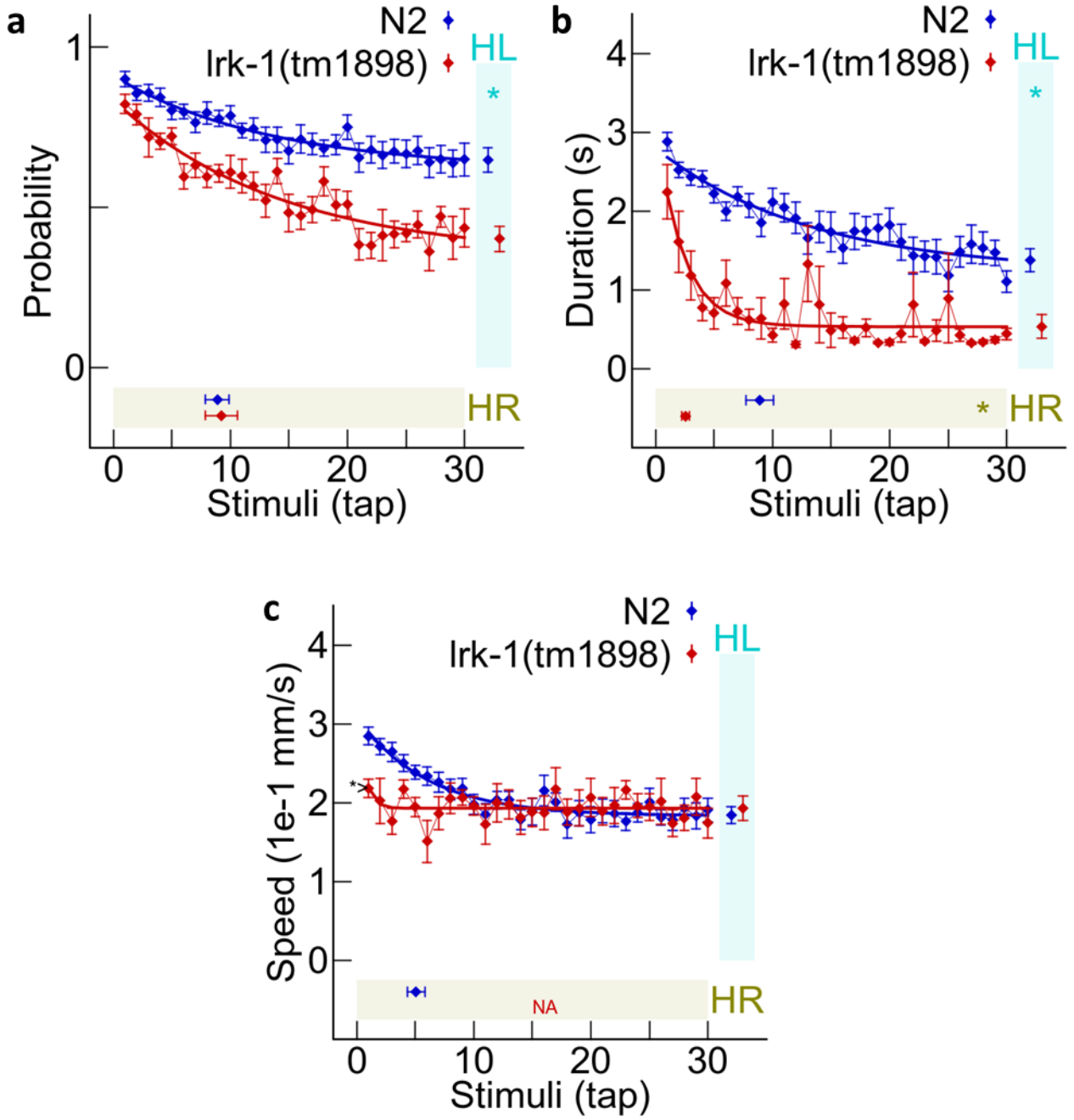

Figure 9:60s ISI Tap Habituation for *lrk-1(tm1898)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

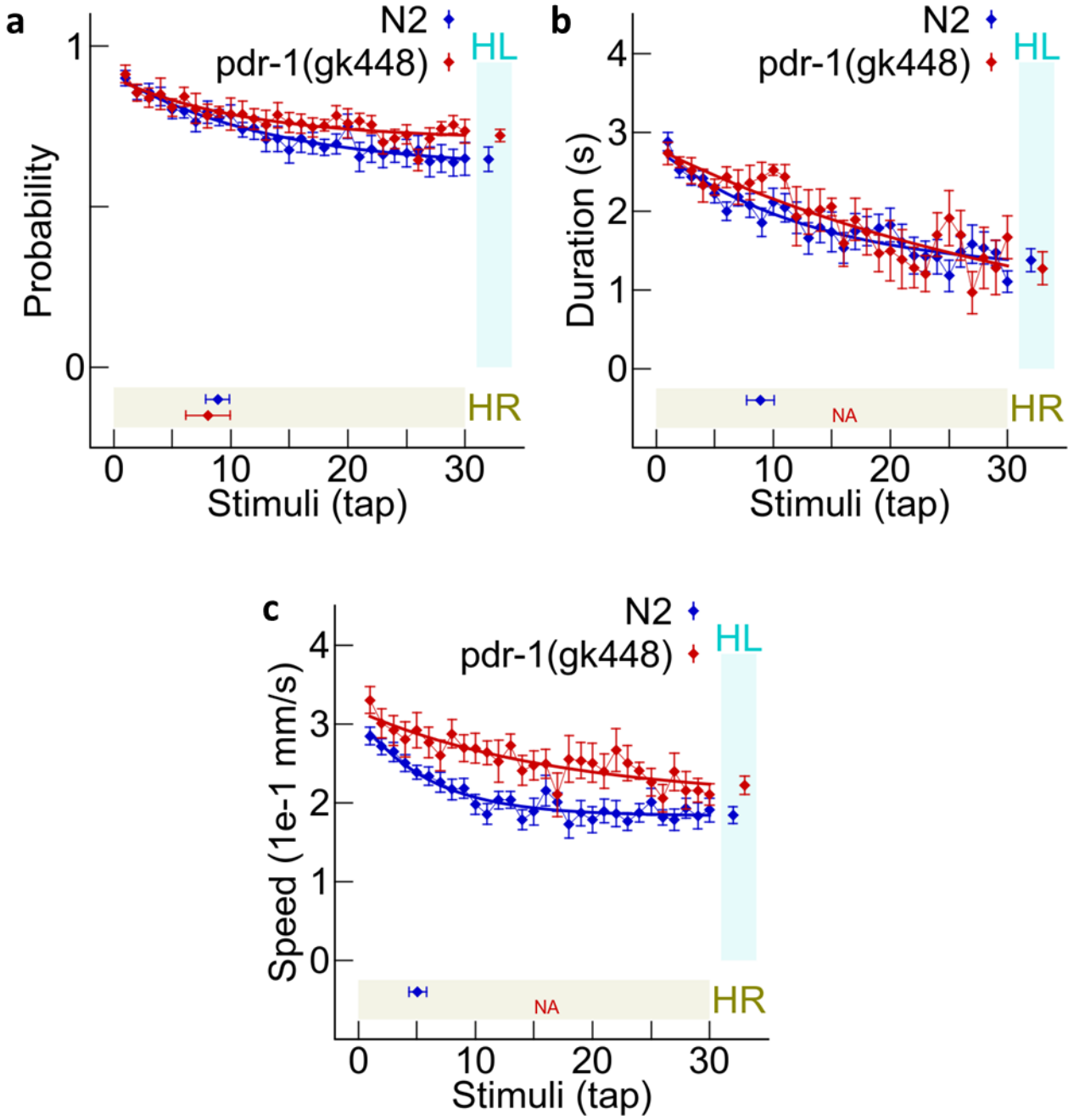

Figure 10: 60s ISI Tap Habituation for *pdr-1(gk448)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

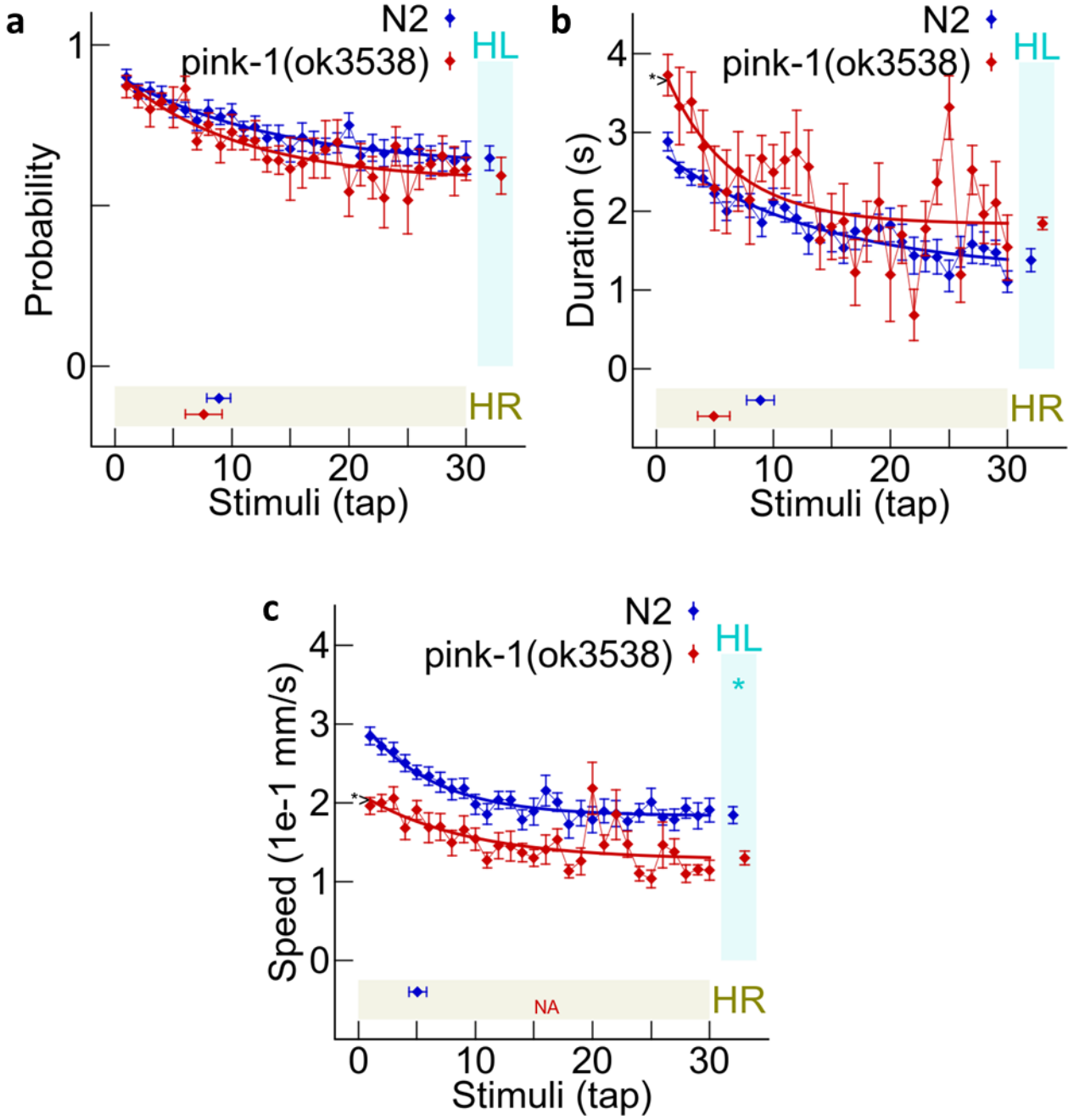

Figure 11: 60s ISI Tap Habituation for *pink-1(ok3538)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

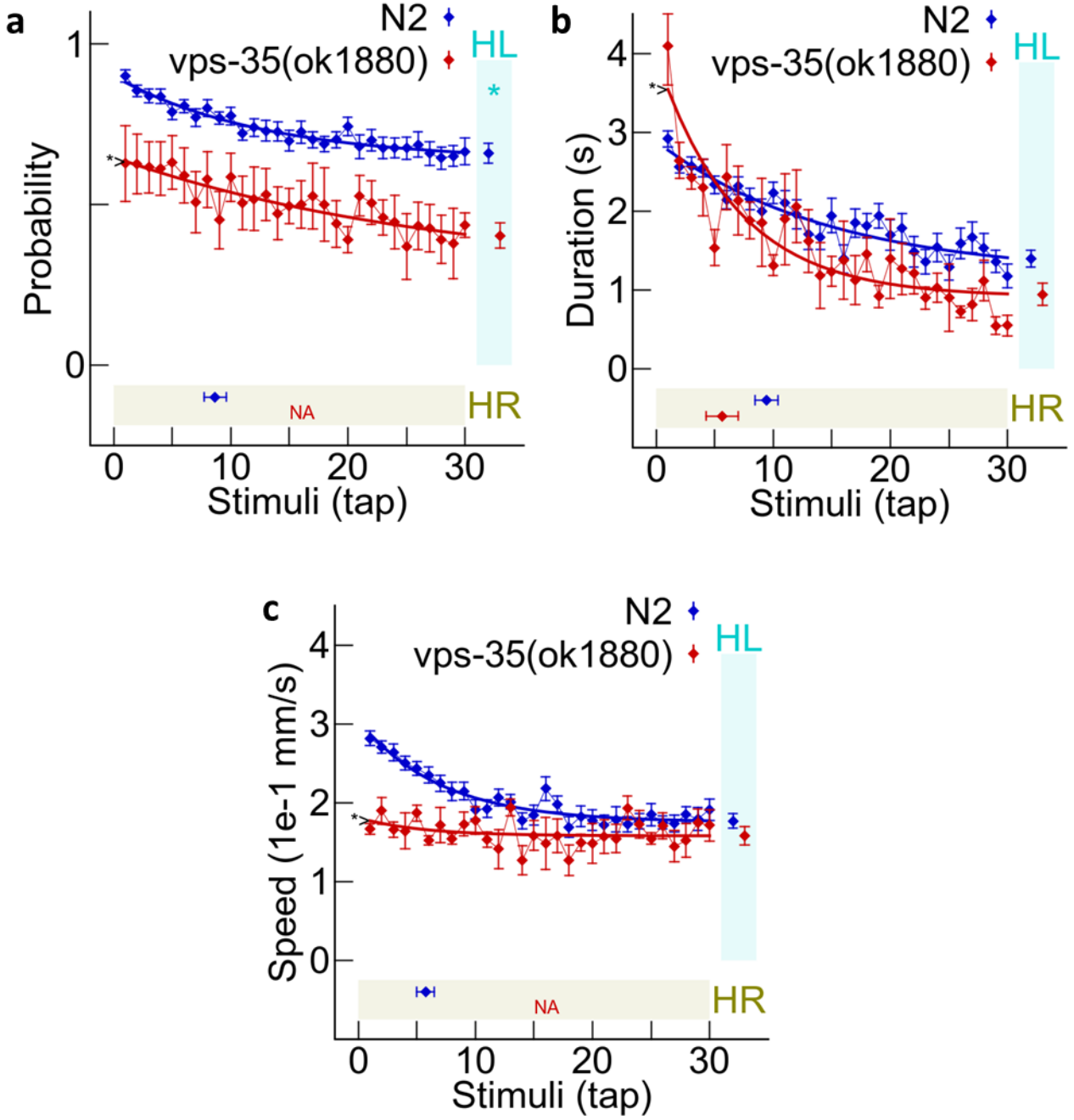

Figure 12: 60s ISI Tap Habituation for *vps-35(ok1880)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

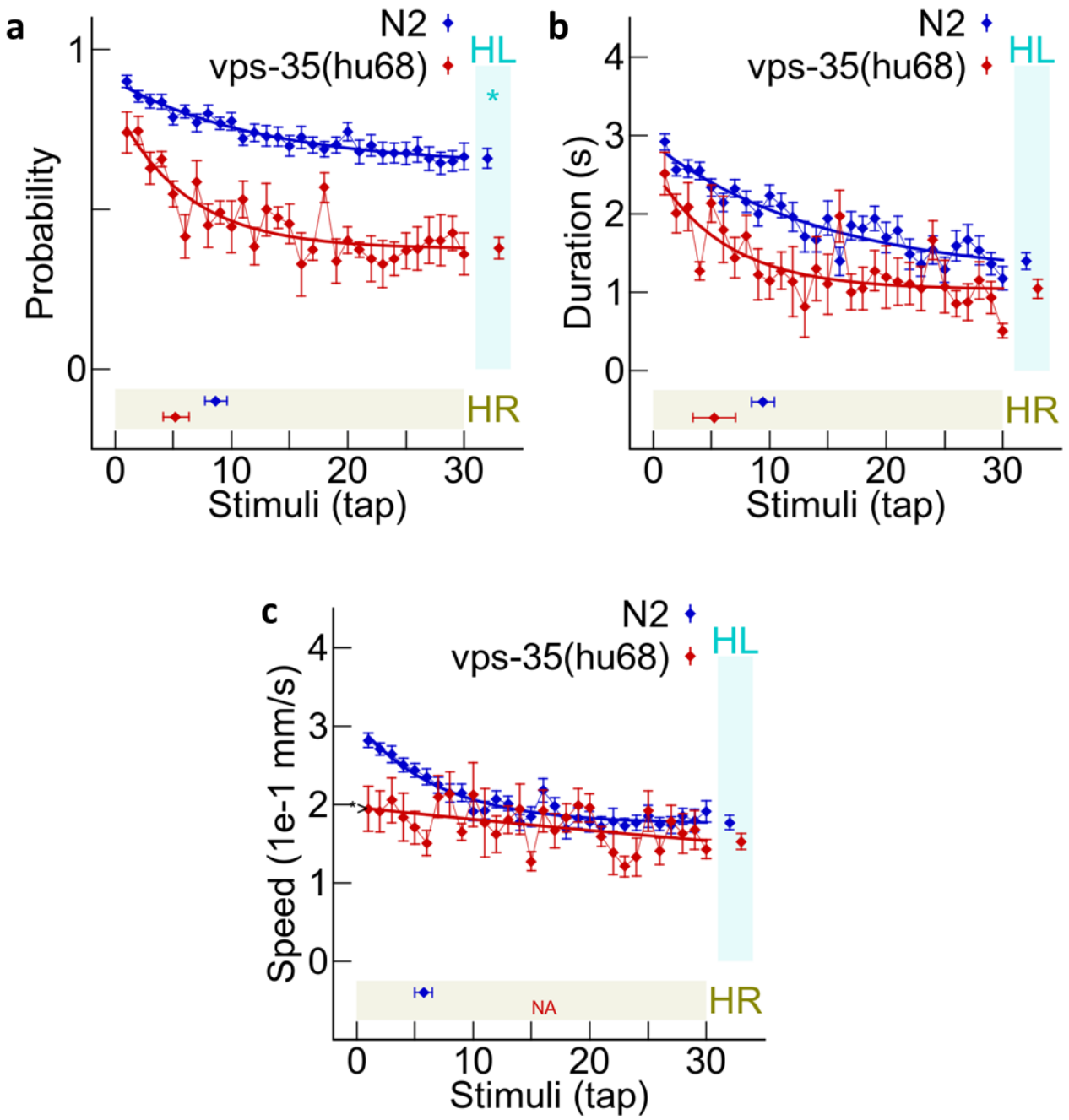

Figure 13: 60s ISI Tap Habituation for *vps-35(hu68)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

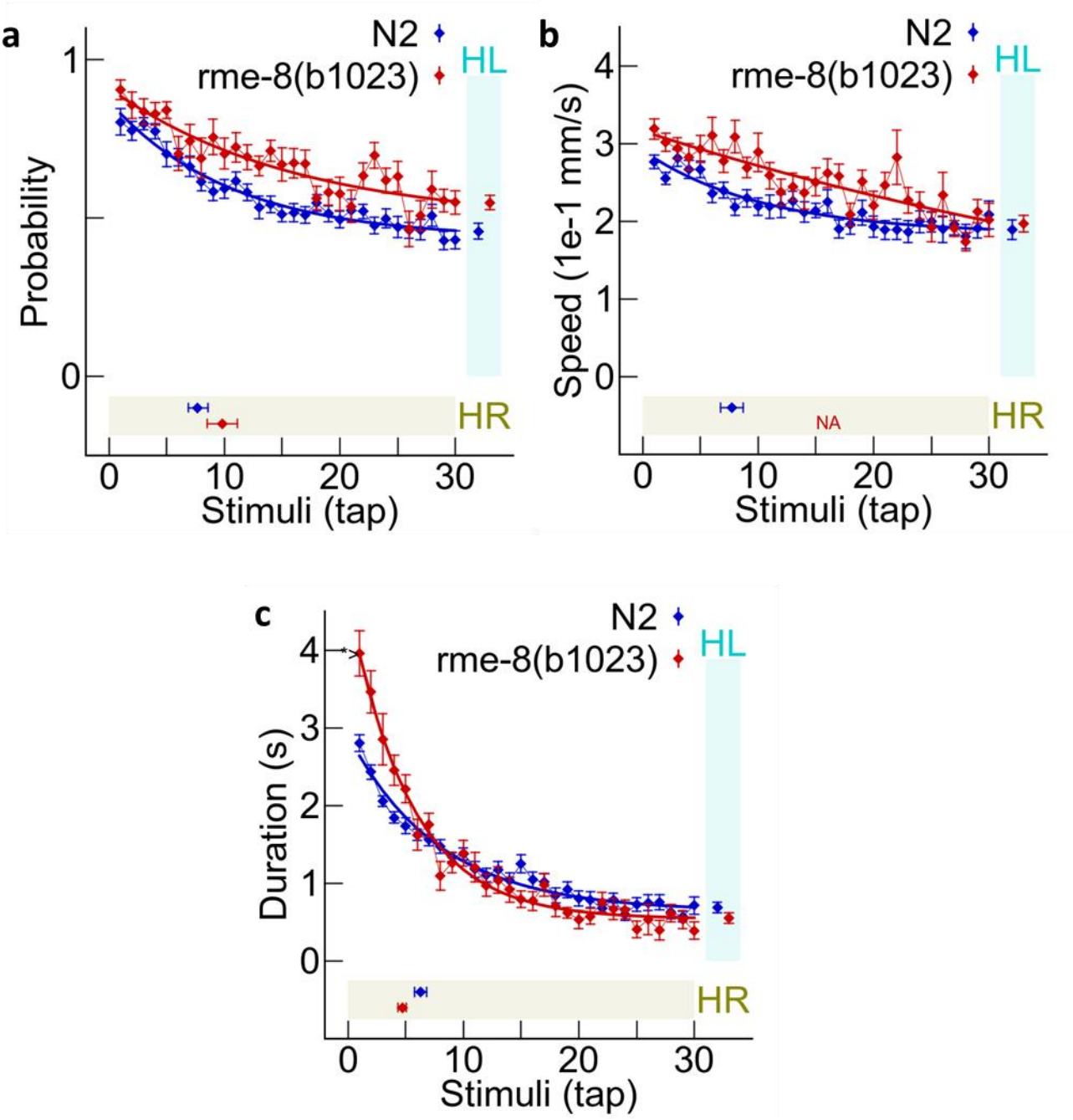

Figure 14: 60s ISI Tap Habituation for *rme-8(b1023)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

Strain	Initial Response Probability	Initial Response Duration	Initial Response Speed	Probability Rate	Duration Rate	Speed Rate	Probability Final Level	Duration Final Level	Speed Final Level
lrk-1(km17)			-				-	-	
lrk-1(tm1898)			-		+		-	-	
pdr-1(gk448)									
pink-1(ok3538)		+	-						-
vps-35(ok1180)	-	+	-				-		
vps-35(hu68)			-				-		
rme-8(b1023)			+				+		

Table 4: Tap Habituation Summary for 60s ISI
Differences noted are as compared to N2, with all noted differences significant with (-) meaning lower than and (+) higher than N2. Specific p values can be found in text.

although the phenotypic differences are not as consistent between alleles.

The issue of differences in the results for two alleles of the same gene have not yet been resolved. When testing genetic mutants, it is important to remember that there is always the chance that the observed behaviors were due to background mutations that were not accounted for in the strain. For example the large difference in the reversals per minute seen for the two alleles for *lrk-1* (*km17* and *tm1898*) may be due to differences in the severity or type of mutation, or they may be due to the presence of additional mutations in one of the strains. The only way to confirm that a given mutation was the cause of the observed phenotype is to rescue the mutant with the gene in question. The other possible explanation for differences is that the nature of the mutations in two alleles may affect the function of the protein in different ways. It is not clear if either of the mutations are true nulls, therefore there could simply be alterations in protein function that lead to different behavioural phenotypes. To test this would require assays of protein function for the proteins in question.

Chapter 3: Assessing the Role of Dopamine

3.1 Introduction

On a cellular level, it is important to understand how genes work together to produce an outcome, both on a behavioral and neuronal level. Once it is known that a behavior is affected, the next question is how that change happens. This of course leads us to how neurons are signaling in their network and what neurotransmitters are involved in that process. Since dopamine is the classic neurotransmitter that has been implicated in PD, this seems like the logical neurotransmitter to investigate. Dopamine has been previously implicated in habituation, making it the ideal target to further this line of questioning. By testing the PD mutant worms, we can hopefully determine whether any of these genes are involved in dopamine signaling.

3.2 Methods

3.2.1 Swimming induced paralysis

In order to screen the mutant strains quickly to determine whether any had higher than normal levels of dopamine (DA), the Swimming Induced Paralysis (SWIP) assay was used. This assay was modified from McDonald et al. (2007) who found that worms with excess DA will become paralyzed as soon as 6 minutes after being placed in a drop of water. Wild-type worms will continue to thrash (swim) for upwards of 30 minutes, with this time being set as the standard for a wild-type response.

Age synchronized 4-day old worms were placed on an agar plate that was fully covered in water. The behaviour of worms on these plates was scored using the MWT for 30 minutes as well as being visually monitored.

3.2.2 ON/OFF food

Habituation behavior is modulated by the presence of food, with behavior differing between whether the worms are on or off an *E. coli* lawn. It has been previously established that this response is modulated by dopamine, with dopamine deficient worms not showing any difference in habituation on or off food.

Age synchronized 4-day old worms were washed off their home plates using 500 ul of M9 buffer and placed in a .5 ml Eppendorf tube. Worms were centrifuged for 15 seconds in a mini centrifuge. Liquid was aspirated from the tube and the wash was repeated an additional two times in order to rid the worms of any bacteria on their bodies. Using cut pipette tips, the worms were then liquid transferred in three 20 ul drops to a new MWT plate with either food or no food. Plates were allowed to air dry for 30 minutes and were then tested using the same 10ISI habituation protocol used previously. Data was analyzed with Beethoven and Matlab in order to look at proportion worms reversing.

3.2.4 Statistics

Data analysis and statistics were run as previously described (see Chapter 2). On/Off food figures were created using Choreography.

3.3 Results

3.3.1 Swimming induced paralysis

All strains tested performed the thrashing behavior in water for the full 30 minutes of testing. Since this test screens for mutants that have an excess of dopamine, this suggests that none of the strains tested had levels of dopamine above normal.

3.3.2 ON/OFF food tap habituation

Previous studies have shown that there are dramatic changes in habituation behavior when worms are tested either on or off of food. The most robust phenotype is the reduction in reversal probability when worms are tested off of food (Kindt et al., 2007). When this shift is not seen, it is consistent with a reduction in dopamine signaling in the worms. In this study a significantly lower initial response was found for reversal probability, duration, and speed ($p<.001$, $p<.01$, and $p<.05$) when worms were tested off of food compared to on food. The reduction in probability off of food was observed in N2 worms ($p<.05$**)**, in addition, reversal duration showed a faster rate of habituation ($p<.05$) and a significantly lower final level habituation ($p<.01$) than when worms were tested on food.

For *lrk-1* alleles, *km17* demonstrated this characteristic drop in final level reversal probability ($p<.05$), as well as a significantly faster rate for reversal probability ($p<.05$). In addition, *km17* also had a lower initial response for reversal probability ($p<.001$) and reversal duration ($p<.05$) off of food as compared to on food. However, for *tm1898* the only difference in response between on and off food was for initial response of reversal duration ($p<.05$).

For *pdr-1*, *gk448* had a lower final level habituation for probability ($p<05$), as well as for reversal speed ($p<.01$). Lower initial responses were also found for reversal probability, duration, and speed ($p<.05$, $p<.01$, and $p<.01$ respectively). For *pink-1(ok3538)* final level habituation was lower off of food ($p<.01$), with initial response being lower for reversal probability and for duration ($p<.001$ and $p<.01$). *rme-8(b1023)* also showed a lower final level habituation for probability ($p<.001$) and for reversal speed ($p<.01$). Initial response for reversal probability, duration, and speed were also significantly different off of food ($p<.001$, $p<.01$, $p<.01$).

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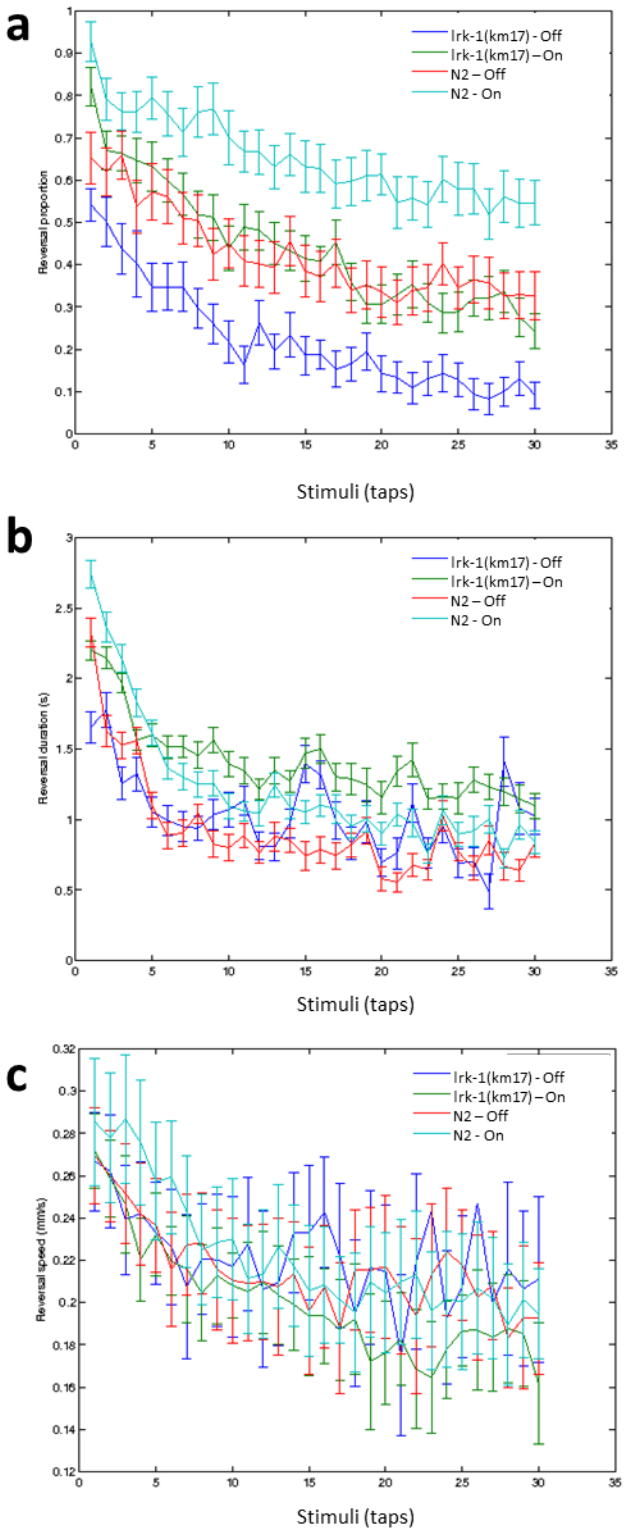

Figure 15: *lrk-1(km17)* ON/OFF Food Tap Habituation
10s ISI. Error bars are s.e.m.

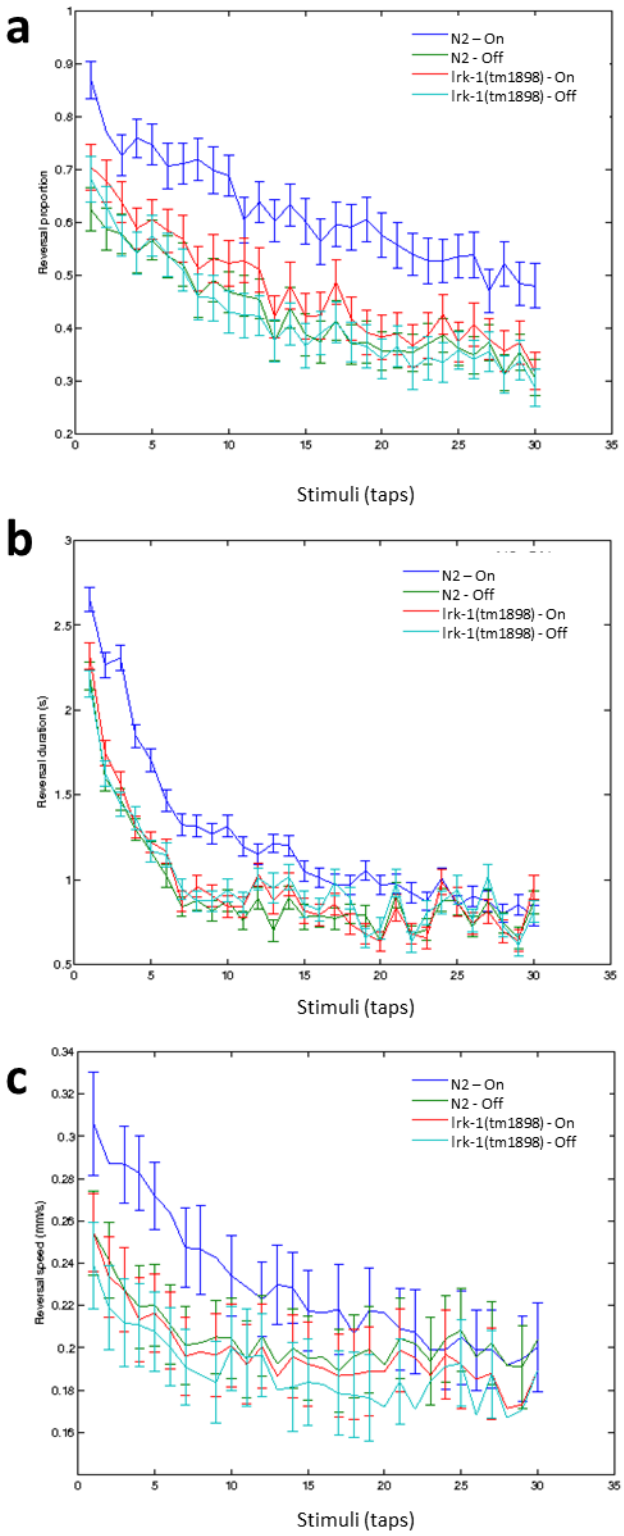

Figure 16: *lrk-1(tm1898)* ON/OFF Food Tap Habituation
10s ISI. Error bars are s.e.m.

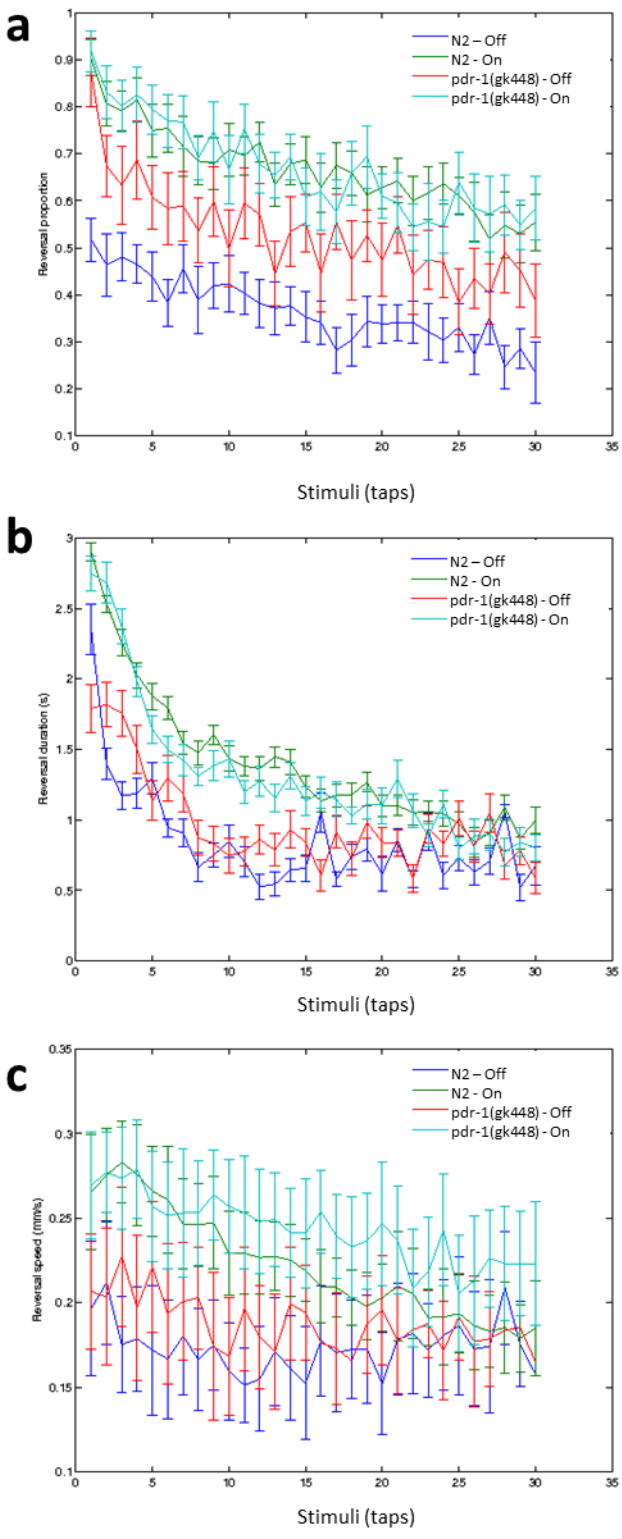

Figure 17: *pdr-1(gk448)* ON/OFF Food Tap Habituation
10s ISI. Error bars are s.e.m.

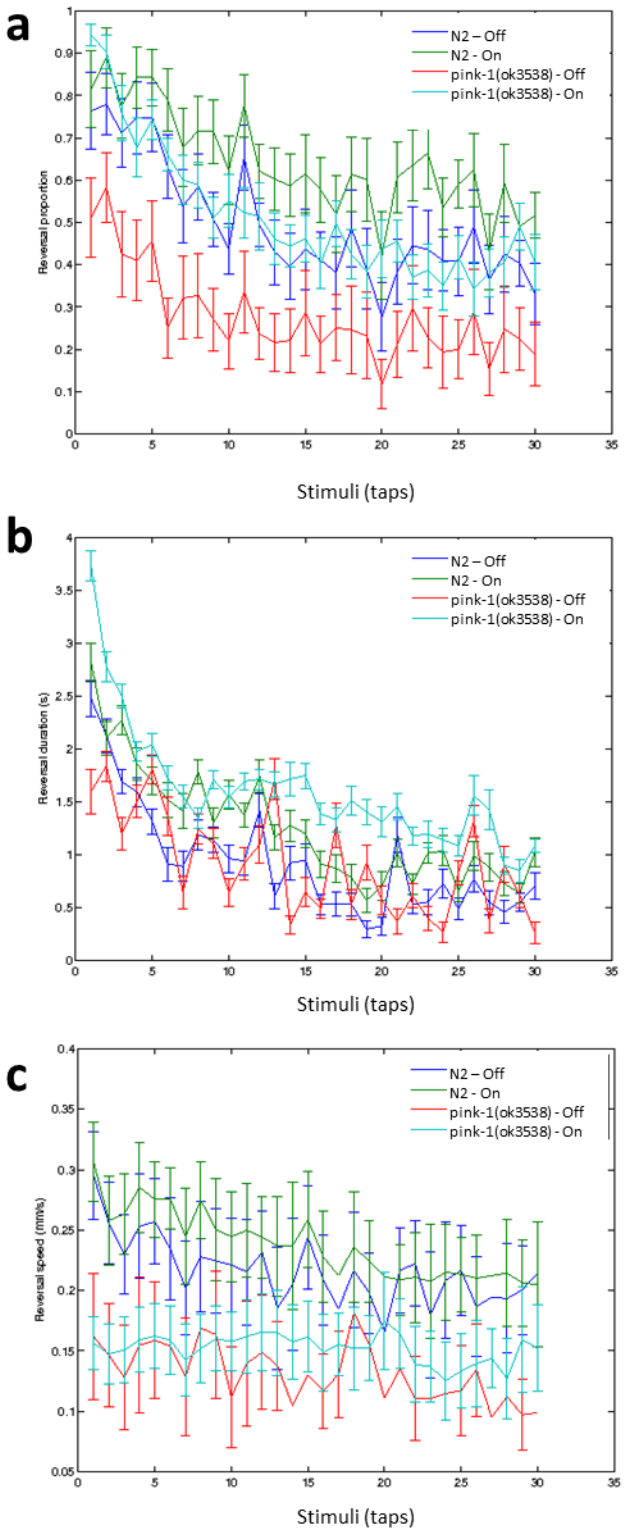

Figure 18: *pink-1(ok3538)* ON/OFF Food Tap Habituation
10s ISI. Error bars are s.e.m.

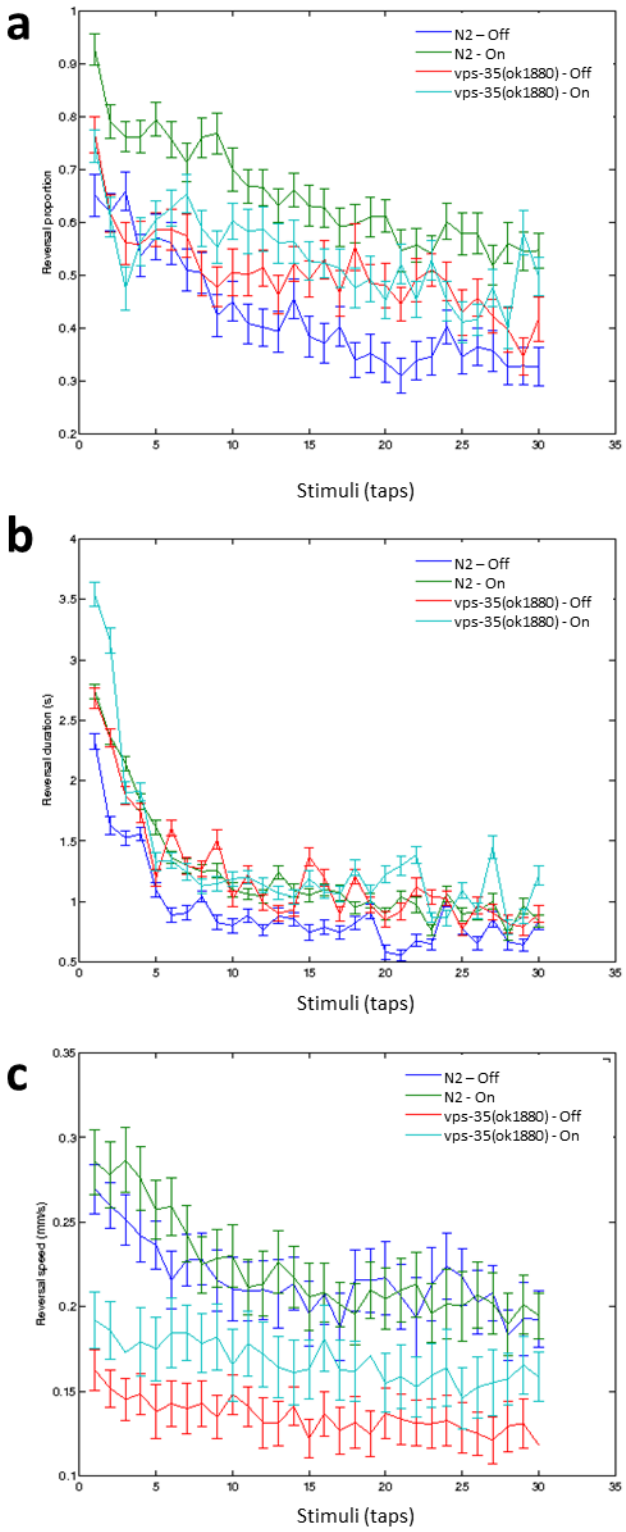

Figure 19: *vps-35(ok1880)* ON/OFF Food Tap Habituation
10s ISI. Error bars are s.e.m.

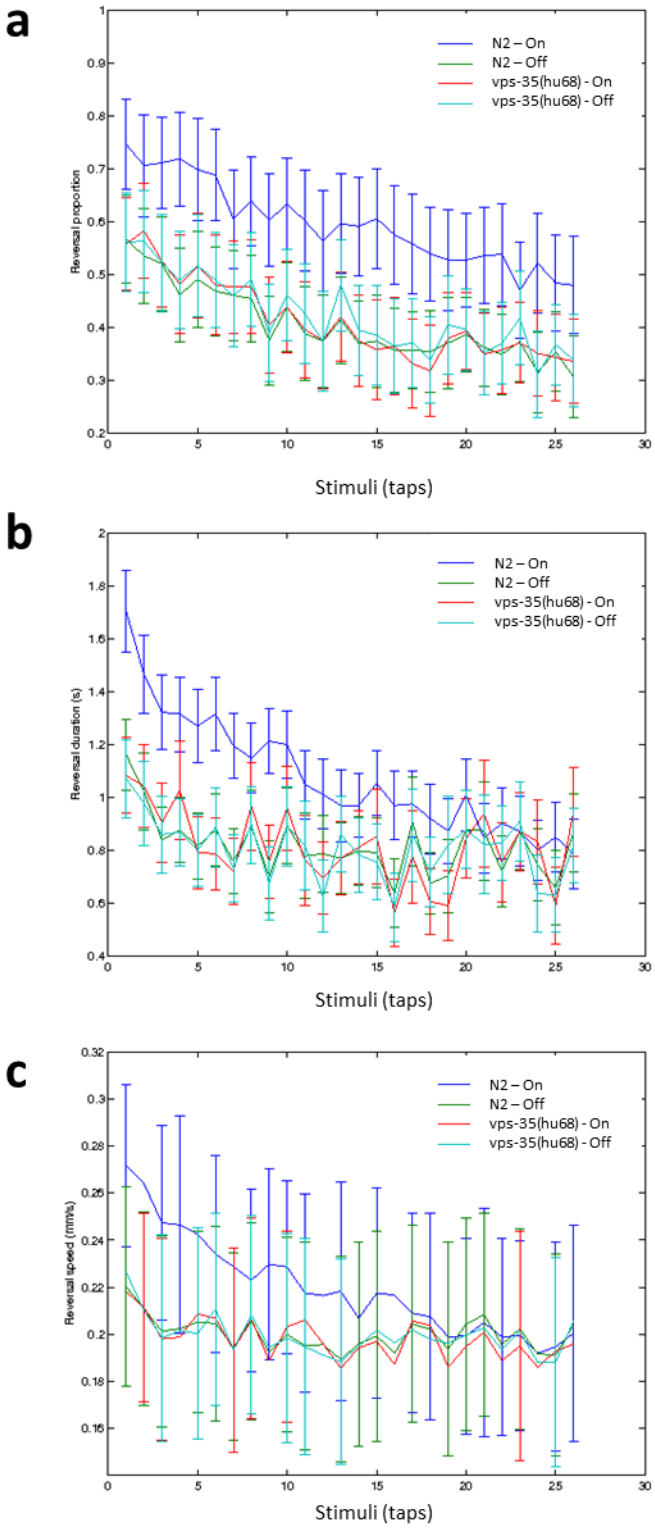

Figure 20: *vps-35(hu68)* ON/OFF Food Tap Habituation
10s ISI. Error bars are s.e.m.

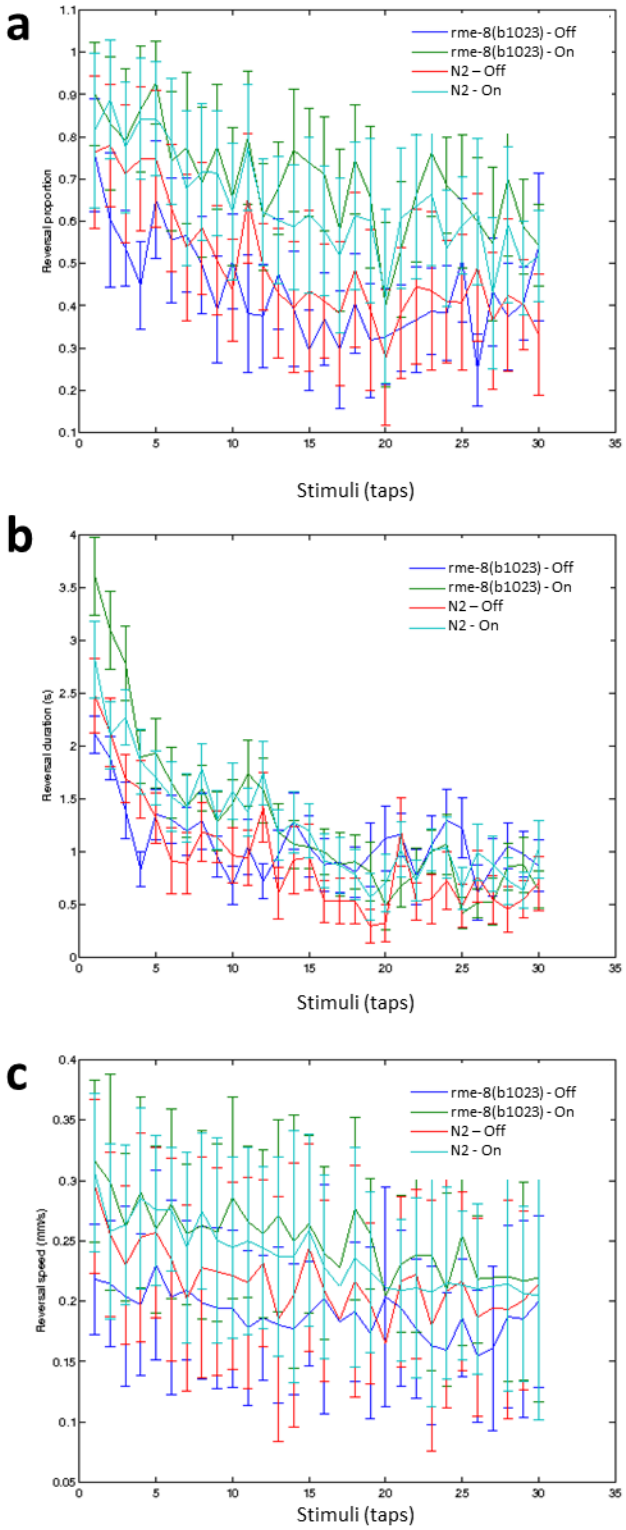

Figure 21: *rme-8(b1023)* ON/OFF Food Tap Habituation
10s ISI. Error bars are s.e.m.

Alleles of *vps-35*, however, did not show the reversal probability difference in final level when comparing on food to off food. *vps-35(hu68)* was not significantly different off of food in any measures. However, *vps-35(ok1180),* did show a lower final level habituation for reversal speed ($p<.05$) phenotype, as well as lower initial response for speed ($p<.05$). Since there was no difference in reversal probability whether on or off food in the *vps-35* mutants, these worms have a phenotype consistent with lower than normal dopamine signaling.

3.4 Discussion

Taken together, this set of experiments suggests that *vps-35* has an important role in dopamine signaling in the cell. The literature has previously established that dopamine is critical for sensing food, and that habituation is affected when food is not present. This is particularly dramatic when it comes to final level habituation for reversal probability. However, previous studies of this effect did not score reversal duration and reversal speed as separate measures of habituation, but focused on reversal distance. Since N2 worms also showed dramatic reductions in final level and rate for reversal speed when off of food, it is possible that the impact that the presence of food has on habituation is larger than previously believed. Initial response was also lower in all three measures of habituation, meaning that the tap response was significantly impacted by the presence or absence of food. However, what these studies show is that the two *vps-35* mutants, *ok1880* and *hu68*; do not change their habituation response when tested on and off of food. This lack of a change in the response has previously been tied with lowered dopamine signaling in the neurons (Kindt et al 2007), which could be due to either depleted signaling or a lack of the proper receptors. Further experiments could be performed to test whether the sensory neurons are functioning normally, and that the ability to sense food in these animals is normal. It is also important to note that since the SWIP test did not yield any

significant results and the food habituation did not show any high probability phenotypes, none of the tested strains are likely to have excess dopamine signaling.

Chapter 4: Focusing in on VPS35

4.1 Introduction

Because *vps-35* was the only gene of interest to show a distinct dopamine phenotype, it was investigated in more depth. Two additional genes, *dpy-23* and *mig-14*, were also tested because they are thought to interact with *vps-35* and *rme-8*(Pan et al., 2008). *dpy-23* codes for the adaptin mu2 ortholog of the adapt protein complex 2 (AP2). This is required for receptor-mediated endocytosis, and localizes with SNB-1 at synaptic vesicles (Gu et al., 2008; Pan et al., 2008). *mig-14* is orthologous to Wntless, meaning it is involved in all major Wnt processes and has a key role in development (Bänziger et al., 2006). *vps-35(hu68)* has been shown to interact directly with *egl-20* (*wnt*) whose secretion is mediated by *mig-14* (Harterink et al., 2013; Coudreuse et al., 2006). Since no WASH complex homologs are currently identified in *C. elegans*, these proteins provided the best avenue for studying some of the proteins that could be downstream in these gene circuits.

4.2 Methods

4.2.1 *vps-35* rescue and overexpression

Cosmid constructs *38dH01* and *22cA09* containing the wild-type copy of *vps-35* were acquired from the Moerman Lab (UBC). *E. coli* stocks of the cosmids were grown up and purified using the GeneJet Plasmid Miniprep kit. An injection mixture of Cosmid DNA at 10 ng/ul, *myo-3* mcherry marker at 5 ng/ul, and puc-19 at 95 ng/ul was used for injections. Microinjection was performed on an inverted scope. First, N2 worms were removed from their home petri plate and moved onto a glass slide with a 2% agarose pad. This pad had a drop of 700 Halocarbon oil which immobilizes the worms, and allows them to be injected. 1 ul of injection fluid was pipetted into a small glass capillary that has been pulled to a needle-point. Once the

needle was flowing, worms were injected with this pressurized needle, making sure that it was placed at the distal core cytoplasm of one gonad arm. The worms were then recovered with M9 buffer and moved back to agar plates with an E. coli lawn.

At approximately 3 days post injection, plates were screened for F1 worms expressing the mCherry co-injection marker using a Leica fluorescent microscope. Each progeny expressing the marker was moved to a new plate and cloned out. These plates were then screened several days later, with any plates bearing a large number of expressing worms being considered a stable line of N2 expressing extra-chromosomal arrays of *vps-35*. This strain allowed us to look at the effects of overexpression of *vps-35* on a wild-type background.

Male worms from this N2+transgene line were created via heat shock, in which young L4 hermaphrodites are placed at 35C for 3-4 hours. Plates were then screened 3-4 days after this procedure, selecting male worms expressing mCherry. These worms were then placed on a new plate containing young hermaphrodites of the strain VC1390 in a ratio of 10:4. Several days later, plates were screened for hermaphrodites expressing the mCherry marker. These worms were placed on individual plates and were cloned out. Several progeny for each of these plates were lysed and genotyped using PCR. VC1390 worms expressing the transgene demonstrated a proper genetic cross and were maintained as a stable line. Worms from these two lines were subsequently age synchronized for behavioral testing. Pre testing, the Leica fluorescent scope was used to find expressing worms and move them onto MWT plates for testing. Worms were subsequently tested on the MWT for 10 ISI habituation and analyzed as was previously described.

4.2.3 Cross and testing pdat-1::ChR2 x *vps-35(ok1880)*

In order to further investigate the effect the *vps-35* mutation had on the DA neurons, a simple assay was used that took advantage of the ChR2 expressed in the DA neurons using the dopamine transporter promoter (*pdat-1)*. To do this a genetic cross was made using strain AQ2028 *pdat-1*::ChR2; *unc-126*::yfp. Males were generated using heat shock at 35C for 3-4 hours. Males expressing YFP were picked using a Leica Fluorescent scope and put on a plate with VC1390 hermaphrodites in a 10:4 ratio. Plates were screened 3-4 days later, with F1 worms expressing the YFP marker moved to new plates and cloned out on individual plates. Two worms from each of these plates were lysed and genotyped using PCR. Crosses that were successful bore both the marker and had the mutant allele of *vps-35*. The most successful cross was maintained and named VG577. VG577 worms were age syncheronized on MWT plates seeded with E. coli that contained retinol at a 1:80 ratio. Four-day old worms were tested on the tracker, with a 3 minute pre-plate followed by three 3 second blue light pulses. Data was analyzed using Beethoven, with Matlab used to look at speed over the course of the trial.

4.2.3 Behavioral characterization of *dpy-23* and *mig-14*

The MWT was used to test strains at a 10s ISI for tap habituation. Four-day old age synchronized worms were placed on the MWT and tracked for 5 minutes to establish baseline behavior. The habituation protocol was then performed, with 30 taps being administered to the side of the petri plate. “Beethoven” was used to analyze the habituation data for proportion reversing, reversal duration, and speed over the course of the experiments. Data collected during the 10ISI habituation experiment was further analyzed using the program “Maestro”. Body size, speed, and spontaneous reversals were found to be of special interest.

4.2.4 Data analysis and statistics

All habituation data was analyzed as was described in previously (see Chapter 2). For the ChR2 experiment data as first analyzed using Choreography to create summary files for each plate. These files were then imported into Matlab and combined into summary files. The script "rastor_analysis" was run in three, 3 second bins to describe the times before stimulation, during the 3s stimulation, and after. This analysis gave values for forward velocity, which were subsequently imported into R. A one-way ANOVA and Tukey's honest significant difference test were used to compare the statistical significance of these values

4.3 Results

4.3.1 *vps-35* rescue and overexpression

Since transformation of the rescue construct was not originally successful in the mutant *vps-35(ok1180)* worms, the transgene was injected into N2 animals. These modified N2 worms expressed more *vps-35* than wild-type worms and can be studied to assess how overexpression of this gene affects worm behavior. First, the *vps-35(+)* had a lower final level reversal probability compared to wild-type ($p<.01$), as well as a higher final level for reversal duration ($p<.05$). This strain also had a lower final level for reversal speed as compared to wild-type ($p<.05$), a characteristic shared with *vps-35(ok1180).vps-35(ok1180)* and *vps-35(+*) also shared a lower initial response for probability and speed as compared to N2 ($p<.001$ and $p<.001$). The *vps-35(+)* worms were successfully crossed with *vps-35(ok1880)*, with the *vps-35(rescue)* worms showing no differences from N2 for habituation with the exception of initial response for reversal speed ($p<.001$). Body size was found to be 1.46 mm2, with basal speed at .218 mm/s, 3.94 reversals per minute ($p<.001$), with reversal duration at .253s.

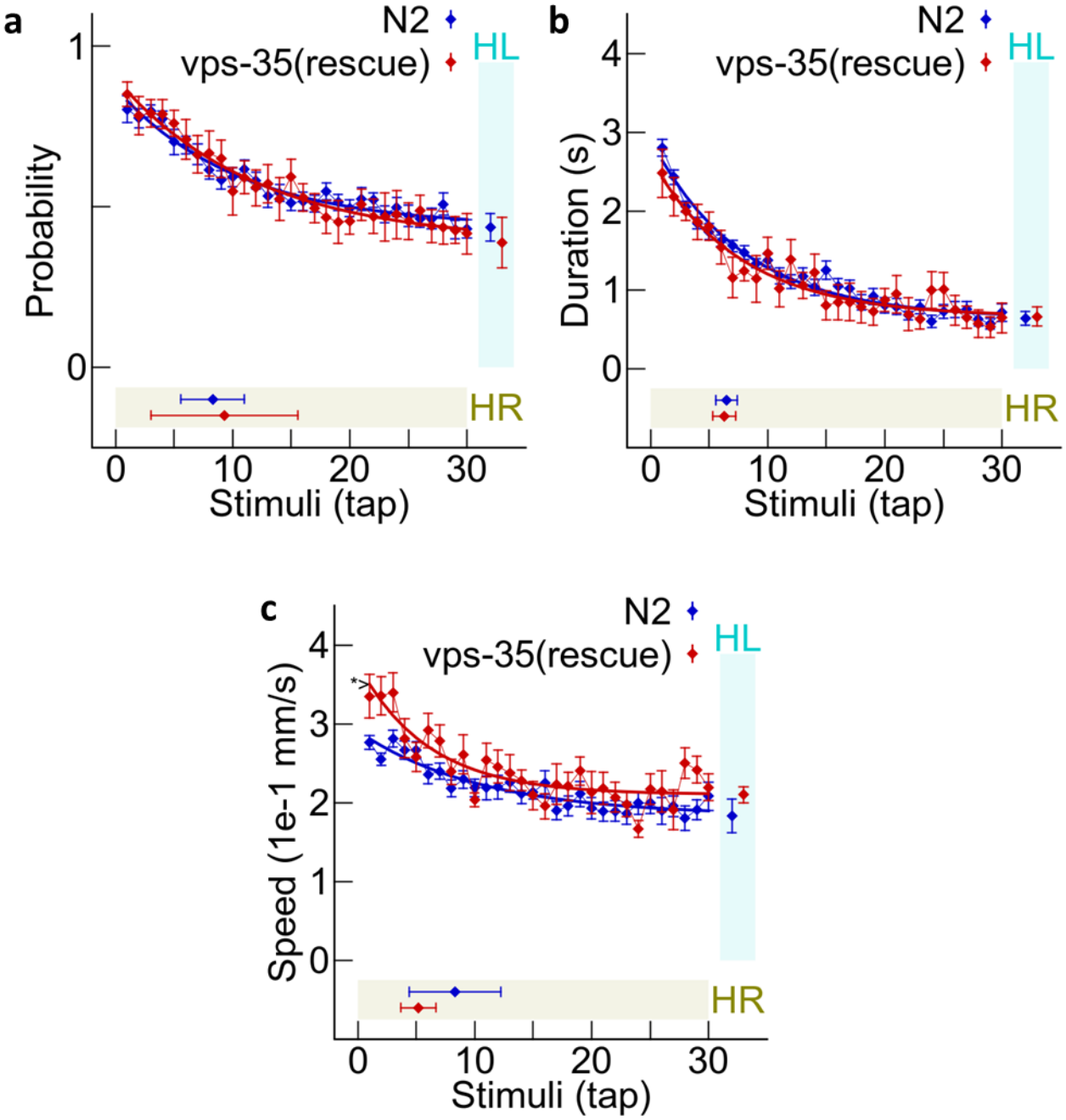

Figure 22: 10s ISI Tap Habituation for *vps-35(rescue)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

Strain	Initial Response Probability	Initial Response Duration	Initial Response Speed	Probability Rate	Duration Rate	Speed Rate	Probability Final Level	Duration Final Level	Speed Final Level
vps-35(ok1180)	-	+	-		+	-			-
vps-35(rescue)			+						
vps-35 (+)	-		-				-	+	

Table 5: 10s ISI Tap Habituation Characterization Comparing *vps-35(ok1880)*, N2 *vps-35(+)* Overexpression and *vps-35(rescue)*

Differences noted are as compared to N2, with all differences significant if noted, with (-) meaning lower than and (+) higher than N2. Specific p values can be found in text.

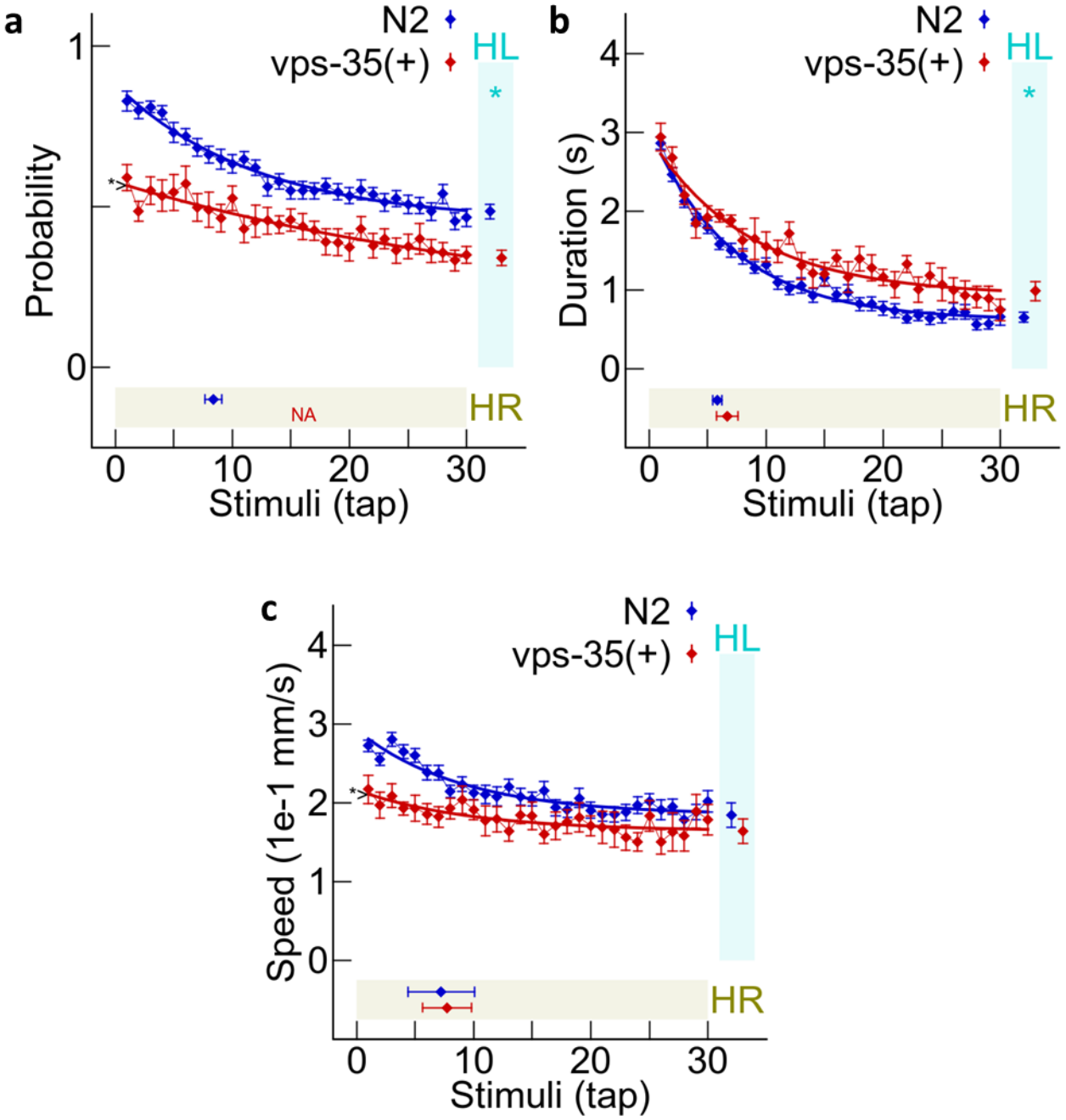

Figure 23: 10s ISI Tap Habituation for *vps-35(+)* Overexpression
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

4.3.2 Cross and testing *pdat-1*::ChR2 x *vps-35(ok1880)*

In order to gain a better understanding of how the dopamine neurons function in a *vps-35* mutant background we took advantage of a strain expressing channelrhodopsin (ChR2) in dopamine neurons (driven by the *pdat-1* promoter). Previous studies have shown that activating these ChR2 expressing dopamine neurons with a single blue light leads to an immediate locomotor slowing response (Ezcurra et al., 2011).This slowing response will only occur if the dopamine neurons are healthy and intact; worms with abnormal dopamine signaling will not perform a slowing response. The control strain, *pdat-1*::CHR2, shows a drop in forward velocity when a 3 second blue light pulse is given ($p<.001$). This depression in velocity is result of activated dopamine neurons causing a slowing response. Forward velocity remained depressed for the 3 second period post light-pulse as well ($p<.001$). However, in the strain VG577 (*vps-45(ok1880)* x pdat-1::ChR2), there was no significant drop in velocity during or following the blue light pulse. Although VG577 does have a lower baseline velocity ($p<.001$), there was no characteristic slowing as the dopamine neurons were activated.

4.3.3 Behavioral testing of *vps-35* and *rme-8* interactors

VPS35 and DNAJC13 are involved in the same system of cargo recognition and receptor mediated endocytosis and have been shown to interact via FAM21 of the WASH complex in mammalian cells (Zavodszky et al., 2014). Although in *C. elegans* no homologs for these genes have been identified it has been shown that *vps-35* and *rme-8* can interact via two other genes, *mig-14* and *dpy-23* (Park et al., 2008). For *dpy-23(e840)*, initial response was found to be lower for probability($p<.01$), duration ($p<.01$), and speed ($p<.01$; Figure 24). Additionally, probability of habituation final level was lower compared to N2 ($p<.05$). For *mig-14*, both alleles, *ga62* and *mu71*, had the same phenotypes. Initial response was lower for probability ($p<.001$; $p<.01$) and

speed(p<.05; p<.01), while probability final level was also lower (p<.01; p<.05) as compared to N2(Figure 25; Figure 26).

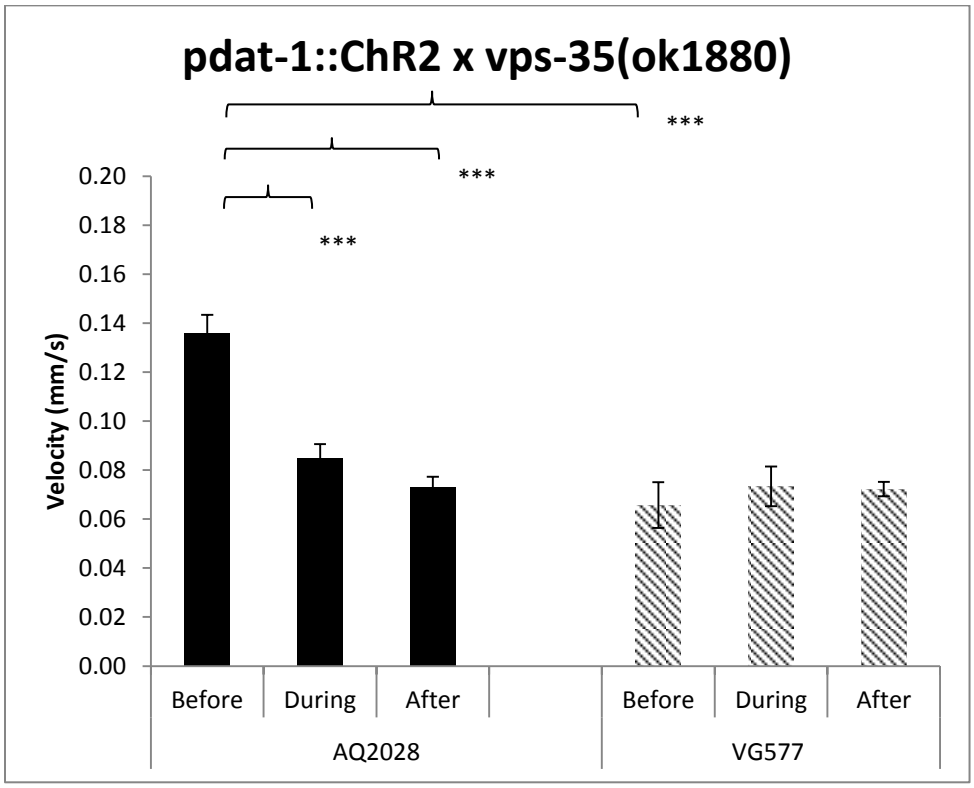

Figure 24: Dopamine Neuron Reactivity
Each of the three blocks (Before, During, After) are the average forward velocity over a 3s time period. *** denotes significant difference of p<.001.

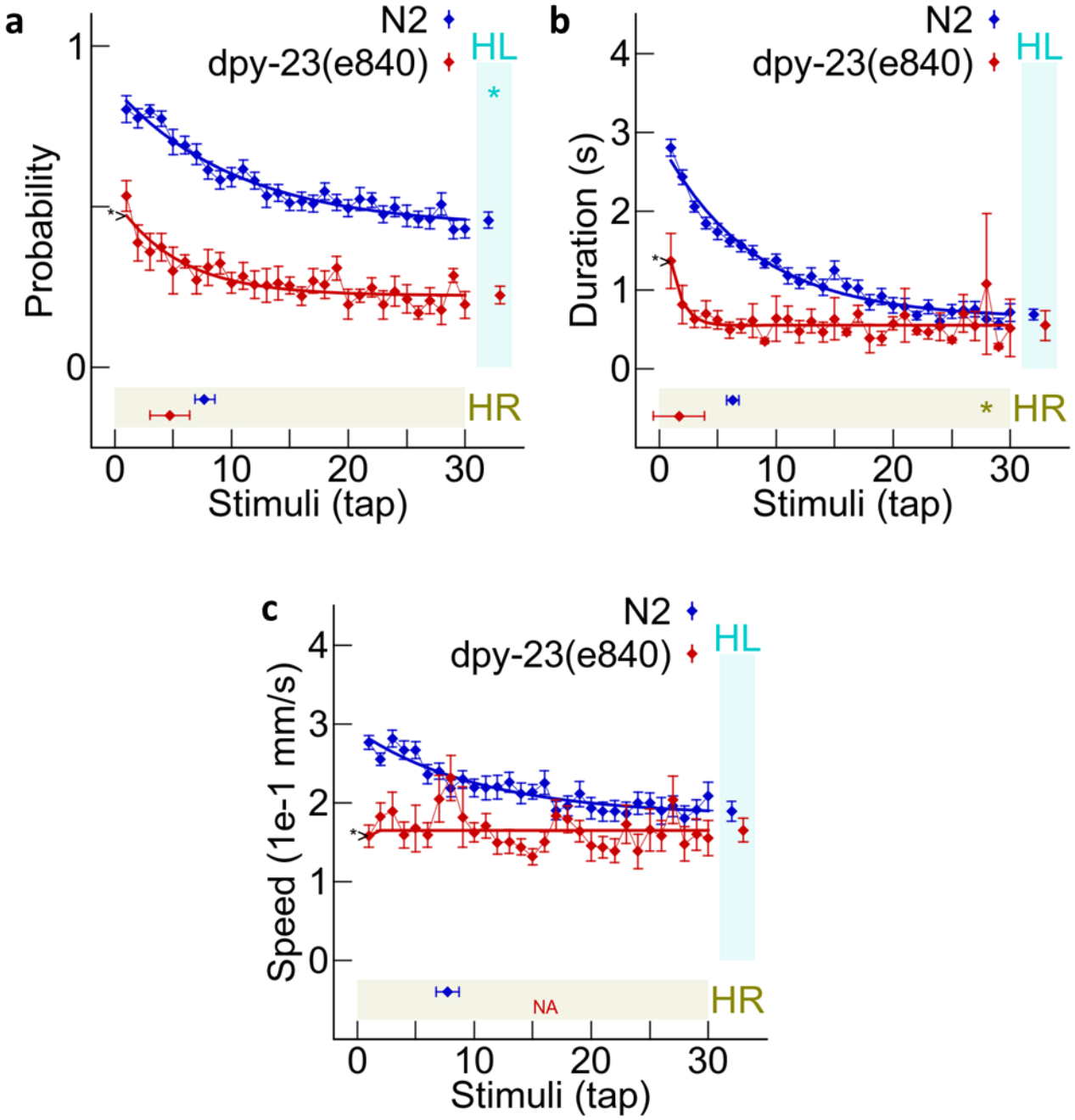

Figure 25: 10s ISI Habituation for *dpy-23(e840)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

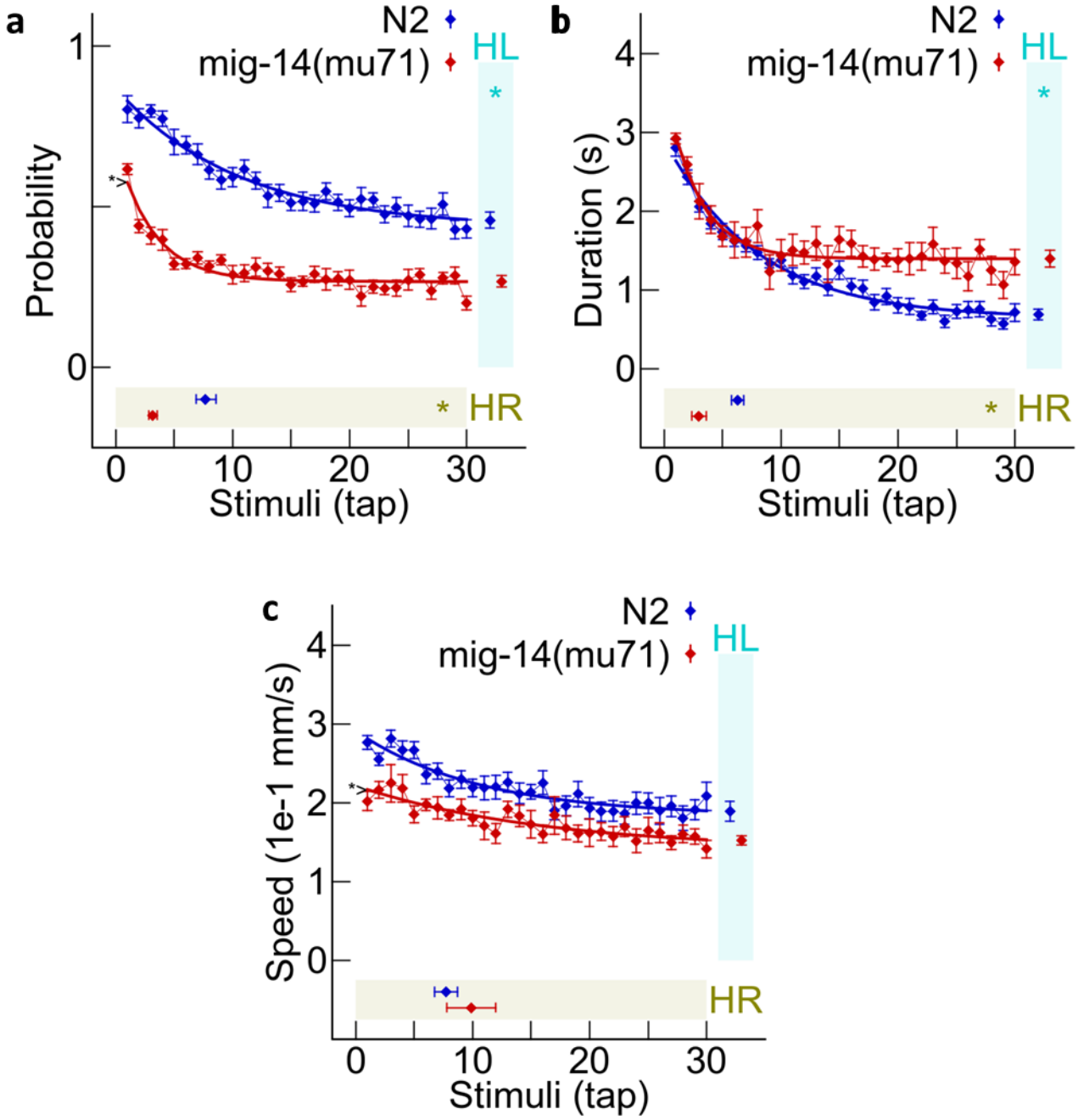

Figure 26: 10s ISI Tap Habiutation for *mig-14(mu71)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

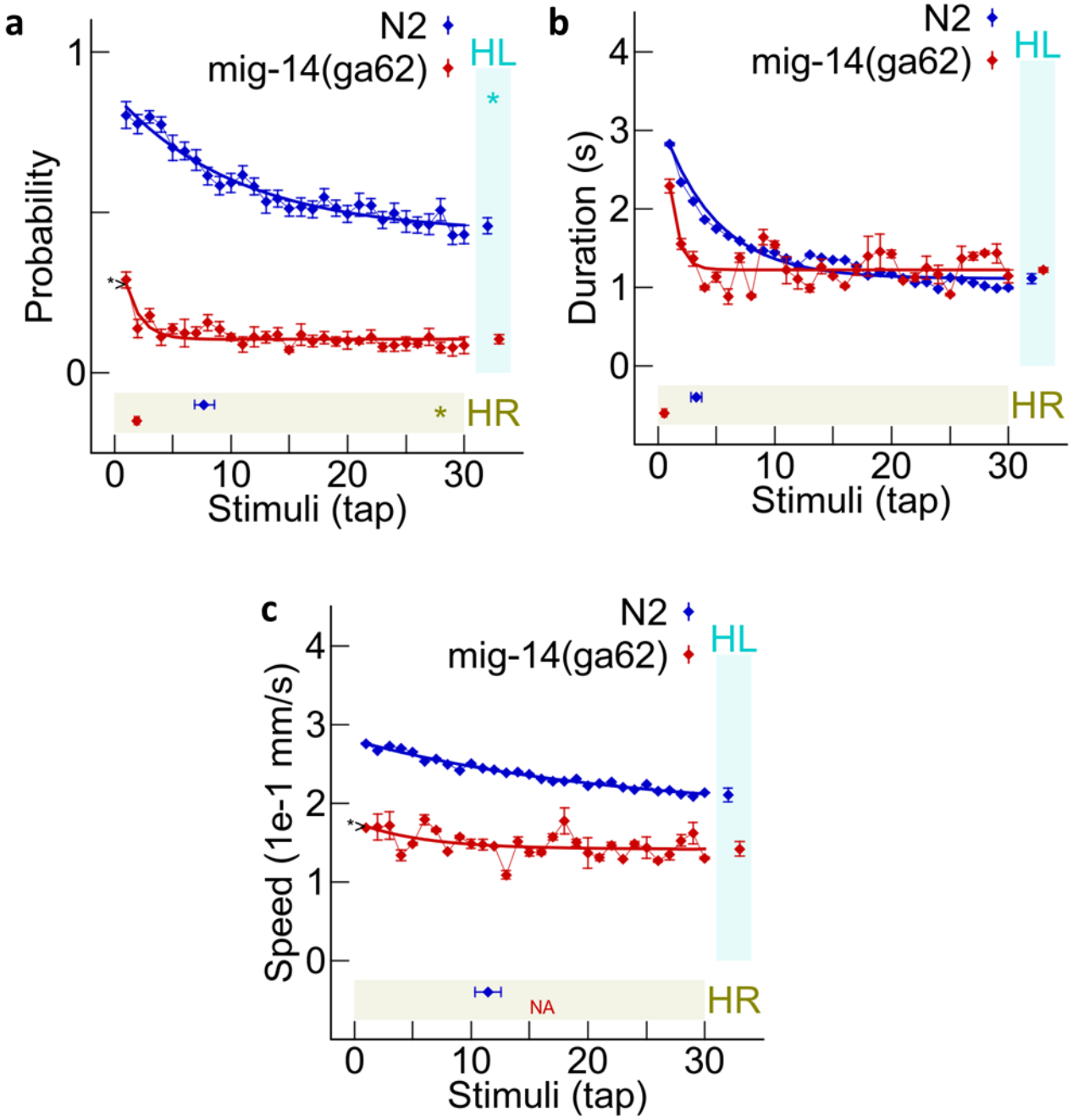

Figure 27: 10s ISI Tap Habituation for *mig-14(ga62)*
Habituation of reversal probability (a), reversal duration (b), and reversal speed (c). Error bars represent s.e.m., with (*) denoting significant differences from wild-type. Habituation level (HL) and habituation rate (HR) are the final measures of habituation as calculated by the asymptotic curve.

Strain	Initial Response Probability	Initial Response Duration	Initial Response Speed	Probability Rate	Duration Rate	Speed Rate	Probability Final Level	Duration Final Level	Speed Final Level
vps-35(ok1180)	-	+	-		+	-			-
vps-35(hu68)	-	-	-			-	-	+	
rme-8(b1023)		+							
dpy-23(e840)	-	-	-		+		-		
mig-14(ga62)	-		-	+	+		-	+	
mig-14(mu71)	-		-	+			-		

Table 6:10s ISI Habituation Phenotypes for *mig-14* and *dpy-23* as Compared to *vps-35* and *rme-8*
Differences noted are as compared to N2, with all noted differences being significant, with (-) indicates lower than and (+) higher than N2. Specific p values can be found in text.

4.4 Discussion

Because the *vps-35* mutants showed the most consistent evidence for having abnormal dopamine signaling, this gene was the candidate we chose for follow up studies. To investigate dopamine neurotransmission in *vps-35* we took advantage of ChR2 technology. By crossing a *pdat-1* ChR2 strain, expressing channelrhodopsin in the dopamine neurons with our *vps-35(ok1880)* mutant, we were able to confirm that dopamine neurotransmission is altered in *vps-35*. Because we observed no slowing of locomotion in response to stimulation of the dopamine neurons we hypothesize that this strain has lower levels of dopamine as compared to wild-type worms. This suggests that *vps-35* plays a role for maintaining dopamine signaling in the cell. This makes sense since *vps-35* is a key component of the retromer complex and has been suggested as a possible contributor to dopaminergic depletion in PD (Vilariño-Güell et al., 2011). However, it is possible that this protein regulates other neurotransmitters as well, It appears that acetylcholine and GABA transmission in motor neurons must be relatively normal as the mutant *vps-35* worms do not crawl at a slower speed than N2.Serotonin may be affected by *vps-35* mutations since the worms are egg-laying defective. To test whether serotonin signaling is altered a serotonin dependent behavioural assay called Enhanced Slowing Response, could be used to test worms response when entering an E. coli lawn (Sawin et al., 2000). In addition, since *vps-35* has been implicated in *glr-1* trafficking (Zhang et al., 2012), glutamate mediated behaviours such as nose-touch (Hart et al , 1995) would be an ideal candidate for follow-up experiments It will be important to use both alleles of *vps-35* in all subsequent experiments.

When investigating any mutant that has a specific phenotype, it is always important to create a rescue of the strain to confirm that it is that specific mutation that is contributing to the behavior of interest. Since *vps-35(ok1880)* has a deficit in egg-laying behavior, this proved to be

quite a challenge. However, a rescue strain has been generated and pilot data suggests it did rescue most of the *vps-35* habituation phenotypes (Figure 22). We believe this will confirm that a mutation *vps-35* is responsible for the habituation and basal phenotypes. However, since *vps-35(hu68)* does largely phenocopy *vps(ok1880*), we are confident that this gene is largely responsible for the habituation and dopamine phenotypes that have been recorded. In addition, the overexpression of *vps-35* in N2 tells us some interesting information about the effect that this protein has on an animal. Initial response for probability and speed are lowered, as are final level for probability (See Table 4). This is very similar to the *vps-35(ok1880)* mutant, suggesting that initial response is lower when the equilibrium of this protein is abnormal. In addition, final level for duration is higher than for N2. This suggests that the speed circuit is over activated when there is excess *vps-35(ok1880)*, while this same circuit is depressed in the mutant animals. Overall, these experiments led to an understanding of the importance of the delicate balance of levels of expression for this gene, with too little having certain effects on the animal, while having too much has others.

The identified genetic interactors of *rme-8* and *vps-35* showed a combination of phenotypes that suggests they may be affecting neurons in similar ways. Both *mig-14* and *dpy-23* show the characteristic initial response differences observed in both *vps-35* and *rme-8*. However *mig-14* uniquely demonstrates a faster rate of habituation for reversal probability. These results support the hypothesis that *dpy-23* and *mig-14* are working in the same pathway as *vps-35* to affect habituation. However, the other habituation characteristics could be due to some of their other functions. *mig-14* is the homolog of *Wntless*, and is therefore responsible for many developmental processes as well as regulating the cell on a more large-scale level. Since *mig-14* is regulated by the retromer complex, the overlapping phenotypes make sense (Coudreuse et al.,

2006). *dpy-23* is much closer to *vps-35* in its phenotype, which makes sense since it is necessary for receptor mediated endocytosis. This new information about these genes can hopefully serve as new avenues of research, focused on their involvement in the dopamine system and how they could interact with the PD associated genes.

Chapter 5: Conclusion

5.1 Full behavioral characterization of PD associated genes

This set of experiments has behaviourally characterized the homologs of genes that have been associated with PD in the model organism *C. elegans*. The basal measures, such as body size, speed, and spontaneous reversals have given us information about the importance of a particular gene on a whole organism level. On the other hand, habituation experiments have given us insights into how a gene affects the animals' ability to learn. This will help us generate additional questions that can be explored to fully understand how these genes effect both movement and cognitive function.

5.1.2 Focusing in on the retromer complex

The initial characterization of the PD associated genes made it clear that the next avenue that needed to be explored would be in regards to the neurotransmitter systems that could be altered in these mutant worms. By testing the PD strains on and off of food, we were able to ascertain which of the mutations led to altered dopamine signaling. This further pointed to mutations in the retromer complex, specifically *vps-35*, being consistent with lower than normal dopamine signaling. However, further work will need to be done in order to see if other neurotransmitter systems are affected by mutations in *vps-35*. By creating strains that both overexpressed and rescued *vps-35*, we are able gain even more information about how this gene is effecting habituation. Testing *mig-14* and *dpy-2* provided an interesting snapshot on some of the downstream effects this circuit can have on habituation when proteins that have several roles are mutated.

It is this type of information that gives new insight into complicated systems and provides new avenues to explore in other model systems. By using a model system such as *C. elegans*, it

is possible to rapidly and efficiently answer questions that would be impossible to answer in any other way. The high-throughput approach of studying habituation with the MWT, allowed us to test a group of genes and make connections between various phenotypes. This allows us to better understand how proteins are working together to give a behavioral output. Hopefully, this new information about the role of *vps-35* in dopamine signaling will be of assistance in piecing together what role an abnormal retromer complex plays in producing PD.

5.2 Future Directions

5.2.1 Imaging (DA, *snb-1*, *glr-1*) to determine differences in expression

Since *C. elegans* are both translucent and microscopic, they can very easily be imaged using confocal microscopy. Transgenic worms that have been engineered to express fluorescent proteins can be imaged using this technique, looking for differences in the amount of protein and how it is expressed between different strains. Using this technology, we plan to create transgenic worms in which our mutant strains express markers for proteins that are of particular interest. For example, the DA system is of particular interest for PD, with our results showing that *vps-35* has decreased dopamine signaling. Crossing *vps-35* with *dat-1*::GFP would allow us to see the morphology of the dopamine neurons and could provide us with important information about changes taking place in the DA system as a result of this mutation. *C. elegans* possess eight dopaminergic neurons, all of which are mechanosensory and are thought to be crucial for sensing changes in the texture of their environment, such as those produced by food (Chase & Koelle, 2007; Sawin et al, 2000). Studies have also shown that *vps-35* is involved in the recycling of the *C. elegans* AMPA-type glutamate receptor subunit *glr-1*. Crossing the *vps-35* strains and the other PD mutants in with the *glr-1*::GFP would allow us to look at changes that are happening in the recycling system and how they affect localization and density of receptors at the synapse.

Finally, synaptobrevin (*snb-1*) is a protein involved in vesicle release that has also been shown to play an important role in DA release. Crossing a *pdat-1*:: *snb-1*::GFP strain with our PD mutant to image the presynaptic terminals of the DA neurons would give us interesting information about whether the presynaptic terminals are normal. By using fluorescent confocal microscopy and closely examining morphology of the cells and the pre- and post-synaptic terminals, we hope to piece together an understanding of some of the changes that take place in the neurons due to these PD related mutations that affect the worms behavior.

5.2.2 Expression of human forms of PD Genes

One of the advantages to using *C. elegans* for research is their easily manipulated genome. As has been previously described, gene expression can be changed in the worm by injection of a plasmid, creating progeny that express the desired form of a gene. This means that constructs containing the human form of a gene can also be expressed in *C. elegans*. Using this technique, forms of the genes with known mutations or SNPs that have been linked to PD can be injected into the worm and used to discover new information about how that particular mutation changes gene function. Testing these new transgenic animals using the same battery of tests can give us clues about how the protein is functioning and pose additional questions that need to be answered.

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