Genetics in Invertebrates: Modeling Dopaminergic Signaling and Neurodegeneration

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Dopamine (DA) is an important modulatory neurotransmitter, mediating complex human processes such as arousal, learning, reward and motor control; those same behaviors that go awry in neural disorders such as attention-deficit-hyperactivity disorder(ADHD), bipolar disorder, schizophrenia and Parkinson's Disease (PD). Owing to the anatomical and genetic complexity of vertebrate model systems, the invertebrate model systems *Caenhorhabditis elegans* and *Drosophila melanogaster* are ideal systems to study genetic contributions within DA networks and, in the case of flies, genes that contribute to or suppress DA neuron degeneration. In worms, the use of forward and reverse genetics has revealed how a modest dopaminergic nervous system can regulate locomotion, and how a simple locomotory phenotype can be employed to identify novel regulatory genes and their functions within its DA network. Continued genetic study of these networks may reveal novel genes involved in regulating DA biosynthesis, release, uptake and signaling. Many genes have been identified in familial-associated PD (FAPD), and these candidate genes have been used in flies to study neurodegeneration. This review will describe how flies have been employed to dissect the function of three FAPD genes: α-synuclein, parkin, and PINK1. In the case of parkin and PINK1, reverse genetic approaches in the fly have revealed the importance of mitochondrial dynamics to DA neuron susceptibility in PD. The impact of these findings may define a true departure from classical forward and reverse genetics in invertebrates, and that a clearer understanding of DA neural networks will be revealed through the mutual employment of both with convergent genetics.

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INTRODUCTION

From worms to humans, dopamine(DA) is one of the most important neurotransmitters in regulating complex definitive behaviors. In humans, DA's actions extend to processes like arousal, learning, motor control and reward, the defects of which are evident in disorders such as ADHD, PD, bipolar disorder, and schizophrenia. Our understanding of these processes and disease states has advanced much in recent years, but our understanding of the molecular circuitry that regulate dopaminergic pathways is still incomplete. To uncover the proteins that regulate key processes such as DA biosynthesis, release, reuptake and signaling, a combination of genetic approaches must be taken to: (1) enrich our functional understanding of known genes, (2) discover novel genes, or (3) link existing genes to dopaminergic signaling processes. While still possessing the unbiased nature of classical forward genetics, forward genetics today is often paired with candidate-gene approaches, the use of which creates the opportunity to model dopaminergic states and determine, through mutagenesis, what novel genes can mimic or suppress these states. Conversely, the exclusive use of reverse genetics is, by definition, limited to the study of genes that have already been implicated in DA signaling. Drawing from both classical forward and reverse genetic approaches, "convergent genetics" may be the best way to appreciate the sophisticated gene networks that regulate DA signaling. What follows is an overview of efforts in invertebrate model systems that have augmented our understanding of dopaminergic circuitry in *C. elegans* and the functional contributions of three FAPD genes: α-synuclein, parkin, and PINK1 to dopaminergic diseases like PD using *Drosophila*.

Due to the ease of genetic manipulations, a complete genome sequence, simple behaviors, and a growing list of powerful experimental techniques, *C. elegans* is an effective system with which to study the regulation of a network of DA-releasing and responsive cells. *C. elegans* have a 100 megabase (Mb) genome that consists of five pairs of autosomal chromosomes and a pair of X chromosomes in the case of self-fertilizing hermaphrodites (XO in the rare males)[1, 2]. The transparent worm has a very precise and reproducible cell lineage program, thus, the origin and development of the cellular DA network can be traced and visualized from embryo to adult[3-6]. In addition, serial electron microscope reconstructions of the entire worm have revealed the distinct

morphology, location, electrical and synaptic connections of all neurons[7]. Therefore, the role of DA can be studied within multi-neuronal circuits and its influence mapped to specific cells without reservation about unknown connections extant in vertebrates. Moreover, genetic manipulations of *C. elegans* can reveal the cell autonomous or non-autonomous function of one gene within these simple, multi-neuronal circuits.

A forward genetic approach in the worm is not new, but its application, combined with the development of *in vivo* electrophysiology, continues to reveal genes important for neuronal function, especially those essential for synaptic transmission. Erik Jorgensen and colleagues have characterized previously identified uncoordinated(*unc*) mutants as important components of synaptic vesicle docking, priming, and fusion[8-12]. The same group has also identified the function and localization of several genes in glutamatergic and GABAergic neurons within the worm[13-15]. The functional conservation of these synaptic genes in mammals demonstrates that the use of *C. elegans* is a valid approach to identify novel dopaminergic genes *in vivo*.

The study of DA signaling in *C. elegans* began in 1975 with the work of Nobel Laureate John Sulston. Using Nomarski microscopy and a technique known as formaldehyde-induced fluorescence(FIF)[16], Sulston identified all of the dopaminergic neurons in the hermaphrodite and male worms[17]. The hermaphrodite possesses eight dopaminergic neurons consisting of two pairs of cephalic (CEP) neurons, a single pair of anterior deirid neurons (ADE), and one pair of posterior deirid (PDE) neurons[17, 18]. Sulston also noted the presence of three additional pairs within the sexually dimorphic male tail in sensory rays five, seven + nine, the loss of which were later shown to cause defects in male mating coordination[17, 19]. Through the use of transgenic DA specific reporters, these neurons have been shown to specifically express genes associated with the biosynthesis of DA such as tyrosine hydroxylase (cat-2) and its uptake via the presynaptic DA transporter (dat-1)[20, 21].

After identifying these neurons, Sulston then performed a small forward genetic screen isolating mutants that showed a decrease in FIF, mutants that he deemed *cat* or catecholamine deficient. In his screen, he isolated five mutants (cat-(1-5)) that showed a reduction or complete loss of FIF, and mapped the mutations to chromosomal intervals[17]. Despite the modesty of his screen, Sulston identified three important genes involved in the maintenance of proper DA signaling in the worm. The cloning and sequencing of the cat-1 locus demonstrated that it encodes the reserpine sensitive *C. elegans* vesicular monoamine transporter (vMAT), responsible for the packaging of biogenic amines, including DA and serotonin (5-HT), into synaptic vesicles[22]. Cat-4 encodes GTP cyclohydrolase I, a necessary cofactor that participates with the aforementioned cat-2 in the biosynthesis of biogenic amines, including DA[23]. The importance of this early work cannot be overstated, as later investigators have used these mutants to study behaviors altered by hypodopaminergic and hyposerotenergic states[23], but techniques mimicking a hyperdopaminergic state in the worm have also borne fruit.

The use of exogenous DA, in combination with genetic approaches, has revealed important components of the dopaminergic network and how they function together to produce distinct behaviors in the worm, most notably locomotion. In a landmark study by Schafer and Kenyon, it was found that wild-type worms paralyze when plated on exogenous DA or 5-HT[24]. After a four-hour period, these same worms will adapt and begin to move normally, but worms lacking a functional voltage-sensitive calcium channel subunit unc-2 fail to adapt to exogenous DA and remain paralyzed. DA regulation of worm locomotion is also evident in a behavior known as the basal slowing response, a behavior where well-fed wild type worms reduce their locomotory rate upon entering a lawn of bacteria[23]. It is believed that DA, by slowing locomotion in the presence of food, maximizes the opportunity for feeding and the continued survival of the worm – as DA also modulates food search[25]. Worms lacking DA due to a mutation in cat-2 do not slow in response to food, and a later study demonstrated that worms lacking a particular DA receptor dop-3 also fail to reduce their locomotory rate[26]. With a repertoire of dopaminergic locomotory phenotypes in hand, more detailed genetic studies of the dopaminergic network were possible.

The observation that *C. elegans* will paralyze when plated on exogenous DA offered a simple, robust phenotype for researchers to exploit using both forward and reverse genetic approaches[24, 27]. Using bioinformatics, Chase *et al.* identified a new DA receptor dop-3, characterized the locomotive behaviors of worms lacking dop-3 and other known receptors, and found that dop-3 and the antagonistic actions of dop-1, mediate DA's effects on locomotion. Worms lacking dop-3 fail to demonstrate the basal slowing response and do not paralyze on exogenous DA, but both behaviors are normal in worms lacking both receptors dop-1 and dop-3. They determined that these receptors are coexpressed within groups of cholinergic and GABAergic motor neurons along the ventral cord of the animal, acting antagonistically in the same cells to either promote or inhibit locomotion. Further experiments have demonstrated that the cholinergic neurons are the most important for DA's modulation of locomotion (Daniel Chase, personal communication). Expressing fluorescently tagged

Nomarski Microscopy
A microscopy technique that enhances contrast in an unstained, transparent sample.

Formaldehyde-induced Fluorescence
The formation of fluorescent isoquinolines upon incubation of catecholamines with gaseous formaldehyde.

dop-1 and dop-3 in these neurons has shown that dop-3 is expressed diffusely along the plasma membrane in these neurons, but dop-1 is localized at the neuromuscular junctions of these neurons, expression data that supports the hypothesis that dop-1 promotes locomotion through activation of muscle and that paralysis can occur with an excess of extrasynaptic DA that hyperactivates dop-3 (Daniel Chase, personal communication). Following description of the receptors that mediate this phenomenon, they used forward genetics to look for mutants that phenocopy the exogenous DA-resistant strain *dop-3(vs106)*. They isolated nine mutants in four genomic loci, and, using these mutants and candidate based analysis they determined that dop-1 is acting through the *C. elegans* $G\alpha_q$ protein egl-30 and dop-3 is acting through the $G\alpha_o$ protein goa-1, and mutants that decrease goa-1 or increase egl-30 signaling increase exogenous DA resistance. Having elegantly described the postsynaptic actions with a convergent genetic approach, study of the presynaptic machinery has capitalized on these findings.

The aforementioned presynaptic dopaminergic neurons express components that regulate the temporal and spatial actions of DA, most importantly, the presynaptic DA transporter (DAT) or dat-1 in *C. elegans.* The study of dat-1 in *C. elegans* began with the cloning of an antidepressant and cocaine-sensitive cDNA that exhibits DA-specific uptake and encodes a protein with 47% and 43% homology to the human norepinephrine transporter (hNET) and hDAT respectively[28]. It is expressed specifically in DA neurons and will mediate the *in vivo* transport of neurotoxins such as 6-OHDA, while worms lacking a functional dat-1 are resistant to these effects[21, 29]. Dat-1 is also important in controlling DA's influence on locomotion, but, unlike the use of exogenous DA to identify DA receptors, its influence is most apparent in regulating endogenous DA levels. When placed in a small volume of water, wild-type worms will increase their locomotory rate and thrash at a sustained level for 20-25 min., but worms lacking a functional DA transporter (*dat-1(ok157))* will swim at first and paralyze within 10 minutes[30]. This phenotype, known as swimming induced paralysis, or SWIP, is presynaptically rescued by pretreatment with the vMAT inhibitor reserpine or knockout of cat-1 or cat-2, and postsynaptically by loss of dop-3. In addition, the SWIP phenotype can be induced by the application of tricyclic anti-depressants and amphetamine, which may help reveal genes important for drug action (unpublished observations and Carvelli *et al.*, in press). This robust phenotype is now being used in forward and candidate based screens to look for novel presynaptic regulators of dat-1, DA release, and postsynaptic components in motor neurons (Hardie *et al.*, in press and Daniel Chase, personal communication). The continued application of forward genetics with a robust phenotype will yield important functional insights into presynaptic genes involved in DA release and uptake, but the application of reverse genetics in invertebrate systems enables the study of one gene of interest in DA neurons.

Beginning in the mid 1990's, several genes were identified in pedigrees of FAPD, but their role in mediating DA neuron susceptibility is still unclear. One of the pathological hallmarks of PD is the progressive age-dependent loss of dopaminergic neurons in the substantia nigra, but animal models were required to study genetic contributions to this neurodegenerative process. To that end, researchers employed invertebrate and vertebrate model systems to study these candidate genes. Although this strategy has recently been used in *C. elegans* (rev. in [31, 32]), the fruit fly, *Drosophila melanogaster*, is the most common invertebrate model for modeling PD and using genetics to understand the susceptibility of dopaminergic neurons.

With its history of use dating back to Charles Woodworth and Thomas Hunt Morgan in the early 1900s, *Drosop*hila may be the most well studied and understood of all genetic model systems. Currently, its power as a tool to study neurodegeneration stems from our understanding of the organism's genome. The fly has a fully sequenced genome of 180 Mb that is contained on three pairs of autosomes and a pair of X/Y chromosomes and is predicted to encode ~13,601 genes[33]. During development some cells replicate their DNA without undergoing cell division or separation of sister chromatids, forming giant polytene chromosomes that can be seen under a microscope and have a characteristic and reproducible black and white banding pattern, which has facilitated the mapping of mutations in mutagenesis screens[33]. *Drosophila* also possess practical advantages of other invertebrates, which are short generation times, large population sizes, and a simple and consistent nervous system. The adult fly is thought to contain around 300,000 neurons, about 10^3 fold greater than the worm, so much effort has been expended to characterize dopaminergic neural populations in the fly throughout development[34]. Earlier work used immunoreactivity to dopaminergic components such as TH or dopa decarboxylase(Ddc, a.k.a – aromatic acid decarboxylase) to identify these cells, and more recent work has used the Gal4/UAS system to specifically identify cells that express these genes using fluorescent reporters[35, 36]. In studying the adult fly brain, these studies identified six pairs of clusters of dopaminergic neurons in and around the protocerebrum of the fly and a ventral medial pair[35-38]. In addition to behavioral and survival analysis, the loss of these protocerebral dopaminergic clusters has been the primary measure of PD models in the fly.

Following the linkage of the chromosomal locus 4q21-q23 in a large Italian kindred with autosomal dominant PD[39], and its subsequent identification as α-synuclein (PARK1)[40]; many researchers sought to create animal models of PD to study α-synuclein's role in pathogenesis. Using the Gal4/UAS system in flies, Feany and Bender overexpressed human WT α-synuclein and FAPD alleles (A30P and A53T) pan-neuronally and in dopaminergic neurons[41]. In all cases, they saw an age dependent loss of dopaminergic neurons, restricted involvement to the nervous system, and the formation of filamentous inclusions reminiscent of Lewy Bodies (LBs), one of the pathological hallmarks of PD. Furthermore, the overexpression of these genes in flies caused an age-dependent decline in climbing ability, which parallels motor symptoms such as bradykinesia in patients with PD. The same group reported that the loss of DA neurons was attenuated by the overexpression of the chaperone Hsp70[42] or the pharmacological activation of chaperones[43], and also showed that chaperones localize to LBs of post-mortem PD patient samples. The post-translational modification of α-synuclein may also be important, as mutant flies overexpressing phosphorylation-null (S129A) α-synuclein do not show DA neuron degeneration and, surprisingly, show increased inclusion formation. Currently a subject of considerable debate, these results are consistent with the hypothesis that inclusions may be a neuroprotective mechanism.

Now equipped with a model that recapitulates the core pathology and behavior of the disease, many investigators sought to study the genetic influences that might suppress neurodegeneration in a model of PD. Genetic studies of FAPD have identified several genes that produce autosomal recessive juvenile parkinsonism (ARJP)[44-46], but it is the candidate gene approach in the fly that has revealed much about the function of two ARJP genes PTEN induced kinase 1 or PINK1(PARK6) and parkin(PARK2) *in vivo* and how they may regulate the susceptibility of dopaminergic neurons.

Through study of its function in *Drosophila*, we now better understand the function of PINK1 in dopaminergic neurons. PINK1 was first identified in a large Sicilian kindred with four family members afflicted by ARJP[44], who were later found to have nonsense or missense loss of-function single nucleotide polymorphisms (SNPs) in the coding region of PINK1[47]. In 2006, three groups published studies characterizing the neurodegenerative phenotypes of flies with deletions in dPINK1, and found very similar phenotypes[48-50]. These mutant strains showed decreased longevity, male sterility, and mitochondrial defects in energy demanding regions of the fly such as the male testis, thorax, and flight muscle. Such defects included vacuolarization of the mitochondria, disorganization, and a decrease in total ATP levels, suggesting that PINK1 has a role in mitochondrial maintenance. In both studies, these defects could be suppressed by the overexpression of parkin, which is consistent with an epistatic role of parkin to PINK1.

Parkin was first identified in a large deletion in a Japanese patient with ARJP, and was mapped to the long arm of chromosome 6(6q25.2-q27)[45]. The cloning and sequencing of this gene revealed an N-terminal ubiquitin-like domain, a motif similar to a RING-finger at its C-terminus, and would later be identified as a cytoplasmic E3 ubiquitin ligase[5,1]. Consistent with its role in ARJP and as a neurodegenerative suppressor, the knockdown of parkin in flies (*parkin*) could augment PaeI-R-mediated loss of dopaminergic neurons, and, conversely, its overexpression could protect against both PaeI-R and α-synuclein-mediated DA neurodegeneration[52]. A second group showed that a complete loss-of-function resulted in a reduced lifespan, locomotor defects and male sterility; the underlying basis for which was shown to be mitochondrial dysfunction[53]. Using reverse genetic approaches in an invertebrate system, these studies, and those of PINK1, have shown that mitochondrial dysfunction may underlie susceptibility of DA neurons in PD.

Mitochondria are highly dynamic organelles, and the importance of PINK1 and parkin in regulating these dynamics may be a pivotal process in maintaining neural integrity and preventing neurodegeneration (rev in. [54]). Genetic interactions have demonstrated that PINK1/parkin acts in a linear pathway in mitochondrial fission, and that mitochondrial defects and behavioral phenotypes evident in these mutants are rescued by reduced gene dosage of proteins that promote mitochondrial fusion[55]. A similar study confirmed the same phenomenon concerning these fission/fusion regulatory proteins, and, using mitoGFP, demonstrated that the loss of PINK1 causes the formation of mitochondrial aggregates and tubules not seen in WT[56]. These data suggest that mitochondrial fission is somehow neuroprotective in *Drosophila* DA neurons, or, alternatively, that neurodegeneration in the case of flies and patients with ARJP results from the inability of these neurons to adapt to their dynamic energetic needs through either fission or fusion. For example, it has been shown that mitochondrial fusion protects against neurodegeneration in the cerebellum, so the neuroprotective dynamics of mitochondria may not be so one-sided[57]. In addition, a recent study using SH-SY5Y cells showed that mitochondrial morphological defects and a decrease in ATP production upon loss of function of parkin or PINK1 were rescued by the

Lewy Bodies
Proteinaceous neural aggregates that typify PD patients, but are also seen in other neurological disorders.

Bradykinesia
Slowness in the execution of movement, one of the primary motor symptoms of PD.

E3 ubiquitin ligase
Enzyme that pairs with an E2 ubiquitin-conjugating enzyme to attach ubiquitin moieties to particular substrate lysine residues.

Mitochondrial fission
Division of mitochondria into two smaller parts.

Mitochondrial fusion
The combination of two mitochondria into one that occurs at the tips or sides of the mitochondria.

mitoGFP
Green fluorescent protein (GFP) fused to a mitochondrial localization sequence, effectively labeling only mitochondria with GFP.

overexpression of proteins that promote mitochondrial fusion, Mfn2 and Opa1, or the expression of a dominant negative Drp1, which inhibits fission[58]. The precise roles of PINK1/parkin in the fission machinery are unknown, so the continued study of these genes in invertebrates may help refine their function in regulating mitochondrial dynamics.

In summary, the use of convergent genetics in invertebrate model systems has revealed much about dopaminergic signaling components in *C. elegans* and DA neurodegenerative susceptibility genes in *Drosophila*. The ease of genetic manipulations, transgenic expression and knockdown in these systems make them a simple, tractable system to study candidate genes. Furthermore, genomic annotation, conserved anatomical phenotypes, and simple behaviors in these two systems create the opportunity to use forward genetics to identify novel components of dopaminergic networks or proteins that may suppress or induce neurodegeneration in these systems.

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FURTHER INFORMATION

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