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Acute blockade of the *Caenorhabditis elegans* dopamine transporter DAT-1 by the mammalian norepinephrine transporter inhibitor nisoxetine reveals the influence of genetic modifications of dopamine signaling *in vivo*

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ABSTRACT

Modulation of neurotransmission by the catecholamine dopamine (DA) is conserved across phylogeny. In the nematode *Caenorhabditis elegans*, excess DA signaling triggers Swimming-Induced Paralysis (Swip), a phenotype first described in animals with loss of function mutations in the presynaptic DA transporter (*dat-1*). Swip has proven to be a phenotype suitable for the identification of novel *dat-1* mutations as well as the identification of novel genes that impact DA signaling. Pharmacological manipulations can also induce Swip, though the reagents employed to date lack specificity and potency, limiting their use in evaluation of *dat-1* expression and function. Our lab previously established the mammalian norepinephrine transporter (NET) inhibitor nisoxetine to be a potent antagonist of DA uptake conferred by DAT-1 following heterologous expression. Here we demonstrate the ability of low (μM) concentrations of nisoxetine to trigger Swip within minutes of incubation, with paralysis dependent on DA release and signaling, and non-additive with Swip triggered by *dat-1* deletion. Using nisoxetine in combination with genetic mutations that impact DA release, we further demonstrate the utility of the drug for demonstrating contributions of presynaptic DA receptors and ion channels to Swip. Together, these findings reveal nisoxetine as a powerful reagent for monitoring multiple dimensions of DA signaling *in vivo*, thus providing a new resource that can be used to evaluate contributions of *dat-1* and other genes linked to DA signaling without the potential for compensations that attend constitutive genetic mutations.

1. Introduction

The modulation of behavior by the neurotransmitter dopamine (DA) is evolutionarily conserved, evident in animals that range by orders of magnitude in complexity, from humans to the soil-dwelling nematode *Caenorhabditis elegans*. As in humans, DA signaling in *C. elegans* regulates multiple behaviors including locomotion (Chase et al., 2004; Omura et al., 2012; Sawin et al., 2000) and associative learning (Voglis and Tavernarakis, 2008). The conservation of genes encoding proteins that support DA biosynthesis, vesicular packaging, release, and response, makes the worm a powerful tool to elucidate novel mechanisms that regulate DA signaling across phylogeny (McDonald et al., 2006). Particularly useful is the amenability of this organism to rapid genetic manipulation and behavioral characterization. Additionally, pharmacological agents have been utilized successfully to elicit behavioral responses through pathways shared with more complex vertebrates (Choy and Thomas, 1999; Dwyer et al., 2014; Miller et al., 1996; Weinshenker et al., 1995). As in vertebrates, the latter agents offer the opportunity to manipulate chemical signaling at specific time points in development and, when activity is evident with acute exposure, lessen concern for the compensations that arise from constitutive genetic manipulations.

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A powerful example of a rapidly acting drug that has been successfully used in the worm to manipulate a specific chemical signaling pathway is the acetylcholinesterase inhibitor, aldicarb, which has been used extensively to evaluate the capacity for cholinergic signaling (Bany et al., 2003; Iwasaki et al., 1997; Miller et al., 1996; Mullen et al., 2007). Acetylcholine (ACh) is released at the neuromuscular junction in *C. elegans* to trigger muscle contraction. Aldicarb, by blocking the major determinant of extracellular ACh inactivation, acetylcholinesterase (AChE), induces rapid, hypercontracted, motor paralysis due to excessive activation of neuromuscular ACh receptors. Genetic and pharmacological modifiers of ACh signaling, such as proteins that regulate vesicular ACh release, can be studied via their ability to enhance or suppress aldicarb-induced paralysis (Jorgensen et al., 1995; Nonet et al., 1997). This enhancement or suppression can be used to determine how a particular genetic mutation or drug might alter ACh signaling, even if there is no obvious phenotype in the absence of drug. Dozens of genes have been identified or studied based on the presence of a Ric or Hic (**R**esistance or **H**ypersensitivity to **I**nhibitors of A**C**hE) phenotype, and include genes that act both pre- and postsynaptically (For review, see (Rand, 2007)).

To date, examples of potent and selective agents that act like aldicarb at non-cholinergic synapses are limited, in part due to the high concentrations (typically mM) needed for many substances to afford penetration through the worm cuticle. Additionally, inactivation of small molecule neurotransmitters besides ACh is determined by transporter-mediated clearance, and many mammalian transporter antagonists lose potency as inhibitors of their *C. elegans* orthologs (Jayanthi et al., 1998; Ranganathan et al., 2001). Thus, the *C. elegans* DA transporter (DAT-1) is one to two orders of magnitude less sensitive *in vitro* to the mammalian DAT inhibitors GBR12909 and nomifensine, respectively (Jayanthi et al., 1998). Interestingly, the mammalian norepinephrine (NE) transporter (NET)-specific antagonist nisoxetine (NIS) exhibits low nanomolar potency for inhibition of DAT-1 mediated DA uptake (Jayanthi et al., 1998). Since the worm lacks a NET ortholog (worms do not make NE), we reasoned that NIS might prove a potent and selective antagonist of DAT-1 *in vivo*.

Here, we describe our efforts to demonstrate the utility of NIS as a potent pharmacological modulator of DA signaling-dependent behavior in the worm. The behavior monitored in our studies, Swimming-Induced Paralysis (Swip), was first described in worms bearing a *dat-1* deletion (McDonald et al., 2007). We demonstrated that loss of DAT-1 expression leads to premature paralysis when worms are placed in water, with paralysis emerging in a few minutes vs. the stable (>10 min) swimming evident in wild-type (N2) worms. Swip produced by *dat-1* mutation can be rescued by mutation of DA biosynthetic and vesicular packaging genes (*cat-2* and *cat-1* respectively). Additionally, Swip in dat-1 animals is lost in a cross to animals with a loss of function mutation in the gene encoding the D2 receptor *dop-3* (McDonald et al., 2007). These and other findings support the use of Swip as a behavioral readout of hyperdopaminergia. In this report, we demonstrate that Swip can be induced rapidly by incubation of worms with low concentrations of NIS, that Swip induction by NIS is DA-dependent, and that we can positively and negatively modify NIS-induced Swip by genetic manipulation of presynaptic regulators of DA release. We discuss the potential for NIS to serve as a useful tool for the identification and characterization of novel and potentially conserved regulators of DA signaling.

2. Materials and methods

2.1. C. elegans strains

Strains were maintained at 15–20 °C using standard methods as described previously (Brenner, 1974). N2 (Bristol) served as our wild-type strain. Additional strains used in this work as follows: *dat-1(ok157); dop-2(vs105); asic-1(ok415); cat-2(e1112); dop-3(vs106); dat-1(ok157), dop-2(vs105); dat-1(ok157), dop-2(vs105) vtEx58 (Pdat-1::dop-2).*

dat-1(ok157), dop-2(vs105) vtEx58 (Pdat-1::dop-2) transgenic animals were generated by microinjection of *dat-1(ok157); dop-2(vs106)* worms with *pdat-1::dop-2* transgene and co-injection markers *punc-122::GFP* and *pdat-1::mCherry*. Stable transgenic line was selected based on presence and high transmission of *punc-122::GFP* fluorescence, and *pdat-1::mCherry* fluorescence was used to pick animals for behavioral experiments.

2.2. Plasmid construction

The *pdat-1::dop-2* rescue construct was generated in the backbone pRB1106 which contains the *dat-1* promoter and unique restriction enzymes for subcloning of open reading frames (ORFs). *dop-2S* cDNA was amplified from *ceh-17::dop-2S* plasmid (provided by Satoshi Suo, University of Tokyo, Tokyo, Japan) with AscI and KpnI engineered into the 5′ and 3′ ends, respectively. This PCR fragment was digested and subcloned into digested backbone. Successful cloning was verified by sequencing of the plasmid prior to microinjection.

2.3. Swip assays

Swip assays were performed as previously described (Hardaway et al., 2012; McDonald et al., 2007). Synchronous L4 worms were generated by hypochlorite treatment of adult animals, and plating of synchronized L1 animals. All manual assays were carried out by picking 10 L4 animals into a well of 100 μL of water plus or minus nisoxetine hydrochloride (Sigma–Aldrich, St. Louis, MO) or methylphenidate (Sigma–Aldrich, St. Louis, MO) and scoring the number of animals paralyzed after 10 min. For osmosuppression studies, solutions of 100, 200, and 300 mOsm were generated with sucrose. Eight wells were scored per genotype/treatment, and were repeated on at least 3 separate days with 1–2 experimenters per day for an N = 24–48 per condition. Experimenters were blind to genotype and/or drug. Automated analyses were performed using 10 min videos of individual worms captured from at least 25 worms per genotype/treatment using Tracker software and analyzed using SwimR software as previously described (Hardaway et al., 2015, 2014). Latency to paralysis was measured as the time until the thrashing frequency for each animal dropped below 20% of the maximum thrashing value for at least 20 s. Probability of reversion to swimming was measured as the percent of paralyzed animals that displayed bouts of thrashing above 20% of the maximum thrashing value after a bout of paralysis (defined as above).

For *dop-2* rescue experiments, worms expressing the *pdat-1::dop-2* transgene were selected based on the presence of the co-injection marker *pdat-1::mCherry* and were picked as L2-L3 animals the day before video acquisition.

2.4. Graphical and statistical methods

Data was analyzed and graphed using either SwimR software (described above), or using Prism 6.0 (GraphPad, Inc., La Jolla, CA). All statistical analyses and curve fits were performed in Prism 6.0. Descriptions of all statistical tests are noted in the figure legends.

3. Results

Wild-type worms placed in water supplemented with increasing concentrations of NIS demonstrated a dose-dependent