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Research Paper

A rapid chemical-genetic screen utilizing impaired movement phenotypes in *C. elegans*: Input into genetics of neurodevelopmental disorders

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ABSTRACT

Autism spectrum disorder (ASD) is the most common neurodevelopmental disorder with a constantly increasing prevalence. Model organisms may be tools to identify underlying cellular and molecular mechanisms, as well as aid the discovery and development of novel therapeutic approaches. A simple animal such as the nematode *Caenorhabditis elegans* may provide insights into the extreme complexity of ASD genetics. Despite its potential, using *C. elegans* in ASD research is a controversial approach and has not yet been used extensively in this context. In this study, we present a screening approach of potential *C. elegans* mutants as potential ASD models. We screened these mutants for motor-deficiency phenotypes, which can be exploited to study underlying mechanisms of the disorder. Selected motor-deficient mutants were then used in a comprehensive drug screen of over 3900 compounds, including many FDA-approved and natural molecules, that were analyzed for their ability to suppress motility defects caused by ASD-associated gene orthologues. This genetic-chemical approach, *i.e.* establishing *C. elegans* models for ASD and screening of a well-characterized compound library, might be a promising first step to understand the mechanisms of how gene variations cause neuronal dysfunction, leading to ASD and other neurological disorders. Positively acting compounds could also be promising candidates for preclinical studies.

1. Introduction

Neurodevelopmental disorders (NDD) are a clinically heterogeneous group that describes heritable psychiatric illnesses caused by aberrant brain development and growth (APA, 2013). They comprise cognitive, motor, language and affective disabilities and include autism spectrum disorder (ASD), intellectual disabilities, communication disorders, attention deficit/hyperactivity disorders (ADHD), specific learning disorder, as well as motor disorders (APA, 2013). Disordered development affects neurogenesis, glia/neuronal proliferation/migration, synapse formation and myelination (Ding, 2015; Hu et al., 2014), leading to physiological and behavioral deficits in both children and adults. NDD overlap with other brain disorders and co-occurrence is common (for instance, ADHD goes often along with specific learning disorder, or ASD with intellectual disabilities), which forms a complex net of neuropsychiatric comorbidities (Dalsgaard et al., 2013; Lala and Sajatovic, 2012). NDD remain hard to treat, since no specific and effective treatments like pharmacological therapies that successfully treat core NDD deficits are available (Dalsgaard et al., 2013; Elder et al., 2016).

ASD has the highest prevalence under all NDD with 1.47% (1 in 68) of US children aged 8 years affected in 2014 (Network, 2014), and with the constantly rising numbers they have a huge socio-economic impact (Dykens, 2015). ASD was previously grouped into autistic disorder, Asperger syndrome and not otherwise specified pervasive developmental disorders, but the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5, 2013) suggests that all patients should be diagnosed with ASD, relinquishing sub-classification (APA, 2013). ASD is characterized by abnormalities in brain function and morphology, ultimately resulting in abnormal social behavior, difficulties in

Abbreviations: ASD, autism spectrum disorder; NDD, neurodevelopmental disorders; ADHD, attention deficit hyperactivity disorders; WT, wildtype; SNPs, single nucleotide polymorphism; SFARI, Simons Foundation Autism Research Initiative; GABA, γ-aminobutyric acid; RNAi, RNA interference; DMSO, dimethyl sulfoxide; NGM, Nematode growth medium; ROS, Reactive oxygen species; Nrf2, Nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; ANK2, ankyrin B; NMDA, N-methyl-d-aspartate; MAO-A, Monoamine oxidase A; CACNA1A, Voltage-dependent calcium channel, P/Q type, alpha-1A subunit; CACNA1C, Voltage-dependent calcium channel, P/Q type, alpha-1C subunit.

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communication, stereotypies and repetitive behavior, seizures, obsession with routines, and delays in cognitive development (Gadke et al., 2016; Grzadzinski et al., 2016). As the name indicates, individual ASD cases range on a spectrum and symptoms can vary extremely, such as cognitive function that reaches throughout the spectrum from high functioning patients to severely neurodevelopmentally disabled individuals (Postorino et al., 2016). There is also a substantial phenotypic variation for social communication and interaction in the healthy population, which complicates diagnoses and the search for the causes.

Underlying ASD are most likely a variety of causes and evident points towards mutations in multiple genes, epigenetics, and environmental factors (Kubota and Mochizuki, 2016; Robinson et al., 2016; Sealey et al., 2016). Screenings of ASD patients' genomes have not revealed a common genetic background, indicating that the genetics are complex and remain scarcely understood. Almost all genetic risk factors for ASD can be found in unaffected individuals, and the genetic relationship between neuropsychiatric disorders and typical social and behavioral variation remains unclear (Hanson et al., 2015). It is likely that mutations in multiple genes responsible for neurodevelopment, organization and connection of neurons and synapses are important contributors (Gaugler et al., 2014; Robinson et al., 2016). It has been reported that common genotyped SNPs are a major cause in at least 20% of all ASD cases (Bulik-Sullivan et al., 2015; Cross-Disorder Group of the Psychiatric Genomics, 2013). *De novo* variations are responsible for <5% of the overall liability to ASD, but are still found in 10–20% of cases (Gaugler et al., 2014; Iossifov et al., 2014). Studies have shown that there is an increased risk among first-degree family members of patients to be diagnosed with ASD, which is higher in monozygotic twins, demonstrating that ASD cases are partially inherited (Hallmayer et al., 2011; Lundstrom et al., 2012). An evidence-based database and risk-scoring estimation on all ASD associated genes known hitherto is provided by the Simons Foundation Autism Research Initiative (SFARI; http://sfari.org).

Translational research to develop novel therapeutics in the management of ASD and other NDD is strongly required. One research focus hereby lies in the development of novel preclinical models that could help identify biological targets and biomarkers to gain mechanistic insights into ASD and possible therapeutic approaches.

Model organisms have made major contributions to biomedical science and are essential models for studying the neurobiology of human brain disorders, including ASD. Given the complex genetics in ASD, not to mention the complexity of the human brain in general, model organisms can help provide insights into the underlying network of genetic variations, epigenetic effects, and molecular mechanisms resulting in behavioral changes.

The nematode *C. elegans* was introduced in 1974 as a model for investigating the development and function of the nervous system (Brenner, 1974). Due to its short lifespan, easy handling, and high number of conserved genes, *C. elegans* has become a popular animal model to investigate a wide variety of biological topics (Kennedy, 2008). These animals are especially well suited for neuroscience research thanks to the comprehensively detailed neuronal lineage and interconnectivity of synapses that resembles very well those of the vertebrate nervous system. The *C. elegans* nervous system contains 302 neurons, divided into 118 neuronal classes, 56 glia cells, and about 7600 synapses (Hobert, 2010). *C. elegans* is transparent, which allows the visualization of neurons *in vivo* and in freely moving animals. The worms are highly amenable to genetic manipulation making it possible to identify genes that are important for neuronal formation, migration, and activity (Vashlishan et al., 2008). Their behavioral plasticity is surprisingly complex and the underlying neuronal circuits are well established (Hobert, 2003). *C. elegans* movement is coordinated by head and ventral nerve cord circuits, where cholinergic motor neurons excite muscles *via* input from acetylcholine on either side of the body, while they indirectly inhibit muscles on the opposite side due to excitation of γ-aminobutyric acid (GABA)ergic motor neurons (Jospin et al., 2009; McIntire et al., 1993). In liquid culture, worms display a stereotypical swimming behavior, with great activity of the neuromuscular junction to maintain functioning of the body wall muscles at high levels.

C. elegans possesses a wide variety of phenotypes that may be used to study ASD and other NDD. Behavioral as well as cellular or molecular phenotypes, for instance the migration and connectivity of synapses or deficits within these as a proposed fundamental event in the etiology of ASD can be examined (reviewed in Bessa et al., 2013). Altered cognition resulting from perturbed synapse development and function has been reported as a hallmark of ASD, and mutations in the genes coding for neurexin, neuroligin, and shank have been shown to be responsible for these events (Hu et al., 2012; Sudhof, 2008). *C. elegans* orthologues of these genes are *nrx-1*, *nlg-1*, and *shn-1*, respectively, and mutants of these genes have proven to be valuable tools to study neurodevelopmental processes (Calahorro, 2014). NRX-1 and NLG-1 are mediators of a retrograde synaptic signal to inhibit neurotransmitter release at the neuromuscular junction (Hu et al., 2012), and loss of NLG-1 leads to a strong reduction of GABA(A)R (positioning type A GABA receptors) levels at GABAergic inhibitory synapses (Tu et al., 2015). Both NRX-1 and NLG-1 stabilize diffusing GABA(A)R and mediate the organization of GABAergic postsynapses (Maro et al., 2015; Tong et al., 2015). *C. elegans* mutants of *nrx-1* and *nlg-1* show deficits in sensory processing, touch response and osmotic avoidance (Calahorro et al., 2009).

In this study, we used *C. elegans* in a comprehensive chemical-genetic screen to identify novel nematode models for the purpose of studying ASD and underlying mechanisms of the disorder. We used the simple approach of monitoring locomotion (swimming behavior) in mutant worm strains for genes that are orthologues of human genes associated with ASD. Mutant strains with motility defects were considered candidates for small molecule screens to restore wild type movement behavior. For the selected mutant strains, we performed comprehensive drug screens where we tested >3900 molecules (many FDA-approved) per screen for their ability to rescue motor phenotypes.

Our chemical-genetic approach links small molecules to gene function for several genes associated with ASD in humans. The positively-acting compounds, especially when classified into functional categories, suggest novel mechanisms underlying ASD and could potentially be used in future preclinical studies in more advanced models.

2. Methods

2.1. Nematode strains and maintenance

Standard conditions were applied for handling *C. elegans* (Stiernagle, 2006). Briefly, worms were kept on NGM agar streaked with *E. coli* OP50 as food source at 15 °C for maintenance and 20 °C for all assays. Mutant strains used for this publication (see Table 1) were provided by the Caenorhabditis Genetics Center at the University of Minnesota and outcrossed four times to N2 wildtype (WT).

The transgenic 'rescue' strain was generated by injecting cosmid T01C3 (15 μg/ml), which contains an open reading frame encoding *nmr-2* (Kano et al., 2008), along with co-injection 'marker' plasmid L4640 (0.5 μg/ml) that expresses YFP under the control of a *myo-2* promoter into the germ line of adult hermaphrodites, as described previously (Mello and Fire, 1995). YPF positive progeny were selected and checked for the presence of T01C3 *via* PCR. Two resulting lines were used in experiments.

2.2. RNAi experiments

RNAi experiments were performed according to (Kamath et al., 2001). RNAi clones (*E. coli* HT115) of *skn-1* and *ced-3* were taken from the ORFeome RNAi library (Open Biosystems) and compared to empty vector (L4440). To confirm correct clones sequencing was performed before use. Worms were grown on RNAi from the egg stage on NGM