Neuroligin-deficient mutants of *C. elegans* have sensory processing deficits and are hypersensitive to oxidative stress and mercury toxicity

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**SUMMARY**

Neuroligins are postsynaptic cell adhesion proteins that bind specifically to presynaptic membrane proteins called neurexins. Mutations in human neuroligin genes are associated with autism spectrum disorders in some families. The nematode *Caenorhabditis elegans* has a single neuroligin gene (*nlg-1*), and approximately a sixth of *C. elegans* neurons, including some sensory neurons, interneurons and a subset of cholinergic motor neurons, express a neuroligin transcriptional reporter. Neuroligin-deficient mutants of *C. elegans* are viable, and they do not appear deficient in any major motor functions. However, neuroligin mutants are defective in a subset of sensory behaviors and sensory processing, and are hypersensitive to oxidative stress and mercury compounds; the behavioral deficits are strikingly similar to traits frequently associated with autism spectrum disorders. Our results suggest a possible link between genetic defects in synapse formation or function, and sensitivity to environmental factors in the development of autism spectrum disorders.

**INTRODUCTION**

Neuroligins are a family of postsynaptic cell adhesion proteins that were originally isolated on the basis of their binding to presynaptic proteins called neurexins (Ichchenko et al., 1995; Ichchenko et al., 1996; Boucard et al., 2005; Chih et al., 2006). Although early studies demonstrated that, under certain conditions, the interaction between neuroligin and neurexin was capable of inducing synaptogenesis (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004), recent studies suggest that neuroligins function primarily in the maturation, stability and/or maintenance of synapses, rather than synaptogenesis per se (Varoqueaux et al., 2006; Südhof, 2008).

There are four neuroligin genes in mammals, and several important studies have shown that mutations in the human genes encoding neuroligin 3 and neuroligin 4 are associated with autism spectrum disorders (ASDs) (Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005). Although subsequent studies have shown that neuroligin mutations are only associated with ASDs in a limited number of family pedigrees (Vincent et al., 2004; Gauthier et al., 2004; Ylisaauko-oja et al., 2005), these data provide a crucial connection between defects in a specific type of synaptic molecule and a pervasive developmental disorder of the nervous system.

Based on the association between neuroligin mutations and autism, a model emerged that emphasized the importance of genetic perturbations of synaptic structure and/or synaptic transmission in the etiology of autism (Zoghbi, 2003; Garber, 2007). Support for this model increased with reports that mutations in the genes encoding Shank 3 (a postsynaptic scaffolding protein) and neurexin 1 are also associated with autism (Durand et al., 2007; Moessner et al., 2007; The Autism Genome Project Consortium, 2007; Kim et al., 2008). It is now generally accepted that mutations affecting structural components of synapses provide a significant risk factor for the development of ASDs.

In the present study, we have examined the expression, localization and biological function of the synaptic protein neuroligin in a simple model organism, the nematode *Caenorhabditis elegans*. Historically, studies of *C. elegans* synaptic proteins and mutants have been instrumental in elaborating the roles of synaptic proteins in neuronal function and development (Piechotta et al., 2006; Jin and Garner, 2008). We now report that neuroligin-deficient mutants of *C. elegans* are defective in a subset of sensory behaviors and sensory processing, and are hypersensitive to oxidative stress and heavy metal toxicity; the behavioral deficits are strikingly similar to traits frequently associated with ASDs. Our data thus provide clear connections between the nematode equivalent of an autism-associated synaptic mutation, altered sensory behaviors and hypersensitivity to environmental toxins. We believe that these data support an important model for how both genetic and environmental contributions to a neurological disorder can have a single underlying basis.

**RESULTS**

**The nlg-1 gene encodes the C. elegans homolog of vertebrate neuroligins**

The *C. elegans* genome contains a single gene (cosmid designation C40C9.5) encoding a homolog of vertebrate neuroligins. This gene, now designated *nlg-1*, spans approximately 5.7 kb of genomic sequence (Fig. 1). The reading frame corresponding to the longest possible transcript (see below) encodes an 847-amino acid protein (including a 17-amino acid N-terminal signal sequence). The structure of the predicted NLG-1 protein is similar to those of the mammalian neuroligins: a single-pass type I membrane protein with a large extracellular cholinesterase-like domain, and a small intracellular domain terminating in a PDZ-binding motif (Fig. 2).

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NLG-1 is 26-28% identical (45-47% similar) to the four human neuroligins and is most similar overall (28% identical, 47% similar) to human neuroligin 4 (supplementary material Fig. S1).

Through a combination of cDNA sequencing and reverse transcription PCR (RT-PCR) analysis of transcripts, we documented several types of nlg-1 alternative splicing (Figs 1, 2). Exons 13 and 14 are variably present in nlg-1 transcripts; the skipping of these two exons occurs independently, and we have detected transcripts containing only exon 13, only exon 14, both exons, and neither exon. In addition, we have identified tandem alternative splice acceptor sites at the 5′-ends of exons 4 and 16, and tandem alternative splice donor sites at the 3′-end of exon 14. If these splicing events are independent, there could be as many as 24 distinct NLG-1 isoforms.

nlg-1 is expressed in a subset of neurons and muscle cells

We used a transgenic transcriptional reporter, with the nlg-1 promoter driving YFP expression (FRM77, Fig. 1), to examine the cellular expression of nlg-1. We found that nlg-1 is expressed in a subset of neurons in C. elegans adults, including ~20 cells in the ventral nerve cord and ~20 cells in the head (Fig. 3). We identified the nlg-1-expressing cells in the ventral nerve cord as the cholinergic VA and DA motor neurons (Fig. 3). We also identified the two AYI and two URB interneurons and the four URA motor neurons in the head, and the two PVD mechanosensory and two HSN motor neurons in the body, as nlg-1-expressing cells. Of these cells, the AYI interneurons are cholinergic (Altun-Gultekin et al., 2001), the PVD neurons are glutamatergic (Lee et al., 1999), and the HSN neurons release both serotonin and acetylcholine (ACh) (Desai et al., 1988; Duerr et al., 2001). Neurotransmitter assignments have not been reported for the remaining nlg-1-expressing neurons; however, they do not express GABAergic, dopaminergic, serotonergic or glutamatergic reporters (see Methods). Finally, we also observed faint Pnlg-1::YFP expression in body wall muscles (Fig. 3G).

The NLG-1 protein is localized to synapses

To examine the subcellular localization of the NLG-1 protein, we generated a transgenic NLG-1::YFP fusion protein under the control of the nlg-1 promoter (FRM253, Fig. 1). This NLG-1::YFP fusion protein rescues all nlg-1 mutant behaviors (see below). Confocal microscopy revealed that NLG-1::YFP is present at or near synapses (Fig. 4A–C); localization in the synapse-rich nerve ring and ventral nerve cord is observed in embryos, and persists throughout development. NLG-1::YFP was also present in some neuronal cell bodies (such cells also express the FRM77 Pnlg-1::YFP transcriptional reporter); this may reflect modest overexpression of the NLG-1::YFP transgene.
We compared the distribution of NLG-1::YFP with that of the presynaptic protein UNC-10/RIM (an active zone protein originally identified as a Rab3-interacting molecule) in the four sublateral nerve cords. In adult animals, each of these nerve cords contains five axons: SIA, SIB, SMB and SMD axons projecting from cell bodies in the head, and ALN/PLN axons projecting from cell bodies in the tail (White et al., 1986). These axons make periodic en passant synapses (neuromuscular junctions) onto the adjacent body wall muscles. None of the neurons with axons in the sublateral nerve cords expresses *nlg-1*, but the gene is expressed in the body wall muscles (Fig. 3G). We observed NLG-1::YFP-containing puncta along the sublateral nerve cords (Fig. 4D); these are of necessity muscle-derived, and therefore postsynaptic. Furthermore, these NLG-1::YFP puncta were apposed to presynaptic active zones (UNC-10/RIM-containing puncta) present in the axons (Fig. 4D-F). We conclude that, at least in some cells, NLG-1::YFP is localized to postsynaptic regions.

**Fig. 3. Neuroligin-expressing cells.** Confocal images of young adult transgenic animals expressing a P*nlg-1::YFP* reporter (FRM77, see Fig. 1). Anterior is to the left and ventral is down. The reporter is expressed in ~45 neurons in the head and body (out of the adult complement of 302 neurons). An adult head view is shown in A-C. The *nlg-1* reporter is shown in green and a ttx-3 reporter [specific for AIY neurons (Altun-Gultekin et al., 2001)] is shown in red (nr, nerve ring; vnc, ventral nerve cord). A portion of the ventral nerve cord is shown in D-F. The neuroligin reporter is shown in green and a cholinergic reporter is shown in red. The neuroligin reporter is expressed in a subset of cholinergic motor neurons in the ventral cord. A region of an adult body is enlarged in G, showing that the neuroligin reporter is expressed in body wall muscles. Bars, ~10 μm.

**Fig. 4. A functional neuroligin-YFP fusion protein is localized to synaptic regions.** Transgenic animals (expressing FRM253) were stained with anti-green fluorescent protein (GFP) (green; A,B,D,E) and anti-UNC-10/RIM (red; B,C,E,F). (A-C) The head of a young adult hermaphrodite. The positions of the nerve ring (nr), dorsal nerve cord (dnc) and ventral nerve cord (vnc) are indicated. Anterior is to the left and ventral is down. (D-F) An enlarged section of a sublateral nerve cord. Bars, ~10 μm (A-C); ~2.5 μm (D-F).
Neuroligin-deficient mutants are viable and superficially wild type
We characterized the phenotypes associated with two independent nlg-1 alleles: ok259 and tm474. The nlg-1(ok259) mutation removes approximately half of the nlg-1 coding sequence (2341 base pairs; Figs 1, 2) and is almost certainly a null mutation. The tm474 mutation is associated with a smaller (583-base pair) deletion, which removes exon 7 and part of exon 8 (Figs 1, 2). Animals homozygous for either of these alleles are viable and superficially wild type in their appearance, development and behavior. In addition, the nervous system of nlg-1 mutants is grossly normal: expression of neuronal reporters in live animals and indirect immunofluorescence for several different synaptic antigens in fixed specimens revealed no apparent difference between nlg-1(ok259) mutants and wild-type animals (data not shown).

We used two quantitative assays to measure general synaptic function in nlg-1 mutants. The response to the acetylcholinesterase inhibitor aldicarb is commonly used as a measure of cholinergic neurotransmission in C. elegans; resistance to aldicarb is typically associated with decreased ACh release (Miller et al., 1996). Locomotory behavior in swimming assays provides a general measure of motor neuron function and neurotransmitter release. We found that nlg-1 mutants did not differ from wild-type animals in their response to aldicarb in an acute response assay (not shown) and were not appreciably deficient in swimming behavior (wild type: 157±4 body bends/minute; nlg-1(ok259): 143±11 body bends/minute; details in Methods). We therefore conclude that elimination of nlg-1 function does not lead to dramatic deficits in synapse formation or function. However, as described below, we have identified a number of significant behavioral and biochemical differences between nlg-1 mutants and wild-type animals.

nlg-1 mutants have specific deficits in chemosensation and the processing of chemosensory cues
Wild-type C. elegans hermaphrodites respond to a wide variety of volatile and water-soluble chemical cues (Bargmann et al., 1993; Bargmann, 2006), and nlg-1 mutants responded normally to most attractants and repellants. However, we were able to identify some specific chemosensory deficits in nlg-1 mutants. For example, nlg-1 mutants are not repelled by the normally aversive chemical 1-octanol: the chemotaxis index for wild type=−0.68±0.11; nlg-1=−0.04±0.08; and transgenic rescue by FRM253=−0.52±0.05 (negative values indicate repulsion; details in Methods). The difference between nlg-1 and wild type is statistically significant (P<0.0001). The octanol-sensing deficit can not be ascribed to a general insensitivity to volatile chemicals (nlg-1 mutants respond normally to the volatile attractant diacetyl), or to an insensitivity to repellants in general (the mutants have a normal response to the repellent cupric acetate), or even to a general insensitivity to volatile repellants (the mutants have a normal response to the volatile repellant nonanone). The behavior seems instead to be a specific lack of response to 1-octanol.

In addition to defects in response to specific chemical cues, nlg-1 mutants also exhibit defects in the processing of two conflicting chemosensory inputs. For example, wild-type animals and nlg-1 mutants are comparably attracted to diacetyl and repelled by cupric acetate (Fig. 5A,B). However, when animals are presented with these two compounds simultaneously (i.e. with a cupric acetate barrier between the animals and the attractant; see Fig. 5A,B), nlg-1 mutants are far more likely than wild-type animals to cross the barrier in response to the attractant.

Defects in thermal response
When well-fed wild-type nematodes are placed in a thermal gradient, they preferentially accumulate at the temperature at which they were raised (Hedgecock and Russell, 1975). However, nlg-1 mutants do not accumulate at a specific temperature, but instead move independently of temperature (Fig. 5C). The mutants appear to lack completely a thermal response; this atactic behavior was independent of the temperature at which the animals were grown or their feeding state (not shown). A normal thermal response was restored by transgenic expression of an NLG-1::YFP fusion protein (Fig. 5C). We conclude that nlg-1 mutants are either unable to sense temperature, or are indifferent to changes in ambient temperature.

Spontaneous reversal frequency is decreased in nlg-1 mutants
For approximately 20 minutes after wild-type C. elegans are removed from food, they display a stereotypic locomotory behavior (Hills et al., 2004; Gray et al., 2005): the animals move forward for 30–40 seconds, and then spontaneously reverse direction and back up for approximately three body lengths. They then start moving forward again, usually in a different direction. We found that nlg-1 mutants move forward for a much longer time than wild-type animals before initiating backward movement (Fig. 5D). This decrease in spontaneous reversal rate, sometimes called a ‘hypo-reversal’ phenotype (Tsalik and Hobert, 2003), was rescued by transgenic expression of the NLG-1::YFP fusion protein (Fig. 5D). However, although the likelihood of a reversal event was greatly reduced in nlg-1 mutants, the duration of backing, once initiated, was approximately normal (wild type: 5.8±0.9 seconds; nlg-1(ok259): 7.2±1.8 seconds). We also observed that the decrease in reversal likelihood was progressive: the decrease was significant in young larvae, but was far more pronounced in adults (Fig. 5D). We note that other phenotypes of nlg-1 mutants described above (e.g. the lack of response to 1-octanol and insensitivity to temperature) do not appear to be progressive.

Sensitivity to oxidative stress and heavy metals
The progressive nature of the spontaneous reversal phenotype in nlg-1 mutants suggested that the absence of neuroligin might trigger some type of degenerative process. We therefore evaluated the sensitivity of nlg-1 mutants to oxidative stress. Exposure to paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) is commonly used as a paradigm for oxidative stress in C. elegans (Ishii et al., 1990). We found that nlg-1 mutants were significantly more sensitive to paraquat than wild-type animals, and that this hypersensitivity was rescued by transgenic expression of a NLG-1::YFP fusion protein (Fig. 6A). In most organisms, including C. elegans, hypersensitivity to oxidative stress is associated with decreased life span (Ishii et al., 1998; Senoo-Matsuda et al., 2001; Kondo et al., 2005), and this was also true for nlg-1 mutants (supplementary material Fig. S2).

We extended these studies by examining the toxic effects of mercury compounds, and we found that nlg-1 mutants were significantly more sensitive than wild-type animals to both inorganic (HgCl₂) and organic [thimerosal (sodium ethylmercurithiosalicylate)] forms of mercury (Fig. 6B). The nlg-1 mutants were also more sensitive than wild-type animals to the
toxic effects of copper (cupric acetate) (supplementary material Fig. S3). However, there was no difference between the mutants and wild-type animals in their survival on cadmium (CdCl₂) (supplementary material Fig. S3); nlg-1 mutants are therefore not hypersensitive to all heavy metals.

An oxidative biomarker is elevated in nlg-1 mutants

To examine oxidative damage directly, we measured protein carbonylation (an irreversible oxidative modification) in wild-type, nlg-1 and mev-1 strains. This approach has been used previously to assess oxidative damage in C. elegans (Adachi et al., 1998; Yasuda et al., 1999). The mev-1 gene encodes a subunit of succinate dehydrogenase cytochrome b, part of complex II of the mitochondrial electron transport chain (Ishii et al., 1998); it was included in our analysis because mev-1 mutants are hypersensitive to paraquat (Ishii et al., 1990) and exhibit elevated levels of carbonylated proteins (Adachi et al., 1998; Yasuda et al., 1999). We found that nlg-1 mutants had a significantly higher level of protein carbonylation than wild-type animals (Fig. 6C). Exposure to paraquat significantly increased the protein carbonylation levels of all of the strains tested, although the levels remained much higher in nlg-1 mutants than in wild-type animals. Transgenic expression of the NLG-1::YFP fusion protein reduced protein carbonylation levels to those of the wild type (Fig. 6C), confirming the specificity of this biochemical phenotype. Levels of carbonylated proteins in untreated and treated mev-1 mutants were comparable to those observed in similarly treated nlg-1 mutants. We conclude that, even in the absence of paraquat, nlg-1 mutants experience elevated oxidative stress.

DISCUSSION

In this study we report that neuroligin is expressed in a subset of neurons and in muscles of C. elegans, and is localized to synaptic regions. Neuroligin-deficient mutants of C. elegans are defective in specific sensory behaviors and sensory processing, and are hypersensitive to oxidative stress and heavy metal toxicity. Our data provide clear connections between the nematode equivalent of an autism-associated synaptic mutation, altered sensory behaviors, and hypersensitivity to environmental toxins.

The C. elegans neuroligin gene and protein structure

The C. elegans neuroligin homolog (C40C9.5), encoded by the nlg-1 gene. The nlg-1 transcripts undergo extensive alternative splicing, giving rise to a number of
NLG-1 isoforms. However, in contrast to vertebrate neuroligins, where alternative splicing primarily affects the extracellular domain of the protein and modulates binding interactions with neurexins (Comoletti et al., 2006), alternative splicing of C. elegans nlg-1 transcripts leads almost exclusively to diversity in the intracellular domain of the protein (Fig. 2). It is possible that the isoform diversity generated by alternative splicing of nematode neuroligin corresponds to some of the diversity provided by multiple neuroligin genes in mammals. For example, the intracellular domain of mammalian neuroligin 2 has a proline-rich region that is not found in the other mammalian neuroligins. Similarly, exon 14 of C. elegans nlg-1 encodes a proline-rich region; perhaps nematode isoforms containing this exon correspond to mammalian neuroligin 2. However, we note that transgenic expression of a single isoform containing both exons 13 and 14 (Fig. 1) provided complete rescue of the thermosensory, chemosensory, spontaneous reversal, toxin sensitivity and protein oxidation phenotypes associated with nlg-1 mutants (Fig. 5B-D; Fig. 6A-C).

Neuroligin expression and localization

Our reporter studies indicate that neuroligin is expressed in approximately 45 of the 302 neurons present in adult hermaphrodites. Aside from the DA and VA motor neurons in the ventral nerve cord, there is no clear pattern (e.g. neurotransmitter, circuitry, etc.) linking the neuroligin-expressing neurons. In addition to neurons, we observed nlg-1 expression in the body wall muscles (Fig. 3); muscle expression has also been reported for human neuroligin 4 (Bolliger et al., 2001).

We report that neuroligin is localized to synaptic regions in C. elegans, and is present in postsynaptic puncta in at least a subset of cells. Interestingly, a recent study found that neuroligin is present in both presynaptic and postsynaptic regions of C. elegans neurons (Feinberg et al., 2008). In the DA9 motor neuron, for example, strong NLG-1::YFP fluorescence was present in the ventral postsynaptic region and weaker fluorescence was present in the dorsal presynaptic region. The significance of this presynaptic localization is unclear, although we note that rodent neurexins are localized to both presynaptic and postsynaptic regions (Taniguchi et al., 2007), and the same may be true for neuroligins.

Integration of multiple sensory inputs

Although sensory problems are not part of the official diagnostic criteria for ASDs, difficulties with the processing and/or integration of sensory inputs are often part of the presentation (American Psychiatric Association, 1994; Filipek et al., 1999). It is therefore particularly intriguing that nlg-1 mutants have deficits in the processing of conflicting sensory inputs, as measured in an approach-avoidance paradigm. nlg-1 mutants respond normally to the volatile attractant diacetyl and the repellent cupric acetate; however, their response to the simultaneous presentation of these two compounds is clearly not normal (Fig. 5A,B). We believe that the mutant phenotype should not be interpreted as a failure to process the two conflicting signals; rather, the discrete sensory inputs are processed, but the processing appears to use an alternative algorithm. This may reflect functional deficits in one or more of the processing interneurons.

Neuroligin and behavioral circuits

The circuits mediating C. elegans thermotaxis and spontaneous reversal behaviors have been described in considerable detail. Thermotaxis involves input from the AFD sensory neurons, and processing by the AIY, AIZ and RIA interneurons (Mori and...
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Ohshima, 1995); control of ‘spontaneous’ reversal involves input from many sensory neurons (including the AFD thermosensory neurons), followed by processing through the AY, AIZ, RIB and RIM interneurons (Zheng et al., 1999; Tsalik and Hobert, 2003). Although the AFD sensory neurons and the AY and AIZ interneurons participate in both circuits, only the AIY cells appear to express neuroligin. However, laser ablation of the AIY neurons leads to cryophilic (cold-seeking) behavior (Mori and Ohshima, 1995) and a ‘hyper-reversal’ phenotype (Tsalik and Hobert, 2003), whereas nlg-1 mutants are athermotactic and have a ‘hypo-reversal’ phenotype. The lack of neuroligin in the AIY neurons may alter the strength of specific synaptic connections rather than simply render these cells non-functional.

**Oxidative stress and autism**

We utilized an established model of oxidative stress in *C. elegans* (Yamamoto et al., 1996; Yanase et al., 2002) to demonstrate that neuroligin-deficient mutants are hypersensitive to paraquat (Fig. 6A). We believe that this sensitivity results from an increased basal level of oxidative stress, and we have shown that nlg-1 mutants exhibit a higher level of biochemical protein modifications characteristic of oxidative stress (Fig. 6C). We do not yet understand how the lack of neuroligin in only a subset of muscle cells and neurons is able to sensitize the entire organism to toxins such as paraquat and mercury.

There is a body of literature documenting the presence of biomarkers associated with oxidative stress in individuals with autism. These biomarkers include a significant decrease in the ratio of reduced to oxidized glutathione in plasma (James et al., 2006; Geier et al., 2009), an increase in 3-nitrotyrosine in cerebellar extracts (Sajdel-Sulkowska et al., 2008), increased urinary excretion of 8-iso-prostanet-Fe(II) (a non-enzymatic oxidation product of arachidonic acid) (Ming et al., 2005), and increased plasma levels of malon dialdehyde (an end product of peroxidation of polyunsaturated fatty acids and related esters) (Chauhan et al., 2004). Although it is unclear how the absence of neuroligin leads to oxidative stress in *C. elegans*, it is plausible that a comparable mechanism might link autism-associated mutations in humans to the autism-associated oxidative phenotypes that have been reported.

**Implications for ASDs**

Human twin studies have shown that the concordance among monozygotic twins for a strict diagnosis of autism is 60%, which is up to 12-fold higher than the concordance among dizygotic twins (Bailey et al., 1995; Muhle et al., 2004). This clearly provides strong evidence for a hereditary basis for autism, but also highlights the importance of non-hereditary (environmental) factors. Our data on the sensitivity of neuroligin-deficient mutants to oxidative stress (e.g. paraquat) and mercury compounds demonstrate a clear connection between the nematode equivalent of an autism-associated synaptic mutation and hypersensitivity to environmental toxins. We believe that this provides an important model for understanding how both genetic and environmental contributions to a neurological disorder can have a single underlying basis.

**METHODS**

**Strains and strain maintenance**

Standard laboratory methods for *C. elegans* were described by Brenner (Brenner, 1974). Worms were grown on NGM-Lite solid medium (Sun and Lambie, 1997), modified by the addition of streptomycin and mycostatin to reduce contamination, and the use of the streptomycin-resistant bacterial strain OP50/1 (Johnson et al., 1988). The nlg-1(ok259) mutant was provided by the *C. elegans* Gene Knockout Consortium, and the nlg-1(tm474) mutant was obtained from Shohei Mitani (Tokyo Women’s Medical College, Tokyo, Japan). The nlg-1(ok259) allele was used for these studies, and observations were confirmed with tm474. The pha-1(e2123) mutant (see below) was obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN).

**Sequencing of mutants and transcript analysis**

nlg-1 deletion mutations were analyzed by amplification of specific nlg-1 genomic regions from mutant animals (Barstead and Waterston, 1989), followed by sequencing of the purified PCR product. The cDNA clone yk497a9 was obtained from Yuji Kohara (National Institute of Genetics, Mishima, Japan) and the insert was fully sequenced (GenBank accession FJ825295); it includes 51 nucleotides of 5’-untranslated region (UTR) (out of a predicted 71 nucleotides), 2526 nucleotides of coding sequence (including both exons 13 and 14; see Fig. 1), 276 nucleotides of 3’-UTR, and a 19-nucleotide polyA sequence. RNA was isolated from wild-type *C. elegans* using standard procedures, and analyzed using RT-PCR with exon-specific primers, followed by DNA sequencing with internal primers. All DNA sequencing was performed at the OMRF DNA Sequencing Core Facility, using oligonucleotide primers obtained from IDT (Coralville, IA).

**Reporter constructs and transgenic methods**

Plasmids containing the cyan fluorescent protein (CFP), YFP or GFP coding sequences were derived from the pPD95.67, pPD132.12 or pPD133.48 plasmids (Miller, D. M. et al., 1999). A plasmid containing a modified mCherry gene (‘wormCherry’) (McNally et al., 2006; Green et al., 2008) was a generous gift from Anjon Audhya and Karen Oegema (Ludwig Institute for Cancer Research, La Jolla, CA). Some reporter constructs were generated using a PCR fusion method (Horton et al., 1989; Hobert, 2002). The neuroligin transcriptional fusion construct (FRM77) was generated by PCR amplification of the 5’-end of the nlg-1 gene (from 3563 base pairs upstream of the SL1 trans-splice site through the first 45 base pairs of exon 3), and fusing this product in-frame to the YFP coding sequence followed by the *unc-54* 3′-UTR. The neuroligin functional fusion protein (FRM253) had the YFP inserted after amino acid E661 (using the protein sequence derived from the yk497a9 cDNA; GenBank accession ACO52513). FRM253 was generated by amplifying a PCR product containing the same upstream region that was used to make FRM77, but extending downstream to exon 11. This amplification product was fused in-frame to the YFP coding sequence, followed by nlg-1 exons 12-16 and the nlg-1 3′-UTR, which were derived from the yk497a9 cDNA. The structures of these reporters are shown in Fig. 1.

Transgenic nematodes were obtained by microinjection of DNA (plasmids and/or PCR products), essentially as described by Mello (Mello et al., 1991). Transformation markers included the pBX plasmid (Heinke and Ralf Schnabel, Max-Plank-Institute für Biochemie), which rescues the temperature-sensitive lethality of pha-1(e2123) mutants (Granato et al., 1994). For experiments utilizing the pBX plasmid, we constructed appropriate recipient
strains for transformation containing the *pha-1(e2123)* mutation. For some DNA injections, we used a modified *unc-122* promoter to drive reporter expression only in coelomocytes (Loria et al., 2004). Extrachromosomal arrays were integrated by gamma irradiation (Schade et al., 2005).

**Cell identification**

*nlg-1*-expressing cells were identified on the basis of position and morphology (Altun and Hall, 2008), and also through the use of specific transgenic reporters for colocalization and/or to provide cellular landmarks. The cells identified as *nlg-1* positive were found to express the *Pnlg-1::YFP* reporter (FRM77) in 100% of animals observed, with at least 10 animals examined for each genotype.

The promoters used for these studies included *unc-25* for GABAergic neurons (Eastman et al., 1999), *unc-17* for cholinergic neurons (Alfonso et al., 1993; Duerr et al., 2008), *eat-4* for a subset of glutamatergic neurons (Lee et al., 1999), *dat-1* for dopaminergic neurons (Sulston et al., 1975; Nass et al., 2002), *tpk-1* for serotonergic neurons (Sze et al., 2000), *acr-5* for DB and VB motor neurons (Winnier et al., 1999), *ttxs-3* for AY interneurons (Altun-Gultekin et al., 2001), *gcy-8* for ADF sensory neurons (Yu et al., 1997), *glr-3* for RIA interneurons (Brockie et al., 2001a), *odr-2(2b)* for AIB, AIZ, ASG, AVG, IL2, PVP, RIF, RIV and SIAV neurons (Chou et al., 2001), and *nmr-1* for AVA, AVD, AVE, RIM, AVG and PVC interneurons (Brockie et al., 2001b). Dye filling of ciliated sensory neurons with DiI (Hedgecock et al., 1985) provided additional cellular landmarks in the head.

**Behavioral assays**

All behavioral measurements were performed on young adults at ~22°C on NGM-Lite plates unless stated otherwise; statistical significance was determined using the Student's *t*-test. Swimming rates were measured using hermaphrodites raised at 20°C, as described previously (Miller, K. G. et al., 1999). Acute response to aldicarb (2-methyl-2-[methylthio]proprionaldehyde-O-[[methylcarbamoy]oxime] (Chem Service, West Chester, PA) was assayed, as described previously (Lackner et al., 1999), using 2 mM aldicarb. To score reversal behavior, worms were grown at 20°C and transferred to unseeded plates at room temperature. After 2 minutes of equilibration, the animals were scored visually for changes in direction for 10 minutes, and the data were recorded using the Etho 1.2.2 program (provided by Dr James H. Thomas, Genome Sciences, University of Washington). Thermal response assays were performed after establishing a thermal gradient on an unseeded 100-mm plate by placing a vial of frozen glacial acetic acid (16.7°C) in the center of an inverted plate in a 25°C incubator (Hedgecock and Russell, 1975). Approximately 50 worms were transferred to the thermal gradient plate and allowed to move freely for 30 minutes; their positions were then scored on an overlay of concentric circles demarking eight equal areas.

For chemotaxis assays, 2 μl of a chemo-attractant or repellent was placed on one side of a 100-mm plate (the ‘A’ side) and diluent was placed on the opposite side of the plate (the ‘B’ side). Approximately 50 worms were placed on the middle of the plate and were allowed to move freely for 20 minutes, and then scored. Only worms within 2 cm of the test or control spots were scored, and therefore worms in neutral areas were disregarded. A chemotaxis index (C.I.) was calculated using the formula C.I. = (A−B)/(A+B). A positive C.I. value (up to 1.00) indicates attraction, a negative value (down to −1.00) indicates repulsion, and a value near 0.00 indicates neutrality.

To test the integration of simultaneous attractive and repellent cues, a repellent barrier (50 μl of 0.5 mM cupric acetate) was placed in a line across the middle of a 100-mm plate (Ishihara et al., 2002). After ~16 hours, a chemo-attractant (2 μl of 0.1% diacetyl in ethanol) was placed on one side of the barrier, and approximately 75 worms were placed on the opposite side. The worms were allowed to move freely on the plate for 30 minutes, and the fraction of worms that had crossed the barrier was then scored. In control experiments, when either cupric acetate or diacetyl was presented without the other, we did not observe significant differences between wild-type animals and *nlg-1* mutants in their responses to varying concentrations of either compound (data not shown).

**Life span measurements**

Synchronized populations were initiated by transferring adults to a plate, letting them lay eggs at 20°C for ~2 hours, and then removing the adults. The eggs were permitted to hatch, and the resulting populations of synchronized animals (~25 animals per plate in triplicate for each strain) were used for life span measurements. Animals were grown at 20°C and transferred away from their progeny to new plates as necessary (usually three times). Animals were assessed each day for viability; those that had ceased pharyngeal pumping and failed to move, even after repeated prodding, were scored as no longer viable. Ages are given as days from hatching.

**Immunofluorescence staining**

Antibodies used in this study included a rabbit polyclonal α-GFP antibody (which also recognizes YFP; Invitrogen/Molecular Probes, Carlsbad, CA), a mouse monoclonal α-UNC-17/VACHT antibody (mAb1403) (Lickteig et al., 2001; Duerr et al., 2008) and a chicken polyclonal α-RIM antibody (pAb271) (Koushika et al., 2001). Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Nematodes were stained using a modified freeze-fracture procedure, as described previously (Duerr et al., 1999; Mullen et al., 2006).

**Microscopy and imaging**

Confocal images were collected on a Leica TCS NT confocal microscope. Low-resolution images were collected with a 40× Plan Fluor 1.0 NA oil immersion objective, at 512×512 or 1024×1024 pixels, with 0.5 micron Z-steps. Higher-resolution images were collected with a 63× Plan APO 1.4 NA oil immersion objective, at 512×512 pixels, with 4× zoom and 0.2 micron Z-steps. Images were cropped to size, assembled, and annotated using Adobe Photoshop CS2. Digital manipulations were limited to rotating and cropping (Photoshop Bicubic) of images, as well as minor level adjustments.

**Toxicity studies**

Stock solutions of paraquat (Sigma-Aldrich), HgCl₂, CdCl₂, cupric acetate or thimerosal (Sigma-Aldrich) were added to molten growth medium. Final concentrations in the medium were: paraquat, 1.8 mM; HgCl₂, 7.3 μM; CdCl₂, ranged from 4 to 28 mM; cupric acetate, 0.9 mM; thimerosal, 91 nM. Toxicity was assessed by placing 20...
Neuroligin mutants and oxidative stress

**Clinical issue**

Autism spectrum disorders (ASDs) are common neurological disorders that include autistic disorder, Asperger disorder and PDD-NOS (pervasive developmental disorder-not otherwise specified). The Centers for Disease Control and Prevention now estimate an overall ASD prevalence of 1 case per 110 children. The three essential criteria for an ASD diagnosis are: (1) impaired verbal and nonverbal communication; (2) impaired social interaction; and (3) restricted, repetitive and stereotyped patterns of behavior, interests and activities. Family studies show that ASDs have a strong hereditary basis, apparently involving a large number of genes. However, it is also clear that environmental factors play a significant role in the etiology and severity of these disorders.

Mutations affecting structural components of synapses are significant risk factors for developing ASDs. The best-studied of these are the neuroligins: postsynaptic adhesion/signaling proteins that bind specifically to a set of presynaptic membrane proteins called neurexins. There are four neuroligin-encoding (NLGN) genes in humans, and mutations disrupting NLGN3 and NLGN4 are associated with autism. However, it is not clear how disruption of a broadly expressed synaptic protein results in the relatively specific behavioral deficits associated with ASDs.

**Results**

Here, the authors investigate the effects of neuroligin-disrupting mutations in *Caenorhabditis elegans*. *C. elegans* neuronal proteins are structural and functional homologs of mammalian proteins, making it a powerful model for analyzing synapse structure, function and development. Worms have a single neuroligin gene (*nlg-1*), and *C. elegans* neuroligin is structurally similar to mammalian homologs. Mutants lacking the neuroligin protein have superficially normal growth and behavior, and apparently normal nervous systems. However, the authors show that *nlg-1* null mutants have several sensory deficits: they do not respond normally to some chemical cues, they are insensitive to temperature changes and they have altered processing of conflicting sensory inputs.

*nlg-1* mutants also have increased levels of oxidative stress, which results from excessive free radicals and reactive oxygen species (ROS) that can damage cellular components (e.g. proteins, lipids and DNA). *nlg-1* mutants are hypersensitive to paraquat toxicity (an herbicide that produces excess free radicals and ROS) suggesting elevated levels of endogenous free radicals. Oxidative damage to proteins in *nlg-1* mutants is elevated compared with wild-type animals, and mutants are also hypersensitive to the toxic effects of copper- and mercury-containing compounds.

**Implications and future directions**

The relationship between ASDs and oxidative stress is unclear, although it has been proposed that oxidative stress, from environmental toxins, may contribute to the disorders. These studies show that loss of the synaptic protein neuroligin causes oxidative stress. This raises the possibility that specific types of neuronal disruption might be the cause, rather than the result, of oxidative stress. In addition, these data demonstrate a clear connection between an autism-associated synaptic mutation in *C. elegans* and hypersensitivity to environmental toxins (e.g. paraquat, mercury compounds, etc.). This provides an important example of how both genetic and environmental contributions to a neurological disorder can have a single underlying basis.

ASDs are often associated with changes in sensitivity to sensory inputs, as well as the ability to process and integrate these inputs. It is therefore intriguing that *nlg-1* mutants have specific sensory deficits, as well as deficits in the processing of conflicting sensory inputs. This characterization of a *C. elegans* ASD model indicates a role for neuroligin in processing sensory information and the importance of proper synaptic function in regulating oxidative stress.

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**Analysis of protein carbonylation by ELISA**

To quantify protein carbonylation, we used an ELISA method described by Alamdari et al. (Alamdari et al., 2005), with some modifications. Approximately 50 L4 worms were transferred to NGM-Lite plates with or without paraquat, and allowed to grow for 2 days prior to harvest, with the exception of *mev-1* mutants, which were harvested after 1 day. Nematode proteins were extracted in lysis buffer [20 mM 2-(N-morpholino)-ethanesulfonate (MES) buffer, pH 5.5, containing 0.1% Triton X-100, complete protease inhibitor cocktail (one tablet/50 ml) (Roche Diagnostics), and 100 μM butylated hydroxytoluene (BHT)] on ice, and sonicated on ice with a Sonic Dismembrator model 100 (Fisher Scientific). The protein concentrations of the samples were measured by the Micro BCA spectro-photometric assay (Pierce), and initially adjusted to 10 μg/ml with lysis buffer (some samples needed to be diluted further to stay within the linear working range of the assay). The diluted standards (10 μg protein in 1 ml PBS), samples (10 μg protein in 1 ml PBS), and PBS without protein (blank) were added in duplicate to wells of an ELISA plate. DNPH (2,4-dinitrophenylhydrazine) (0.05 mM, pH 6.2, freshly prepared) solution was added, incubated for 45 minutes at room temperature in the dark, and then neutralized with 0.1 N NaOH. Samples were transferred to a new ELISA plate, and ELISAs were performed using rabbit anti-DNPH and goat anti-rabbit HRP-conjugated IgG antibodies (Chemicon), and standard methods.

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**COMPETING INTERESTS**

The authors declare no competing financial interests.

**AUTHOR CONTRIBUTIONS**

J.W.H., G.P.M. and J.B.R. designed the experiments. J.W.H., G.P.M., J.R.M., J.M.H. and A.D. performed the experiments. J.W.H., G.P.M. and J.B.R. analyzed the data and wrote the manuscript.

**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.003442/-/DC1

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**Figure S1. Sequence alignment of the *C. elegans* NLG-1 and the human NLGN4 proteins.** The sequences were aligned using the VNTI AlignX program. Identical amino acids are indicated with red letters on a yellow background, and structurally similar amino acids with black letters on a grey background. Predicted signal sequences are underlined in blue and transmembrane domains are double-underlined in black. Accession numbers: NLG-1: ACO52513; hNLGN4: NP_065793.
Figure S2. *nlg-1* mutants have reduced lifespan. For each genotype (N2 and *nlg-1*), three groups of 25 animals were grown at 20°C as described in Methods, and assessed each day for survival; data points represent the mean percent survival ± standard deviation. The difference in mean lifespan between *nlg-1* and wild type is statistically significant (*P* < .0001).

Figure S3. *nlg-1* mutants are hypersensitive to copper, but not cadmium toxicity. Young adults (N2, *nlg-1* mutants, and *nlg-1* mutants expressing the FRM253 rescuing transgene) were transferred to plates containing either 0.9 mM cupric acetate (left panel) or 9.1 mM CdCl$_2$ (right panel), and survival was scored every 24 hours. Each data point represents the mean of four trials (with at least 20 animals in each trial) ± standard deviation. The difference in mean survival times on cupric acetate between *nlg-1* (1.8 ± 0.2 days) and wild type (4.2 ± 0.2 days) is statistically significant (*P* < .0001).
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