Long chain polyunsaturated fatty acids are required for efficient neurotransmission in C. elegans

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Summary

The complex lipid constituents of the eukaryotic plasma membrane are precisely controlled in a cell-type-specific manner, suggesting an important, but as yet, unknown cellular function. Neuronal membranes are enriched in long-chain polyunsaturated fatty acids (LC-PUFAs) and alterations in LC-PUFA metabolism cause debilitating neuronal pathologies. However, the physiological role of LC-PUFAs in neurons is unknown. We have characterized the neuronal phenotype of C. elegans mutants depleted of LC-PUFAs.

The C. elegans genome encodes a single Δ6-desaturase gene (fat-3), an essential enzyme for LC-PUFA biosynthesis. Animals lacking fat-3 function do not synthesize LC-PUFAs and show movement and egg-laying abnormalities associated with neuronal impairment.

Expression of functional fat-3 in neurons, or application of exogenous LC-PUFAs to adult animals rescues these defects. Pharmacological, ultrastructural and electrophysiological analyses demonstrate that fat-3 mutant animals are depleted of synaptic vesicles and release abnormally low levels of neurotransmitter at cholinergic and serotonergic neuromuscular junctions. These data indicate that LC-PUFAs are essential for efficient neurotransmission in C. elegans and may account for the clinical conditions associated with mis-regulation of LC-PUFAs in humans.

Key words: C. elegans, Neuromuscular junction, Neurotransmitter release, Polyunsaturated fatty acids, Synapse

Introduction

One of the central challenges in biology is to understand the cellular functions of the wide variety of complex lipids present in animal cells. Long-chain polyunsaturated fatty acids (LC-PUFAs), fatty acids with multiple double bonds, are synthesized from dietary precursors and are localized to cell membranes as phospholipid esters. Both the absolute LC-PUFA levels and their relative concentrations are strictly controlled in mammalian neurons (Lauritzen et al., 2001) implying that LC-PUFAs have critical neuronal functions. Indeed, mutations in enzymes involved in LC-PUFA metabolism cause a form of X-linked mental retardation (Meloni et al., 2002) and two forms of macular dystrophy (Zhang et al., 2001) and diets deficient in essential LC-PUFAs are associated with deficits in infant brain function (Anderson et al., 1999; Helland et al., 2003; Lauritzen et al., 2001; Willatts et al., 1998). In addition, LC-PUFAs induce oligomerization of α-synuclein, a protein found as insoluble aggregates in α-synucleinopathies including Parkinson’s disease (Sharon et al., 2003). Although these and other studies suggest an important role of LC-PUFAs in nervous system function, their precise role in neurons remains unclear. To address the neuronal functions of these molecules we generated Caenorhabditis elegans mutants depleted of LC-PUFAs and analyzed their neuronal phenotypes.

The nematode C. elegans synthesizes many of the LC-PUFAs found in humans (Wallis et al., 2002) and represents a good model organism to systematically study the neuronal roles of these molecules. First, the location, structure and function of virtually all C. elegans neurons are known. Second, pharmacological assays can be used to test synaptic function of certain neurons such as cholinergic motor neurons and serotonergic neurons (see Jorgensen et al., 1995; Lackner et al., 1999; Miller et al., 1999). Third, it is possible to study the role of LC-PUFAs independently of the known eicosanoid-mediated signaling pathways, since the C. elegans genome does not apparently encode orthologs of cyclooxygenase, lipoxygenase, thromboxane synthase or any orthologs of prostaglandin, leukotriene or thromboxane receptors. Finally, C. elegans mutants depleted of LC-PUFAs have been isolated by inactivating the gene fat-3, which encodes Δ6-desaturase, an enzyme essential for LC-PUFA biosynthesis (Fig. 1); this study and Watts and Browse (Watts and Browse, 2002). Initial analysis revealed that some of the defects displayed by fat-3
mutants are suggestive of neuronal impairment (Watts et al., 2003), yet the molecular basis of these effects are unknown.

Here we characterize in detail the neuronal phenotypes displayed by fat-3 mutants and demonstrate that LC-PUFAs are essential for normal neurotransmitter release at cholinergic and serotonergic neuromuscular junctions (NMJs). These defects are not developmental but are functional, since exogenous LC-PUFAs can rescue mutant adults. Consistent with these deficits we provide pharmacological and electrophysiological evidence that animals lacking LC-PUFAs release abnormally low levels of neurotransmitter. In addition, ultrastructural analysis reveals that synapses in these animals are severely depleted of synaptic vesicles. We conclude that LC-PUFAs are required for efficient neurotransmitter release.

Materials and Methods

Strains and isolation of fat-3 deletions

C. elegans strains were cultured at 20°C as described (Brenner, 1974). The strain GR1333 (IsPtph-1::gfp) was provided by I. Y. Sze and G. Ruvkun (University of California Irvine, CA and Harvard University, Cambridge, MA) (Sze et al., 2000).

To isolate deletion of the fat-3 gene, we constructed DNA libraries of approximately 5,400,000 haploid genomes from wild-type (N2) animals. The strains were mutagenized with ethylmethane sulfonate or with trimethylpsoralen. Using the polymerase chain reaction (PCR) with fat-3 primers (Jansen et al., 1997) we identified two deletions: fat-3(lg8101) and fat-3(qa1811). Both deletions confer a fully recessive phenotype. We used PCR-based genotyping to verify that the deleted strains do not contain duplications of the intact fat-3 gene. Analyses were carried out in fat-3(lg8101) homozygous, in fat-3(lg8101)dpy-20(e1282)unc-24(e138)fat-3(qa1811)dpy-20(e1282) heterozygous or in fat-3(wa2) homozygous animals. fat-3 mutants were outcrossed to wild-type animals at least six times before analysis.

Total RNA for RT-PCR was extracted from mixed C. elegans populations (Chomczynski and Sacchi, 1987). 1 µg of total RNA was reverse transcribed and PCR amplified using Ready-to-go RT-PCR beads (Amersham Biosciences, Chalfont St. Giles, UK). RT-PCR for unc-22 mRNA was used as positive control (data not shown). Primers used (Fig. 2A): a, 5’-CTCGAATTTTAAAACACTCGGCCG-3’; b, 5’-GGGACCTTTAAGGATGTC-3’.

Rescue experiments

A region comprising 1 kb 5’ of the fat-3 start, the entire fat-3 coding sequence (Napiér et al., 1998) and 0.9 kb 3’ of the fat-3 end rescued the defects associated with fat-3 mutants. Primers containing KpnI and SacI restriction sites were used to PCR amplify the entire fat-3 coding sequence. This KpnI-SacI fragment and a HindIII-KpnI fragment from Punc-119::gfp (Maduro and Pilgrim, 1995) were cloned into pPD49.26 (provided by A. Fire, Carnegie Institution of Washington, Baltimore, MD) to generate Punc-119::fat-3. To generate Pmyo-3::fat-3, a HindIII-BamHI fragment including the myo-3(+) promoter and the KpnI-SacI PCR fragment including the entire fat-3 coding sequence were cloned into pPD49.26. This KpnI-SacI fat-3 fragment and a Pflm-Smal fragment containing the elt-2 promoter were cloned into pPD49.26 to generate Pelt-2::fat-3. Constructs were injected (Mello et al., 1991) at 1-100 ng/μl in dpy-20(e1282) backgrounds with dpy-20(+) pMH86 (20 ng/μl). The presence of fat-3 mRNA was verified in all transgenic lines by RT-PCR (data not shown).

Fig. 1. fat-3 encodes a Δ6-desaturase. (A) The LC-PUFA synthetic pathways (Lauritzen et al., 2001; Spychalla et al., 1997). ALA, α-linolenic acid; LIN, linoleic acid; DGLA, dihom-γ-linolenic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DHA, docosahexaenoic acid. (B) The fat-3 gene (W082D.4) is located on chromosome IV, between unc-24 and dpy-20. A 4.7 kb genomic fragment including 977 bp 5’ and 862 bp 3’ of the fat-3 coding region rescues fat-3 mutants. The coding regions are in boxes and the non-coding regions are shown as lines. The cytochrome bs-like domain is in gray. Asterisks indicate histidine-rich regions. The fat-3(lg8101) and fat-3(qa1811) deletions and their breakpoints are shown. T indicates an A to T substitution. Dp indicates a 17 bp duplication (GAAAATGGTTGAATCAT). fat-3(wa22) is a C to T point mutation that changes S186 to F. (C) ClustalX alignment of FAT-3 protein with human and plant (Borago officinalis) Δ6-desaturases. The triangle indicates the fat-3(wa22) mutation. Single-letter abbreviations for amino acid residues are used. Identical and similar amino acids are identified by gray and light gray shading, respectively. The putative cytochrome bs-like domain is indicated with a line. Histidines important for catalytic activity are marked by asterisks.
Motility, egg-laying and paralysis assays

These assays were carried out in *fat-3(lg8101)dpy-20(e1282)/unc-24(e138)fat-3(qa1811)dpy-20(e1282), dpy-20(e1282), fat-3(wa22), fat-3(lg8101) mutant transgenic for the indicated constructs, or N2 animals. Motility was quantified by placing adult worms in the centre of a bacterial lawn on a Petri dish and allowing them to move. After 30 seconds worms were immobilized with heat. Pictures of the tracks left on the bacterial lawn were taken using a Leica MZ 125 microscope (Leica Microsystems, Milton Keynes, UK) equipped with a Photometrics CoolSnap digital camera (Roper Scientific, Tucson, AZ) and Openlab software (Improvement, Coventry, UK). Single tracks were highlighted with Adobe Illustrator (Adobe Systems, Uxbridge, UK) and their pixels counted using NIH Image (National Institutes of Health, Bethesda, MD). 20–47 tracks from at least two independent transgenic stable lines per genotype were analyzed.

Egg-laying-defective animals were determined by cloning L4 hermaphrodites to single plates and by scoring them every day for 4 days for embryos that hatched inside the mother.

The egg-laying assay in the presence of drugs was performed in microtiter wells as described previously (Trent et al., 1983) using serotonin or fluoxetine (Sigma) dissolved at the indicated doses in M9 buffer. 12-36 animals for each dose were analyzed.

For the paralysis assay, plates containing 1 mM acridine orange (Greyhound Chromatography, Birkenhead, UK) or 0.2 mM levamisole (Sigma) were prepared fresh for each set of assays as described previously (Miller et al., 1999). To allow comparisons between assays, wild-type controls were included in each assay. The acetylcholine (ACh) sensitivity was tested by spreading 1 M ACh on plates (final concentration ~10 mM). To minimize ACh hydrolysis, the assay was started within 10 minutes. 35-40 animals per experiment were scored for complete paralysis (Lackner et al., 1999) and 3-6 independent experiments per dose and per drug were carried out.

Statistical analysis was performed using the Mann-Whitney test with InStat software (GraphPad Software, San Diego, CA).

**ACh and fatty acid quantification**

ACh was quantified in *fat-3(lg8101) dpy-20(e1282)/unc-24(e138)fat-3(qa1811)dpy-20(e1282) or dpy-20(e1282) animals as described previously (Nonet et al., 1993).

The genotypes of animals used for fatty acid quantification were as follows: *fat-3(lg8101), N2, fat-3(lg8101)dpy-20(e1282);* *fat-3(qa1811)dpy-20(e1282) or dpy-20(e1282)*. Lipids were extracted and partitioned (Hargreaves and Clandinin, 1988). Phospholipid-derived fatty acid methyl esters were separated by capillary gas liquid chromatography using a fully automated Varian 6000 GLC (Varian Instruments, Mississauga, Ontario). Data were expressed as a percentage of the area count for each individual fatty acid relative to all fatty acids combined.

**Electrophysiology**

Electrophysiological methods were performed as previously described (Richmond et al., 1999; Richmond and Jorgensen, 1999) with minor adjustments. Briefly, the animals were immobilized in cyanacrylic glue and a lateral incision was made to expose the ventral medial body wall muscles. The preparation was then treated with collagenase (type IV; Sigma) for 15 seconds at a concentration of 0.5 mg/ml. The muscle was then voltage clamped using the whole cell configuration at a holding potential of ~60 mV. All recording were made at room temperature (21°C) using an EPC-9 patch-clamp amplifier (HEKA, Southboro, MA) run on an ITC-16 interface (Instrutech, Port Washington, NY). Data were acquired using Pulse software (HEKA).

The extracellular solution contained: 150 mM NaCl, 5 mM KCl, 0.5 mM CaCl₂, 4 mM MgCl₂, 10 mM glucose, 15 mM Heps, pH 7.4.
7.35, and sucrose to 340 mOsm. The pipette solution contained: 120 mM KCl, 20 mM KOH, 4 mM MgCl₂, 5 mM N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid, 0.25 mM CaCl₂, 4 mM NaATP, 36 mM sucrose, 5 mM EGTA, pH 7.2, sucrose to 335 mOsm. All data analysis and graph preparation was performed using Pulsefit (HEKA), Mini Analysis (Synaptosoft, Decatur, GA), and Igor Pro (Wavemetrics, Lake Oswego, OR).

Results
Generation of mutants depleted of LC-PUFAs
LC-PUFAs are synthesized from dietary precursors by sequential double bond insertion (desaturation) and elongation. Δ6-desaturase catalyses desaturation at the Δ6 position of α-linolenic acid (ALA) and LIN (Los and Murata, 1998; Nakamura et al., 2001) (Fig. 1A). The C. elegans gene fat-3, also called W08D2.4 (Fig. 1B), is a Δ6-desaturase. This protein contains three histidine clusters distinctive of desaturases (Los and Murata, 1998), harbors a cytochrome-b5-like domain observed in other Δ6-desaturases (Napier et al., 1999), is homologous to human and plant Δ6-desaturases (Fig. 1C), and has Δ6-desaturase enzymatic activity on C18 fatty acids (Napier et al., 1998).

fat-3(wa22) results in a serine to phenylalanine substitution at position 186 (Fig. 1B,C) (Watts and Browse, 2002); this position is not conserved in human or plant desaturases and it is not clear that this mutation is a null allele. To determine the allele is tightly associated with fat-3(qa1811) (Jansen et al., 1997). Both of these mutations are likely to strongly reduce or completely eliminate fat-3 activity: fat-3(qa1811) lacks 1,324 bp that include the three histidine clusters necessary for desaturation activity (Los and Murata, 1998). fat-3(lg8101) lacks 2,076 bp that include the start codon, an invariant heme-binding site indispensable for enzymatic activity (Los and Murata, 1998).

Using a highly sensitive and quantitative chromatographic method, we demonstrated that fat-3 deletion mutants are defective in LC-PUFA production and display a fatty acid composition similar to that reported for fat-3(wa22) mutants (Watts and Browse, 2002). Wild-type worms produce significant levels of dihomo-γ-linolenic acid (DGLA), AA and eicosapentaenoic acid (EPA; Table 1), three LC-PUFAs synthesized only in the presence of active Δ6-desaturase. Consistent with loss of Δ6-desaturase activity, fat-3(lg8101) mutant animals have drastically reduced levels of these three LC-PUFAs and accumulate two Δ6-desaturase substrates, ALA and LIN (Table 1). The residual levels of DGLA, AA and EPA detected in these animals may reflect the activity of another desaturase or a dietary contribution to the animal. These observations suggest that the defects observed in fat-3 animals are caused by depletion of LC-PUFAs.

The behavioral defects observed in fat-3 mutants are caused by LC-PUFA depletion
fat-3 mutants display a variety of phenotypes that include both behavioral and non-behavioral defects (Watts et al., 2003). Since we were interested in clarifying the role of LC-PUFAs in the nervous system, we focused our analysis on the two most prominent fat-3 behavioral phenotypes, namely, the deficits in movement and egg laying. fat-3 mutants show deficiencies in both forward and backward movements, and are particularly unable to respond to head-touch, that is, when touched gently near the head, wild-type animals respond by reversing direction and proceeding rapidly away from the stimulus. However, when fat-3 mutants are stimulated in the same manner, they stop or proceed backwards only very slowly. In addition, while fat-3 mutants do lay eggs, they frequently retain eggs in the uterus abnormally as they age. Some of these eggs hatch before being laid, causing the mother to eventually be consumed by hatched embryos. This phenotype is both qualitatively and quantitatively similar to the egg-laying defects caused by mutations in the egl-1 gene, which cause a specific developmental disruption of the hermaphrodite specific neurons (HSNs) (Desai and Horvitz, 1989; Trent et al., 1983), a pair of serotonergic neurons innervating the egg-laying muscles. In particular, 29% of fat-3(lg8101)/fat-3(qa1811) (n=24), 39% of fat-3(lg8101) (n=23) and 47% of egl-1(n487) (n=34) animals were consumed by hatched embryos late in adult life. Based on these observations and on the fact that these behavioral defects were rescued by selective expression of fat-3 in the nervous system (see below) we hypothesized that fat-

Table 1. LC-PUFA composition

| LC-PUFA | Wild type | fat-3(lg8101) | Wild type | fat-3(lg8101) | fat-3(lg8101) | fat-3(lg8101) |
|---------|-----------|--------------|-----------|--------------|--------------|--------------|
|         |           |              | +DHA      |              | +DHA         | Exfat-3(+)   |
| LIN     | 3.7±0.06  | 16.3±1.4     | 9.5±1.0   | 10.7±0.6     | 3.4±0.3      |
| DGLA    | 5.8±0.2   | 0.7±0.0      | 3.6±0.7   | 2.0±0.2      | 8.7±0.8      |
| AA      | 2.9±0.3   | 0.7±0.3      | 0.6±0.08  | 0.4±0.1      | 2.2±0.3      |
| ALA     | ND        | 7.5±0.8      | 1.4±0.2   | 2.1±0.5      | ND           |
| EPA     | 21.8±1.8  | 1.0±0.2      | 11.4±2.3  | 10.2±1.3     | 12.0±0.9     |
| DHA     | ND        | ND           | 7.1±1.4   | 7.5±0.8      | ND           |

LC-PUFA composition of total phospholipids isolated from wild-type and fat-3(lg8101) C. elegans grown in normal growth medium or medium containing DHA. Transgenic fat-3(lg8101)Exfat-3(+) worms carry cosmid C24G5, which contains coding region and regulatory sequences of fat-3. Data are the mean±s.e.m. of 3–4 independent measurements. ND, not detected (<0.2%).

Percentage (w/w) of total fatty acids
systems, AA is a precursor of eicosanoids, bioactive lipids responsible for the observed phenotypic rescue. In other animals (Table 1), suggesting that AA by itself is not animals rescued with DHA. We observed that exogenous AA, we measured the levels of each lipid in To test whether exogenous LC-PUFAs rescue the movement defects of fat-3(lg8101)/fat-3(qa1811) mutants. Animals were exposed to fatty acids from egg to adult. *P<0.0001 versus wild-type animals. (B) fat-3 expressed under the control of the neuronal promoter unc-119 (Exfat-3(+)) but not under the control of the muscular promoter myo-3 (Exfat-3(+)) muscle, completely rescues the egg-laying defect of fat-3(lg8101)/fat-3(qa1811) animals. Egl+ indicates hermaphrodites that were not consumed by embryos by the fourth day after reaching adulthood. (C) fat-3 expressed under the control of the neuronal promoter but not under the control of the muscular promoter or the intestinal promoter elt-2 (Exfat-3(+)) intestine), almost completely rescues the movement defects of fat-3(lg8101) homozygous animals.

Fig. 3. Rescue of the behavioral defects associated with loss of fat-3 activity. (A) Exogenous AA and DHA, but not LIN, rescue the movement defects of fat-3(lg8101)/fat-3(qa1811) mutants. Animals were exposed to fatty acids from egg to adult. *P<0.0001 versus wild-type animals. (B) fat-3 expressed under the control of the neuronal promoter unc-119 (Exfat-3(+)) neuron) but not under the control of the muscular promoter myo-3 (Exfat-3(+)) muscle), completely rescues the egg-laying defect of fat-3(lg8101)/fat-3(qa1811) animals. Egl+ indicates hermaphrodites that were not consumed by embryos by the fourth day after reaching adulthood. (C) fat-3 expressed under the control of the neuronal promoter but not under the control of the muscular promoter or the intestinal promoter elt-2 (Exfat-3(+)) intestine), almost completely rescues the movement defects of fat-3(lg8101) homozygous animals. *P<0.0001 versus fat-3Exfat-3(+) animals. Data in A and C are plotted as mean ± s.e.m.

To determine whether the behavioral defects observed in fat-3 mutants are caused by deficits in LC-PUFA levels, we asked whether exogenous LC-PUFAs rescue the movement defects associated with loss of fat-3 function. We grew fat-3(lg8101)/fat-3(qa1811) hermaphrodites from egg to adult in the presence of AA, whose synthesis is dependent on Δ6-desaturase (Fig. 1A). AA fully rescued the lack of coordination of fat-3 mutants (Fig. 3A). Similarly, exogenous application of DHA, another LC-PUFA product of Δ6-desaturase activity, was also sufficient to rescue the locomotion defects associated with fat-3 mutant animals. As a negative control we used LIN (Fig. 1A). Since fat-3 mutants have inactive Δ6-desaturase and can convert LIN to LC-PUFAs only very poorly, if at all (Table 1), LIN administration is not expected to rescue the fat-3 phenotype. Indeed, exogenous LIN did not have any effect on the motility of fat-3 mutants (Fig. 3A).

DHA or its metabolic products mediate Δ6-desaturase function

To test whether Δ6-desaturase function is mediated by DHA or AA, we measured the levels of each lipid in fat-3 mutant animals rescued with DHA. We observed that exogenous application of DHA did not increase AA levels in fat-3 mutant animals (Table 1), suggesting that AA by itself is not responsible for the observed phenotypic rescue. In other systems, AA is a precursor of eicosanoids, bioactive lipids involved in a variety of biological functions. However, eicosanoids do not appear to be produced or used in C. elegans. Thus, AA exerts its function via a distinct as yet uncharacterized lipid pathway. Conversion of AA into DHA precursors by a C. elegans enzyme has been previously observed (Spychalla et al., 1997). Therefore, the active LC-PUFA species are DHA or its related metabolic products and precursors, which could be generated catabolically.

fat-3 expressed in neurons rescues the behavioral defects of fat-3 mutants

The FAT-3 protein is expressed in the intestine, body-wall muscles, pharynx and several neurons (Watts et al., 2003). To determine where fat-3 expression is required, we generated constructs that drive gene expression in specific tissues and tested their ability to rescue the behavioral defects associated with loss of fat-3 activity. As expected, a construct comprising the endogenous promoter, the coding sequence and the 3′ untranslated region of fat-3 (Fig. 1B) restored normal LC-PUFA levels (Table 1) and rescued the egg-laying impairment and the reduced motility of fat-3(lg8101) homozygous animals (Fig. 3B,C). Since the uncoordinated and egg-laying phenotypes of fat-3 mutants are suggestive of neuronal defects, we investigated whether fat-3 selectively expressed in the nervous system could rescue the impaired motility and the egg-laying defect of fat-3 mutant animals. We made a chimeric construct, Punc-119::fat-3, in which the fat-3 coding sequence is placed under the control of a promoter driving gene
expression in the entire nervous system (Maduro and Pilgrim, 1995). Transgenic fat-3 mutants bearing this construct recovered normal egg-laying capability (Fig. 3B) and almost normal motility (Fig. 3C). Conversely, expression of the fat-3 coding sequence under the control of the muscle-specific myo-3 promoter did not rescue the egg-laying defect (Fig. 3B) and only minimally rescued the motility defect (Fig. 3C) associated with the fat-3 mutation. We also tested whether intestine-specific expression was sufficient to rescue the fat-3 mutant phenotype. We placed fat-3 under the control of the intestine-specific promoter elt-2 (Fukushige et al., 1998), which is expressed in the intestine and its precursors cells from early embryonic stages. This chimeric construct could not rescue the uncoordinated phenotype of fat-3 mutants (Fig. 3C). Since we restored normal egg-laying and near-normal motility only when we expressed fat-3 in the nervous system, it is likely that fat-3 activity is required in neurons for their normal function. However, we cannot exclude the possibility that fat-3 may have additional normal functions in other cells.

Loss of fat-3 activity causes functional, not developmental defects

The behavioral deficits observed in fat-3 mutants could reflect developmental defects in the nervous system. As a first approach to this issue, we analyzed the neuronal morphology of fat-3 mutants at the light microscope level. We generated animals in which specific subsets of neurons were fluorescently labeled with green fluorescent protein (GFP) and could not detect any morphological defect in HSN structure or in the attachments of the HSN to its egg-laying muscle target (Fig. 4A). We also could not detect morphological alterations in serotonergic neurons (Fig. 4B). Moreover, when we visualized the entire nervous system using a pan-neuronal GFP marker (Punc-119::gfp) we were also unable to detect any morphological defect in fat-3 mutant animals (data not shown). Finally, when we analyzed the ultrastructure of the nervous system, we found that the general arrangement, structure and positioning of neurons as well as synaptic morphology are normal in fat-3 mutants (data not shown). These results suggest that the neuronal impairments associated with loss of fat-3 activity are likely to be functional rather than developmental.

If the behavioral defects observed in fat-3 mutants are indeed functional it should be possible to rescue these phenotypes by providing adult animals, in which the nervous system is fully developed, with the metabolic products of fat-3 activity. We therefore exposed adult fat-3 mutants to exogenous LC-PUFAs and analyzed movement. Adult homozygotes exposed to AA for 24 hours recovered almost normal motility and were completely rescued after 48 hours (Fig. 4C). These results are consistent with the notion that LC-PUFAs are required for neuronal function rather than development.

fat-3 mutants display defects in neurotransmitter release

To better assess these defects in neuronal function, we measured the transmission efficiency of both serotonergic NMJs involved in egg-laying and cholinergic NMJs involved in body wall muscle contraction and movement. Egg-laying is mainly controlled by the serotonergic HSN motor neurons (Trent et al., 1983). Both exogenous serotonin and fluoxetine induce wild-type animals to lay eggs (Desai and Horvitz, 1989; Trent et al., 1983; Weisshenker et al., 1995). Fluoxetine potentiates the effect of endogenous serotonin by selectively inhibiting its presynaptic re-uptake (Hyttel, 1994). Animals missing the HSN neurons are sensitive to serotonin and insensitive to fluoxetine (Trent et al., 1983; Weisshenker et al., 1995). Animals with defective egg-laying muscles are insensitive to both drugs. The egg-laying response of fat-3 mutant animals exposed to serotonin was normal (Fig. 5A). For example, at 5 mg/ml, the dose eliciting the highest response, wild-type animals laid 16.42±1.17 eggs and fat-3(lg8101)/fat-3(qa1811) mutants laid 13.00±1.04 eggs. This demonstrates that muscle function in these animals is not disrupted. However, fat-3 animals responded very poorly to fluoxetine (Fig. 5B). Wild-type animals laid an increasing number of eggs when exposed to higher doses of fluoxetine. They reached a peak of 11.88±0.97 eggs laid at 0.5 mg/ml fluoxetine. However, fat-3(lg8101)/fat-3(qa1811) animals laid an almost constant number of eggs whatever the dose of fluoxetine (from 3.29±0.64 eggs at 0.1 mg/ml fluoxetine to 5.00±0.94 eggs at 1 mg/ml). Similarly, fat-3 mutants were only inefficiently stimulated by imipramine, another potentiator of endogenous serotonin (Fig. 5C). These observations suggest...
that fat-3 mutant animals release abnormally low levels of serotonin into the synaptic cleft.

Cholinergic NMJ function was probed using the agonist levamisole, which binds to muscular ACh receptors, and the ACh esterase inhibitor aldicarb, which enhances the effect of endogenously released ACh. Treatment of wild-type animals with either drug results in muscle hypercontraction and paralysis. However, mutants with decreased ACh release are resistant to aldicarb (Jorgensen et al., 1995; Nonet et al., 1993; Nonet et al., 1998). Consistent with reduced ACh release into the synaptic cleft, fat-3 mutant animals were significantly less responsive to aldicarb than wild-type animals. For example, exposure to 1 mM aldicarb for 60 minutes resulted in 90.5±3.3% of wild-type animals and only 54.5±7.4% of fat-
3(lg8101)/fat-3(qa1811) mutant animals being paralyzed (Fig. 5D). Both fat-3(lg8101)/fat-3(qa1811) and fat-3(wa22) animals responded to aldicarb in similar fashion (Fig. 5D,E). This reduced response to aldicarb is comparable in severity to that observed in the “weak” synaptic transmission mutant rab-3 (Fig. 5D) (Nonet et al., 1997). To verify that this response is associated with loss of fat-3 activity, we exposed fat-3 mutants, expressing fat-3 in neurons, to aldicarb. These, but not animals expressing fat-3 in muscles, recovered almost normal sensitivity to aldicarb (Fig. 5F). This decreased response is not due to ACh receptor impairment because fat-3 mutants were hypersensitive to both levamisole (Fig. 5G,H) and ACh (data not shown) as compared to wild-type animals. For example, exposure to levamisole for 40 minutes resulted in paralysis of 47.5±7.1% of wild-type and 94.7±1.5% of fat-3(lg8101)/fat-3(qa1811) mutant animals (Fig. 5G). Normal response to levamisole was restored in fat-3 mutants grown in the presence of AA (Fig. 5I), confirming that the hypersensitivity to levamisole is dependent upon LC-PUFA levels. Such hypersensitivity may reflect an adaptive response to decreased ACh availability and is observed in other mutant backgrounds defective in cholinergic transmission (Nonet et al., 1993). Lower levels of ACh at the synaptic cleft of fat-3 mutants could arise from either inefficient release of ACh or decreased ACh biosynthesis. To discriminate between these possibilities we quantitatively assessed the level of ACh produced in fat-3 mutants. Since total ACh levels do not diminish in fat-3 mutants (65.1±10.8 fmoles per µg of protein in fat-3 mutants (n=20) versus 48.7±4.0 in wild-type (n=20), P=0.3040), defects in ACh biosynthesis are unlikely to account for the movement defects displayed by fat-3 mutants. Taken together, these results suggest that the fat-3 lesion causes inefficient ACh release from cholinergic neurons.

To test directly whether fat-3 mutants have decreases in ACh release, we measured both evoked excitatory postsynaptic currents and endogenous miniature excitatory postsynaptic currents from voltage-clamped muscles at the C. elegans NMJ. fat-3(wa22) mutants displayed a decrease in evoked amplitude (417±41 pA) compared to the wild type (830±144 pA; Fig. 6A,B). In addition, the frequency of miniature postsynaptic currents, caused by the fusion of one or a few synaptic vesicles, was reduced in fat-3(wa22) mutants (10±2 fusions/second) compared to the wild type (15±1 fusions/second; Fig. 6C,D). The amplitude of the miniature currents was not significantly different in fat-3(wa22) (41±3 pA) compared to wild type (37±2 pA; Fig. 6E). These data suggest that in fat-3 mutants synaptic vesicles are correctly filled with neurotransmitter and the postsynaptic receptor field is normal. However, synaptic vesicles are either reduced in number or in release probability at fat-3 mutant synapses.

Presynaptic sites are depleted of synaptic vesicles in fat-3 mutants

Decrease in neurotransmitter release in fat-3 mutants could be caused by a number of possible mechanisms. For example, synaptic vesicles could be assembled and localized normally to NMJs but undergo exocytosis only inefficiently. In this view, the number of vesicles at NMJs is expected to increase. Alternatively, fat-3 mutants could localize fewer synaptic vesicles at the nerve terminal or could be defective in endocytosis. In this view, the number of synaptic vesicles is expected to decrease (Harris et al., 2000; Jorgensen et al., 1995). To distinguish between these possibilities, we examined the synaptic ultrastructure of fat-3 mutants and determined the distribution of synaptic vesicles at NMJs. We found that while synapses in wild-type animals had clusters of vesicles in the proximity of the active zone (Fig. 7A), synapses in fat-3 mutants were depleted of vesicles (Fig. 7B). The number of synaptic vesicles per synaptic terminal (within 300 nm of the active zone) was 2.7-fold smaller in fat-3 mutants than in wild-type animals (10.32±0.96 versus 28.07±3.22, P<0.0001; Fig. 7C). In addition, both the number of synaptic vesicles docked to the presynaptic membrane (0.72±0.15 versus 2.00±0.36, P<0.001) and the number of those in its vicinity (within 100 nm: 3.40±0.30 versus 6.88±0.87, P=0.0001) were significantly decreased in fat-3 mutant animals (Fig. 7C). Moreover, our ultrastructural analysis did not reveal accumulation of vesicles in neuronal cell bodies in fat-3 mutants (data not shown). These results indicate that LC-PUFAs are required to maintain a normal pool of synaptic vesicles at NMJs.

**Fig. 6.** fat-3 mutants have reduced evoked amplitude and reduced rates of spontaneous fusion but normal quantal size. (A) Representative evoked responses from wild-type and fat-3 animals. (B) The mean amplitude of the evoked responses is reduced in fat-3 (n=6) compared to wild-type (n=7) animals (P<0.03). (C) Representative traces of spontaneous fusion events in wild-type and fat-3 mutants. (D-E) The mean frequency of spontaneous fusion is reduced in fat-3 mutants (n=13) compared to wild-type (n=14; *P<0.02), while the mean amplitude of the individual events is normal. Data is plotted as mean ± s.e.m.
LC-PUFAs and neurotransmission

The complex lipids LC-PUFAs are highly enriched and precisely regulated in neurons. By inactivating the gene fat-3, we have generated animals depleted of LC-PUFAs and analyzed their neuronal deficits. We show that depletion of LC-PUFAs causes functional rather than developmental defects in the nervous system. These defects can be rescued by selective FAT-3 expression in the nervous system or by dietary supplementation of LC-PUFAs to adult worms. Using pharmacological techniques, we identify neurotransmitter release defects in both cholinergic and serotonergic neurons of fat-3 mutants. Electrophysiological studies suggest that a decrease in neurotransmitter release rather than neurotransmitter loading is responsible for the neuronal defects of fat-3 mutants. Finally, ultrastructural analysis of synaptic terminals demonstrates that synapses are depleted of synaptic vesicles. We conclude that LC-PUFA depletion results in insufficient neurotransmitter release.

LC-PUFAs and neurotransmitter release

The locomotion and egg-laying defects observed in fat-3 mutants are neuronal in nature and could be rescued by expressing fat-3 in the nervous system but not by expressing it in muscles or intestine. This suggests that LC-PUFAs are produced and act in neurons. The defects associated with loss of fat-3 activity were also rescued by providing exogenous LC-PUFAs. Free fatty acids are known to diffuse across biological membranes from intercellular spaces (Frohnert and Bernlohr, 2000). The observation that fat-3 expressed in intestine and muscles did not result in rescue, suggests that fat-3 expression in these tissues does not provide enough LC-PUFAs in intercellular spaces to support normal neuronal function. Therefore it is likely that fat-3 activity is required in neurons for their normal function.

Although we cannot rule out that fat-3 mutants might have subtle neuronal developmental defects, several lines of evidence suggest that the neuronal developmental defects do not contribute significantly to the behavioral phenotypes observed in C. elegans depleted of LC-PUFAs. First, in fat-3 mutants we could not observe gross morphological defects in neurons visualized with GFP markers. Second, at the ultrastructural level we found that both the general organization of the nervous system and neuronal specializations such as the NMJs appeared normal in animals without fat-3 activity. Third, the locomotion defects of adult fat-3 mutants were rescued acutely by providing exogenous LC-PUFAs. Therefore, our results indicate that LC-PUFAs are important for neuronal function rather than neuronal development.

Two lines of evidence indicate the defects in neuronal function observed in fat-3 mutants are due to decreases in neurotransmitter release. First, fat-3 mutant animals displayed presynaptic defects in neuronal function at both cholinergic and serotonergic neurons in pharmacological assays. Second, electrophysiological recordings demonstrate that fat-3 mutants release abnormally low levels of neurotransmitter. This impaired neurotransmission is probably caused by decreases in synaptic vesicle number rather than neurotransmitter loading or release probability. In fat-3 mutants an abnormally low quantity of neurotransmitter is released upon stimulation. This reduction in neurotransmitter release does not result from decreases in loading of neurotransmitter into synaptic vesicles as fat-3 mutants have similar amplitudes of mini currents as wild-type animals. Thus, a decrease in the number of synaptic vesicles undergoing exocytosis is probably responsible for the impaired neurotransmission. This reduced number of vesicles
undergoing exocytosis is probably due to a decrease in available vesicles rather than defects in release since our ultrastructural analysis of fat-3 mutant synapses showed reductions in both total and morphologically docked synaptic vesicles. The decrease in available vesicles being responsible for fat-3 mutant defects is also supported by the correlation between the extent of synaptic vesicle depletion and the decrease in synaptic vesicle fusion. fat-3 mutants carry approximately 40% of the synaptic vesicles of wild-type animals. Similarly, the evoked responses in fat-3 mutants is approximately 50% that of wild-type animals. We conclude that the abnormally low number of synaptic vesicles present at NMJs of fat-3 mutants is insufficient to support normal neurotransmission.

The decrease in synaptic vesicle number at synaptic terminals could be due to defects in transport, endocytosis, or synaptic vesicle biogenesis. fat-3 mutants are unlikely to have significant defects in synaptic vesicle transport, since in our ultrastructural analysis we did not observe vesicle accumulation in the proximity of the Golgi apparatus or in the rest of the neuronal cell body (data not shown). Therefore it is likely that fat-3 mutants are defective in synaptic vesicle biogenesis and/or synaptic vesicle recycling. Further work is needed to clarify in detail the mechanism(s) responsible for the synaptic vesicle depletion observed in fat-3 mutant animals.

Link with human brain function

LC-PUFAs have been associated with normal neuronal and retinal function (Lauritzen et al., 2001; Martinez et al., 2000; Meloni et al., 2002). In addition, it has been suggested that AA and DHA are required for normal cognitive development in infants (e.g. Anderson et al., 1999; Helland et al., 2003; Willatts et al., 1998). This hypothesis derives from studies comparing cognitive function of breast-fed infants with those of formula-fed infants. In most of these studies children who had been breast-fed scored better than children who had been formula fed. Since human milk naturally contains AA and DHA, while infant formulas do not, it was concluded that AA and DHA are responsible for the better cognitive skills observed in breast-fed infants. However, it has been difficult to unequivocally determine whether this difference is due to LC-PUFAs or to confounding variables such as socio-economic status or parental education. Moreover, no specific cellular process has been unambiguously identified for LC-PUFAs. Here we have demonstrated pharmacologically and electrophysiologically that LC-PUFA depletion leads to decreased neurotransmitter release and have therefore proved that LC-PUFAs have a direct effect on neuronal function. Thus LC-PUFA depletion is likely to impair communication among many types of neurons. This could well have a dramatic effect on cognition and memory and could account for the psychomotor retardation associated with diseases altering LC-PUFA metabolism (Martinez et al., 2000; Meloni et al., 2002).

It is still controversial as to whether LC-PUFA deficiency leads to functional or developmental defects in humans (Lauritzen et al., 2001). For example, conflicting data exist on the recovery of retinal function after repletion of LC-PUFA depleted animals. Connor and Neuringer reported that restoration of normal LC-PUFA levels was not sufficient to rescue functional retinal abnormalities (Connor and Neuringer, 1988). Conversely, Weisinger and colleagues reported a complete functional recovery (Weisinger et al., 1999). Here we have demonstrated that LC-PUFA depletion leads to functional but not developmental defects in C. elegans neurons. Our findings are consistent with recent reports showing that supplementation of certain LC-PUFAs can improve neurological conditions associated with LC-PUFA mis-regulation (Martinez et al., 2000).

These experiments provide a foundation for a genetic analysis of LC-PUFA function. We anticipate that genetic screens aimed at identifying proteins regulated by LC-PUFAs in C. elegans will uncover the cellular targets and define the molecular mechanisms underlying the roles of LC-PUFAs in neurotransmission.

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