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, lipooxygenase, 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.

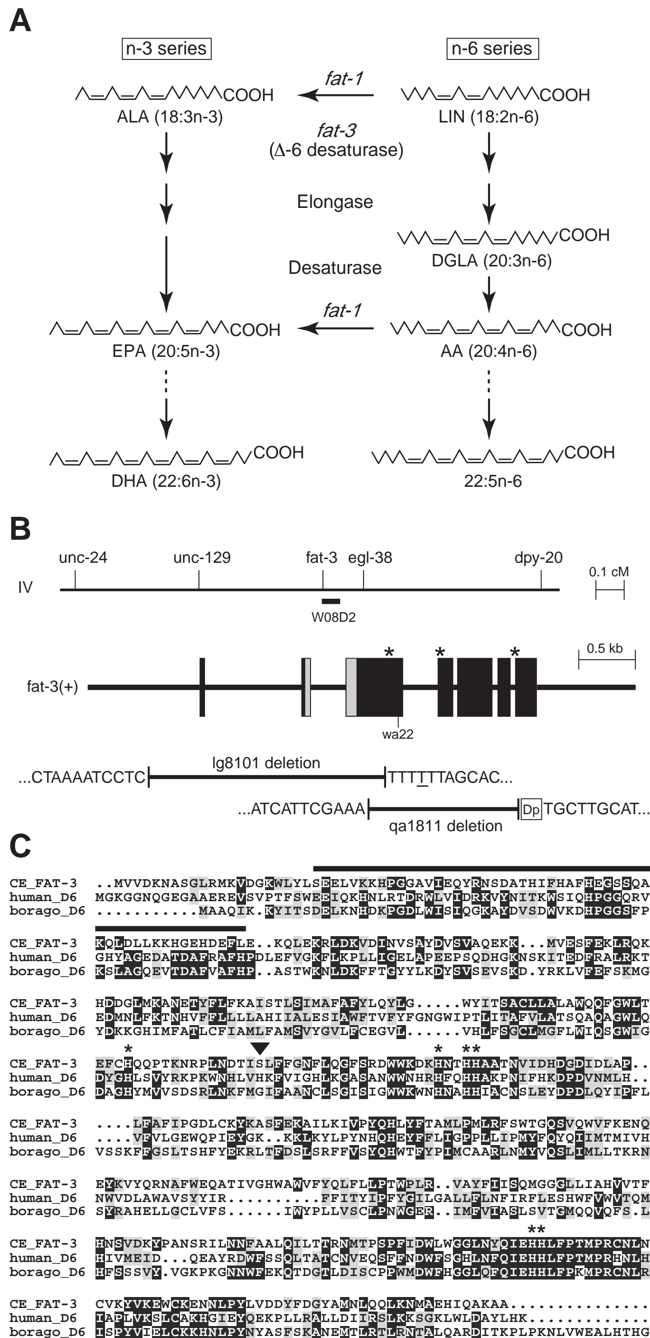

Fig. 1. *fat-3* encodes a Δ6-desaturase. (A) The LC-PUFA synthetic pathways (Lauritzen et al., 2001; Spychalla et al., 1997). ALA, α-linolenic acid; LIN, linoleic acid; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DHA, docosahexaenoic acid. (B) The *fat-3* gene (W08D2.4) 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 b_5-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 S_{186} 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 b_5-like domain is indicated with a line. Histidines important for catalytic activity are marked by asterisks.

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 mutagenized with ethylmethane sulfonate or with trimethyl psoralen. Using the polymerase chain reaction (PCR) with *fat-3* primers (Jansen et al., 1997) we isolated 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(wa22)* 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′-CTCGAATTTTAAACAACTTCGCCGC-3′; b, 5′-GGCAGCTTTAGCTTGAATGTGCTC-3′; c, 5′-CAGAAGCTTC-ATGATGATGG-3′.

Rescue experiments

A region comprising 1 kb 5′ of the *fat-3* start, the entire *fat-3* coding sequence (Napier et al., 1998) and 0.9 kb 3′ of the *fat-3* end rescued the defects associated with *fat-3* mutants. Primers containing *Kpn*I and *Sac*I restriction sites were used to PCR amplify the entire *fat-3* coding sequence. This *Kpn*I-*Sac*I fragment and a *Hin*dIII-*Kpn*I 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 *Hin*dIII-*Bam*HI fragment including the *myo-3(+)* promoter and the *Kpn*I-*Sac*I PCR fragment including the entire *fat-3* coding sequence were cloned into pPD49.26. This *Kpn*I-*Sac*I *fat-3* fragment and a *Pst*I-*Sma*I 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).

For fatty acid rescue experiments, arachidonic acid (AA), docosahexaenoic acid (DHA) or linoleic acid (LIN; Sigma, Poole, UK) were prepared as 100 mg/ml solution in 95% ethanol and 100 µl were spread over nematode growth medium plates. *E. coli* OP-50 were then added as a food source and 3-20 L4-adult *fat-3(lg8101)dpy-20(e1282)/unc-24(e138)fat-3(qa1811)dpy-20(e1282)* hermaphrodites were grown on each plate. These hermaphrodites or their progeny (thus exposed to LC-PUFAs from hatching), were analyzed.

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)* mutants 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 (Improvision, 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 aldicarb (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).

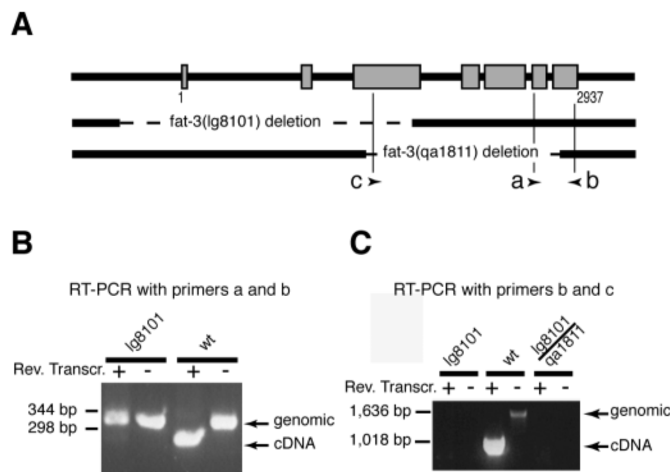

Fig. 2. *fat-3* deleted mutants do not synthesize *fat-3* mRNA. (A) Schematic of the *fat-3(lg8101)* and *fat-3(qa1811)* deletions and the primers used for *fat-3* mRNA analysis. (B,C) No wild-type *fat-3* mRNA is detected in *fat-3(lg8101)* or *fat-3(lg8101)/fat-3(qa1811)* mutant animals. (B) RT-PCR of total mRNA from *fat-3(lg8101)* or wild-type animals with primers a and b, in the presence (+) or in the absence (–) of reverse transcriptase. Predicted PCR products: cDNA, 281 bp; genomic DNA, 326 bp. (C) RT-PCR with primers b and c of total mRNA from *fat-3(lg8101)*, wild-type or *fat-3(lg8101)/fat-3(qa1811)* animals. Predicted PCR products: cDNA, 999 bp; genomic DNA, 1,451 bp.

Visualization of neurons and synapses

To visualize serotonergic neurons in *fat-3* mutants, we constructed the strain *fat-3(lg8101);Is(Ptph-1::gfp,rol-6*^{D}*)*. *tph-1* encodes a tryptophan hydroxylase and is expressed in serotonergic neurons (Sze et al., 2000). Animals were immobilized with 10 mM levamisole, mounted on a 2% agarose pad and observed under a 40× or a 63× objective on a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkoken, Germany).

For electron microscopy, young adult nematodes were fixed by immersion in buffered aldehydes and stained in osmium tetroxide (Hall, 1995) or fast frozen under high pressure followed by freeze substitution into osmium tetroxide in acetone (McDonald, 1999). Samples were embedded into plastic resin, thin sectioned using a diamond knife, counterstained with uranyl acetate and lead citrate, and examined on a Philips CM10 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). We used 2 fixation conditions because *fat-3* mutants are, for some unknown reason, difficult to fix well and the quality of the images obtained was not optimal. However, we found similar results with either fixation method. Both, *fat-3(lg8101)* and *dpy-20(e1282)fat-3(lg8101)/unc-24(e138)fat-3(qa1811)dpy-20(e1282)* mutants displayed similarly depleted synapses. Morphometric analysis was carried out in animals stained with osmium tetroxide. Vesicles of approximately 30 nm diameter were counted. Statistical analysis was performed using SAS/STAT software (SAS Institute Inc., Cary, NC). Significance values were calculated using Student's *t*-test.

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, or *fat-3(lg8101)dpy-20(e1282);Ex(fat-3(+)dpy-20(+))*. 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 cyanoacrylic 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 $CaCl_2$, 4 mM $MgCl_2$, 10 mM glucose, 15 mM Hepes, pH

7.35, and sucrose to 340 mOsm. The pipette solution contained: 120 mM KCl, 20 mM KOH, 4 mM $MgCl_2$, 5 mM N-*tris* (hydroxymethyl) methyl-2-aminoethane-sulphonic acid, 0.25 mM $CaCl_2$, 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-b_5-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 serine is not conserved in human or plant desaturases and it is not clear that this mutation is a null allele. To determine the null phenotype we generated two *fat-3* deletion mutations using PCR to screen chemically mutagenized *C. elegans* libraries with *fat-3* specific primers (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 desaturase 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 (Sayanova et al., 1999) and a large portion of the predicted promoter (Fig. 1B) and results in no detectable *fat-3* mRNA transcripts (Fig. 2B,C). *fat-3(lg8101)* homozygous animals develop very slowly and their phenotype is more severe than that of *fat-3(lg8101)/fat-3(qa1811)*, *fat-3(lg8101)/fat-3(wa22)* and *fat-3(wa22)* homozygotes (data not shown). Therefore, it is likely that *fat-3(lg8101)* is a molecular null while *fat-3(qa1811)* and *fat-3(wa22)* are severe loss-of-function mutations. The phenotype of *fat-3(qa1811)* homozygous animals could not be directly assessed because the *qa1811* allele is tightly associated with an independent lethal mutation.

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

	Percentage (w/w) of total fatty acids				
LC-PUFA	Wild type	*fat-3(lg8101)*	Wild type +DHA	*fat-3(lg8101)* +DHA	*fat-3(lg8101) 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	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	2.1±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%).

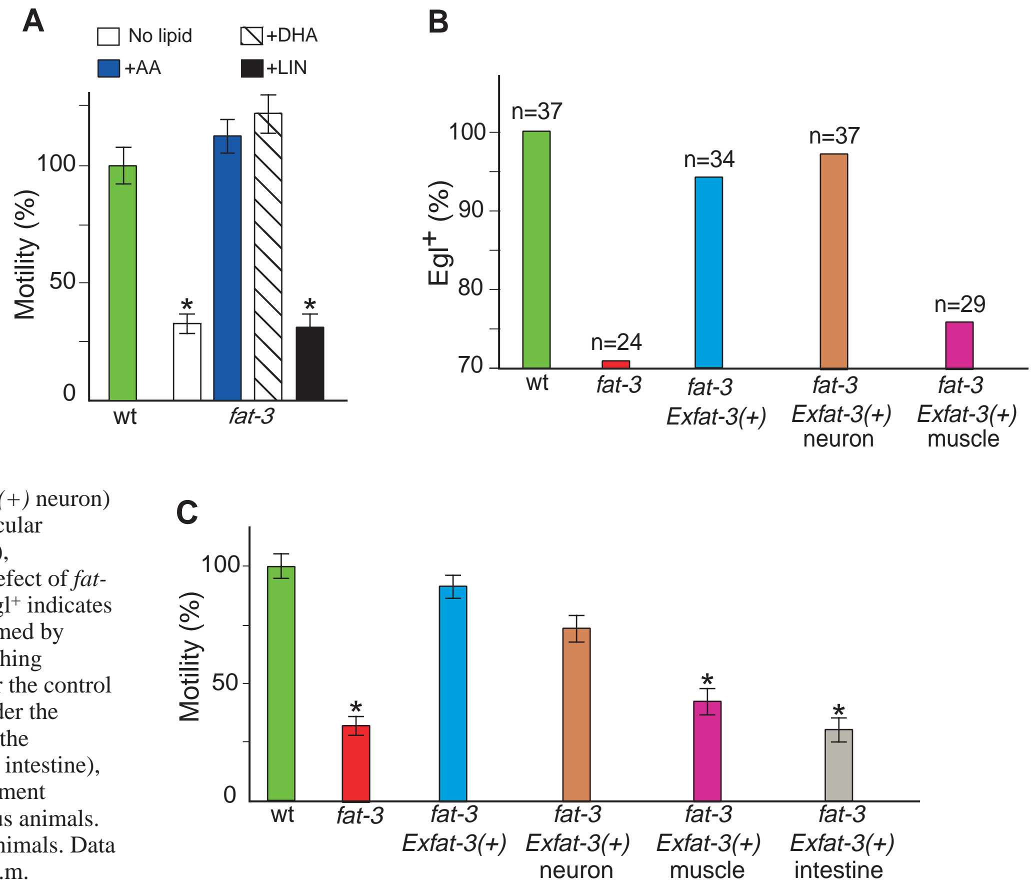

Fig. 3. Rescue of the behavioral defects associated with loss of *fat-3* activity. (A) Exogenous AA and DHA, but not LIN, rescue the movement defects of *fat-3(lg8101)/fat-3(qa1811)* mutants. Animals were exposed to fatty acids from egg to adult. **P*<0.0001 versus wild-type animals. (B) *fat-3* expressed 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.

3 activity might be required for normal neuronal development or function.

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.

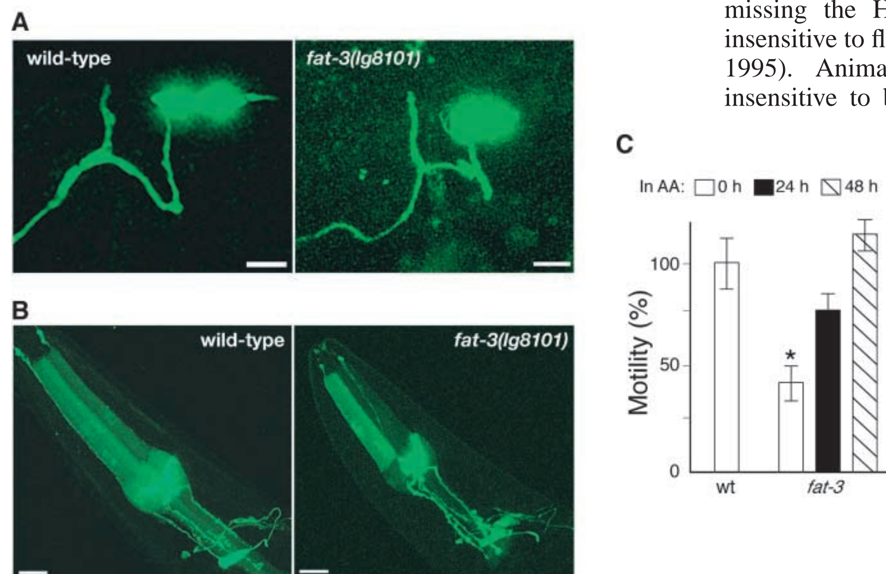

Fig. 4. *fat-3* mutant animals display functional and not morphological neuronal defects. (A,B) Serotonergic neurons visualized with GFP under the control of a tryptophane hydoxylase promoter (*tph-1*), which is expressed in serotonergic neurons (Sze et al., 2000). (A) An HSN motor neuron in proximity of the vulva. Bars, 5 μm. (B) Serotonergic neurons in the head. Images shown are projections of confocal xy sections. Bars, 10 μm. (C) Exogenous arachidonic acid (AA) restores wild-type movement in adult *fat-3(lg8101)/fat-3(qa1811)* mutant animals within 48 hours. **P*=0.0017 versus wild-type animals. Data is plotted as mean ± s.e.m.

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; Weinshenker 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; Weinshenker 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

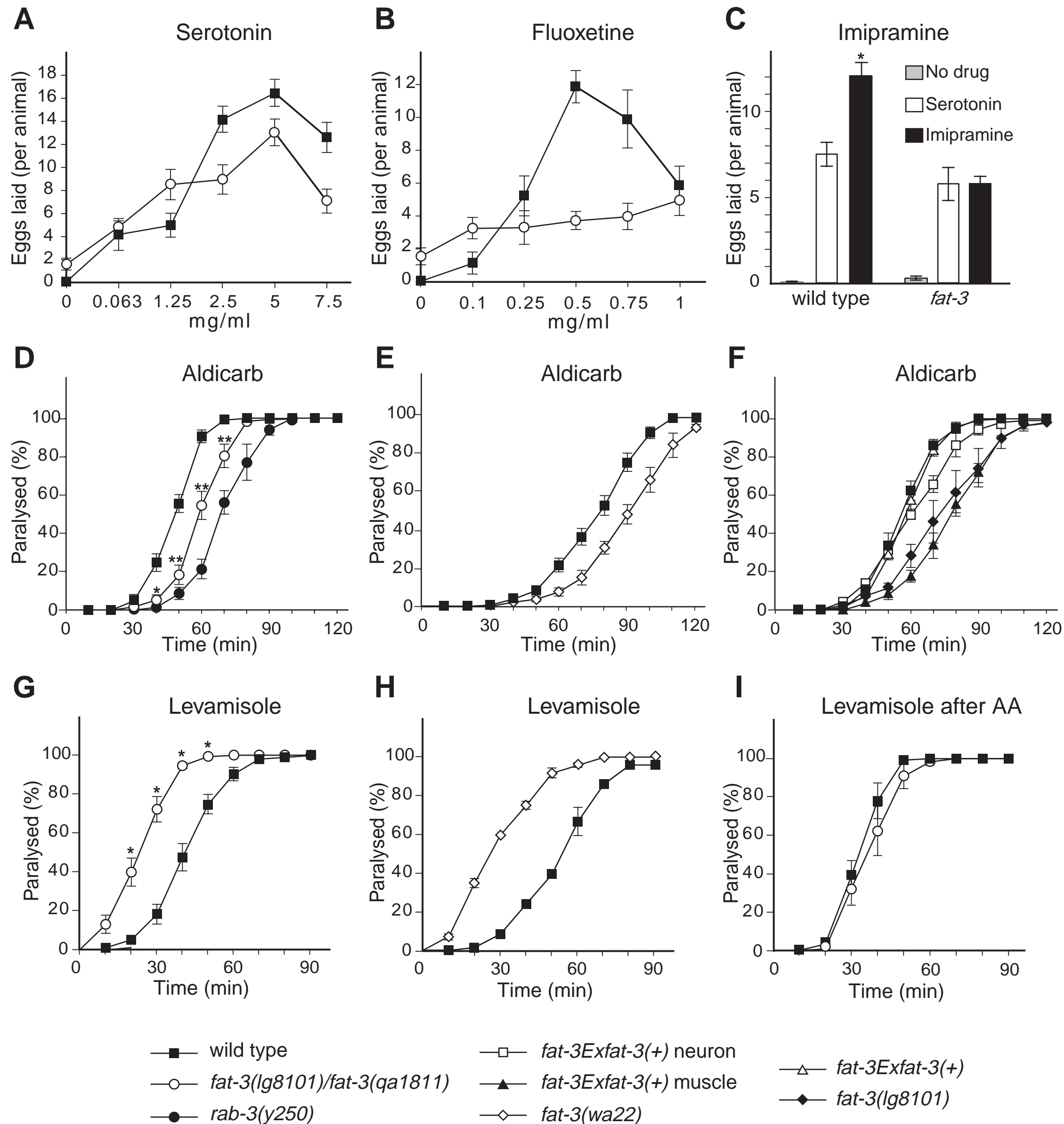

Fig. 5. Cholinergic and serotonergic synapses are functionally disrupted in *fat-3* mutant animals. (A-C) Synaptic release of endogenous serotonin was measured by determining the egg-laying response of *fat-3(lg8101)/fat-3(qa1811)* mutants to serotonin, fluoxetine and imipramine. Muscles are functional in *fat-3* mutants because they respond well to serotonin (A,C). However, they are defective in serotonin release, because they respond only inefficiently to the endogenous serotonin potentiators fluoxetine (B) and imipramine (C). In C, a single dose of serotonin (5 mg/ml) and imipramine (0.75 mg/ml) were used. (D,E) Synaptic release of endogenous ACh was measured by determining the response to the endogenous ACh potentiator aldicarb. *fat-3* mutants are defective in ACh release because aldicarb induces spastic paralysis faster in wild-type animals than in *fat-3(lg8101)/fat-3(qa1811)* (D) or in *fat-3(wa22)* (E) mutants. The defect of *fat-3* mutants is weaker but comparable to that of *rab-3(y250)*, a synaptic vesicle transmission mutant. *$P<0.01$; **$P<0.005$. (F) The altered aldicarb sensitivity of *fat-3* mutants is of neuronal origin. *fat-3* expressed in neurons, but not in muscles, restores normal aldicarb sensitivity to *fat-3(lg8101)* mutants. (G-H) The ACh mimetic levamisole induces paralysis faster in *fat-3(lg8101)/fat-3(qa1811)* (G) and in *fat-3(wa22)* (H) mutants than in wild-type animals. *$P<0.003$. (I) Normal sensitivity to levamisole is restored in *fat-3(lg8101)/fat-3(qa1811)* mutant animals exposed to AA from egg to adult. In all panels, data points show the mean ± s.e.m.

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.

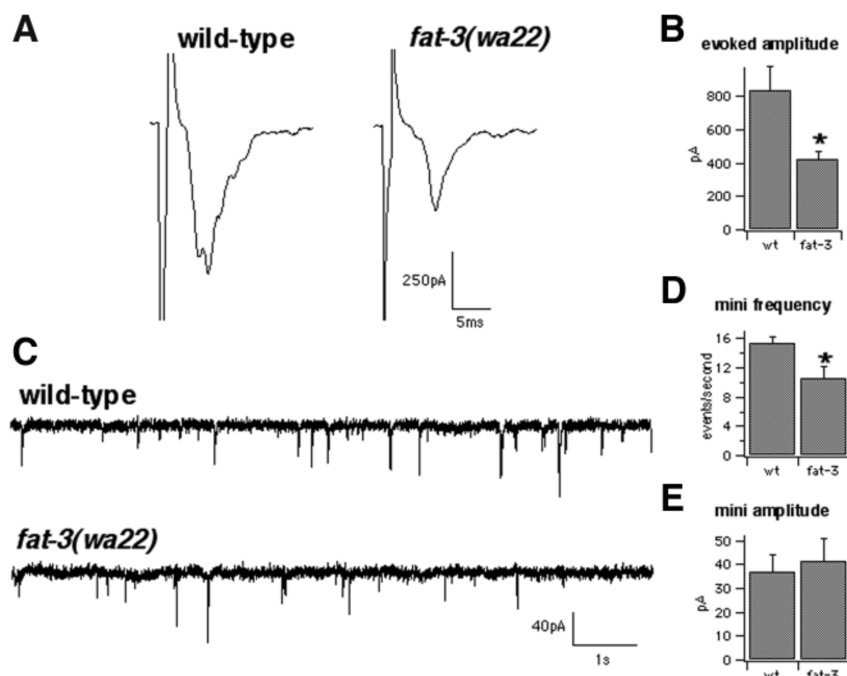

Fig. 6. *fat-3* mutants have reduced evoked amplitude and reduced rates of spontaneous fusion but normal quantal size. (A) Representative evoked responses from wild-type and *fat-3* animals. (B) The mean amplitude of the evoked responses is reduced in *fat-3* (*n*=6) compared to wild-type (*n*=7) animals (*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.

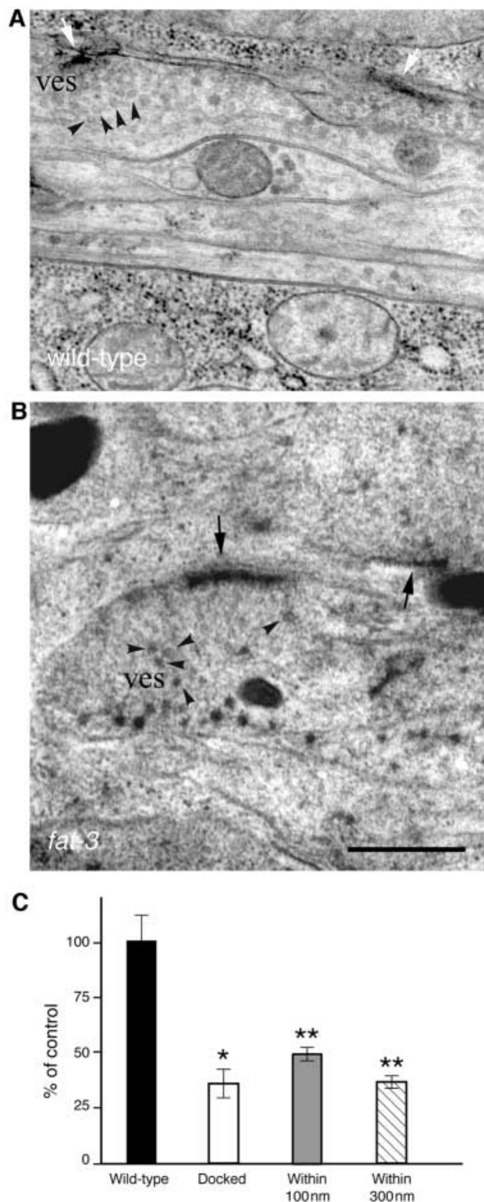

Fig. 7. Synapses are partially depleted of synaptic vesicles in *fat-3* mutant animals. Electron micrographs of wild-type (A) and *fat-3(lg8101)* (B) synapses fixed by fast-freezing. (A) Clusters of vesicles (ves and arrowheads) in ventral nerve cord neurons are localized close or docked to the active zone (white arrows). (B) Ventral ganglion synapses are depleted of most vesicles close to the active zone (black arrows). A few vesicles lie at a distance (ves and arrowheads). (C) Quantification of synaptic vesicles in *fat-3(lg8101)/fat-3(qa1811)* mutant animals. The average fraction of synaptic vesicles per section at given distances in *fat-3* mutants compared to wild-type animals (100%). Vesicles were considered docked when they were touching the active zone. Total number of synapses scored: 50, *fat-3*; 42, wild-type. $*P<0.001$; $**P<0.0001$. Scale bar: 0.5 μm.

Discussion

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 collegues 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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