Polyunsaturated fatty acids and neurotransmission in *Caenorhabditis elegans*

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Abstract

Changes in PUFA (polyunsaturated fatty acid) metabolism can cause mental retardation and cognitive impairment. However, it is still unclear why altered levels of PUFAs result in neuronal dysfunction. Recent studies on the nematode *Caenorhabditis elegans* suggest that PUFA depletion may cause cognitive impairment by compromising communication among neurons. Pharmacological and electrophysiological experiments showed that animals devoid of most PUFAs release abnormally low levels of neurotransmitters. In addition, ultrastructural analysis revealed that synapses in these mutants are severely depleted of synaptic vesicles. The conclusion of these studies is that PUFAs are required to maintain a normal pool of synaptic vesicles at pre-synaptic sites, thus ensuring efficient neurotransmission.

Introduction

One of the central challenges in modern biology is to understand the precise function and regulation of the huge variety of complex lipids present in cell membranes. PUFAs (polyunsaturated fatty acids), fatty acids with two or more double bonds, are synthesized in the endoplasmic reticulum from dietary precursors (Figure 1) and are mostly stored in cell membranes as phospholipid esters. A lipase can hydrolyse these esters and release non-esterified ('free') fatty acids in the cytoplasm. Both the absolute PUFA levels and their relative concentrations are strictly controlled in mammalian neurons implying that PUFAs have critical neuronal functions. Indeed, many correlative studies suggest that PUFAs are essential for normal brain development and function, particularly during the first months of life [1–4]. This is strongly supported by the findings that (i) PUFAs modulate electrical currents in neurons by interfering with a variety of ion channels [5–9], (ii) deprivation of PUFAs results in behavioural deficits [10] and (iii) mutation of enzymes that regulate PUFA metabolism causes serious neuronal diseases. For example, a 5 bp deletion in a PUFA elongase causes macular dystrophy [11] (degeneration of the retina, a type of neuronal tissue) and mutations in a fatty acid–CoA ligase result in an X-linked form of mental retardation [12]. However, until recently, no specific neuronal process had been unambiguously identified to explain the effects of PUFAs on neuronal function or cognition. Now, studies on the nematode *Caenorhabditis elegans* have identified neuronal defects caused by PUFA depletion, which might explain why low levels of PUFAs result in neuronal and cognitive impairment [13,14].

C. elegans offers several advantages to study the role that PUFAs play in the nervous system. First, the location, structure and function of virtually all *C. elegans* neurons are known, and well-established pharmacological assays are available to test synaptic function of specific neurons [15–17]. Secondly, it is possible to study the role of PUFAs independently of the known complex signalling pathways activated by eicosanoids (derivatives of 20-carbon PUFAs involved in many processes, particularly the inflammatory response; see legend to Figure 1), since no genes from these pathways are present in the *C. elegans* genome. Thirdly, PUFAs are largely conserved between humans and nematodes [18] and are therefore likely to have evolutionarily conserved functions. In the present paper, we describe a series of experiments carried out in nematodes, which demonstrate that PUFAs are required for efficient neurotransmitter release.

PUFA-depleted animals display behavioural defects

We, and others, have generated *C. elegans* strains depleted of PUFAs by inactivating the gene *fat-3*, which encodes Δ^6 desaturase, an enzyme that is essential for PUFA biosynthesis [19,20]. These strains are unable to synthesize long-chain PUFAs and accumulate the Δ^6 desaturase substrates linoleic acid and α-linolenic acid (Figure 1). *fat-3* mutants display a range of abnormalities, including egg-laying and locomotion defects. These two phenotypes are often seen in animals with impaired muscle or neuronal function; however, muscle function in *fat-3* mutants is normal. This suggests that the *fat-3* phenotypes are due to impaired neuronal function, a

Key words: *Caenorhabditis elegans*, endocytosis, nervous system, neurotransmitter, polyunsaturated fatty acid (PUFA), synaptic vesicle.

Abbreviation used: PUFA, polyunsaturated fatty acid.

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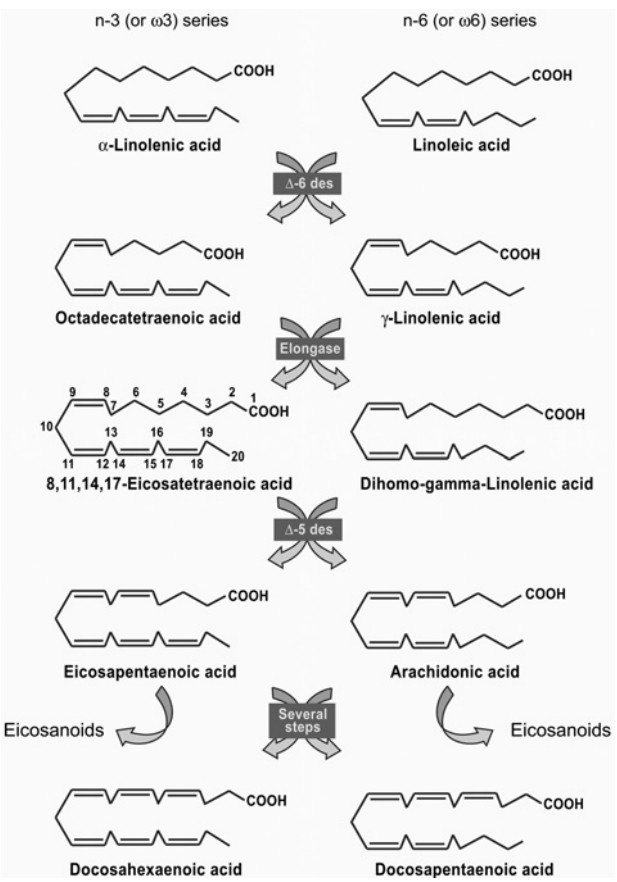
Figure 1 | Simplified PUFA biosynthetic pathway

While *C. elegans* can synthesize the fatty acids α-linolenic acid and linoleic acid, mammals cannot and must ingest these fatty acids with food. Two series of PUFAs exist, the $n-3$ and the $n-6$ series. $n-3$ and $n-6$ indicate the number of carbon atoms separating the last double bond from the terminal methyl group, or ω carbon. Carbon atoms are numbered starting from the carboxy group, as shown for eicosatetraenoic acid. Eicosanoids (prostaglandins, prostacyclins, thromboxanes and leukotrienes) are cyclic compound derivatives of arachidonic acid and eicosapentaenoic acid, which in mammals have critical roles in a variety of processes, including inflammation, fever, regulation of blood pressure and tissue growth. *C. elegans* is unable to synthesize eicosanoids. Δ-5 des, Δ^5 desaturase; Δ-6 des, Δ^6 desaturase.



hypothesis that is supported by the fact that the egg-laying and locomotion defects were rescued by expressing wild-type copies of *fat-3* specifically in the nervous system and not in other tissues. The *fat-3* mutant phenotypes were also rescued by providing exogenous PUFAs, confirming that the neuronal defects observed in *fat-3* mutants are caused by PUFA depletion. Surprisingly, these neuronal defects are not developmental; they were rescued by supplementing PUFAs to adult animals in which the nervous system is fully developed. Moreover, neurons in PUFA-depleted animals are morphologically normal, as visualized with green fluorescent protein or at the ultrastructural level. Therefore PUFAs are required for neuronal function, rather than for the correct development of the nervous system.

Pharmacological evidence of neurotransmission impairment in PUFA-depleted animals

One measurement of neuronal function is the efficiency of neurons in converting physiological stimuli into neurotransmitter release. There are two pharmacological assays commonly used to measure neurotransmitter release into the synaptic cleft in *C. elegans*. The first is the rate of paralysis in response to the chemical aldicarb, which is proportional to the levels of acetylcholine at the synapse [21–23] (Figure 2). The second is the number of eggs laid in response to fluoxetine treatment, which correlates with levels of 5-hydroxytryptamine (serotonin) present at the synapse [24,25]. Results from these pharmacological assays indicated that *fat-3* mutants release abnormally low levels of both acetylcholine and 5-hydroxytryptamine in the synaptic cleft; these animals were less sensitive to aldicarb and laid fewer eggs in response to fluoxetine. Moreover, mutant animals selectively expressing the *fat-3* gene in muscles still displayed locomotion impairment in response to aldicarb, indicating that the neuronal defects of *fat-3* mutants are pre-synaptic. One possible explanation for the decreased amounts of acetylcholine and 5-hydroxytryptamine available post-synaptically is that *fat-3* mutants do not synthesize enough neurotransmitters. However, acetylcholine levels are normal in *fat-3* mutants, suggesting that the neuronal defects observed in PUFA-depleted animals are unlikely to be caused by defects in neurotransmitter synthesis.

The fact that both neurotransmitters, acetylcholine and 5-hydroxytryptamine, are released inefficiently in *fat-3* mutants suggests that the effect of PUFA depletion is not specific, but is likely to affect processes common to both (and probably all) neurotransmitters.

Electrophysiological and ultrastructural evidence suggests that PUFAs are involved in neurotransmitter release

To test directly whether *fat-3* mutants are defective in neurotransmitter release, post-synaptic currents in response to nerve stimulation were measured from voltage-clamped muscles. Evoked currents displayed decreased amplitude in *fat-3* mutants. Endogenous miniature excitatory post-synaptic currents (or minis) were also measured. Minis represent the current generated by the fusion of one or very few synaptic vesicles with the pre-synaptic membrane in the absence of stimulus. The frequency of minis was reduced in *fat-3* mutants by approx. 50%. However, the amplitude was not significantly different between *fat-3* and wild-type animals, suggesting that synaptic vesicles in *fat-3* mutants are filled with normal levels of neurotransmitter. In addition, the fact that currents generated in response to a stimulus as well as the frequency of minis decreased indicated that the number of synaptic vesicles undergoing exocytosis is strongly reduced in

Figure 2 | **Example of a pharmacological test used to compare neurotransmitter release between wild-type and mutant animals**

(**A**) Following a stimulus, synaptic vesicles in cholinergic neurons fuse with the active zone, a specialization of the pre-synaptic membrane, and release acetylcholine into the synaptic cleft. (**B**) In the presence of aldicarb, which inhibits acetylcholinesterase, the same stimulus results in more acetylcholine being available to acetylcholine receptors. This causes muscle hypercontraction and paralysis. The fraction of animals becoming paralysed over time is proportional to the amount of neurotransmitter being released. SYN, synaptic cleft.

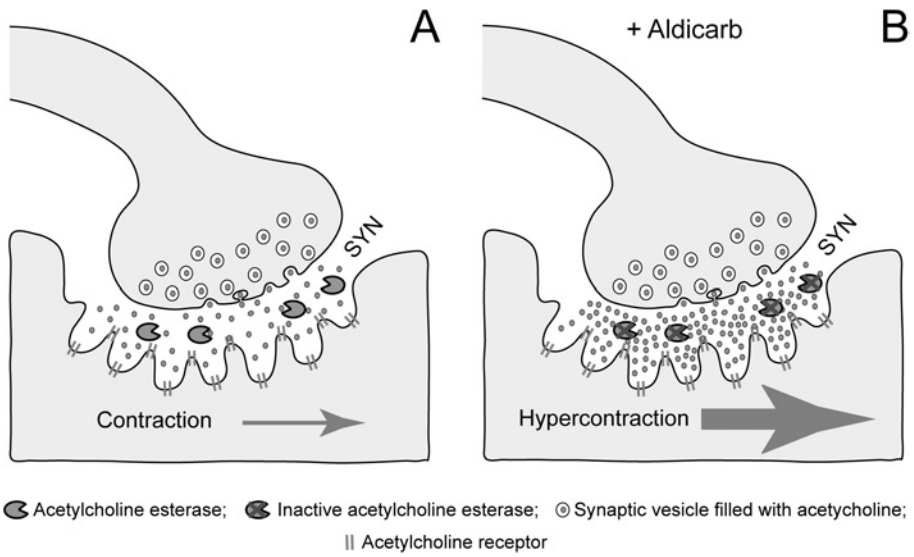
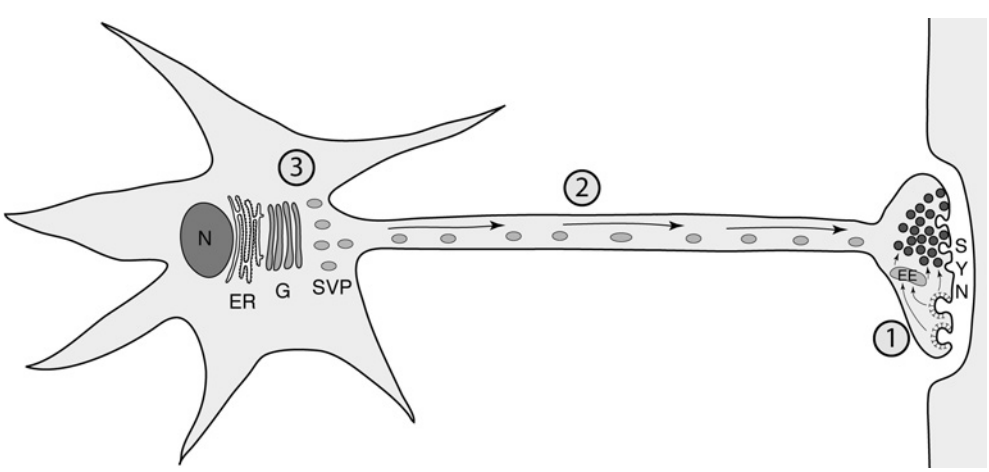


Figure 3 | **Possible mechanisms causing depletion of synaptic vesicles in *fat-3* mutants**

(1) Impaired endocytosis. Synaptic vesicle components are retrieved from the pre-synaptic membrane, endocytosed via coated pits and re-assembled into new synaptic vesicles, in part directly and in part via the early endosome (EE). (2) Hampered transport of synaptic vesicle precursors from the cell body to the sites of release, possibly caused by altered interaction with motors. (3) Insufficient formation of synaptic vesicle precursors in the neuronal cell body. ER, endoplasmic reticulum; G, Golgi apparatus; N, nucleus; SVP, synaptic vesicle precursor; SYN, synaptic cleft. See text for details.



fat-3 mutants. Ultrastructural analysis of synapses confirmed that *fat-3* mutants had fewer synaptic vesicles when compared with wild-type animals. This indicates that animals devoid of PUFAs release less neurotransmitter because they are depleted of synaptic vesicles, rather than because of a defect in vesicle fusion [26]. Indeed, a defect in fusion would have caused an accumulation of synaptic vesicles at pre-synaptic sites [27].

Possible mechanisms of synaptic vesicle depletion in PUFA-depleted animals

The mechanisms underlying the depletion of synaptic vesicles observed in *fat-3* mutants have not been clarified yet. However, it is likely that one or more of the following defects are responsible for the abnormally low number of synaptic vesicles at pre-synaptic sites (Figure 3). First, *fat-3* mutants

could be defective in endocytosis, the process which retrieves synaptic vesicle components from the plasma membrane [28]. Decreased recovery of such components would result in formation of fewer releasable vesicles [15]. Secondly, synaptic vesicle precursors [29] could be transported inefficiently from the *trans*-Golgi apparatus to pre-synaptic sites. For example, the altered membrane composition in *fat-3* mutants could impair the interaction of synaptic vesicle precursors with motors [30]. This would result in accumulation of vesicular components in the cell body, and possibly in axons. Thirdly, *fat-3* mutants could be defective in the biogenesis of synaptic vesicle precursors. These precursors mature while they move from the endoplasmic reticulum through the Golgi apparatus [31], a process that involves budding and fusion of vesicles. It is known that specific lipids affect membrane curvature differently [32]. The lack of PUFAs in *fat-3* mutants may alter the membrane curvature or the sorting or functioning of proteins involved in vesicle budding/fusion, thus slowing down vesicle biogenesis. Future experiments should discriminate among these mechanisms.

Although *fat-3* mutants display both neuronal and non-neuronal defects, the most prominent deficiencies appear to be of neuronal origin. Why would neurons be more affected than other cells by the lack of PUFAs? In mammals, long-chain PUFAs are highly enriched in neurons [2], suggesting that these cells would suffer most when PUFAs are not available. In addition, it has been reported that certain PUFAs are particularly abundant in synaptic vesicles [33,34]. Therefore, on the basis of these findings, one would predict that *fat-3* neurons, which lack most PUFAs, would be especially impaired in their synaptic vesicle function. Indeed, this is exactly what was observed. However, the details on how PUFAs contribute to synaptic vesicle function remain to be elucidated. No doubt, this will keep researchers occupied for many years.

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