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Mutations in the dystrophin-like *dys-1* gene of *Caenorhabditis elegans* result in reduced acetylcholinesterase activity

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Abstract Mutations of the *Caenorhabditis elegans* dystrophin/utrophin-like *dys-1* gene lead to hyperactivity and hypercontraction of the animals. In addition *dys-1* mutants are hypersensitive to acetylcholine and acetylcholinesterase inhibitors. We investigated this phenotype further by assaying acetylcholinesterase activity. Total extracts from three different *dys-1* alleles showed significantly less acetylcholinesterase-specific activity than wild-type controls. In addition, double mutants carrying a mutation in the *dys-1* gene plus a mutation in either of the two major acetylcholinesterase genes (*ace-1* and *ace-2*) display locomotor defects consistent with a strong reduction of acetylcholinesterases, whereas none of the single mutants does. Therefore, in *C. elegans*, disruption of the dystrophin/utrophin-like *dys-1* gene affects acetylcholinesterase activity.

Key words: Dystrophin; Duchenne muscular dystrophy; Nematode; Acetylcholine

1. Introduction

Duchenne and Becker muscular dystrophies are allelic forms of progressive myopathies caused by the disruption of the dystrophin gene. This gene encodes a 3685 amino acid protein found in skeletal and cardiac muscles and in the nervous system [1,2]. The function of the dystrophin protein is not clearly established yet. It has been proposed that dystrophin is a structural protein whose function is to increase membrane stability during repeated cycles of muscular contraction [1,3]. The physiological function of dystrophin overlaps with that of utrophin, a protein very similar to dystrophin and expressed in most cell types [4]. Dystrophin is present under the sarcolemma of skeletal muscles, where it binds cortical actin via its N-terminal region. Through its C-terminal region, dystrophin binds to a complex of membrane proteins termed the dystrophin-associated proteins (reviewed in [5]).

The genome of *Caenorhabditis elegans* contains a dystrophin/utrophin homologue named *dys-1*. We previously reported that loss-of-function mutations of the *dys-1* gene lead to a complex phenotype including hyperactivity, hypercontraction, and hypersensitivity to acetylcholine and to the acetylcholinesterase inhibitor aldicarb [6]. The *dys-1* gene was shown to be expressed and required in muscle tissue [6] where its absence may result in increased muscle excitability.

C. elegans acetylcholinesterases are encoded by at least four genes differing in their substrate and inhibitor specificities (reviewed in [7,8]). Most of acetylcholinesterase activity is contributed by the genes *ace-1* and *ace-2*, while *ace-3* and *ace-4* play only a minor role. Work performed on both *C. elegans* and the closely related species *Steinernema carpocapsae* suggests that *ace-1* generates a tetrameric form similar to the mammalian amphiphilic G4 form, and *ace-2* generates a glycolipid-anchored dimer [7,9]. The genetic tractability of *C. elegans* has permitted the isolation of mutants of *ace-1*, *ace-2* and *ace-3* [10–12]. None of these mutants displays a major developmental or behavioral phenotype, indicating that the worm is not dramatically affected by the loss of one of these genes. However, *ace-1 ace-2* double mutants, lacking approximately 90% of acetylcholinesterase activity, display severely uncoordinated locomotion [11]. In contrast, *ace-1 ace-3* and *ace-2 ace-3* double mutants appear normal. The triple mutant *ace-1 ace-2 ace-3* is paralyzed and developmentally arrested, dying at the hatching stage [12]. Genetic mosaic analysis suggests that *ace-1* activity is required in the muscles of the animal, presumably at the neuromuscular junction [12,13].

C. elegans animals lacking the functional dystrophin homologue *dys-1* do not display any obvious muscular degeneration phenotype, but are hyperactive and hypercontracted. The ability of the human dystrophin cDNA to partly suppress this phenotype indicates that a common function has been conserved between the two proteins [6]. Moreover, the presence of several known partners of dystrophin in the *C. elegans* genome further suggests that an ancestral function of dystrophin has been maintained through evolution. Our current work with *C. elegans* dystrophin aims at determining both the nature of this ancestral function and the common denominator that could lead to phenotypes as different in appearance in mammals (muscle degeneration) and nematodes (hyperactivity and hypercontraction).

In this study, we investigated whether the phenotypes observed in *C. elegans dys-1* mutants could be explained by a decrease in acetylcholinesterase activity. We report that worm extracts obtained from three different *dys-1* alleles show a significant reduction of total acetylcholinesterase activity, compared to wild-type. This indicates that, in *C. elegans*, the absence of dystrophin affects acetylcholinesterases.

2. Materials and methods

2.1. Animal strains

Strains used in this study were obtained from the Caenorhabditis Genetic Center (CGC), Minneapolis, MN, USA. *dys-1* alleles were isolated in our laboratory. The reference N2 strain was used as a

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wild-type control. PR1000: *ace-1(p1000)*; GG201: *ace-1(p1000) ace-2(g72)*; GG202: *ace-2(g72)*. *dys-1* alleles *cx18, cx26* and *cx35* are described in [6]. Their molecular nature suggests that these are severe loss-of-function or null mutations of the gene. *dys-1(cx26) ace-1(p1000)* and *dys-1(cx26) ace-2(g72)* double mutants were constructed following standard genetic techniques [14], and their genotype was ascertained by molecular analysis.

2.2. Acetylcholinesterase (AChE) assay

AChE activity was assayed in total worm extracts by the method of Ellman et al. [15]. Worms were extracted using silica beads in low-salt buffer (10 mM Tris, pH 7.5) containing 1% Brij 97 (10-oleyl-ether, Sigma) and antiproteolytics (aprotinin, 7.5×10^{-3} TIU, bacitracin, 0.1 mg/ml, benzamidine, 1 mM, EDTA, 1 mM). After centrifugation (30 min at $12\,000\times g$), AChE activity was determined by the spectrophotometric method of Ellman et al. [15] using 1 mM acetylthiocholine as a substrate. Protein concentration was estimated with the Bradford assay (Bio-Rad, DC Protein Assay).

3. Results and discussion

3.1. Reduced AChE activity measured in dys-1 mutants

We assayed AChE activity on worm extracts of wild-type and *dys-1* animals. *ace-1, ace-2* and *ace-1 ace-2* mutants were used as internal controls. The results are presented in Fig. 1. All three *dys-1* alleles tested, *cx18, cx26* and *cx35*, showed a significant reduction of the AChE activity ($P<0.05$), ranging from 59% to 69% of wild-type activity. In an attempt to determine which class of acetylcholinesterase was affected by the *dys-1* mutations, we then assayed the AChE activity of *ace-1 dys-1(cx26)* and *ace-2 dys-1(cx26)* double mutants (Fig. 1). The double mutants showed a reduced activity compared to *ace-1* and *ace-2* single mutants, albeit non-cumulative.

We previously showed that *dys-1* phenotypes are probably due to a lack of *dys-1* expression in muscles, since *dys-1* is detected only in muscles, and *dys-1* animals can be rescued when this gene is driven by a muscle-specific promoter [6]. This leads to two major (and non-exclusive) hypotheses for the interpretation of the phenotype: either an increase of muscle excitability or a decrease of acetylcholinesterase activity. In this study, we have tested the second hypothesis, and show that it is consistent with the data obtained.

Unlike mammals and *Drosophila*, *C. elegans* has four acetylcholinesterase genes [10,8]. *ace-1* and *ace-2* contribute approximately 95% of the total AChE activity. The *ace-1* mutation *ace-1(p1000)* is likely to be a null mutation because it is caused by a premature stop codon leaving a predicted protein of 98 instead of 620 amino acids [16]. The molecular nature of *ace-2(g72)* and of *ace-3(dc2)* is unknown.

The fact that single mutants in each of these genes, *ace-1(p1000)* and *ace-2(g72)*, have no behavioral phenotype suggests that they functionally overlap. However, *ace-1* is expressed mainly in muscle cells [12,13,17] whereas *ace-2* is expressed mainly in neurons [18]. Once synthesized, both enzymes are likely to be transported and anchored to synaptic sites where both are able to hydrolyze acetylcholine. We speculate that these steps might be affected by the lack of the *dys-1* product. We then tested the significance of our in vitro results by an in vivo assay.

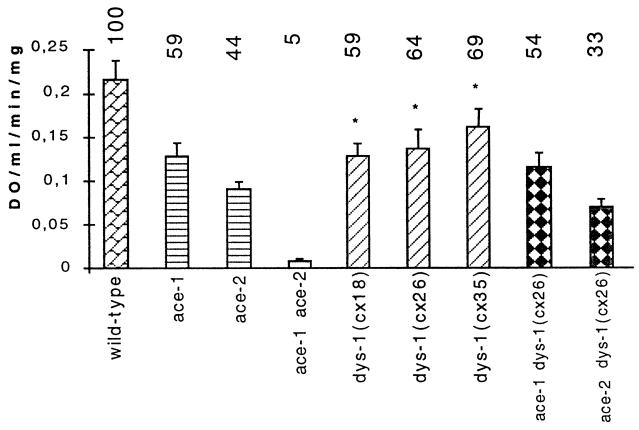

Fig. 1. Acetylcholinesterase-specific activity in wild-type and mutant backgrounds. Bars represent the mean AChE-specific activity for each genotype. Error bars indicate the S.E.M. Numbers above each bar indicate the percentage of wild-type activity. Each bar represents a minimum of eight measurements. Values were compared by a paired Student's *t*-test. Asterisks indicate statistically significant differences to wild-type with $P<0.05$. All three *dys-1* alleles tested showed reduced AChE activity.

3.2. Behavioral defects of ace-1 dys-1(cx26) and ace-2 dys-1(cx26) double mutants

The rationale for this experiment was as follows: if *dys-1* mutations reduce acetylcholinesterase activity in the animal, then combining a *dys-1* mutation with either the *ace-1(p1000)* or the *ace-2(g72)* mutation may impair animal locomotion by bringing acetylcholinesterases under a critical threshold.

Our results show that this is the case: whereas *ace-1* and *ace-2* mutants locomote normally, *ace-1 dys-1(cx26)* and *ace-2 dys-1(cx26)* double mutants are uncoordinated and hypercontracted in a way reminiscent of *ace-1 ace-2* double mutants (also similar to worms exposed to acetylcholinesterase inhibitors), but with a milder phenotype (Table 1). In particular, the animals have difficulties moving backwards and often fail to produce the appropriate locomotor response when prodded either anteriorly or posteriorly. They instead hypercontract

Table 1
Behavioral phenotypes of *dys-1 ace-1* and *dys-1 ace-2* mutants

Genotype	Locomotor activity	Forward locomotion	Backward locomotion	Hypercontraction
N2	+++	+++	+++	−
dys-1(cx26)	++++[a]	+++	++	+
ace-1(p1000)	+++	+++	+++	−
ace-2(g72)	+++	+++	+++	−
ace-1(p1000) ace-2(g72)	−	+	−	+++
dys-1(cx26) ace-1(p1000)	+	+	+	++
dys-1(cx26) ace-2(g72)	++	++	+	+

Semi-quantitative description of the locomotor pattern of the animals. (wild-type is rated +++, excepted for column 'hypercontraction' where wild-type is rated −). Locomotor activity describes the spontaneous activity of the animals. Forward and backward locomotion describe the ability of the animals to produce wild-type-like sinusoidal tracks. Hypercontraction describes the tendency to hypercontract when prodded.
[a]*dys-1* animals are spontaneously hyperactive.

for a few seconds before relaxing. This behavioral defect is common to most hypercontracting mutations in *C. elegans*.

Locomotor defects in *C. elegans* can be of many different types. Strikingly, the ones exhibited by the double mutants *ace-1 dys-1* and *ace-2 dys-1* resemble those of *ace-1 ace-2* animals, as well as phenocopies obtained by exposing wild-type animals to the AChE inhibitor aldicarb.

These results, as well as the reduction of AChE activity found in *ace-1 dys-1* and *ace-2 dys-1* mutants, suggest that the *dys-1* mutation affects both *ace-1* and *ace-2*. The nature of the interaction between *ace* genes and *dys-1* remains to be explained.

In the mouse model of Duchenne muscular dystrophy, the *mdx* mouse, Oliver and coworkers have reported a modification of the distribution of AChE patches in muscles observed by fluorescence microscopy [19]. Another notable effect of the *mdx* mutation is an elevated level of acetylcholinesterase in the serum [19]. It is likely that this excess of AChE activity originates from the muscles.

How can the mouse and *C. elegans* data be reconciled? One common interpretation is to say that dystrophin absence leads to local disorganizations of the muscle membrane. As a consequence, acetylcholinesterase anchoring might be perturbed, leading to elevated serum levels in mice and reduced total activity in the worm. For example, some forms of AChE bind to perlecan, which itself binds to α-dystroglycan, a component of the dystrophin complex [20]. One could speculate that the lack of dystrophin, known to perturb the dystrophin complex, would in turn modify membrane and extracellular matrix clues necessary for proper AChE attachment. Such a local disorganization would also explain why other proteins, like the neuronal nitric oxide synthase, are mislocalized in *mdx* mice [21].

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