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ORIGINAL PAPER

# Blocking of striated muscle degeneration by serotonin in *C. elegans*

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**Abstract** Prevention of muscle fiber degeneration is a key issue in the treatment of muscular dystrophies such as Duchenne Muscular Dystrophy (DMD). It is widely postulated that existing pharmaceutical compounds might potentially be beneficial to DMD patients, but tools to identify them are lacking. Here, by using a *Caenorhabditis elegans* model of dystrophin-dependent muscular dystrophy, we show that the neurohormone serotonin and some of its agonists are potent suppressors of muscle degeneration. Inhibitors of serotonin reuptake transporters, which prolong the action of endogenous serotonin, have a similar effect. Moreover, reduction of serotonin levels leads to degeneration of non-dystrophic muscles. Our results demonstrate that serotonin is critical to *C. elegans* striated muscles. These findings reveal a new fonction of serotonin in striated muscles.

**Keywords** Serotonin · *C. elegans* · Muscular dystrophy · Striated muscles · Dystrophin

## Introduction

Duchenne muscular dystrophy (DMD) is a progressive disorder affecting striated and cardiac muscles. It is caused by mutations in the dystrophin gene, and there is still no efficient treatment against this disease (Koenig et al. 1987; Ahn and Kunkel 1993; Dubowitz 2000; Khurana and Davies 2003). Alongside gene therapy and other novel approaches, random screens of bioactive molecules still represent a serious therapeutic option for the treatment of DMD. To conduct such large screens, the invertebrate model *Caenorhabditis elegans* is an apealling system, since it has striated muscles (Waterston 1988), a dystrophin-like gene (*dys-1*) (Bessou et al. 1998), and can be grown at low cost. Moreover, since *C. elegans* is unable to regenerate lost muscle cells, muscle cell survival can be quantitated rapidly and unambiguously (Gieseler et al. 2000).

Although null mutations of the *C. elegans dys-1* gene occasionally lead to muscle cell death in aging animals (Grisoni et al. 2003), such a low penetrance phenotype offers only a limited window for the search of suppressing effects. To circumvent this problem, we combine a null *dys-1* mutation with a weak and almost silent mutation of the *CeMyoD/hlh-1* gene (encoding a transcription factor involved in the development of muscles), which fragilizes the muscles (Gieseler et al. 2000). In the double mutant, the synergistic effect of the two mutations results in a progressive muscle degeneration affecting up to 30% of the body wall muscle cells responsible for locomotion (Gieseler et al. 2000). *dys-1*; *CeMyoD* mutant animals are therefore severely paralysed as they age (Gieseler et al. 2000). We are making use of this phenotype to screen random molecules that may be active against DMD. By following this approach, we found that, quite unexpectedly, the neuro-hormone serotonin is a potent blocker of striated muscle degeneration.

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## Materials and methods

### Strains and culture conditions

*C. elegans* strains were cultured at 15°C on 6 cm Petri dishes containing NGM agar and a lawn of *E. coli* OP50 unless stated otherwise. The *dys-1(cx18)*; *CeMyoD(cc561)* strain was grown at 15°C, which is the permissive temperature for the ts mutation *CeMyoD(cc561)* (Gieseler et al. 2000). All subsequent strains carrying *CeMyoD(cc561)* were also grown at 15°C. The wild-type N2 strain and other mutant strains were obtained from the *Caenorhabditis* Genetics Center.

### Pharmacological compounds

All pharmacological compounds were obtained from Sigma Chemical Co. (St. Louis, MO). Concentrated solutions of each drug were prepared by dissolving compounds in water (M9 medium for serotonin). Compounds were added to liquid NGM that had been autoclaved and cooled to 55°C, and the media was immediately dispensed into Petri dishes. Drugs used in this study were: serotonin creatinine sulfate (4 mg/ml); serotonin hydrochloride (2 mg/ml); Fluoxetine hydrocloride (0.05 mg/ml); Imipramine hydrocloride (0.1 mg/ml); Trimipramine maleate (0.1 mg/ml); m-chlorophenyl piperazine (0.05 mg/ml); CGS12066A (0.012 mg/ml); α-methyl-5-hydroxytryptamine maleate (0.2 mg/ml); N-methyl quipazine (0.2 mg/ml); m-chlorophenyl biguanide (0.5 mg/ml); 2-methyl-5-hydroxytryptamine (0.3 mg/ml). These concentrations were chosen because they were the highest concentration obtainable on plates which does not slow worm development. 5–7 adult gravid worms were put on plates in the presence of the drug for one night and removed, so as the progeny were exposed to drug from hatching to fixation. L4 larvaes were picked to a new drug containing plate, allowed to develop into adults, and fixed 72 h later.

### Dosing of serotonin inside the animals

To dose serotonin levels inside the serotonin-treated animals, we measured the serotonin concentration by HPLC. After overnight exposure to 10 mM serotonin in liquid, animals were quickly washed with water, and an extract was made by three passes through a French Press (12,000 psi). The supernatant (3000 g, 5 mn) was injected into a C18 liquid chromatography column, 18 cm, $3.9 \times 150$ mm, and serotonin was detected by fluorometry as in Yamada et al. (1983). The column was previously calibrated with serial dilutions of serotonin creatinine sulfate (Sigma H-7752).

### Muscle observation and analysis

Muscle observation was performed by rhodamine coupled-phalloidin staining, a marker of filamentous actin. Staining was done according to Waterston et al. (1984). Slides were observed on a Zeiss Axioplan microscope. A body wall muscle cell is considered degenerating when its actin fibers are fragmented or destroyed (Gieseler et al. 2000). Only the 2 most visible muscle quadrants of each animal were scored (40 cells per animal). Numbers were compared by a Student's *t*-test.

## Results

We exploited the *C. elegans* model of dystrophin-dependent muscle degeneration in a large screen of hundreds of bioactive molecules to identify potential blockers of muscle degeneration. The relevance of such a screen has been previously demonstrated (Gaud et al. 2004). During this screen, we observed by visual inspection that adult animals grown on 10 mM serotonin plates moved better than non-treated animals, suggesting that the function of body wall muscles in these animals was partially restored. Serotonin was active both as a creatinine–sulfate salt and a hydrochloride salt. A dose–response experiment showed that the effect of serotonin was qualitatively visible down to 2.5 mM. Examination of the musculature of *dys-1*; *CeMyoD* animals by phalloidin staining, which labels actin fibers, revealed a dramatic reduction of muscle degeneration in treated animals (Fig. 1). The number of missing or degenerating cells was significantly reduced from $12.3 \pm 3.8$ per animal to $2.5 \pm 1.2$ (Table 1). Visualization of muscles with anti-myosin antibodies (Miller et al. 1983) confirmed this finding (data not shown). Since the animal model analysed contains both a null mutation of *dys-1* and a mild mutation of *CeMyoD*, which act synergistically to produce muscle degeneration, we needed to determine on which of the two mutations serotonin was active. *dys-1(cx18)* animals could not be tested directly because muscle degeneration is not enough penetrant in these animals. Instead, we used *dys-1(cx18)*; *egl-19(ad695)* animals, which are subject to a limited muscular degradation when they age (Mariol and Segalat 2001). This degradation was completely suppressed by the serotonin treatment ($0.7 \pm 0.2$ down to $0.1 \pm 0.1$; Table 1), demonstrating that serotonin is

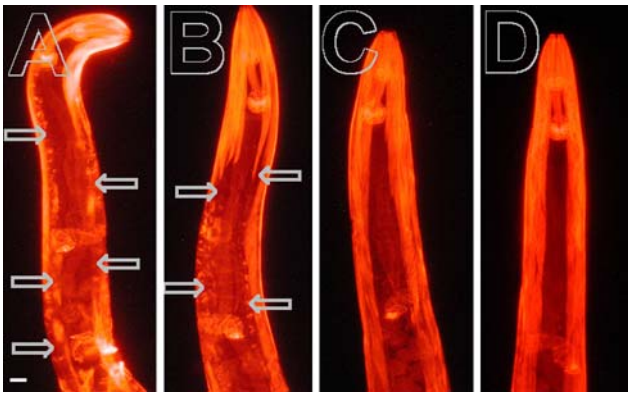


**Fig. 1** Representative examples of *C. elegans* muscles in absence and in presence of serotonin. Striated body-wall muscles of dystrophin-deficient animals (genotype *dys-1(cx18); CeMyoD(cc561)*) shown after fixation and phalloidin-rhodamine incubation to visualize the actin fibers. Body-wall muscles appear as 2 stripes of diamond-shaped cells on each side of the animal. Non-treated animals (**A**, **B**) show a high number of degenerating cells (arrows) rarely seen in animals treated with serotonin at 4 mg/ml (**C**, **D**). Animals were grown in constant presence of food at 15°C and fixed 3 days after the L4-stage. Images are approximately 300 × 100 micrometers. Bar in **A** is 10 micrometers

beneficial to dystrophin-deficient animals independently of the CeMyoD mutation. In the reciprocal experiment, we tested the response of *CeMyoD(cc561)* animals to serotonin. Muscle degeneration was also slightly reduced from 0.8 ± 0.1 to 0.3 ± 0.1, indicating that serotonin is also beneficial to muscles with impaired *MyoD* function.

To confirm the action of serotonin, we hypothesized that drugs known to increase endogenous serotonin levels should produce results similar to serotonin. Endogenous serotonin levels can be increased by drugs inhibiting the reuptake of serotonin. We tested the effect of three molecules of this class: Fluoxetine (Prozac), Imipramine and Trimipramine. All three drugs also significantly reduced muscle degeneration, albeit less significantly than serotonin (Table 1). Other amines (Dopamine and Octopamine) showed no effect (data not shown).

Along the same lines, we applied to *dys-1*; *CeMyoD* animals various serotonin agonists representative of the major serotonin receptor types. Only m-chlorophenyl piperazine and N-methyl quipazine significantly reduced the muscular degeneration (Table 1). However, their effect was lower than that of serotonin, suggesting that serotonin might act through several pathways in parallel to protect *C. elegans* muscles from degeneration. In mammals, m-chlorophenyl piperazine, and N-methyl quipazine are 5-HT1 and 5-HT3 agonists respectively. Since the typology of 5-HT agonists is probably different in *C. elegans*, it is difficult to infer at this point which serotonin receptor type is involved in the response to serotonin. We attempted to block the serotonin response by inhibiting the expression of all known serotonin receptors (Olde and

**Table 1** Effect of serotonin and related compounds on the muscle of dystrophin-deficient mutants

| Compound (concentration in medium) | Class | Number of missing or degenerating muscle cells per animal ± s.e.m. *dys-1(cx18); CeMyoD (cc561)* animals | Number of missing or degenerating muscle cells per animal ± s.e.m. *dys-1(cx18); egl-19(ad695)* animals |
|---|---|---|---|
| Control (water) | | 12.3 ± 0.7 (30) | 0.7 ± 0.2 (30) |
| Serotonin (creatinine, 10 mM) | | 2.5 ± 0.2* (40) | 0.1 ± 0.1* (30) |
| Serotonin (hydrochloride, 10 mM) | | 2.7 ± 0.2* (40) | N.D. |
| Fluoxetine (150 μM) | Reuptake blocker | 4.6 ± 0.5* (20) | N.D. |
| Imipramine (300 μM) | Reuptake blocker | 6.2 ± 0.6* (20) | N.D. |
| Trimipramine (250 μM) | Reuptake blocker | 4.5 ± 0.3* (20) | N.D. |
| m-chlorophenyl piperazine (250 μM) | 5HT1 agonist | 3.9 ± 0.5* (20) | N.D. |
| CGS12066A (26 μM) | 5HT1B agonist | 10.1 ± 0.7 (20) | N.D. |
| a-methyl-5-hydroxytryptamine (650 μM) | 5HT2 agonist | 7.8 ± 0.6 (20) | N.D. |
| N-methyl quipazine (400 μM) | 5HT3 agonist | 4.5 ± 0.4* (20) | N.D. |
| m-chlorophenyl biguanide (2 mM) | 5HT3 agonist | 9.1 ± 0.5 (20) | N.D. |
| 2-methyl-5-hydroxytryptamine (1 mM) | 5HT3 agonist | 9.5 ± 0.5 (20) | N.D. |

Drugs were mixed with the medium at the concentration indicated. Drugs were dissolved in water, excepted for serotonin which was dissolved in M9. Muscle cells were observed after phalloidin staining of animals fixed 3 days after the L4-stage. Animals were grown in constant presence of food and drugs at 15 °C

Mean ± s. e. m.: * different from control at $p < 0.05$. N.D.: not determined. The number in parentheses is the number of scored animals

McCombie 1997; Hamdan et al. 1999; Hobson et al. 2003; Ranganathan et al. 2000). These experiments gave only modest results with respect to serotonin response inhibition (data not shown). Thus, our current intrepretation is that serotonin blocks muscle degeneration either by acting through several receptors simultaneously or by acting through a so far uncharacterized receptor.

Short-term exposure to serotonin is known to result in reduced locomotor activity in *C. elegans (17)*. Therefore, one could wonder whether the protective effect of serotonin and agonists observed in this study is caused by a reduction of muscle activity. Two strong arguments rule out this hypothesis. First, modulation of locomotion by serotonin in *C. elegans* is subject to habituation after a few hours, a negligible time period compared to the length of the experiments presented here. Second, dystrophic animals treated with serotonin actually did move better than control animals (4.5 ± 1 vs. 0.5 ± 0.4 body bends per minute). Therefore, the action of serotonin on *C. elegans* muscles is not a general property of antagonists of muscle function.

We next wondered what is the critical period for serotonin treatment. To address that question we transferred a synchronized culture of animals from control plates to serotonin plates at various time points. Animals were fixed and examined at day 7, after having spent 0, 1, 2, 3, 4, 5, or 7 days on serotonin containing plates. Serotonin was active in reducing muscle degeneration in animals that had been exposed for as little as 1 day (i.e. transferred at day 6) (Fig. 2). Day 6 corresponds to the peak of degeneration in absence of treatment (Gieseler et al. 2000). The reciprocal experiment, in which animals were transferred from serotonin plates to control plates, was carried out in parallel. Interestingly, animals exposed to serotonin for only the first four days of their life and then cultured on control plates were protected (Fig. 2), despite the fact that degeneration begins only at day 5 in this model (Gieseler et al. 2000). Since serotonin is probably metabolized rapidly in *C. elegans*, this result suggests either that serotonin induces a protective mechanism that lasts several days, or that dystrophin-dependent muscle degeneration in *C. elegans* is determined during larval stages although it occurs only later in adulthood.

Since increasing serotonin levels is beneficial to *C. elegans* striated muscles in the absence of DYS-1, we wondered whether reduced serotonin levels would have a deleterious effect on the same muscles. To test this hypothesis, we looked at the muscles of *tph-1(mg280)* animals, which have reduced serotonin levels due to a mutation in the serotonin biosynthetic pathway (Sze et al. 2000). No muscle degeneration was observed in *tph-1(mg280)* animals. However, when placed in the sensitized background *CeMyoD(cc561)*, the *tph-1* mutation resulted in a severe muscle degradation (Table 2). Thus, the negative effect of the *tph-1* mutation on *C. elegans* muscles was revealed in the *CeMyoD(cc561)* background. As we previously observed for the *dys-1* mutations, the muscle degradation caused by *tph-1(mg280)* was time-dependent. Since serotonin is a negative regulator of locomotion in *C. elegans* (Horvitz et al. 1982; Ségalat et al. 1995), *tph-1* mutants are hyperactive (Sze et al. 2000). To determine whether *tph-1(mg280)*; *CeMyoD(cc561)* animals displayed muscle degradation because of the reduction of serotonin *per se*, or only as a consequence of hyperactivity, we constructed a *goa-1(n1134)*; *CeMyoD(cc561)* strain. *goa-1* mutants are amongst the most hyperactive mutants known in *C. elegans* (Ségalat et al. 1995). The level of muscle degeneration in *goa-1(n1134)*; *CeMyoD(cc561)* animals was no different from that of *CeMyoD(cc561)*, indicating that hyperactivity by itself was not the cause of muscle degeneration in *tph-1(mg280)*; *CeMyoD(cc561)* animals (Table 2). Therefore, we conclude that muscle degeneration occurring in *tph-1(mg280)*; *CeMyoD(cc561)* is the result of reduced serotonin levels. Altogether these

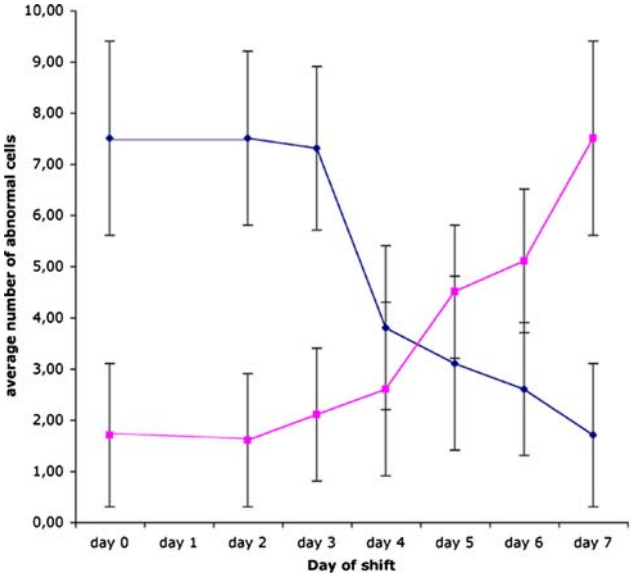


**Fig. 2** Kinetics of serotonin action. Number of missing or degenerating body-wall muscle cells observed at day 7, after animals had been exposed to serotonin during various periods of time. The abscissa value represents the day animals were transferred from solvant-containing plates to serotonin-containing plates (pink squares), or the opposite (blue diamonds). Standard deviation is indicated by the vertical lines. Concentration of serotonin was 4 mg/ml. In non-treated animals, extensive muscle degeneration occurs between days 5 and 7 under these growth conditions

**Table 2** Muscle defects in mutants with altered serotonin metabolism

| Genotype | Number of missing or degenerating muscle cells per animal ± s.e.m. |
|---|---|
| N2 | 0 ± 0 (40) |
| *dys-1(cx18)* | 0.1 ± 0.05 (40) |
| *CeMyoD(cc561)* | 0.8 ± 0.1 (40) |
| *dys-1(cx18); CeMyoD(cc561)* | 12.3 ± 0.6* (40) |
| *tph-1(mg280)* | 0.0 ± 0.0 (40) |
| *dys-1(cx18); tph-1(mg280)* | 0.5 ± 0.1 (20) |
| *tph-1(mg280); CeMyoD(cc561)* | 5.2 ± 0.3* (20) |
| *goa-1(1134)* | 0.0 ± 0.0 (40) |
| *goa-1(n1134); CeMyoD(cc561)* | 1.3 ± 0.2 (20) |

Muscle cells were observed after phalloidin staining of animals fixed 3 days after the L4-stage

Animals were grown in constant presence of food without drugs at 15°C

Mean ± s. e. m. *different from *CeMyoD(cc561)* at $P < 0.05$. The number in parentheses is the number of scored animals

results show that serotonin facilitates striated muscle survival in *C. elegans*, and that reduction of serotonin levels leads to muscle degeneration in a sensitized background.

## Discussion

In this paper we show that serotonin and compounds increasing serotonin levels block muscle degeneration in *C. elegans*. Our findings also shed light on a muscle signaling mechanism that has likely been overlooked. We previously demonstrated that prednisone, a steroid used as a palliative treatment against DMD, can also reduce dystrophin-dependent muscle degeneration in *C. elegans* (Gaud et al. 2004). Serotonin and molecules described in this paper are much more efficient than prednisone in blocking the muscle degeneration. An active concentration of 2–10 mM serotonin might seem high, but one has to keep in mind that this is the concentration in the semi-solid agar medium, and only a small fraction of the molecules diffuse through the cuticle of the animals. This serotonin concentration range is usual in *C. elegans* studies (Dempsey et al. 2005; Chao et al. 2004). We determined by HPLC that the concentration of 5-HT in whole animal is approximately 0.5 mM. This is a global estimate since it is not possible to dissect tissues from adult *C. elegans*. 5-HT concentration within the cells is probably lower than this. Other non-related compounds active on the nematode model of myopathy, such as prednisone, also work at high concentrations in the agar medium (Gaud et al. 2004; and unpub. results). However, 5-HT agonists and fluoxetine-like molecules are active at a much lower concentration. Given the variety of molecules used, we believe that the effect we observe is specific.

Do *dys-1* mutations alter serotonin signaling in *C. elegans* muscles? Only circumstantial evidence can support this hypothesis at the moment. First, *dys-1* mutants are hyperactive (Bessou et al. 1998), a trait shared by mutants in which serotonin signaling or metabolism is affected (Sze et al. 2000; Ségalat et al. 1995). Second, the muscles of *dys-1(cx18); tph-1(mg280)* double mutants do not degenerate more than those of *dys-1(cx18)* (Table 2). Such an absence of a cumulative effect is often the consequence of mutations affecting a common biological pathway. There is no indication so far that *dys-1* mutations affect the activity of *C. elegans* serotonin receptors. However, it is possible that the absence of a functional DYS-1 protein affects components of the signaling machinery downstream of serotonin receptors, since dystrophin mutations in vertebrates destabilize the membrane and lead to mislocalization of signaling proteins (Brenman et al. 1995; Blake et al. 2002). Some genetic suppressors of *dys-1(cx18); CeMyoD(cc561)* muscle degeneration are known components of metabotropic receptor signaling in other systems (L.S., unpub. observations).

Although the effect of serotonin on vertebrate smooth muscles has been largely documented, little is known about serotonin action on skeletal muscles. Serotonin receptors have been detected in skeletal muscles of mouse embryos and adult rats, but their function in these tissues remains unknown (Lauder et al. 2000; Hajduch et al. 1999). The presence of serotonin receptors on the surface of *C. elegans* body wall muscles remains to be investigated. Injections of serotonin and imipramine at high concentration were once used to produce myopathy in rat skeletal muscles (Mendell et al. 1976). Although this might seem contradictory with the results presented here, these data indicate nonetheless that skeletal muscles are indeed responsive to serotonin. Our results are the first report that serotonin is beneficial to striated muscles and reduces dystrophy.

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