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Synaptic defects associated with s-inclusion body myositis are prevented by copper

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Abstract Sporadic-inclusion body myositis (s-IBM) is the most common skeletal muscle disorder to afflict the elderly, and is clinically characterized by skeletal muscle degeneration. Its progressive course leads to muscle weakness and wasting, resulting in severe disability. The exact pathogenesis of this disease is unknown and no effective treatment has yet been found. An intriguing aspect of s-IBM is that it shares several molecular abnormalities with Alzheimer's disease, including the accumulation of amyloid-β-peptide (Aβ). Both disorders affect homeostasis of the cytotoxic fragment A$\beta_{1\text{-}42}$ during aging, but they are clinically distinct diseases. The use of animals that mimic some characteristics of a disease has become important in the search to elucidate the molecular mechanisms underlying the pathogenesis. With the aim of analyzing Aβ-induced pathology and evaluating the consequences of modulating Aβ aggregation, we used *Caenorhabditis elegans* that express the Aβ human peptide in muscle cells as a model of s-IBM. Previous studies indicate that copper treatment increases the number and size of amyloid deposits in muscle cells, and is able to ameliorate the motility impairments in Aβ transgenic *C. elegans*. Our recent studies show that neuromuscular synaptic transmission is defective in animals that express the Aβ-peptide and suggest a specific defect at the nicotine acetylcholine receptors level. Biochemical analyses show that copper treatment increases the number of amyloid deposits but decreases Aβ-oligomers. Copper treatment improves motility, synaptic structure and function. Our results suggest that Aβ-oligomers are the toxic Aβ species that trigger neuromuscular junction dysfunction.

Keywords Inclusion body myositis · Amyloid-β-peptide · Copper · *C. elegans* · Neuromuscular junction

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Introduction

Inclusion body myositis (IBM), described by (Adams et al. 1965) is the most common myopathy in people over 50 years of age. To this day, no effective

treatment has been developed for this disease. Most of the known cases of IBM are sporadic (s-IBM) and therefore do not seem to involve a clear and unique hereditary component. This disease affects the skeletal muscle and its progression leads to severe muscle weakness (Lotz et al. 1989), especially in the limbs. In more advanced stages it can even affect the respiratory muscles (Askanas and Engel 2006, 2007).

Sporadic-inclusion body myositis has a striking resemblance with Alzheimer's disease (AD). Among the many similarities at the molecular level, the most remarkable is the presence, in skeletal muscle fibers, of intracellular amyloid-β-peptide (Aβ) aggregates, which are positive for Congo Red and Thioflavine-S stainings (Th-S) (Askanas et al. 1993; Askanas and Engel 2001). In the case of s-IBM, this accumulation occurs inside the muscle cells and is considered to be the prelude to muscle deterioration. The similarities between AD and s-IBM, coupled to the large body of research done on AD, provide us with clues that may help to elucidate the molecular mechanisms that trigger s-IBM. The intracellular accumulation of Aβ-peptide has been proposed to have a leading role in the pathogenesis of this disease, as is the case in the development of AD (LaFerla and Oddo 2005; LaFerla et al. 2007). There is a significant resemblance between IBM and AD that goes beyond the presence of the Aβ-peptide (Askanas and Engel 2002, 2006, 2008). To the already mentioned accumulation of Aβ; IBM is characterized by the abnormal presence, structure, or abundance of many other molecules that are also related to AD (phosphorilated Tau, ERK, Presenilin-1, ApoE, BACE, Ubiquitin, Prion, Myostatin, etc.) (Rebolledo et al. 2008). These cellular and molecular abnormalities, as well as the late onset of the disease, show the extraordinary similarities between AD and IBM. They also reveal the complexity of these multifactorial disorders.

During the last few years we have been working with an invertebrate model of s-IBM (Sattelle and Buckingham 2006; Rebolledo et al. 2008). We have chosen the invertebrate *Caenorhabditis elegans* because it has been used very successfully in experimental studies of the molecular mechanisms of programmed cell death, RNA interference, the nervous system, the neuromuscular junction (NMJ), the aging process and as a model for studying human diseases such as Parkinson's disease and AD, among many others (Link 2005; Sattelle and Buckingham 2006; Harrington et al. 2011).

The available *C. elegans* model of s-IBM consists of transgenic animals that express the human Aβ-peptide intracellularly in body wall muscles (Link 1995, 2005). This model shows phenotypes resembling the human disease: Aβ aggregate formation, increased oxidative stress, etc. Furthermore, previous work shows that these animals present locomotory impairments and synaptic alterations before muscle deterioration can be detected (Rebolledo et al. 2008; Minniti et al. 2009). Our work is based on modulating Aβ aggregation in vivo through chronic copper exposure. The results show that copper treatment ameliorates the synaptic defects observed in the Aβ *C. elegans* strains.

Materials and methods

Nematode strains and culture

Transgenic strains CL2120 (*dvIs14*[*unc-54*/Aβ1-42 (pCL12) + *mtl-2::GFP* (pCL26)]), CL2122 (*dvIs15* [pPD30.38 (*unc-54* vector) + *mtl-2::GFP* (pCL26)]) and ANM30 were described previously (Link 1995; Fay et al. 1998; Rebolledo et al. 2011). Briefly, CL2120 expresses the human A$\beta_{3\text{-}42}$ peptide under the control of the *unc-54* (myosin heavy chain) promoter that drives expression in the body wall muscle cells. These animals form intracellular amyloid deposits constitutively in their muscle cells (Link 1995). CL2122 is the control strain for CL2120, and does not express Aβ.

The worms were cultured on regular culture plates with NGM agar seeded with the bacterial strain OP50 (Brenner 1974). Strains were maintained at 20 °C. For copper treatments, the agar was supplemented with $CuCl_2$ (Sigma) 150 μM, and the worms were treated from the embryo stage.

Th-S staining

Thioflavine-S staining was performed as described previously (Fay et al. 1998). Briefly, the worms washed from the plates were fixed with 4 % paraformaldehyde in PBS pH 7.4 for 24 h at 4 °C. The fixative solution was removed, replaced by permeabilization solution (125 mM Tris, pH 7.4, 1 % Triton X-100, 5 % β-mercaptoethanol) and incubated at 37 °C for 24 h. The animals were washed three times

in PBS-T (PBS + Triton X-100 0.1 %), stained in 0.125 % Th-S (Sigma) in 50 % ethanol for 2 min and destained for another 2 min in 50 % ethanol. The stained samples were resuspended in PBS and mounted in fluorescence mounting medium (DAKO).

BSB staining

(*Trans,trans*)-1-Bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy) Styrylbenzene (BSB) was purchased from AnaSpec Inc. (San Jose, CA, USA) and prepared according to the manufacturer's instruction. The assay was performed according to the protocol establish by (Toledo and Inestrosa 2009), with the following modifications. Briefly, synchronized worms were cultured and collected at different stages, fixed with paraformaldehide 4 % and permeabilized. Then, the worms were incubated with BSB (0.02 % resuspended in ethanol 50 %), for 4 h at 37 °C. The worms were then washed with lithium carbonate (1 M), washed with water and finally mounted for microscopy observation.

X-34 staining

X-34 dye was kindly provided by Dr. William Klunk. Live transgenic animals were incubated in X-34 following the recommendations establish by Link et al. (2001).

Microscopy

Fluorescence images were acquired using the same exposure parameters with a 40 or 100× objective in an Olympus BX51 microscope (Tokyo, Japan). The microscope was equipped with a digital camera Micropublisher 3.3 RTV (JH Technologies, San Francisco, CA, USA). For ACR-16::GFP distribution analysis, 1–2 day-old worms were anesthetized with 20 mM NaN_3 and photographed.

Image quantification and statistical analysis

Digital quantification of Th-S fluorescent intensity was estimated using the WCIF ImageJ software.

Thrashing assays

Individual animals of the same age were placed on an 80 µl drop of M9 buffer. After a 2-min recovery period the worms were recorded for 1.5 min with a digital camera (Nikon Coolpix-4500) and the thrashes counted while replaying the video in slow motion. A thrash is defined as a change in the direction of bending at the mid body (Miller et al. 1996). For the copper treatment experiments, the worms to be assayed were exposed to copper from the embryo (egg) stage in agar plates until the thrashing experiments were performed 72 (1 day-old adults), 120 (3 day-old adults) and 168 h later (5 day-old adults).

Results

We used *C. elegans* that express Aβ human peptide 3-42 (McColl et al. 2009) in muscle cells as a model of IBM (Fig. 1), with the aim of analyzing Aβ-induced pathology and of evaluating the consequences of modulating Aβ aggregation.

These animals show a significant amount of Aβ-peptide inside their muscle cells. We previously showed that these aggregates are reactive to Th-S (Fay et al. 1998; Rebolledo et al. 2011). The intramuscular Aβ deposits are also reactive to X-34 dye (Link et al. 2001) (Fig. 1b) and to anti-Aβ antibodies (Fig. 2d). With these tools we are able to analyze the aggregation of the Aβ-peptide.

Caenorhabditis elegans model of IBM shows synaptic defects

Our previous work shows that the transgenic strain expressing the Aβ-peptide presents a strong impairment in motility that is linked with defective neuromuscular synapse transmission, suggesting a specific defect on nicotine sensitive acetylcholine receptors (AChR) (Rebolledo et al. 2008). Table 1 shows frequency of bending during swimming in liquid media (Thrashes/min) of young adult individuals. The transgenic Aβ strain (CL2120) shows a 50 % decrease in its swimming capacity with respect to the Wt (see supplemental movie 1 and 2). We have also reported that Aβ transgenic worms show significant resistance to nicotine exposure compared to the wild type animals (Fig. 2).This behavior is specific to nicotine; exposure to levamisole (another AChR agonist that specifically affects the *C.elegans* nAChRs sensitive to levamisole) does not show differences between the Aβ strain and the control (Rebolledo et al. 2011). These

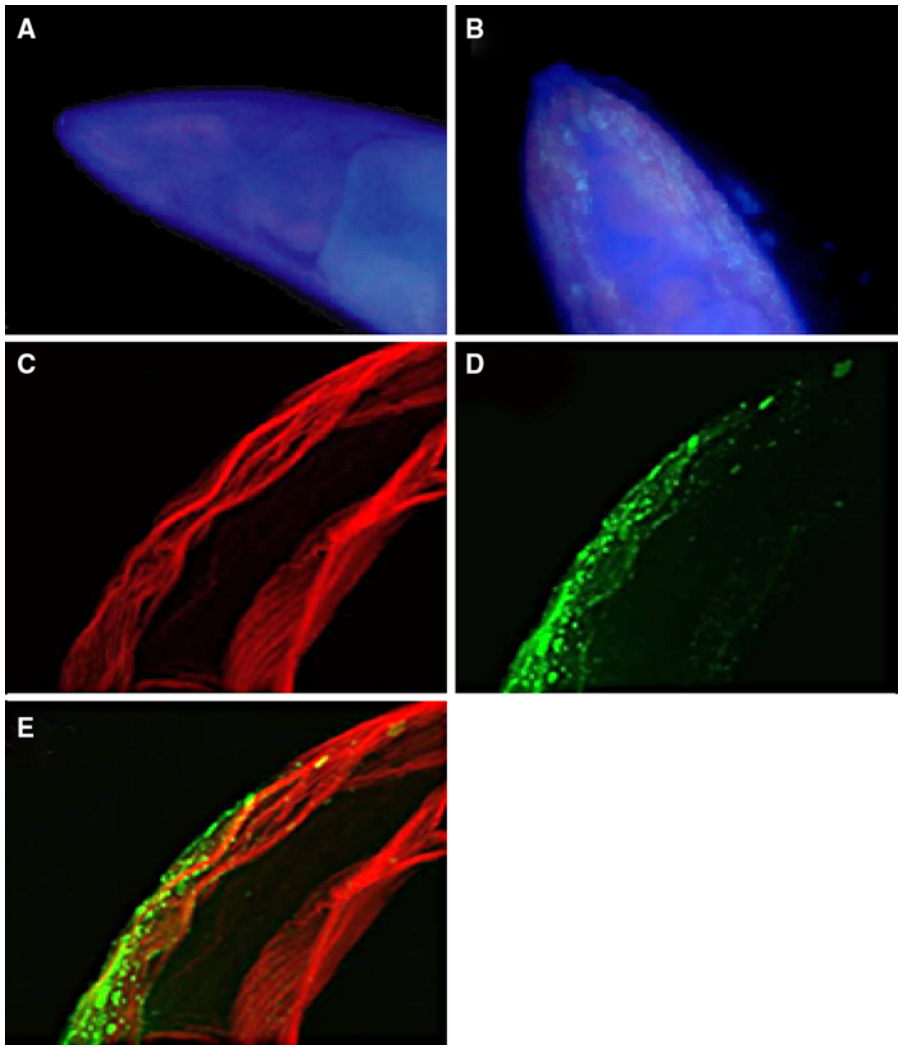

Fig. 1 The *C. elegans* IBM model. **a** Control worm stained with the amyloid dye X-34. **b** Amyloid deposits can be observed in the head of the transgenic Aβ worm. **c** *C. elegans* muscle cells stained with phalloidin to show the actin cytoskeleton. **d** The same as in "**c**", stained with anti-Aβ antibodies shows amyloid and non-amyloid Aβ aggregates. **e** Merge of pictures shown in "**c**" and "**d**"

results suggest that the Aβ transgenic worms had synaptic defects associated, directly or indirectly, with specific nAChRs at the NMJ.

Copper treatment increases amyloid deposits and decreases oligomeric Aβ species

With the purpose of modulating Aβ-peptide aggregation in vivo we exposed the worms to different copper concentrations. Histological and biochemical analyses allowed us to determine that copper treatment increases the amyloid deposits (Rebolledo et al. 2011) and decreases Aβ-oligomers in this model (Fig. 3). Th-S positive aggregates increased 166 % in larvae and 161 % in adults (Table 2) and oligomeric species decreased 56 and 35 % in young and older adult, respectively (Table 3). Moreover, chronic copper treatment improves motility (Supplemental movie 3) and the response to nicotine, indicative of an improvement in synaptic transmission (Rebolledo et al. 2011).

In agreement with the synaptic transmission defects observed in the Aβ transgenic worms we found that synaptic dysfunction correlates with misslocalization of ACR-16 (Fig. 4c), the AChR subunit essential for

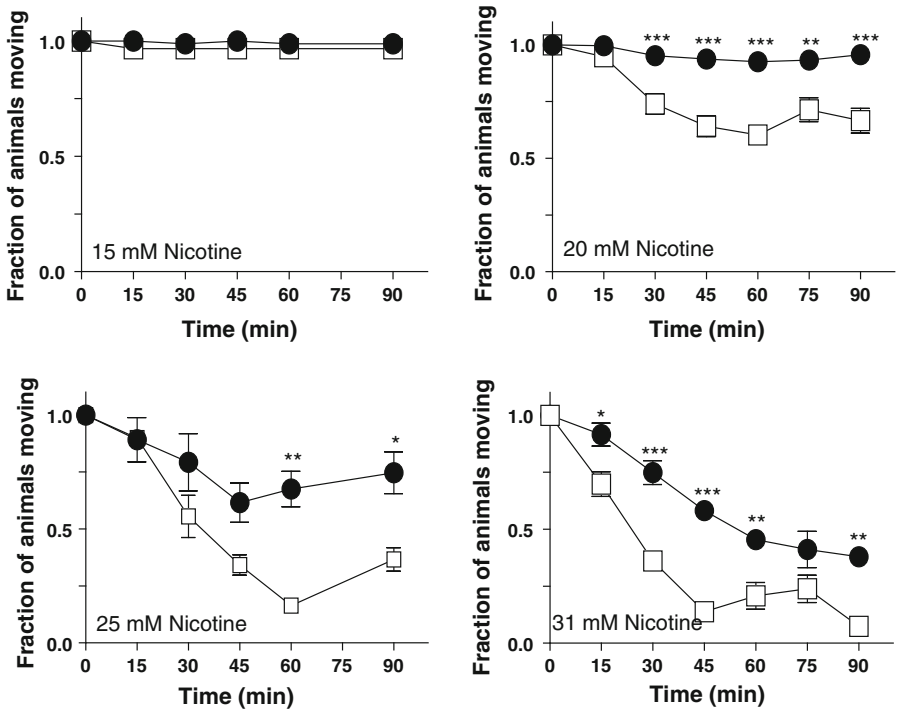

Fig. 2 *Caenorhabditis elegans* that express Aβ are resistant to nicotine. The worms were exposed to increasing nicotine concentrations. The differential effect of the drug on the Aβ worms is clearly observed with exposures to 20, 25 and 31 mM nicotine. Aβ worms: *black circles*. Control worms: *white squares*

Table 1 Aβ-peptide muscle expression in *C. elegans* strain affects locomotion in liquid media

Strain	Thrashes/min
CL2122 (no Aβ)	203.7 ± 4.09
CL2120 (Aβ)	107.8 ± 3.85

Thrashing assays of different strains were performed in 1 day-old adult worms after being cultured on regular agar plates

nicotine triggered currents (Francis et al. 2005). Interestingly, copper treatment restores the wild type ACR-16 distribution at the NMJ (Fig. 4d) (Rebolledo et al. 2011).

Discussion

Our results indicate that copper modulates Aβ-induced pathology and suggest that Aβ-oligomers are the toxic Aβ species that trigger neuromuscular dysfunction in this *C. elegans* model of IBM (Fig. 5 model). Our findings emphasize the importance of neuromuscular synaptic dysfunction in s-IBM and the relevance of modulating the amyloidogenic component as an alternative therapeutic approach for this disease.

Aβ-peptide and its effects on the synapse

It is currently known that the neurotoxic effects of Aβ-peptide in AD compromise synaptic function well before the onset of cell death (Selkoe 2002). For instance, there is a decrease of synaptic proteins before the onset of plaque formation in murine models of AD (Mucke et al. 2000) and also in AD patients (Masliah et al. 2001a, b) while misslocalization of NMJ proteins has been observed in cellular cultures from IBM biopsies (McFerrin et al. 1998).

The compromised functionality of vertebrate nAChRs containing the α7 subunit has been related to several neuropathologies, including AD (Perry et al. 1987, 1995). α7-nAChRs colocalizes with AD plaques (Wang et al. 2000a, b). Moreover, this subunit localizes in neurons that are susceptible to Aβ toxicity (D'Andrea and Nagele 2006). There are also several reports showing interaction between Aβ and α7nAChR (Wang et al. 2000a, b). It is unknown, and still a matter of controversy, what the consequences of this interaction are. Chronic exposure to Aβ can lead to desensitization of the receptors (Dineley et al. 2002; Buckingham et al. 2009). It can also generate intracellular accumulation through enhanced internalization (Nagele et al. 2002).

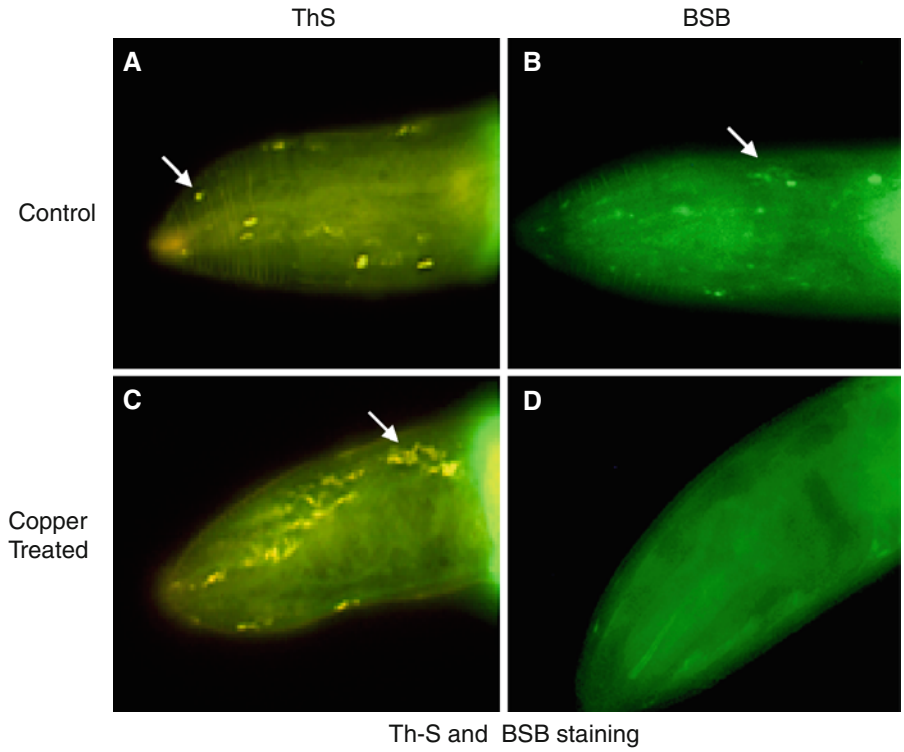

Fig. 3 Copper treatment increases amyloid aggregates and decreases Aβ oligomeric species. **a** Control animals stained with Th-S show amyloid deposits. **b** Control animals stained with BSB show accumulation of Aβ-oligomers. **c** Th-S staining shows that copper treatment increases the number of amyloid deposits. **d** BSB staining shows that copper treatment decreases oligomeric Aβ species

Table 2 Cu^{2+} modulates the aggregation state of intracellular Aβ-peptide in transgenic *C. elegans*

	Number Th-S positive aggregates		
	Larvae (L4)	Adult (4 day-old)	Th-S positive aggregates increment during aging
Aβ strain (CL2120)	2.13 ± 0.48	10.7 ± 0.41	5.02 times
Aβ strain + Cu^{2+}	3.55 ± 0.71	17.3 ± 0.44	4.87 times
Th-S positive aggregate increment with copper treatment	1.67 times	1.62 times	

Table 3 Cu^{2+} decreases the amount of Aβ oligomeric species in transgenic *C. elegans*

	Number BSB positive oligomers		
	Adult (1 day-old)	Adult (4 day-old)	Oligomeric species increment during aging
Aβ strain (CL2120)	0.21 ± 0.02	0.35 ± 0.03	0.71 times
Aβ strain + Cu^{2+}	0.12 ± 0.02	0.13 ± 0.02	0.10 times
Oligomeric species decrease with copper treatment	0.56 times	0.35 times	

The closest *C. elegans* homologue of the vertebrate α7nAChR is the ACR-16 protein that localizes to the NMJ. An Aβ/ACR-16 interaction could modify the activity of ACR-16 receptors and cause an altered response to nicotine. Mutations in the ACR-16 gene eliminate the cell's response to nicotine in electrophysiology

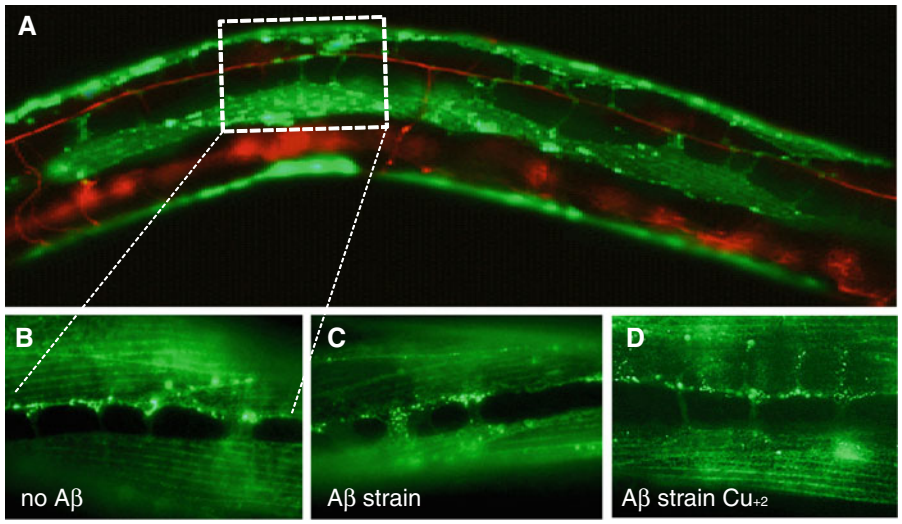

Fig. 4 ACR-16-contaning AChRs are misslocalized in transgenic *C. elegans* expressing Aβ, and copper treatment prevents receptor misslocalization. **a** Structure of the *C. elegans* neuromuscular system. Some muscle cells are stained green and the nervous system is stained red. The muscle arms can be seen as thin green structures that project form the muscle cells (*green*) and reach the nerve cord (*red*) (strain RP247) where the NMJ is formed. **b** Control worms (no Aβ) expressing ACR-16::GFP show fluorescent clusters aligned over the ventral nerve cord, while the muscle arms are free of ACR-16::GFP clusters (Francis et al. 2005). **c** Worms that express Aβ have a markedly altered localization of ACR-16::GFP clusters that can be seen inside several muscle arms. **d** Copper treatment improves ACR-16 localization (Rebolledo et al. 2011). (Color figure online)

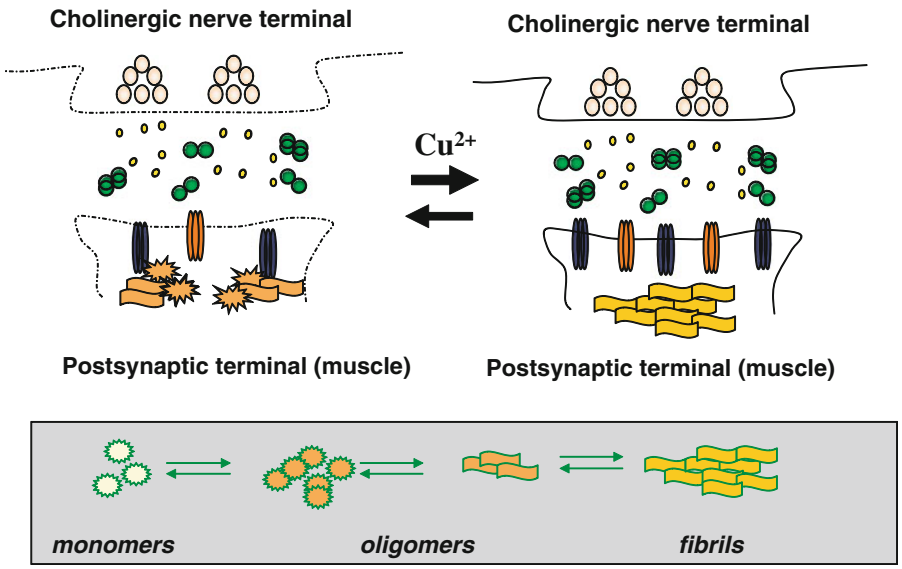

Fig. 5 Working model. The early pathological events in a *C. elegans* model of IBM are associated with the neuromuscular synapse and are mainly caused by the accumulation of specific Aβ species in muscle cells. Metals, such as copper, modulate the Aβ species inducing the accumulation of Aβ fibrils and eventually amyloid, and decreasing the oligomeric species in vivo improving synaptic structure and function

experiments (Francis et al. 2005). The Aβ/ACR-16 interaction could be directly modifying the properties of ACR-16. Alternatively, Aβ could be affecting the intracellular trafficking of the receptor, leading to misslocalization that triggers the synaptic dysfunction observed in Aβ transgenic *C. elegans*. Our in vivo analysis of the ACR-16::GFP fusion protein expressed in the presence of Aβ shows receptor clusters localized in

the muscle arms instead of at the NMJ. *C. elegans* has numerous nAChRs and it is possible that other receptors could also be affected by Aβ.

Aβ-peptide, Aβ-oligomers and plasticity of amyloid aggregates: role of copper in Aβ aggregation

Current evidence shows that, in AD, Aβ-oligomers are the toxic species (Cerpa et al. 2004; Lesne et al. 2006; De Felice et al. 2007; Lacor et al. 2007). Current studies on the effect of different Aβ species (oligomers vs amyloid aggregates) in neuronal cells are mainly based on cell culture in vitro models where the Aβ species are provided exogenously to the cells mimicking the AD situation. To analyze in vivo the role of Aβ aggregation in NMJ dysfunction we attempted to modulate the Aβ species present in the muscle tissue of our IBM model using exposure to copper. The aggregation state of Aβ-peptide is known to be modulated by metals (such us Cu^{2+}or Zn^{2+}) (Bush et al. 1994a, b, 2003; Inestrosa et al. 2005) and by metal chelators (Miller et al. 1996; Cherny et al. 2001; Ritchie et al. 2003); however, the role of copper as deleterious or beneficial in the context of amyloidogenic and neurodegenerative diseases is controversial (Strausak et al. 2001; Bayer et al. 2003; Bellingham et al. 2004; Cerpa et al. 2005; Kessler et al. 2005).

In our previous work we demonstrated that treatment with copper increases the formation of amyloid deposits *in vivo* in the IBM *C. elegans* model and that the treated worms improved their locomotory capacity (Minniti et al. 2009). Since the total amount of Aβ in these worms remained constant, it was possible that the increase in aggregates translated into a decrease in oligomeric species. Indeed, our results show that certain oligomeric species are decreased in Aβ expressing animals treated with copper. A general decrease of oligomers is also observed when the Aβ animals are treated with copper and then stained with the high affinity oligomeric Aβ dye BSB (Fig. 3b, d). Interestingly, we found that copper treatment also improves synaptic transmission in the Aβ IBM model, shown as a reversion of the resistance to nicotine (Rebolledo et al. 2011).

IBM is a multifactorial disease and is therefore difficult to study; however, our experimental approach allowed us to explore specifically those events related with Aβ expression that seem to be significant in the development of the disease. In addition, our results identified the type of postsynaptic molecules that might be affected early on during the development of IBM and therefore may suggest novel approaches to treatment including development of new drugs.

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