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RESEARCH ARTICLE

Pharmacological assays reveal age-related changes in synaptic transmission at the *Caenorhabditis elegans* neuromuscular junction that are modified by reduced insulin signalling

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SUMMARY

Frailty is a feature of neuromuscular ageing. Here we provide insight into the relative contribution of pre- and postsynaptic dysfunction to neuromuscular ageing using the nematode *Caenorhabditis elegans*. Assays of *C. elegans* motility highlight a precipitous decline during ageing. We describe a novel deployment of pharmacological assays of *C. elegans* neuromuscular function to resolve pre- and postsynaptic dysfunction that underpin this decreased motility during ageing. The cholinergic agonist levamisole and the cholinesterase inhibitor aldicarb elicited whole worm contraction and allowed a direct comparison of neuromuscular integrity, from 1 to 16 days old: measurements could be made from aged worms that were otherwise almost completely immobile. The rapidity and magnitude of the drug-induced contraction provides a measure of neuromuscular signalling whilst the difference between levamisole and aldicarb highlights presynaptic effects. Presynaptic neuromuscular transmission increased between 1 and 5 days old in wild-type but not in the insulin/IGF1 receptor mutant *daf-2* (*e1370*). Intriguingly, there was no evidence of a role for insulin-dependent effects in older worms. Notably in 16-day-old worms, which were virtually devoid of spontaneous movement, the maximal contraction produced by both drugs was unchanged. Taken together the data support a maturation of presynaptic function and/or upstream elements during early ageing that is lost after genetic reduction of insulin signalling. Furthermore, this experimental approach has demonstrated a counterintuitive phenomenon: in aged worms neuromuscular strength is maintained despite the absence of motility.

Key words: aldicarb, ageing, insulin, levamisole, synapse.

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INTRODUCTION

Ageing is the biggest risk factor for neurodegenerative disease and frailty. During 'healthy ageing' (characterized by functional decline below the threshold of disease) humans and other species exhibit cognitive decline (reviewed in Yankner et al., 2008). Morphological changes in the brain correlate with this functional decline. In particular, synaptic density declines during ageing (Bertoni-Freddari et al., 1990; Terry and Katzman, 2001) in the absence of widespread neuronal loss (reviewed by Morrison and Hof, 1997). Synaptic dysfunction and loss precedes the clinical symptoms of neurodegenerative diseases, and there is evidence that many diseases begin at the synapse (Selkoe, 2002; Koffie et al., 2011), and as such are referred to as 'synaptopathies' (Li et al., 2003; Forero et al., 2006; Gray et al., 2009; Asuni et al., 2010; Brose et al., 2010). The 'aged' state of neurons in the brain makes them vulnerable to neurodegenerative disease (Herrup, 2010).

The peripheral nervous system is also affected by ageing. Indeed there is a striking decrease in motor units in aged humans, and morphological alterations at surviving motor units (Campbell et al., 1973). Although muscle integrity is important, it is clear that upstream elements including the motor neurons and neuromuscular synapses play an important role in defining muscle function and the onset of frailty. Resolving cause and effect remains difficult and there is good evidence to suggest that muscle disruption is preceded by dysfunction of motor neurons and neuromuscular synapses (Delbono, 2003; Deschenes et al., 2010).

Investigations, particularly those at the interface between nerve function and ageing, are facilitated by model organisms. The fundamental biology of the ageing process is conserved between humans and the model organism *Caenorhabditis elegans* (Kenyon, 2010). The major neurotransmitter systems are present in *C. elegans* (Dimitriadi and Hart, 2010), and at least 42% of all human disease-related genes have a *C. elegans* orthologue (Culetto and Sattelle, 2000). Aside from the high levels of conservation with humans, there are a number of additional advantages of using the worm, chief among these being the well-characterized molecular and anatomical understanding of synaptic structure and function in the worm (White et al., 1986; Richmond and Jorgensen, 1999; Stigloher et al., 2011).

The motor system of *C. elegans* provides a tractable route through which to drive investigations into synaptic function. The motor neurons run along the length of the worm in tracts called the nerve cords (White et al., 1986). The muscle cells send projections called muscle arms that make contact with the motor neurons and become the site of neuromuscular junctions (White et al., 1986). The neuromuscular junctions use either acetylcholine or γ-aminobutyric acid (GABA) as their primary excitatory and inhibitory neurotransmitters, respectively. Acetylcholine causes contraction of the muscle (Lewis et al., 1980), and GABA causes relaxation (McIntire et al., 1993; Richmond, 2007). The motor system controls a range of stereotypic behaviours that can be quantified as a measure for neuromuscular function, including locomotion, swimming and

feeding behaviours (Albertson and Thomson, 1976; Avery and Horvitz, 1989; Pierce-Shimomura et al., 2008).

Early studies showed a remarkable preservation of the worm nervous system with age (Herndon et al., 2002). Recent studies have shown that motor neurons and sensory neurons exhibit subtle morphological alterations with age (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). A recent morphological study extended these observations to the synapse and correlated reduced nerve terminal size and synaptic vesicle number with increased age and decreased mobility (Toth et al., 2012). In addition to evidence that ageing may affect synaptic function (Glenn et al., 2004; Toth et al., 2012), a converse relationship in which synaptic function affects ageing has been noted (Evason et al., 2005; Kornfeld and Evason, 2006; Ch'ng et al., 2008). The interactions between the presynapse and postsynapse of the worm neuromuscular junction are also complex. Stimulation of the postsynapse with acetylcholine from the presynapse leads to a modulation of nicotinic acetylcholine receptors on the postsynaptic membrane, and a retrograde signal that results in further modulation of acetylcholine release from the presynapse (Simon et al., 2008). Muscle contraction resulting from acetylcholine release also activates stretch-activated neurons in the tail that further increase the amount of acetylcholine release from the presynapse (Hu et al., 2011). Such co-dependency is not surprising. It does, however, add a layer of difficulty in interpreting how the effect of a chronic process such as ageing affects the synapse.

In an effort to overcome some of the complexity of interaction between neuromuscular function as the organism ages and shows reduced motility, we have optimized classic pharmacological assays of neuromuscular function that make use of the mechanisms of action of aldicarb and levamisole. Aldicarb is an acetylcholinesterase inhibitor, and when applied to the worms over an extended period of time (1 h or longer) results in spastic paralysis of the worm due to excess acetylcholine build-up at the neuromuscular junction (Nonet et al., 1993; Mahoney et al., 2006). The amount of acetylcholine that builds up, and hence the speed of onset of paralysis, is dependent on the rate of acetylcholine release from the presynapse. The onset of paralysis also depends on the competency of the postsynapse in translating the cholinergic signal into a muscle contraction, and the ability of the muscle itself to contract. Levamisole is another drug that acts at the neuromuscular junction to cause spastic paralysis. Levamisole is a selective agonist of the major subtype of ligand-gated acetylcholine receptors (AChRs) located at the postsynaptic side of the neuromuscular junction, and acts as the major determinant of synaptically evoked muscular contraction (Fleming et al., 1997; Richmond and Jorgensen, 1999). Hence the action of levamisole is largely independent of any acute interaction with presynaptic release of acetylcholine. Therefore, whilst the aldicarb assays give an insight into the presynapse and postsynapse combined, the levamisole assays inform only on the postsynapse. The effects of these drugs on the worms is still dependent on muscle function, but as they affect different parts of synaptic transmission, a comparison between the drugs at different ages helps pinpoint where in neuromuscular transmission the ageing processes may have an impact.

Using this approach we provide evidence for an initial increase in presynaptic signalling during early ageing. This presynaptic effect is lost in a mutant with reduced insulin signalling. This is followed by a decline in strength as the worms continue to age, although even aged worms can still contract in response to drug treatment. Remarkably, aged worms can still exhibit the same maximal contraction as young worms. This suggests that neuronal and synaptic function are important contributors to the age-dependent decline in motility that is observed during ageing.

MATERIALS AND METHODS

Strains

The wild-type strain used was the N2 Bristol strain. Additional strains used include CB113 [*unc-17(e113)*], CB1370 [*daf-2(e1370)*], RP247 [*him-4p::MB::YFP; hmr-1b::DsRed2; unc-129nsp::DsRed2*], and a cross between CB113 and RP247 [*unc-17(e113)*; *him-4p::MB::YFP; hmr-1b::DsRed2; unc-129nsp::DsRed2*].

Lifespan

Worms were maintained under standard culture conditions, at 20°C (Brenner, 1974). Lifespan and ageing experiments were performed in parallel. Worms were passaged onto new plates every 1–2 days during their reproductive period (in order to prevent contamination by progeny), and as required afterwards. Worms were synchronized by picking at the L4 stage. Many ageing experiments in *C. elegans* are done in the presence of 5-fluoro-2′-deoxyuridine (FUDR), which prevents progeny from hatching (Hosono, 1978). The experiments detailed in this paper do not use this chemical, as it can modify lifespan (Aitlhadj and Stürzenbaum, 2010; Van Raamsdonk and Hekimi, 2011), morphology and motility (Bolanowski et al., 1981; Glenn et al., 2004). Worms that exploded, bagged (contained larval progeny) or crawled up the side of the plates were censored, as this results in death uncoupled from the ageing process. Censored worms were included in the analyses (Henis-Korenblit et al., 2010).

Behavioural assays

All behavioural analyses were done at room temperature (20–21°C). All comparisons were done in parallel.

For chemotaxis assays worms were picked into a 20 µl spot of M9 buffer 5 cm away from OP50 on a 9 cm diameter nematode growth medium (NGM) plate. The assay was started when the buffer absorbed into the plate/evaporated and the worms were free to move about the plate. The number of worms that reached the OP50 was quantified at 10 min intervals for up to 2 h. Worms that crawled up the side of the plates were excluded from analyses.

Thrashing assays were performed in 24-well plates using M9 buffer with bovine serum albumin (BSA, 0.01% w/v; Sigma-Aldrich, Gillingham, UK). The worms were transferred from their staged population to each well and left for 5 min to settle. Thrashing is a rhythmic pattern of activity where the worm oscillates side-to-side around its midpoint. A single thrash was defined by a movement through the midpoint and back (Mitchell et al., 2007).

Pharyngeal pumping assays were undertaken on seeded NGM plates. Briefly, worms were transferred from their population plate to the assay plates and left for 10 min to recover from the mechanical stimulation of the picking. A pump was defined as a backward movement of the pharyngeal grinder (Albertson and Thomson, 1976; Raizen et al., 1995).

Pumps and thrashes were quantified using a dissecting microscope and a hand-held counter.

Pharmacological analysis

Drug plates were made by adding 200 µl levamisole (Sigma-Aldrich) or aldicarb (Sigma-Aldrich) to unseeded NGM plates, giving a final concentration of either 100 or 250 µmol l^{-1}, respectively [drugs were dissolved in distilled water (dH_2O)]. Control plates were made by adding the same volume of vehicle (dH_2O). These plates were left overnight at room temperature to allow for the drugs to

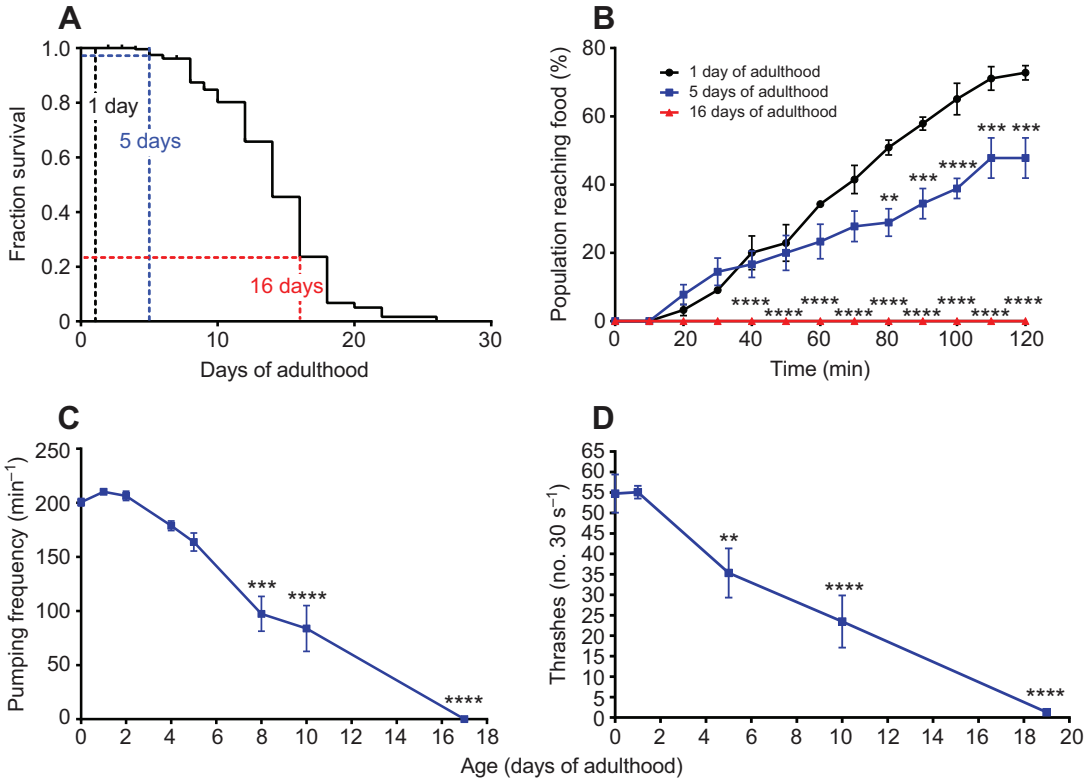

Fig. 1. Wild-type *C. elegans* exhibit deficits in a range of neuromuscular-dependent behaviours with age. (A) Wild-type *C. elegans* live for a mean of approximately 14 days of adulthood (N=69 deaths). (B) *C. elegans* are unable to chemotax towards a food source at advanced age (27–40 worms per assay; N=3; two-way ANOVA with Bonferroni *post hoc* test; **P<0.01; ***P<0.001; ****P<0.0001). (C) Aged *C. elegans* exhibit a lower rate of pharyngeal pumping on food (one-way ANOVA with Bonferroni *post hoc* test; ***P<0.001; ****P<0.0001). (D) Wild-type *C. elegans* exhibit an age-related reduction in thrashing behaviour (one-way ANOVA with Bonferroni *post hoc* test; **P<0.01; ****P<0.0001). Statistical significance symbols (*) indicate a difference when compared with worms on the first day of adulthood. Data are means ± s.e.m.

equilibrate into the agar. For the assay, worms were taken from their staged population and left on an unseeded 'cleaning plate' plate for 30 s. At the 30 s time point, a reference image was taken of the worm. The worm was then transferred to either a drug plate or a control plate, and images were taken over a set time course (5 min for levamisole and 5 h for aldicarb). Worm lengths were determined using ImageJ software. Worms were binarized and skeletonized. The length of the skeleton was used to define the length of the worm. The lengths of individual worms whilst on the drug (or control) plates were compared with the length of the same worm prior to treatment (i.e. the reference image taken on the cleaning plate) to give a fraction of the initial length of the worm. Worms transferred to control plates showed a small contraction in response to the picking, and then returned to normal length.

To plot fractional response graphs, response (contraction) of worms on drug plates was normalized to the control population (see Fig. 2).

Imaging

Imaging of muscle arms was done using the RP247 strain, expressing the *trIs30* transgene (*him-4p::MB::YFP; hmr-1b::DsRed2; unc-129nsp::DsRed2*) (Dixon and Roy, 2005). Imaging was performed using a confocal microscope, and worms were immobilized and orientated using Histoacryl glue (Braun, Tuttingen, Germany). Muscle arm counts were carried out only on muscle cells that were not obscured by excess autofluorescence, and could be seen clearly. For the quantification of muscle arm counts in the *unc-17(e113)* background, RP247 male worms were generated by heat shock treatment at 31°C for 5–6 h and crossed with CB113. Successful crosses were confirmed by the presence of fluorescence and severe unco-ordinated phenotype (Shakir et al., 2008).

RESULTS

Motility and feeding behaviours decline with age

We chose three simple behavioural assays as a whole organism surrogate of neuromuscular junction (NMJ) competence as a function of age: (1) 'chemotaxis' or locomotion of the worm towards a food source [note that chemotaxis also incorporates sensory function (Bargmann, 2006)]; (2) 'thrashing' or the swimming motion the worms exhibit when in a liquid medium; and (3) 'pharyngeal pumping' muscular contraction that is stimulated by the presence of bacteria and underpins the filter feeding mechanism by which worms ingest food (Albertson and Thomson, 1976). This was performed on synchronized populations of worms that represent distinct stages of the ageing process. In particular, ageing assays suggest that the wild-type worms show a median life span of around 14 days of adulthood (Fig. 1A), similar to previously published data (Klass, 1977; Bolanowski et al., 1981). We found that all three of these behaviours decline from early adulthood as the worms age (Fig. 1B–D). This agrees with previous observations from a range of other laboratories (reviewed by Huang et al., 2004; Collins et al., 2008). As well as the reduction in frequency, the rhythmic pattern of thrashing in aged worms became irregular compared with the smooth thrashing of young adult worms (data not shown). Interestingly, all behaviours show a decline in their function from the first day of adulthood, which indicates that intact behaviours

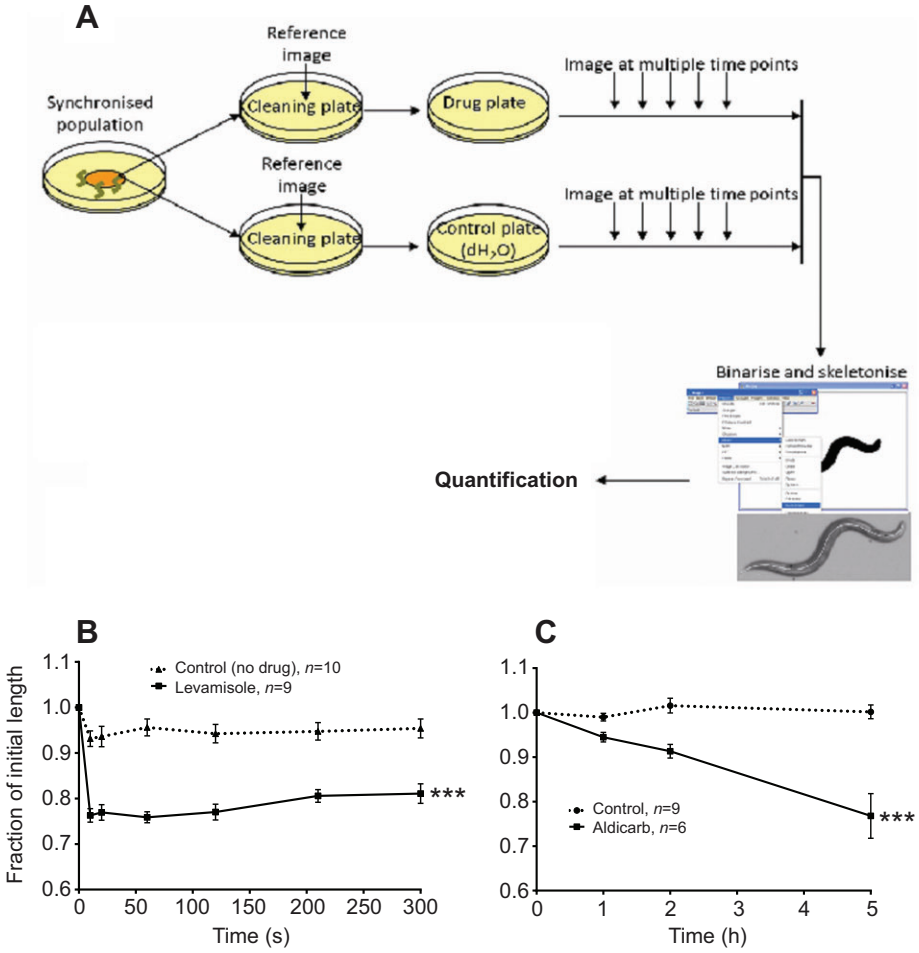

Fig. 2. Methodology for quantification of aldicarb- and levamisole-induced drug contraction. (A) A single worm is picked from a synchronized population onto a cleaning plate (unseeded nematode growth medium; NGM) for 30 s before a reference image is taken to represent untreated worm length. The worm is moved onto the treatment plate loaded with drug or water (vehicle). Images are taken at various time points over 5 min for levamisole assays and 5 h for aldicarb assays. Images are skeletonized using ImageJ and the length of the skeleton determined to define worm length. Each of these images is compared against the reference image to give a fraction of the initial (untreated) length. This can be plotted, as in B and C. (B) Rapid levamisole-induced contraction of wild-type worms compared with control (vehicle) plates; C shows the same for aldicarb-induced contraction, which is due to the gradual build-up of acetylcholine in the synaptic cleft. On both graphs, asterisks indicate statistical significance (***P<0.001) between control and drug plates using a two-way ANOVA to analyse the total time course. Data are means ± s.e.m. In subsequent figures the raw contractile response seen here is normalized to control worms and expressed as 'fractional response'. To calculate 'fractional response' a ratio of the fraction of initial length of the worms on the drug and vehicle is made. This controls for the effects of mechanical stimulation during picking, and the effect of being left on an unseeded plate for hours in the case of aldicarb assays. Fractional response is shown in Figs 3–5.

that are underpinned by NMJ function appear to be reduced prior to the precipitous decline in lifespan. However, there is evidence that the integrity of *C. elegans* muscle cells is altered with age (Herndon et al., 2002; Glenn et al., 2004; Chow et al., 2006), and may exhibit sarcopenia at advanced age. This analysis in which animals undergo increasing immotility with age confounds the use of locomotion to more precisely detail changes in synaptic function with age.

Pharmacological assays investigate the efficacy of neuromuscular function during ageing

To refine insight into how neuromuscular transmission is affected in aged worms, we compared how aldicarb and levamisole impacted on neuromuscular function. Assays using these drugs traditionally use time-to-paralysis as an end-point (Nonet et al., 1993; Mahoney et al., 2006; Rand, 2007). However, as shown by Fig. 1D, aged worms beyond their median life expectancy are largely immobile. We circumvented this potential confounding effect by using the contraction of the worms in response to the drug as a measure of its effects (Fig. 2A) (Glenn et al., 2004). We measured contraction over a protracted time course, allowing us to use rate of onset as a measure of mode of action. Levamisole has a very fast onset (within seconds; Fig. 2B), consistent with rapid crossing of the cuticle and/or ingestion, and which reaches a steady-state contraction that is largely sustained for a number of hours (data not shown). In contrast, aldicarb has a much slower onset reaching peak contraction within hours of exposure (Fig. 2C). Fig. 2B,C shows that worm length changes on the drug plate relative to the initial length measured on a cleaning plate. In subsequent experiments, the levamisole- and aldicarb-induced contractions were normalized against these control contractions to give 'fractional response' (Figs 4, 5). The fractional response can then be used as a measure of contraction efficacy and provides a comparative representation of the effects of the drugs at different ages and across distinct genotypes. Furthermore, as indicated by the kinetics of the drug-induced contraction, both the rate and the ability to produce maximal contraction serve as useful measures of contraction efficacy. We have combined these to form a contraction index based on the area under the curve of the fractional response graphs (Fig. 4E, Fig. 5E, Fig. 6C,D). Prior to using these optimized assays on cohorts of ageing worms, we tested them on a strain with a mutation affecting synaptic function in order to assess their suitability to identify alterations in neuromuscular signalling.

Contraction-based pharmacological assays correlate with morphological alterations of the neuromuscular system

unc-17 encodes a vesicular acetylcholine transporter similar to the vesicular monoamine transporters (Alfonso et al., 1993; Rand, 2007). The *unc-17(e113)* mutation affects a promoter region outside the coding regions of the gene and in this way also disrupts *cha-1* (choline acetyltransferase-1), which is part of the same 'cholinergic locus' (Rand, 1989). Hence this strain has reduced acetylcholine release. We have identified that *unc-17(e113)* worms have fewer muscle arms than wild-type (Fig. 3A). Muscle arms are thin projections sent out by *C. elegans* muscle cells to make contact with motor neurons and form the site of neuromuscular junctions (Fig. 3B). The fact that *unc-17* worms have fewer muscle arms

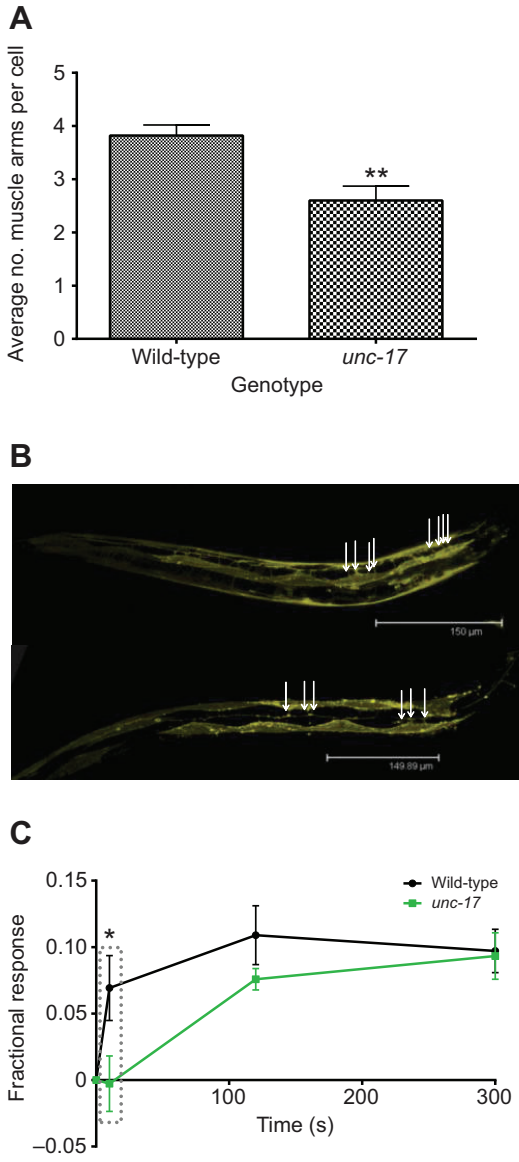

Fig. 3. Drug-induced contractile assays can identify functional changes that have an anatomical basis. (A) *unc-17(e113)* worms genetically defective in cholinergic neurotransmission have fewer muscle arms than wild-type counterparts (wild-type: *N*=9 worms, 36 muscle cells, 134 muscle arms; *unc-17*: *N*=3 worms, 15 muscle cells, 41 muscle arms; ***P*<0.01; Student's unpaired *t*-test). (B) Representative images of wild-type (top) and *unc-17(e113)* (bottom) worms expressing the *trIs30* transgene (*him-4p::MB::YFP; hmr-1b::DsRed2; unc-129nsp::DsRed2*), with the muscle cells and muscle arms in yellow. White arrows indicate muscle arms. (C) *unc-17(e113)* worms are less responsive to levamisole than age-matched wild-type counterparts (*N*=7 and 9, respectively; **P*<0.05; Bonferroni *post hoc* test). The graph shows fractional response, in which the contraction of worms on drug plates was normalized to the vehicle control population. Data are means ± s.e.m.

suggests that there is less area for neuromuscular synapse formation, and hence fewer sites at which the levamisole can act to bring about a contraction. In support of this, Fig. 3C shows that the *unc-17* mutants contract more slowly in response to levamisole. Thus, this simple contraction-based pharmacological assay can identify changes at the neuromuscular level that can be correlated with modified morphological characteristics of the NMJ.

Wild-type *C. elegans* neuromuscular signalling is altered with age

Pharmacological assays were used to investigate whether the wild-type neuromuscular synapse is altered with age. During levamisole treatment, worms on the third day of adulthood were not significantly different from those on the first (Fig. 4A). As worms reached the fifth day of adulthood, they became more responsive to levamisole when compared with the first day (Fig. 4B). This is reflected in the rate at which they achieve the maximal response. Such increased efficacy of contraction is consistent with improved postsynaptic function. This could reflect increased receptor number, better coupling between the receptor and its downstream targets, or an improved ability of the muscle to contract in response to neuromuscular stimulation. As the worms aged from the fifth day of adulthood to the tenth day, this increased responsiveness to levamisole disappeared, and they were similar to worms on the first day of adulthood (Fig. 4C). The worms on the sixteenth day of adulthood were still not significantly different from those on the first day (Fig. 4D) in terms of their maximal contraction, suggesting that the ability of the muscle to contract is maintained at this age. There is, however, a trend towards slower contraction on the sixteenth day of adulthood compared with the first, although it does not reach statistical significance. As summarized by Fig. 4E, worms on the sixteenth day of adulthood were significantly worse at contracting than on the fifth day, suggestive of decreased function at the postsynaptic level compared with that seen during early ageing (Fig. 4E).

Fig. 5 shows that upon aldicarb treatment, worms on the third, fifth and tenth days of adulthood exhibit a more rapid contraction than worms on the first day of adulthood. This is in agreement with the observations made with levamisole, although the increased responsiveness to aldicarb with ageing is more marked. The more rapid contractions observed in the 'middle-aged' worms may be either due to a presynaptic or postsynaptic mechanism. However, the absence of an increased responsiveness to levamisole at 3 and 10 days old points to a primarily presynaptic contribution. Again, such an observation supports the notion of a maturation of the neuromuscular junction between the first and fifth days of adulthood (Fig. 5E).

In contrast, more aged worms (on the sixteenth day of adulthood) responded with a time course that is very similar to worms on the first day of adulthood (Fig. 5D). Therefore acetylcholine release continues into advanced age, although the aldicarb-triggered contraction is less than that observed on the third, fifth and tenth days of adulthood (Fig. 5A–C; summarized in Fig. 5E).

Thus, as the worms age from their first day of adulthood they initially increase in neuromuscular strength (Fig. 4E, Fig. 5E). This is particularly apparent at the level of the presynapse. This is then followed by a progressive reduction in the efficacy of neuromuscular function as worms progress beyond their median lifespan and are representative of an aged organism (day sixteen).

Muscle arms do not disappear with age in *C. elegans*

The *unc-17* data in Fig. 3 indicate that changes in cholinergic signalling can be indicative of changes in gross morphology of the neuromuscular system, specifically the density of muscle arms. Therefore, as the data summarized in Fig. 4E and Fig. 5E suggest age-related changes in synaptic strength, we investigated the effect of ageing on muscle arm density. This analysis contrasted with the previous mutant analysis in that the number of muscle arms in the wild-type worms remained constant throughout age, regardless of whether there was an increase or decrease in the efficacy of drug-

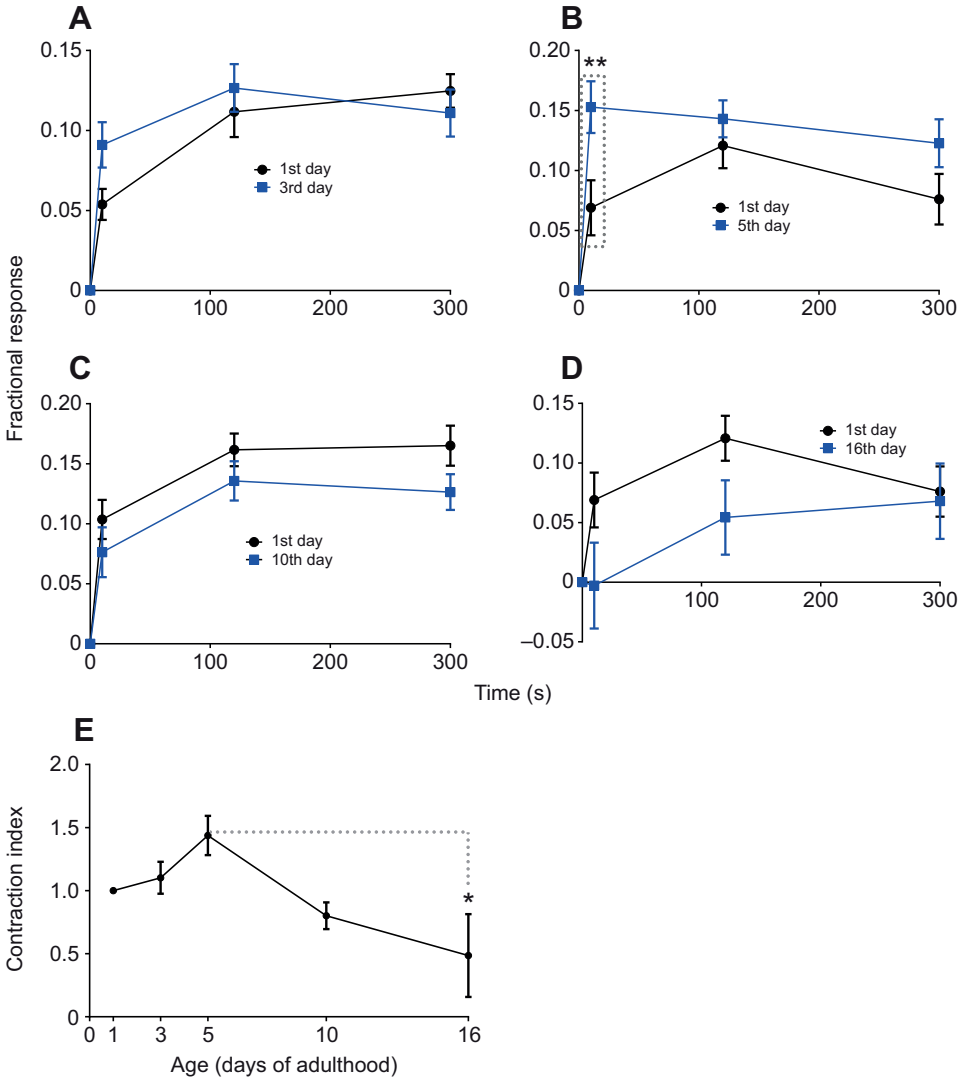

Fig. 4. The response of wild-type *C. elegans* to levamisole is altered with age. (A–D) The fractional response of wild-type worms placed on levamisole on the third, fifth, tenth and sixteenth days of adulthood, respectively, plotted against worms on the first day of adulthood assayed in parallel (two-way ANOVA with Bonferroni *post hoc* test; $^{**}P<0.01$). (E) The contraction index of different ages of worm in response to aldicarb. The contraction index utilizes the area under the curve in the fractional response graphs as a measure of the effect of the drug. *Significant difference of $P<0.05$ between worms on the fifth and sixteenth days of adulthood, using a Bonferroni *post hoc* test. Data are means ± s.e.m.

induced contraction (one-way ANOVA, $P=0.32$). On the first day of adulthood, there were 3.821±0.1988 muscle arms per muscle cell ($N=9$), on the fifth day 3.558±0.1248 ($N=4$), and on the sixteenth day 3.343±0.2835 ($N=7$). Therefore, the changes revealed by the drug assays are not underpinned by a reduction in the number of muscle arms, but rather reflect alterations of neuromuscular transmission.

Synaptic ageing is modified by reduced insulin/IGF-1 signalling

We next used the pharmacological assays to investigate how the age-related differences in neuromuscular function are affected in a long-lived mutant, *daf-2(e1370)*. *daf-2* encodes the *C. elegans* insulin/IGF-1 receptor orthologue (Kimura et al., 1997). The *e1370* mutation consists of a substitution in the tyrosine kinase domain, and results in a reduction of function (Kimura et al., 1997). As is well documented in the literature (Kenyon et al., 1993), the *daf-2* worms lived for over twice as long as wild-type (Fig. 6A). Although thrashing was reduced in the *daf-2* mutants relative to the wild-type worms during the early time points of the lifespan (Fig. 6B, days 1 and 5), this relationship was reversed as the worms aged. Accordingly, when the aged wild-type worms showed a reduced thrashing competence, *daf-2* worms sustained thrashing rates at days 10 and 16 (Fig. 6B). This agrees with previous observations that motility is preserved in these long-lived *daf-2* mutants (Kenyon et al., 1993). When wild-type and *daf-2* worms at different ages were subjected to pharmacological assays, we observed distinct age-dependent contraction competence. In particular, *daf-2* worms show a gradual decline in their aldicarb-induced contraction with age (Fig. 6C). This contrasts with the data obtained in the wild-type background, where we observed an increase in response during middle age, followed by a subsequent decrease (Fig. 5E). Although there is a mismatch in the levamisole-induced contraction between wild-type and *daf-2* worms at day 1, we found that both genotypes showed a peak levamisole-induced contraction on the fifth day of adulthood. This common peak was followed by a subsequent decline in the more aged worms (the sixteenth day of adulthood).

In the *daf-2* background, the loss of increased response to aldicarb seen in wild-type worms during early ageing, coupled with the retention of a similar pattern to wild-type in the levamisole experiments, suggests that reduction of insulin signalling has a major effect on the presynaptic component of early ageing.

DISCUSSION

Caenorhabditis elegans motility declines from the first day of adulthood (reviewed by Collins et al., 2008). We have modified

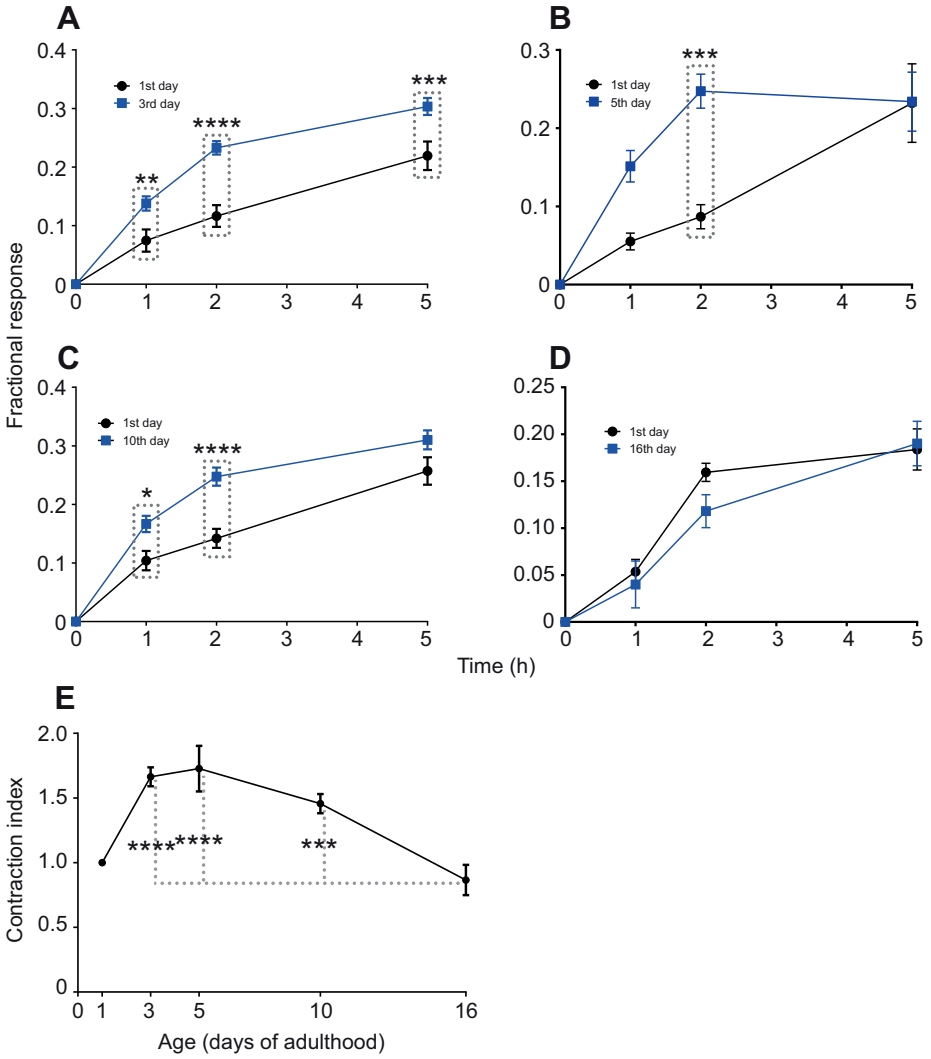

Fig. 5. The response of wild-type *C. elegans* to aldicarb is altered with age. (A–D) The fractional response of wild-type worms placed on aldicarb on the third, fifth, tenth and sixteenth days of adulthood, respectively, plotted against worms on the first day of adulthood assayed in parallel (two-way ANOVA with Bonferroni *post hoc* test; **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001). (E) The contraction index of different ages of worm in response to aldicarb. The contraction index utilizes the area under the curve in the fractional response graphs as a measure of the effect of the drug. ****Significant difference of *P*<0.0001 when compared against worms on the sixteenth day of adulthood, using a Bonferroni *post hoc* test; *** indicates the same with the exception that *P*<0.001. Data are means ± s.e.m.

established pharmacological assays of neuromuscular function to better resolve changes in the efficacy of synaptic transmission. Immobility is the traditional measurement used in these pharmacological assays (Mahoney et al., 2006), but as aged worms are already immobile we used a contraction-based analysis that measures the contraction of the worm (Glenn et al., 2004) over a time course of drug treatment.

In order to assess the utility of such assays in informing on synaptic function they were first tested on a mutant worm strain with reduced acetylcholine release and severe locomotion defects (*unc-17*). The levamisole assay (an assay for postsynaptic function) identified a phenotype in these mutants that can be explained by the presence of fewer muscle arms, probably leading to fewer sites at which the drug can act. This phenotype has not been previously described for *unc-17*, or to our knowledge for any worm that has decreased neurotransmitter release. We find it interesting that these worms deficient in acetylcholine release have fewer muscle arms, as this may suggest that the formation of muscle arms is activity dependent, and the deficiency in muscle arms seen in Fig. 3 may be developmental in origin. Supporting this, it has been shown in a mutant worm with excess exocytosis that there is a higher density of synaptic puncta in the dorsal nerve cord than in a wild-type background (Guthmueller et al., 2011).

As worms went through ‘early ageing’ (from the first to the fifth day of adulthood), there was a strengthening of the synapse both pre- and postsynaptically. This may be due to increased acetylcholine release, an increased efficiency of the postsynapse in translating presynaptic signals into muscle contraction, or a combination of both. The presence of an effect during the aldicarb assays on the third, fifth and tenth days of adulthood (before, during and after the effect in the levamisole assays, seen only on the fifth day of adulthood) points to the presynapse having a primary role in this synaptic strengthening. It is possible that the postsynaptic effect is secondary to the presynaptic effect. From here until the sixteenth day of adulthood, late in the lifespan of the worm, the response to the drugs decreased back to the levels they were on the first day of adulthood. This suggests that the ability of the neuromuscular synapse to initiate a muscle contraction in response to excess pharmacological stimulation is maintained into the late stages of ageing, but reduced relative to what it was on the fifth day of adulthood. This analysis at distinct stages of worm ageing suggests a biphasic modulation of neuromuscular function as the worm ages from a young adult to mature adult, to an organism beyond the median point of its life expectancy. The observation of a stronger neuromuscular system during early ageing is supported by a recent study on male *C. elegans*, suggesting an increased excitability of

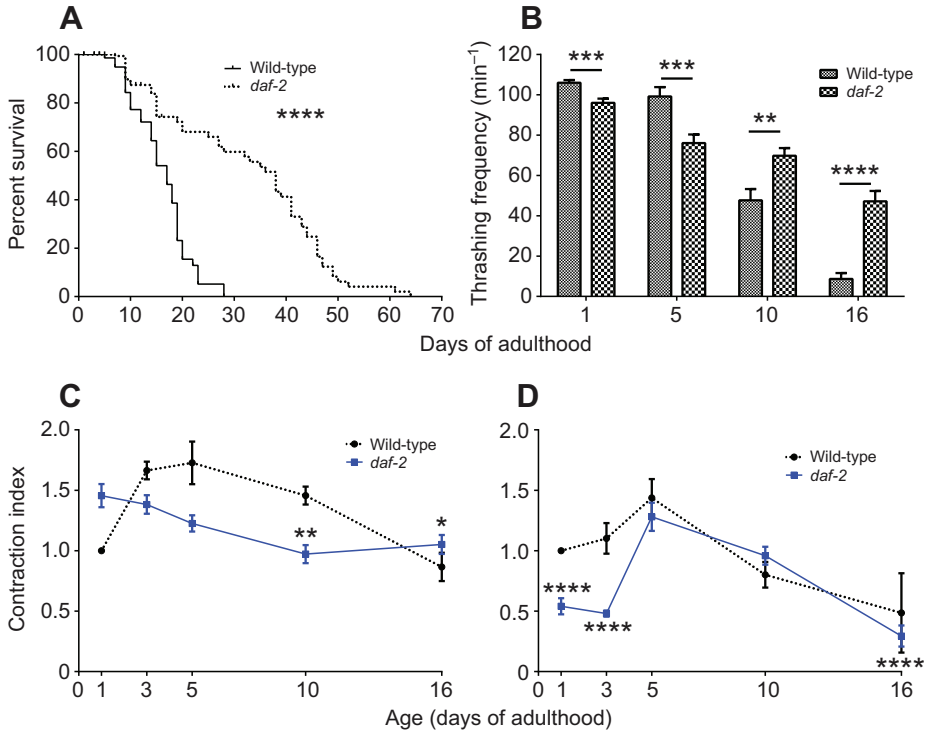

Fig. 6. Perturbation of insulin/IGF-1 signalling modifies the effects of ageing on *C. elegans* neuromuscular transmission. (A) *daf-2* worms are long-lived compared with wild-type worms (log rank test; **P*<0.0001). (B) *daf-2* worms show less of a decline in thrashing frequency than wild-type worms during ageing (unpaired *t*-test; ***P*<0.01; ****P*<0.001; *****P*<0.0001). (C) The aldicarb-induced contraction index of *daf-2* worms declines steadily with age. For comparison, the contraction index for wild-type worms on aldicarb is included as a dashed line. Statistical significance was calculated by comparing each age with the first day of adulthood (Bonferroni *post hoc* test; **P*<0.05; ***P*<0.01). (D) The levamisole-induced contraction index of *daf-2* worms first increases and then decreases with age. Again, the contraction index for wild-type worms on levamisole is included as a dashed line as a reminder. Here, statistical significance was calculated by comparing each age with the fifth day of adulthood (Bonferroni *post hoc* test; *****P*<0.0001). Data are means ± s.e.m.

the muscle cells used during mating in early ageing (Guo et al., 2012). This was coupled with the observation that the spicule muscles of males on the third day of adulthood are hypersensitive to levamisole (Guo et al., 2012).

There is an interesting discontinuity in the observations derived from the pharmacological and behavioural assays. In the latter, motility and pharyngeal nerve–muscle function appear to be in decline during a period that the pharmacological assays of neuromuscular function are suggesting improved efficacy that peaks on the fifth day of adulthood. In male worms, Guo et al. (Guo et al., 2012) suggest that the hyperexcitability seen in the muscle cells used for mating during early ageing may be the basis for the decline in mating behaviour. Indeed, by genetically reducing the excitability of the muscle cells they delayed the behavioural decline (Guo et al., 2012). Therefore, an increased strength of neuromuscular transmission does not necessarily correlate with the ability to execute intact behaviours. More important is the co-ordination of a network activity between different types of neurons to effect the contraction and relaxation of muscle cells in the appropriate temporal and spatial patterns to support the behaviours. This co-ordinated network activity may play a role in the behavioural decline seen during ageing in *C. elegans*. Supporting this, the pattern of thrashing becomes irregular in aged worms (data not shown).

We cannot rule out the possibility that there may be subtle defects at the *C. elegans* neuromuscular junction that are only apparent at physiological concentrations of acetylcholine, as these may be overcome by the excess stimulation seen in the drug assays. This is supported by a previous study that documented behavioural changes that occur as *C. elegans* age (Glenn et al., 2004). They performed an interesting experiment with arecoline, an agonist of muscarinic acetylcholine receptors that stimulates acetylcholine release from the neuromuscular synapse. Elevation of acetylcholine release in aged worms by arecoline resulted in an increase in spontaneous locomotion levels (Glenn et al., 2004), suggesting that locomotory behaviour in aged worms is not limited by defective muscle function (consistent with our observations of aged worms upon aldicarb treatment). It is possible that this may be reflective of reduced acetylcholine release, a reduced ability of the postsynapse to respond to the same amount of cholinergic input, or a combination of both. In addition, we cannot rule out the effects of body mechanics as a variable in the contraction assays (Herndon et al., 2002; Park et al., 2007).

The above observations suggest that developmental processes probably dominate the improving neuromuscular transmission up to the fifth day of adulthood, and it is only at points beyond these periods that one can expect to model age-related decline. This is supported by the observation that both the intact behaviours and the reduced drug-induced measures of neuronal function decline in worms that have progressed beyond their median lifespan. Importantly, the decline in neuromuscular function identified by the pharmacological assays in aged worms appears to occur without changes in the ability of the organism to reach and sustain a maximal contraction, supporting the notion that modification of drug sensitivity across age may reflect modifications in underlying neuronal and/or synaptic competency, rather than muscle function. This is supported by the observation that despite the ultrastructural changes that appear in muscle cells with age, they do not fully disintegrate (Herndon et al., 2002).

Counting the number of muscle arms across different ages of worms did not reveal quantitative changes with age, but this analysis does not resolve more qualitative changes in the fine structure or molecular complement of the synapse at these advanced ages. A recent study identified fewer synaptic vesicles and smaller presynaptic terminals in aged *C. elegans* (Toth et al., 2012). There has been limited investigation of synaptic plasticity in the worm (Rose et al., 2003; Grunwald et al., 2004; Simon et al., 2008; Davis et al., 2010; Hu et al., 2011; Jensen et al., 2012), but there is clear precedent for structural and molecular plasticity at the neuromuscular junction. Further investigation is required to assess the morphological and molecular changes that occur at synapses in *C. elegans* with age.

After characterizing the wild-type neuromuscular junction across age using the optimized pharmacological assays, we examined a *daf-2* reduction of function mutant. Similar to previous reports (Kenyon et al., 1993), we observed a preservation of motility with age (Fig. 6B). However, the interpretation of the pharmacological assays is more complex (Fig. 6C,D). In the *daf-2* mutants there is a selective loss of the increased contraction in response to aldicarb during early ageing seen in wild-type worms. However, during the same period the response to levamisole follows a similar pattern to wild-type. Therefore genetic reduction of insulin signalling has a selective impact on presynaptic alterations seen during early ageing. This could be due to direct modulation by the insulin signalling pathway or a secondary, downstream effect of enhanced cellular maintenance in this genetic background. Supporting the possibility of a direct effect is the presence of an increased response to levamisole during early ageing in the *daf-2* mutant background, similar to wild-type. If the effect was a global one, downstream of cellular maintenance, one would expect both aldicarb and levamisole measurements to be modified similarly. In addition, one would expect the aldicarb-induced contraction index of *daf-2* mutants to show a similar pattern to wild-type, simply occurring in more aged animals, a pattern we did not see. There is also evidence of a role for insulin signalling in modulation of presynaptic function in other systems. Acute insulin signalling has been shown to modify neurotransmitter release processes at presynaptic terminals in the mammalian brain (reviewed in Zhao and Alkon, 2001). IGF-1 has also been shown to potentiate quantal secretion at developing motor neurons in *Xenopus* cell culture, with acute application enhancing spontaneous acetylcholine release (Liou et al., 2003). In the *Xenopus* model, it has been postulated that the IGF-1 is released from the myocytes to support presynaptic development (Liou et al., 2003). However, further studies are required to distinguish whether the effects of *daf-2* reduction of function on presynaptic function during early ageing are direct or indirect.

On the sixteenth day of adulthood, the pharmacological assays indicate similar strengths of neuromuscular transmission in both wild-type and *daf-2* mutants, even though at this age the *daf-2* mutants thrash better than wild-type. This supports the role for neuronal network co-ordination at a hierarchical level above the neuromuscular junction in age-related behavioural decline.

daf-2 mutants do have differences from wild-type in the expression of a range of genes involved in synaptic function, including genes involved in neurotransmitter release (Shen et al., 2007). Such biochemical analysis may be of value at different ages in the wild-type and *daf-2* backgrounds to get a clearer picture of the molecular mechanisms involved in the alterations of synaptic strength we see during ageing.

In summary, the data suggest that mobility in aged worms is not limited by muscle function, and the immobility evidenced by the behavioural assays may be a result of altered signal transduction at the level of the neuromuscular synapse, or the integration of neuronal signals in the hierarchical regulatory neuronal network. This observation resonates with emerging studies from human frailty, which reveal that age-related immobility is underpinned by dysfunction at the neuromuscular junction and upstream neural pathways that control its output (Campbell et al., 1973; Delbono, 2003; Deschenes et al., 2010). The ability to resolve neuromuscular integrity during ageing in wild-type *C. elegans* and mutants in genetic pathways that may selectively control neuromuscular competence should improve the understanding of the fundamental biology of neuromuscular ageing, and assist in identification of pathways for disease intervention.

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