Overexpression of tau results in defective synaptic transmission in Drosophila neuromuscular junctions

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Abstract
Synaptic dysfunction is believed to be an early pathological change in neurodegenerative diseases and may cause the earliest clinical symptoms. We have used Drosophila to model a tauopathy in order to analyse the earliest neuronal and synaptic dysfunction. Our work has shown that overexpression of human tau (0N3R) in larval motor neurons causes a disruption of axonal transport and a morphological and functional disruption of NMJs (neuromuscular junctions). Tau-expressing NMJs are smaller with an abnormal structure. Despite abnormal morphology, tau-expressing NMJs retain synaptotagmin expression and can form active zones. Tau-expressing NMJs are functionally abnormal and exhibit disrupted vesicle cycling and synaptic transmission. At low-frequency stimulation (1 Hz), ESPs (evoked synaptic potentials) produced by tau-expressing motor neurons were indistinguishable from wild-type; however, following high-frequency stimulation (50 Hz), ESPs from tau-expressing NMJs were significantly decreased in amplitude. To investigate the mechanism underlying the change in ESPs, we analysed the relative numbers and distribution of mitochondria. This revealed that motor neurons expressing tau had a significant reduction in the number of detectable mitochondria in the pre-synaptic terminal. Our results demonstrate that tau overexpression results in synaptic dysfunction, associated with a reduced complement of functional mitochondria. These findings suggest that disruption of axonal transport and synaptic transmission may be key components of the pathogenic mechanism that underlie neuronal dysfunction in the early stages of tauopathies.

Introduction
It is becoming clear that synaptic loss/dysfunction occurs in the early stages of a variety of neurodegenerative diseases including AD (Alzheimer’s disease; reviewed in [1]), HD (Huntingdon’s disease; reviewed in [2]) and prion diseases [3]. Although these diseases are characterized by extensive neuronal loss, abnormalities in the synapse have been reported prior to cell death in a number of human studies [4]. This is replicated in animal models of these diseases: synaptic electrophysiological defects are seen in the absence of cell death in scrapie, a mouse model of prion disease (J.R.T. Greene, personal communication), and in transgenic mouse and Drosophila models of AD [5–7] and HD [8,9]. In all these models, the synaptic dysfunction manifested in behavioural abnormalities, implying that early symptoms of many diseases are likely to be attributable to defects in synaptic transmission and not to neurodegeneration itself. This is supported by studies that show that clinical symptoms precede overt neuronal loss and pathological hallmarks in HD [10] and AD [11]. The mechanism(s) by which synaptic dysfunction occurs is not understood, and although there may be disease-specific pathogenic processes that underlie dysfunction in neurodegenerative diseases, common mechanisms are also likely to operate in all neurodegenerative diseases.

For example, Drosophila has been used to model aspects of synapse degeneration. For example, Drosophila has been used to model aspects of AD [12,13], Parkinson’s disease [14] and HD [15].

Tau and neurodegeneration
We have shown that targeted overexpression of tau in neurons of Drosophila induces a progressive degeneration with many of the attributes associated with neurodegeneration in...
human tauopathies and mammalian models of tauopathies [7,13,16]. This ability to faithfully replicate aspects of human disease, together with the availability of superior tools for experimental manipulation, make *Drosophila* an ideal experimental organism in which to analyse the mechanisms of neurodegeneration that underlie the pathogenesis of the human disease.

We have overexpressed mutant and wt (wild-type) forms of tau protein in motor neurons to induce a progressive degeneration in a population of identified and well-characterized neurons. During larval stages of development, motor neurons have long peripheral axons that can be imaged live and in situ, and have a peripheral synapse that is amenable to electrophysiological, functional and morphological analyses. These attributes allow us to study the effects of tau on the function of a single neuron and analyse the functional decline as it degenerates.

**Tau disrupts axonal transport**

Tau overexpression may result in neuronal dysfunction in a myriad of ways, and one obvious possibility is the disruption of axonal transport. *In vitro* studies have shown that tau interferes with kinesin activation [17] and disrupts the transport of various kinesin cargoes [18–20]. To analyse the effects of tau expression on axonal transport, we expressed a vesicle-targeted GFP (green fluorescent protein) to visualize the distribution of vesicles in tau-overexpressing and wt motor axons. This revealed that tau was having a devastating effect on axonal transport. In wt neurons, GFP-tagged vesicles were evenly distributed throughout the axons and NMJs (neuromuscular junctions). In tau-overexpressing neurons, the organization of vesicles was severely disrupted and the GFP-tagged vesicles accumulated into ‘vesicular aggregations’ along the axons. The aggregations were remarkably similar to the organelle ‘jams’ described in *Drosophila* axonal transport mutants. These results provided clear *in vivo* evidence to support the hypothesis that overexpression of tau potently inhibits axonal transport. It is noteworthy that these effects were seen before any evidence of neuronal death or formation of neurofibrillary tangles. These results highlight that the breakdown of axon transport can play a major part in the early pathogenic process in tauopathies.

**Tau expression in motor neurons produces a locomotor phenotype**

As well as showing clear dysfunction of axonal transport, larvae overexpressing tau in motor neurons exhibited a locomotor impairment. This was typically manifested by a sluggish locomotion and a loss of body tone. The most severely affected larvae showed paralysis of the posterior end and a characteristic tail flipping phenotype qualitatively similar to that described for mutations known to affect axonal transport. To further analyse the loss of locomotor function, we measured locomotor performance using several assays (crawling speed, rates of peristaltic contractions, righting behaviour and line crossing), all of which showed that locomotion of tau-expressing larvae is abnormal.

It is possible that this loss of locomotor function could be attributed to the death of motor neurons. A detailed count of neuronal cell bodies in the central nervous system, however, showed no loss of motor neurons in tau-overexpressing larvae, suggesting that the locomotor effects cannot be attributed to neuronal death and are likely to reflect a dysfunction of the neuron. To further analyse the locomotor dysfunction, we studied the consequences of tau overexpression on the motor neuron synapse – the NMJ.

**The morphology of NMJs overexpressing tau is abnormal**

Examination of the NMJs showed that in tau-overexpressing larvae, the overall pattern of innervation to the body wall musculature was identical with wt larvae and confirmed that overexpression of tau did not cause neuronal loss. Despite the overall pattern of innervation appearing normal, detailed observations showed that the NMJs of tau-overexpressing motor neurons were abnormal. Tau-overexpressing NMJs were significantly smaller than age-matched wt NMJs. Furthermore, NMJs overexpressing tau also showed abnormal morphological features not seen in wt NMJs. In tau-overexpressing NMJs, synaptic boutons were irregular in size and shape and surrounded by numerous small bouton-like structures which we called ‘mini boutons’. These mini boutons resemble the abnormal boutons observed in NMJs from motor neurons overexpressing the *Drosophila* APP (amyloid precursor protein) homologue APLP (APP-like protein) [21], in which axonal transport defects have also been reported [22,23].

Localization of the synaptic vesicle protein synaptotagmin in wt NMJs typically reflects the position of a readily releasable pool of synaptic vesicles and appears as a ring of staining in the periphery of synaptic boutons. In tau-expressing NMJs, this distribution was significantly changed, with synaptotagmin staining being localized largely within the core of the bouton and not the periphery. We also analysed whether tau affected pre-synaptic active zones using the nc82 antibody, a marker of active zones [24,25]. nc82 immunostaining of wt NMJs revealed discrete, punctate active zones distributed throughout the NMJ, predominantly located within boutons. In tau-overexpressing NMJs, although the overall structure of the NMJ was aberrant, discrete nc82 active zones were detected. Active zones were also seen in mini boutons. These results indicated that, although the NMJs from motor neurons overexpressing tau appeared morphologically abnormal, the presence of synaptotagmin I and nc82 (albeit abnormal) indicated that these synapses might be capable of neurotransmitter release.

**Exo/endocytosis is abnormal in NMJs overexpressing tau**

Given that tau-expressing NMJs have abnormal morphology, we analysed whether tau overexpression resulted in aberrant endo- and exo-cytosis using the styryl dye FM1-43. In wt NMJs loading with FM1-43 produced a high level of staining comparable with that seen in previous studies [26]. Similarly,
the induction of exocytosis to release the dye resulted in almost complete clearance of the dye from the NMJ.

Imaging of FM-143 uptake in tau-overexpressing motor neurons revealed a range of abnormalities. In the most extreme cases, there was a massive reduction in the amount of dye taken up compared with wt neurons. In most cases (75%), however, there was measurable FM1-43 uptake, but less than that seen in wt. Furthermore, tau-overexpressing NMJs displayed incomplete exocytosis of dye compared with wt. Taken together, these results suggest that, in tau-overexpressing preparations, endocytosis during transmitter recycling appears to take place relatively normally, but that following endocytosis the vesicles fail to return to the readily releasable pool.

**Evoked excitatory postsynaptic potentials**

We concluded from the FM1-43 uptake work that tau overexpression is disrupting the function of the synapse. To test this, we undertook an electrophysiological study of neuromuscular transmission. Surprisingly, this showed that, at low-frequency stimulation, synaptic transmission in tau-overexpressing motor neurons was indistinguishable from wt. However, when tau-overexpressing NMJs were exposed to high-frequency stimulation, the ESPs (evoked synaptic potentials) were significantly reduced compared with wt. These results indicate that unlike the wt synapses, NMJs overexpressing tau are not capable of sustaining the same level of neurotransmission.

**Motor neurons overexpressing tau have fewer functional mitochondria at the NMJ**

Since overexpression of tau disrupts mitochondrial transport in vitro [18,27] and mitochondrial function is critical for synaptic transmission, we postulated that changes in synaptic mitochondria may underlie the synaptic changes seen in the tau-overexpressing larvae. We examined mitochondria in the NMJ using TMRE (tetramethylrhodamine ethyl ester) to label functional mitochondria. In wt NMJs, uptake of TMRE into mitochondria revealed numerous intensely fluorescent clusters of mitochondria throughout the entire NMJ, with labelling evident in every bouton. Although NMJs from some motor neurons overexpressing tau showed TMRE labelling comparable with wt, the majority of boutons in tau-overexpressing NMJs contained smaller clusters of labelled mitochondria, with many boutons devoid of staining. These results indicated that there were significantly fewer functional mitochondria in the NMJs of motor neurons overexpressing tau.

**Conclusions**

In our work, we demonstrate that tau overexpression induces neurodegeneration in *Drosophila* and that this system provides an excellent method for analysing the cellular and molecular mechanisms that underlie the progression of tau-induced neurodegeneration. We have been able to show that one of the earliest manifestations of neuronal dysfunction is a disruption of axonal transport and synaptic function. Furthermore, our results indicate that the most likely cause of this synaptic dysfunction is a reduction in the number of functional mitochondria at the synapse. These phenotypes were seen in the absence of neurofibrillary tangles or neuronal death.

These results prove that tau can mediate neuronal and synaptic dysfunction in the early stages of tauopathies, before overt neurodegenerative events are evident. These effects may be responsible for preclinical symptoms in tauopathies such as AD. Recognition of the role played by these early events in disease may pave the way for novel therapeutic intervention to halt the degenerative process.

**References**


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