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Tauopathies in Drosophila: Do Tau isoforms have the same toxicity and can Neurexin rescue Tau induced neurodegeneration?

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Tauopathies in *Drosophila*: Do Tau isoforms have the same toxicity and can Neurexin rescue Tau induced neurodegeneration?

A thesis submitted to Bangor University by

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Master of Science by Research

Supervised by

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Declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

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For Glossop Grandma, whose diagnosis and experience with dementia inspired me to pursue tauopathy research.

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Abstract

Tauopathies are a group of heterogeneous dementias and movement disorders characterised by intracellular accumulations and hyperphosphorylation of the microtubule-associated protein Tau. The best known tauopathy is Alzheimer's Disease, once thought to develop solely by the aggregation of β-amyloid into plaques. It is now understood to also be a result of the misfolding of Tau, alongside the aggregation of β-amyloid plaques. Neurodegeneration cannot be reversed, and the effects of these diseases can only currently be alleviated, not cured. With increases in technology and scientific capabilities, tauopathy research has exponentially grown and it is hoped that a treatment for neurodegenerative diseases will be developed. Tau typically plays a role in maintaining the stability of microtubules and axons in the neurons. When Tau becomes hyperphosphorylated, it loses its normal physiological function and aggregates into toxic neurofibrillary tangles, a hallmark of tauopathies. This work uses the larval neuromuscular junction of the fruit fly, *Drosophila melanogaster*¸ a well-established model system for studying tauopathies, to analyse two aspects of tauopathies. The first aim sought to compare the effects of overexpressing each of the six human isoforms. The results showed that UAS-hTau^{0N3R}, UAS-hTau^{2N3R} and UAS-hTau^{0N4R} produced statistically significant changes in NMJ morphology, with a decrease in average bouton size, and that UAS-hTau^{0N3R} was the only isoform to produce a significant change in average bouton number. These results were surprising and do not align with previous work which suggest that all Tau isoforms are equally toxic. The second aim was a proof of principle study to see if co-expression of the synaptic proteins known as Neurexins could ameliorate the toxic effects of Tau. Neurexins are presynaptic cell adhesion proteins which alongside Neuroligins, their post-synaptic partners, promote and stabilise synapse formation. The effects of Neurexin expression on the Tau phenotypes was inconclusive, with the crosses designed to generate co-expression of Neurexin and Tau proving lethal. The implications of these results are discussed and suggestions for further experimental work proposed.

Key words: *Drosophila melanogaster*, Tauopathies, Neuromuscular Junction, Tau, Neurexin

Introduction

Neurodegenerative diseases represent one of the major healthcare challenges of the 21st Century. By 2015 there were 850,000 people living with dementia in the UK, with dementia costing the UK £26 billion a year (Prince *et al.,* 2014). This context places a huge imperative on research to develop treatments to mitigate the financial and personal implications of these diseases. Alzheimer's Disease (AD), the best known of the dementias, is an irreversible and progressive disease that slowly erodes cognitive functions. The progression of AD is associated with two classes of abnormal structures: extracellular amyloid plaques and intraneuronal neurofibrillary tangles (Goedert, 1998; Hernández and Avila, 2007; Lee *et al.*, 2001; O'Neill *et al.*, 2001; Williams, 2006; Wood *et al.,* 1986). The building blocks of these respective structures include amyloid-β (Aβ) peptides and the microtubule-associated protein Tau (DeTure and Dickson, 2019; Goedert and Spillantini, 2006; Stelzmann *et al.*, 1995; Wenk, 2003).

The focus of AD research since the early 1990s has been on the role of Aβ, however there is increasing evidence for a greater role of Tau in AD pathogenesis, with neurofibrillary tangles appearing before Aβ plaques (Kanaan *et al.,* 2015). Whilst Tau is often seen as being pivotal to AD progression, the relationship between Tau and Aβ is not simple and Tau abnormalities are not solely responsible for AD pathogenesis. Two models exist in regard to the relationship between Tau and Aβ. The first, the amyloid cascade hypothesis, postulates that Aβ is the causative agent in AD pathology, acting in a series upstream of Tau therefore triggering Tau activity (Gulisano *et al.*, 2018; Hardy and Higgins, 1992; Puzzo *et al.*, 2017). The second suggests Aβ and Tau work cooperatively and in parallel to each other, and it is this which is currently disbanding the original hypothesis (Fá *et al.,* 2016; Ondrejcak *et al.,* 2018; Pickett *et al.,* 2019; Puzzo *et al.,* 2017). In support of this notion Puzzo et al. (2020) suppressed Tau in knockout mice. This caused a reduction in long-term potentiation (LTP) – the process of strengthening the synapses leading to a long-lasting increase in signal transmission, an important quality that determines synaptic plasticity (Cohen *et al.,* 1999; Fu and Jhamandas, 2020, Lynch, 2004). This proved that Aβ continues to cause synaptic defects independent of the microtubule-associated protein, suggesting the need to encourage therapies that simultaneously target the downstream action of Aβ and Tau to improve LTP and memory in AD patients (Puzzo *et al.,*

2020). Despite this subtle interaction between Tau and Aβ, Tau is recognized to be at the heart of many neurodegenerative diseases, known collectively as tauopathies, all of which are characterized by the abnormal intracellular accumulation of Tau, in the form of tangles. (Hernández and Avila, 2007; Lee *et al.,* 2001; O'Neill *et al.,* 2001; Williams, 2006; Wood *et al.,* 1986).

To study the mechanisms of tauopathies numerous vertebrate and invertebrate animal models have been developed. One of the most widely used invertebrate animal models is the fruit fly, *Drosophila melanogaster*, with its powerful molecular genetic toolbox providing understanding into the mechanisms of Tau toxicity in tauopathies (Sivanantharajah *et al.,* 2019). One of the key outcomes of tauopathy work with *Drosophila* is the demonstration that abnormal Tau can rapidly result in the breakdown of the axonal cytoskeleton, synaptic structure and related functions (Chee *et al.,* 2005).

Tauopathies

Tauopathies are a group of dementias that are neuropathically characterised by prominent intracellular accumulations of abnormal Tau and appear to share common mechanisms of disease. Examples of tauopathies include AD, Primary Age-Related Tauopathy, Frontotemporal Dementia, Pick's Disease, Progressive Supranuclear Palsy, Corticobasal Generation and Argyrophilic Disease (Kovacs, 2018). Tauopathies are characterised by the hyperphosphorylation of Tau, where Tau disassociates from microtubules to form insoluble aggregates, creating the neurofibrillary tangles common of tauopathies (Arendt *et al.,* 2016; Binder *et al.,* 2005; Hernández and Avila, 2007; Lee *et al., 1*989; O'Neill *et al.,* 2001). Tau (tubulin associated unit), also known as microtubule-associated protein Tau, is encoded by the *MAPT* gene on chromosome 17, in humans, and is predominantly expressed in brain cells. Its primary role is the assembly and stabilization of microtubules (Bachmann *et al.,* 2020; Lee *et al.,* 1989). Microtubules are required for the transport of molecular cargo (axon transport) between the cell body and synapses (Wolfe, 2009).

Mechanisms in disease

There is not thought to be one single trigger of how Tau causes disease. Abnormal hyperphosphorylation of Tau can lead to the polymerization of neurofibrillary tangles, due to the

presence of too much Tau, and it is these filamentous aggregates of Tau which result in a toxic gain of function (Gendron and Petrucelli, 2009). Too much or too little Tau can affect microtubule dynamics, axon transport and microtubule stability, through hyper/hypo-phosphorylation, either through over-stabilization or destabilization, respectively, thereby impacting axon stability and synaptic function (Barbier *et al.*, 2019; Cai and Tammineni, 2017; Chauhan *et al.*, 2022; Cheng and Bai, 2018; Wolfe, 2009). In AD, Tau is hyperphosphorylated, and abnormal hyperphosphorylation and altered Tau metabolism can stimulate neurofibrillary tangle formation (Wolfe, 2009). This aggregation of Tau into filamentous tangles causes a deleterious gain of function similar to that of Aβ accumulation in AD, and it is this that is thought to result in synaptic damage and early axonal transport defects, affecting microtubule assembly and disassembly, triggering neurodegeneration (Alonso *et al.*, 1994; 1996; 1997; Binder *et al.*, 2005; Gendron and Petrucelli, 2009; Spillantini and Goedert, 1998; Spillantini *et al.*, 1998).

Glycosylation is also linked to Tau phosphorylation, with glycosidic bonds being either N-linked or O-linked depending on which terminal the sugars are linked to (Gendron and Petrucelli, 2009). Hyperphosphorylated Tau in human AD brains are glycosylated, through N-linkage, but this is not the case in Tau extracted from controls, suggesting glycosylation enhances Tau hyperphosphorylation (Gendron and Petrucelli, 2009; Takahashi *et al.*, 1999; Wang *et al*., 1996). O-linkage glycosylation creates the O-linked monosaccharide β-N-acetylglucosamine (O-GlcNAc), which is involved in controlling the regulation of Tau phosphorylation (Gendron and Petrucelli, 2009; Liu *et al.*, 2004). O-GlcNAc levels are lower in the AD brain, causing the hyperphosphorylation of Tau to occur more frequently (Gendron and Petrucelli, 2009). It isn't all about phosphorylation, however, alterations in Tau protein quantity and missense mutations have also been shown to be a cause of tauopathies (Binder *et al.*, 2005; Hernández and Avila, 2007; Lee *et al.*, 1989).

Mutations in the *MAPT* gene in humans, and *Mapt* gene in murine patients, produces structural aberrations alongside increased levels of aneuploidy (Rossi *et al.*, 2014). This aneuploidy caused by mutant Tau is therefore conceivably present in all animal models, due to the perseverance of evolutionary conservation, and its existence within the central nervous system, creating pathological neurodegeneration (Rossi *et al.*, 2014). Mutations in the *MAPT* gene affecting

splicing mechanisms or Tau functionality can lead to the formation of various isoforms that are more likely to form the neurofibrillary tangles seen in all animal models. This is because alternative splicing of *MAPT* transcripts determines the ratio of 3R and 4R Tau isoforms in the adult brain, and if an imbalanced ratio occurs, an altered number of binding sites available for the isoforms to bind to arises, potentially leading to increased filament assembly due to the free – floating – Tau (Gendron and Petrucelli, 2009). Missense and deletion mutations are another set of mutations which can infer toxicity and cause disease and have been found to be involved in tangle acceleration in frontotemporal dementia (Arrasate *et al.,* 1999; Goedert *et al.,* 1999; Nacharaju *et al.,* 1999).

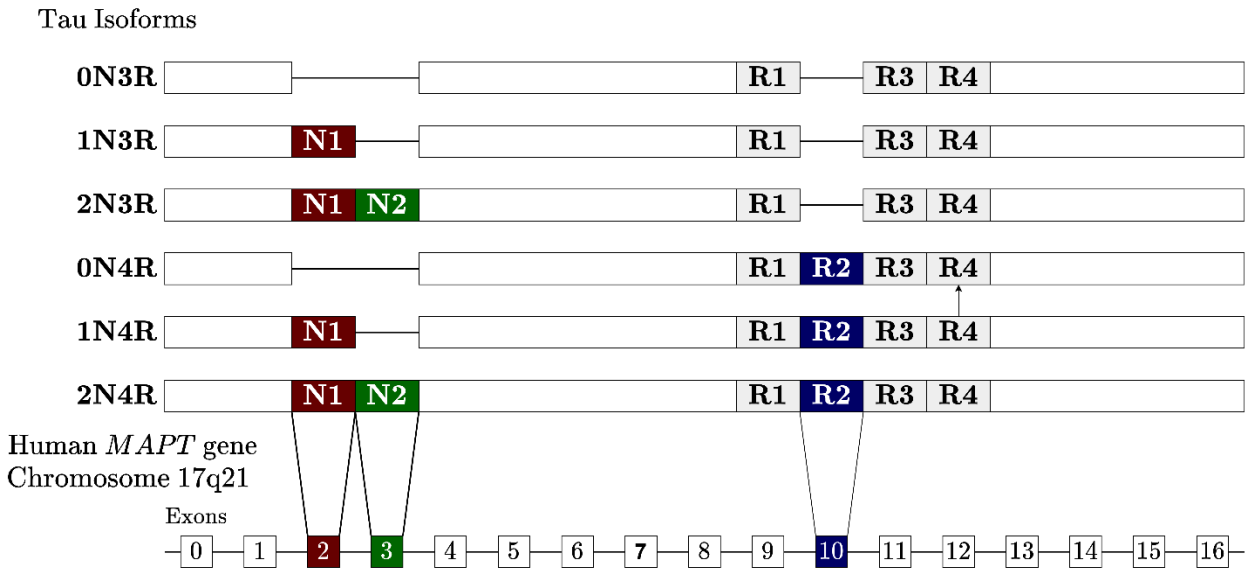
Loss of function Tau mutants also cause adverse effects in Tau-knockout mice, phenotypes corresponding to frontotemporal dementias and parkinsonism's are seen (Ikegami *et al.,* 2000). Tau-knockout can disrupt neuron development, mitochondrial transport, create motor deficits and impair learning abilities in mice (Lei *et al.,* 2012, 2014; Regan *et al.,* 2015; Sapir *et al.,* 2012). Despite these evidently toxic phenotypes, the evidence is contradictory, with some suggesting that Tau-knockout does not produce changes in behaviour and locomotor function, hence suggesting these animals appear reasonably normal, implying other proteins compensate for the lack of Tau (Ahmed *et al.,* 2014; Dawson *et al.,* 2001; Lei *et al.,* 2014; Morris *et al.,* 2013; Tucker *et al.,* 2001). Here, standardization between experiments and their complexities have caused bias and produced these conflicting results. Tau toxicity is therefore complicated with numerous mechanisms of disease existing. Some of these mechanisms are genetics-related, whilst others are not. One complication is the existence of multiple isoforms of Tau, each conferring differing effects. Alternative splicing creates different isoforms of Tau, and it is these isoforms that exhibit varying levels of expression within the adult brain.

Tau isoforms

There are six isoforms of Tau expressed in the adult human brain and each of the six isoforms are not equally expressed in all neurons, differing in their microtubule-binding affinity, aggregation capability, microtubule bundling ability and their process of regulating microtubule dynamics (Buée *et al.,* 2000; Cox *et al.,* 2016; Goedert and Jakes, 1990; Levy *et al.,* 2005; Panda *et al.,* 2003; Trinczek *et al.,* 1995). The different isoforms are formed by alternative splicing of exons 2, 3 and 10 on the *MAPT* gene on chromosome 17, which consists of 16 exons in total (Fig 1) (Bachmann *et al.,* 2020; Couchie *et al.,* 1992; Goedert and Jakes, 1990; Ruiz-Gabarre *et al.,*

2022; Vourkou *et al.*, 2022). Exons 2 and 3 at the N terminus are called N1 and N2 respectively, and Tau isoforms can include none (0N isoforms), one (1N isoforms) or both (2N isoforms) of these exons (Vourkou *et al.*, 2022) (Fig 1). Exon 10 is one of four so called R domains (exons 9, 10, 11 and 12) and all Tau isoforms include the R domains encoded by exons 9, 11 and 12, whereas integration of exon 10 is variable (Fig 1) (Ruiz-Gabarre *et al.*, 2022). Isoforms lacking exon 10 are referred to as 3R Tau, and those that include exon 10 as 4R Tau (Fig 1) (Bachmann *et al.*, 2020; Couchie *et al.*, 1992; Goedert and Jakes, 1990; Vourkou *et al.*, 2022). These different Tau isoforms exist in humans and have different cellular functions.

Figure 1. The six isoforms of Tau, expressed in the adult human brain, as a result of alternative



splicing of the *MAPT* gene. Each isoform consists of a differing N-terminal insert (0N, 1N, 2N), produced by exons 2 and 3, and C-terminal repeat domain, encoded for by exon 10 on the human *MAPT* gene. The presence of the R2 domain determines whether the isoform becomes 3R or 4R. These isoforms are formed by alternative splicing of pre-RNA at exons 2, 3 and 10 of the *MAPT* gene.

Typically, 4R Tau isoforms have higher affinity for aggregation due to the wide range of filament lengths they produce and the increased microtubule stability they generate after synaptic connections have been assembled (Andreadis *et al.*, 1992; Bunker *et al.*, 2004; Cox *et al.*, 2016; Feinstein and Wilson, 2005; Goedert and Jakes, 1990; Goode *et al.*, 2000; Levy *et al.*, 2005; Panda *et al.*, 2003; Peck *et al.*, 2011; Trinczek *et al.*, 1995). In contrast, 3R Tau isoforms form

globular oligomeric aggregates, and rarely long filaments (Cox *et al.,* 2016). 3R isoforms play key roles in axonal outgrowth during development and bind much less efficiently than 4R isoforms, likely due to their lack of an inter-repeat sequence unique to isoforms with four repeats, thus causing fewer aggregation of neurofibrillary tangles (Arendt *et al.,* 2016; Goedert and Jakes, 1990).

A large proportion of studies have used murine models to study tauopathies and discover the structural integrity of the Tau isoforms. Mice are valuable models in the study of diseases as they are key to demonstrating the effects on models which are anatomically, physiologically, and genetically similar to humans. They are also advantageous due to their small size, short life cycle and the abundant genetic research surrounding them. However, a smaller model might be necessary when first attempting to conduct proof of principle studies, and in labs where resources will not yet allow for the use of rodents. A key model here is *Drosophila melanogaster,* the fruit fly.

Drosophila as a model organism to study tauopathies

There are many species of *Drosophila,* including *D. simulans, D. suzukii* and *D. bifurca,* each with their own unique characteristics and traits, but *Drosophila melanogaster* is undoubtedly the most well-known and widely used in biological research. *Drosophila melanogaster,* or the fruit fly, has been a valuable model organism in genetics for over a century. *Drosophila* is easy and inexpensive to breed within a laboratory setting, due to their short life cycle of approximately 12 days and a rapid generation time of two weeks. *Drosophila's* main attribute is its genetics. Its genome has been well-characterised, and with only four pairs of chromosomes, opposed to the twenty-three pairs in humans, *Drosophila's* genes have been easily mapped allowing for the study of genes and genetic pathways that are conserved across animal species (Csink and Henikoff, 1996; Trieu and Cheng, 2014). The fruit fly has been extensively studied, creating a vast body of knowledge on its genetics, behaviour, development, and physiology, all of which can be linked to human biological processes and pathways. Due to its impressive phenotypic plasticity, *Drosophila* can adapt to changes in their environment, providing insights into how environmental factors can influence the progression of genetic pathways and consequently affect *Drosophila's* development, physiology and behaviour (David *et al.,* 1997; Gibert *et al.,* 2004; Mathur and Schmidt, 2017; Schlesener *et al.,* 2020; Zhou *et al.,* 2012).

In the late 1800s, Castle and Woodworth discovered the ease of breeding flies in laboratory conditions, introducing the fly as a potential model organism in genetic research (Carlson, 2013). In the 1910s, Thomas Hunt Morgan and his team used *Drosophila melanogaster* to identify key principles of modern genetics, including genetic linkage, the concept of genetic recombination, and most notably, sex-linked inheritance, establishing chromosome theory of inheritance and debuting the species as a prominent and powerful model organism (Morgan, 1910). Since then, *Drosophila* has contributed hugely to the understanding of genetics and biology. Alfred Sturtevant developed the first genetic map of the *Drosophila* genome, paving the way for the entire genome sequencing in the early 2000s (Adams *et al.,* 2000; Sturtevant, 1920). The ground-breaking discovery of the homeobox gene family was initiated by the work of Wieschaus and Nüsslein-Volhard who identified the key genes involved in patterning the developing embryo, revealing the genetic basis of embryonic patterning (Nüsslein-Volhard and Wieschaus, 1980). The astonishing ability to manipulate its genome is what has led to the pre-eminence of the fly in modern biological research. The fly has been subjected to widespread and systematic programs of mutagenesis, identifying many genes that have subsequently proven to be highly conserved and of fundamental importance in all biological systems (Konopka *et al.,* 1994; Nüsslein-Volhard and Wieschaus, 1980; Roberts, 2006; Yamaguchi and Yoshida, 2018). The *Drosophila* genome has also proven to be amendable to targeted manipulation of genes, and the development of directed gene expression systems that allow the targeted, tissue and cell specific misexpression of new or modified genes, including tagged genes such as fluorescent proteins (Brand and Perrimon, 1993). Such tools have not only allowed for the analysis of gene function, but also to visualize the growth and structure of specific tissues and cells in animals using the expression of green fluorescent proteins (GFP) and GFP-fusions, for example, to study the development and function of the *Drosophila* larval NMJ (Salvaterra and Kitamoto, 2001; Schmid and Sigrist, 2008).

Animal nervous system structures and functions exhibit evolutionary conservation across all vertebrate species (Reichert, 2009). Many cellular and molecular homologies exist between humans and *Drosophila* with processes such as axon transport, synaptic transmission, signal transduction and neuron excitability all being highly conserved (Sivanantharajah *et al.*, 2019). This conservation is not just cellular, but also present at the molecular level, with the sequencing of the *Drosophila* genome by Adams et al. (2000) revealing remarkable levels of conservation of

genes from flies to humans (Adams *et al.,* 2000). Such that approximately 60% of *Drosophila* genes have clear homologs in the human genome, with approximately 75% of human genes known to be associated with diseases also having homologs in *Drosophila* (Mirzoyan *et al.,* 2019; Ugur *et al.,* 2016). Such homology allows *Drosophila* to be used as a model for human diseases, for instance; neurodegenerative diseases and cancers (Greenspan and Dierick, 2004). Using *Drosophila* as a model for human disease allows us to analyse and manage how disease-causing genes can disrupt complex biological systems, especially in the development of dementias.

Contributions of *Drosophila* for Tauopathies analysis

Work on *Drosophila* has made major contributions to the understanding of Tau-mediated neurodegeneration (Lee *et al.,* 2005). The first demonstration that human Tau (hTau) is pathogenic in flies came from the work of Wittmann et al. (2001), showing that hTau over-expression in *Drosophila* neurons produces neurodegeneration in the absence of filamentous aggregates. It was originally believed that the filamentous tangles, which occur due to Tau hyperphosphorylation, were acting as physical barriers inhibiting the transport mechanisms required for a cell to function correctly (Wittmann *et al.,* 2001). Further work has now confirmed that neurotoxicity is a result of Tau modifications prior to the formation of filamentous tangles, indicating Tau toxicity alone can cause neurodegeneration (Wittmann *et al.,* 2001). These studies show that simple over-expression of Tau itself is enough to cause neurodegeneration, whilst hyperphosphorylation and filament formation exacerbate Tau induced neurodegeneration (Chee *et al.,* 2005; Jackson *et al.,* 2002; Mudher *et al.,* 2004; Wittmann *et al.,* 2001). However, there are caveats to using over-expression to study protein properties and functionality; expression levels vary and are often not standardised across different laboratory experiments and resource overload could occur where cellular resources may be stretched, resulting in cellular defects (Moriya, 2015). Both of these have the potential to create a biased result in comparison to the wild type (WT) (Moriya, 2015).

Another significant attribute of working with *Drosophila* is that it allows the study of the effects of Tau expression on individual identified neurons in living animals. It is therefore possible to study the early stages of Tau induced degeneration, to understand the earliest cellular and physiological stages of the disease. Taking advantage of this, Mudher et al. (2004) showed that expression of Tau in *Drosophila* motor neurons disrupted axonal transport, causing vesicles to

aggregate, with a subsequent decline in locomotor function. They also showed that the Tau-kinase, glycogen synthase kinase 3 beta (GSK-3β) activity could enhance the Tau phenotype, suggesting that the effects of Tau are regulated by phosphorylation. Building on this, Chee et al. (2005) analysed the effects of Tau expression on the structure and function of the neuromuscular synapse in *Drosophila,* showing that expression of human 0N3R Tau in larval motor neurons causes morphological and physiological disruption of NMJs. The results showed Tau expressing NMJs were reduced in size, the synaptic bouton structure was abnormal and that the NMJs had disrupted synaptic vesicle trafficking, impaired synaptic transmission, and a reduction in functional mitochondria in the presynaptic terminal. This large and growing body of research using *Drosophila* to study the pathogenesis of Tau serves to demonstrate the enormous value of the fly as a model system.

The effects of expressing the different Tau isoforms have been tested in *Drosophila* neurons but due to the differing experimental paradigms their relative toxicities remain unclear. The earliest studies focused on 0N3R Tau, showing that expression of this isoform in motor neurons decreases synaptic function by causing structural defects in motor neurons (Chee *et al.,* 2005; Mudher *et al.,* 2004). Interestingly, the $hTau^{0N3R}$ isoform is expressed during foetal development in humans, and is more likely to have a unique role compared to the other five hTau isoforms, which are only expressed in fully developed adults (Buée *et al.,* 2000; Goedert and Jakes, 1990; Kosik *et al.,* 1989).

A more extensive study of the different Tau isoforms in *Drosophila* expressed different hTau isoforms within the *Drosophila* mushroom body (MB) showing that expression of 0N3R, 0N4R and 2N4R isoforms all negatively affect MB structure, resulting in abnormal olfactory learning and memory, with 0N4R having the most severe effects and 0N3R have minimal (Kosmidis *et al.,* 2010). Using the Giant Fiber system of *Drosophila*, Kadas et al. (2019) tested the effects of expressing different Tau isoforms on axonal and synaptic physiology, showing 3R and 4R isoforms affecting different aspects of synaptic function. They showed that expression of 0N3R Tau affected synaptic transmission resulting in increased synaptic failure at high stimulation frequencies whereas 0N4R Tau adversely affected axonal conduction and the speed of synaptic transmission.

Vourkou et al. (2022) studied the effect of expressing the six Tau isoforms on the *Drosophila* MBs, and whilst analysing both anatomical and behavioural effects showed that the expression of 0N3R, 1N3R, 2N3R and 0N4R isoforms all affected MB morphology by decreasing MB size, but strikingly only 0N4R produced learning deficits. Collectively, these observations suggest that the behavioural deficit is a specific effect of the 4R domain isoforms. Expresion of none of the isoforms had any impact on protein synthesis-independent memory formation, although the 4R isoforms negatively affected protein synthesis-dependent memory formation, as it is comprised of by only the 4R isoforms, implying these isoforms may have a damaging effect on translational abilities, and it is translation which forms the basis of protein synthesis-dependent memory, therefore impacting an animals learning capabilities (Tully *et al.*, 1994; Vourkou *et al.*, 2022). Expession of 0N3R and 2N4R had no effect on habituation, whereas 1N3R inhibited it, whilst 2N3R, 0N4R and 1N4R promoted premature habituation. Successful habituation is dependent upon regulated neurotransmission from numerous different types of MB neurons, suggesting that each of the hTau isoforms that cause habituation alterations have different functional specificity (Acevedo *et al.*, 2007; Roussou *et al.*, 2019; Vourkou *et al.*, 2022). In addition to this they also showed that expression of all isoforms increase the animals locomotion and vulnerability to oxidative stress, which consequently leads to the expression of all hTau isoforms causing a decreased lifespan compared to WT.

Drosophila NMJ as a model system to study neurodegeneration

My project uses the *Drosophila* NMJ as a model system to analyse the early stages of induced tauopathies. The *Drosophila* NMJ is commonly used to study synaptic function, making it an ideal structure to study tauopathies, as they are easily accessible and therefore effortlessly facilitate anatomical and physiological study in living animals (Beramendi *et al.*, 2007; Keshishian *et al.*, 1996). NMJs are specialized synapses where motor neurons communicate with muscle fibres to initiate muscle contractions and is also the locus to many neurodegenerative diseases (Engel, 2008; Hughes *et al.*, 2006). There are two main types of NMJs: type 1 and type 2. The classical structure of a type 1 NMJ is to have the appearance of simple axonal branching structure, with more or less evenly spaced swellings or boutons giving the NMJ a ‘beads on a string’ appearance (Fig 2D.). These have larger numbers of synaptic vesicles and a higher density of acetylcholine receptors, whilst the opposite is true for type 2 NMJs (Atwood *et al.*, 1993; Broadie and Bate, 1995; Johansen *et al.*, 1989). The structural differences of the two types

of NMJs reflect their functional specialisations. Type 1 NMJs are specialised for fast and powerful contractions, and are usually found on larger muscles, whilst type 2 are specialised for sustained, low-intensity contractions on smaller muscles (Atwood *et al.,* 1993; Broadie and Bate, 1995; Johansen *et al.,* 1989). This thesis focuses only on the type 1 NMJs as these are the most widely studied and have been previously used in the study of tauopathies (Chee *et al.,* 2005; Mudher *et al.,* 2004).

To access the *Drosophila* NMJ a larva (Fig 2A) must be obtained for dissection and analysis. Following this it can be easily imaged and analysed using high through-put techniques, making it possible to perform large-scale studies of synaptic function and morphology, identifying any changes in activity that may trigger neurodegenerative diseases. Due to its genetic tractability and neuronal conservation, the *Drosophila* NMJ is ideal in the implication of human diseases. As the molecular and cellular mechanisms of synaptic function and plasticity are highly conserved across animal species, with many genes involved in human neurodegenerative diseases having orthologs in *Drosophila*, the study of the *Drosophila* NMJ can provide valuable insights into neurodegeneration, giving rise to potential target therapies.

Each abdominal hemisegment of the larva contains thirty somatic muscles, each uniquely identifiable by shape and position and segmentally repeated (Fig 2B), creating a stereotypic pattern and repeatable structure, with each muscle individually identifiable, perfect for individual muscle analysis (Fig 2C.) (Bate, 1990; Budnik, 1996; Gramates and Budnik, 1999; Hoang and Chiba, 2001; Jan and Jan, 1976; Johansen *et al.,* 1989; Keshishian *et al.,* 1996). The muscles are controlled by an array of thirty two motor neurons in each abdominal hemi-neuromere of the ventral nerve cord, which project their axons peripherally to synapses on their specific muscle (Menon *et al.,* 2013; Prokop, 1999). Each muscle is innervated by a single type 1 motor neuron which produces a highly stereotyped NMJ on each muscle, thus not only is each muscle identifiable, each NMJ is also. This is such that it is possible to analyse single unique NMJs which have invariant NMJ morphologies in WT flies.

Boutons, otherwise known as axon terminals, are specialized bulbous structures positioned at the end of an axon, and have a unique size and distributional spread individual to each neuron (Knodel *et al.,* 2014; Pack-Chung *et al.,* 2007). They are responsible for the release of neurotransmitters into the synaptic cleft – the gap bridging the presynaptic and postsynaptic

membrane (Atwood *et al.,* 1993; Knodel *et al.,* 2014; Pack-Chung *et al.,* 2007). Boutons are an essential structure establishing the connection between the CNS and neurons and are present across the entire animal kingdom. Two types of boutons are present on the larval body wall muscles of *Drosophila*; type I and type II, with type I subdividing into two further classes - Type Ib (larger, spherical and variable) and Is (smaller and regular) boutons (Atwood *et al.,* 1993). Type II boutons are much smaller than type I, extend the muscle length and can be stained and identified by the 'Small Synaptic Bouton' antigen (Budnik and Gorczyca, 1992). Both types of boutons contain numerous glutamatergic release sites (Atwood *et al.,* 1993; Meinertzhagen *et al.,* 1998; Knodel *et al.,* 2014; Sigrist *et al.,* 2003).

Glutamate is the most abundant excitatory neurotransmitter present in both the *Drosophila* and mammalian brain, is involved in the majority of excitatory functions and consequently, by binding to glutamate receptors, controls muscle contractions throughout the animals body (DiAntonio, 2006). Glutamate receptors are required during synapse formation and for efficient synaptic function, but also allow for synaptic plasticity in response to altered neural activity (DiAntonio, 2006). This plasticity is advantageous as the synapse will evolve and strengthen neuronal connections in response to change, important for learning and memory (Citri and Malenka, 2008). Similarly, if the NMJ begins to degrade, the glutamatergic property of the synapse will adapt to this and learning and memory function may also disintegrate. A further disadvantage of glutamatergic synapses is the potential of toxic levels of glutamate being released, causing neuronal death (Choi, 1988).

A method of monitoring neuronal health is via the GAL4 expression system. GAL4 driven expression of Tau in *Drosophila* larval motor neurons has been previously shown to affect NMJ structure and function. Typically, the overexpression of hTau in motor neurons results in defective NMJ structures characterised by irregular and abnormal bouton structure, consequently causing an increased number of smaller, spindlier and abnormally shaped boutons than in WT NMJs (Fig 2E) (Chee *et al.,* 2005).

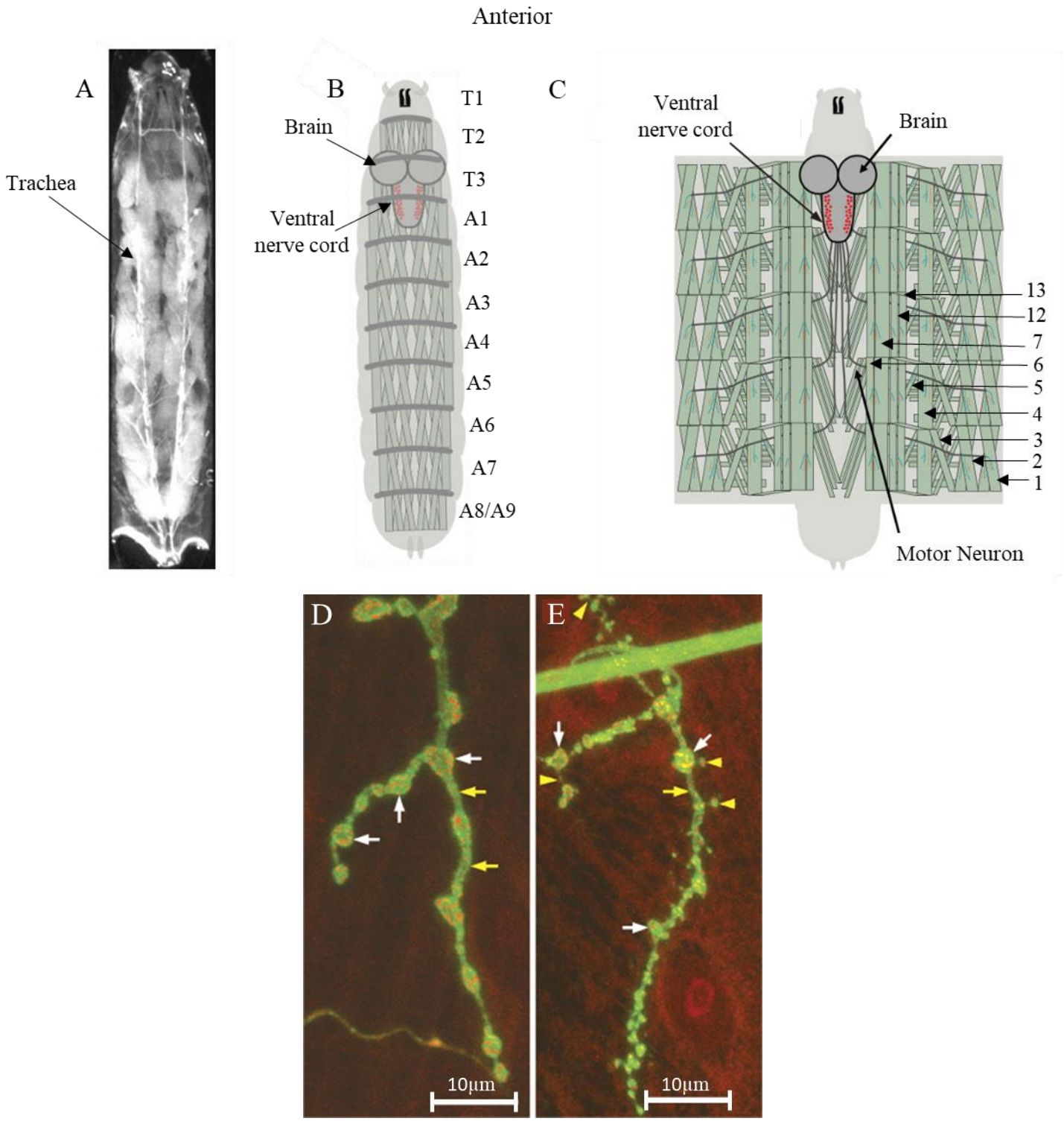


Figure 2. (A) Dorsal view of a *Drosophila* larva, transparent cuticle is showing the trachea (arrow) (Image from Newman, 2016). (B) Diagram of *Drosophila* larva showing segmentation (T1-T3 represent the thoracic segments and A1-A9 the abdominal segments), the brain and the ventral nerve cord. (Image from Newman, 2016). (C) Diagram of a dissected open larva; the two

lobes of the brain are visible, with the ventral nerve cord, motor neurons, and body wall muscles (green). Individually identifiable muscles (1, 2, 3, 4, 5, 6, 7, 12 and 13) in a stereotypic innervation, with each muscle having its own motor neuron. (Image from Newman, 2016) (D) Normal NMJ morphology with the classic bouton 'beads on a string' organisation with much larger boutons (Image from Chee *et al.,* 2005). (E) Tau-affected NMJ (Image from Chee *et al.,* 2005). The red dots present in both D and E indicate the active zones – presynaptic structures revealed by the anti-synaptotagmin antibody. The white arrows indicate the labelling of anti-nc82 in boutons, yellow arrows indicate the inter-bouton regions and the yellow arrowheads indicate satellite boutons.

The UAS/GAL4 Expression system

This study makes use of the UAS/GAL4 expression system first developed by Brand and Perrimon (1993). The system is one of the most powerful experimental tools in *Drosophila* making it possible to drive the expression of any desired gene in selected and specific sub-sets of cells and tissues. This GAL4 system utilizes a tissue/cell specific driver line (GAL4) and a responder line, an upstream activation sequence (UAS) linked to the gene to be expressed (Brand and Perrimon, 1993). The GAL4 line uses a cell or tissue specific enhancer element to drive the expression of GAL4, a yeast transcriptional activator, which in turn binds to the UAS enhancer – a cis-acting regulatory sequence and binding site for GAL4, thereby activating expression of the downstream gene (Fig 3) (Brand and Perrimon, 1993; Cowan *et al.,* 2011). When flies are produced that have the specific GAL4 and UAS linked target gene, the tissue specific expression of GAL4 will activate transcription of the UAS linked transgene in a cell/tissue specific pattern (Brand and Perrimon, 1993; Cowan *et al.,* 2011). This is achieved by simply crossing flies carrying the required GAL4 driver with the desired UAS responder line. In this study, a larval motor neuron specific GAL4 line was used to drive expression of UAS linked hTau isoforms in larval motor neurons. There are a number of GAL4 lines that can be used for larval motor neuron specific expression, but this study utilises the OK6 enhancer-trap line which has been widely used to drive expression in larval motor neurons and is the most specific of the motor neuron specific GAL4 lines (Sanyal, 2009).

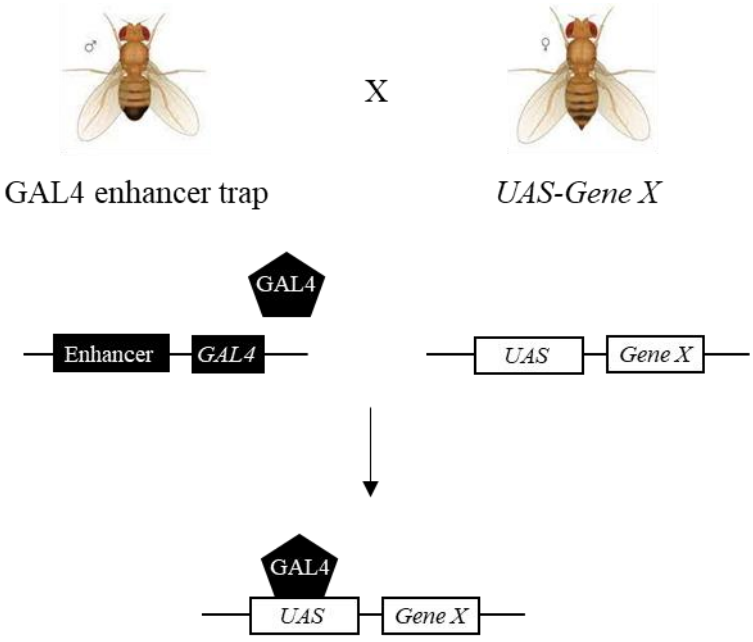


Figure 3. GAL4/UAS expression system. Flies expressing GAL4 are crossed with flies carrying the target UAS-gene. The GAL4 transcriptional activator binds to the UAS enhancer sequences. Transcription and translational activity occur to induce gene expression downstream.

This thesis builds on *Drosophila* models with the use of this expression system to address the following question.

Hypothesis 1 - Does targeted expression of the six different isoforms of human Tau have varying effects on the *Drosophila* neuromuscular junction?

To test this, the UAS-hTau lines created by Fernius et al. (2017), who made a collection of lines in which UAS linked transgenes for each the 0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R Tau isoforms were inserted into the same genomic location, were utilised. The location of the transgenes at a common genomic location should result in less variability in expression levels of the UAS driven hTau isoforms and facilitate a comparison of the differing toxicities of the different Tau isoforms. To compare the different isoforms, the motor neuron specific GAL4 line was used to exclusively drive hTau expression in motor neurons and analyse the phenotypic effects on the morphology of the larval neuromuscular junction (NMJ). Preceding the work of Fernius et al. (2017), who post-mitotically expressed hTau in the *Drosophila* nervous system, these fly stocks were used to express hTau in the *Drosophila* NMJ, under the hypothesis that each hTau isoform will have varying levels of toxicity.

Can a synapse rescue itself from Tau-induced neurodegeneration?

Neurexin and Neuroligin interactions

In vertebrates, Neurexins (NRXNs) act as cell adhesion proteins and bind to proteins on the surface of synaptically connected neurons (Reissner *et al.,* 2013). They are localized to the presynaptic membrane and induce postsynaptic differentiation and clustering of receptors and proteins to form the postsynaptic terminal (Dean and Dresbach, 2006; Trotter *et al.,* 2019). In *Drosophila,* NRXNs are also involved in synapse function and growth of the NMJ, and defining the cytoarchitecture of synaptic bouton active zones (Li *et al.,* 2007b; Trotter *et al.,* 2019).

The mammalian genes are controlled by two alternative promoters, and encode two transcripts: α and β, which result in the formation of six possible transcripts (NRXN1α, NRXN2α, NRXN3α, NRXN1β, NRXN2β, NRXN3β) (Baudouin and Scheiffele, 2010; Dalva *et al.,* 2007). α-NRXNs have a long extracellular portion, allowing α-NRXNs to participate in extracellular interactions, whilst β-NRXNs have a shorter extracellular portion (Fig 4.) (Dalva *et al.,* 2007; Dean and Dresbach, 2006; Lisé and El-Husseini, 2006; Missler and Südhof, 1998; Reissner *et al.,* 2013). α-NRXNs also possess six LNS-domains (laminin/neurexin/sec hormone-binding globulin-domain), and three EGF-like domains, whilst β-NRXNs have a single LNS-domain, suggesting α-NRXNs can bind a diverse range of extracellular proteins and mediate cell adhesion (Fig 4.) (Rudenko *et al.,* 2001; Südhof, 2008).

A major part of NRXN function is their interaction with Neuroligins (NLGNs). NLGNs are transmembrane proteins localised on the postsynaptic membrane, and act as NRXN ligands mediating heterophilic adhesion and the formation of synaptic contacts (Fabrichny *et al.,* 2007; Südhof, 2008). The NLGN-NRXN complex bidirectionally triggers synapse formation and provides a direct way of coordinating synaptic connectivity by uniting the pre-and postsynaptic domains (Baudouin and Scheiffele, 2010; Dean and Dresbach, 2006; Chen *et al.,* 2008; Craig and Kang, 2007). Together NRXNs and NLGNs regulate excitatory and inhibitory synapses by organizing and controlling the function and maturation of synapses (Chen *et al.,* 2008; Craig and Kang, 2007; Dalva *et al.,* 2007; Dean and Dresbach, 2006; Südhof, 2008; Varoqueaux *et al.,* 2006).

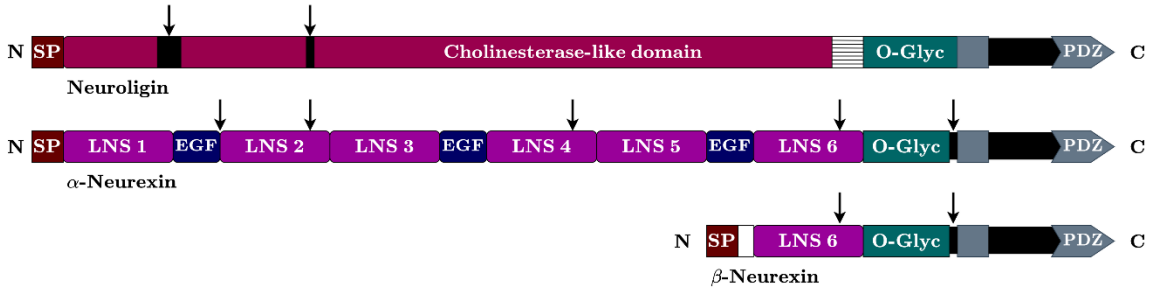


Figure 4. Domain structure of NLGNs and NRXNs. NLGNs consist of two splice sites, whilst α-NRXN and β-NRXN consist of five and two respectively (arrowheads) with a N-terminal and C-terminal. The Cholinesterase-like domain mediates NLGN-NRXN binding. NRXNs are also composed of a N-terminal and C-terminal domain, with α-NRXN possessing six LNS domains and an EGF-like domain, and β-NRXN with just one LNS domain. The extracellular region at the N-terminal of both NLGNs and NRXNs is composed of a signal peptide (SP), whilst the extracellular region at the C-terminal consists of an O-linked glycosylation (O-Glyc) region with a PDZ-interaction site.

Neurexins and Neuroligins in disease

As well as being essential for synapse formation NRXNs and NLGNs are implicated in human disease, with mutations in both resulting in disease pathogenesis, causing a loss of synaptic connectivity and terminal loss, followed by neural dysfunction, cognitive impairments and dementias, like AD (Craig and Kang, 2007; Reddy *et al.*, 2005; Sindi *et al.*, 2014; Tannenberg *et al.*, 2006). Impairments in the NRXN-NLGN complex are also associated with autism spectrum disorder (ASD) and schizophrenia (Bourgeron, 2009; Reichelt *et al.*, 2012; Südhof, 2008; Wang *et al.*, 2018). It has been reported that heterozygous deletions of the NRXN-1α, NRXN-1β promoter and exons are found in individuals with cognitive impairment and autistic features (Friedman *et al.*, 2006; Glessner *et al.*, 2009; Kim *et al.*, 2008; Marshall *et al.*, 2008; Morrow *et al.*, 2008). Likewise, mutations in NLGN3 and NLGN4 alter the synaptic function and lead to the common idiosyncrasies of ASD (Bourgeron, 2009). As with ASD, heterozygous deletions that eliminate the promoter and exon of NRXN-1α have been associated with schizophrenia (Kirov *et al.*, 2009; Rujescu *et al.*, 2009; Vrijenhoek *et al.*, 2008; Walsh *et al.*, 2008). These truncating mutations prevent NRXN1 and NRXN2, meaning they cannot bind to their respective ligands, and the NRXN-NLGN complex is not formed and synaptic differentiation is impaired (Gauthier *et al.*, 2011). All in all, this indicates that NRXN-NLGN interactions are important components of the promotion of synapse formation.

Neurexin and Neuroligins in *Drosophila*

NRXN and NLGN have homologs in *Drosophila* (DNrx and DNlg, respectively) and, like in vertebrates, play a major role in synapse organization and growth (Banerjee *et al.,* 2017). The *Drosophila* genome contains a single NRXN – CG7050 (NRXN-1), a homolog of vertebrate α-NRXN, and is expressed in the CNS (Zeng *et al.,* 2007). Contrastingly, the *Drosophila* genome contains four NLGN proteins - DNlg1, DNlg2, DNlg3, DNlg4, which correspond to the equivalent genes in humans (NLGN1, NLGN2, NLGN3 and NLGN4, respectively) (Baudouin and Scheiffele, 2010; Bolliger *et al.,* 2001; Dean and Dresbach, 2006; Ichtchenko *et al.,* 1996; Lisé and El-Husseini, 2006). Humans also have a fifth gene; NLGN4Y (Baudouin and Scheiffele, 2010).

Loss of function mutations of both DNrx and DNlg results in reduced synaptic growth at the NMJ (Banovic *et al.,* 2010; Li *et al.,* 2007b). These mutations, especially deletions of both *dnrx* and *dnlg1,* cause reductions in synaptic bouton number, decreased synaptic transmission and disruption of the active zones (Banerjee *et al.,* 2017). DNrx over-expression in motor neurons results in amplified synaptic bouton growth illustrating its role in synapse formation and active zone development (Banerjee *et al.,* 2017; Li *et al.,* 2007a; Zeng *et al.,* 2007). DNlg1 over-expression results in synaptic overgrowth, and an absence of *dnlg1* function causes reduced synaptic bouton numbers, thus reducing synaptic transmission (Mozer and Sandstrom, 2012). In vivo biochemical analysis on DNrx and DNlg1 shows a direct molecular association between the two proteins, suggesting they work at the synapse as part of a large macromolecular complex at the active zone (Banerjee *et al.,* 2017). Without the presence of this macromolecular complex regulating synapse development, reduced synaptic transmission would likely occur. If Tau overexpression coincided with loss of function DNrx and DNlg, it is also hypothesised that this would result in a much larger disturbance to the NMJ, with severe synaptic transmission disruption.

The question at the heart of this study asks whether manipulation of NRXN and NLGN function can counteract Tau induced synapse loss using the *Drosophila* NMJ as the test platform. NRXNs and NLGNs trigger postsynaptic and presynaptic differentiation respectively, alongside the hippocampus and its related structures, which communicates with the hippocampal system to aggravate synapse formation (Craig and Kang, 2007; Wible, 2013). By co-expressing these proteins in disease models, such as tauopathies, it is possible that the role in promoter synapse

formation could arrest the negative effects of Tau over-expression on synaptic function. *Drosophila* is an ideal model organism for this.

Hypothesis 2 – Can Neurexins ameliorate the toxic effects of Tau on NMJ morphology?

NRXNs play a key role in stabilising the synaptic and axonal cytoskeleton (Dalva *et al.*, 2007). The second part of this thesis was based around this and designed to see if co-expressing human NRXN1 proteins with hTau would rescue elements of the Tau phenotypes. It was hypothesised that human Neurexin-1 (NRXN1) overexpression in motor neurons would rescue the negative effects of hTau expression on the *Drosophila* axonal cytoskeleton and NMJ, by driving synapse formation. Human NRXN1 was used, employing the pre-existing UAS-NRXN::GFP (NRXN1-GFP) line which encodes a GFP tagged NRXN1 that retains NRXN activity in *Drosophila,* with a useful GFP tag to localise the expressed protein (Chen *et al.*, 2010). The NRXN1-GFP fusion and hTau were selectively co-expressed in *Drosophila* motor neurons and the effects on the morphology of the larval NMJ analysed, believing, if successful, the work would pave way for a deeper exploration of the role of the NRXNs and determine if they can offer a route to future therapeutics.

Experimental Overview

The over-expression of hTau in *Drosophila* causes neurodegeneration (Wittmann *et al.,* 2001) and as Chee et al. (2005) showed, the NMJs of Tau expressing motor neurons were smaller and spindlier compared to WT, with more, smaller, and abnormally shaped synaptic boutons.

In contrast, NRXNs promote synaptic differentiation and, with NLGNs, can increase synapse numbers, therefore promoting NMJ development. This leads to the hypothesising that the expression of NRXNs and NLGNs might rescue the NMJ from Tau induced degeneration.

To test this, hTau will be expressed in motor neurons of *Drosophila* larvae, to establish whether the phenotypic effects of the six hTau isoforms are identical or vary in their toxicity. In the second part of this study, NRXN1 will be co-expressed with the different hTau isoforms in motor neurons, to test if the synapse forming abilities of NRXN1 can counteract the pathological effects of Tau. NMJ morphology will be assayed, analysed, and compared against the effects of expressing hTau alone, NRXN1 alone, and co-expressing both to establish whether NRXN1 can rescue the effects of hTau. This will be completed on *Drosophila* 3rd instar larvae, due to the continuous development of the larva prior to this stage, where bouton numbers and muscle area are increasing dramatically, changing the cytoskeletal structure of the larva (Menon *et al.,* 2013). The morphology of the NMJ on muscle 4 will then be analysed.

Materials and Methods

Fly stocks

All fly stocks and crosses were raised on standard cornmeal medium in tubes maintained at a constant 25°C temperature, in a 16/8-hour dark/light cycle incubator. Motor neuron specific expression of different hTau isoforms was achieved using the motor neuron specific OK6 GAL4 line, to drive expression of different upstream activating sequence (UAS) linked hTau isoforms (Aberle *et al.*, 2002). To separate phenotypes caused by overexpression of any protein, OK6 control flies expressed beta-galactosidase (β-gal). The UAS-Tau stocks (Fernius *et al.*, 2017) were obtained from Bloomington Stock Center, Indiana (Table 1). A lab-maintained Oregon-R (OreR) was used as a wild-type control.

Once the crosses were established - the NRXN1 crosses created with the assistance of Lovesha Sivanantharajah - they were maintained and raised at 25°C, and processed and dissected at room temperature (18°C-21°C). All crosses involved the use of OK6 males, with the UAS-Tau and UAS-NRXN1 *Drosophila* being virgin females.

Table 1. Fly Stocks

Stock Number	Genotype
3703	w[1118]/Dp(1;Y)y[+]; CyO/nub[1] b[1] sna[Sco] lt[1] stw[3]; MKRS/TM6B, Tb[1]
64199	P{w[+mW.hs]=GawB}OK6
1776	p{UAS-lacZ.B}
78846	y[1] w[1118]; PBac{y[+mDint2] w[+mC]=UAS-h*MAPT*.0N3R.F}VK00018
78847	y[1] w[1118]; PBac{y[+mDint2] w[+mC]=UAS-h*MAPT*.0N4R.F}VK00018
78848	y[1] w[1118]; PBac{y[+mDint2] w[+mC]=UAS-h*MAPT*.1N3R.F}VK00018
78849	y[1] w[1118]; PBac{y[+mDint2] w[+mC]=UAS-h*MAPT*.1N4R.F}VK00018
78850	y[1] w[1118]; PBac{y[+mDint2] w[+mC]=UAS-h*MAPT*.2N3R.F}VK00018/CyO*
78861	y[1] w[1118]; PBac{y[+mDint2] w[+mC]=UAS-h*MAPT*.2N4R.F}VK00018
DAZ 1502	UAS-Nrx::GFP/FM7, GFP; +; Sp, e/TM6b, Ub-GFP
	UAS-Nrx-GFP;2N3R/CyO; +
	UAS-Nrx-GFP;2N4R/CyO; +

* The line was made homozygous for tau in this study

Crossing Scheme

hTau expression

Virgin female hTau expressing *Drosophila* were crossed with male OK6 lines to create the six hTau lines. Sex was determined via visual observation of animal size and genitalia.

NRXN1 expression

Virgin female NRXN1; +; $\frac{+}{TM6B-GFP}$ flies were crossed with male $\frac{CyO}{sco}$; $\frac{MKRS}{TM6B}$, creating either NRXN ; $\frac{CyO}{+}$ or NRXN1 ; $\frac{Sco}{+}$ flies (Fig 5). This was to ensure all of the flies in the next generation have NRXN1 on the X chromosome. If these sexes were opposite, only half of the flies would have NRXN present. The males of these were crossed back to the original female (NRXN1; +; $\frac{+}{TM6B-GFP}$), to ensure NRXN1 remained. These were done in parallel. NRXN1 is now homozygous, with either CyO or Sco. These two stocks were then crossed together to

produce one of four outcomes on the second chromosome: $\frac{Sco}{Cyo}$, $\frac{Sco}{+}$, $\frac{+}{CyO}$, $\frac{+}{+}$. One quarter of the flies were therefore NRXN1 ; $\frac{Sco}{CyO}$; $\frac{MKRS}{+}$. This is the desired stock which is built up.

NRXN1 ; $\frac{Sco}{CyO}$; $\frac{MKRS}{+}$ female virgins were next double balanced and crossed with the desired male hTau isoform ($\frac{hTau\ isoform}{CyO}$) to establish a NRXN1 line with $\frac{hTau}{CyO}$. This produced four possible outcomes: $\frac{Sco}{hTau}$, $\frac{Sco}{CyO}$, $\frac{hTau}{CyO}$ and $\frac{CyO}{CyO}$, with the latter causing fatalities. The females here are heterozygous for NRXN1, and are not desirable as over time the flies will lose NRXN1 and become homozygous for the WT. Male NRXN1; $\frac{hTau}{CyO}$; + are therefore wanted (where + is either TM6B, MKRS, E or Sp). This is the second stock that is built up.

To express this hTau isoform, Male NRXN1; $\frac{hTau}{CyO}$ was then crossed with OK6 female virgins, to express NRXN1 and hTau with the GAL4 UAS system. Six crosses were set up in parallel – each of the six hTau isoforms. CyO is not an identifiable trait in larvae so, to ensure hTau was present, flies were further stained with Tau AT8, to further indicate the presence of hTau.

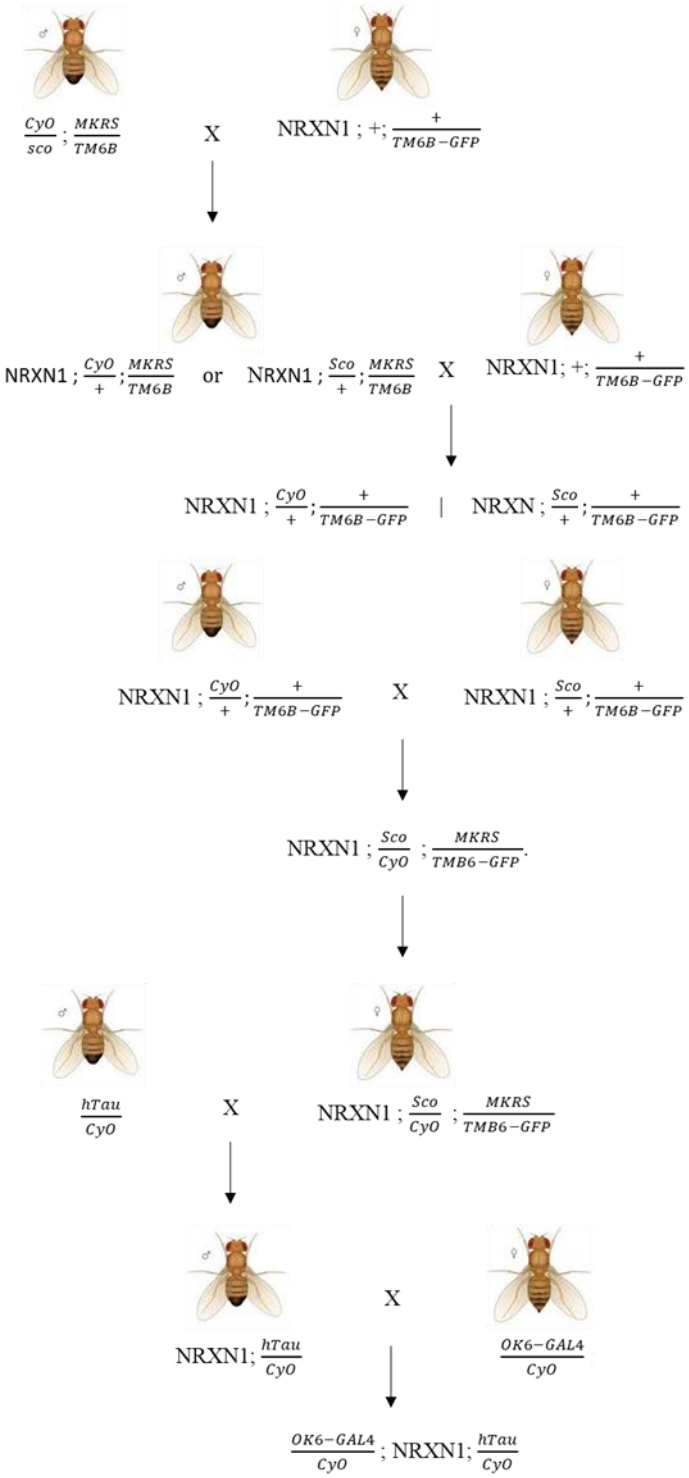


Figure 5. Crossing scheme to express NRXN1 and hTau in *Drosophila melanogaster*.

Dissection and immunocytochemistry

Wandering 3rd stage instar larvae were anaesthetised on ice and dissected in ice-cold 1X Dulbecco's Phosphate Buffered Saline (Gibco, Invitrogen, Fisher – 0.901mM $CaCl_2$ and 0.493mM $MgCl_2$). A mid-dorsal longitudinal incision was made, and the cuticle stretched and pinned flat onto a sylgard dish. The viscera were removed to expose the CNS and musculature.

Pinned larvae were fixed in 4% formaldehyde for thirty minutes, and, after fixation, the tissues were washed with PBS, five times, over a fifteen-minute period. Washed tissues were transferred in a solid watch glass and further washed in a 0.3% PBS Triton X-100 solution (PBSTX), for a further five times in a fifteen-minute window. Tissues were then incubated in a block serum (Heat Inactivated Donkey Serum [dilution: 1:50]) for one hour at room temperature.

For the study of the controls and hTau, Rabbit Anti-HRP (Jackson ImmunoResearch – Promega, AB_2315781 [dilution: 1:1000]) was added after blocking. Tissues were then left at room temperature for thirty minutes before a further five washes, in fifteen minutes, with PBS. The addition of the secondary antibodies; Alexafluor 488 Goat anti-rabbit (Fisher [Invitrogen-Molecular Probes], AB_2576217 [dilution: 1:500]) and Alexafluor 568 Goat anti-mouse (Fisher [Invitrogen-Molecular Probes], AB_2534072 [dilution: 1:500]), then occurred and the specimens were incubated at 4°C for two hours, devoid of light. For the study of the effects of NRXN1 alone, Rabbit Anti-HRP and Chicken GFP (Fisher [Invitrogen-Molecular Probes], AB_2534023 [dilution: 1:1000]) were used, alongside the secondary antibodies; Alexafluor 568 Goat anti-rabbit (Fisher [Invitrogen-Molecular Probes], AB_10563566 [dilution: 1:500]) and Alexafluor 488 Goat anti-chicken (Fisher [Invitrogen-Molecular Probes], AB_2534096 [dilution: 1:500]). The co-expression of NRXN1 and hTau also required the use of Rabbit Anti-HRP and Chicken GFP, in addition to Mouse Anti-AT8 (Fisher [Invitrogen-Molecular Probes], AB_223647 [dilution: 1:1000]), with the secondary antibodies Alexafluor 568 Goat anti-rabbit, Alexafluor 488 Goat anti-chicken and Alexafluor 647 Goat anti-mouse (Fisher [Invitrogen-Molecular Probes], AB_2535804 [dilution: 1:500]). Tissues were then washed in PBS, mounted onto microscope slides in 80% glycerol/PBS, covered slipped and sealed with nail varnish.

Tissues were imaged on a Zeiss LSM 710 confocal microscope at 63x magnification, using identical imaging parameters. LSM files were contrast-enhanced as necessary. Z-projected images created, and analysis of bouton size (μm^2) and numbers were all performed using ImageJ

(http://rsbweb.nih.gov/ij/). Bouton size was measured by tracing the circumference of the boutons using ImageJ software.

Mann-Whitney U Test

A Mann-Whitney U test was performed using SPSS to compare bouton sizes between each of the treatment groups (wildtype/control, wildtype/unexpressed, wildtype/expressed and unexpressed/expressed) to determine whether a statistically significant difference exists between the groups. The null hypothesis (H_0) states that the means are not significantly different, whilst the alternative hypothesis (H_1) states they are. To reject the H_0, and to confirm that the treatment groups are significantly different, the p-value was required to be below 0.05, at the 95% confidence level.

Chi-Square test

A chi-square test was performed to determine if the distribution of the expressed and non-expressed hTau isoforms matched the distribution of the WT (OreR). OreR and OK6 were compared against each other, as were the expressed and non-expressed hTau isoforms, with the latter of each group being the ‘observed’ result.

Results

To assess the relative toxicity of the different hTau isoforms, the expression of six different hTau isoforms was targeted to *Drosophila* larval motor neurons using the OK6 GAL4 driver. The effects of the expression of the different hTaus were analysed on a single identified NMJ on muscle 4, in abdominal segments (A2-A5), which is readily identifiable and has a simple and stereotypical morphology. Based on previous studies, NMJ were analysed by statistically quantifying average bouton size and average bouton number with Mann-Whitney *U* analysis, (where N = bouton number per NMJ, n = number of data values used in Mann-Whitney *U* analysis).

Wild type NMJ structure

The NMJs of WT larvae, revealed by anti-HRP antibody probe, show that the NMJ on muscle 4 exhibits the classical structure of the type 1 NMJ, with the smooth and rounded boutons, characteristic of the classic 'beads on a string' structure, with little variation in individual bouton sizes (Fig 6A). Comparable morphology is also seen in the OK6 GAL4 driver line when driving the inert protein LacZ (Fig 6B), the OK6/LacZ cross was used as the OK6 control for all subsequent experiments.

The quantification of average NMJ structures in WT and OK6 show no statistical differences in average bouton size (Fig 6D) (OreR = 12.63µm^2 ± 0.70µm^2 and OK6 = 12.06µm^2 ± 0.81µm^2, Mann-Whitney $U = 6803.5$, $P > 0.05$ two-tailed, $n_1 = 143$, $n_2 = 108$) (where the error is the standard error of the mean). Similarly, analysis of the average bouton number reveals no significant difference between WT and GAL4 driver line (OreR N = 20.43 ± 3.64, OK6 18 ± 2.84, Mann-Whitney $U = 20$, $P > 0.05$ two-tailed, $n_1 = 7$, $n_2 = 7$) (Fig 6A, 6B, 6E) or between the distribution of small boutons (OreR/OK6 [<5µm^2], $P > 0.05$ – chi-square) (Fig 6C).

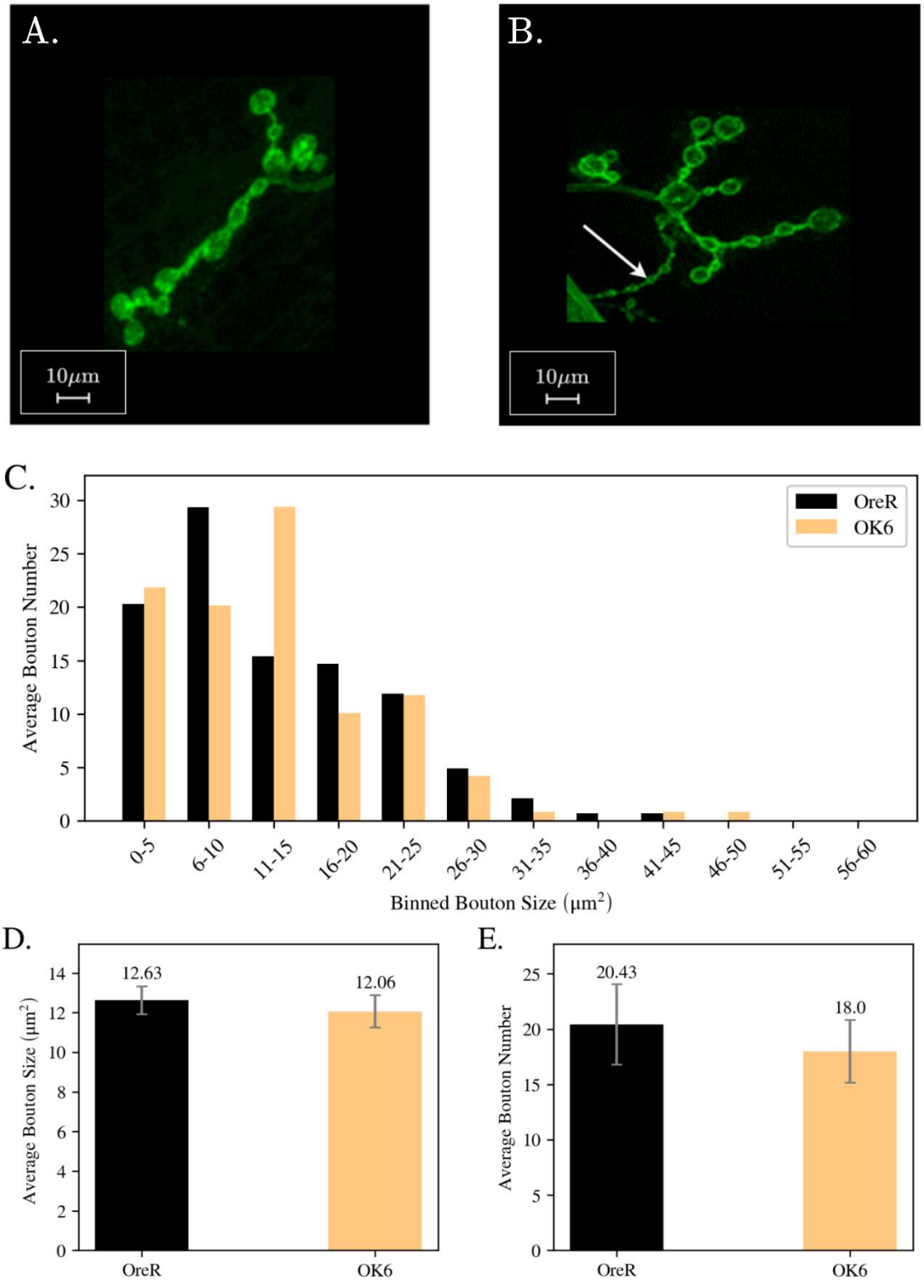


Figure 6. Structure of the NMJ in muscle 4 in wild type and non-expressing GAL4 driver line larvae. Morphology of the NMJ on muscle 4 revealed with anti-HRP antibody of A) wild type and B) OK6 GAL4 shows the classic 'beads on a string' morphology with smooth rounded and

comparably sized boutons. Analysis of C) the distribution of bouton sizes per NMJ, D) the average bouton size per NMJ and E) the average bouton number per NMJ reveals no statistically significant differences between WT and the non-expressing OK6 GAL4 line. The white arrow indicates an adjacent and more ventral type 2 NMJ that is not a part of this study.

Effects of hTau expression on NMJ structure

UAS-hTau^{0N3R}, UAS-hTau^{2N3R} and UAS-hTau^{0N4R} produce structural aberrations on the *Drosophila* NMJ

0N3R

To confirm the results of Chee et al. (2005), the first analysis was to repeat the expression of 0N3R using OK6 to confirm that the expression of this isoform in motor neurons causes significant changes in NMJ morphology with irregular bouton sizes evident (Fig 6A, 7B).

Morphometric analysis of bouton structures shows that 0N3R Tau expressing NMJs do have a statistically smaller average bouton size than WT (OreR = 12.63µm^2 ± 0.70µm^2, Exp-0N3R = 8.47µm^2 ± 0.57µm^2, Mann-Whitney $U = 10796$, $P < 0.05$ two-tailed, $n_1 = 143$, $n_2 = 244$) (Fig 7D). This is present alongside a statistically significant increase in average bouton number per NMJ compared to WT (OreR N = 20.43 ± 3.64, Exp-0N3R N = 40.67 ± 6.83, Mann-Whitney $U = 5$, $P < 0.05$ two-tailed, $n_1 = 7$, $n_2 = 6$) (Fig 6A, 7A, 7E). There is also a statistically significant increase in the number of smaller boutons expressing NMJs in comparison to WT (OreR/Exp-0N3R [<5µm^2], $P < 0.05$ – chi-square) (Fig 7C).

The non-expressing NMJs however also show significant differences from WT in terms of average bouton size (OreR = 12.63µm^2 ± 0.70µm^2, 0N3R = 10.91µm^2 ± 0.81µm^2, Mann-Whitney $U = 7526.5$, $P < 0.05$ two-tailed, $n_1 = 143$, $n_2 = 126$) (Fig 7D), with no significance difference in average bouton number per NMJ than the WT (OreR N = 20.43 ± 3.64, 0N3R N = 25.20 ± 3.09, Mann-Whitney $U = 10.5$, $P > 0.05$ two-tailed, $n_1 = 7$, $n_2 = 5$) (Fig 6A, 7A, 7E), These are not expected results and suggests an effect in the unexpressed controls. No significant difference is present in bouton distribution (OreR/0N3R [<5µm^2], $P > 0.05$ – chi-square) (Fig 7C), indicating the distribution of boutons <5µm^2 is similar between non-expressing 0N3R larvae and WT.

0N3R Tau expressing NMJs were also compared against unexpressed 0N3R and a statistically significant difference can be seen for average bouton size (0N3R = 10.91µm^2 ± 0.81µm^2, Exp-0N3R = 8.47µm^2 ± 0.57µm^2, Mann-Whitney $U = 11730$, $P < 0.05$ [1.80×10^{-04}] two-tailed, $n_1 = 126$, $n_2 = 244$) (Fig 7A, 7B, 7D). Alongside this, expressing NMJs produced a much greater average bouton number compared to non-expressing NMJs, but due to how the Mann-Whitney U test produces significant values, this was not a statistically significant difference (0N3R N =

25.20 ± 3.09, Exp-0N3R N = 40.67 ± 6.83, Mann-Whitney $U = 5.5$, $P > 0.05$, two-tailed, $n_1 = 5$, $n_2 = 6$) (Fig 7A, 7B, 7E) Similarly, there was not a significantly different distribution of small boutons in expressing NMJs compared to non-expressing (0N3R/Exp-0N3R [<5μm^2], $P > 0.05$ – chi-square) (Fig 7A, 7B, 7C),

The results of expressed 0N3R confirm the work of Chee et al. (2005), indicating that UAS-hTau^{0N3R} does in fact have an effect on bouton size in *Drosophila* NMJ. To further evaluate the effect of hTau expression on *Drosophila* NMJ, other 3R hTau isoforms were analysed to determine if a correlation exists between the number of microtubule binding domain repeats.

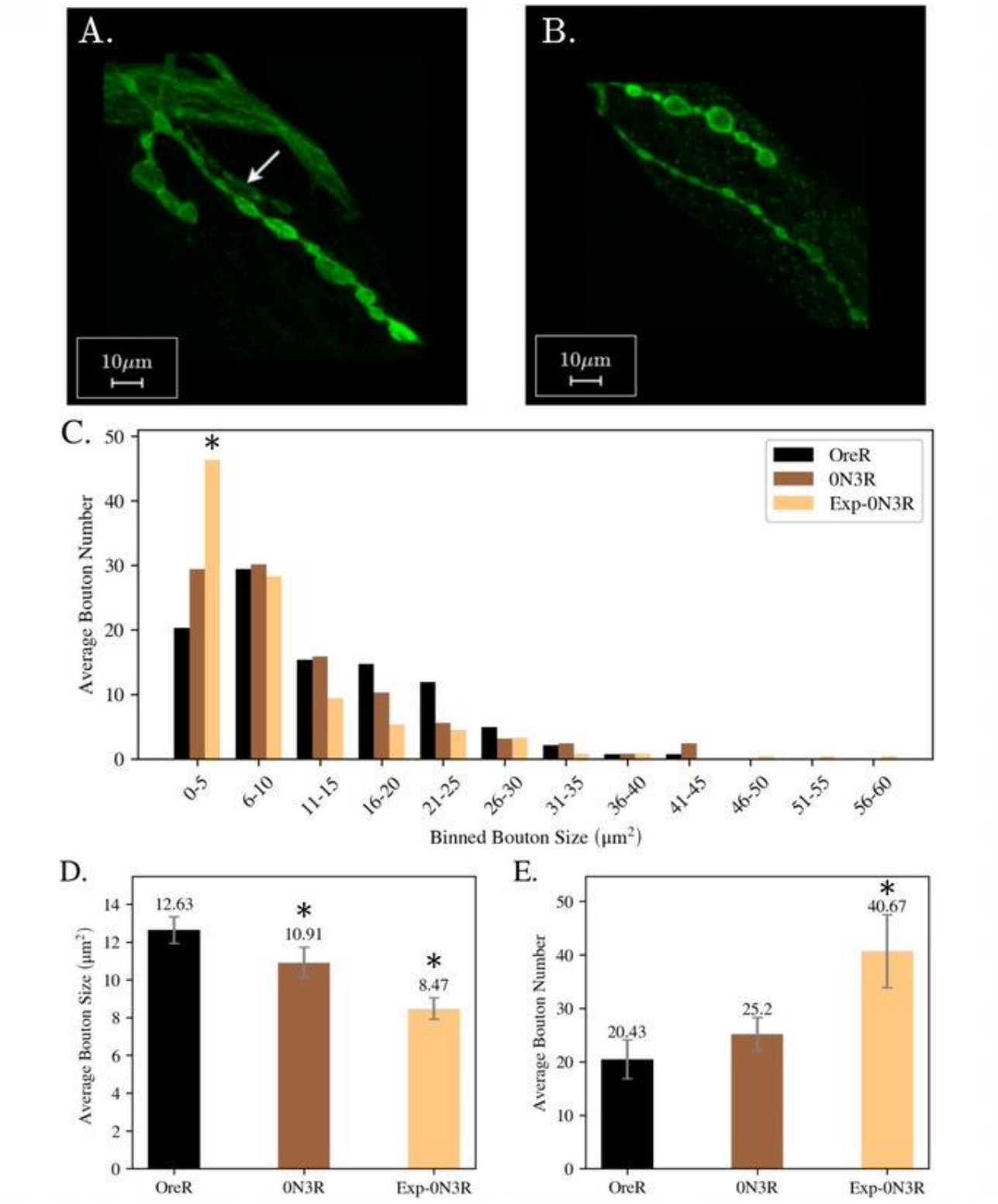


Figure 7. Structure of the NMJ in muscle 4 in wild type, non-expressing 0N3R and expressing 0N3R larvae. Morphology of the NMJ on muscle 4 revealed with anti-HRP antibody of A) 0N3R and B) expressed-0N3R. Analysis of C) the distribution of bouton sizes per NMJ, D) the average

bouton size per NMJ and E) the average bouton number per NMJ. *Statistically significant difference of average bouton size between OreR/0N3R ($P = 0.0198$, Mann-Whitney U) and OreR/Exp-0N3R ($P = 1.00 \times 10^{-5}$, Mann-Whitney U), average bouton number between OreR/Exp-0N3R ($P = 0.0271$, Mann-Whitney U) and distribution of boutons <5μm^2 of OreR/Exp-0N3R ($P = 2.32 \times 10^{-04}$, chi-square), The white arrow indicates an adjacent and more ventral type 2 NMJ that is not a part of this study.

2N3R

Following 0N3R, 2N3R was analysed to locate the effects of the 3R domain isoforms. The expressing NMJs appear spindlier than WT and the expression of 2N3R hTau causes NMJs to exhibit significantly smaller average bouton sizes, compared to WT (OreR = 12.63μm^2 ± 0.70μm^2, Exp-2N3R = 11.78μm^2 ± 0.89μm^2, Mann-Whitney $U = 8484.5$, $P < 0.05$ two-tailed, $n_1 = 143$, $n_2 = 139$) (Fig 6A, 8B, 8D) and a larger number of boutons per NMJ than WT, yet this was not a significant result (OreR = 20.43 ± 3.64, Exp-2N3R = 27.8 ± 5.19, Mann-Whitney $U = 10.5$, $P > 0.05$ two-tailed, $n_1 = 7$, $n_2 = 5$) (Fig 6A, 8B, 8E). An increased number of smaller boutons in the expressing NMJs is also present (Fig 6A, 8B, 8C) however this difference is not statistically significant (OreR/Exp-2N3R [<5μm^2], $P > 0.05$– chi-square) (Fig 6A, 8A, 8C).

The non-expressed 2N3R also showed a significant difference from WT in terms of average bouton size (OreR = 12.63μm^2 ± 0.70μm^2, 2N3R = 11.23μm^2 ± 0.85μm^2, Mann-Whitney $U = 8500.5$, $P < 0.05$ two-tailed, $n_1 = 143$, $n_2 = 141$) (Fig 6A, 8A, 8C). The number of boutons per sample was also greater than the WT, but again was not a significant result (OreR = 20.43 ± 3.64, 2N3R = 28.20 ± 1.69, Mann-Whitney $U = 8.5$, $P > 0.05$ two-tailed, $n_1 = 7$, $n_2 = 5$) (Fig 6A, 8A, 8E). This also did not produce a significantly greater distribution of smaller boutons (OreR/2N3R [<5μm^2], $P > 0.05$– chi-square) (Fig 6A, 8A, 8C).

When expressed NMJs were compared to non-expressed, no statistical difference was seen for bouton size (2N3R = 11.23μm^2 ± 0.85μm^2, Exp-2N3R = 11.78μm^2 ± 0.89μm^2, Mann-Whitney $U = 9584$, $P > 0.05$ two-tailed, $n_1 = 141$, $n_2 = 139$) (Fig 8A, 8B, 8C), nor did expressing NMJs produce a significant difference in average number of boutons (2N3R = 28.20 ± 1.69, Exp-2N3R = 27.8 ± 5.19, Mann-Whitney $U = 8.5$, $P > 0.05$ two-tailed, $n_1 = 5$, $n_2 = 5$) (Fig 8A, 8B, 8E) or significantly larger distributions of smaller boutons (2N3R/Exp-2N3R [<5μm^2], $P > 0.05$– chi-square) (Fig 8A, 8B, 8C).

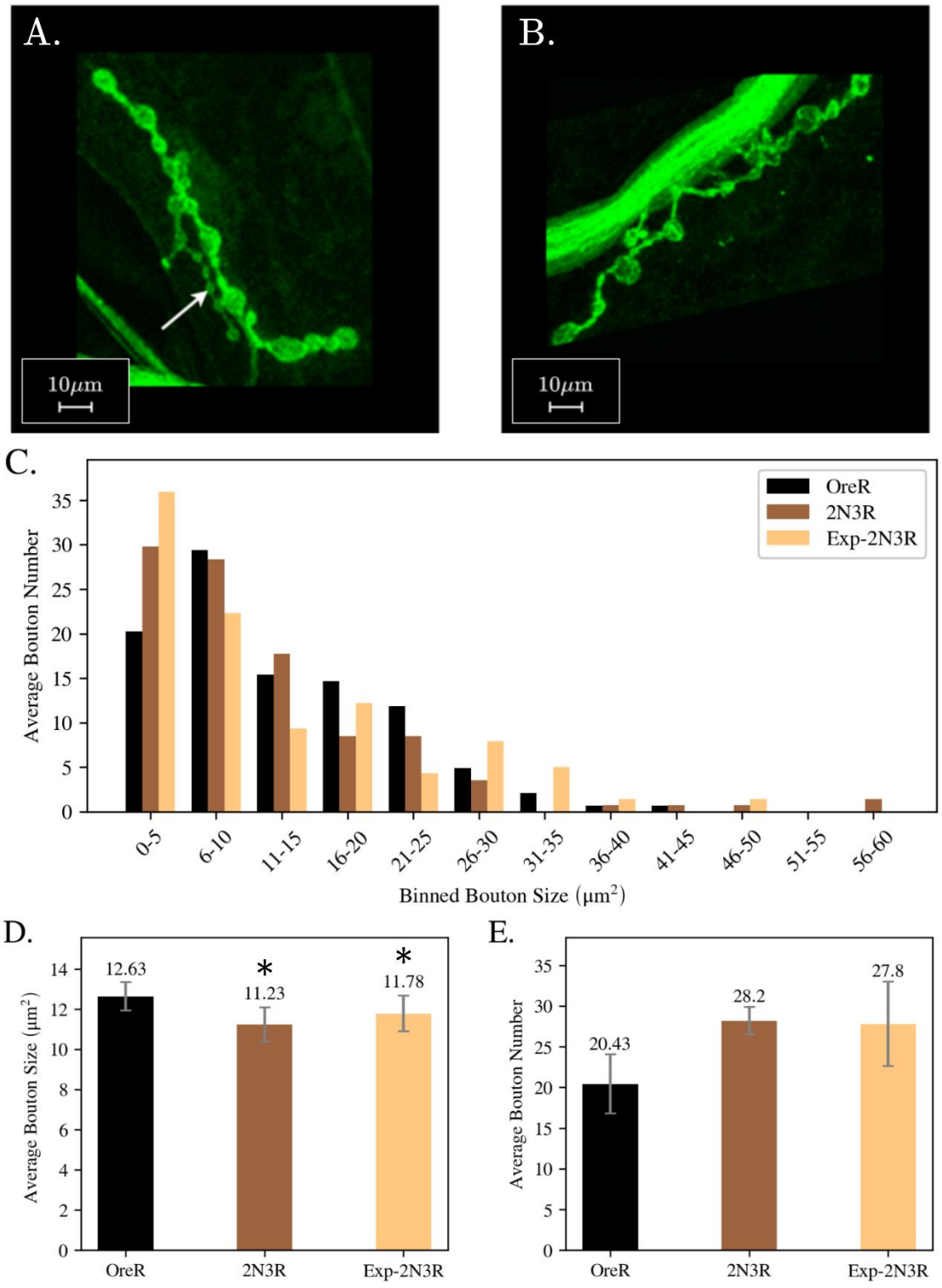


Figure 8. Structure of the NMJ in muscle 4 in wild type, non-expressing 2N3R and expressing 2N3R larvae. Morphology of the NMJ on muscle 4 revealed with anti-HRP antibody of A) 2N3R, showing the classic 'beads on a string' morphology, and B) expressed-2N3R, showing smaller and more sporadically spread boutons. Analysis of C) the distribution of bouton sizes per

NMJ, D) the average bouton size per NMJ and E) the average bouton number per NMJ. *Statistically significant differences between average bouton size of OreR/2N3R (P = 0.0226, Mann-Whitney *U*) and OreR/Exp-2N3R (P = 0.034, Mann-Whitney *U*). The white arrow indicates a type 2 NMJ that is not a part of this study.

0N4R

Expression of 0N4R shows effects on NMJ morphology, with expressing NMJs visually showing irregular bouton sizes and numbers compared to WT (Fig 6A, 9B). Quantification of expressed NMJs revealed significant differences in average bouton size compared to the WT (OreR = 12.63µm^2 ± 0.70µm^2, Exp-0N4R = 10.42µm^2 ± 0.77µm^2, Mann-Whitney $U = 8098.5$, $P < 0.05$ two-tailed, $n_1 = 143$, $n_2 = 144$) (Fig 6A, 9B, 9D). Expressing NMJs boutons are therefore significantly smaller than WT, but this is not combined with a significantly different number of boutons in the NMJs of expressing flies (OreR N = 20.43 ± 3.64, Exp-0N4R N = 24.00 ± 3.44, Mann-Whitney $U = 15.5$, $P > 0.05$ two-tailed, $n_1 = 7$, $n_2 = 6$) (Fig 6A, 9B, 9E). There is also variation in bouton distribution between Exp-0N4R and WT with a greater number of smaller boutons in Exp-0N4R NMJs than WT (Fig 9C), but this is not statistically significant (OreR/Exp-0N4R [<5µm^2], $P > 0.05$ – chi-square) (Fig 6A, 9B, 9C).

There is no significant difference in average bouton size between non-expressing NMJs and WT (OreR = 12.63µm^2 ± 0.70µm^2, 0N4R = 10.69µm^2 ± 0.57µm^2, Mann-Whitney $U = 7053$, $P > 0.05$ two-tailed, $n_1 = 143$, $n_2 = 108$) (Fig 6A, 9A, 9D), or in average bouton number (OreR N = 20.42 ± 3.64, 0N4R N = 21.60 ± 2.99, Mann-Whitney $U = 14.5$, $P > 0.05$ two-tailed, $n_1 = 7$, $n_2 = 6$) (Fig 6A, 9A, 9E), but this does not correspond with a greater distribution of smaller boutons (OreR/0N4R [<5µm^2], $P > 0.05$ – chi-square) (Fig 6A, 9A, 9C).

When expressing NMJs are compared against non-expressing, a significant difference in average bouton size is seen with expressing NMJs being smaller (0N4R = 10.69µm^2 ± 0.57µm^2, Exp-0N4R = 10.42µm^2 ± 0.77µm^2, Mann-Whitney $U = 6470$, $P < 0.05$ two-tailed, $n_1 = 108$, $n_2 = 144$). This is present alongside a visual increase in average bouton number per NMJ, but not a statistically significant increase (0N4R N = 21.60 ± 2.99, Exp-0N4R N = 24.00 ± 3.44, Mann-Whitney $U = 12.5$, $P > 0.05$ two-tailed, $n_1 = 5$, $n_2 = 6$) (Fig 9A, 9B, 9E). Expressing NMJs produced a statistical difference from the non-expressing control in bouton distribution and the presence of an increased number of smaller boutons (0N4R/Exp-0N4R [<5µm^2], $P < 0.05$ [6.17×10^{-3}] – chi-square) (Fig 9A, 9B, 9C). This is the only isoform which exhibits a pattern

where the expressed isoform is significantly different from the unexpressed NMJ in regard to $<5\mu m^2$ bouton distribution.

Despite these significant results, not all isoforms proved to exhibit significant effects on the *Drosophila* NMJ.

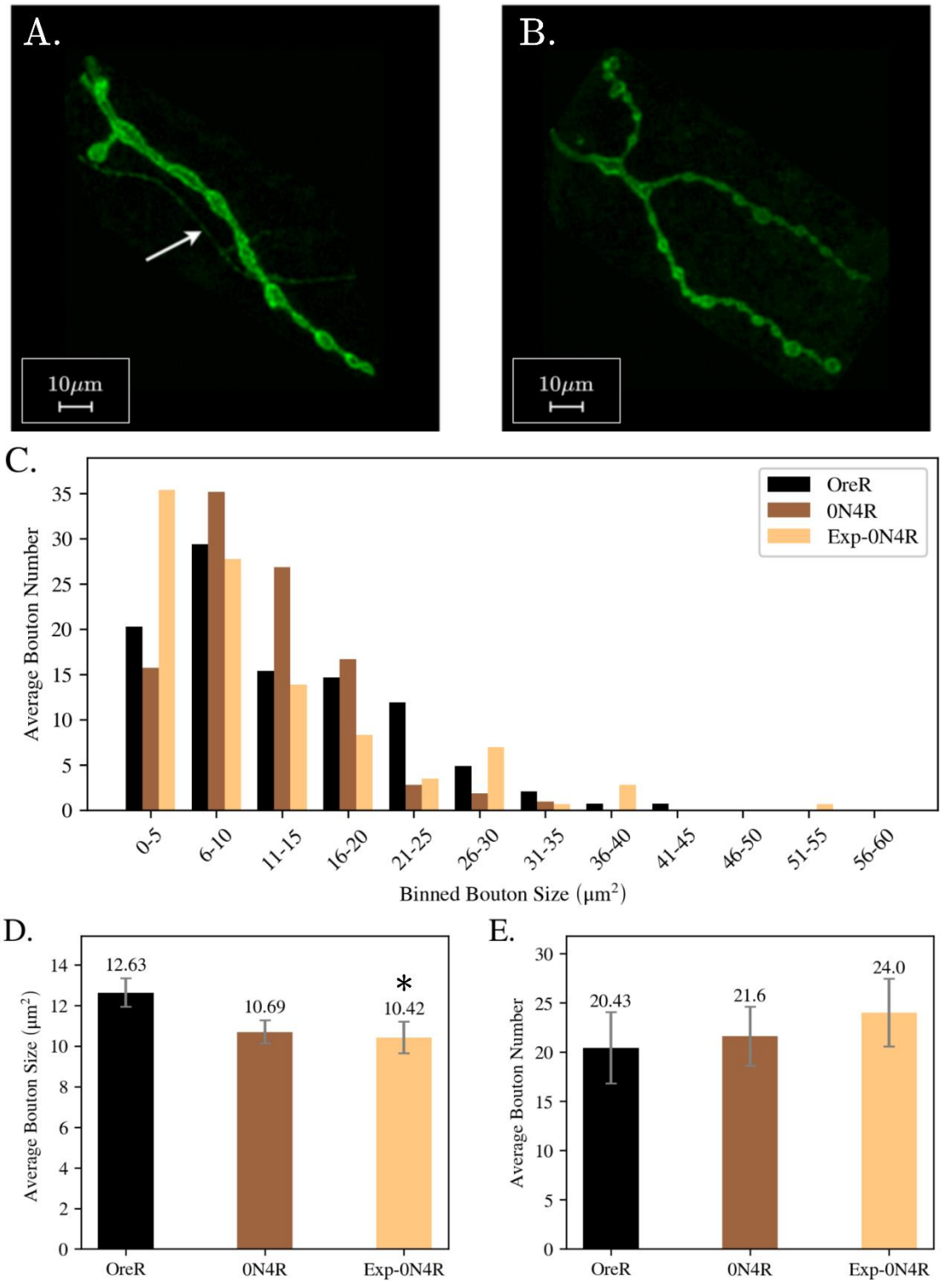


Figure 9. Structure of the NMJ in muscle 4 in wild type, non-expressing 0N4R and expressing 0N4R larvae. Morphology of the NMJ on muscle 4 revealed with anti-HRP antibody of A) 0N4R, showing the classic 'beads on a string' morphology, and B) expressed-0N4R, showing

smaller and more sporadically spread boutons. Analysis of C) the distribution of bouton sizes per NMJ, D) the average bouton size per NMJ and E) the average bouton number per NMJ. *Statistically significant differences between average bouton size of OreR/Exp-0N4R ($P = 1.74\times10^{-3}$, Mann-Whitney *U*). The white arrow indicates a type 2 NMJ that is not a part of this study.

UAS-hTau^{1N3R}, UAS-hTau^{1N4R} and UAS-hTau^{2N4R} do not exhibit statistically significant alterations in *Drosophila* NMJ morphology

1N3R

Expression of 1N3R hTau did not produce a significant difference compared to WT (OreR = 12.63µm^2 ± 0.70µm^2, Exp-1N3R = 12.56µm^2 ± 0.95µm^2, Mann-Whitney $U = 6587.5$, $P > 0.05$ two-tailed, $n_1 = 143$, $n_2 = 95$) (Fig 6A, 10B, 10D). There is also no significant difference in bouton number between the expressing NMJs and WT (OreR N = 20.43 ± 3.64, Exp-1N3R N = 19.00 ± 5.22, Mann-Whitney $U = 15.5$, $P > 0.05$, $n_1 = 7$, $n_2 = 5$) (Fig 6A, 10B, 10E). Expression of 1N3R Tau also does not create a significantly larger proportion of small boutons (OreR/Exp-1N3R [<5µm^2], $P > 0.05$ – chi-square) (Fig 6A, 10B, 10C).

Surprisingly the non-expressing NMJs are significantly different from WT with smaller average bouton sizes than WT (OreR = 12.63µm^2 ± 0.70µm^2, 1N3R = 8.98µm^2 ± 0.62µm^2, Mann-Whitney $U = 5184$, $P < 0.05$ two-tailed, $n_1 = 143$, $n_2 = 97$) (Fig 6A, 10A, 10D). The average bouton number per non-expressing NMJs is not significantly different from WT (OreR N = 20.43 ± 3.64, 1N3R N = 16.17 ± 2.27, Mann-Whitney $U = 16.5$, $P > 0.05$, $n_1 = 7$, $n_2 = 5$) (Fig 6A, 10A, 10E), and non-expressing NMJs do not have a significantly greater distribution of smaller boutons (OreR/1N3R [<5µm^2], $P > 0.05$ – chi-square) (Fig 6A, 10A, 10C).

When the expressing NMJs were compared to the non-expressing NMJs a statistically significant result is seen, with non-expressing NMJs having smaller average bouton sizes than the expressing NMJs (1N3R = 8.98µm^2 ± 0.62µm^2, Exp-1N3R = 12.56µm^2 ± 0.95µm^2, Mann-Whitney $U = 3665.5$, $P < 0.05$ [0.01428] two-tailed, $n_1 = 97$, $n_2 = 95$) (Fig 10A, 10B, 10D). Despite this, average bouton number per NMJ did not exhibit a significant difference in expressing NMJs (1N3R N = 16.17 ± 2.27, Exp-1N3R N = 19.00 ± 5.22, Mann-Whitney $U = 14.5$, $P > 0.05$ two-tailed, $n_1 = 6$, $n_2 = 5$) (Fig 10A, 10B, 10E). This also did not produce a significantly greater number of small boutons (1N3R/Exp-1N3R [<5µm^2], $P > 0.05$ – chi-square) (Fig 10A, 10B, 10C).

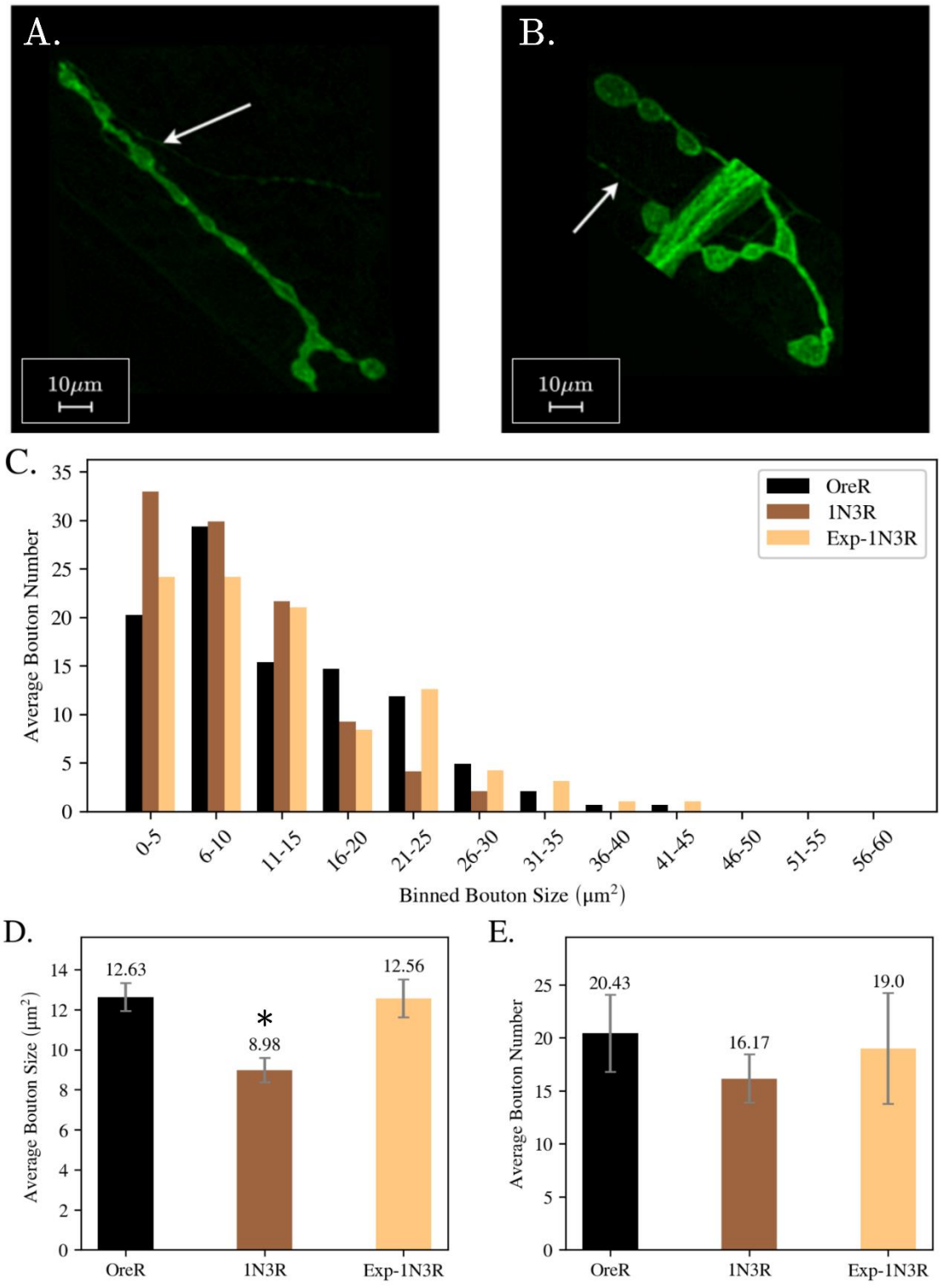


Figure 10. Structure of the NMJ in muscle 4 in wild type, non-expressing 1N3R and expressing 1N3R larvae. Morphology of the NMJ on muscle 4 revealed with anti-HRP antibody of A) 1N3R, showing the classic 'beads on a string' morphology, and B) expressed-1N3R, showing

larger boutons. Analysis of C) the distribution of bouton sizes per NMJ, D) the average bouton size per NMJ and E) the average bouton number per NMJ reveals no statistically significant differences between WT and expressed larvae. *Statistically significant difference between average bouton size of OreR/1N3R ($P = 9\times10^{-4}$, Mann-Whitney U). The white arrows indicate type 2 NMJs that are not a part of this study.

1N4R

With the expression of the second 1N isoform, 1N4R, the NMJs look anatomically WT (Fig 6A, 11B). Morphometric analysis confirms this - with the average bouton sizes per NMJ not producing significant differences compared to WT (Fig 11D) (OreR = 12.63μm^2 ± 0.70μm^2, Exp-1N4R = 13.71μm^2 ± 1.76μm^2, Mann-Whitney $U = 4227$, $P > 0.05$ two-tailed, $n_1 = 143$, $n_2 = 60$). 1N4R expressing NMJs produce a smaller number of boutons per NMJ than WT, but a Mann-Whitney *U* was unable to be performed for this comparison due to too small of a sample size ($n < 5$) (OreR = 20.43 ± 3.64, Exp-1N4R = 15.00 ± 2.80) (Fig 6A, 11B, 11E). Expressing NMJs did not produce a significant difference in distribution of smaller boutons (OreR/Exp-1N4R [<5μm^2], $P > 0.05$ – chi-square) (Fig 6A, 11B, 11C).

There is also no significant difference between unexpressed 1N4R NMJs and WT (OreR = 12.63μm^2 ± 0.70μm^2, 1N4R = 12.16μm^2 ± 0.74μm^2, Mann-Whitney $U = 6994$, $P > 0.05$ two-tailed, $n_1 = 143$, $n_2 = 98$) (Fig 6A, 11A, 11D). Non-expressing NMJs were not significantly different from WT (OreR = 20.43 ± 3.64, 1N4R = 17.25 ± 2.66, Mann-Whitney $U = 17.5$, $P > 0.05$, $n_1 = 7$, $n_2 = 6$) (Fig 6A, 11A, 11E), nor did they produce a significantly greater distribution of smaller boutons (OreR/1N4R [<5μm^2], $P > 0.05$ – chi-square) (Fig 6A, 11A, 11C).

The 1N4R expressing NMJs were not significantly different from the non-expressed NMJs, producing a similar average bouton size per NMJ (1N4R = 12.16μm^2 ± 0.74μm^2, Exp-1N4R = 13.71μm^2 ± 1.76μm^2, Mann-Whitney $U = 2839.5$, $P > 0.05$ two-tailed, $n_1 = 98$, $n_2 = 60$) (Fig 11A, 11B, 11D), with a decreased observable number of boutons per NMJ (1N4R = 17.25 ± 2.66, Exp-1N4R = 15.00 ± 2.80) (Fig 11A, 11B, 11E). A Mann-Whitney *U* test was unable to be performed here due to inadequate sample sizes. There was no significant difference in the distribution of smaller boutons (1N4R/Exp-1N4R [<5μm^2], $P > 0.05$ – chi-square) Fig 11A, 11B, 11C). This isoform was the only one to produce insignificant results across all of the statistical analyses.

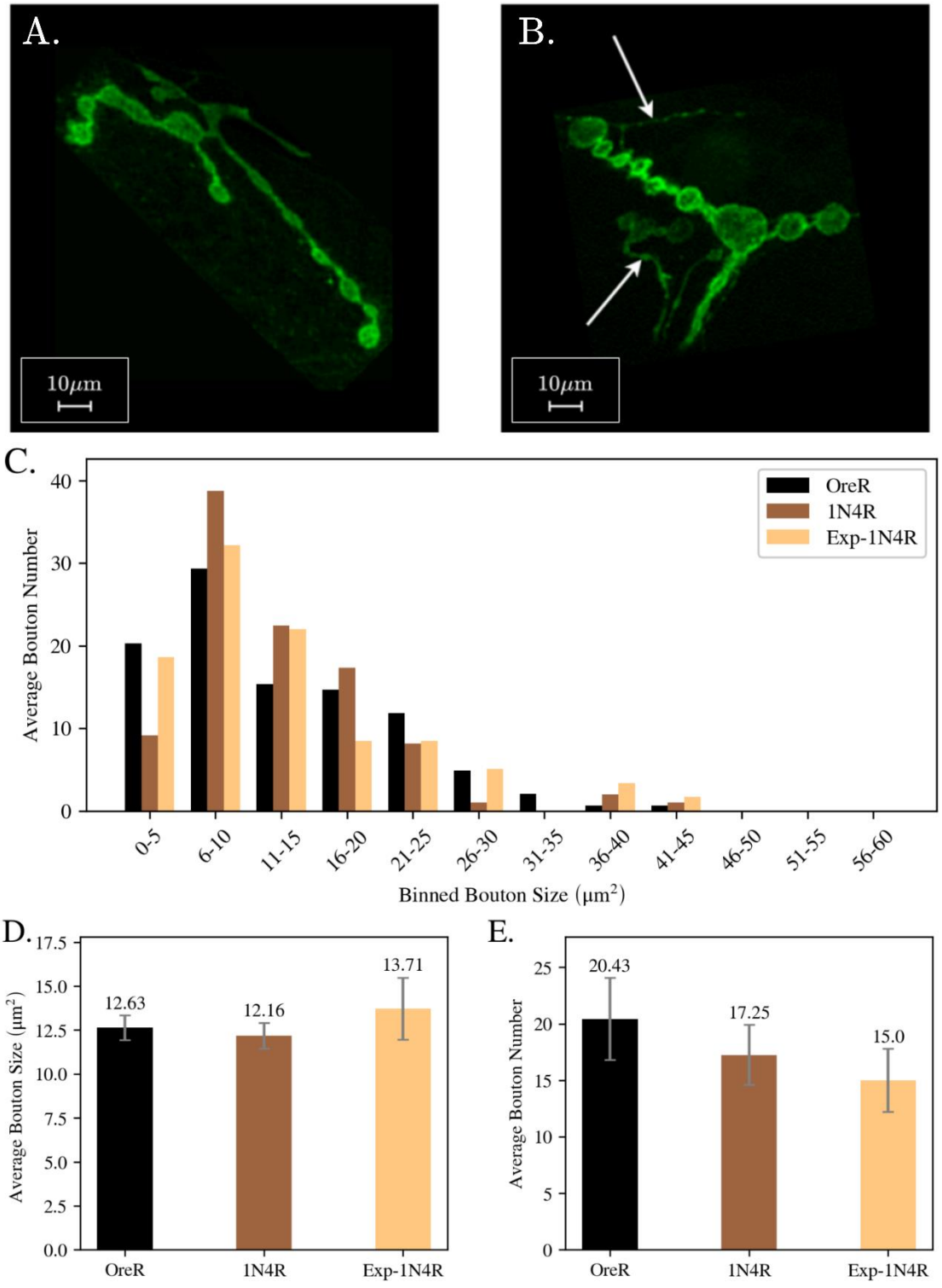


Figure 11. Structure of the NMJ in muscle 4 in wild type, non-expressing 1N4R and expressing 1N4R larvae. Morphology of the NMJ on muscle 4 revealed with anti-HRP antibody of A) 1N4R and B) expressed-1N4R. Analysis of C) the distribution of bouton sizes per NMJ, D) the average

bouton size per NMJ and E) the average bouton number per NMJ reveals no statistically significant differences between WT and the expressed larvae. The white arrows indicate type 2 NMJs that are not a part of this study.

2N4R

Similar to the expression of 1N4R, 2N4R expressing NMJs did not produce a statistically significant difference from WT for average bouton size (OreR = 12.63μm^2 ± 0.70μm^2, Exp-2N4R = 10.35μm^2 ± 0.79μm^2, Mann-Whitney $U = 4876$, $P \geq 0.05$ two-tailed, $n_1 = 143$, $n_2 = 81$) (Fig 6A, 12B, 12D) but, with $P = 0.05$, this result teeters on the threshold of the 95% confidence level. The expressing 2N4R NMJs also have a smaller average number of boutons than WT, but this is not significantly different (OreR = 20.43 ± 3.64, Exp-2N4R = 16.2 ± 2.18, Mann-Whitney $U = 14.5$, $P > 0.05$, $n_1 = 7$, $n_2 = 5$) (Fig 6A, 12B, 12E). Likewise, there were no significant differences in the distribution of bouton sizes (OreR/Exp-2N4R [<5μm^2], $P > 0.05$ – chi-square) (Fig 6A, 12B, 12C).

Unexpressed 2N4R had significantly larger boutons than WT (OreR = 12.63μm^2 ± 0.70μm^2, 2N4R = 15.39μm^2 ± 1.08μm^2, Mann-Whitney $U = 5491.5$, $P < 0.05$ two-tailed, $n_1 = 143$, $n_2 = 91$) (Fig 6A, 12A, 12D). Average bouton number was also observably smaller than WT, but not a significant result (OreR = 20.43 ± 3.64, 2N4R = 15.17 ± 3.11, Mann-Whitney $U = 13$, $P > 0.05$, $n_1 = 7$, $n_2 = 6$) (Fig 6A, 12A, 12E), and did not produce a significant difference in distribution of smaller boutons (OreR/2N4R [<5μm^2], $P > 0.05$ – chi-square) (Fig 6A, 12A, 12C).

This unexpected pattern of statistical significance continues with the comparison of average bouton size of expressing and non-expressing NMJs. The expressing NMJs produced significantly smaller boutons than the non-expressing NMJs (2N4R = 15.39μm^2 ± 1.08μm^2, Exp-2N4R = 10.35μm^2 ± 0.79μm^2, Mann-Whitney $U = 2482$, $P < 0.05$ [3.18×10^{-3}] two-tailed, $n_1 = 91$, $n_2 = 81$) (Fig 12A, 12B, 12D), but with no significant difference in average number of boutons per NMJ (2N4R = 15.17 ± 3.11, Exp-2N4R = 16.20 ± 2.18, Mann-Whitney $U = 11$, $P > 0.05$ two-tailed, $n_1 = 6$, $n_2 = 5$) (Fig 12A, 12B, 12E). There were, however, no significant differences in the distribution of smaller boutons (2N4R/Exp-2N4R [<5μm^2], $P > 0.05$ – chi-square) (Fig 12A, 12B, 12C).

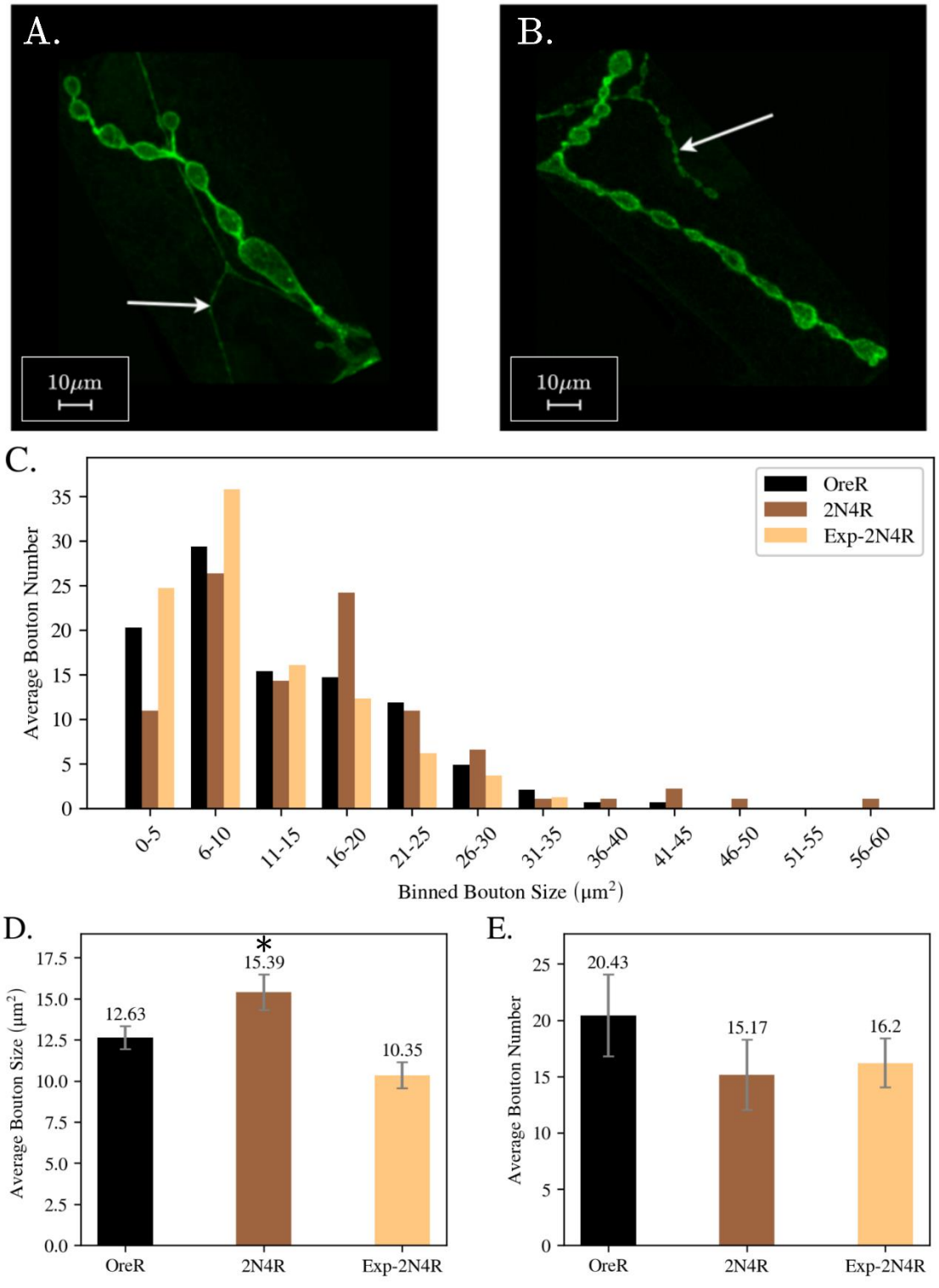


Figure 12. Structure of the NMJ in muscle 4 in wild type, non-expressing 2N4R and expressing 2N4R larvae. Morphology of the NMJ on muscle 4 revealed with anti-HRP antibody of A) 2N4R and B) expressed-2N4R, both showing the classic 'beads on a string' morphology with smooth

rounded and comparably sized boutons. Analysis of C) the distribution of bouton sizes per NMJ, D) the average bouton size per NMJ and E) the average bouton number per NMJ reveals no statistically significant differences between WT and the expressed larvae. *Statistically significant differences between average bouton size of OreR/2N4R (P = 0.0444, Mann-Whitney *U*). The white arrows indicate type 2 NMJs that are not a part of this study.

After thorough analysis of the effect on expressing the various hTau isoforms on the *Drosophila* NMJ, it is evident that each of the isoforms have varying toxicities. UAS-hTau^{0N3R}, UAS-hTau^{2N3R} and UAS-hTau^{0N4R} undoubtedly exhibit structural aberrations on the *Drosophila* NMJ, whilst UAS-hTau^{1N3R}, UAS-hTau^{1N4R} and UAS-hTau^{2N4R} do not produce significant morphological irregularities on the *Drosophila* NMJ.

Effects of human Neurexin-1 overexpression on NMJ structure

Human Neurexin-1 expression alone does not affect NMJ structure

NRXNs primary function is as a binding molecule to its transsynaptic partner NLGN. These molecules bridge the synaptic cleft promoting dendrite and axon adhesion, promoting synapse development (Craig and Kang, 2007; Dean and Dresbach, 2006; Li *et al.*, 2007b). Following this, NRXN1 alone induces GABA post-synaptic differentiation and provokes dendrite receptor clustering (Graf *et al.*, 2004). To test this, NRXN1-GFP was expressed in motor neurons using the OK6 GAL4 driver line. Confirming earlier studies, (Dean and Dresbach, 2006; Chen *et al.*, 2010; Sun *et al.*, 2009), it was shown that NRXN1-GFP has no detrimental effects on NMJ structure. NRXN1-GFP expressing NMJs were normally organised with significantly smaller boutons than WT (OreR = 12.63µm^2 ± 0.70µm^2, NRXN1-GFP = 9.3µm^2, Mann-Whitney U = 545.5, $P < 0.05$ [1×10^{-5}] two-tailed, $n_1 = 143$, $n_2 = 19$) (Fig 6A, 13A), and a comparable bouton number (OreR = 20.43 ± 3.64, NRXN1-GFP = 19.00), A Mann-Whitney U test could not be performed on average bouton number due to a NMJ sample size <5. This work also confirmed previous observations that expressed NRXN1 is localised primarily to the active zones within the terminal regions of the NMJS, and does not accumulate in the axonal segments (Fig 13A, 13B) (Li *et al.*, 2007b).

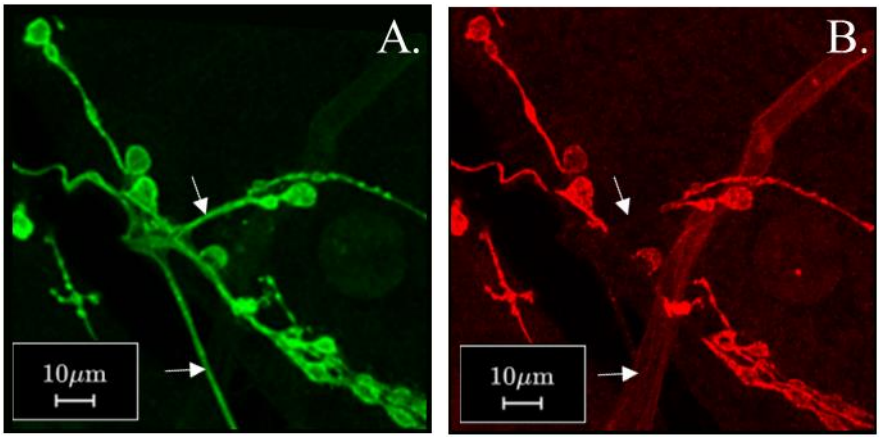


Figure 13. Structure of the NMJ in muscle 4 expressing GFP-tagged human NRXN1 in 3rd instar larvae. A) Structure of the NMJ revealed with anti-HRP. B) Localisation of NRXN1-GFP. The white arrows indicate the absence of NRXN1 in axonal regions, visible with anti-HRP but lacking NRXN1-GFP.

NRXN1 and hTau co-expression proves lethal and significantly reduces bouton size in surviving larvae

To test whether co-expression of NRXN1 and hTau could rescue elements of Tau induced NMJ phenotypes, stocks of flies were created that allowed simultaneous expression of different hTau isoforms and NRXN1 using the OK6 GAL4 driver.

Of the six isoforms tested, only two (UAS-hTau^{2N3R} and UAS-hTau^{2N4R}) when co-expressed with NRNX1 produced larvae. A quick observation analysis of egg laying showed that UAS-hTau^{2N3R} and UAS-hTau^{2N4R} were the only co-expressing flies that laid eggs. This suggests that the co-expression of the 0N3R, 1N3R, 0N4R and 1N4R hTau isoforms and NRXN1 with the OK6 GAL4 driver created flies that were in some ways infertile.

With UAS-hTau^{2N3R}, larvae only survived to 1st instar, and were too fragile to dissect. The NRXN1-T.2N4R larvae were also sick with a small number that survived to 3rd instar larvae.

Analysis of NMJ morphology in the NRXN1-T.2N4R co-expressing surviving larvae revealed spindly NMJs (Fig 4A) with significantly smaller and irregularly positioned boutons that were abnormal in comparison to the WT (OreR = 12.63μm^2 ± 0.70μm^2, NRXN1-T.2N4R = 4.56μm^2 ± 0.49μm^2, Mann-Whitney $U = 1034$, $P < 0.05$ [1×10^{-5}] two-tailed, $n_1 = 143$, $n_2 = 44$) (Fig 6A, 13A). A Mann-Whitney *U* test could not be performed on average bouton number due to a NMJ sample size <5, but bouton number is almost identical when comparing the mean average (OreR = 20.43 ± 3.64, NRXN1-T.2N4R = 20.5 ± 2.50).

Immunocytochemical analysis of the NMJs confirms that in these crosses NRXN1-GFP is localised to the NMJ (Fig 14B) and that hTau, detected with the anti-Tau AT8 antibody, which detects highly phosphorylated Tau, is also expressed, and localised primarily within the axonal segments (Fig 14C).

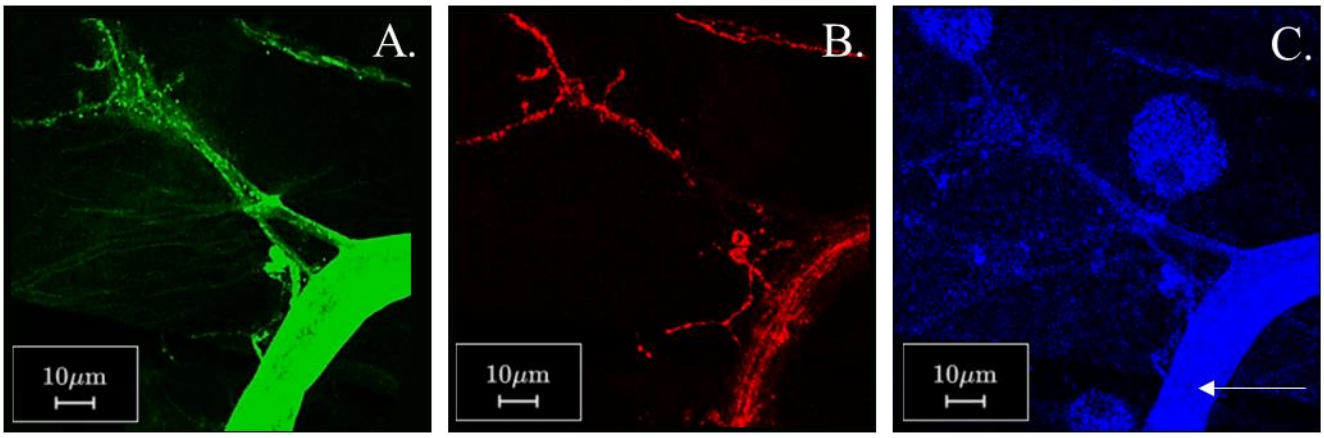


Figure 14. Structure of NMJ in muscle 4 in co-expressed NRXN1 and UAS-hTau^{2N4R} larvae. A) NMJ structure revealed with anti-GFP, B) localisation of the NRXN1-GFP and C) expression of hTau with anti-Tau-AT8. The white arrow indicates the presence of highly phosphorylated Tau in the peripheral nerve.

Discussion

This thesis had two aims. The first was to compare the effects of different hTau isoforms on the morphology of the larval NMJ. This work was taking advantage of the lines produced by Fernius et al. (2017) who created a set of fly stock in which UAS linked versions of the various hTau isoforms had been inserted at the same genomic location to reduce the impact of positional effects on the expression levels often associated with randomly inserted transgenes (Kirchhoff *et al.,* 2020; Manivannan and Simcox, 2016).

The second aim was a proof of principle experiment seeking to evaluate whether the co-expression of hTau and NRXN1 would have a beneficial effect on larval NMJ morphology. The NRXNs are neuronal cell-surface proteins involved in axonal regulation and cytoskeleton assembly in vertebrates and invertebrates, and are the presynaptic partner to NLGNs, postsynaptic molecules who play a vital role in synaptic function (Banerjee and Riordan, 2018; Li *et al.,* 2007b; Reichelt *et al.*, 2012; Xing *et al.,* 2018). The cross-synaptic interaction between DNrx and DNlg1 has been shown to regulate the actin cytoskeleton in NMJs, and given how the hyperphosphorylation of Tau disrupts microtubule dynamics, it is hypothesised that co-expressing NRXN1 with hTau may be able to counteract the effects of Tau, rescuing the NMJ from the collateral damage of hTau (Li *et al.,* 2007a; Xing *et al.,* 2018). Each piece of work will be discussed separately.

Tau isoform expression

Whilst Fernius et al. (2017) suggests all UAS-hTau isoforms are toxic to a similar degree in *D. melanogaster,* when expressed post-mitotically using the pan-nervous system driver *n*-Syb-GAL4, other studies, alongside the one presented in this thesis, have varying results.

The effect of hTau expression on the *Drosophila* NMJ

Of the six isoforms tested in this study, only the expression of hTau^{0N3R} had a significant negative effect on all the morphology parameters, showing smaller and fewer boutons and a greater number of smaller boutons. Of the others, only hTau^{2N3R} and hTau^{0N4R} had any significant effects on *Drosophila* NMJ morphology, with the production of smaller boutons than WT.

The remaining isoforms, hTau^{1N3R}, hTau^{1N4R} and hTau^{2N4R}, revealed no significant difference in any parameter (average bouton size, average bouton number and distribution of <5µm^2 boutons) (Table 2). Due to a small sample size, there was not a sufficient number of data values for expressing 1N4R, and therefore a Mann-Whitney *U* could not be performed. It is pertinent to note that due to the small size of individual samples for average bouton number, minute deviations can cause significant differences in the *P* value of the Mann-Whitney *U*.

Table 2. Effects of non-expressing and expressing hTau isoforms on varying parameters on NMJ morphology

	Average Bouton Size (µm^2)		Average Bouton Number		Bouton Distribution (µm^2)	
	Non-expressing	Expressing	Non-expressing	Expressing	Non-expressing	Expressing
0N3R	*	*	-	*	-	*
2N3R	*	*	-	-	-	-
0N4R	-	*	-	-	-	-
1N3R	*	-	-	-	-	-
1N4R	-	-	-	N/A	-	-
2N4R	*	-	-	-	-	-

Table 2. A summary of the significant severity of each parameter on non-expressing and expressing NMJ morphology against WT. * indicates a significant difference, - indicates an insignificant difference. Expressing 1N4R did not have enough values to perform a Mann-Whitney *U* test for the average bouton number (N/A).

Numerous studies have indicated that specific isoforms might have greater impacts on *Drosophila* NMJ morphology. An indication that UAS-hTau^{0N4R} has the largest degenerative effect is proposed by a *Drosophila* eye sensory neuron assay performed by Sealey et al. (2017). The Sealey lab observed Tau-dependent neurodegeneration on a timed basis, comparing hTau^{0N4R} and hTau^{0N3R} isoforms. At the midpoint of the study, day 20, hTau^{0N4R} showed a much greater sign of degeneration than that of the hTau^{0N3R} isoform, with more irregularities in morphology, and by day 40, hTau^{0N3R} isoforms had a greater quantity of axons remaining compared to hTau^{0N4R}. Whereas Chee et al. (2005) discovered that UAS-hTau^{0N3R} isoforms had the most significant effects on structure and morphology. In a different study, Vourkou et al.

(2022) reported that hTau^{0N3R}, hTau^{1N3R}, hTau^{2N3R} and hTau^{0N4R} expressing flies produce significant reductions in MB size in the NMJ. This largely corresponds with the results of this thesis where hTau^{0N3R}, hTau^{2N3R} and hTau^{0N4R} cause the biggest effects on bouton size and bouton number but contrasts markedly with the lack of effects produced by the expression of 1N3R in this study.

Given the relative lack of effects produced by hTau^{1N3R} and hTau^{1N4R}, on all parameters tested, raises a possibility that the 1N isoforms may have some features that might prevent or limit their pathological effects on the *Drosophila* NMJ. In murine models the 1N isoforms are reported as being localised to the nucleus, cell body and dendrites of neurons, but not to the axons (Liu and Götz, 2013). If this localisation is conserved in *Drosophila* neurons it is conceivable that the 1N isoforms do not localise in the axons of the motor neurons and therefore do not produce the classic effect on NMJ bouton size, number, and distribution.

Tau expression levels

One surprising result that arose from this work was that some of the Tau isoforms had relatively little effect on NMJ morphology. Whilst this suggest that Tau toxicity of the different isoforms can vary it could also simply reflect other elements of the experimental set up. One reason for the reduced toxicity of some Tau isoforms is that the levels of expression produced by the different UAS-Tau lines is variable, and that in some lines the levels of Tau expression may be too low to produce a measurable effect on NMJ morphology. The lines were deliberately chosen to post-mitotically express hTau in the *Drosophila* nervous system, with the UAS-Tau inserted at the same genomic location, to reduce site specific effects on levels of expression (Fernius *et al.,* 2017). For this study, Gal4 expression was targeted to motor neurons so the effects of Tau would be restricted to a specific set of neurons rather than globally affecting nervous system function, producing indirect effects on NMJ function. Despite the use of specific lines, there is no certainty that this will guarantee identical levels of UAS-Tau expression in all circumstances. This postulates a number of questions; is the expression of the different UAS-Tau isoforms variable? Have these unknown expression levels resulted in the inconsistent effects on NMJ morphology? Is it possible hTau was interacting with the native *Drosophila* Tau?

Fernius et al. (2017) used Western blot analysis to show that expression of all six isoforms showed comparable expression levels. Based on this work we chose not to quantify hTau

because previous studies had not indicated a need to do this. Alongside this, there was not time to do so. It is now evident that Tau expression levels should have been quantified and localisation of each of the isoforms using immunocytochemistry, or more precisely with quantitative western blotting or qPCR analysis (Cowan *et al.*, 2010). It would also be pertinent to assess the relative expression levels of each of the isoforms as they may reflect mRNA instability, hTau isoform instability or translational efficiency differences (Vourkou *et al.*, 2022). With this knowledge we could eliminate any effects that reflect lower toxicity as a result of lower levels of Tau expression. Further improvements to the power of this study would be to take a blinded approach where the hTau isoforms were unknown prior to dissection and analysis.

What this study has shown is that the expression of the different isoforms of hTau in the *Drosophila* NMJ affect NMJ morphology and structure to varying extents. $hTau^{0N3R}$ affects it as expected, whilst $hTau^{2N3R}$ and $hTau^{0N4R}$ negatively affected some parameters.

The effect of the non-expressing controls on the *Drosophila* NMJ

A major problem with this study however was that in some of the experiments the unexpressed Tau controls also showed negative effects on NMJ morphology. In fact, 0N4R was the only isoform to have the expected result, with non-expressing NMJs having no change in morphology.

Non-expressing 0N3R, 1N3R, 2N3R and 2N4R isoforms all exhibited statistically significant effects when compared to WT. These results undermine the impact of significant effects observed in the hTau expressing NMJs. Given that by design the non-expressing controls should not produce any morphological aberrations from the WT, as there should be no hTau expression, these results point to an unknown issue in the background of the flies. Most likely this is because there is some "leaky" (non GAL4-driven) expression of Tau in the UAS-hTau stocks. It is therefore vital that before further work is done that the non-expressing UAS-Tau stocks are checked for Tau expression.

Overexpression vs CRISPR

Another way to improve the approach to study hTau toxicity would be to consider other newer expression systems. For example, Di Maria et al. (2020) utilised an adapted Cas9 system, the S*p*dCas9 and S*a*dCas9-based activator approach created by Ma et al. (2018), to overexpress

genes involved in neuronal activity, opening new doors to the use of CRISPR in neurodegenerative diseases. This approach may be key to gene insertion, and a more appropriate method for future analysis of hTau isoforms in *Drosophila* over standard overexpression methods.

The traditional approach of gene expression clones and inserts the coding sequence into the cells of interest using a plasmid, via complementary DNAs (cDNA) or open reading frames (ORF), to exogenously expresses the gene of interest (Harbers, 2008). This technique is widely accessible in all labs if the desired cDNA/ ORF is commercially available. However, if not available, the process is very time consuming. The insertion of non-native DNA, using an artificial transcriptional promoter and signalling elements, can create expression issues, as they do not align with the natural expression method of the native gene (Harbers, 2008). This is where the use of CRISPR technology is perfect. CRISPR (clustered regularly interspaced short palindromic repeats), when linked with the Cas9 nuclease has many advantages over the common over-expressive technique involved in the expression of hTau within *Drosophila*. Transgenic expression, despite being site-specific, is an unpredictable method of gene insertion, and due to the random integration of the expression vector, its' reproducibility decreases, whereas CRISPR-Cas9 allows for successful transmission through the germline due to its sequence-specific capacity (Hunt *et al.,* 2021). CRISPR also provides the possibility of activating multiple genes within the same cell, which is often limited in standard overexpression to one gene at a time (Dawson *et al.*, 2018; Götz and Ittner, 2008; Harvey *et al.*, 2008, 2011; Kunieda *et al.*, 2002).

Data consistency

Whilst the statistics did not reveal significant toxic effects for all of the isoforms, it is possible that expression of these hTau isoforms was impacting NMJ morphology and that my analysis did not have the statistical rigour to reveal this. One reason to suspect this is that for all the hTau isoforms it was possible to observe some of the typical characteristics of Tau expression in the NMJs, but the analysis was not supported by the statistics. Whilst this could perhaps have been alleviated with a larger sample size, previous studies have been able to reveal significant effects using similar sample sizes (Chee *et al.*, 2005). Also, when undertaking the data analysis, human errors may also have arisen in identifying type I and type II boutons. This could be overcome by the co-expression of Bruchpilot (Brp) to discriminate between bouton types.

When deciding which statistical tests to run, Mann-Whitney *U* was chosen for the average bouton size. A Mann-Whitney *U* test was also performed on the average bouton number per NMJ, but it is worth noting that due to the small sample size obtained during the course of this experiment the results of this test can be extremely sensitive to small deviations within individual samples. With a smaller sample size, these tests have lower statistical power – meaning there is a reduced ability to detect significantly statistical differences in a population.

The results induced by the expression of $hTau^{2N4R}$ were the most intriguing. Expressing NMJs were not proven to be significantly smaller than WT, despite the numerical average bouton size almost guaranteeing it. When a Mann-Whitney *U* analysis was undertaken, a *P* value of precisely 0.05 was produced. With this result balancing on the threshold of the 95% confidence level, it reinforces the continued proposition of the need for an improved experimental procedure, as these results may have been different if a larger sample size had been utilised.

In this respect, it is pertinent to point out that some of weakness in the statistics could be due to inconsistency in the dissection of NMJ preparations. Consistent dissection of larvae to produce analysable preparations is a skilled procedure and requires months of practice and training to ensure the preparations are consistent with minimal artefactual impacts on the NMJs. Given the compressed timescale of this project, it was inevitable that some of the materials used in the analysis were produced during the 'learning' stage, and that the quality of preparations was not consistent, especially during the learning phases of the study. As some results contradict previous studies, it is important that the analysis presented here is repeated with greater dissection consistency and experience to reduce any artefactual errors before attempting to suggest that previous studies are incorrect.

Future Perspectives

Drosophila is not the only animal model which has been extensively used to analyse the effects of Tau expression. Vertebrate models are extremely valuable in the research of neurodegenerative diseases, due to their closer proximity to the human genome and the continuation of evolutionary conservation.

Vertebrate animal models

A step up from *Drosophila* on the evolutionary tree is the zebrafish (*Danio rerio*), and, despite rodent models being the most common in the study of tauopathies, additional animal models are required to broaden our understanding of the diseases. *D. rerio* interestingly contain two paralogous *MAPT* genes; *Mapta* and *Maptb*, with the first being capable of being alternatively spliced into isoforms with between four and six tubulin-binding repeats, and the second being spliced into isoforms with three binding repeats (Chen *et al.*, 2009). This larger range of isoforms makes the zebrafish a useful tool in studying the interactions between the various Tau isoforms. Wu et al. (2016) drove 3R and 4R isoforms in zebrafish, resulting in an increase in neuronal death matched with increased phosphorylation of Tau (Wu *et al.*, 2016). Few studies have been undertaken expressing hTau in zebrafish, whilst a large proportion of Tau studies have been executed in murine models.

Murine models are a powerful tool for the development of new treatments and an insight into the mechanisms of diseases. Mice are especially popular for studying the development of the nervous system due to their genome being very similar to the human genome, and because of their cost effectiveness and ease of maintenance. Previous murine studies have indicated that 3R-Tau isoforms are the prominent isoforms in foetal and new-born mice, with 0N3R being the only one expressed during foetal brain development and therefore likely having a unique role in comparison to that of the other five isoforms (Buée *et al.*, 2000; Goedert and Jakes, 1990; Janke *et al.*, 1999; Kampers *et al.*, 1999; Kosik *et al.*, 1989; McMillan *et al.*,2008; Takuma *et al.*, 2003).

Despite the success of tauopathy research in murine models, there is scope that they do not serve as true models for the human aged-brain, due to their short lifespan. This is where primate models are valuable. There is divergence in the expression of Tau isoforms across primate species, with certain species showing greater similarities with humans than others. Sharma et al.

(2019) interestingly found the marmoset expression pattern to be more like that of mice, than humans, Humans, chimpanzees, and gibbons express both 3R and 4R Tau in the adult brain (Goedert *et al.,* 1989b; Kosik *et al.,* 1989), whereas marmosets only express 4R in the adult brain (Sharma *et al.,* 2019). This is interesting as marmoset Tau consists of the identical nucleotide sequence within the stem-loop of that of hTau, but a slight difference in the nucleotide sequence of the intron-splicing modulator region is what differentiates it from other primates (Grover *et al.,* 1999; Sharma *et al.,* 2019; Qian and Liu, 2014).

In mice, 3R Tau is the abundant isoform, whilst in the adult human brain both the 3R and 4R isoforms are equally plentiful (Goedert *et al.,* 1989a; McMillan *et al.,* 2008; Takuma *et al.,* 2003). It is interesting to observe a gradual divergence in the presence of the R domains as you move up the evolutionary tree. To further develop tauopathy research in humans, a wider scope of models needs to be utilized. Alongside this, further studies need to be conducted to determine if there are other genomic co-expressions that could alleviate the symptoms of AD.

What implications do these results have in humans?

Research on the NMJ in *D. melanogaster* can provide important insights into the mechanisms of human neuromuscular function and disease. It has been instrumental in elucidating the fundamental mechanisms of human synaptic transmission due to the evolutionary conservation conveyed between the species. The use of *Drosophila* models of tauopathies has allowed researcher to study the impact of Tau on neuronal function and structure, identifying genetic causes of Tau toxicity. Animal models often lack the full spectrum of Tau isoforms that are present in humans. Utilising hTau in various transgenic animal models, allows for the exploration of the different toxicities of hTau. If continuous consistencies arise in the data of these studies, researchers can refine and determine which hTau isoform is the most toxic, or if one isoform has a greater involvement in specific diseases.

For example, both 3R and 4R Tau have been found to impact cholinergic neurons in the human brain (Barron *et al.,* 2020). The accumulation of Tau can lead to the degeneration of these neurons, contributing to the cognitive decline common of tauopathies (Simón *et al.,* 2013). In healthy neurons, Tau is mainly phosphorylated at specific sites, whereas in AD it hyper-phosphorylates in NFTs, of which 3R Tau is preferentially incorporated (Cherry *et al.,* 2021). 4R

Tau is associated more with Frontotemporal Dementias, Pick's Disease and Corticobasal degeneration, due to its localization in the basal ganglia, brainstem, and cortex (Mott *et al.*, 2005; Williams, 2006; Yoshida, 2006).

In addition to cholinergic dysfunction, glutamate toxicity, or excitotoxicity, is another mechanism by which Tau pathology contributes to neuronal degeneration. Glutamate is the primary excitatory neurotransmitter in the human brain, and excess glutamate can lead to excitotoxicity (DiAntonio, 2006). Abnormal Tau can impair axonal transport, resulting in the accumulation of excess glutamate, and thus excessive release of the neurotransmitter, causing excitotoxicity (Ackerley *et al.*, 2000; Vandenberg and Ryan, 2013).

Overall. hTau research on any model organism provides scientists with the knowledge to create potential therapeutic targets for the treatment of tauopathies, helping to identify candidate drugs to prevent or reverse Tau pathology, such as microtubule-binding drugs and even the controversial use of NSAIDS (In'T Veld *et al.*, 2001; Szekely *et al.*, 2008; Zhang *et al.*, 2005). It is therefore imperative that these types of studies continue.

Future studies

Several elements of the experimental procedure executed in this study would undeniably need to change in order to identify the potential issues and develop viable results.

OK6 reduces longevity

The OK6 GAL4 driver line was chosen due to its immediate availability and the compressed laboratory time available for the experimental work. However, it may not have been the best choice. Following the completion of this project, Lovesha Sivanantharajah reported (pers comm) that the OK6 GAL4 driver line reduced longevity in her work. This points to a previously unknown negative effects on viability in the driver line that could explain some of the unexpected results presented here.

It is conceivable that the results of this study when compared to others is a product of the use of different driver lines in different studies. The GAL4 system is well known as an impressive cell and tissue specific driver, but it isn't perfect and can have other unforeseen effects, e.g., including a salivary-gland specific enhancer which expresses GAL4 in the salivary glands of

larvae and adults (Brand and Perrimon, 1993), not to mention other undocumented expression, however transient, in other tissues and cells. To counteract this, Fernius et al. (2017) used the GAL80 driver instead. GAL80 can be used to refine and repress GAL4 activity to ensure expression is restricted to the desired tissues and cells, e.g., supressing GAL4 in the salivary glands, and ensuring more specific expression of hTau (Suster and Seugnet, 2004). Whilst the GAL4 line chosen in this study was based on its availability, the results do point to the need for more careful driver selection and the possibility of using GAL80 to refine the expression as needed.

There are also other motor neuron specific GAL4 drivers available, for example D42 (James and Broihier, 2011). It would be interesting to test if a different driver produced different outcomes, removing any potential effects which may have arisen due to the use of the OK6 GAL4 driver line.

Neurexin and hTau Co-expression

The crosses designed to produce co-expression of NRXN1, which was chosen due to its immediate availability, and hTau struggled to produce viable larvae. With four of the isoforms (0N3R, 0N4R, 1N3R and 1N4R) rendering the female flies apparently sterile and unable to lay eggs, only $hTau^{2N3R}$ and $hTau^{2N4R}$ produced viable larvae, but even these were weak and struggled to thrive. The NRXN1-T.2N4R larvae did not survive past the 1st instar stage, but a small number of the NRXN1-T.2N4R larvae did survive to the 3rd instar stage, but even these failed to pupate, were fragile and very difficult to dissect. At one level this result clearly indicates that there is some unforeseen interaction in these crosses that can render the adult female sterile. Although the fact that the two crosses that produced fertile females, and some sickly larvae, were the two 2N hTau isoforms, suggests that there is a potential interaction involving the 2N isoforms that allow for this fertility. There could also be a possible toxic effect between the 0N and 1N isoforms that when co-expressed with NRXN1 causes the stocks to be sterile. Either way it does point to an interesting avenue for further exploration.

Interestingly, Liu et al. (2016) reported that a larger number of Tau-interacting proteins bind to the 2N isoforms, indicating these isoforms have a more profound role in neurodegenerative diseases. The abundance of Tau-interacting proteins in the 2N isoforms could have been

interacting with NRXN1, thereby preventing these proteins from interacting with Tau as efficiently and reducing Tau-induced degeneration in the 2N isoforms crosses.

There are however other factors to consider in this experiment. Why the fertile crosses produced sick offspring is not immediately clear, as only 25% of the progeny would have been co-expressing NRXN1 and hTau regardless, begging the question; how come the other 75% did not survive? This suggests that the performance of the cross was not simply the result of the co-expression. There may also be something else in the background of these crosses that is having a negative impact on reproducibility and survivability. Despite the fact that the stock would be inherently weak due to the presence of the balancer CyO, this alone should not produce such a poor outcome. Clearly this result suggests that before exploring the possible roles of the 2N isoforms, in some kind of rescue effect, the crosses and the stocks used to establish these crosses should be reassessed to eliminate or minimise other background effects.

Alternative protein co-expressions

Whilst this work was designed to see if expression of NRXN1 could rescue a Tau phenotype, this was only a proof of principle study. If this was to be continued further, it is pertinent to acknowledge that NRXN1 does not exert its effects alone.

Its main mode of action also involves binding with its transsynaptic partner, NLGN. It is therefore conceivable that even if these stocks had produced viable larvae, the NRXN would be inert in the absence of its binding partner. To truly execute this experiment, the simultaneous co-expression of NLGN in the post synaptic muscles would be required. Whilst this is theoretically possible, it would require more effort than available for this study, and the need to use two different expression systems; GAL4, to drive NRXN, and the muscle specific Lex A line to drive NLGN in muscles.

Other signalling components are also involved in DNlg1 transsynaptic functions and the regulation of their recruitment to synapses, for example the binding of postsynaptic density protein 95 kDA (PSD-95) and N-Methyl-D-aspartate (NMDA) receptors (Barrow *et al.,* 2009; Dresbach *et al.,* 2004). This highlights how the hypotheses postulated in this thesis are not reliant on a simple series of chain reactions, but in fact a complex system with many variables.

Genetic and immunocytochemistry alterations

A pertinent change in how the larvae were sexed would be necessary. Rather than just visually sexing the flies, an appropriate method would be to perform heat-shock on them. A lethal gene is present on the Y-chromosome, which is activated under an increase in temperature, due to it being controlled by a heat-shock promoter. Fertile males only have a sole Y-chromosome, making this an efficient technique of removing them and singling out females (Carvalho, 2002). XO males would survive this, but these flies are sterile and would not interfere with the establishment of stocks (Carvalho, 2002).

Delving further into the genetic background of the study, different balancer chromosomes should also have been considered. Given that larvae were worked on, using a 'green' balancer with a larvally detectable GFP expression stage, for example the GFP/CyO balancer, would have meant the correct genotypes for dissection would have been selected for, thus making the experiment easier to execute and eliminate the need to use antibody staining to disregard the unwanted genotypes.

Another element to consider changing is the genetic locus of the UAS-NRXN-GFP in the NRXN1 crosses. The available inserts are located on the X chromosome, and although this isn't a primary cause of the problems, the use of the X chromosome was a complicating factor in the genetics used to create the experimental lines. As such, it may be worthwhile obtaining an insert on the second chromosome, and recombining it with UAS-hTau, so both transgenes can be inherited on the same chromosome.

The antibodies used were a result of accessibility for the lab. Tau AT8 is an antibody that only detects phosphorylation at two residues – Serine 202 and Threonine (Goedert *et al.,* 1995), and this was used in the co-expression of NRXN1 and T.2N4R. In Fig 13C Tau AT8 is staining muscle cell nuclei, reconfirming the need for different identification techniques. Perhaps different antibodies would have been more potent in this experiment to locate the expression of hTau, such as AT180 or AT270, which detects phosphorylation at Threonine 231 and Threonine 181, respectively (Goedert *et al.,* 1994).

Conclusion

This thesis sought to determine whether the different isoforms of hTau have the same levels of toxicity when expressed in the *Drosophila* larval NMJ, and if the expression of NRXN could rescue Tau induced phenotypes.

To assess the first aim of this thesis; Does targeted expression of the six different isoforms of hTau have varying effects on the *Drosophila* NMJ, OreR WT, OK6 GAL4 control and the six hTau isoforms (0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R) stocks were dissected, whilst also crossing the OK6 GAL4 driver line stock with each of the hTau isoforms. Once these crosses were established and 3rd instar were dissected, their NMJs were analysed. The expression of NRXN1 was evaluated to determine whether NRNX1 alone had any effect on the *Drosophila* NMJ. Following this, NRXN1 and hTau were co-expressed to test the second hypothesis; does the co-expression of NRXN1 and hTau rescue Tau induced phenotypes in the *Drosophila* NMJ?

The analysis of the NMJs involved measuring bouton number and bouton size (μm^2) using ImageJ. Mann-Whitney *U* tests were performed using SPSS to compare average bouton size and number, and chi-square tests to compare the distribution of small boutons (<5μm^2) against the Oregon-R wildtype.

This study was only partially successful in confirming the main hypotheses due to inconsistent results. When compared to wild type, 0N3R, 0N4R and 2N3R expressing neuromuscular junctions produced significantly smaller boutons, but only 0N3R expressing NMJs had a significantly different average bouton number and distribution of bouton sizes <5μm^2. Despite this, hTau expressing NMJs do look morphologically different to WT, and that despite the small sample sizes, it cannot be ruled out that the expression of different hTau isoforms does have different and detrimental effects on the *Drosophila* NMJ. It was also discovered that there may be a potential genetic background element that may have cause a reduced life span and compromised the results.

For the NRXN experiments it was confirmed that expression of NRXN1-GFP alone had no effect on NMJ structures. But when NRXN1 is co-expressed with T.2N4R (the only isoform that produced analysable larvae) it produced spindly, irregularly arranged, and abnormal boutons compared to WT.

These problems highlight the need to revaluate some of the lines used in this study, most notably the use of a different GAL4 driver line, alongside balancer chromosomes that are detectable in larvae and the use of inserts on different chromosomes. Furthermore, a larger time frame is required to ensure accurate and consistent dissections occur, in conjunction with larger sample sizes. Analysing the NMJ of NRXN1 alone, without the use of the OK6 GAL4 driver, would be pertinent to determine if OK6 did in fact have negative effects of NMJ morphology.

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Appendix

Chi-Square Test

Table 3. Chi-Square test on distribution of boutons with an average bouton size <5μm^2

Genotype	Chi-Square	*P*
OreR/OK6	0.7099	1.0000
OreR/0N3R	4.0703	0.9441
OreR/Exp-0N3R	33.4153	0.0002
0N3R/Exp-0N3R	9.7797	0.4600
OreR/0N4R	1.0159	0.9998
OreR/Exp-0N4R	11.2983	0.3348
0N4R/Exp-0N4R	24.5949	0.0062
OreR/1N3R	7.9658	0.6322
OreR/Exp-1N3R	0.7619	1.0000
1N3R/Exp-1N3R	2.3363	0.9931
OreR/1N4R	6.0712	0.8092
OreR/Exp-1N4R	0.1319	1.0000
1N4R/Exp-1N4R	9.7455	0.4631
OreR/2N3R	4.4573	0.9244
OreR/Exp-2N3R	12.1414	0.2757
2N3R/Exp-2N3R	1.2838	0.9995
OreR/2N4R	4.2563	0.9350
OreR/Exp-2N4R	0.9597	0.9999
2N4R/Exp-2N4R	17.0856	0.0725

Mann-Whitney *U* Test

Table 4. Mann-Whitney *U* test on average bouton size (μm^2)

	U	*P*	Accept or Reject H_0
OreR/OK6	6803.5	0.83400	Accept
OreR/0N3R	7526.5	0.01980	Reject
OreR/Exp-0N3R	10796	0.00001	Reject
0N3R/Exp-0N3R	11730	0.00018	Reject
OreR/0N4R	7053	0.28462	Accept
OreR/Exp-0N4R	8098.5	0.00174	Reject
0N4R/Exp-0N4R	6470	0.02260	Reject
OreR/1N3R	5184	0.00090	Reject
OreR/Exp-1N3R	6587.5	0.69654	Accept
1N3R/Exp-1N3R	3665.5	0.01428	Reject
OreR/1N4R	6994	0.98404	Accept
OreR/Exp-1N4R	4227	0.87288	Accept
1N4R/Exp-1N4R	2839.5	0.71884	Accept
OreR/2N3R	8500.5	0.02260	Reject
OreR/Exp-2N3R	8484.5	0.03400	Reject
2N3R/Exp-2N3R	9584	0.74896	Accept
OreR/2N4R	5491.5	0.04440	Reject
OreR/Exp-2N4R	4876	0.05000	Accept
2N4R/Exp-2N4R	2482	0.00318	Reject
OreR/NRXN1-GFP	545.5	0.00001	Reject
NRXN1/T.2N4R	1034	0.00001	Reject

Table 5. Mann-Whitney *U* test on average bouton number per NMJ

	U	*P*	Accept or Reject H_0
OreR/OK6	20	0.61006	Accept
OreR/0N3R	10.5	0.28914	Accept
OreR/Exp-0N3R	5	0.02710	Reject
0N3R/Exp-0N3R	5.5	0.10100	Accept
OreR/0N4R	14.5	0.68180	Accept
OreR/Exp-0N4R	15.5	0.47770	Accept
0N4R/Exp-0N4R	12.5	0.71138	Accept
OreR/1N3R	16.5	0.56868	Accept
OreR/Exp-1N3R	15.5	0.81034	Accept
1N3R/Exp-1N3R	14.5	1.00000	Accept
OreR/1N4R	17.5	0.93624	Accept
OreR/Exp-1N4R	N/A	N/A	N/A
1N4R/Exp-1N4R	N/A	N/A	N/A
OreR/2N3R	8.5	0.16758	Accept
OreR/Exp-2N3R	10.5	0.28914	Accept
2N3R/Exp-2N3R	8.5	0.46540	Accept
OreR/2N4R	13	0.28462	Accept
OreR/Exp-2N4R	14.5	0.68180	Accept
2N4R/Exp-2N4R	11	0.52218	Accept