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The BMP signaling pathway at the *Drosophila* neuromuscular junction and its links to neurodegenerative diseases

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Summary

The *Drosophila* neuromuscular junction (NMJ) has recently provided new insights into the roles of various proteins in neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), Spinal Muscular Atrophy (SMA), Multiple Sclerosis (MS) Hereditary Spastic Paraplegia (HSP), and Huntington's Disease (HD). Several developmental signaling pathways including WNT, MAPK and BMP/TGF-β signaling play important roles in the formation and growth of the *Drosophila* NMJ. Studies of the fly homologues of genes that cause neurodegenerative disease at the NMJ have resulted in a better understanding of the roles of these proteins *in vivo*. These studies may shed light on the pathological mechanisms of these diseases, with implications for reduced BMP/TGF-β signaling in ALS, SMA and HD and increased signaling in HSP and MS.

Introduction

Neurodegenerative diseases are among the most common diseases and yet probing the pathological mechanisms has remained a challenge. The diseases typically come in both sporadic and hereditary forms, and mutations in numerous genes in familial cases have been identified. To obtain insights into how these genes and their mutations are involved in specific diseases, one of our best strategies is to study these genes and the corresponding mutations in model organisms like *Drosophila* and mice. The *Drosophila* larval neuromuscular junction (NMJ) is an especially amenable system to study the role of these genes in invertebrates[1]. As shown in Figure 1, the motor neuron's terminals form close spherical connections with the muscle, called boutons[1]. At each bouton, many synapses, the presynaptic regions of which are known as active zones, form between neuron and muscle. These zones have a T-shaped structure known as a T-Bar where synaptic vesicles (SVs, green) cluster, fuse and release glutamate (red lightning) into the extracellular space. The neurotransmitter is then bound by glutamate receptors (purple cylinders) on the muscle,

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Competing Interests
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that triggers calcium influx and subsequent muscle contraction[2]. Given that *Drosophila* permits sophisticated genetic manipulations[3,4], numerous probing experiments can be performed. For example, in a mutant background, proteins can be expressed pre- or post-synaptically to determine where they are required. Electrophysiological experiments on individual nerves targeting specific muscles allow one to derive very valuable information about the ability to release vesicles, endocytose membranes, the function of postsynaptic receptors, etc[5]. FM1–43 dye uptake experiments allow one to establish how much membrane is taken up during endocytosis and how much is released during exocytosis, thus providing real-time analysis of vesicle trafficking[6]. Antibodies against numerous proteins that are present at the NMJ pre- and post-synaptically are available and allow one to assess which proteins may be implicated in the process or pathology that is being studied[7]. These data, combined with Transmission Electron Microscopy and Immuno-EM studies provide very valuable information about the number, distribution and size of SV, and the number and size of active zones[7,8]. Finally, genetic interactions and epistasis with a vast number of mutants as well as the ability to overexpress specific proteins allow for a detailed analysis of genetic pathways[9]. In summary, no synapses are currently more amenable to a diverse set of manipulations *in vivo* than the *Drosophila* NMJ, and by combining the information obtained from different experimental datasets, very valuable biological information can be derived.

The development and growth of the *Drosophila* NMJ requires an anterograde as well as a retrograde input from the muscle[2]. The anterograde signaling is primarily mediated by the Wnt ligand Wingless (Wg)[10], whereas retrograde signaling occurs mostly by a Bone Morphogenetic Protein (BMP) ligand named Glass bottom boat (Gbb) that is released from the muscle and binds to its tetrameric presynaptic BMP receptors containing Wishful Thinking (Wit), Thickveins (Tkv) and Saxophone (Sax) on the neuronal cell membrane (Figure 1)[11]. Binding of Gbb to its receptor has two parallel effects. The first involves the activation of the Williams Syndrome-associated kinase LIMK1 to act in the stabilization of the synapse[12]. In the absence of BMP signaling or LIMK1, one sees many ‘synaptic footprint’ or ‘retraction’ sites at the NMJ, in which one still sees clustered postsynaptic proteins like Discs Large (Dlg) but no longer any presynaptic molecules[12]. The other involves receptor-mediated phosphorylation of the Smad family transcription factor Mad (Mothers against decapentaplegic), signaling the neuron to expand the number of synapses[11]. In the absence of key components of the pathway, including Wit and Gbb, small NMJs with reduced neurotransmission develop, and in the absence of negative pathway components such as Daughters against decapentaplegic (Dad), the NMJs overgrow, with characteristic ‘satellite boutons’ that appear disconnected from the other boutons[13]. In addition to the BMP and WNT pathways, a MAP Kinase pathway regulated by Highwire, a putative RING finger E3 ubiquitin ligase, also controls NMJ growth and branching[2].

Here we will review some of the salient recent work related to several genes that cause familial forms of neurodegenerative diseases whose culprits are conserved in flies and have been studied at the *Drosophila* NMJ. Some of the discussed work was based on forward genetics and only later was it realized that these genes caused or were related to neurological diseases. Other genes were studied specifically because they are known to be mutated in such diseases and the NMJ offers a highly relevant toolkit to study the pathogenesis of neurodegenerative diseases.

Hereditary Spastic Paraplegia

The Hereditary Spastic Paraplegias (HSPs) are a diverse set of diseases that share the primary feature of progressive, severe, lower extremity spasticity, with many causative genes identified so far[14]. Autopsies have revealed, among many other findings, myelin

pallor and axonal loss in the corticospinal tracts, and in the brain, a high density of irregular tau-positive neurofibrillary tangles[15]. Mutations, frequently nonsense, in the gene *spastin* are responsible for almost half of all cases. Some of the earliest studies of *spastin* function *in vivo* were performed at the *Drosophila* NMJ. Spastin was found to localize to the NMJ and to be present both in neuronal and muscle cytoplasm. Loss of Spastin causes an increase in the number of boutons that are smaller than normal and clustered together, in a pattern reminiscent of the 'satellite boutons' seen in some endocytic mutants (Tables 1, 2)[16]. *spastin* mutants also display a thickened microtubule (MT) network, while tissue-specific overexpression results in a loss of the MT network. This work, along with other data and *in vitro* experiments, provided further evidence that Spastin is a microtubule-severing protein[17,18]. Another HSP protein encoded by *atlastin (atl)*, responsible for 10% of HSP cases, was found to localize to punctae throughout the muscle cytoplasm. In mutant *atl* larvae synaptic boutons are clustered and satellite boutons are prominent, similar to *spastin* mutants (Tables 1, 2), and similar to overexpression of BMP pathway components. Furthermore, the Subsynaptic Reticulum (SSR), composed of postsynaptic infoldings of the muscle surrounding the boutons, is greatly reduced. In addition, muscle-specific but not neuron-specific expression of the gene in mutant larvae can rescue the phenotype, indicating that its muscle-specific function is sufficient for NMJ development. This is in contrast to *spastin* mutants, which can be rescued by presynaptic Spastin expression. However, Atlastin binds to Spastin in GST-pulldown assays, and forms a functional complex[19]. Similarities between the *spastin* and *atlastin* mutant and overexpression phenotypes are summarized in Tables 1 and 2. This work supports the idea that these two disease proteins function together to regulate the microtubule network.

A possible connection between HSP proteins and BMP signaling was revealed by the study of *spichthyin* (*spict*), the fly ortholog of another HSP gene, *Nonimprinted in Prader-Willi/ Angelman* (*NIPA1*). *Drosophila* Spict is widely expressed and colocalizes with an early endosomal marker Rab5. Unlike in the case of *atlastin*, however, loss of *spict* is rescued by neuronal-specific driven expression only, indicating that its function is primarily presynaptic. *spict* mutants have twice as many synaptic boutons as normal (Tables 1, 2) and Spict overexpression produces a loss of boutons reminiscent of the loss of *gbb*, *sax*, and *tkv* suggesting that it may inhibit the BMP pathway. Indeed, levels of pMad were 4-fold higher in *spict* mutants and mutations in *tkv*, *sax*, *wit*, *gbb*, and the co-Smad *medea* suppressed the *spict* overgrowth phenotype[20]. Another group, using a frog oocyte expression system, found that NIPA1 encodes a Magnesium transporter that depending upon the Magnesium concentration localizes either to the endosomes or the cell's plasma membrane[21]. Magnesium is an essential cofactor for Rab5 and Rab7 GTPases[22] necessary for endosomal formation and maturation, suggesting the possibility that an endosomal maturation defect may impair the degradation and processing of BMP receptors in *spict* mutants.

Subsequent mammalian cell culture experiments have confirmed that NIPA1 is an inhibitor of BMP signaling. Although the mechanism is still unclear, in that same report, knockdown of Spastin and Spartin resulted in an almost identical increase in phosphorylated Smad levels as observed for NIPA1, suggesting that a common mechanism may be involved[23]. In *Drosophila*, then, it is possible that when Atlastin and Spastin are reduced, secretion of the postsynaptic BMP ligand is increased, whereas in *spict* mutants, the BMP signal is somewhat constitutive because of an endosomal trafficking defect.

Interestingly, pMad has recently been shown to bind to the promoter of the *trio* gene, which encodes an activator of the *Rac* GTPase, essential for MT bundle formation and bouton growth. In the absence of retrograde BMP signaling, very little Trio is transcribed and little Rac is activated[24], which may underlie the phenotype associated with *spict* presynaptic

loss but does not provide a rationale for the observations associated with the loss of Atlastin post-synaptically.

An important new insight into the BMP pathway has recently been uncovered that might explain how a microtubule-severing protein in the muscle may affect BMP signaling in the neuron. The activin family ligand Dawdle plays an important role at the fly NMJ too, with mutants in this activin and its target Type 1 receptor Baboon having strikingly similar small NMJ phenotypes to mutants in the BMP signaling pathway like *mad*. This activin signaling pathway from the neuron to the muscle was found to lie upstream of the BMP signaling pathway that operates in the opposite direction, thus creating a signaling loop between muscle and neuron (Figure 1)[25]. Smads specifically bind to MT and in response to phosphorylation become dissociated from MT and relocate to the nucleus[26]. Hence, the MT impairment in *atlastin*-mutant muscles may result in higher levels of activin signaling and hence Gbb production, a hypothesis that should be tested. Finally, considering that activins play a neuroprotective role and that activin inhibition results in glutamatergic transmission and synaptic plasticity defects in mice, it would be useful to determine if secretion of BMP ligands by postsynaptic neurons in response to Activins occurs in mammals[27].

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis is a devastating motor neuron disease characterized by late-onset progressive upper and lower motor neuron degeneration. About 90% of the cases are sporadic, but mutations in 7 genes have been identified in familial cases, including *VapB*[14]. Histologic and ultrastructural studies of ALS patients' NMJs have provided strong evidence of frequent denervation and reinnervation of the muscle endplates. In addition, synaptic terminals are on average smaller than normal but they are frequently unusually large [28]. Prior to its identification as an ALS locus, loss of *VapB* in *Drosophila* was found to reduce bouton number as well as increase their average size (Table 2). Overexpression of wild-type *VapB* results in the opposite phenotype: an expanded NMJ with many more boutons. Furthermore, loss of VapB causes a depolymerization of the MT in synaptic boutons, while overexpression results in denser, thickened MT[29]. Subsequent studies confirmed this disorganized microtubule phenotype as well as a 'floating T-Bar' phenotype observed in mutants with impaired BMP signaling[30]. Ratnaparkhi et al. (2008) showed that VapB overexpression results in higher levels of pMad while loss of VapB results in lower levels of pMad compared to controls[31]. How VapB affects BMP signaling is still unclear however. The VapB N-terminal domain is cleaved and secreted by neurons, and binds to Eph receptors as well as other receptors present on muscles (paracrine) and probably neurons (autocrine). The latter pathway may affect the MT network in *VapB* mutants via Rac signaling via an unknown receptor, as Eph loss does not affect NMJ morphology[32,33]. Overexpression of the VapB protein with the mutation found in ALS patients, P58S, also results in aggregates in the Endoplasmic Reticulum with a corresponding upregulated Unfolded Protein Response (UPR)[33]. Interestingly, there is increasing evidence from pathology specimens to support the idea of impaired BMP/TGF-β signaling in ALS, with motor neurons from human Sporadic ALS spinal cords possessing severely reduced nuclear pSmad levels and aggregated pSmad in their cytoplasmic Round Hyaline Inclusions (RHIs)[34]. Enhancing BMP/TGF- β signaling could therefore be a promising therapeutic strategy.

Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is a disease in which the lower motor nerves degenerate, resulting in progressive muscular atrophy[14]. SMA shares some key features with ALS,

although SMA affects lower motor neurons, whereas ALS typically affects both upper and lower motor neurons. SMA Type I is a recessive disease often caused by deletions of *Survival Motor Neuron 1* (*Smn1*). Interestingly, abnormal copy numbers (1 or 3) of *Smn1* have also been found in 12% of patients with sporadic ALS as compared to 4.5% in the wider population[35]. Histologic studies of human fetuses with homozygous deletions of the *Smn1* gene have found the nuclei of motor neurons to be frequently small and unusually shaped [36]. In addition, like in ALS, observations from studies of SMA mutant mice have revealed a large accumulation of neurofilaments in the presynaptic terminals. The *Smn1* gene encodes a protein that localizes to both the nucleus and the cytoplasm. It is proposed to play a role in pre-mRNA splicing, but whether or not this is relevant to the NMJ defects found in SMA patients is still unclear. Smn in *Drosophila* localizes to the nucleus, but it is also found to colocalize with Discs large (Dlg) at the NMJ in the muscle, indicating that it may have a non-nuclear function too. Loss of function mutations result in a severe reduction in the number of synaptic boutons, a phenotype enhanced by loss of BMP signaling components like Mad (Table 2). Levels of pMad in *Smn* mutants were also observed to be reduced, providing further evidence that the Smn protein may play a critically important and previously unrecognized role in the BMP signaling pathway[37]. Intriguingly, the same P58S mutation in the previously mentioned VapB, besides ALS, has also been documented to cause SMA, and since VapB affects BMP signaling as well, reduced BMP signaling may be a common theme. It is possible therefore that impaired activation and trafficking of BMP pathway molecules like Smad may be a common theme in these diseases [38].

Multiple Sclerosis

Multiple Sclerosis is a neuroinflammatory disorder in which patients develop demyelinated plaques in their CNS with corresponding neurological deficits. At least five independent genetic studies have linked polymorphisms in the *Clec16A* gene to the disease[39]. In a screen designed to identify new genes that regulate synaptic terminal growth, fly mutants of the homolog of Clec16A, *endosomal maturation defective* (*ema*), were observed to possess dramatically overgrown synapses (Table 2)[40]. This is similar to the mutant phenotype seen in a putative lysosomal sugar carrier *spinster*[41]. *ema* mutant neurons possess enlarged immature endosomal compartments, with the BMP receptor Tkv present at levels almost double those of wild-type, and a more than 4-fold increase in the levels of pMad[40]. In *ema* mutants, the endolysosomal pathway is unable to efficiently degrade Tkv and as anticipated, the overgrowth phenotype is suppressed by mutations in Mad.

The fly neuron must limit how much BMP signaling occurs under normal conditions, as excessive signaling results in overgrown NMJs, as in *ema* mutants. Hence, loss of numerous endocytic genes frequently cause characteristic satellite bouton phenotypes[4,16]. In light of ALS and MS's potential connections with impaired BMP signaling, it is interesting that children of patients with ALS are almost three times as likely to develop MS than the average person[42]. It is interesting too that very high levels of BMPs 4 and 5 have been observed in MS patient lesions[43] as well as elevated BMPs 4, 6, and 7 levels in a mouse model of the disease[44]. Finally, considering the phenotypic similarities of endosomal maturation defects and increased BMP signaling between *ema* and *spinster* mutants[45], it is worth noting that 16p11 and 17p13, the loci containing human *Spinster 1* and *2* respectively, have both shown linkage in patients with Multiple Sclerosis[46,47].

Huntington's Disease

Huntington's Disease is a late-onset progressive disorder characterized by increasingly jerky and uncoordinated movements, rigidity, and neuropsychiatric symptoms[48]. Gradual degeneration of the basal ganglia is a key feature of the disease, which is caused by

polyglutamine expansion of the protein Huntingtin. One of the early primary dysfunctions found in Huntington's Disease is excessive glutamatergic neurotransmission, while later in the disease course, too little glutamatergic transmission becomes evident[49]. The disease is also known to be associated with axonal transport defects, and this has been proposed to be related to the sequestration of Huntingtin-interacting proteins in aggregates[48]. One such Huntingtin-interacting protein is the Arp2/3-interacting F-Bar protein CDC42-interacting protein 4 (CIP4). Patients with HD progressively accumulate high levels of CIP4 in their basal ganglia, and overexpression of CIP4 was shown to be highly neurotoxic[50]. Interestingly, *Drosophila CIP4* was identified in a forward genetic screen, with mutant larvae having an overgrown NMJ (Table 2). It was further discovered that CIP4 acts to downregulate the BMP signaling pathway, but, unusually, it downregulates the pathway specifically in the muscle. *CIP4* mutants secrete excessive Gbb into the extracellular space, thus activating the pathway[51]. Hence, it is possible that excessive CIP4, as found in the Huntington's Disease-afflicted brains, causes a downregulation of BMP signaling. Indeed, low blood and neuronal levels of TGF-β1 were recently reported as the earliest known biomarker in asymptomatic Huntington's Disease patients[52].

Concluding Remarks

In summary, studies at the fly NMJ are rapidly providing a better understanding of the potential pathological mechanisms of fly homologues of human neurodegenerative diseases. The BMP signaling pathway may be playing a central role in many of these diseases. This is important because the BMP signaling pathway is highly conserved in mammals and has been well studied in relation to bone development, angiogenesis and stem cell proliferation. However, there are surprisingly few studies of its role at the vertebrate NMJ so far. Most studies have so far focused primarily on the pathway's role in eye development, neurogenesis and gliogenesis and not on NMJ synapse formation and growth *per say*. Interestingly, a recent *Xenopus* study has drawn a strong parallel. The vertebrate neuromuscular junction is known to require continuous input from terminal Schwann cells to grow in size. In their absence, nerve terminals do not grow and instead retract, perhaps very similar to the constant cycles of motor nerve terminal retraction observed prior to massive neuronal death in ALS and SMA[53]. Schwann cell-conditioned medium (SCTM) allows for this growth to occur. Until recently it was unknown what factor or factors present in this medium were responsible. Based on the work done on the *Drosophila* NMJ, though, the close BMP relative TGF-β1 was proposed to be a key factor and it was subsequently detected in the medium and shown to be necessary and sufficient to mediate NMJ terminal growth[54]. This suggests that similar signaling pathways operate at the NMJ in vertebrates and may possibly be affecting numerous other synapses as well. In addition, using genetic interactions and drug studies, like the phenotypic suppression by the microtubule-destabilizing drug vinblastine in fly *atlastin* mutants, may allow us to efficiently identify means of slowing the progression of some of these diseases[19]. Hence, *Drosophila* studies of the NMJ have provided and will continue to provide new and intriguing insights into potential mechanisms for neurodegenerative disease mechanisms.

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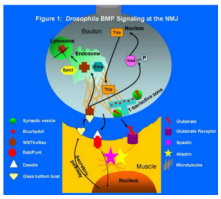

Figure 1.

Table 1

Phenotypic Comparisons between Spastin, Atlastin, and Spichthyin

Null allele	Bouton clustering	Satellite boutons	Bouton number	Temperature-sensitivity	Microtubules	Escaper Sterility	Escaper movement
Atlastin	more clustered	Yes	increased by 17%	Yes	dense network	yes	Highly impaired
Spastin	more clustered	Yes	increased by 60%	Yes	dense network	yes	Highly impaired
Spichthyin	more clustered	No*	increased by 100%	Not tested*	dense network	fertile	Normal*
Overexpression	**Muscle attachment**	**Acetylated Tubulin**	**Microtubules**				
Atlastin	partial/totally detached	significantly less	faint and sparse				
Spastin	partial/totally detached	significantly less	faint and sparse				
Spichthyin	Not discussed	significantly less	faint and sparse				

*Personal communication (Cahir O'Kane, 9 July 2010)

Table 2

Selected diseases discussed	**OMIM #**	***Drosophila* genes discussed**	**Loss of function NMJ phenotypes**
Hereditary Spastic Paraplegia 3	**#182600**	Atlastin	more boutons, bouton clustering, satellite boutons, dense MT network, impaired movement
Hereditary Spastic Paraplegia 4	**#182601**	Spastin	more boutons, bouton clustering, satellite boutons, dense MT network, impaired movement
Hereditary Spastic Paraplegia 6	**#600363**	Spichthyin	more boutons, bouton clustering, dense MT network
Multiple Sclerosis	**#126200**	Ema	more boutons, increased synaptic area, enlarged endosomes
Huntington's Disease	**#143100**	CIP4	more boutons, satellite boutons, increased synaptic area
Amyotrophic Lateral Sclerosis 8	**#608627**	Vap-33-1	fewer and larger boutons, severely disorganized and fragmented MT network
Spinal Muscular Atrophy I, II, III	**#253300, #253550, #253400**	Smn	fewer and larger boutons