Emergence of the Wallerian degeneration pathway as a mechanism of secondary brain injury

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Augustus Volney Waller was a renowned British neurophysiologist who birthed the axon degeneration field in 1850 by describing curdling and fragmentation of the glossopharyngeal and hypoglossal cranial nerves of a frog following a transection injury. The degeneration of axons after a transection injury is now known as Wallerian degeneration (WD). Waller's work was expanded by Santiago Ramón y Cajal who described in detail the morphological stages of WD from monitory fragmentation of the axon and the granular disintegration of the neurofibrils to the final resorption of the axon. Interest in this field burgeoned in the early 1990's with the fortuitous discovery of a mutant mouse, known as the Wallerian degeneration Slow (Wld^S) mouse. Although overtly normal, its remarkable phenotype was discovered when the animals were subjected to a physical nerve injury and a profoundly slowed rate of axonal degeneration was revealed. This slow axon breakdown is intrinsic to neurons, present in central and peripheral nervous system axons, and associated with structural preservation and retention of the ability to conduct axon potentials for up to 2 weeks (a 10-fold delay).

The molecular mechanisms of WD began to be elucidated as the responsible gene (Wld^S) was mapped to mouse chromosome 4 using conventional linkage analysis. The Wld^S mutation was later revealed to involve a genomic rearrangement of two endogenous genes with splicing of resulting mRNAs to create an in-frame fusion protein. The C-terminal comprises the complete protein sequence of nicotinamide mononucleotide adenylyl transferase 1 (NMNAT1), one of three NMNAT proteins, each with a specific subcellular localisation. The NMNAT proteins are a central component of the mammalian nicotinamide adenine dinucleotide (NAD) biosynthetic pathway. Studies on Wld^S revealed that the axonal isoform, NMNAT2, is an essential axon survival protein that is produced in the cell soma and continually shipped into the axon. Due to its rapid turnover rate, a transection injury, or other interruption of transport quickly deprives the distal axon of NMNAT2, causing axon degeneration. NMNAT2 is a labile protein and falling levels in the axon correlates with axon degeneration after a stereotypic latent period. Wld^S is capable of substituting for NMNAT2 in the axon and due to its longer half-life can preserve axonal integrity for a longer period (Coleman and Höke, 2020).

In 2012, a seminal paper was published that demonstrated the versatile power of *Drosophila melanogaster* and applied it to the axon degeneration field. A forward genetic screen of *Drosophila*, combined with rapid direct visualization of axon morphology, was used to identify novel genetic mutations that manifest with delayed WD. In contrast to the overexpression mutant Wld^S this screen identified loss-of-function mutations in the *Drosophila* gene sterile α/Armadillo/Toll-Interleukin receptor homology domain protein (dSarm). dSarm mutants have a remarkably robust axon protective phenotype that endured for the lifespan of the flies. Similarly, a murine knockout of the ortholog *Sarm1* (sterile α and Toll/Interleukin 1 receptor motif containing protein 1) demonstrated robust protection against axotomy induced degeneration in cortical neurons and dorsal root ganglion neurons. *In vivo*, *Sarm1* deletion preserved the distal sciatic nerve morphology, synaptic structure and motor end plate innervation for several days after transection (Osterloh et al., 2012). Generation of a homozygous lethal NMNAT2 gene-trapped loss of function mouse facilitated further experiments to understand the mechanism of WD. These mice had stalled axonal outgrowth that could be rescued in a dose-dependent fashion by expression of Wld^S, consolidating the theory that Wld^S delays axon degeneration by acting as more stable enzymatic substitute for NMNAT2 in the axon compartment. Likewise, the severe truncation of axons seen with the NMNAT2 gene-trap was robustly rescued by crossing with $Sarm1^{-/-}$. In a pivotal experiment, SARM1 deficiency completely rescued lethality and restored NMNAT2 null mice to phenotypic normality (Gilley et al., 2017). These findings placed SARM1 downstream of NMNAT2 depletion as an executor of WD and formed the core of a proposed mechanistic explanation of WD. Recent studies revealed that SARM1 possesses enzymatic NAD cleavage activity which is key for its pro-degenerative function (Essuman et al., 2017), making it an attractive target for drug development programs. SARM1 NAD cleavage activity is further induced following axotomy and NMNAT2 depletion. Several studies suggest that a toxic rise in NMNAT2 substrate NMN as a consequence of NMNAT2 loss is important to regulate and increase SARM1 activity. Downstream of SARM1 are final common execution events such as raised intra-axonal calcium and calpain activation. These terminally dismantle the axon before it is phagocytosed by immune cells. The fine details of the WD process are still being elucidated and it is likely that beyond the core pathway there will be various parallel streams of input and modulating processes. At present known modulatory factors include MAPK, SKP1A, FBXO45, SCG10, Pebbled and the ZNRF1-AKT-GSK3B-CRMP2 pathways. Loss-of-function mutations in the *highwire* gene in *Drosophila* and the mammalian ortholog MYCBP2(PHR-1) is another such factor. The *highwire* gene encodes a large protein with E3 ubiquitin ligase activity that modulates levels of dNMNAT and it is associated with a strong delay in axon degeneration both *in vitro* and *in vivo*. Although the vista of WD has broadened significantly in the past 30 years, the story is by no means complete, and further discoveries will be required to fully describe this evolutionarily conserved death pathway. A simplified linear WD pathway is shown in **Figure 1**.

Our understanding of WD continues to advance at pace. The striking protection achieved with Sarm1 deletion (lifelong rescue in certain circumstances) has encouraged pharmaceutical companies to target the WD pathway, due to its possible role in neurological disorders affecting both the peripheral and central nervous system (Coleman and Höke, 2020). Despite some differences between WD in the peripheral nervous system and central nervous system, namely in the inflammatory response to injury and the time required to clear myelin debris by different glial cells, the axon death pathway discussed above controls degeneration of both peripheral nervous system and central nervous system axons. One of these diseases is traumatic brain injury (TBI) (Hill et al., 2016). An estimated 10 million people per year suffer a TBI; it is one of the leading causes of death in many parts of the world and can have profound individual and socioeconomic consequences. TBI differs from many traditional diseases in so far as it is not primarily driven by genetic mutation or an aberration of normal physiology. Instead it is an acquired insult caused by an external force at a single moment in time. What follows can be understood in terms of subsequent biological consequences of that initial act – known as secondary brain injury. While the cause of the primary neurological injury is usually obvious, the multitude of processes driving secondary injury are concealed at the cellular and subcellular levels (Hill et al., 2016).

In 1956, Sabina Strich described a dementia-like phenotype with histological evidence of white matter degeneration in 5 patients following severe head injury. Experimental evidence of diffuse axonal pathology in large-animals subjected to trauma was reported in the 1980s and is supported by recent evidence of progressive white matter degeneration, years following a single traumatic brain injury in humans. The relationship of these findings to WD has not been validated with certainty. A timeline showing the relative discoveries in WD and TBI science is presented in **Additional Figure 1**.

Not all axonal injury and death is via the WD pathway and acknowledgement of this fact is important if we are to avoid inadvertently ascribing effects to WD when it is actually due to modulation of distinct secondary injury mechanism. Primary traumatic axotomy occurs when an injury leads to direct axon transection. Even in severe TBI this is thought to affect relatively small numbers of axons and so this is unlikely to be a major player in patient outcomes following injury. A common criticism of WD research is that if axon-soma disconnection has occurred (as in transection) then what would be the functional benefit of delaying, or even permanently stopping, as the axon is no longer an intact functional neurological unit. This is a valid statement and it seems logical that those transected neurons are beyond salvage. However, whether temporarily delaying the physical fragmentation of axons, and the associated signaling and glial events that may accompany it, has any beneficial or harmful effect on the long-term health of neighbouring axons is not known. Perhaps more important than transection injury is the case of sub-transection injuries. There is evidence that these partial injuries, including axonal stretch due to rapid acceleration-deceleration forces, result in microtubular fractures that impairs axonal transport and induce WD (Tang-Schomer et al., 2012). However, unlike a transection injury, this might be recoverable if initiation of the WD pathway were delayed as this is a transient process. Hence, there is a mechanistic link between high impact trauma and WD, and a therapeutic rational for blocking it. Additional direct evidence for WD as a secondary brain injury mechanism remains limited. Radiological descriptions of WD in diffuse axonal injury exist across various imaging modalities but robust correlation of advanced technologies such as diffusion-weighted injury and tractography with postmortem tissue are lacking. Therefore,

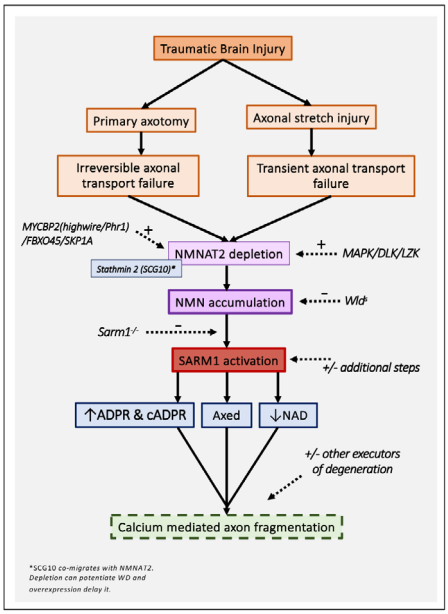

Figure 1 | 1 Molecular control pathway of Wallerian degeneration and their potential relationship to traumatic brain injury.
Outline of a linear Wallerian degeneration pathway based on current experimental evidence, and potential ways that traumatic brain injury may act as an initiating event. Brain injury can result in primary axotomy or an axonal stretch injury that leads to secondary axotomy following axonal transport failure. The core steps in Wallerian degeneration include NMNAT2 depletion, NMN accumulation, prodegenerative SARM1 activation, and finally calcium mediated axon dismantling (Hill et al., 2016). These steps may be modifiable by various ancillary pathways and inputs. It is probable that this picture is incomplete and additional steps will be added in the future. In a non-transected axon, interruption of Wallerian degeneration at any point before terminal initiation of axon fragmentation may have the potential to allow axon preservation. Abbreviations: Axunded (Axed), ADP ribose (ADPR), Cyclic ADP ribose (cADPR), Dual leucine zipper kinase (DLK), F box protein 45 (FBXO45), Leucine zipper bearing kinase (LZK), Mitogen activated protein kinases (MAPK), Nicotinamide adenine dinucleotide (NAD), Nicotinamide mononucleotide (NMN), MYC- binding protein 2 (MYCBP2), Nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2), Superior cervical ganglion 10 (SCG10), S phase kinase associated protein 1A (SKP1A), Sterile- α and Toll/interleukin 1 receptor (TIR) motif containing protein 1 (SARM1).

it is not possible to be certain that reported neuroradiological markers of axon degeneration truly represent WD as opposed to non-Wallerian mediated cell death and axon degeneration. An alternative source of evidence for WD occurring in TBI is sometimes cited based on the examination of biomarkers. Biomarkers are surrogate markers of disease presence and progression/severity. They are often cytoskeletal proteins or other molecules released during neuronal or glial breakdown. An effective biomarker of axonal injury provides a diagnostic tool, a quantitative measure of injury severity, and an indicator to gauge treatment efficacy. There is active research ongoing into biomarkers of axonal injury and generic markers of axonal injury such as neurofilament light chain that can provide an indication of axonal injury. Currently there are no clinically used biomarkers that can differentiate WD from non-WD neuronal death, however a recent study suggest that SARM1 product cADPR might be used as a sensitive biomarker of SARM1 activation (Sasaki et al., 2020).

Perhaps the most convincing evidence for WD in TBI comes from several *in vivo* animal studies. The first concerns a Wld^S expressing mouse that was exposed to a single weight-drop cortical-contusion injury. It was found to have less motor and cognitive impairment than uninjured control animals (Fox and Faden, 1998). Wld^S conferred protection also against blast mediated TBI. Other examples include a closed cortical-injury in a *Sarm1*$^{-/-}$ mouse which demonstrated reduced neuronal loss and cognitive impairment following injury, and an impact acceleration model of Thy1-eYFP-H/*Sarm1*$^{-/-}$ mice that found a reduction in the number of axonal lesions early after injury (Henninger et al., 2016; Ziogas and Koliatsos, 2018). *Sarm1* deletion also reduces axon damage and improves functional outcomes in in models of mild TBI (Marion et al, 2019; Maynard et al., 2020). The modelling of TBI in *Drosophila* is a recent development. We used a *Drosophila* model of trauma to investigate if a loss-of-function model of the *highwire* gene would affect outcomes from TBI and found that it protected the fly against trauma induced premature death and behavioral deficits. We also demonstrated injury induced loss of a subset of dopaminergic neurons related to locomotion that was robustly rescued by *highwire* deficiency (Hill et al., 2020). These neurons degenerate in genetic PINK1^{B9}/*Parkin* loss of function models of Parkinson's disease but can be directly rescued by *highwire* deletion (Loreto et al., 2020). This is intriguing given that Parkinsonism is a well-recognized sequalae of TBI and WD is emerging as a potential therapeutic target in this disease.

The ultimate proof of WD's involvement in human TBI necessitates examination in the human condition. Modelling disease using human organotypic explants, organoids, or human induced pluripotent stem cell lines are an option for *in vitro* experimentation. The expression of NMNAT2 and SARM1 levels varies between individuals – sometimes pathologically so – but the effect of this on outcomes following TBI and other neurodegenerative diseases remains unexplored in humans. Targeted pharmacological blocking of WD offers an opportunity to directly assess the effect of WD modulation on outcomes, and interest in developing safe, brain-penetrant versions of Wallerian degeneration modulating drugs is high. While the role of WD as a significant secondary brain injury mechanism in trauma has yet to be established beyond doubt, preclinical models are suggestive that this may represent an exciting modifiable target that has the potential to improve outcomes from TBI.

We apologize for omissions in citing relevant publications due to the reference limit.

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Additional files:
Additional file 1: *Open peer review report 1.*
Additional Figure 1: *Landmark discoveries in Wallerian degeneration and traumatic axonal injury of the brain research.*

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