Review

Starch-like polyglucosan formation in neuronal dendrites in the Lafora form of human epilepsy: a theory of pathogenesis

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Abstract: Lafora disease is a teenage-onset fatal form of progressive myoclonus epilepsy. In this disease, starch-like polyglucosans are formed in neurons, specifically in the neuronal soma and dendrites. Laforin, the protein product of the disease gene, possesses a CBM20 type of starch-binding domain with which it preferentially binds starch over glycogen. It also contains a dual-specificity phosphatase domain with yet unknown phosphoprotein substrate(s). Since polyglucosans are abnormal in human cells and the probable cause of epilepsy in this disease, we theorize that laforin is designed to detect their appearance and initiate mechanisms to prevent their further formation or to promote their disposal. In this article, we review glycogen metabolism pertinent to neurons and the known pathology and biochemistry of Lafora disease and related rare human conditions with polyglucosan formation. We discuss the likeliest most direct pathways through which laforin may inhibit polyglucosan synthesis. We also discern the probable existence of a carbohydrate shuttle system to clear polyglucosans from dendrites. Finally, we detect that the phosphokinase glycogen synthase kinase 3 (GSK3) is a common thread in the pathways invoked to explain the role of laforin. We propose that GSK3 is likely an important participant in the control of polyglucosan formation and accumulation in human brain.

Key words: Lafora, epilepsy, polyglucosan, glycogen, synthase, EPM2A, EPM2B.

Abbreviations: AM, acid maltase; AMPK, AMP-activated protein kinase; BE, branching enzyme; CA, corpora amylacea; CBM, carbohydrate-binding module; CK1, casein kinase 1; CK2, casein kinase 2; DE, debranching enzyme; ER, endoplasmic reticulum; GP, glycogen phosphorylase; GS, glycogen synthase; GSK3, glycogen synthase kinase 3; G6P, glucose 6-phosphate; G6Pase, glucose 6-phosphatase; LB, Lafora bodies; LD, Lafora disease; PKA, protein kinase A; PP1, protein phosphatase 1; TIRE, thymine-rich insulin response element.

Lafora disease

Lafora disease (LD) is a catastrophic autosomal recessive epilepsy. Seizures start in teenage years, in previously normal adolescents, and rapidly become intractable. Dementia follows within two years, at which time the patient is having very frequent myoclonus (jerk-like seizures). Myoclonus, other seizures and dementia progress inexorably until death within 10 years of first symptoms (Lafora & Gluck, 1911; Minassian, 2002). LD is caused by mutations in the EPM2A (Minassian et al., 1998) or EPM2B (Chan et al., 2003) genes encoding the laforin dual-specificity phosphatase and the malin putative ubiquitin E3 ligase, respectively. There is also evidence for a third yet unidentified locus for LD (Chan et al., 2004b). Beyond its bioinformatically predicted function, little is known about malin. Progress with the earlier identified laforin on the other hand has been substantial and is reviewed as part of this article.

Neuronal dendrites are occupied by Lafora bodies in Lafora disease

Brains of LD patients contain numerous Lafora bodies (LB) of different sizes. The largest ones have a distinctive bilayered spherical appearance and can occupy the entire neuronal cell body, or outgrow and destroy the neuron and float freely in the neuropil (Fig. 1A) (Lafora & Gluck, 1911; Minassian, 2002). A closer look with electron microscopy reveals that the brain is replete with multitudes of smaller LB present in two specific locations, adjacent to neuronal nuclei, and in largest numbers

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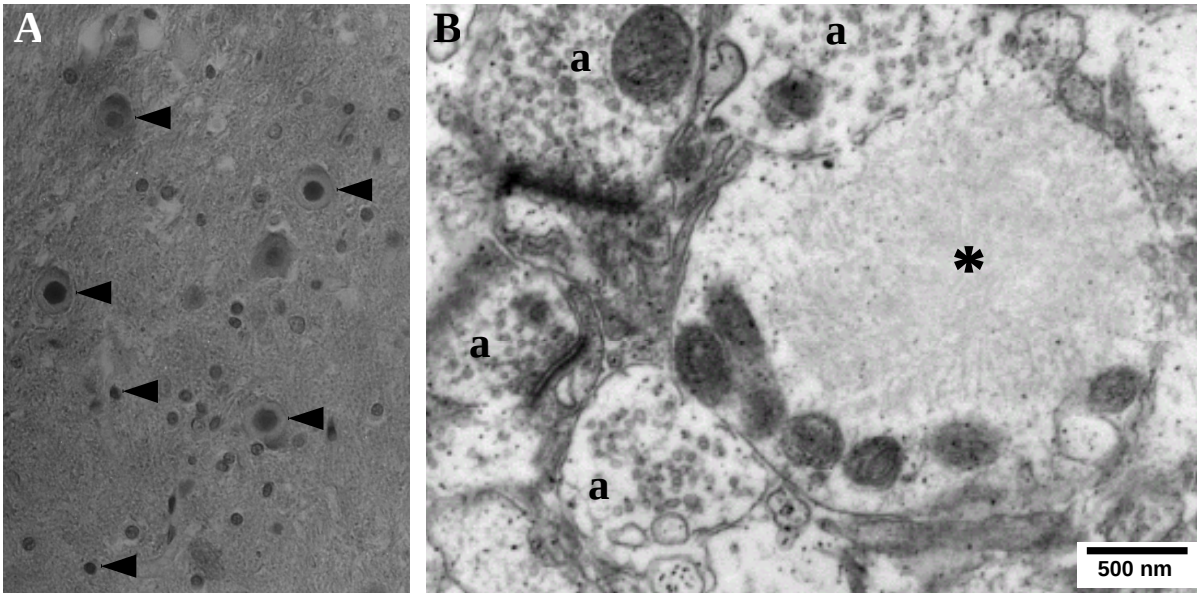

Fig. 1. Lafora bodies (LB) in patient brain biopsies. (**A**) LB of different sizes (arrowheads); original magnification 500×. (**B**) LB in a dendrite (asterisk); note fibrillar nature. Surrounding axons (a), recognizable by their synaptic vesicles do not contain any abnormal storage.

within dendrites (Fig. 1B). Importantly, LB are not present in neuronal axons or in neuroglia (Cavanagh, 1999).

Lafora bodies are masses of starch-like polyglucosans

LB are compact masses of a fibrillar material (Fig. 1B), the chemical composition of which has been resolved and shown to be polyglucosans (polymers of glucose) (Sakai et al., 1970). Whereas glycogen, the glucose storage reservoir molecule in animals, has a strictly regular branching pattern, rendering it globular in shape and allowing it to suspend in the cytoplasm, polyglucosans lack regular (or any) branching and thus are more akin to plant starches. They accordingly precipitate and accumulate into LB as they appear in LD. It is not known whether polyglucosans pathologically form in LD or whether they are a physiologic occurrence in neurons, the normal disposal of which is disturbed in LD. This article contends the latter.

Neurons form glycogen, but lack the usual enzymes to break it down then

Glycogen synthase (GS), the main enzyme of glycogen synthesis, adds glucose units (derived from glucose 6-phosphate (G6P), i.e. the cellular form of glucose) to a six to seven-residue oligosaccharide pre-attached to the glycogenin protein (Roach, 2002). Elongation of the saccharide by GS is linear and if unchecked results in polyglucosan formation (Raben et al., 2001). As GS elongates the strand, glycogen branching enzyme (BE) removes a six to seven-residue oligomer from the end of the extending chain, reattaches it inside the chain creating a second end for GS to extend. The process resumes and continues at each end, and the glycogen molecule expands in globular fashion (Roach, 2002). Neurons possess GS (Kaslow & Lesikar, 1984; Inoue et al., 1988) and BE (Robitaille et al., 1980; Lossos et al., 1998) and do form glycogen (Brown, 2004).

In cells other than neurons including neuroglia, when glucose is required, glycogen phosphorylase (GP) removes glucose units sequentially from all ends of the glycogen particle. When it reaches four residues from a branch point, debranching enzyme (DE) removes three of the four glucoses, places them at a longer end for GP to work on, and digests the branch bond releasing the final glucose of that strand (Roach, 2002). A great enigma in neurobiology is the absence of GP in neurons (Ignacio et al., 1990; Pfeiffer-Guglielmi et al., 2003), which means that neurons make glycogen, but cannot digest it, at least not in the fashion of other cell types. Furthermore, neuronal glycogenosis with GP deficiency has not been reported. Consistent with the absence of GP in neurons is the absence of any report of neuronal DE or neuronal phenotype in DE deficiency.

If neurons indeed do not possess a GP, the only known way in which glycogen could be digested is in the lysosome, with acid maltase (AM). This enzyme is present in rodent neurons (Ponce et al., 1999) and human neurons, as evidenced by neuronal glycogenosis in AM deficiency disease. Although in extraneuronal cells the contribution of AM to glycogen digestion is inconsequential (Konishi et al., 1990; Hesselink et al., 2003) – e.g., only 3% of hepatic glycogen is digested by AM (Konishi et al., 1990) – its presence in greater amounts in neurons than in glia (Ponce et al., 1999), despite the far greater amounts of glycogen in glia, supports the notion that AM may be the neuronal glycogen catabolizing enzyme.

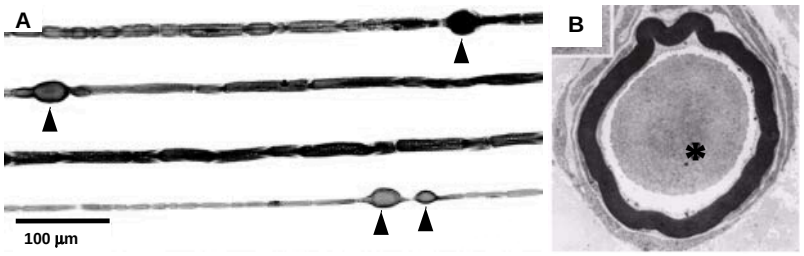

Fig. 2. LB in patients with BE deficiency. (**A**) Axonal enlargements containing LB (arrowheads) in teased-fiber preparations from sural nerve biopsies (from Klein et al. (2004); with permission). (**B**) LB (asterisk) in a myelinated axon (from Ziemssen et al. (2000); with permission); original magnification 9200×.

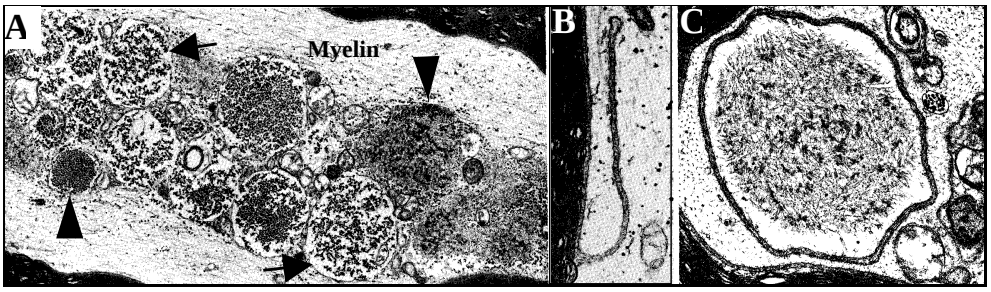

Fig. 3. Sciatic nerves from 24 month-old alloxan chronically diabetic rats. (**A**) The axoplasm is packed with membrane bounded glycogen masses (arrows) and LB (arrowheads); original magnification 8000X. (**B**) Invaginating Schwann cell cytoplasmic sheet in a myelinated axon; original magnification 40,000×. (**C**) A LB segregated from other axonal contents after sequestration by invaginating Schwann cell cytoplasm; original magnification 29,000× (from Powell et al., (1979); with permission).

Evidence for the existence of a polyglucosan shuttling system out of dendrites, towards axons

The distribution of the glycogen synthesizing enzymes GS and BE within neurons has not been studied, but it is almost certain that dendrites contain GS without which polyglucosans could not be made in dendrites in LD. What about BE? Is it present in dendrites or are polyglucosans first moved into the soma where they are then branched? In BE deficiency disease, loss of the normal balance between GS and BE leads to polyglucosan formation and appearance of vast numbers of LB, rivaling those seen in LD. However, unlike in LD, the LB in this condition are invariably located within axons (Fig. 2), and not in dendrites (Robitaille et al., 1980; Lossos et al., 1998). If branching of glycogen does normally take place in dendrites, then the absence of polyglucosans in dendrites in BE deficiency disease indicates that there exists a mechanism to move polyglucosans out of dendrites. Therefore, whether or not BE exists in dendrites, neurons must possess a mechanism for moving polyglucosans out of dendrites.

Parenthetically, patients with BE deficiency have a slow dementia and upper and lower motor neurological signs, likely due to the LB obstructing axons, but they do not have epilepsy (Robitaille et al., 1980; Bruno et al., 1993; Lossos et al., 1998; Klein et al., 2004). This suggests that disturbance of dendritic function by LB underlies the epilepsy in LD (Minassian, 2002).

Is there additional evidence for the presence of a neuronal polysaccharide flux mechanism in the direction of axons? In rats made chronically diabetic with alloxan, masses of glycogen and polyglucosans accumulate with age within neurons (Powell et al., 1979), presumably due to greatly excessive neuronal glucose uptake. These get jammed within axons (Fig. 3A) and have not been reported to be present in dendrites.

Further support comes from the distribution of corpora amylacea (CA) in neurons. CA are polyglucosan masses essentially indistinguishable from LB that normally form in mammalian brain with advanced aging (Cavanagh, 1999). They likely are low-quantity byproducts of failures in glycogen branching that precipitate and with time accumulate. Because astrocytes are the principal glycogen storage cells (Brown, 2004), CA form in greatest amounts in these glia. However, with advanced aging, CA also appear in neurons, where they compartmentalize, again, always within axons (Cavanagh, 1999).

The above observations allow a glimpse into an axoplasmic polysaccharide flux, but what is the fate of polyglucosans or glycogen after they enter axons? They could be digested by lysosomes. Additionally, they could be transferred into astrocytic cytoplasm by astrocytic processes entering into neurons(Cavanagh, 1999). In alloxan diabetic rats it has been possible to observe that the large axonal polyglucosan/glycogen masses are cleared by invaginating Schwann cell cytoplasmic extensions (Fig. 3B,C) (Powell et al., 1979). It is possible that this also happens physiologically, and that uptake of polyglucosans or glycogen from axons by Schwann cells and oligodendroglia serves as one carbohydrate supply route into these cells for the saccharide component of the large amounts of myelin they need to continuously produce and maintain. As an interesting aside in regards to CA, it appears that these bodies transfer from astrocyte to astrocyte with time to finally

deposit, in advanced aging, in huge masses in the subpial feltwork under the meningeal covering of the brain (Cavanagh, 1999).

Dendrites can sequester G6P in the endoplasmic reticulum out of reach of GS

Glucose 6-phosphatase (G6Pase) is a major hepatic enzyme that supplies the bulk of systemic glucose. It is an endoplasmic reticulum (ER) complex, which translocates G6P into the ER lumen and then dephosphorylates it to glucose. Secreted into the blood stream, glucose is taken up by all cell types through glucose transporters and converted by hexokinases back to G6P for glycolysis and energy generation. In times of plenty, G6P is stored into glycogen (Roach, 2002).

G6Pase is present in some extrahepatic cell types. In brain, it is found in neurons (Van Schaftingen & Gerin, 2002), where it is compartmentalized in the cell body and dendritic ER, and is not present in axonal ER (Broadwell & Cataldo, 1983; 1984). Dendrites are therefore able to divert G6P into the ER, away from GS. This would not only reduce GS substrate, but would drastically decrease GS activity, because G6P is an extremely potent allosteric activator of GS (Hays et al., 1981; Roach, 2002; Brown, 2004). G6Pase is not present in astrocytes (Brown, 2004), even though these glia are the main glycogen storage cells in the brain. Astrocytes support neuronal energy needs in times of glucose shortage by generating lactate from their glycogen stores through glycogenolysis and glycolysis and providing it to neurons (Pierre et al., 2002; Brown, 2004; Kasischke et al., 2004).

Laforin localizes in dendrites and detects and controls polyglucosans

In 1998, we identified the *EPM2A* gene and showed that mutations of this gene cause LD (Minassian et al., 1998). Laforin, the *EPM2A* gene product, contains two main domains. In the C-terminus is a dual-specificity phosphatase domain (Minassian et al., 1998; 2001; Ganesh et al., 2000), the specific phosphoprotein substrate of which remains unknown. In the N-terminus, there is a carbohydrate-binding module (CBM), which belongs not to the family of glycogen binding proteins, but into the CBM20 family of starch-binding prokaryotic and early eukaryotic enzymes (Minassian et al., 2000; Janecek, 2002a,b; Coutinho et al., 2003) (http://afmb.cnrs-mrs.fr/CAZY/). We and others have recently confirmed experimentally that laforin does indeed preferentially bind starch versus glycogen (Wang et al., 2002; Chan et al., 2004a; Ganesh et al., 2004; Wang & Roach, 2004). In the same work we showed that *in vivo* laforin localizes in neuronal dendrites (and the soma), but not axons (and not neuroglia), and that it strongly binds LB (Chan et al., 2004a).

The observation that laforin preferentially binds polyglucosans affords crucial insight into the role of the laforin pathway. It indicates that laforin's function is linked to the presence of polyglucosans, i.e. its role starts after the initial appearance of polyglucosans. Since accumulation of polyglucosans is obviously neurotoxic, then the role of the laforin pathway is very likely in the detection of polyglucosans and the initiation of measures to counter their formation or accumulation.

Fig. 4. Phosphorylation sites on rabbit muscle GS (GYS1), and their kinases. Note: brain GS is also encoded by GYS1, but has not been separately studied in nearly the same detail in rabbit or human. Liver GS is encoded by GYS2 and is similarly phosphoregulated with some differences. Modified from Roach (2002); with permission.

Interestingly, the human proteome contains two additional CBM20 family proteins, genethonin-1 (Janecek, 2002b) and FLJ11085 (Coutinho et al., 2003) (http://afmb.cnrs-mrs.fr/CAZY/). Their roles in LD remain unexplored.

Potential pathways of laforin-mediated polyglucosan control

Laforin could control polyglucosan amounts in dendrites by inhibiting their production and/or activating mechanisms for their digestion or their removal out of dendrites. To arrest polyglucosan production, laforin could inhibit GS, remove G6P, and/or activate BE.

GS inhibition

GS is phosphoregulated at nine sites (Fig. 4) by multiple kinases and to date one phosphatase (Roach, 2002). Generally, dephosphorylation activates GS, and hence laforin itself is not expected to act directly on GS.

Glycogen synthase kinase 3 (GSK3) is the principal GS kinase. It downregulates GS activity by phosphorylating four sites (3a, 3b, 3c and 4) in the C-terminus of the enzyme (Roach, 2002) (Fig. 4). GSK3 is activated by phosphorylation in its own C-terminus, and inactivated by phosphorylation in its N-terminus (Hughes et al., 1993; Cross et al., 1995). To date, no phosphatase for the GSK3 N-terminus site is known. Laforin could be a phosphatase for this site and activate GSK3, which would in turn phosphorylate and inhibit GS (Fig. 4).

The activity of GSK3 on GS is dependent on an initial phosphorylation of a fifth C-terminal site on GS (site 5) by casein kinase 2 (CK2) (Roach, 2002). CK2 activity is not affected by phosphorylation (Litchfield, 2003), and hence a laforin effect on GS via CK2 appears unlikely.

AMP-activated protein kinase (AMPK) is allosterically activated by AMP (Hardie, 2003; Woj-

taszewski et al., 2003) and NAD (Rafaeloff-Phail et al., 2004) and inhibited by ATP and NADH, thus sensing the cell's energy status (Hardie, 2003). It is a heterotrimeric ($\alpha\beta\gamma$) complex of catalytic (α), glycogen binding (β), and AMP interaction (γ) subunits, with alternate isoforms existing for each subunit (there are two α, two β and three γ genes in mammals) (Hardie, 2003; Carling, 2004). The AMPK catalytic subunit (α) phosphorylates site 2 on GS and inactivates the enzyme (Fig. 4) (Carling & Hardie, 1989). Its glycogen binding domain (β) is very similar to that of BE (Hudson et al., 2003; Polekhina et al., 2003), raising the possibility that AMPK might compete and displace BE from its sites on glycogen. AMPK may therefore inhibit both synthetic and branching facets of glycogen formation. Recently, mutations in the porcine γ3 subunit (Milan et al., 2000) and human γ2 subunit (Arad et al., 2002) were shown to result in increased glycogen and LB formation in skeletal and cardiac muscle. These mutations decrease AMP activation of AMPK (Daniel & Carling, 2002; Scott et al., 2004), which would decrease AMPK inhibition of GS. They, however, are not expected to affect AMPK binding to BE sites on glycogen. The combined effect of increased GS activity and decreased BE ability would result in polyglucosans and LB.

The formation of LB with AMPK mutations makes it important to consider a role for AMPK in LD. Currently, the only characterized phosphorylation site on AMPK (Thr172, α1 subunit) activates the enzyme upon phosphorylation (Carling, 2004) and would not be a potential target for laforin phosphatase activity. However, there are additional phosphorylation sites on all the subunits (Carling, 2004), yet to be studied. These could have stimulatory effects on AMPK upon dephosphorylation and thus serve as laforin targets to activate AMPK, which would in turn inhibit GS.

Phosphorylation at the GS site 2 serves as a primer for phosphorylation by casein kinase 1 (CK1) of yet another site (2a) nearby (Roach, 2002) (Fig. 4). CK1 is activated by dephosphorylation (Rivers et al., 1998), and hence could be activated by laforin to phosphoinactivate GS.

Protein kinase A (PKA) phosphorylates two C-terminal sites (1a, 1b, Fig. 4) (Roach, 2002). This activity has not been confirmed *in vivo* but raises the possibility of the existence of yet another physiologic GS kinase, perhaps related to PKA.

A single phosphatase, protein phosphatase 1 (PP1), is currently known to act on GS at an unspecified site(s) (Roach, 2002). PP1 is recruited to glycogen and GS by members of a glycogen-targeting family of proteins (Ceulemans & Bollen, 2004). These proteins generally have two phosphorylation sites. Phosphorylation of one site stimulates PP1 activity on GS, and of the other inhibits this activity (Dent et al., 1990; Ceulemans & Bollen, 2004). Laforin was recently shown to bind R5, one of the glycogen-targeting family of proteins, and to bind it at the site of R5 interaction with GS (Fernandez-Sanchez et al., 2003). Laforin could therefore displace the R5-PP1 complex from GS and/or inactivate it by dephosphorylation, which would in turn inactivate GS.

G6P removal

As mentioned, G6P exerts strong allosteric potentiation on GS (Roach, 2002), and hence its removal would have a major impact on polyglucosan synthesis. G6P may be removed by glycolysis, but that energy-generating path is unfavored at times of energy plenty, when GS is "on" and is attempting to store away excess glucose. G6P can also be removed into the ER by G6Pase, converted back to glucose, and returned to the blood stream. Laforin localizes at the external surface of the ER (Ganesh et al., 2000; Minassian et al., 2001) (in addition to binding polyglucosans), and could therefore phosphoregulate G6Pase activity. Currently, phosphoregulation of the complicated ER-membrane spanning G6Pase complex remains unstudied (Foster & Nordlie, 2002; Van Schaftingen & Gerin, 2002). On the other hand, G6Pase regulation at the genetic level has received significant attention. The G6Pase gene promoter contains a thymine-rich insulin response element (TIRE), which is activated by a pathway controlled by GSK3 (Lochhead et al., 2001; Finlay et al., 2004). GSK3 upregulates G6Pase, which removes G6P and thus inhibits GS. Interestingly, laforin has an isoform generated by alternative splicing of the *EPM2A* gene that localizes in the nucleus (Minassian et al., 1998; Ganesh et al., 2002; Ianzano et al., 2004), and that could impact nuclear GSK3 or the GSK3-TIRE pathway.

In the above paragraphs we reviewed the most direct means through which laforin, after detecting polyglucosans with its starch-binding domain, could act, through its phosphatase domain, to inhibit further polyglucosan synthesis. We did not address the wider possibilities of more upstream actions, such as possible inhibition of glucose uptake or gluconeogenesis, both of which would reduce cellular G6P levels.

If branching does take place in the dendrite, then laforin could potentially act on BE and convert the polyglucosans to soluble glycogen. At this time, no phosphoregulation of BE is known.

Laforin may also be involved in the digestion of polyglucosans. As mentioned above, it does not appear that neurons possess the standard glycogen catabolizing enzymes (GP and DE). It is possible that laforin instigates recruitment of lysosomes for this task.

Finally, laforin may regulate the predicted shuttle mechanism, discussed above, that moves polyglucosans out of dendrites towards the soma and into axons. Interestingly, the transport system that supplies proteins to axons is critically controlled by GSK3 (Morfini et al., 2004). If the same microtubular tracks and kinesins are also utilized for polyglucosan transport, then once

again GSK3 would be in a position of polyglucosan control. We theorize that GSK3 is a key effector that responds to the laforin signal and regulates a multifaceted cellular response to counteract dendritic polyglucosan accumulation and LD.

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