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ONTOGENY OF LAFORA BODIES AND NEUROCYTOSKELETON CHANGES IN LAFORIN-DEFICIENT MICE

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Abstract

Lafora disease (LD) is an autosomal recessive, always fatal progressive myoclonus epilepsy with rapid cognitive and neurologic deterioration. One of the pathological hallmarks of LD is the presence of cytoplasmic PAS+ polyglucosan inclusions called Lafora bodies (LBs). Current clinical and neuropathological views consider LBs to be the cause of neurological derangement of patients. A systematic study of the ontogeny and structural features of the LBs has not been done in the past. Therefore, we undertook a detailed microscopic analysis of the neuropile of a Laforin-deficient (epm2a^{-/-}) mouse model. Wild type and epm2a^{-/-} mice were sacrificed at different ages and their encephalon processed for light microscopy. Luxol-fast-blue, PAS, Bielschowski techniques, as well as immunocytochemistry (TUNEL, Caspase-3, Apaf-1, Cytochrome-C and Neurofilament L antibodies) were used. Young null mice (11 days old) showed necrotic neuronal death in the absence of LBs. Both cell death and LBs showed a progressive increment in size and number with age. Type I LBs emerged at two weeks of age and were distributed in somata and neurites. Type II LBs appeared around the second month of age and always showed a complex architecture and restricted to neuronal somata. Their number was considerably less than Type I LBs. Bielschowski method showed neurofibrillary degeneration and senile-like plaques. These changes were more prominent in the hippocampus and ventral pons. Neurofibrillary tangles were already present in 11 days-old experimental animals, whereas senile-like plaques appeared around the third to fourth month of life. The encephalon of null mice was not uniformly affected: Diencephalic structures were spared, whereas cerebral cortex, basal ganglia, pons, hippocampus and cerebellum were notoriously affected. This uneven distribution was present even within the same structure, i.e., hippocampal sectors. Of special relevance, was the observation of the presence of immunoreactivity to neurofilament L on the external rim of Type II LBs. Perhaps,

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type II LB is not the end point of a metabolic abnormality. Instead, we suggest that type II LB is a highly specialized structural and functional entity that emerges as a neuronal response to major carbohydrate metabolism impairment. Early necrotic cell death, neurocytoskeleton derangement, different structural and probably functional profiles for both forms of LBs, a potential relationship between the external rim of the LB type II and the cytoskeleton and an uneven distribution of these abnormalities indicate that LD is both a complex neurodegenerative disease and a glycogen metabolism disorder. Our findings are critical for future studies on disease mechanisms and therapies for LD. Interestingly, the neurodegenerative changes observed in this LD model can also be useful for understanding the process of dementia.

Keywords

Lafora body disease; laforin protein; mouse; cytoskeleton

Introduction

Lafora disease (LD) is a progressive myoclonic epilepsy with an autosomal recessive hereditary pattern (Delgado-Escueta et al., 2009).

Since its early description by Gonzalo Lafora, one century ago (1911), it is known that an enormous collection of PAS-positive (PAS+) inclusions are present in the nervous tissue of these patients, as well as in other organs with high carbohydrate consumption. Therefore, the progressive neurological derangements observed in LD patients have been associated with the accumulation of Lafora Bodies (LBs) (Chan et al., 2004a; Minassian, 2001; Mittal et al., 2007). However, the actual role of LBs is unknown and their specificity in LD has been challenged (Hall et al., 1998; Mitsuno et al., 1999; Suzuki et al., 1978; Yanai et al., 1994).

In 1967, Van Hoof and Hageman-Bal made a significant contribution by describing some features of LBs in a brain biopsy of a 27 year old male patient with LD. Using PAS stain they identified three types of LBs. Most inclusions belonged to type I and consisted of granular, polymorphic, “dust-like”, uniformly stained particles. Type II inclusions were structures characterized by a heavily stained core, surrounded by a less stained circular rim, as revealed by PAS reaction. The third type was like type II inclusions but they show some kind of “fissure” in their dark core, reminiscent of the letter “Y”. This type was rarely observed. These authors corroborated the intracellular location and, at the ultrastructural level, the absence of a membrane encasing these polyglucosan inclusions (Van Hoof and Hageman-Bal, 1967).

The observation of PAS+ neuronal inclusions in LD patients still constitutes a matter of discussion for several reasons: Origin, formation mechanism and apparent lack of specificity, among other issues. Actually, Lafora himself referred to these inclusions as *corpora amylacea* (*Amyloider Körperchen*). Cavanagh wrote a thorough review about *corpora amylacea* and related carbohydrate inclusions. From his work, one can conclude that the specificity of LBs is relative and their biomolecular significance is unknown (Cavanagh, 1999). An early description by Bielschowski of the presence of PAS+ inclusions confined to the globus pallidus in a case of *status marmoratus* (Bielschowski, 1912) and confirmed by other authors in similar cases (Adler et al., 1982; de Leon, 1974; Probst et al., 1980) provide further support to the uncertain specificity of LBs in LD. Furthermore, a handful of publications have described LB-like inclusions in the nervous system of several nonhuman species, without any signs of LD (Antal, 1982; Hall et al., 1998; Mitsuno et al., 1999; Suzuki et al., 1978; Yanai et al., 1994). More recent literature has focused on the clinical, molecular and genetic aspects of LD, leaving aside the systematic structural and

ultrastructural study of LBs (Chan et al., 2004b; Delgado-Escueta et al., 2001; Fernandez-Sanchez et al., 2003; Ganesh et al., 2000; Ganesh et al., 2002; Gomez-Garre et al., 2000; Wang et al., 2002).

A decade ago, our group identified a mutation in the EPM2A gene in LD patients and the corresponding absence of its product: Laforin (Minassian et al., 1998; Serratosa et al., 1999). Laforin is an enzyme with a dual specificity phosphatase site as well as an amino-terminal carbohydrate-binding domain (Delgado-Escueta, 2007; Ganesh et al., 2006). A second mutation was found in the EPM2B gene, which normally produces Malin, an E3 ubiquitin-ligase enzyme (Chan et al., 2003; Gentry et al., 2005). By inducing a selective mutation in the Epm2a gene, we produced an experimental model of LD in mice. In these animals we replicated myoclonias, EEG polyspike-slow wave discharges, cerebellar-damage signs of ataxia and intraneuronal PAS+ inclusions, similar to those LBs found in LD patients. Furthermore, LBs from our homozygous mice immunoreacted to antibodies against human LBs (Ganesh et al., 2002), a similar crossed immunoreaction occurring between canine and human LBs (Yamanami et al., 1992).

Despite the fact that LD has been studied from many perspectives, none have considered the cytoskeleton of damaged neurons.

In this communication, we provide a detailed neuromorphological study of our LD model (laforin-deficient-mice (epm2a^{-/-})). Our observations challenge some classic concepts about LD and shed some light on the molecular biology of this fatal form of epilepsy. We emphasize the relevance of the ontogeny of LBs as well as the structural changes of the cytoskeleton of affected nerve cells. The latter appears to provide support for the involvement of a neurodegenerative process. Some novel structural and putative functional features of LBs are also presented and discussed.

Materials and Methods

Development of Lafora-deficient Mouse model

The dual-specificity phosphatase domain-coding region of the Epm2a gene was deleted to obtain laforin-deficient mice (for a detailed description see Ganesh et al., 2002).

Vivarium care, sacrifice and tissue acquisition

Wildtype and experimental mice were kept under UCLA and VA standard vivarium conditions. The experimental protocol was in accordance with the Animal Act of 1986 for Scientific Procedures and the IRB approval. All experiments conformed to local and international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Animals from both genders and groups were sacrificed under general anaesthesia induced by intraperitoneal barbiturate injection, at 11 days, 2, 3, 9, 16, 20 and 26 months of age (ten mice per age). Using a transthoracic exposure of the heart we perfused all animals through the left ventricle with a buffered solution of paraformaldehyde 1% and glutaraldehyde 0.5%. Afterwards, under a dissection microscope, the encephalon was carefully removed. Finally, after 48 hours of whole-brain fixation, brain fragments were obtained. We studied: lower brainstem, diencephalon, cerebellum, dorsal hippocampus, basal ganglia and cerebral cortex.

The following histology methods were used: Luxol-fast-blue, PAS stain and Bielschowski method. Luxol-fast-blue and PAS methods were used following standard techniques (Luna, 1968). The Bielschowski method was used following the modification by Cajal (Ramon y Cajal and Castro, 1972).

We tried several immunocytochemical techniques to reveal apoptosis: TUNEL stain, Caspase-3, Apaf-1 and Cytochrome-C. For immunohistochemistry, mouse monoclonal antibodies to Caspase-3, Cytochrome-C, the rabbit polyclonal antibody to Apaf-1 and mouse monoclonal antibodies to NF-L, were used in a 1:300 dilution (Santa Cruz Biotechnology USA). Tissue samples were incubated for 30 minutes at room temperature followed by detection of bound antibody using the DAKO LSAB+HRP reporter system (DAKO Corporation, Carpinteria, CA. 93013 USA) and counter-stained with hematoxyline (Elias, 1990; Nadji and Morales, 1986; Trojanowski et al., 1986). The basic protocol for TUNEL stain was as follows: After thorough rinsing of the tissue with Tris-HCl-buffered saline (TBS, pH 7.4), the sections were incubated with a tailing buffer (25 mM Tris-HCl buffer, 1 mM CoCl₂, 200 mM sodium cacodylate, 0.025% bovine serum albumin pH 6.6) containing 0.1 mM dATP, 0.01 mM biotin-16-dUTP and 250 U/ml terminal deoxynucleotidyl transferase from calf thymus (Boehringer-Mannheim, FRG) for 60 min at 37 °C. The enzymatic reaction was stopped by rinsing with PBS. Sections were exposed to avidin-biotin-peroxidase complex (ABC) (Vectastain ABC kit, Vector Laboratories). A nickel ammonium sulfate-intensified DAB was used to make the label evident. Finally, the sections were counterstained with cresyl violet. For control purposes, either the enzyme or biotin-16-dUTP was skipped from the sequence. DAB reaction was not detected in negative controls (Sugimoto et al., 2004).

Results

Phenotype and general features of Laforin-deficient mice

The skin from laforin-deficient mice had a dry appearance. Their hair lost its terse and shiny look and its “furry-like” appearance. With age, some of these animals develop kyphosis and their spontaneous movement appeared to be decreased. They were less reactive when the experimenter tried to pick them up. The macroscopic aspect of *epm2a*^{-/-} mice encephalon was similar to those from controls.

General light-microscopy findings

We studied the lower brainstem, diencephalon, cerebellum, dorsal hippocampus, basal ganglia and cortex of *epm2a*^{-/-} and wild type mice, at 11 days, 2, 3, 9, 16, 20 and 26 months of age. Light microscopy showed that at a very young age (11 days-old) *epm2a*^{-/-} mice already demonstrated degenerating and dead nerve cells without any PAS⁺ inclusions. Distorted, collapsed and hyperdense neuronal somata containing inconspicuous organelles, were frequently seen in these animals (Fig. 1A–C). Older animals showed an increasing number of neurons harbouring LBs. Even though old experimental animals had many nerve cells with abundant PAS⁺ inclusions, most of these neurons did not show any prominent protoplasmic and/or organelle degenerative changes compatible with an advanced degenerative state or cell death.

Brains from *epm2a*^{-/-} mice were characterized by a differential sensitivity to neuropathological changes secondary to the absence of laforin. For example, the hippocampus, some parts of the lower brainstem and cerebellum displayed remarkable anatomical derangement, whereas diencephalic nuclei were spared. The presence of cell death in very young experimental animals, never related to accumulation of LBs, led us to explore some other putative mechanisms. We tried several immunocytochemical techniques oriented to reveal apoptosis however all immunoreactions were negative. After discarding an apoptotic mechanism, necrosis emerged as the alternative cell death process occurring in our experimental animals.

PAS stain observations

-Ontogeny and Morphologic subtypes of Type I and Type II Lafora bodies—

LBs formation has been successfully replicated in *epm2a*^{-/-} mice allowing us to undertake an ontogenetic study of them. We have observed a clear-cut progression from total absence of LBs in many neurons at early age, through large amounts of them at 9 months of life, ending up with enormous polyglucosan deposits in the central nervous system of older animals. Figure 2A–C illustrates this progression in the cerebral cortex. An advanced and prominent state is shown in the cerebellar cortex of a 26-month-old null mouse, displaying large deposits of PAS + inclusions in the Purkinje cell and Granule cell layers (Fig. 2D). As expected, wild type mice neuropile did not show any PAS+ reaction, exception made of that observed in brain capillaries.

At the light-microscopy level, LBs are clearly observed around 2 months of life ($\pm$ 15 days), displaying a patchy cytoplasmic and an intraneurite distribution. From the very early ontogenetic stages of LBs up to the late ones, it is evident that LBs formation does not equally affect all sorts of neurons or all of the central nervous system nuclei. For example, large and medium-sized neurons rather than small ones are most prone to concentrate LBs, with the exception of granule cells from the cerebellum. Similarly, whereas the lower brain stem shows many LBs, diencephalic nuclei remain intact, even at late ages. Furthermore, these non-uniform effects can be observed even within the same neural structure. Such is the case of CA1 and CA2 hippocampal sectors, which consistently display fewer LBs, contrasting with the large amounts observed in CA4 (Fig. 3A–B). A similar phenomenon is illustrated in figure 3-C and D. A total absence of polyglucosan in the pontine periventricular grey matter (Fig. 3-C) contrasts with an enormous amount of LBs in the nearby pontine dorsolateral region (vestibular nucleus) (Fig. 3-D).

In our experimental mice, the Van Hoof and Hageman-Bal's type I, "dust-like" PAS+ inclusions, were the first vestige of polyglucosan to appear and, they were distributed in small amounts in somata and neurites. Later, type I LBs became widely spread and more abundant in the affected neuropile. This type of inclusion constitutes the largest proportion of the total LBs contained by nerve cells. They are inside somata, dendrites and axons (Fig. 4A–B). As shown in figure 2 the number of intracytoplasmic type 1 PAS+ particles increases with time. Quite frequently, type 1 PAS+ particles tend to be coalescent. There are some major qualitative and quantitative differences between type I and type II LBs. The polymorphic, "dust-like" appearance of type I LBs strongly contrasts with the structural complexity of type II LBs. To further substantiate our laboratory replica of human LD, we observed a striking resemblance of type II LBs observed in our null mice, with those observed in human patients with LD (Fig. 4C–D). In *epm2a*^{-/-} animals, type II LBs became evident at a later stage, around 2 months after the appearance of type I LBs. This complex form of LB (Type II) appeared to always be located inside the nerve cell cytoplasm, apparently never inside neurites and certainly never within small nerve cells. The number of type II LBs is considerably smaller than that of type I LBs, at any age. In most cases, we observed just one type II LB inclusion per affected neuron, although nerve cells with bulging appearance containing two Type II LBs were occasionally seen (Fig. 7).

- CNS Nuclei with major structural impact secondary to lack of laforin.

Features and intensity—Brain sections from *epm2a*^{-/-} mice displayed a wide variety of cyto- and myeloarchitectonic changes. For instance, thalamic and hypothalamic structures did not show any major anatomical modification attributed to the genetically induced loss of laforin, whereas adjoining basal ganglia and septal nuclei contained many type I and type II LBs, keeping intact their basic histological features (Fig. 5A–B). On the other hand, going from lesser to very prominent changes, the cerebral cortex (Fig. 2A–C), cerebellum (Fig.

2D), hippocampus (Fig. 3A–B) and basal pons (Fig. 5C–D) showed marked anatomical derangement.

The hippocampus-dentate complex of experimental animals was intensively but non-uniformly affected. The hippocampal sector CA4 displayed a prominent disruption of the pyramid layer as well as large amounts of both types of LBs. In contrast, the CA2 sector was minimally altered (Fig. 3A–B). The closely related dentate gyrus also showed modest changes.

The ventral pontine tegmentum of *epm2a*^{−/−} mice was the most affected structure. The magnocellular region of the pontine reticular formation and the most ventrally located pontine nuclei, showed cyto- and myeloarchitectonic disorganization and enormous amounts of PAS⁺ particles (Fig. 5C–D). Giant and mid-sized reticular formation neurons from the pons displayed prominent type II LBs, accounting for their somatodendritic deformities (Fig. 4 and Fig. 7). Collapsed and deformed dark perikarya were frequently seen in the central and ventral regions of the pons. Myelin sheaths also showed distorted profiles. In some cases, the abundance of large masses of polyhedral type I LBs in the ventral part of the lower brainstem produced a mosaic-like effect on a greatly distorted neuropile (Fig. 5D). In contrast, the closely located periaqueductal grey matter was remarkably well preserved (Fig. 3C).

Bielschowski method observations

We already mentioned that nerve cell degeneration and nerve cell death was detected in our null mice very early. This observation, made with the PAS reaction and some ancillary techniques, was strongly supported by the Bielschowski method, which showed intense argyrophilic (“dark”) and collapsed somata in 11 days-old null mice ventral pons, surrounded by quite normal large nerve cells (Fig. 6A).

At 9 months of age, null animals showed an increment on the number of dark and collapsed neurons and more distorted argyrophilic neurites (Fig. 6B (arrows)). Older experimental animals displayed more dramatic changes as shown in figure 6C–D. With the support of the Bielschowski method we were able to observe somatodendritic distortions. Axons and dendrites displayed conspicuous thickening and nodosities and the delicate linear traces made by the fibrillary proteins of cytoskeleton, were replaced by thick dark bands. Some of the *epm2a*^{−/−} mice somata depicted a translucent cytoplasmic area, which corresponds to polyglucosan inclusions, revealed as a “negative image” by this method (Fig. 6 arrows in C–D).

Our observations made with the Bielschowski method have demonstrated a prominent and progressive impairment of the neurocytoskeleton of many nerve cells in the LD mice, closely resembling what occurs in neurodegenerative processes. Furthermore, we also observed some argyrophilic images that are strongly compatible with senile (neurite) plaques (Fig. 6A–B).

The cytoskeleton changes observed in nerve cells, prompted us to complete our neuromorphological study using a monoclonal antibody against neurofilament L (NF-L). A previously unknown component of type II LB came across during our microscopic analysis of immunoreactivity to NF-L in *epm2a*^{−/−} mice. Immunocytochemistry showed some cytoplasmic ring-like structures, containing a circular arrangement of 12 to 18 globular corpuscles, which are strongly positive to NF-L. These intracytoplasmic ring-like structures have an empty core, surrounded by a circular brownish band, which always contains these dark spherules. These corpuscles are perfectly arranged and equidistant between one and the next (Fig. 8A–D).

These ring-like structures are present in most parts of the null mice encephalon. One ring per neuron is usually seen; however some cytoplasm displayed two of these inclusions (Fig. 8C). Interestingly, these rings containing spherules positive to NF-L are rarely seen in the thalami. These circular corpuscles are not present in all nerve cells, perhaps reflecting some degree of selectivity. Soon, we realized that these ring-like structures were the mirror image of type II LBs, as shown by PAS stain. We also realized that we were, for the first time, revealing the presence of fibrillary proteins NF-L in type II LBs, unveiling a potential structural relationship between them and the neurocytoskeleton.

Discussion

Our laforin-deficient mouse model is a reliable reproduction of LD phenotypes of myoclonias, EEG polyspikes, impaired retention, poor grip strength and ataxia (Ganesh et al. 2002; Delgado-Escueta, 2007). Therefore, it represents a unique opportunity to study the central nervous system of these animals, emphasizing the ontogeny of LBs and their neurocytoarchitectonics. Table 1 illustrates how anatomical alterations of neurodegenerative cell loss, type I and type II LBs, neurofibrillary tangles and senile plaques relate to behavioral and neurological changes.

Our observations demonstrated that nerve cell death in the mouse model of LD is not related to apoptosis. Collapsed and strongly stained neurosomata confirm necrosis as the dominant cell death mechanism in *epm2a*^{-/-} mice. The cardinal importance of this finding becomes more prominent in view of the presence of many dead neurons at very young age and that these cells do not contain LB. Early cell death and involvement of dysfunctional neurons are the initial and main factors for LD neurologic derangement. LBs do not participate in this early process. This observation contradicts current dominant neuropathological views that strongly associate large number of polyglucosan inclusions to nerve cell degeneration, cell death and neurological derangement (Van Heycop Ten Ham, 1975; Minassian, 2001). This is a wrong generalization that emerges from the fact that all human neuropathological studies are made in very advanced stages of LD. For the first time, our laforin-deficient mice model allows a detailed ontogenetic study of LBs. Recent experiments in our laboratory are trying to elucidate the molecular relationship between genetically induced laforin deficiency and nerve cell death.

Despite the fact that with age, the *epm2a*^{-/-} mice showed an increment in both the number of degenerated nerve cells and LBs, the biological impact of the absence of laforin does not display a universal negative effect on all encephalic structures. We have noticed considerable variations among different neural structures and even among nerve cells themselves; it appears that large neurons are more affected than small ones. We observed that the cerebellum, hippocampus and reticular formation neurons showed prominent PAS⁺ inclusions and degenerative changes, whereas diencephalic structures were only slightly damaged and rarely showed LBs or degenerated neurons. Furthermore, this biological dissociation in sensitivity is even present within the same structure as in the case of hippocampus. Lafora himself observed this non-uniform pattern of involvement in human brains (Lafora, 1911). This patchy pattern of neurobiological response to absence of laforin poses important, unanswered questions about the differential effects of “radical” genetic elimination of a specific enzyme. The putative presence, within a specific neuronal population of some factors or molecules protecting against the consequences of laforin deficiency emerges as one possible explanation.

Our data suggest a progressive formation of both types of LBs with clear morphologic, chronologic and topographic differential patterns. The morphologic features displayed by each type make them so different. On the one hand, type I LBs are relatively simple crystal-

like, uniformly stained structures with a wide distribution in affected neuropile. On the other hand, we are facing a complicated cytoplasmic structure with two clear-cut concentric spaces, each with a strongly different stain affinity. Furthermore, when we consider the small number of type II LBs present in our laforin-deficient mice and their selective allocation, one cannot stop thinking of this type of LBs as something structurally and functionally special.

These observations allowed us to consider the following hypothesis. As a result of lack of laforin, an abnormally branched glycogen is formed (polyglucosan) and its progressive accumulation generates minuscule Type I LBs. Because of the small size of these inclusions early in life, they might be easily transported from their cytoplasmic origin to neurites using the dynamic elements of the cytoskeleton. When the still unknown specific metabolic failure increases, so does the amount of polyglucosan and the nerve cells may be facing great difficulty trying to get rid of these insoluble molecules. Thus, it is possible that, at this critical moment, some neurons activate an unknown molecular mechanism to start building up type II LBs.

Type II LBs are complex intracytoplasmic structures with a high content of polyglucosan. A potentially underlying failure in glycosylation allows for an unusual deposit of large and abnormal carbohydrate molecules in nerve cells. Moreover, Type II LBs display a concentric architecture. Up to now, this structure has been considered a simple, passive reservoir (Lafora, 1911; Minassian, 2001). However, it is hard to believe that such a complex entity is simply the direct consequence of a stochastic accumulation of misshapen molecules. We postulate that the core and peripheral ring are actively formed, through some unknown genetic de-repression, allowing some neurons to hyperconcentrate polyglucosans as revealed by the PAS reaction. Furthermore, we envision type II LBs as complex organelles that achieve a high degree of protective compartmentalization and/or an efficient metabolic machinery that breaks down large polyglucosan molecules into small ones to allow their centrifugal transport by the cytoskeleton.

The contour of the outer ring of Type II LBs is uniform and much less reactive to PAS stain. Our immunohistochemical observations on this peripheral rim yielded an extremely interesting and unexpected finding: The presence of 12 to 18 microspherules with a circular and regular arrangement within this rim and displaying an intense immunoreaction against NF-L (Machado-Salas et al., 2005; Machado-Salas et al., 2007). We have not found any previous reference to this observation. We consider that this immunoreactivity to NF-L unveils an unsuspected connection between Type II LBs and the neurocytoskeleton.

This important finding opened a significant question still to be answered: Are type II Lafora Bodies simple reservoirs of polyglucosans, or are they actual complex structures that many nerve cells actively build up as a response to a deranged carbohydrate metabolism?

In this regard, we would like to postulate that when some nerve cells detect that polyglucosans are building up, a protective genetic mechanism is triggered to initiate construction of type II LBs as a survival mechanism. Their central core may actively hyperconcentrate as many polyglucosan molecules as possible, reducing their potentially deleterious effect. Our immunohistochemical observations allow saying that the structure of the external rim involves fibrillary elements (NF-L) possibly establishing a direct relationship with the cytoskeleton. It is tempting to speculate that this feature provides a bidirectional “plasmastrassen”, either to centripetally carry small polyglucosan molecules to hyperconcentrate them, or to centrifugally drive them as “dust-like” inclusions away from the nerve cell cytoplasm (Figure 9).

These Type II LBs appear to emerge as “genetically built organelles”, which might be actively and systematically produced in human and murine LD and in the nervous system of other species under different circumstances (Kamiya et al., 1990; Simmons, 1994; Suzuki et al., 1980). Perhaps, they accomplish a protective rather than a deleterious role. Furthermore, not all LBs are equivalent. The brain LBs display different immunocytochemical reactions when compared to LBs from liver or muscle (Ganesh et al., 2002).

The dramatic change in volume and shape that occur in non-apoptotic cell death imply prominent modifications of the cytoskeleton. Acting as an anatomo-functional unit, it also underlies axonal flow. This highly dynamic quality of the neurocytoskeleton is closely related to an intrinsic notoriously phosphorylated state and a strongly ATP-dependent system. Both features make its components highly sensitive to several kinases and phosphatases (Carden et al., 1987; Eyer and Letierrier, 1988; Lee et al., 1988), including Tau protein.

The argentophylia unveiled by Bielschowsky method revealed that typical thin, fibril-like structures in neuronal somata of wildtype mice had become replaced by coarse, dark bands in several types of neurons from young and old experimental mice. These bands extended into primary dendrites and axon hillocks and were frequently observed inside collapsed somata and distorted neurites. This peculiar pattern, totally unexpected in LD, resembled the neurofibrillary degeneration observed in brains of patients with Alzheimer disease. These abnormalities were not confined to nerve cells themselves. We also observed, some clumps of neurite fragments, with significant argentophylia, reminiscent of neurite (senile) plaques.

In this regard, we recently demonstrated hyperphosphorylation of Tau in the neurocytoskeleton of Lafora mice. Also, our group has shown a close interaction between laforin and Tau protein. Laforin appears as a novel Tau phosphatase and its absence facilitates neurofibrillary tangle formation (Puri et al. 2009). Our study has shown images concordant with neurofibrillary tangles and probably neurite (senile) plaques. How then, does the formation of PAS+, poorly branched insoluble polyglucosan bodies relate to the formation of neurofibrillary hyperphosphorylated tau tangles in *epm2a*^{-/-} mice? One of the normal functions of laforin/dual specificity phosphatase is to selectively dephosphorylate glycogen synthase kinase 3beta (Wang et al., 2008) and thus suppress excessive glycogen phosphorylation. In *epm2a*^{-/-} mice that lack laforin, phosphorylation of glycogen is increased 3 to 4-fold as more poorly branched and insoluble glycogen progressively forms (Tagliabracci et al., 2008). In Alzheimer disease brains, amyloid plaques upregulate taurine protein kinase I, which leads to excessive phosphorylation of Tau destabilizing microtubules, impairing axonal transport and producing neuronal death (Ishiguro et al., 1992; Imahori, 2010). Upregulated Tau protein kinase I in mitochondria also phosphorylates and inactivates pyruvate dehydrogenase, reduces acetylcholine synthesis and contributes to neuronal death by reducing energy production and signal transmission between neurones (Hoshi et al., 1997). Interestingly, Tau protein kinase I (TPKI) is identical to glycogen synthase kinase 3beta (GSK3beta) by cDNA analysis (Imahori, 2010; Yamaguchi et al., 1996). Thus, TPKI/GSK3beta in a hyperphosphorylated state due to the absence of normal laforin function in *epm2a*^{-/-} mice leads to the increased formation of both phosphorylated Tau and phosphorylated poorly branched insoluble glycogen (polyglucosan).

We would like to postulate that human LD and our reliable laforin-deficient model share affinities with neurodegenerative processes. However, the marked and parallel participation of an abnormal metabolism of glucose/glycogen is a major genetic-dependent issue, clearly specific for LD. Perhaps, laforin deficiency upregulates Tau protein kinase I/glycogen synthase kinase 3-beta forming Tau, amyloid and polyglucosan bodies. Necrotic cell death, cytoskeleton derangement, identification of the ontogeny of two main forms of LBs, an

apparent differential structural and functional identity for both forms of LBs, a potential relationship between the external rim of the LB II and the cytoskeleton and an uneven distribution of these abnormalities indicate that LD is both a neurodegenerative disease and a glycogen metabolism disorder. Our findings are critical for future experiments on disease mechanisms and therapies for Lafora disease.

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Highlights

- Lafora bodies appear at different ages
- The brain of null mice were not uniformly affected
- Cell dead is necrotic and appears at very young age
- We observed immunoreactivity to neurofilament L on the external rim of Lafora Bodies type II
- Neurofibrillary degeneration and senile-like plaques were present

Figure 1.

Cell death in very young laforin-deficient mouse. Panoramic views of the cerebral cortex (A), hippocampus (B) and cerebellum (C) from 11 days old laforin-deficient mouse. Notice the presence of collapsed and intensively stained (“dark”) neuronal cytoplasm (white arrows) and the absence of PAS+ inclusions. PS-Cerebral cortex pial surface; CA3-Hippocampal sector; ML-Cerebellum molecular layer; GL-Granule cell layer. PAS stain. Scale bar: A, B and C: 100 μ m.

Figure 2.

Ontogeny of Lafora bodies. (A–C) null mouse cerebral cortex showing progressive increment of LBs from early to late age (A-11 days old; B-9 months old; C 16-months old, laforin-deficient mouse). PAS+ reaction observed in A corresponds to blood vessels wall (arrows). In an older animal B, some LBs are seen in several cortical layers. Interestingly, abundant LBs appear as subpial conglomerates (arrows). Prominent number of PAS + inclusions are seen in the cerebral cortex from a 16 months old null mouse (C arrows). A similarly progressive deposit of LBs was observed in many other central nervous system structures. LBs were very prominent in the granule cell layer of cerebellum, as shown in a 26-months-old null mouse (D). Also, notice an intense PAS reaction within Purkinje cells cytoplasm (arrows). (PS-Cerebral cortex pial surface; GL-Granule cell layer; ML-Molecular layer). PAS stain. Scale bar A, C-100 μ m; B- 40 μ m; D –60 μ m.

Figure 3.

It appears that the final structural and molecular impact, triggered by lack of laforin is not uniform throughout the nervous system. (A) Illustrates a moderate deposit of LBs in hippocampus CA2 sector and a well-preserved neuropile. (B) Illustrates an advanced loss of the cytoarchitecture of the closely related CA4 sector with a prominent accumulation of PAS + inclusions (A and B- 20-month-old null mouse). (C, D) depict a similar contrast between contiguous anatomic structures. In (C), the pontine periaqueductal grey matter, at the level of the VI cranial nerve nucleus shows a minimum amount of LBs. However, the dorsolateral pons, at the level of the vestibular nucleus, depicts an intense reaction to PAS stain (C, D- 9 month old mouse). (PAGM- Periaqueductal Grey Matter; VI- VI Cranial Nerve Nucleus; VN- Vestibular Nucleus; ML- Midline). PAS stain. Scale bar: A 75 μ m; B 60 μ m; C and D 150 μ m.

Figure 4.

Lafora bodies: Distribution and forms. (A, B) show the widely scattered type I LBs. They can be seen inside neurites (A, double arrow) as well as intrasomatic (B). They are more abundant in the neuropile and display different size and shape. The second type (Van Hoof's Type II) are complex ring-like structures, limited in number and always restricted to an intracytoplasmic location (C, arrows). Notice the strong similarities between null mouse type II LBs (C) and an equivalent LB from a patient with LD (D). A, B, C- Nerve cells from the pontine reticular nucleus (Magnocellular). D- LD Human cerebral cortex. PAS stain. Scale bar: A- 20 μm ; B- 10 μm ; C- 30 μm ; D- 15 μm .

Figure 5.

Even though some diencephalic nuclei did not show a noticeable impact due to lack of laforin, most null mice structures showed a variable involvement. (A, B) depict a delicate but widespread deposit of LBs in the neuropile of the striatum and the septal nucleus respectively. By far, most of these polyglucosan inclusions are made of Type I LBs. On the other hand, the ventral part of the pons of experimental animals was characterized by massive deposits of polyglucosans (C, D), creating notorious alterations of the myelo- and cytoarchitectonics of the ventral tegmental region and a mosaic-like appearance. (A-D) 9 months old null mouse. PAS stain. Scale bar A, C- 160 μm ; B, D- 75 μm .

Figure 6.

(A) A prominent argyrophillic and collapsed nerve cell (long arrow) is observed in the ventral pons surrounded by normal neurons (short arrows), in an 11-day old null mouse. (B) Neuropile from a 9-month-old null mouse showing several neurons displaying somatodendritic distortion. One of them is fully collapsed (long arrow). Notice an abnormally large neurite displaying a “corkscrew” argyrophillic pattern (short arrow). (C) A notoriously dilated neuron (arrow) with a translucent cytoplasmic area corresponding to a LB. The remaining dark cytoplasmic content includes a distorted cytoskeleton and nucleus. Around it, many grossly argyrophillic neurites show an unorderly arrangement (20-month-old laforin-deficient mouse). (D) Multiple “negative images” of type I LBs (oval) and LBs type II (square) can be seen. Thick argyrophilic bands are contained in neurites and somata. A moderate argyrophilia is evident in the external ring of type II LBs (inset) 20 months old null mouse, ventral pons. Bielschowski method. Scale bar: A, B and C- 50 μ m; D- 100 μ m.

Figure 7.
Two LBs type II are present in a nerve cell from the pontine reticular nucleus, pars magnocellularis. A centrally located nucleus separates them. 20 month-old null mouse. Bielschowski method. Scale bar: 30 μ m

Figure 8.

Presence of Neurofilament L in the external rim of LBs. (A) a ring-like structure eventually identified as type II LB, shows an intense immunoreaction to neurofilament L surrounding its “empty” core. (B) When a green filter was used, an “spheroidal” or punctuated appearance was unveiled, emphasizing their individuality. (C) Occasionally, we observe a nerve cell containing two of these immunoreactive rings. Even though the diameter is different, their structural resemblance is high. Notice a peripherally displaced and collapsed nucleus (arrow). (D) Another example of immunoreaction to neurofilament L within a type II LB, contained in an apparently normal superior colliculus neuron. (A, B, C) Cortical neurons. Antineurofilament L immunostaining. Scale bar: A, B- 10 μ m. C, D- 20 μ m.

Figure 9.

Type II Lafora Body, a simple reservoir or highly specialized metabolic machinery? The punctated immunoreactive structures forming a ring-like intracytoplasmic structure (A), perfectly fit into the external rim of a type II LB, as seen with PAS stain (B). In (C), a large neuron from the ventral pons displays a LB II containing darkly stained structures as revealed by Bielschowski method. (IC- Inner core; ER- External rim; N- Nucleus). (A, C) Laforin-deficient mouse cerebral cortex and ventral pons, respectively. (B) LD Human cerebral cortex. Scale bar: A, B- 5 μm; C- 10 μm.

Table 1

Morphological alterations and phenotype timeline in laforin deficient mice.

Age	Cell Death	Neurofibrillary tangles	PAS+ Lafora Bodies		Senile plaques	Myoclonic Seizures	Ataxia	Retention	Grip strength
			Type I	Type II					
11 d	+	+	--	--	--	nd	nd	nd	nd
2 month	+	+	+	--	--	nd	nd	nd	nd
3 months	+	+	+	+	+	+	--	+	--
9 months	+	+	+	+	+	+	+	+	+
16-26 months	+	+	+	+	+	nd	nd	nd	nd

Ataxia-rotarod: Retention- passive avoidance test; Wire hang test-motor-grip strength.

+ - present
-- - abstent
nd- not done