Mouse cerebellar Purkinje cell damage induced by diphenylhydantoin acute intoxication

Orlando J. Castejón*

Biological Research Institute and Faculty of Medicine, Zulia University, Maracaibo, Venezuela.

ABSTRACT: Twenty one days old Swiss albino mice that received diphenylhydantoin (25 mg/kg, i.p., daily for 15 days) progressively developed gait alterations, changes of behavior and cerebellar ataxia. Cerebellar slices were processed by conventional transmission electron microscopy. The body of Purkinje cells exhibited fragmented limiting plasma membranes, dilated nuclear envelopes, swelling and disassembly of nuclear pores, enlargement of rough and smooth endoplasmic reticulum and a notable detachment of membrane associated ribosomes, together with distorted vacuoles of smooth endoplasmic reticulum, bizarre shaped and swollen mitochondria with dilated cristae, as well as disrupted limiting lysosomal membranes. Degenerated axosomatic synapses apparently corresponding to basket cell axonal endings were recognized. Degenerated Purkinje cell axon initial segments exhibited vacuolar degeneration of myelin sheath, dilated axoplasmic tubular bundles, fragmented axonal membranes, swollen mitochondria, and disassembly of cytoskeletal structures. Some edematous and clear secondary and tertiary dendrites exhibited areas of dilated cisterns of smooth endoplasmic reticulum, clear and dark multivesicular bodies, and coated vesicles. Other dendritic ramifications exhibited an electron dense dendroplasm. Degenerated and large climbing fiber endings were observed making axodendritic synapses with edematous Purkinje dendrites. These presynaptic endings appeared depleted or containing few synaptic vesicles. These synapses did not exhibit pre- and postsynaptic densities. At the molecular layer, the edematous synaptic varicosities of parallel fibers containing pleomorphic synaptic vesicles and dense extravesicular substance were observed making asymmetric synaptic contacts with swollen Purkinje dendritic spines. These findings are postulated as pathogenic mechanisms of mouse cerebellar ataxia.

Introduction

Purkinje's cell density after diphenylhydantoin intoxication in rats was early reported by Dam in experimental animals and epileptic patients (1970 a,b,c). Later, the effects of diphenylhydantoin on activity of rat cerebellar Purkinje cells was described by Puro and Wooward (1973). Takeichi (1981, 1983) reported neurobiological and electron microscopic investigations of the rat cerebellum with chronic diphenylhydantoin intoxication. The oral administration of diphenylhydantoin for a period of more than nine months produced ataxia and muscle weakness in rats (Tashiro *et al.*, 1982). Chronic administration of diphenylhydantoin in the daily diet of mice induced on presynaptic segments of Purkinje cell axons massive enlargement and swelling in the deep cerebellar nuclei due to accumulation of spherical particles and tubular structures in the axoplasm (Volk and Kirchgässner, 1985). Kiefer *et al.* (1989) reported Purkinje cell axonal swellings in deep cerebellar nuclei of mice following orally administered phenytoin from 3 to 46 days.

Imamura *et al.* (1992), Villa and Sica (1994) and Pulliainen *et al.* (1997) subsequently described that diphenylhydantoin yields cerebellar ataxia in chronically treated epileptic patients due to cerebellar atrophy with loss of Purkinje cells. Later, Awada *et al.* (1999) reported that high doses of phenytoin can be toxic to human cerebellar cortical cells and induce mild cerebellar atrophy. Altered synaptic organization in the cerebellum and ataxia was observed in pogo/pogo mutant mice. In these mutant mice, parallel fiber varicosities were larger than normal and a single fiber often established synaptic contacts with up to four dendritic spines of a Purkinje cell (Jeong and Hyun, 2000).

However, a detailed submicroscopic study of pathological changes of Purkinje cell body, dendritic ramifications and their synaptic connections in Swiss albino mouse with diphenilhydantoin acute intoxication has not been carried
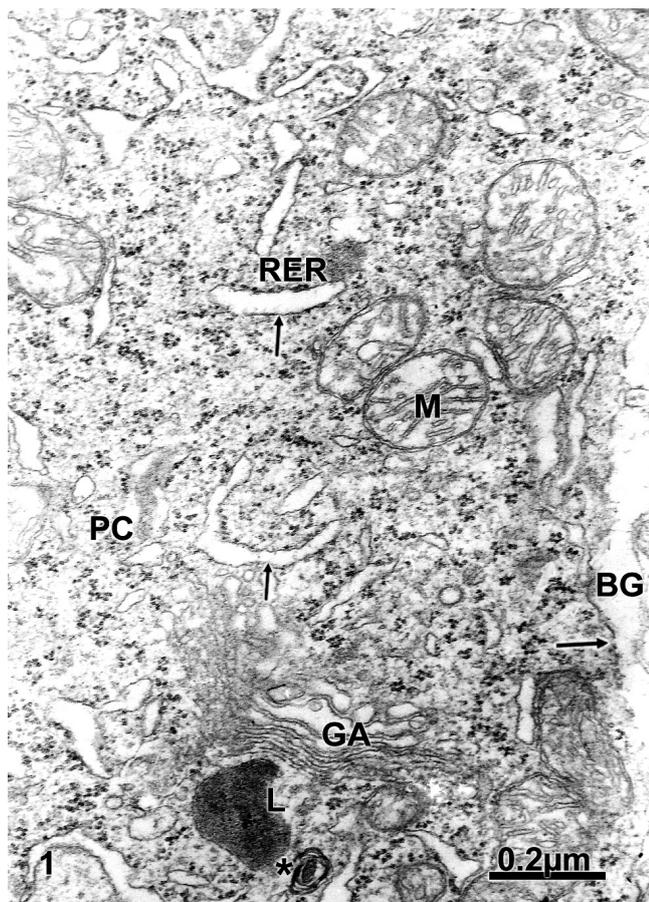
FIGURE 1. Swollen Purkinje cell cytoplasm (PC) showing the enlarged endoplasmic reticulum cisterns (RER), and the areas of detachment of associated ribosomes (short arrows), edematous mitochondria (M), fragmented limiting plasma membrane (long arrow), swollen and fragmented Golgi apparatus (GA), and a lysosome (L) with disrupted limiting membrane (asterisk). A portion of a swollen Bergmann glial cell cytoplasm (BG) is also is seen.

out up to the present time. Such study is basically important for further understanding the pathogenic mechanisms of experimental and human cerebellar ataxia.

Materail and Méthods

Swiss albino mice 21 days postnatal received intraperitoneal injection of diphenylhydantoin (Dilantin) at 25 mg/kg daily for 15 days developing progressively gait alterations, changes of behavior and cerebellar ataxia. Mice were decapitated after intraperitoneal injection of sodium pentobarbital. The cerebellar cortex was first fixed in 0.1M glutaraldehyde-buffer phosphate solution by two hours, and later fixed by 1 hour in similarly buffered 1% osmium tetroxide solution. The samples were dehydrated and embedded in Epon. Ultrathin sections were obtained in MT-2B Sorvall ultramicrotome, and

stained with uranyl salts and lead hydroxide. Observations were made in a JEOL 100B at 80 kV at intermediate magnifications (20.000 to 60.000 X) (Castejón, 2012).

Results

Clear and edematous Purkinje cell bodies exhibited fragmented limiting plasma membrane, dilated nuclear envelope, swollen and disassembly of nuclear pores, enlargement of rough endoplasmic reticulum and a notable detachment of membrane associated ribosomes, smooth enlarged cisterns and distorted vacuoles of the smooth endoplasmic reticulum, bizarre shaped and swollen mitochondria with dilated mitochondrial cristae, lysosomes with disrupted limiting membrane, and swollen Golgi apparatus, with fragmentation and vacuolar necrosis (Figs.1 and 2). Areas of dilated smooth endoplasmic reticulum surrounding small islands of Purkinje cell cytoplasm containing numerous vesicular profiles and free ribosomes were observed (Fig. 3). Fine and coarse electron dense precipitate of dense matrix lysosomes was observed as well as disrupted lysosomal limiting membranes (Fig. 4). Degenerated axosomatic synapses presumably corresponding to basket cell axonal endings were recognized on Purkinje cell bodies, which appeared surrounded by the edematous Bergmann glial cell cytoplasm (Fig. 5).

The degenerated Purkinje cell axonal initial segment exhibited vacuolar degeneration of myelin sheath, dilated axoplasmic tubular bundles, fragmented axonal membranes, swollen mitochondria, and disassembly of cytoskeletal structures (Fig. 6). At the molecular layer, some edematous and clear Purkinje primary dendritic trunks (Fig. 7) exhibited areas of dilated cisterns of smooth endoplasmic reticulum,
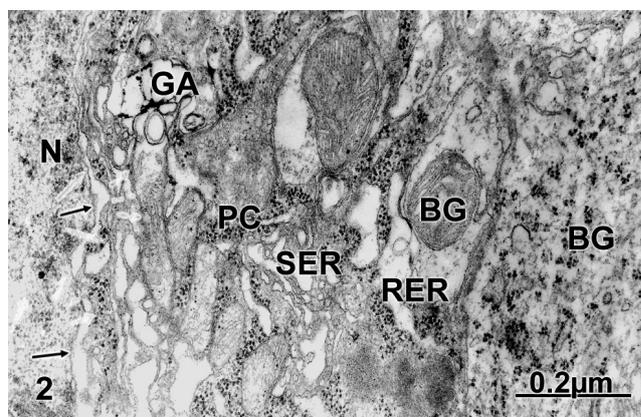
FIGURE 2. Swollen Purkinje cell cytoplasm (PC) exhibiting the nucleus (N) and the enlarged perinuclear cistern (arrows), a vacuolated and necrotic Golgi apparatus (GA), dilated rough (RER) and smooth (SER) endoplasmic reticulum cisterns, and the satellite Bergman glial cell cytoplasm (BG).
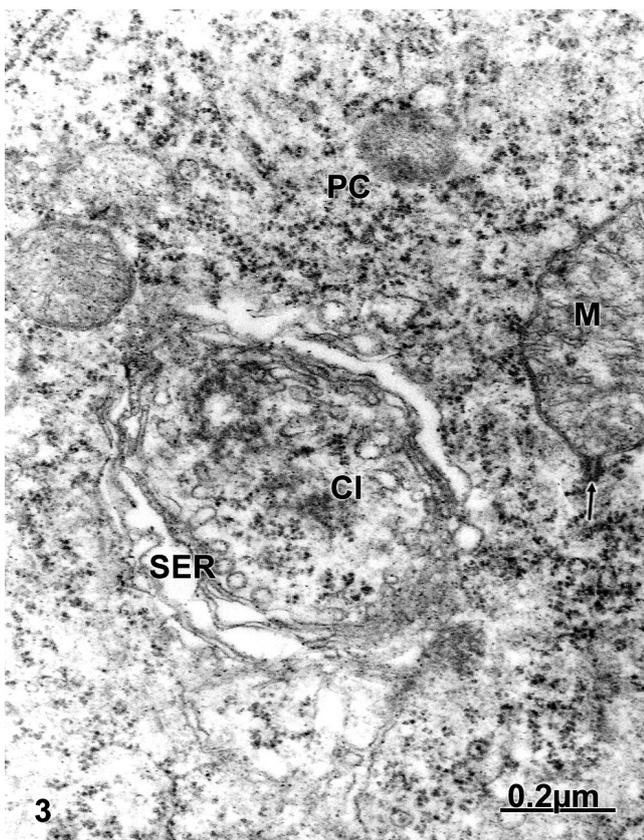
FIGURE 3. Swollen Purkinje cell cytoplasm (PC) depicting a cytoplasmic island (CI) surrounded by vacuolated smooth endoplasmic reticulum cisterns (SER). A swollen mitochondrion (M) shows its outer surface fused with a short rough endoplasmic reticulum cistern (arrow).

dense and bizarre-shaped mitochondria, lamellar bodies associated to mitochondria, and disrupted limiting plasma membranes.

Large and degenerated climbing fiber endings were observed making degenerated axodendritic synapses with edematous Purkinje dendrites. These swollen presynaptic endings appeared depleted of synaptic vesicles or containing few of them, and the synaptic membrane contacts did not exhibit pre- and postsynaptic densities. Swollen presynaptic endings of stellate neuron also were observed containing flattened synaptic vesicles (Fig. 8).

The swollen parallel fiber synaptic varicosities contained spheroidal and ellipsoidal synaptic vesicles and established asymmetric synaptic contacts with clear and edematous Purkinje dendritic spines (Fig. 9). The synaptic membrane contacts did no exhibit presynaptic density, and only remnants of postsynaptic density. These partially degenerated synaptic junctions appeared covered by the perisynaptic and swollen Bergmann glial cell cytoplasm.

The edematous and vacuolated stellate neurons displayed fragmented microtubules and vacuolated and severely damage lysosomes with fragmented limiting membrane.

The neighboring neuropil showed the swollen non-synaptic segments of parallel fibers and the en passant parallel fiber varicosities establishing asymmetric synapses with Purkinje dendritic spines (Fig. 10).

Discussion

In the present paper we have found edematous changes of mouse Purkinje cell body, axonal and dendritic processes and degenerated intracortical synaptic contacts induced by diphenylhydantoin intoxication. Similar electron microscopic findings in altered Purkinje cells (edematous cisternae of endoplasmic reticulum, swollen mitochondria and cytoplasmic bodies, along with the synaptic and axonal changes) were earlier reported by Breiden-Arends and Gullotta
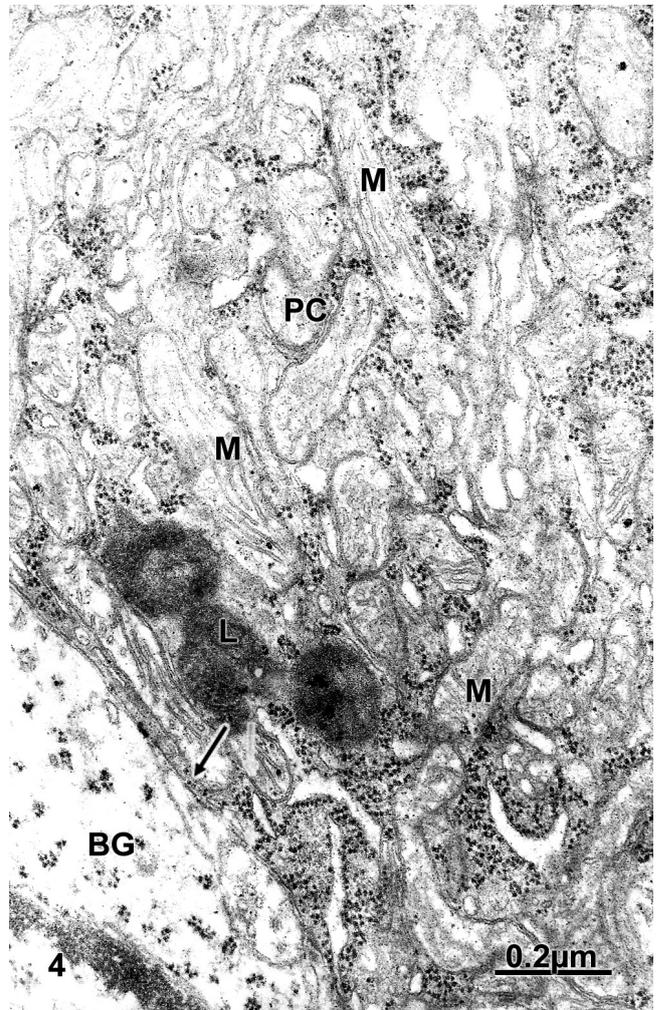
FIGURE 4. Swollen Purkinje cell cytoplasm (PC) showing irregularly shaped- lysosomes (L) with dark matrix granular deposit, bizarre-shaped mitochondria (M), and surrounded by the swollen perineuronal Bergmann glial cell cytoplasm (BG).
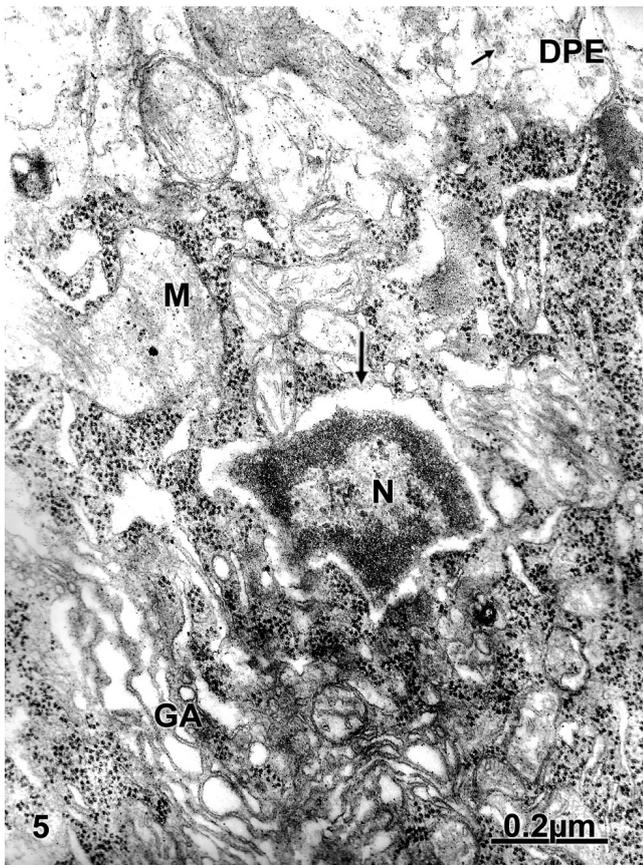
FIGURE 5. Swollen Purkinje cell body showing a partial nuclear (N) pole surrounded by a dilated nuclear envelope (long arrow), notably swollen mitochondria (M), and a fragmented and vacuolar Golgi apparatus (GA). A degenerated presynaptic ending (DPE), surrounded by swollen Bergmann glial cell cytoplasm, exhibits few dark presynaptic vesicles (short arrow), and depleted clear synaptic vesicles. Note the absence of pre- and postsynaptic densities.

(1981) in two epileptic patients who had been treated over years with diphenylhydantoin. Biggiogera *et al.* (1983) studied under the electron microscope the effect on Purkinje cells of diphenylhydantoin administration on postnatal ontogenesis in the rat, and reported signs of neuronal immaturity, both at cytoplasmic and nuclear levels, particularly evident between the 4th and 17th day of postnatal life. Volk and Kirchgässner (1985) earlier reported in male C57/BL6J mice the occurrence of marked dystrophic changes in Purkinje cell axons in the cerebellar vermis after chronic phenytoin administration. According to these authors, the presynaptic segments of Purkinje cell axons in the deep cerebellar nuclei showed massive enlargement and swelling due to accumulation of spherical particles and tubular structures in the axoplasm. They suggested that tubular structures represent a proliferation of smooth endoplasmic reticulum. Kiefer *et al.* (1989) postulated that phenytoin-induced axonal pathology of Purkinje cells is a dynamic process characterized by the

progressive accumulation of proliferating membranous material arranged in an increasingly complex fashion. However, they presumably represent edematous enlargement of these substructures since bundles of tubular structures in the axoplasm of Purkinje cell initial axonal segment are also found in normal mice (Castejón, 2012).

Ohmori *et al.* (1997) reported that phenytoin induces neurotoxic damage to granule cells and Purkinje cells in the developing cerebellum and impairs selected aspects of motor coordination ability. Tauer *et al.* (1998) also described in adult mice focal swellings along the Purkinje cell axon correlated with ataxia and incoordination of movements following administration of phenytoin. Later, Fonnum and Lock (2000) found that Purkinje cells are very sensitive to ischemia, bilirubin, ethanol and diphenylhydantoin. We have also reported similar findings in severe and complicated human traumatic brain injuries (Castejón 1985, Castejón *et al.*1995, 1997), suggesting a common and non-specific Purkinje cell reaction.
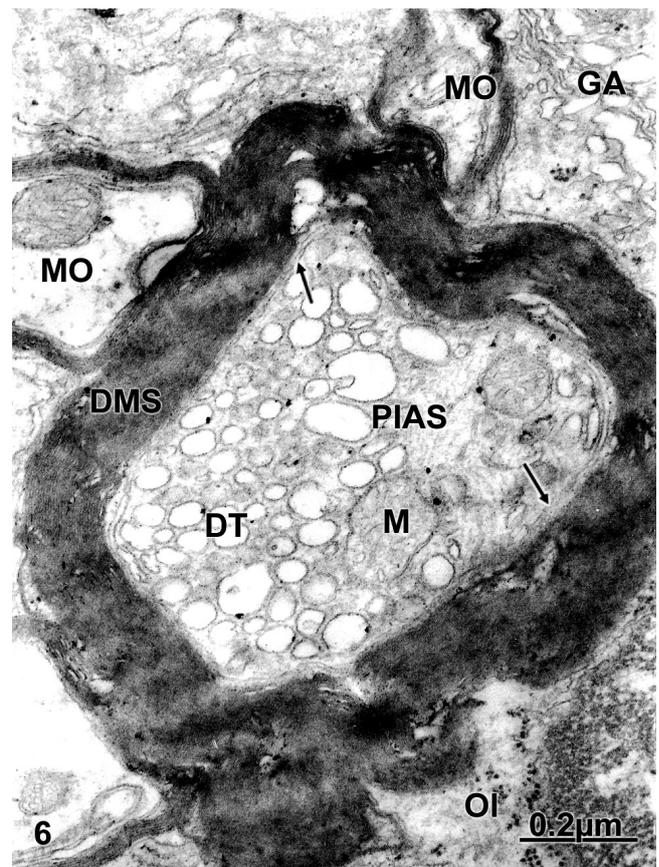
FIGURE 6. Degenerated Purkinje cell initial axonal segment (PIAS) showing the cross section of dilated axoplasmic tubular bundles (DT), edematous mitochondria (M), myelin sheath degeneration (DMS) with loss of myelin periodicity and myelin ovoids (MO). A swollen associated oligodendrocyte (OI) with vacuolar degeneration of Golgi apparatus (GA) also is seen.

The alteration of the Golgi complex is apparently related with neuronal, axonal and synaptic degeneration (Castejón *et al.,* 1995., Castejón *et al.,* 1997; Castejón, 2011). The diphenylhydantoin-induced ischemic and anoxic conditions of cerebellar tissue also alter the post-translational modifications of proteins within the Golgi apparatus. Consequently the insertion of integral membrane proteins, including ion channels and receptor molecules, into the plasma membrane is disturbed causing fragmentation and necrosis of the plasma membrane, as observed in the electron micrographs. The fragmentation of the mitochondrial cristae suggest that oxidative phosphorylation of ADP, the precursor of the high-energy phosphate bond of ATP, no longer occurs. In addition, we suppose an interruption of mitochondrial membrane intracellular transport, which causes respiration-dependent extrusion of H^+ and accumulation of Ca^{2+} from the cytoplasm (Castejón, 2004a).

Diphenylhydantoin-induced coarse and dense granulation of lysosomal matrix and disruption of the limiting
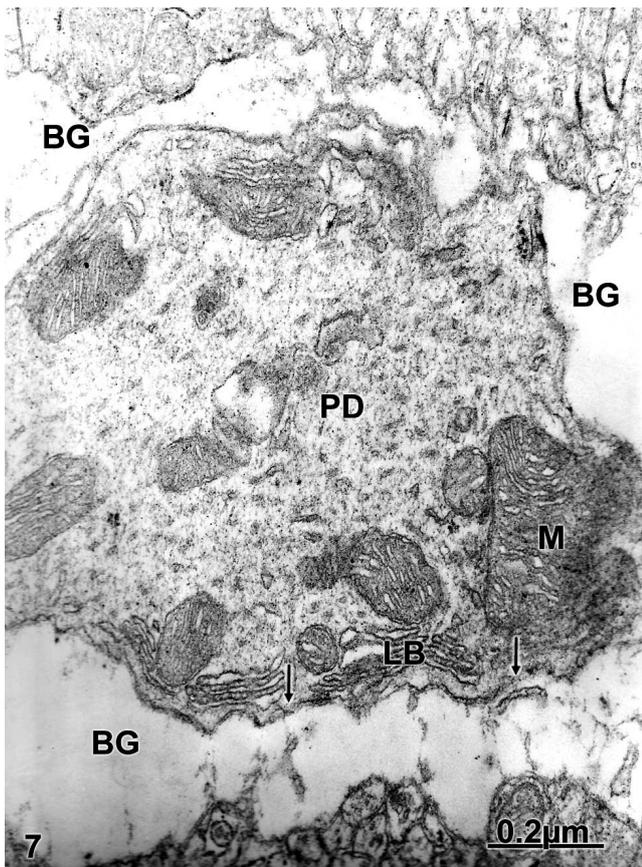
FIGURE 7. Swollen Purkinje primary dendritic trunk (PD) surrounded by swollen Bergmann glial cell cytoplasm (BG), containing dense and edematous mitochondria (M), and lamellar bodies (LB) associated to mitochondria, and exhibiting disrupted limiting plasma membrane (arrows).
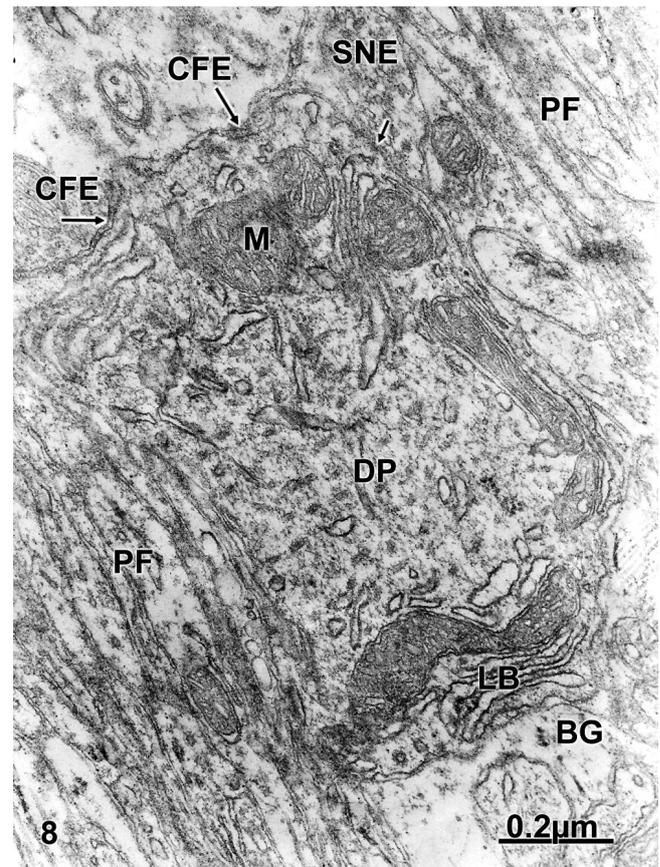
FIGURE 8. Swollen and degenerated climbing fiber endings (CFE) in the molecular layer, making axodendritic synaptic junction (long arrow) with a secondary and edematous Purkinje cell dendrite (PD). The latter exhibits the lamellar bodies (LB) and swollen dense mitochondria (M). The climbing fiber endings show few synaptic vesicles and vestiges of pre- and postsynaptic densities. A swollen stellate neuron presynaptic ending (SNE) containing flattened synaptic vesicles makes an axodendritic contact (short arrow) lacking pre-and postsynaptic densities. The neighboring non-synaptic segments of parallel fibers (PF) and the swollen peridendritic Bergmann glial cell (BG) also are noted.

membrane were interpreted as abnormal protein aggregation of released lysosomal enzymes and adsorbed cytosolic proteins. In addition, the multiple factors involved in cytotoxic edema should be considered (Castejón, 2004b).

We observed cytoplasmic islands surrounded by smooth endoplasmic reticulum in Purkinje cells, suggesting diphenylhydantoin-induced proliferation of smooth endoplasmic reticulum. We also found a fine and coarse electron dense precipitates over the lysosomal dense matrix. This finding is probably due to diphenylhydantoin activation of lysosome-bound enzymes, and to a decrease of thiamine pyrophosphatase activity in Purkinje cells (Lettmann *et al.,* 1987).

Hitchcock and Gabra-Sanders (1977) reported the effect of diphenylhydantoin upon gamma aminobutyric acid (GABA) and succinic dehydrogenase levels in rat Purkinje
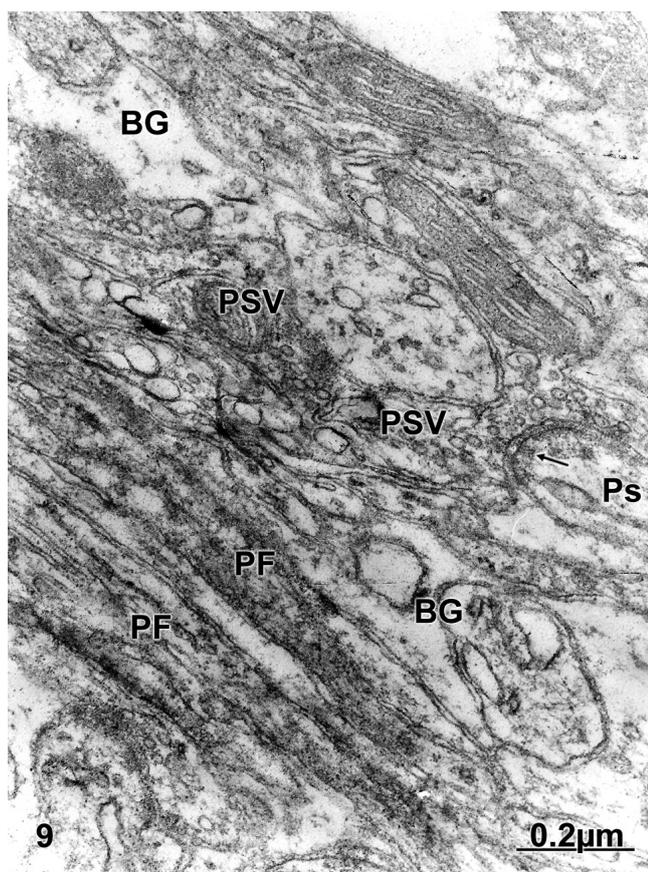
FIGURE 9. Molecular layer. Swollen synaptic varicosities of parallel fibers **(PSV)** making asymmetric synaptic contact (arrow) with clear and edematous Purkinje dendritic spines **(Ps)** in the molecular layer. The non-synaptic segments of a parallel fiber **(PF)** and the surrounding swollen Bergmann glial cell cytoplasm **(BG)** also are distinguished.

cells, and found a significant progressive loss of GABA with increasing dosage of diphenylhydantoin. These earlier studies are in agreement with the cytotoxic edema reported in the present study.

Bergmann glial cells showed a remarkable edema after diphenylhydantoin treatment, as illustrated in our electron micrographs, which indicates a severe disruption of the Purkinje cell-Bergmann glial cell unit. Bergmann glial cells contain a large repertoire of transmitter receptors, such as glutamate receptors and transporters (Kainate/Ampa receptors, NMDA receptors) and GABA receptors, allowing them to sense the activity of neighboring synapses (Somogyi *et. al.,* 1990; Chaudry *et al.,* 1995, *et al.,* Castejón, 2002). These receptors have distinct biophysical and pharmacological features activating second-messenger pathways in the Bergmann glial cells. These molecular pathways are altered by diphenylhydantoin action contributing to the pathogenic mechanisms of mouse cerebellar ataxia.

The degenerated climbing fiber and parallel fiber-Purkinje cell dendrite synaptic relationship, and the degenerated

Purkinje cell axon reported herein suggest a diphenylhydantoin-induced decreased in excitability of Purkinje cells at the molecular layer. Cytotoxic edema, damage of Bergmann glial cell-Purkinje cell unit, synaptic degeneration of axodendritic and axospinodendritic junctions, and Purkinje cell axonal degeneration appear as essential components of the pathogenic mechanisms of mouse cerebellar ataxia.

References

Anderson WA, Flumerfelt BA (1980). A light and electron microscopic study of the effects of 3-acetylpyridine intoxication on the inferior olivary complex and cerebellar cortex. *Journal of Comparative Neurology* **190:** 157-174.

Awada A, Amene P, al Jumah M, Beladi K (1999). Residual cerebellar ataxia following acute phenytoin intoxication. *Review of Neurology (Paris)* **155:**306-308.
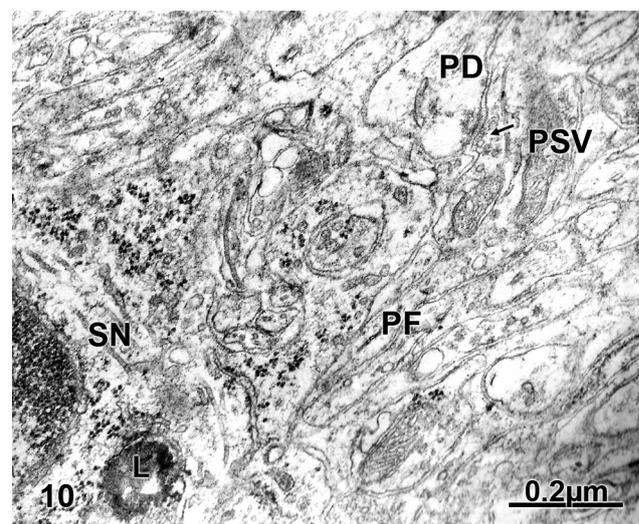
FIGURE 10. Notably edematous stellate neuron **(SN)** showing fragmented microtubules and vacuolated and degenerated lysosome **(L)** in the molecular layer. The neighboring neuropil exhibits the non-synaptic segments of parallel fibers **(PF)**, and their synaptic varicosities **(PSV)** containing few synaptic vesicles (arrow), and making asymmetric axodendritic contact with a Purkinje dendritic spine **(PD)**.

Biggiogera M, Scherini E, Barni S, Bernocchi G (1983). Effects of diphenyl-hydantoin on postnatal ontogenesis in the rat. Ultrastructural analysis of Purkinje neurons and hepatocytes. *Journal of Submicroscopic Cytology* **15:**1027-1040.

Breiden-Arends C, Gullotta F (1981). Diphenylhydantoin, epilepsy, cerebellar atrophy-histological and electron microscope examinations. *Fortschritte der Neurologie-Psychiatrie* **49:** 406-414.

Castejón OJ (1985). Electron microscopic study of central axonal degeneration in traumatic human brain edema. *Journal of Submicroscopic Cytology* **17:** 703-718.

Castejón OJ, Valero, C, Díaz, M (1995). Synaptic degenerative changes in human traumatic brain edema. *Journal Neurosurgical Sciences* **39:** 47-65, 1995.

Castejón OJ, Valero, C, Díaz, M (1997). Light and electron microscopy of nerve cells in traumatic oedematous human cerebral cortex. *Brain Injury* **11:** 363-388.

Castejón OJ (1999). Ultrastructural pathology of Golgi apparatus of nerve cells in human brain edema associated to brain congenital malformations, tumors and trauma. *Journal of Submicroscopic Cytology and Pathology* **31:** 203-213.

Castejón OJ, Dailey ME, Apkarian RP, Castejón HV (2002). Correlative microscopy of cerebellar Bergmann glial cells. *Journal of Submicroscopic Cytology and Pathology* **34:** 131-142.

Castejón OJ (2004a) Structural pattern of injured mitochondria in oedematous human cerebral Castejón cortex. *Brain Injury* **18:** 1107-1126.

Castejón, OJ (2004b). Lysosome abnmormalities and lipofucsin content of nerve cells of oedematous human cerebral cortex. *Journal of Submicroscopic Cytology and Pathology* **36:** 263-271.

Castejón OJ (2011). Contributions of Golgi apparatus and myelinated axon derangement to the synaptic degeneration in traumatic human brain edema. An hypothesis. *Acta Microscopica* **20:** 91-92.

Castejón OJ (2012). Correlative microscopy of Purkinje cells. *Biocell* **36:**1-29.

Fonnum F, Lock EA (2000). Cerebellum as a target for toxic substances. *Toxicology Letter.* **112:** 9-16.

Dam M. (1970a). Number of Purkinje cells in patients with grand mal epilepsy treated with diphenylhydantoin. *Epilepsia* **11:** 313-320.

Dam M (1970b). The number of Purkinje cells after diphenylhydantoin intoxication in monkeys. *Epilepsia* **11:**199-205.

Dam M (1970c). Number of Purkinje's cells after diphenylhydantoin intoxication in pigs. *Archives of Neurology.* **22:** 64-67.

Hitchcock E, Gabra-Sanders T (1977). Effect of diphenylhydantoin on gamma aminobutyric acid (GABA) and succinate activity in rat Purkinje cells. *Journal of Neurolology. Neurosurgery and Psychiatry* **40:** 565-569.

Imamura T, Ejima A, Sahara M, Saito H, Tsuburaya K (1992). Cerebellar atrophy and persistent cerebellar ataxia after acute intoxication of phenytoin. *No To Shinkei.* **44:**149-153.

Iwasaki Y, Mori K, Ito M, Tatsumi S, Mimuro M, Yoshida M (2013). An autopsied case of progressive supranuclear palsy presenting with cerebellar ataxia and severe cerebellar involvement. *Neuropathology* **33:**561-567.

Jeong YG, Hyun BH (2000). Abnormal synaptic organization between granule cells and Purkinje cells in the new ataxic mutant mouse, pogo. *Neuroscience Letter* **294:** 77-80.

Kiefer R, Knoth R, Anagnostopoulos J, Volk B. 1(989). Cerebellar injury due to phenytoin. Identification and evolution of Purkinje cell axonal swellings in deep cerebellar nuclei of mice. *Acta Neuropathology* **77:** 289-298.

Lettmann E, Rune GM, Korfsmeier KH (1987). Neurotoxicity of diphenylhydantoin (DPH) in the cerebellum of Mongolian gerbils. An enzyme histochemical study. *Journal of Environmental Pathology Toxicology and Oncology* **7:** 17-26.

Ohmori H, Yamashita K, Hatta T, Yamasaki S, Kawamura M, Higashi Y, Yata N, Yasuda M (1997). Effects of low-dose phenytoin administered to newborn mice on developing cerebellum. *Neurotoxicology and Teratology* **19:** 205-211.

Pulliainen V, Jokelainen M, Hedman C, Pammo O (1997). Cerebellar damage caused by phenytoin intoxication. *Duodecim* **113:** 2309-2313.

Puro DG, Woodward DJ (1973). Effects of diphenylhydantoin on activity of rat cerebellar Purkinje cells. *Neuropharmacology* **12:** 433-440.

Takeichi M (1981). Neurobiological study of experimental diphenylhydantoin intoxication. I. Electron microscopic investigations of the rat cerebellum with chronic diphenylhydantoin intoxication. *Folia of Psychiatry and Neurology Japan* **35:** 487-99.

Takeichi M (1983). Neurobiological studies of experimental diphenylhydantoin intoxication-III. Electron microscopic studies on development and disintegration mechanism of altered axon terminals and synaptic endings in rat cerebellum with chronic diphenylhydantoin intoxication. *Folia of Psychiatry and Neurology Japan* **37:** 455-464.

Tashiro N, Takeichi M, Matsushima M, Kono R (1982). Neurobiological studies of experimental diphenylhydantoin intoxication. II. Electrophysiological and electronmicroscopic studies of the rat cerebellum with chronic diphenylhydantoin intoxication. *Folia of Psychiatry and Neurology Japan* **36:** 421-430.

Tauer U, Knoth R, Volk B. (1998). Phenytoin alters Purkinje cell axon morphology and targeting in vitro. *Acta Neuropathologica* **95:** 583-591.

Villa AM, Sica RE (1994). Persistent cerebellar ataxia after toxic administration of diphenylhydantoin. *Arquivos de Neuropsiquiatria* **52:** 572-574.

Volk B, Kirchgässner N (1985). Damage of Purkinje cell axons following chronic phenytoin administration: an animal model of distal axonopathy. *Acta Neuropathologica* **67:** 67-74.