Virchows Archiv A Pathol Anat (1991) 419:403–408

*Virchows Archiv A*
*Pathological Anatomy and Histopathology*
© Springer-Verlag 1991

# Purkinje cell toxicity of β-aminopropionitrile in the rat

**J.E. Martin, J.A. Sosa-Melgarejo, M. Swash, K. Mather, P.N. Leigh, and C.L. Berry**

Department of Morbid Anatomy, The Royal London Hospital, Whitechapel, London E1 1BB, UK

Received November 15, 1990 / Accepted May 31, 1991

**Summary.** Compounds causing neurolathyrism are putative aetiological agents in neurodegenerative disorders including amyotrophic lateral sclerosis. β-Aminopropionitrile (BAPN) is one such compound. We have administered this lathyrogenic agent at a dose of 1 g/kg by the intraperitoneal route in experiments in adult Sprague-Dawley rats during a period of 10 weeks. The rats developed marked kyphoscoliosis, ataxia with paralysis and muscle wasting of the hind limbs. Vacuolation and loss of Purkinje cells developed, but no anterior horn cell degeneration was noted. Immunohistochemical studies of phosphorylated neurofilaments and the 72 kDa heat shock protein were normal and no intraneuronal ubiquitinated inclusions were seen. High-dose intraperitoneal BAPN in the rat causes Purkinje cell changes, but no other central nervous system abnormalities.

**Key words:** Amyotrophic lateral sclerosis – Cerebellum – β-Aminopropionitrile – Heat shock proteins – Ubiquitin

## Introduction

β-Aminopropionitrile (BAPN) is a toxic compound found in certain species of *Lathyrus*, particularly sweet pea, singletary pea and caley pea (Rao et al. 1964). The compound is teratogenic in fetal rats (Simpson et al. 1987) and because of its effects on collagen metabolism, it has been used as an experimental lathyrogen (Selye 1957; Simpson et al. 1987). BAPN and the metabolically and structurally related compound β-*N*-oxalyl-L-α,β-diaminopropionic acid (BOAA) have been implicated in the development of neurolathyrism (Rao et al. 1967; Roy et al. 1985; Spencer 1987), although recent evidence (Spencer et al. 1986) suggests that the latter compound is much more neurotoxic than BAPN itself. We have studied the effects of intraperitoneal BAPN in the rat in order to address its potential neurotoxicity.

*Offprint requests to:* J.E. Martin

## Materials and methods

One-month-old female Sprague-Dawley rats were maintained singly or in pairs on a standard diet. The animals were weighed and examined daily. Seven animals were innoculated using the intraperitoneal route for 5 days a week with BAPN, as the fumarate (99% purity; Sigma, Poole, Dorset, UK), in doses of 1000 mg/kg. This dose represents a dose equivalent to 20% of the $LD_{50}$ in rats of this age and weight (Wiley and Joneja 1976). This dosage regime was continued for 10 weeks. Seven control animals were treated with equal volumes of intraperitoneal saline. The rats were killed immediately after the end of the experiment by perfusion fixation with 2.5% glutaraldehyde under deep benzodiazepine anaesthesia. Muscle, brains and spinal cords were removed after fixation and post-fixed in formol saline to permit immunohistochemical studies.

Serial sections were taken through the cerebrum, cerebellum, spinal cord, muscle and nerve roots. Cerebellar sections were also taken in the sagittal and horizontal planes in some animals to determine the distribution of Purkinje cells (Fabregues et al. 1985; Ghetti et al. 1985). Tissue was processed from post-fixation in formol saline to paraffin wax in the usual manner. Serial sections for light microscopy were cut at 5 μm and stained using haematoxylin and eosin (H&E), luxol fast blue/cresyl violet, periodic acid-Schiff (PAS) and the Glees and Marsland silver impregnation methods.

Sections for immunohistochemical studies were cut at 5 μm. A standard indirect immunoperoxidase method was used for each antibody (Sternberger 1979). Positive and negative controls were included in which the pattern of staining was known, e.g. sections known to contain ubiquitinated inclusions in anterior horn cells in a case of amyotrophic lateral sclerosis (ALS) (Leigh et al. 1988) and negative controls in which the primary antibody was omitted were included in the histological series. Blocking with normal serum was performed as a control procedure. Sections were incubated with primary antibody overnight at 4° C [except glial fibrillary acidic protein (GFAP) which was incubated for 1 h at room temperature].

The pattern of staining with the following antibodies was studied:

1. mAb 147, a monoclonal antibody with specificity in normal tissue for the highly phosphorylated 210 kDa neurofilament protein which is found in axons but not neuronal cell bodies (Anderton et al. 1982). Diluted 1 in 1.

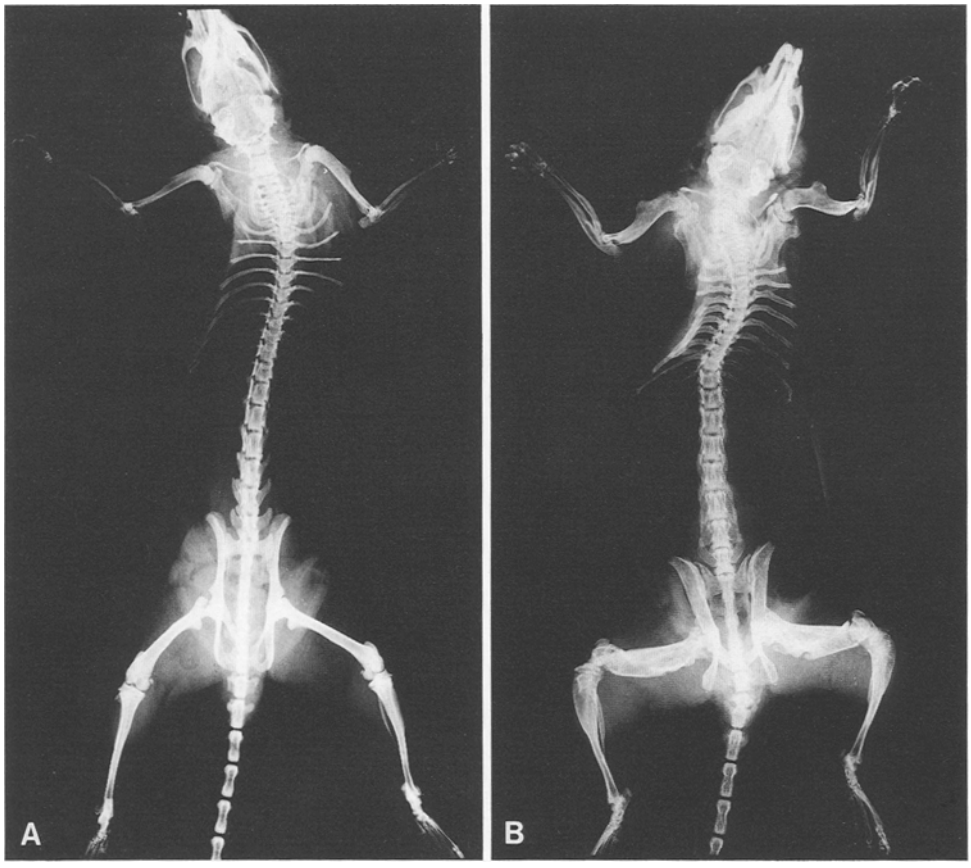



**Fig. 1.** Radiographs of control (**A**) and β-aminopropionitrile (BAPN)-treated (**B**) rats. In the treated animal there is generalised osteoporosis, bowing of the long bones, bone cyst formation near the epiphyses, and a marked thoracic kyphoscoliosis

2. RT 97, a monoclonal antibody with specificity similar to mAb 147, but which also recognises the neurofibrillary tangles in Alzheimer's disease (Anderton et al. 1982). Diluted 1 in 1000.
3. Anti-GFAP, a commercially available monoclonal antibody raised against the bovine glial protein (Dako, UK). This antibody demonstrates astrocytes, but not neurons or microglia. Diluted 1 in 250.
4. Anti-ubiquitin, an affinity-purified polyclonal antibody (Leigh et al. 1988) which demonstrates accumulations of ubiquitin such as those found in association with neurofibrillary tangles in Alzheimer's disease, intraneuronal inclusions in ALS, and other cellular inclusions (Leigh et al. 1988; Manetto et al. 1988). Diluted 1 in 1000.
5. Anti-72 kDa heat-shock protein (HSP), a commercially available monoclonal antibody raised against the purified protein (Ashburner and Bonner 1979; Bond and Schlesinger 1985) (Amersham, UK). Diluted 1 in 800.

## Results

### *Clinical features*

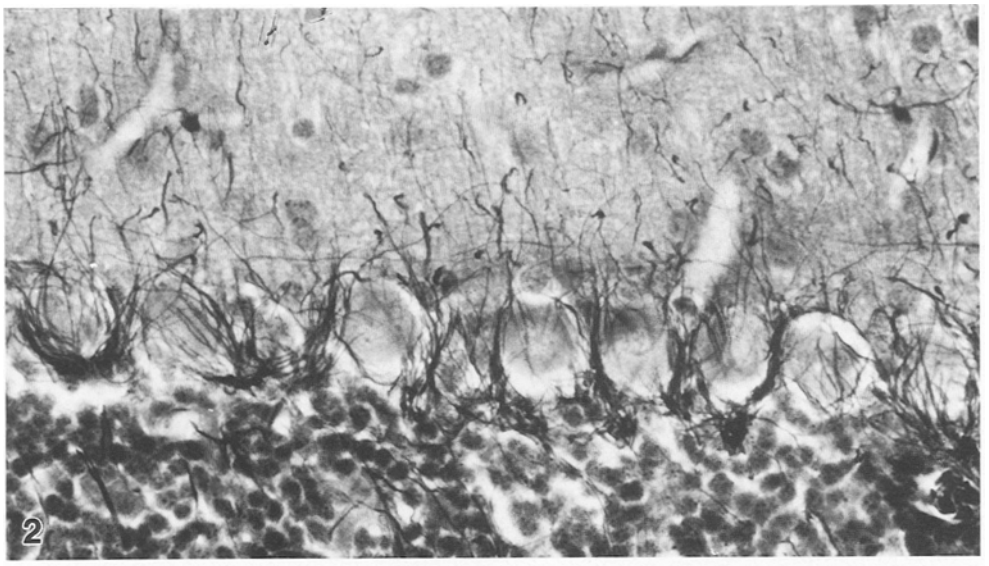
Rats in groups treated with BAPN developed a severe kyphoscoliosis, with ataxia and muscle wasting. Their hind limbs were extended with atrophic weak muscles. These abnormalities were not seen in control animals. X-rays films of the rats, taken at the end of each experiment, showed that the bone deformities of osteolathyrism were prominent in every BAPN-treated rat (Fig. 1). There was hyperostosis, especially in the femur and humerus, with proximal bone cysts. All the treated animals developed severe kyphoscoliosis.

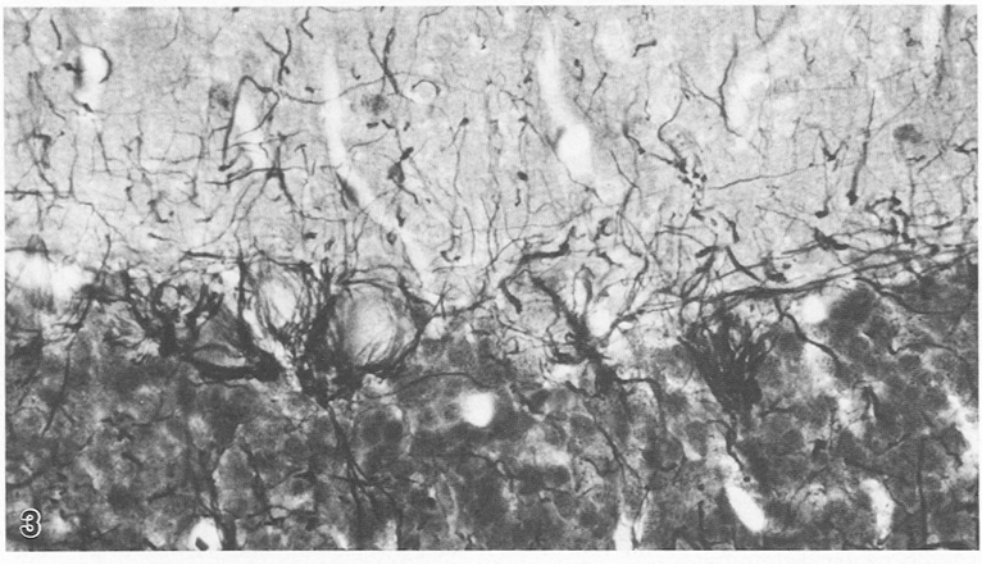
### *Macroscopical findings*

The gross findings noted above were confirmed. No arterial aneurysms were noted. The cerebral cortex appeared normal in size in both treated and untreated animals. There was a pale brown discoloration of the meninges and central nervous system tissue in all treated animals. The spinal cord from treated animals was fixed in an undulating double curve, related to the kyphoscoliosis. There was no necrosis of the cord or haemorrhage into the spinal canal.

### *Light microscopy*

The first microscopical change was osteoporosis of the vertebrae and long bones. The cortex of the vertebral bones was very thin. The trabeculae were also thin and widely separated from each other by haemopoietic bone marrow. Similar changes were observed in long bones. The thickened and cellular periosteum overlaid islands of vascular connective tissue which contained irregular areas of intramembranous new bone. The epiphyseal growth plates were widened and the cartilage cells were disorganised and irregularly aligned. Columns of carti-



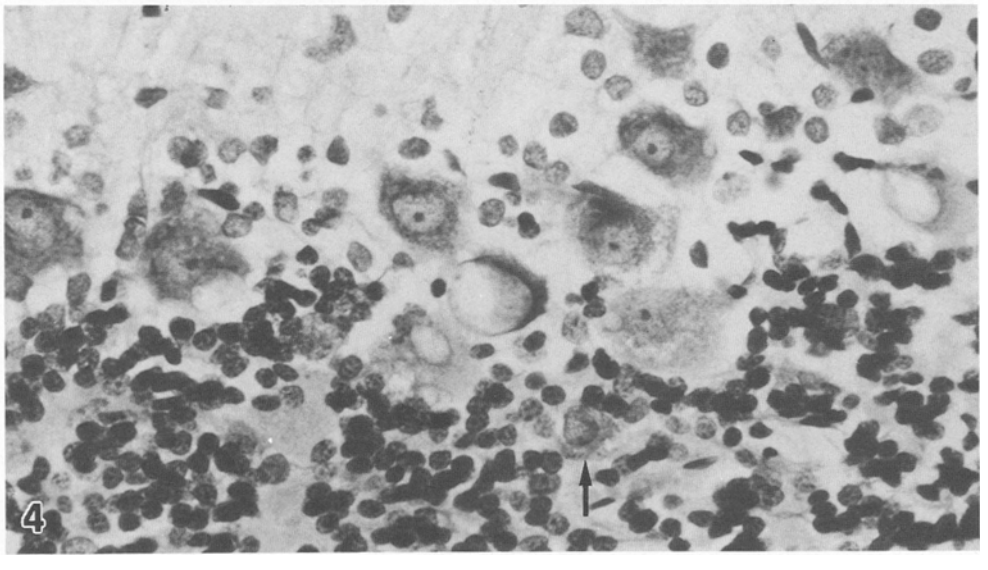




**Fig. 2.** Normal Purkinje cells in the cerebellar vermis of a control animal. Glees and Marsland, ×400

**Fig. 3.** Empty baskets in the cerebellar vermis of an animal treated with BAPN. Glees and Marsland, ×400

**Fig. 4.** Vacuolation of a Purkinje cell and reactive glial cells (*arrowed*) in the cerebellar vermis of an animal treated with BAPN. Glees and Marsland, ×500

lage cells frequently extended deeply into the metaphysis, so that the boundary between metaphysis and epiphysis was irregular. The bodies of the vertebrae at the level of the kyphoscoliosis showed severe wedging and osteoporosis, with no signs of new bone formation. Large masses of fibrous tissue were observed adjacent to and between the vertebrae, so that the intervertebral discs were distorted and narrowed. No aneurysmal dilatation of arteries was noted.

No abnormalities in the cerebral cortex were seen in treated animals or in controls. The spinal cord was normal in all rats, with no evidence of axonal damage secondary to cord compression in treated animals. The cerebellar cortex was of normal thickness in all animals. The molecular layer appeared normal. In treated rats there was an increase in the number of "empty baskets" where basket cells formed processes around an area in which no Purkinje cells were present (Figs. 2, 3). Focal areas of Purkinje cell loss were seen in the vermis, with a reactive astrocytosis and microglial cell activation. Elsewhere in the cerebellum the Bergman glial layer was normal and the granular layer showed no abnormalities. Occasional Purkinje cells in BAPN-treated cells showed vacuolation of their cytoplasm, especially in the vermis (lobules I-III, IX and X) (Fig. 4). Affected cells showed no positivity with PAS methods or abnormal structures in silver impregnations. The content of the vacuoles was not demonstrated by any histochemical method used. The nerve roots were normal and the muscle, despite apparent wasting clinically, showed normal muscle fibres with no features of neurogenic atrophy or fatty replacement.

*Immunohistochemistry*

Staining for phosphorylated neurofilament epitopes with mAb 147 and RT97 showed no abnormalities. No axonal swellings were seen, and no abnormal perikaryal staining was present. No staining for ubiquitin was present in cortical or anterior horn cells. No staining for HSP72 was seen. The GFAP preparations showed a normal complement of astrocytes, with decoration of the reactive astrocytes in the cerebellar vermis of treated animals. No abnormal staining of Purkinje cells was seen using any antibody.

## Discussion

The severe skeletal abnormalities observed in the BAPN-treated rats are similar to those reported by others (Ponseti and Baird 1952; Selye 1957; Coulson et al. 1969). The molecular basis of BAPN-induced osteolathyrism depends on its tendency to bind lysyl oxidase, the enzyme that initiates cross-linking in both collagen and elastin formation (Simpson et al. 1987). The dose of BAPN administered in our experiments was 20% of the $LD_{50}$. BAPN has been shown to accumulate rapidly in brain and cartilage in the rat (Waddell et al. 1974). BAPN is found in the *Lathyrus* species of sweet pea, singletary pea and caley pea (Rao et al. 1964), and has been implicated in human and experimental lathyrism (Rao et al. 1964, 1967; Roy et al. 1985). BAPN is teratogenic in rats and has been suspected to be neurotoxic (Selye 1957; Mato and Uchiyama 1974; Roy et al. 1985; Martyn 1987; Simpson et al. 1987). BAPN is closely related to BOAA, a possible cause of neurolathyrism and perhaps of certain neurodegenerative disorders such as Guamanian motor neuron disease (Spencer 1987; Spencer et al. 1987; Martyn 1987). More recent studies have suggested that BOAA and cycad neurotoxin *β-N*-methylamino-L-alanine (BMAA) are more neurotoxic than BAPN itself (Nunn et al. 1968; Seawright et al. 1990). Ultrastructural abnormalities induced by BAPN are similar to those produced by BMAA (Olney et al. 1976; Seawright et al. 1990).

BAPN is known to cause defects in Schwann cell basal lamina in cell culture (Okada and Bunge 1981; Ninomiya and Kobayashi 1985). However, the clinical relevance of this finding is unknown. This class of compounds is also known to chelate copper, zinc and nickel (Nunn et al. 1989). Ultrastructural abnormalities have been described in the cerebellum of the rat following administration of BAPN (Mato and Uchiyama 1974), with the appearance of lamellar structures in Purkinje cell cytoplasm. In the latter study, however, the dosage of BAPN was lower than in our experiments, and the drug was administered orally rather than by intraperitoneal injection. The ultrastructural changes included proliferation of the Golgi apparatus and the appearance of membranous sacs, stacked to form lamellar structures (Mato and Uchiyama 1974). In our light microscopical study we noted intracytoplasmic vacuoles in Purkinje cells, but these vacuoles did not stain with any of the methods employed, suggesting loss of contents during processing of the tissue, or a fluid composition.

Lamellar structures within Purkinje cells very similar to those induced by BAPN (Mato and Uchiyama 1974) have also been seen at electron microscopy after treatment with BMAA (Seawright et al. 1990) and similar structures have been described in studies of drug and bilirubin toxicity (Snider and Del Carro 1967; Schutta and Johnson 1967; Jung and Suzuki 1978). These structures are thought to represent cisterns of the Purkinje cell endoplasmic reticulum (Seawright et al. 1990). The 72 kDa HSP plays an essential role in the transport of proteins across intracellular membranes, in particular the endoplasmic reticulum (Lindquist and Craig 1988). No localised staining for HSP72 was found in Purkinje cells, suggesting that although the endoplasmic reticulum is reduplicated, the associated wheat shock response is not an aggregated or particulate response as in the "stress granule" formed in reaction to repeated heat shock to cells in culture (Neumann et al. 1987; Collier et al. 1988) or in endothelial cells in vivo in aluminium toxicity (Martin et al. 1991). Ubiquitin is another stress protein that plays a role in the degradation of normal and abnormal proteins via non-lysosomal pathways (Bond and Schlesinger 1985). Ubiquitinated inclusions are seen in the brain and spinal cord in ALS (Leigh et al. 1988) and in other disorders in which certain types of intracellular inclusions are seen (Mannetto et al.

1988). No ubiquitinated inclusions were seen in our BAPN-treated rats. The nature of the cellular damage caused by BAPN must therefore be of a type that does not provoke up-regulation or aggregation of ubiquitin or HSP 72.

BMAA, unlike BAPN, causes Purkinje cell changes with vacuolation of the granular cell layer and of the cerebellar roof nuclei (Seawright et al. 1990). BOAA is a compound derived from *Lathyrus sativus* (Rao et al. 1964). BOAA is an excitatory neurotoxin and convulsant agent resembling glutamate in structure, which is also structurally and metabolically related to BAPN (Rao et al. 1964; Olney et al. 1976). This compound causes brain and retinal damage (Olney et al. 1976) and a flaccid paraplegia with neuronal degeneration in rhesus monkeys when administered intrathecally (Rao et al. 1967). The changes seen after administration of BOAA or BMAA however, are more marked and more typical of an excitatory neurotoxin than the changes induced by BAPN.

In BAPN-treated rats the presence of focal Purkinje cell loss in the cerebellum is unlikely to be responsible for the marked motor system abnormalities seen clinically. The lack of abnormality in the central nervous system outside the cerebellum after BAPN administration raises the possibility that abnormalities of muscular function might have been due to peripheral neuropathy. However, no histological abnormalities were found in peripheral nerves or muscles. Indeed, the forelimbs were relatively unaffected, and it is therefore probable that the weakness was due to the kyphoscoliosis and distal skeletal deformities, resulting in a functional rather than a morphological disturbance.

*Acknowledgements.* J.S.-M. is supported by a grant from CONACYT, Mexico, and holds an award from the Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom. J.E.M. is a Medical Research Council Fellow and holds the British Medical Association Very Down Award and the Gillson Scholarship in Pathology of the Society of Apothecaries of London. We would like to thank the patients, relatives and staff of St. Christopher's Hospice, Mr. J.C. Lewis and Mr. G.J. Martin for their kind help. These data were presented in part to the Swiss Neuropathological Society at a meeting in St. Moritz in 1990.

## References

Anderton BH, Breinburg D, Downes MJ, Green PJ, Tomlinson BE, Ulrich J, Wood JN, Kahn J (1982) Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. Nature 298:84–86

Ashburner M, Bonner JJ (1979) The induction of gene activity in Drosophila by heat shock. Cell 17:241–254

Bond U, Schlesinger MJ (1985) Ubiquitin is a heat shock protein in chicken embryo fibroblasts. Mol Cell Biol 5:949–956

Collier NC, Heuser J, Levy MA, Schlesinger MJ (1988) Ultrastructural and biochemical analysis of the stress granule in chicken embryo fibroblasts. J Cell Biol 106:1131–1139

Coulson WF, Linker A, Bottcher E (1969) Lathyrism in swine. Arch Pathol 87:411–417

Fabregues I, Ferrer I, Gairi JM, Cahuana A, Giner P (1985) Effects of prenatal exposure to ethanol on the maturation of the pyramidal neurons in the cerebral cortex of the guinea-pig: a quantitative Golgi study. Neuropathol Appl Neurobiol 11:291–298

Ghetti B, Musicco M, Norton J, Bugiani O (1985) Nerve cell loss in the progressive encephalopathy induced by aluminium powder. A morphologic and semi-quantitative study of the Purkinje cells. Neuropathol Appl Neurobiol 11:31–53

Jung HJ, Suzuki K (1978) Morphological changes in the CNS of rats treated with perhexiline maleate (pexid). Acta Neuropathol (Berl) 42:159–164

Leigh PN, Anderton BH, Dodson A, Gallo J-M, Swash M, Power DM (1988) Ubiquitin deposits in anterior horn cells in motor neuron disease. Neurosci Lett 93:197–203

Lindquist S, Craig EA (1988) The heat shock proteins. Annu Rev Genet 22:631–677

Manetto V, Perry G, Tabaton M, Mulvihill P, Fried VA, Smith HT, Gambetti P, Autilio-Gambetti L (1988) Ubiquitin is associated with abnormal cytoplasmic filaments characteristic of neurodegenerative diseases. Proc Natl Acad Sci USA 85:4501–4505

Martin JE, Mather K, Swash M, Dodd SE, Dale GE, Garofalo O, Leigh PN (1991) Stress protein inclusions in cerebral vessels in dialysis encephalopathy. Neuropathol Appl Neurobiol 17:105–111

Martyn C (1987) Neurological clues from environmental neurotoxins. Br Med J 295:346–347

Mato M, Uchiyama Y (1974) Appearance of lamellar structures in the Purkinje cells of rat cerebellum after administration of β-aminopropionitrile. Experientia 30:1030–1032

Neumann D, Nieden U zur, Manteuffel R, Walter G, Scharf K-D, Nover L (1987) Intracellular localisation of heat-shock proteins in tomato cell cultures. Eur J Cell Biol 43:71–81

Ninomiya T, Kobayashi EO (1985) Studies of deficiency of Schwann cell basal lamina and deformation of collagen fibres induced by beta-aminopropionitrile in cultures of developing rat peripheral neurons. J Anat 143:1–15

Nunn PB, Vega A, Bell EA (1968) Neurotoxic effects of α-amino-β-methyl-aminopropionic acid. Biochem J 106:15

Nunn PB, O'Brien P, Pettit LD, Pyburn SI (1989) Complexes of zinc, copper and nickel with the nonprotein amino acid L-α-amino-β-methylaminopropionic acid: a naturally occuring neurotoxin. J Inorg Chem 37:175–183

Okada E, Bunge RP (1981) Basal lamina deficiency in Schwann cells induced by beta-aminopropionitrile (BAPN) in rat dorsal root ganglion culture. Brain Res 226:326–333

Olney JW, Misra CH, Rhee V (1976) Brain and retinal damage from lathyrus excitotoxin, β-*N*-oxalyl-L-α,β-diaminopropionic acid. Nature 264:659–661

Ponseti I, Baird WA (1952) Scoliosis and dissecting aneurysm of the aorta in rats fed with lathyrus odoratus seeds. Am J Pathol 28:1059–1077

Rao SLN, Adiga PR, Sarma PS (1964) Isolation and characterisation of β-oxalyl-L-alpha,beta-diaminopropionic acid; a neurotoxin from the seeds of *Lathyrus sativus*. Biochemistry 3:432–436

Rao SLN, Sarma PS, Mani KS, Ragunatha Rao TR, Sriramachari S (1967) Experimental neurolathyrism in monkeys. Nature 214:610–611

Roy DN, Spencer PS, Nunn PB (1985) Toxic components of *Lathyrus*. In: Kaul AK, Combes D (eds) Proceedings of the International Symposium on LAthyrus and Lathyrism, Universite de Pau et des Pays de l'Adour, September 1985. Third World Medical Research Foundation, New York, pp. 287–296

Schutta HS, Johnson L (1967) Bilirubin encephalopathy in the Gunn rat: a fine structure study of the cerebellar cortex. J Neuropathol Exp Neurol 26:377–396

Seawright AA, Brown AW, Nolan CC, Cavanagh JB (1990) Selective degeneration of cortical neurons caused by cycad neurotoxin, L-β-methylaminoalanine (L-BMAA), in rats. Neuropathol Appl Neurobiol 16:153–169

Selye H (1957) Lathyrism. Rev Can Biol 16:1–82

Simpson MN, Freeman SJ, Steele CE (1987) Embryotoxic action of β-aminopropionitrile during early organogenesis in rats. Arch Toxicol [Suppl] 11:140–142

Snider RS, Del Carro MP (1967) Drug induced dendritic sprouts on Purkinje cells in the adult cerebellum. Exp Neurol 17:466–480

Spencer PS (1987) Guam ALS/Parkinson-dementia: a long latency neurotoxic disorder caused by 'slow toxin(s)' in food? Can J Med Sci 14:347–357

Spencer PS, Roy DN, Ludolph A, Hugon J, Dwivwdi MP, Schaumberg HH (1986) Lathyrism: evidence for the role of neuroexcitatory aminoacid BOAA. Lancet II:1066–1067

Spencer PS, Nunn PB, Hugon J, Ludolph AC, Ross SM, Roy DN, Robertson RC (1987) Guam amyotrophic lateral sclerosis linked to a plant excitant neurotoxin. Science 237:517–521

Sternberger LA (1979) Immunocytochemistry, 2nd edn. Wiley, New York

Waddell WJ, Wilk AL, Pratt RM, Steffek AJ (1974) Whole-body autoradiography of the distribution of $\beta$-aminopropionitrile in pregnant rats and rat fetuses. Teratology 9:211–216

Wiley MJ, Joneja MG (1976) The teratogenic effects of beta-aminopropionitrile in hamsters. Teratology 14:13–52