

NEUROPATHOLOGICAL ASSESSMENT IN ACUTE NEUROTOXIC STATES. THE “DARK” NEURON

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Pathological examination of the nervous system is an important component of neurotoxicology, but features of the brain and spinal cord can make this assessment challenging. Among these are the cellular complexity, regional variation of structure and function and multiplicity of reactions to injury of these organs. In addition, there is a propensity for histological artifacts to occur in nervous system tissue samples without scrupulous attention to dissection, sampling, fixation, processing, sectioning and staining of this material. Since artifacts are so common, neurotoxicologists need to be aware of their nature, and avoid misinterpreting these often confusing changes.

Prominent among the histological neuronal artifacts is the so-called “dark” neuron, a not uncommon finding in histological sections of brain and spinal cord (Graeber *et al.* 2002; Summers *et al.* 1995). By light microscopy, these cells are shrunken, and have intense (dark) staining of the perikaryal, dendritic and axonal cytoplasm (hence the appellation “dark” neuron) (figure 1). Dendrites may have an irregular, corkscrew appearance. In the cell body, the shrunken, dark-stained nucleus often blends into the compacted perikaryal cytoplasm. Affected neurons may be separated from adjacent neuropil, and are often scattered among their histologically intact fellows. This neuronal artifact nature has long been recognized. In 1903, Turner produced such cells by post-mortem compression of spinal ganglia of normal animals. Scharrer (1938) indicated that “dark” neurons were related to pressure on fresh (unfixed) brain during removal from the skull at necropsy. The issue was highlighted in a series of papers by Cammermayer (1960, 1961, 1972, 1978). This body of work demonstrated that the “dark” neuron artifact was produced by post-mortem trauma either prior to fixation or following inadequate perfusion-fixation of the brain. This post-mortem trauma can be quite subtle and the related histological artifact evolves quite rapidly (Cammermayer, 1961). The change is clearly related to a contraction process in a perturbed neuron at the time of fixation (Auer and Sutherland, 2002), whose biochemical basis is not fully defined. Some of the suggestions for this contraction include post-mortem mechanically-induced tissue depolarization, glucose deprivation, disruption of neuronal membranous attachments allowing shrinkage during subsequent dehydration for paraffin embedding and reduced cytoplasmic osmolarity (Auer and Sutherland, 2002; Cammermayer, 1960, 1972, 1978).

Readers of the Journal of Medical Chemical, Biological and Radiological Defense may wonder as to the need to review such an arcane bit of neuropathology. What such an audience should have no doubt about is the need for careful histopathological evaluation of

neuronal lesions evolving following exposure to convulsion-inducing doses of anticholinesterase nerve agents such as soman (pinacolyl methylphosphonofluoridate) or sarin (isopropyl methylphosphonofluoridate). Such dosages of these organophosphorus nerve agents elicit a sequence of events leading to acute neuronal necrosis. Solberg and Belkin (1997) indicate that these include inhibition of brain acetylcholinesterase, excess acetylcholine, over-stimulation of muscarinic receptors triggering seizures in susceptible regions, recruitment of non-cholinergic systems (especially glutaminergic pathways) and release of excess glutamate with damage to regional neurons. Such glutamate-induced neuronal necrosis is mediated via *N*-methyl-d-aspartate (NMDA) receptor activation, with resulting alteration of ion and water fluxes, diminished cell energy metabolism, elevation of cytosolic free calcium ions and resulting activation of catabolic enzymes (Solberg and Belkin, 1997). Thus, an important effect of such seizure-inducing exposures is rapidly evolving neuronal death in susceptible brain regions. There is a relationship of the severity of neuronal injury and the degree and duration of toxicant-induced convulsions (Shih *et al.*). Since such acute neuronal changes may be subtle, critical histological examination of brain sections is essential in determining the nature and extent of such changes.

A number of papers describe brain lesions in rats following exposure to 1LD₅₀ or more of sarin or soman, dosages producing prolonged, profound seizures (Le Mercier *et al.* 1983; Kadar *et al.* 1995; McLeod *et al.* 1984). Study of hematoxylin and eosin-stained sections of the brains of such rats 24 hours after dosing revealed a rather consistent spectrum of lesions. These included shrunken neurons with eosinophilic perikaryal cytoplasm, pyknotic basophilic nuclei and the presence of spongiform (microvacuolated) changes in the adjacent neuropil (Le Mercier *et al.* 1983; Kadar *et al.* 1995; McLeod *et al.* 1984). Such changes are indicative of acute neuronal death, and are consistent with neuronal necrosis as seen in early stages of ischemic, hypoxic/ischemic, hypoglycemic and excitotoxic states (Auer and Sutherland, 2002; Graeber *et al.*, 2002; Summers *et al.*, 1995). In the soman or sarin exposed rats these lesions were most severe in the cerebral cortex, especially in layer III and in the pyriform region, and in the hippocampus, mainly affecting the pyramidal cells of CA1 and CA3 (Le Mercier *et al.* 1983; Kadar *et al.* 1995; McLeod *et al.* 1984).

The “dark” neuron artifact may complicate interpretation of nerve agent-induced neuronal necrosis. In my view, one example of this is the report of Abdel-Rahman *et al.* (2002), which describes such “neuropathological” events following acute exposure of rats to sarin. A dosage of sarin administered in this study elicited rapidly evolving convulsions lasting approximately three hours, which as noted in the neuropathologic studies cited above, could well give rise to acute neuronal necrosis. They reported acute neurotoxic effects such as inhibition of brain acetylcholinesterase and plasma butyrylcholinesterase, compromise of the blood-brain-barrier, along with what is described as extensive neuronal degeneration in the brains of rats 24 hours after a single intramuscular exposure to 1 LD₅₀ of sarin. Affected neurons were stated to have eosinophilic staining of the cell body and proximal dendrites, and were noted in cerebral cortical, thalamic, hippocampal and cerebellar cortical regions. However, the images of putative degenerating/dying neurons in photomicrographs of hematoxylin and eosin stained brain sections shown by Abdel-Rahman *et al.* (2002) did not demonstrate the neuronal shrinkage, cytoplasmic eosinophilia, nuclear pyknosis and surrounding spongiosis which characterized the nerve agent-induced acute neuronal necrosis

(Le Mercier *et al.* 1983; Kadar *et al.* 1995; McLeod *et al.* 1984). Instead, these neurons appeared as basophilic, contracted cells in the absence of neutrophil vacuolization, and were entirely consistent with “dark” neuron artifact described above (figure 1). Admittedly, this interpretation does not explain the restriction of such changes to sarin-exposed animals as reported by Abdel-Rahman *et al.* (2002).

To support the contention that these cells are artifactual, I have produced similar “dark” neurons in normal male Sprague-Dawley rats by manual manipulation of unfixed brains obtained shortly after euthanasia (figures 1 and 2). This replicates the work of Cammermayer (1960, 1961). These “dark” neurons are quite similar to the neuronal changes illustrated in sarin-exposed rats reported by Abdel-Rahman *et al.* (2002). An example is changes in the cerebellar cortical “dark” Purkinje neurons generated in my control rats, which closely resemble the cells described as undergoing degeneration following exposure to sarin by Abdel-Rahman *et al.* (2002). To illustrate this similarity, I have included a relevant photomicrograph from the Abdel-Rahman (2002) paper and contrast it with one of “dark” neurons generated in a control rat (figure 2). Interestingly, the cerebellar Purkinje cells were relatively spared in other studies of sarin- or sarin-induced acute neuronal injury in rats (Le Mercier *et al.* 1983; Kadar *et al.* 1995; McLeod *et al.* 1984). My contention is that not every histologically observed contracted, darkly stained brain cell is a necrotic neuron, even in an experimental system that should elicit such a change. The “dark” neuron artifact is a common finding. It needs to be considered by both investigators and editorial reviewers when interpreting acute neuronal injury, to help provide unambiguous interpretations of the neurotoxic potential of chemicals.

FIGURES

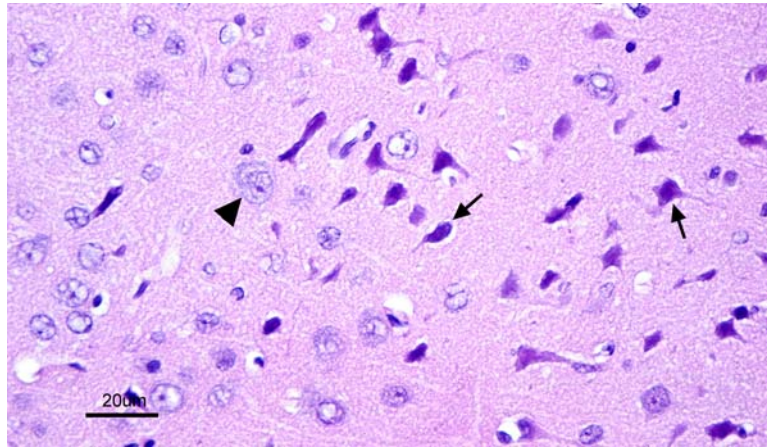


Figure 1. Many intensely stained “dark” neurons are present in the cerebral cortex of a control rat, following manipulation of the unfixed brain. Note the shrunken dark blue stained (basophilic) cell bodies and proximal dendrites in these cells (arrows). Adjacent histologically intact neurons are present (arrowhead). Hematoxylin and eosin stain.

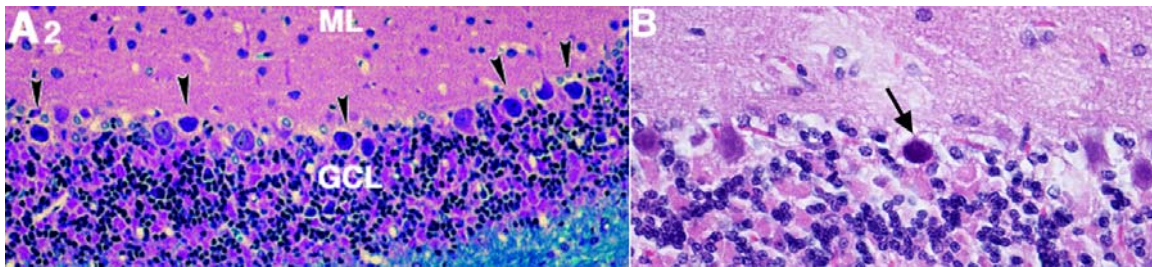


Figure 2A. Cerebellar cortex of a rat from the study of Abdel-Rahman *et al.* (2002), which had been dosed with 1 LD₅₀ of sarin 24 hours prior to sacrifice. The arrowheads indicate contracted, dark-staining cell bodies described as “degenerating” Purkinje neurons (reprinted from Neuroscience, volume 113, Abdel-Rahman, A., Shetty, A.K. and Abou-Donia, M.B., Acute exposure to sarin increases blood brain barrier permeability and induces neuropathological changes in the rat brain: dose-response relationships, figure 15A2, 2002, with permission from Elsevier).

Figure 2B. Cerebellar cortex of a normal rat subject to post-mortem manipulation of brain tissue prior to fixation. A similar contracted, dark-staining Purkinje cell is seen (arrow). Hematoxylin and eosin stain (both images, 2A and 2B).

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