

Commentary-Forum Position Paper

The return of the dark neuron. A histological artifact complicating contemporary neurotoxicologic evaluation

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Received 22 December 2005; accepted 2 March 2006

Available online 22 March 2006

Abstract

A common histological artifact of brain is the presence of contracted, intensely stained neurons. Such cells are termed dark neurons, and can be produced by post-mortem manipulation or trauma in brain tissue. In a number of recently published experimental neurotoxicology reports cells of this nature are interpreted as representing dying or degenerating neurons, thus assigning inappropriate neurotoxic potential to the compounds administered. The latter are the commonly used drugs and pesticides DEET (*N,N*-diethyl *m*-toluamide), malathion, permethrin and pyridostigmine bromide. In this paper I review the nature and genesis of the dark neuron, critically discuss the neuropathologic validity of indicated studies and make a plea for increased awareness of this common artifact.

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Keywords: Dark neuron; Neurotoxicology; Artifact; Neuropathology

Neurotoxic effects of chemicals and drugs in experimental animals have been evaluated by a number of approaches including clinical assessment, neurochemistry, neurophysiology and neuropathology. The latter, comprising microscopically assessed morphological and immunohistochemical changes, is a foundation of neurotoxicology, and has long been employed to enhance the understanding of chemical injury to the nervous system. In addition, regulatory agencies require detailed neuropathologic evaluation in animal studies as part of their assessment of chemical agents to ensure the public safety (OECD, 2004; USEPA, 1998a,b). While pathologic examination of the nervous system is an important component of neurotoxicology, features of the brain (and spinal cord) can make this assessment challenging. Among these are its cellular complexity, regional variation in structure and function, and the multiplicity of reactions to injury. In addition, there is a propensity for histological artifacts to occur in nervous system tissue samples unless scrupulous attention is paid to dissection, sampling, fixation, processing, sectioning and staining of this material. Since artifacts are so common, neurotoxicologists and pathologists need to be aware of their nature, and avoid misinterpreting these often confusing spurious changes as real lesions.

Prominent among the histological neuronal artifacts is the so-called dark neuron. Recent neuropathology textbooks and review papers have drawn attention to this common artifact, including the problem of its being confused with real lesions of the neuronal cell body (Fix and Garman, 2000; Summers et al., 1995). By light microscopy, these cells have a monotonous appearance, being shrunken with an enhanced affinity for histologic dyes (hence the appellation ‘dark neuron’). They are most often basophilic, having a dark blue color of the perikaryal and dendritic cytoplasm with the commonly employed hematoxylin and eosin (H&E) stain (Figs. 1 and 2). At times slight eosinophilia may be superimposed (Summers et al., 1995), giving a dark blue–red tint with the H&E stain. Apical dendrites may have an irregular, corkscrew-shaped appearance (Fig. 2). The shrunken, dark-stained nucleus may be indistinct within the cell body, since it blends into the compacted perikaryal cytoplasm (Fig. 2). Affected neurons may be separated from adjacent neuropil, especially with paraffin-embedded tissue (Cammermeyer, 1978), and are often scattered among their histologically intact fellows (Figs. 1 and 2).

This dark neuron artifact has long been recognized. Turner (1903) produced such cells by post-mortem compression of spinal ganglia of normal animals (dog and cat). Scharrer (1938) indicated that this neuronal hyperchromaticity was related to pressure on fresh (unfixed) rat and

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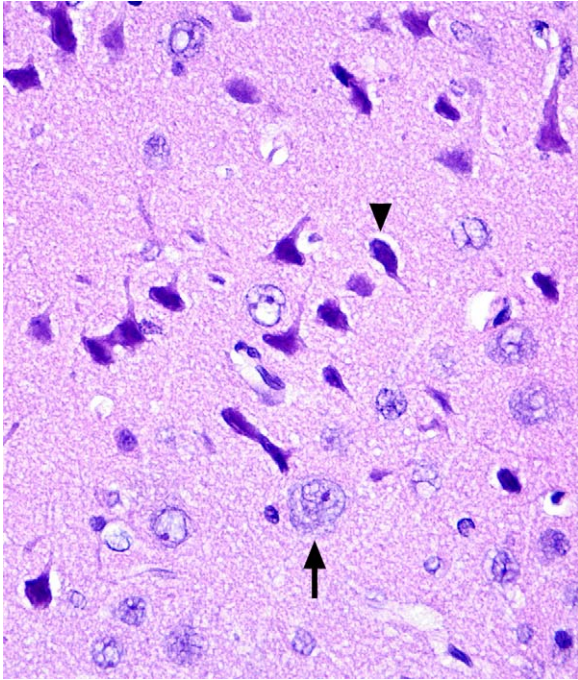


Fig. 1. Cerebral cortex of a normal rat. The brain was removed after CO₂ euthanasia, manually compressed and then immersion-fixed in 10% neutral buffered formalin. Numerous dark neurons are present (arrowhead), adjacent to histologically intact ones (arrow). Hematoxylin and eosin stain.

opossum brain during removal from the skull at necropsy. The issue was highlighted in a series of papers by Cammermeyer (1960, 1961, 1962, 1972, 1978). This body of work demonstrated that the dark neuron artifact could be readily produced in animals such as pigeons, mice, rats, guinea pigs, squirrels, rabbits, cats, dogs, and monkeys by post-mortem manipulation or trauma either prior to fixation or following inadequate perfusion–fixation of the brain (Fig. 2). While the perfusion–fixation is the method of choice in experimental neuropathology, adequately perfused material may still contain dark neurons if sufficient time (several hours) is not allowed between perfusion and brain removal (Cammermeyer, 1960, 1978) (Fig. 2). The post-mortem manipulation of the brain which gives rise to the dark neuron artifact can be subtle and the related histological artifact evolves rapidly (Cammermeyer, 1961). The change is clearly related to a contraction process in a perturbed neuron at the time of fixation (Auer and Sutherland, 2002), the biochemical basis of which is not fully defined. Some of the suggestions for this contraction include post-mortem mechanically induced tissue depolarization, glucose deprivation, disruption of peri- and intra-neuronal attachments allowing shrinkage during fixation and subsequent dehydration for paraffin embedding, and reduced cytoplasmic osmolarity (Auer and Sutherland, 2002; Cammermeyer, 1960, 1962, 1972, 1978). Dark neurons are seen in human cerebral cortical biopsy specimens, related to surgical manipulation during tissue resection (Kepes et al., 1995).

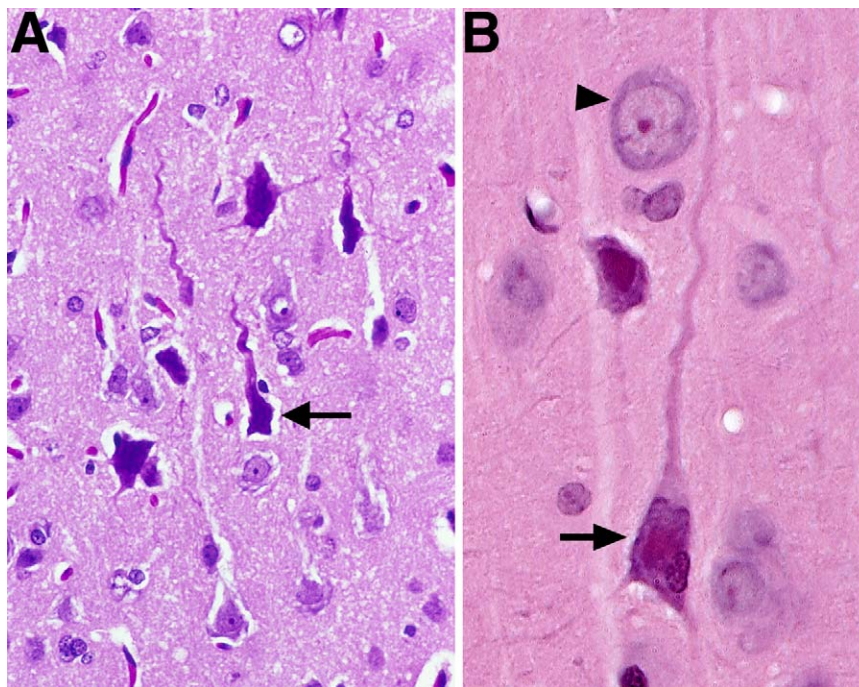


Fig. 2. (A) Several dark neurons are present in the cerebral cortex of this immersion-fixed normal rat brain, induced during removal of the organ from the skull. Features of these cells such as the shrunken, hyperchromatic (darkly stained) basophilic perikaryon and prominent corkscrew-shaped dendrite are noted in affected cells (arrow). The latter are separated from adjacent brain parenchyma (neuropil). Hematoxylin and eosin stain. Reproduced from Fix and Garman (2000) with permission of the Society of Toxicologic Pathologists. (B) Two dark neurons are noted in this perfusion-fixed normal rat cerebral cortex. Hyperchromatic, shrunken perikaryon and a corkscrew-shaped dendrite are seen in one cell (arrow). The appearance of the cell body is distinctly different from that of a normal neuron (arrowhead). Hematoxylin and eosin stain. Image courtesy of Dr. Robert Garman.

Unfortunately this arcane bit of pathologic lore has become pertinent to contemporary neurotoxicology. A recent series of papers have reported this artifact as representing “degenerating or dying neurons” in rats exposed to commonly employed compounds of differing chemical structure and modes of action. These include the Type I pyrethroid insecticide permethrin (Abdel-Rahman et al., 2001, 2002b, 2004a,b), the insect repellent DEET (*N,N*-diethyl *m*-toluamide) (Abdel-Rahman et al., 2001, 2002b, 2004a,b), the carbamate reversible cholinesterase inhibitor pyridostigmine bromide (Abdel-Rahman et al., 2001, 2002b, 2004a), the organophosphate anticholinesterase insecticide malathion (Abdel-Rahman et al., 2004b), the triazole fungicide tebuconazole (Moser et al., 2001), and the organophosphate nerve agent sarin (Abdel-Rahman et al., 2002a). The series of papers by Abdel-Rahman et al. (2001, 2002b, 2004a,b) were largely based upon study of rat brains which had been perfusion-fixed using 4% paraformaldehyde and 0.1% glutaraldehyde, paraffin-embedded, sectioned and stained with H&E. The latter was supplemented in some of these studies by the glial fibrillary acidic protein (GFAP), microtubule associated protein 2 (MAP-2) and silver degeneration staining procedures (Abdel-Rahman et al., 2001, 2002a,b, 2004a). The issue of reported dark neurons in sarin-exposed rats (Abdel-Rahman et al., 2002a) has been addressed by Jortner (2005) and is not further considered here. Relative to apparent misinterpretation of such cells in the other cited studies, Abdel-Rahman et al. (2001) report that daily dermal application of DEET at 40 mg/kg, permethrin at 0.13 mg/kg or a combination of both compounds for 60 days produced “a diffuse neuronal death in the motor cortex, different subfields of the hippocampal formation and the Purkinje cell layer of the cerebellum.” More active putative

neuronal degeneration/death was seen with exposure to the individual compounds than to the combination, which was interpreted by the investigators to mean that the latter dosing scheme led to earlier cell death, largely completed at sacrifice. The “degenerating” or “dying” neurons are described as having “dense eosinophilic staining of the cell body and proximal dendrites.” However, study of photomicrographs from the paper raises questions as to whether the authors are in fact observing truly injured neurons (Abdel-Rahman et al., 2001). Images of H&E-stained sections in the paper demonstrate that these cells are contracted, darkly stained with an indistinct nucleus, and often have a corkscrew-shaped apical dendrite, features entirely consistent with the artifactual dark neuron described above (Fig. 3). Supporting this contention is the fact that morphologically identical neurons can be readily induced by post-mortem manipulation of unfixed normal rat brains (Fig. 3).

A striking feature of the “affected” neurons reported by Abdel-Rahman et al. (2001) is that they have a consistent morphology (Fig. 3), and thus lack the expected sequence of degradative evolutionary changes seen when the cells were dying due to toxicant exposure over a 60-day period. In addition, neither an inflammatory response, such as activated microglia, nor vacuolization of surrounding neuropil is seen. These reactions are seen in cases of extensive acute neuronal necrosis, which this paper purports to demonstrate. The extent of neuronal injury presumably related to the exposures indicated is astounding. As an example, reported quantitative data (Abdel-Rahman et al., 2001) shows that some 45% of neurons in layer III of the motor cortex are “dying” in rats dermally exposed to 40 mg/kg/day DEET for 60 days. Amazingly, these animals demonstrate neither clinical deficits

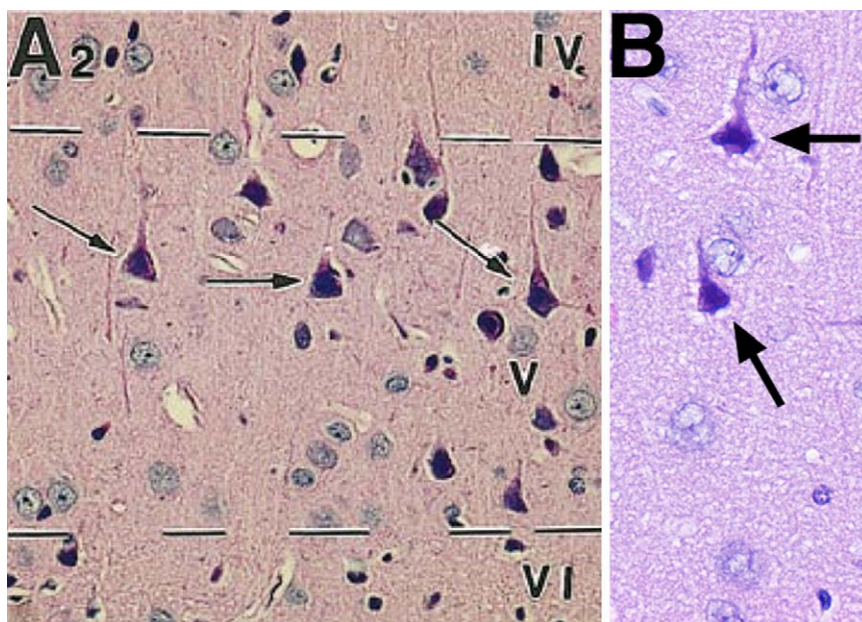


Fig. 3. (A) Deeper layers of the motor cortex from a rat dermally dosed with DEET at 40 mg/kg/day for 60 days, showing cells interpreted as degenerating pyramidal neurons (arrows) by Abdel-Rahman et al. (2001). Hematoxylin and eosin stain. Reproduced from Abdel-Rahman et al. (2001), with permission from Elsevier. (B) Cerebral cortical pyramidal cells showing the dark neuron changes. These were generated by modest post-mortem compression of a normal rat brain, and appear identical to the putative degenerating neurons demonstrated in (A). Immersion-fixation in 10% neutral buffered formalin, hematoxylin and eosin stain.

nor significant alteration in body weight relative to controls over the exposure period (Abdel-Rahman et al., 2001).

In subsequent studies, the same group continues to interpret this histological artifact as neuronal degeneration or death. Abdel-Rahman et al. (2002b, 2004a) report that while 28 daily dosages of pyridostigmine bromide (1.3 mg/kg orally), DEET (40 mg/kg dermally), and permethrin (0.13 mg/kg dermally) did not elicit significant neuronal changes, the addition of 5 min of daily restraint stress made this combination neurotoxic to rats. An important component of this effect was histological changes the authors interpreted as neuronal death, which regionally involved the cerebral cortex, hippocampus, thalamus, hypothalamus and cerebellum. The extent of this change was exemplified by the presence of some 11% of cerebral cortical layer III motor neurons showing the above-noted contracted, hyperchromatic appearance after 30 days of dosing (Abdel-Rahman et al., 2004a). Another study by the same authors reports that daily dermal exposure to malathion (44.4 mg/kg), DEET (40 mg/kg) or permethrin (0.13 mg/kg), alone or in various combinations for 30 days induced this neuronal change in the hippocampus, brainstem and cerebellum of rats (Abdel-Rahman et al., 2004b). As in the 60-day study cited above (Abdel-Rahman et al., 2001), examination of photomicrographs from these papers indicates that the interpretation of toxicant-induced neuronal injury is largely based upon questionable assessment of the histological changes (Abdel-Rahman et al., 2002b, 2004a,b). Specifically, these authors have again considered cells consistent with the common histological dark neuron artifact to represent death of affected cells.

The changes referenced above, which purport to represent neuronal injury and death, do not match any of the classically described histopathological forms of neuronopathy. Use of the adjective ‘eosinophilic’ for cell body staining (Abdel-Rahman et al., 2001, 2002b, 2004a,b) suggests the authors considered these dark neurons to represent ischemic (acidophilic) neuronal necrosis. This is a common response of neurons to conditions such as hypoxia and ischemia, characterized by nuclear pyknosis or karyorrhexis, perikaryal cytoplasmic contraction and affinity for acid dyes, and often vacuolation of adjacent neuropil (Graeber et al., 2002). With the routine hematoxylin and eosin stain, affected perikarya are intensely red, not the more basophilic (blue) stain seen with the dark neuron artifact (Figs. 1, 2 and 4). Acidophilic neurons characteristic of true neurodegeneration thus have features which differ from those demonstrated by Abdel-Rahman et al. (2001, 2002a,b, 2004a,b) (Figs. 3 and 4).

As noted above, neuronal artifacts of this nature are associated with post-mortem pressure on brain tissue, as may occur during removal of the organ from the cranial cavity. This is seen with unfixed tissue or with perfused, but not fully fixed material. Although the animals reported by Abdel-Rahman et al. (2001, 2002a,b, 2004a,b) were, as noted above, transcardially perfused, there is photographic evidence in their papers that fixation was suboptimal in at least some of their animals. Successful systemic perfusion fixes the capillaries and other vessels in a distended state. Examination of many of the

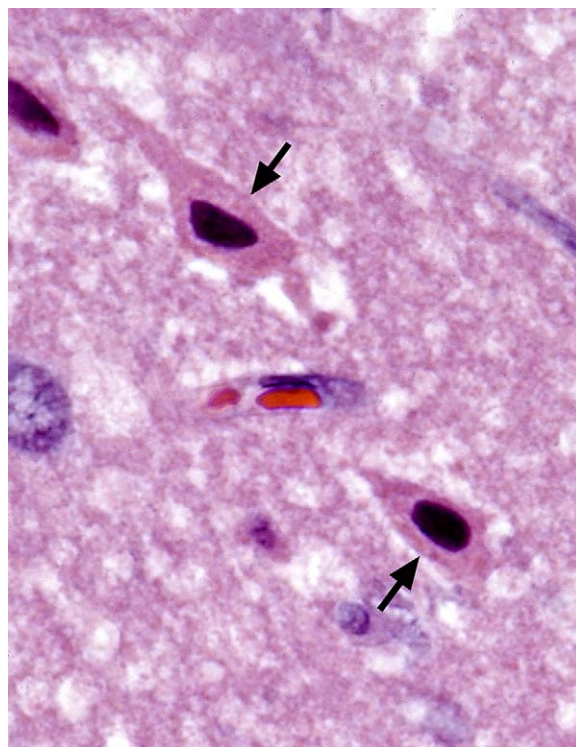


Fig. 4. Acidophilic necrosis of cerebral cortical neurons in a rat exposed to remifentanyl, as reported by Kofke et al. (2002). Note the intense acidophilia (red stain) of the shrunken perikarya and the contrasting basophilia (dark blue stain) of the pyknotic nuclei in two affected cells (arrows). This lesion is clearly different from the dark neuron artifact (Figs. 1 and 2). Hematoxylin and eosin stain. Image courtesy of Dr. Robert Garman.

photomicrographs demonstrates collapsed brain microvessels, which are highly suggestive of inadequate perfusions (Abdel-Rahman et al., 2001, 2002b, 2004a,b). The dark neuron artifact is readily induced without careful removal in suboptimally perfused brains (Cammermeyer, 1961). The significance of other neurohistological findings noted by Abdel-Rahman et al. (2001, 2002b, 2004a), namely alterations demonstrated using MAP-2 or GFAP immunostaining and silver degeneration staining, needs to be carefully interpreted in the presence of the above-noted artifacts.

The issue in question relates to the neuropathologic assessment of neuronal death. As noted above, Abdel-Rahman et al. (2001, 2002b, 2004a,b) have interpreted contracted hyperchromatic (dark) neurons as dying or degenerating in rats given 28–60 days exposures to DEET (40 mg/kg/day dermally), permethrin (0.13 mg/kg/day dermally), malathion (44.4 mg/kg/day dermally) and/or pyridostigmine bromide (1.3 mg/kg/day orally). Since neuronal death is a major, irreversible event, such findings stigmatize these compounds as being highly neurotoxic. This conclusion is not supported by other toxicology studies of these compounds, when using histopathologically detected neuronal necrosis as the endpoint. As examples, rats fed permethrin (40/60 *cis/trans*), a route with greater absorption than the dermal one, at 500, 1000, or 2500 parts per million (ppm) for 2 years had no evidence of neuronal necrosis (Ishmael and Lithfield, 1988). Similar results

were obtained following 3 weeks of daily dermal exposure to permethrin at 100, 300 or 1000 mg/kg/day to rabbits (Lithfield, 1985). Two years feeding studies of malathion to rats at 100, 1000, or 5000 ppm elicited dose-related inhibition of plasma, red blood cell and brain cholinesterase, in the absence of any reported histologically detected neuronal necrosis (Hazleton and Holland, 1953). Detailed neuropathologic examination of the spinal cord, medulla and cerebellum of rats 21 days after a single oral (gavage) exposure to 600, 1000 or 2000 mg/kg malathion failed to discern any neuronal lesion (Ehrich et al., 1993).

Schoenig et al. (1993) performed a chronic neurotoxicity study of DEET in rats. The animals were from dams exposed to DEET during gestation and nursing. After weaning, the rats were provided a diet containing 0, 500, 2000 or 5000 ppm of DEET for an additional 9 months. No neuropathologic changes were seen in the central or peripheral nervous system following this prolonged exposure. Neuronal lesions were absent in rats following 13 weeks of daily dermal exposure to 1000 mg/kg/day DEET (Johnson [unpublished study], 1987, cited by Schoenig and Osimitz, 2001). Pyridostigmine bromide was administered to rats at 5, 15, 30 or 60 mg/kg/day by gavage, for 13 weeks (Levine et al., 1991). Although the higher three doses elicited cholinergic signs, no histopathological evidence of neuronal injury was found in the brain at the conclusion of the study.

The above-noted compounds have been widely and safely used, providing significant benefit. DEET is a personal insect repellent applied dermally or to clothing (Schoenig and Osimitz, 2001). In addition to its nuisance prevention value, it has significant public health benefit. The latter relates to DEET's efficacy against arthropod vectors of some important infectious diseases (Fradin and Day, 2002). This includes the etiologic agents of conditions of recent public health concern such as West Nile virus encephalitis, yellow fever, malaria, rocky mountain spotted fever, and Lyme disease (Pollack et al., 2002; Schoenig and Osimitz, 2001). Given its wide use (an estimated 30% of the United States' population annually), the number of reported adverse events is small and generally related to gross overuse of the product (Bell et al., 2002; Osimitz and Murphy, 1997; Pollack et al., 2002). Thus, the risk of serious medical effects from labeled use of DEET-containing products appears to be low (Bell et al., 2002; Schoenig and Osimitz, 2001). The anticholinesterase drug pyridostigmine bromide is used for diagnostic and therapeutic purposes in man. These include its role in tests to determine the degree of growth factor release from the hypothalamus and in the treatment of myasthenia gravis (Institute of Medicine, 2000). As regards the latter, this drug has been used for long-term treatment of adult humans with myasthenia gravis in oral doses in the range of 60–1500 mg/day, without eliciting regulatory concern (Wickersham and Novak, 2003). Malathion and permethrin are widely used, effective insecticides, with a variety of agricultural, vector control, household and personal applications. While these do have toxic potential and instances of poisoning have occurred, they are considered among the safer insecticides. Malathion is rated as a slightly toxic compound in the U.S.

Environmental Protection Agency Class III toxicity classification, having an oral LD₅₀ of 1000 mg/kg in rats (Extension Toxicology Network; Merck Index, 2001). As regards the potential to induce the cholinergic syndrome, malathion is classified as slightly hazardous (the lowest such classification level) (Lotti, 2000). Permethrin is the most widely used pyrethroid, a class of insecticides posing little hazard to humans by natural routes of exposure at levels likely to be encountered in the environment, or from normal, approved use (Agency for Toxic Substances and Disease Registry, 2003). The oral LD₅₀ for permethrin in rats is over 3000 mg/kg (Merck Index, 2001).

From the foregoing it appears that pyridostigmine bromide, permethrin, DEET and malathion are valuable compounds that have been safely employed to enhance human health and commerce. Toxicologic studies indicate these agents do not readily induce neuronal necrosis at exposures equivalent to or greater than those employed by Abdel-Rahman et al. (2001, 2002b, 2004a,b). Yet these authors report that alone or in combination they cause widespread death of such cells, admittedly an effect not reported in the controls. If those findings were valid, these compounds would be among the most potent known neurotoxicants. Although unable to account for the reported absence of this change in control brains, the foregoing review of the work of Abdel-Rahman et al. (2001, 2002a,b, 2004a,b) demonstrates that histologic changes they interpret as degenerating or dying neurons are entirely consistent with the dark neuron artifact. In light of this, neurotoxicological inferences based upon these publications need to be reassessed.

While the above-cited examples of recent misinterpretation of dark neurons as dying or degenerating cells are from one laboratory (Abdel-Rahman et al., 2001, 2002a,b, 2004a,b), other published studies exist. In one of these, Moser et al. (2001) reported the results of a developmental neurotoxicity study of the triazole fungicide tebuconazole. In this study pregnant F₀ rats were daily orally dosed at 0, 6, 20 or 60 mg/kg from gestational day 14 to post-natal day 7. The F₁ pups were then similarly exposed until post-natal day 42. Neurobehavioral assessment 1 month after the last tebuconazole exposure revealed diminished spatial learning using the Morris water maze in the high dose F₁ animals.

Histopathological study was performed using brains collected 110–112 days after the last dose of tebuconazole. These had been immersion-fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with cresyl violet. This study demonstrated what were described as pyknotic neurons in the hippocampus, sometimes also involving the neocortex. The incidence and intensity of this change were greater in tebuconazole-exposed F₁ rats relative to the vehicle controls. Specifically, while Moser et al. (2001) recognized the existence of an immersion fixation related dark neuron artifact, they felt that the incidence and extent of these pyknotic cells in the compound-exposed rats was both above background and dose related. In addition, they felt there was a qualitative difference between the artifact and what was considered a lesion, namely the absence of perineuronal retraction and corkscrew-shaped dendrites in the latter. Thus,

the pyknotic neurons were considered to represent injured or dying cells, which might provide a morphological basis for the learning deficits seen in the study. When questions were raised regarding this interpretation of the dark-staining neurons in the study, the authors, to their credit, convened a group of neuropathologists to re-examine the study histologic material. This re-examination concluded that the dark-staining neurons were fixation or processing induced artifacts rather than injured cells related to tebuconazole exposure, and the authors thus withdrew all neuropathological conclusions of the study (Barone and Moser, 2004).

The preceding discussion of the artifactual nature of dark neurons, supported by the appearance of such cells induced by post-mortem manipulation of normal rat brains (Fig. 1), should be convincing. However, the occurrence of similar contracted, darkly stained neurons, sometimes with corkscrew-shaped apical dendrites, in some acute neuropathologic states must be noted. The latter include experimentally induced hypoglycemia, status epilepticus and ischemia reperfusion (Auer et al., 1985; Ingvar et al., 1988; Jenkins et al., 1981; Söderfeldt et al., 1981, 1983). While the issue of whether these papers deal with real lesions or artifacts has been debated (Agardh et al., 1981; Brierley and Brown, 1981), the descriptive and photographic data in these reports demonstrate that in these acute insults there is neuronal contraction similar to those of the dark neuron. If one accepts these changes as real, the question of whether these acute lesions can be differentiated from the dark neuron artifact needs to be addressed. Review of the indicated papers (Auer et al., 1985; Ingvar et al., 1988; Jenkins et al., 1981; Nedergaard, 1987; Söderfeldt et al., 1981, 1983) shows this to be the case, since there are distinct neuropathologic changes in hypoglycemia, status epilepticus and ischemia that are not seen with the artifactually induced change. As might be expected in these acute injuries, there is marked edematous change in affected regions of the brain reflected by astrocytic swelling leading to status spongiosus. In addition, these contracted cells are part of a spectrum of neuronal changes in these acute injuries. As opposed to the monotonous cytological pattern seen in the post-mortem mechanically induced artifact, these acute injuries feature a number of evolutionary neuronal changes, not just the contracted darkly stained neuron. Numbers of the latter appear to evolve to recognized forms of neuronal necrosis such as acidophilic change (Graeber et al., 2002), described above. Thus there are clear differences between the neuronal contraction in these acute energy deprivation states and the dark neuron artifact, which can be ascertained by careful neuropathologic evaluation.

Interestingly, many contracted neurons in the above-noted acute neuropathic states revert to normal with time after cessation of the insult, as shown in status epilepticus and hypoglycemia (Auer et al., 1985; Ingvar et al., 1988; Söderfeldt et al., 1981, 1983). This led Auer et al. (1985) to urge caution in equating a contracted, darkly stained neuron with cell death. In a related finding, Kepes et al. (1995) were able to rapidly induce both swollen and contracted, dark-staining neurons by manual compression of the cerebral cortex of anesthetized rats. These cells, which were thought to be a reflection of acute alteration of

neuronal osmoregulation, appeared to revert to normal over a 6-week post-compression period.

It is my contention that, as noted above, several recent studies published in reputable journals have misinterpreted the dark neuron artifact as evidence of dying or degenerated cells. This has led to the attribution of inappropriate neurotoxic potential to the compounds under study. Such a state does not reflect well on the science of neurotoxicology, and more importantly may negatively impact the health of the public. This can be avoided in the future if investigators, journal editors and reviewers, and regulatory officials are aware of this common artifact, and see that it is properly interpreted.

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