Abnormal Neuritic Architecture Identified by Di-I in Pick's Disease

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Abstract. The fluorescent stain Di-I combined with confocal microscopy identified an abnormal neuritic pattern in the cortices of frontal and anterior temporal lobes in postmortem tissues from patients with Pick's disease (PD). Focal, dense neuritic aggregates 50 to 200 µm in diameter were scattered throughout all cortical layers. The three-dimensional analysis provided by confocal microscopy revealed the neuritic clusters to consist of enlarged, randomly arrayed, dystrophic neurites that were not associated with amyloid deposits, astrocytic processes or capillaries. In the intervening neuropil, there were fewer neurites compared to controls. The occipital cortex, which is unaffected in PD, showed a neuritic architecture comparable to normal controls as did affected brain tissues from patients with Alzheimer's disease or remote, ischemic infarction. This neuritic pattern is, thus far, unique to PD and may reflect the loss of specific subpopulations of cortical neurites and proliferation of neurites of the remaining neurites.

Key Words: Confocal microscopy; Di-I; Pick's disease.

INTRODUCTION

Pick's disease (PD) is a rare, dementing disorder with degeneration of neuronal subpopulations in the cerebral cortex and hippocampus. Pick bodies, the intracytoplasmic, argyrophilic neuronal inclusions pathognomonic of this disease, are predominantly located in the somas of non-pyramidal and some pyramidal neurons within layers 2, 3 and 6 in portions of the frontal and temporal cortices. Dentate granule neurons and some pyramidal neurons of the hippocampus also show these neuronal changes (1, 2). Neurofibrillary tangles and amyloid deposits, characteristically seen in Alzheimer's disease (AD), are not a component of PD.

To date, there is a paucity of documentation of the neuritic response in the neocortex in tissues from patients with PD. Using the Golgi impregnation method, Ferrer et al. (3) observes random, degenerating neurons accompanied by dendritic swellings. Pyramidal neurons in layer 2 of the affected neocortex show decreased numbers of dendritic spines (4). In brain homogenates, decreased synaptophysin levels also suggest synaptic loss (5).

A recently described method of studying the detailed neuronal architecture is the microinjection of Di-I (1,1-diotadecyl-3,3,3 3-tetramethylindocarbocyanine perchlorate), a member of the family of fluorescent cyanine dyes (6, 7). It is of particular value when applied to the human central nervous system as the dye can diffuse along neurons in aldehyde-fixed, postmortem tissue while glial and vascular structures remain unstained (7–10). Di-I has two long fatty acyl chains which insert in the lipid fraction of the plasma membrane (11–14). The dye diffuses laterally along the neuronal plasma membrane in both anterograde and retrograde directions. When applied in vivo to developing vertebrate central nervous system, the outline of the soma, axon and dendrites of a few neurons are delineated (7, 8). These properties of Di-I are potentially useful in the study of diseased tissues, especially where there may be changes of the neuritic tree.

In this study, the diffusion of Di-I in postmortem cortical brain tissues with PD is compared to tissues from patients with AD, ischemic infarction and young, age-matched normal controls. Using standard light microscopy and epifluorescence and confocal fluorescence microscopy, morphologic changes in the neuropil, especially dendritic profiles, are examined.

MATERIALS AND METHODS

Subjects

Brain tissues were obtained postmortem from the McLean Hospital Brain Tissue Resource Center, Belmont, MA. These included four patients with PD (two male and two female), ranging in age from 46 to 95 (mean 68 ± 20) years. All patients were selected after neuropathological confirmation of a clinical diagnosis of PD. The mean postmortem interval was 10 hours (range 4 to 23).

Control tissues were obtained from five subjects with no neurological disorders that came to autopsy at the Los Angeles County/University of Southern California Medical Center, and four patients with neurological diseases from the Alzheimer's Disease Research Consortium of Southern California. The five normal subjects included four older adults (age 79 ± 4 years) and one younger, 40 year old adult. The four neurologically diseased controls included two patients with AD (ages 69 and 81), one 86 year old patient with remote, ischemic infarction, and one 44 year old patient with amyotrophic lateral sclerosis (ALS) as a non-demented control with a neurodegenerative dis-
Fig. 1. (a) In PD, Di-I focally illuminates the deeper neuropil (white arrows) in the affected frontal cortex. The arrowhead indicates the subpial injection site. There is comparatively less fluorescence in the deeper neuropil surrounding the fluorescent foci. (b) In the occipital cortex of PD tissues, (c) frontal cortex of normal control, and (d) frontal cortex of AD tissues (bar = 100
ease. All four PD patients showed the characteristic frontal and temporal cortical atrophy grossly, and Pick bodies microscopically. In all patients, the affected cortical sites were fairly densely gliotic with reduced numbers of neurons, including ballooned cells, and relatively few Pick bodies. For AD patients, a clinical diagnosis of AD was based on NINCDS-ADRDA criteria (15). The neuropathologic diagnosis of AD was confirmed using the CERAD (16) modification of the diagnostic criteria of Khachaturian (17). Tissues from severely affected AD patients were chosen for this study to optimize comparisons of neuronal and neuropil changes with those of the PD brain tissues.

Tissue Preparation

All brain tissues were fixed at the time of autopsy in 10% phosphate buffered formalin (EM Science, Gibbstown, NJ). Tissue blocks (2 cm × 2 cm × 1 cm) of cerebral cortex were dissected from frontal, temporal and occipital lobes (Brod- mann’s areas 9, 21 and 19, respectively). Blocks were oriented perpendicular to the pial surface and included the entire width of the gray and superficial white matter.

Microinjection of Di-I

Di-I was injected into two sites: either the junction of the gray and white matter, or approximately 1 mm from the pial surface, close to layer II. Two preparations of Di-I (Molecular Probes, Eugene, OR) were used; one as free crystals, and the other in solution of 100 μg/ml of Di-I dissolved in 100% ethanol. The tip of a glass micropipette was loaded with two or three Di-I crystals and injected with a micromanipulator in the above sites in the tissue block. Other tissue blocks were microinjected with 1 μl of Di-I solution.

To evaluate effects of tissue fixation on Di-I diffusion, one group of blocks remained in 10% neutral buffered formalin and a second group was transferred to 3% paraformaldehyde in 1 mM phosphate buffered saline (PBS), pH 7.4, immediately after injection of Di-I. Each group of blocks was divided so that half were incubated at 21°C and the remainder at 37°C in the dark. To assess rate of dye diffusion, samples were sectioned weekly for 6 weeks.

Confocal and Epifluorescence Microscopy

The tissues were sectioned with a vibratome at 30-50 μm and examined by epifluorescence using rhodamine filters optimally at 563 nm with a Zeiss fluorescence microscope. Selected sections stained with Di-I were also scanned with a Biorad Model MRC 600 laser scanning confocal microscope, using a Zeiss axiovirt inverted microscope. The optical sectioning was done at focal planes of 5 μm intervals, up to 40 μm depths. Photographs of the epifluorescence, phase and “merging” images were obtained directly from the color videomonitor.

Histology and Immunocytochemistry

To detect senile plaques and neurofibrillary tangles, vibratome sections serial to those viewed for Di-I fluorescence were stained with either thioflavin S and viewed by epifluorescence optimally at 480 nm, or by a modified Bielschowsky silver impregnation method. Pick bodies were detected by the Bielschowsky method and anti-tau immunocytochemistry. To evaluate neuronal compartments, additional serial sections were reacted with antibodies to neurofilament (68, 150 and 200 kDa) to detect neurites (ICN ImmunoBiological, Costa Mesa, CA), tau to detect axons (Sigma, St. Louis, MO), synaptophysin (DAKO Corporation, Carpinteria, CA) and GAP 43 to detect pre-synaptic terminals (Boehringer Mannheim, Indianapolis, IN), glial fibrillary acidic protein (GFAP) to identify astrocytes (Sigma) and factor VIII for vascular endothelium (Sigma).

Vibratome sections were washed in PBS, incubated 30 minutes in 10% hydrogen peroxide in methanol to eliminate endogenous peroxidases, and exposed to primary antibody for 1 hour in dilutions recommended by the manufacturer. Subsequent incubations were with biotinylated goat anti-mouse IgG or IgG (1:200 in PBS) followed by the avidin-peroxidase solution (Vector Labs, Burlingame, CA) for 30 minutes each. Sections were washed in PBS between each step, and were finally reacted with 3-amino-9-ethylcarbazole (0.04%) and H2O2 (0.015%) for 10–20 minutes and counterstained with hematoxylin. Sections of normal tissue from this study were used as positive controls and sections with primary antibody incubation omitted were used as negative controls.

RESULTS

In all normal control and diseased tissues, Di-I diffused maximally over a 6 week period. The dye spread most rapidly during the first 2 weeks at a rate of 400 μm per day with intense staining of a meshwork of neuritic processes (Fig. 1). By 4–6 weeks after injection, a 12 mm circumscribed area of fluorescence extended across the cortex to the superficial margin of the white matter with no spread into the underlying white matter. Within the diffusion perimeter, numerous neurites were intensely stained in normal tissues and ranged from 0.5 μm to 1.5 μm in diameter. These processes had varicosities and small, lateral projections suggestive of dendritic spines (Fig. 1g). Surprisingly, few neuronal somas, including pyramidal and non-pyramidal neurons, could be distinguished (not shown).

Dye diffusion was more extensive when injected at the subpial site compared to the junction of the gray and white matter. With the deeper injection, staining remained lo-

Fig. 2. (a) In the frontal cortex of PD, the blood vessel (arrows) as seen by phase contrast imaging is not topographically associated with (b) the Di-1 fluorescent, neuritic bundles (left) in the same section viewed by confocal fluorescence microscopy. Arrows in (b) correspond to the location of the blood vessel in (a) (×8,000).

calized within the gray matter. There was virtually no extension into the subcortical white matter and no staining of axons when transversely oriented. The intensity of fluorescence was strongest with implantation of the solid crystal rather than the dye in solution. Diffusion of the dye was slightly enhanced at 37°C compared to incubation at 21°C. The rate of diffusion was similar in both formalin- and paraformaldehyde-fixed tissues and unrelated to the postmortem interval.

The most striking findings were in the brains of all four patients with PD. Circumscribed, brightly fluorescent, neuritic aggregates, 50–200 μm in diameter, were randomly scattered throughout the gray matter subjacent to every microinjection site and extended to the margin of the subcortical white matter in the frontal and temporal lobes. These clusters were found only in the areas of PD histopathology (Pick bodies and ballooned neurons) in the frontal and temporal lobes (Fig. 1a) and in all four PD patients were absent from the occipital cortex which is unaffected in this disease (Fig. 1b). There was no apparent relationship of these structures to the overall density of Pick bodies or ballooned neurons. The intervening neuropil showed a marked paucity of neurites compared to the normal, AD, ALS and ischemic infarction controls, as well as to the occipital cortices of the brains with PD. In the normal and diseased control tissues, the dye spread diffusely and evenly around the injection site and did not contain regions of clustering (Fig. 1c, d).

At higher magnification, the affected PD tissues revealed brightly fluorescent neurites projecting randomly from the margins of the structures. The central portions were diffusely and homogeneously stained (Fig. 1f). There was no granular or fibrillar central core such as those found in neuritic plaques in AD tissues.

The high resolution provided by confocal microscopy on the Di-1-stained tissues allowed for sequential 5 μm optical sections into the core of the aggregate. The structures consisted entirely of aggregates of randomly oriented, dystrophic neurites (Fig. 1e). Most of the neurites were twisted and focally enlarged measuring up to 3 μm in width (Fig. 1h). In contrast to controls, the PD-affected neurites showed fewer spine-like structures. Because few somas stained, continuity of the neurites in these clusters with neuronal somas was not observed. At high magnification, we confirmed that the intervening neuropil of the affected PD tissues contained strikingly fewer neurites than normal controls (Fig. 1e, g). Parenchymal vessels showed no anatomical relationship to these aggregates, as demonstrated by immunostaining of vascular endothelium with anti-factor VIII (not shown) and by phase contrast combined with Di-1 fluorescence confocal imaging (Fig. 2).

Further characterization of the Di-I-stained, dystrophic processes was undertaken to determine whether they were either axons, dendrites or both. Double-labeling of a section with immunocytochemical markers was not possible as the Di-I fluorescence was markedly quenched by the immunostaining or silver impregnation procedures. On serial sections of the Di-I-injected blocks, neither monoclonal antibodies that primarily stain somas of normal and distended (ballooned) neurons and axons (neurofilament 200 and 150 and 68 kDa forms) (Fig. 3a) nor the Bielschowsky stain (Fig. 3b) were concentrated within the neuritic bundle-like structures. Anti-GFAP showed dif-
fuse staining of the tissue and no aggregated processes (Fig. 3c). Thioflavin-S staining was also negative for diffuse and focal amyloid deposits and angiopathy (Fig. 3d). No stained clusters or hypodense areas were seen with anti-tau, anti-GAP 43, or anti-synaptophysin immunostains (not shown). All PD sections showed a diffuse, rather than focal, decrease in synaptophysin immunoreactivity compared to AD tissues. Pick bodies were identified by Bielschowsky and anti-tau immunostaining (Fig. 3b). Antibody to MAP-2, a dendritic marker, was not informative as we confirmed by preliminary testing that immunoreactivity is destroyed by formalin fixation (18).

DISCUSSION

Aggregates of twisted, dystrophic neurites and the hypodense, adjacent neuropil revealed by Di-I fluorescence provide additional parameters that distinguish neuropathological changes in PD from other dementing disorders that affect the cerebral cortex, including AD and multiple infarcts. Dystrophic clusters of neurites were
scattered throughout the affected cortices of PD brains, but were not present in the brains from patients with AD, ALS, ischemic infarction, or normal, aged controls. A subset of non-pyramidal neurons immunoreactive with anti-parvalbumin is reported to be resistant to degeneration in these affected necortical regions in PD (19). Preliminary observations in our laboratory indicate a marked loss of anti-calbindin D-28K-reactive interneurons (not shown). It is possible that the loss of the latter interneurons and their dendritic fields in PD may result in focal neurite-poor areas.

By exclusion, we postulate that the Di-I-stained structures are most likely to be dendritic. In all brain sites, diffusion of Di-I along axons appears limited by the myelin sheath, with no staining of the axon-rich, underlying white matter. The presence of spines and varicosities along these remaining neurites is also in keeping with dendritic morphology. The decreased immunoreactivity in PD brains by anti-GAP 43 and anti-synaptophysin, which selectively stain pre-synaptic terminals, suggests axonal loss. These results are confirmed by the observations of Weiler et al. (5, 20) of decreased levels of synaptophysin in PD and AD brain homogenates compared to age-matched controls, with PD tissues showing the greatest loss.

In affected areas in PD brains, local sprouting and increased arborization of neurites may possibly be a regenerative attempt of surviving cells of the vulnerable subpopulation or the remaining non-vulnerable adjacent neurons, including some pyramidal neurons. With only a few neuronal somas stained with Di-I, distinguishing the remaining neuronal subtypes contributing neurites to the clusters cannot be resolved with this method.

Amyloid deposits are associated with focal neuritic sprouting in AD (21). Amyloid is not observed in patients with PD and, thus, unrelated to any possible sprouting contributing to the neuritic structures. The diffuse GFAP immunoreactivity demonstrated that the gliosis in PD tissues was not specifically related to the neuritic aggregates or the intervening neurite-poor regions.

Our observations of dendritic clusters are also in contrast to the diffuse neuropil in age-matched controls. Dendritic growth, including sprouting of synaptic spines, may continue during the normal aging process. Age-related dendritic growth has been reported in the moderately old rodent (22-24), non-human primate (25, 26) and human brain (27-30). Changes in the dendritic architecture of neurons may occur differentially in the apical and basilar trees. Sprouting, as well as degeneration of neurites in normal aging, also varies depending on the brain region, the cortical layer and age of the patient. In layer 3 pyramidal cells of the human neocortex, apical dendrites are lost while the apical shaft remains intact (31). Age-related growth of basilar dendrites of pyramidal neurons in layers 2 and 3 of the neocortex is found in the rat (22).

Conversely, pyramidal cells in layer 2 of the parahippocampal gyrus show proliferation of the apical dendritic trees, but little change in the basilar dendrites (27, 28). In the elderly, the initial dendritic growth of dentate granule neurons is followed by dendritic regression of terminal segments as aging progresses (30).

While there are relatively few reports on the dendritic response in PD, dendritic changes in AD tissues have been extensively compared to age-matched, normal controls. The results are somewhat contradictory; Golgi studies in AD tissues show no branching or elongation of apical dendrites of affected pyramidal neurons in layers 2 and 3 of the neocortex while basilar dendrites continue to be comparable to controls (27, 28). Non-pyramidal neurons in these layers show diffuse neuritic regression (3). Dendritic sprouting of adjacent remaining pyramidal cells in neocortical layer 3 has been reported (32-34) and may therefore compensate for the dendritic losses of the affected neurons. The levels of GAP 43, a marker of neuritic growth, are decreased in the AD neocortex but increased in the hippocampus. GAP 43 is also found in dystrophic neurites, suggesting a role in regeneration (21).

More recently, these results have been clarified using immunocytochemical staining along with Golgi preparations. As shown by McKee et al. (35), apical dendrites of pyramidal neurons degenerate while there is distal and perisomatal dendritic sprouting. These authors also confirm that basilar dendrites of hippocampal pyramidal neurons are dystrophic as shown by positive staining by anti-MAP-2. This marker was not used in our study as it is non-reactive on formalin-fixed tissues (18). Dendritic swellings, 1-4 μm in diameter, in the basilar and apical dendrites of neocortical pyramidal neurons and the proximal dendrites of non-pyramidal neurons are reported by Ferrer et al. (3). Granule cells of the dentate gyrus also respond in AD with increased dendritic proliferation (30, 35) but with reduced dendritic length (30, 36). Neurofibrillary pathology in AD may also include neuronophiles which may reflect an abnormal cytoskeleton in either axons or dendrites (37, 38). Anti-parvalbumin-positive pyramidal neurons are lost in AD, particularly in neocortical layers 2 and 3 (39).

The sprouting and regression of neurites leading to degeneration in AD is also variable depending on the duration of disease and severity of neuronal loss and gliosis (29). The loss of synaptic connections accompanying the neuronal degeneration (4, 27, 29, 39), as well as in regenerating neurites, has also been strongly implicated in AD (35, 40, 41). A decrease in synaptophysin immunoreactivity has been demonstrated in affected areas of cortex (5, 20).

Application of Di-I is particularly valuable in that it shows discrete neuritic morphology in formalin-fixed tissues (7). In contrast to immunocytochemical methods, its easy diffusibility throughout a tissue block allows for
a three-dimensional analysis of the neuropil in a thick section when combined with confocal microscopy. As DI-I does not stain glial processes, assessment of neuritic proliferation in PD could be done where a glial response is also to be expected.

Comparing formalin- and paraformaldehyde-fixed tissues, as well as differences in postmortem interval, there were no obvious differences in the staining pattern or diffusibility. The effect of different incubation temperatures (21°C versus 37°C) on the diffusion of DI-I was not remarkable, although the rate of spread seems to be slightly faster at 37°C as suggested by greater fluidity of the lipids. Similarly, faster transport at physiologic temperatures (38°C) has been indicated in the developing rat brain (8) and in the human optic nerve (10). The highest local concentration was achieved with DI-I crystals rather than the ethanol-solubilized form.

The rate of diffusion decreased as DI-I moved further from the microinjection site. Godement et al (7) demonstrated that 2 weeks was sufficient for the diffusion of DI-I through the neurites to a distance of about 5 mm from the injection site, and Mufson et al (42) showed that DI-I extended 8 mm in 6 months. In our study, diffusion occurred at the rate of approximately 400 μm per day. Prolonged fixation (6–12 months) of some of the tissues in formaldehyde prior to microinjection of DI-I could possibly contribute to delayed spread of the probe along neurites.

Our studies show DI-I combined with confocal microscopy to be useful for the three-dimensional analysis of the neuritic architecture in normal and diseased human brain tissues. With these methods, a striking and unique modification of neuronal architecture is revealed in PD. The paucity of somas stained renders it necessary to combine the use of DI-I with other methods, such as immunochemistry to define the vulnerable neuronal subpopulations. Prospectively, frozen tissues and the use of fixative other than formalin will be required for further examination of the neurites contributing to the altered neuronal array in PD.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Sue D’Maggio, of the University of California-Irvine, and the advice of Dr. Scott Frazier of the California Institute of Technology. The authors thank Jeanette Espinosa for secretarial assistance.

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(Received November 4, 1992/Accepted February 16, 1993)