Temporal Sequence of Plaque Formation in the Cerebral Cortex of Non-Demented Individuals

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Abstract. One of the hallmarks of Alzheimer’s disease is the presence of argyrophilic plaques (arg-P) accompanying dementia and other forms of cognitive alterations. In the present investigation 195 non-demented, cognitively normal patients were grouped according to the presence or absence of critical coronary artery disease (cCAD), defined as a 75% or greater stenosis of one of the epicardial arteries. None of the subjects had significant cerebral vascular disease. The parahippocampal gyrus (PHG) and frontal pole were analyzed for the presence of arg-P, A4 deposition, ALZ-50 immunoreactive (IR) neurons and neuropil threads (NT). Individuals with cCAD have a significantly greater incidence of plaques than non-heart disease (non-HD) subjects. Every cCAD subject had ALZ-50 IR neurons in the PHG and a greater incidence of NT as compared to the non-HD subjects. Every subject with plaques also had IR neurons and NT in the PHG. Based on the presumption that early neurodegeneration labeled by ALZ-50 antibody and amyloid deposition are in some way linked, then the sequence of plaque formation is initiated by the presence of ALZ-50 IR neurons followed in order by NT, A4 deposition and diffuse form arg-P.

Key Words: Alzheimer-like pathology; ALZ-50 immunoreactivity; A4 immunoreactivity; Coronary artery disease; Cortical plaques; Plaque formation.

INTRODUCTION

The most consistent diagnostic neuropathologic lesion in Alzheimer’s disease (AD) is reported to be the senile plaque (SP) (1). Four forms of argyrophilic plaques (arg-P) have been described: 1) dense core, 2) neuritic, 3) compact, and 4) diffuse (2, 3). All arg-P forms have been shown to contain amyloid by immunocytochemical reaction with numerous A4 antibodies (4, 5). It has been suggested that the initial form of arg-P is the diffuse type (3) and that deposits of A4 (A4-P), which do not have argyrophilic components, are “pre-plaques” (3, 6) and eventually become arg-P (6). The difference may also be due solely to “stain” sensitivity.

We have recently reported that the incidence of arg-P occurring in the cerebral cortex of non-demented subjects with critical coronary artery disease (cCAD; defined as 75% or greater stenosis of any of the four major epicardial arteries) is significantly increased compared to non-demented subjects without heart disease (non-HD) (7). Many cCAD subjects had numerical densities of plaques diagnostic of AD (8), without overt dementia. All forms of arg-P have been found in cCAD subjects, but appreciably more diffuse form plaques occur in the younger individuals, while more dense core and neuritic forms occur in older cCAD subjects (7).

The neuritic components of arg-P are reactive with ALZ-50 antibody. There is some controversy whether a-68 is the protein tau, although it is well established that ALZ-50 will recognize aberrant forms of tau. Nevertheless, besides exhibiting reactivity with neurites of plaques, ALZ-50 is also immunoreactive (IR) with neuropil threads (NT) (9), with neurons in the process of degenerating (9–11), and with degenerating neurons destined to become neurofibrillary tangles (NFT) (12).

Recently it was reported that the expression of ALZ-50 IR neurons in Down’s syndrome may antedate the presence of arg-P (13). These findings raised the possibility that ALZ-50 IR NT constitute the residuum of ALZ-50 neurons (13). Considerable evidence indicates that individuals with Down’s syndrome living to middle age will invariably develop the clinical manifestations and neuropathologic features characteristic of AD (14, 15). In the present studies we have utilized cCAD subjects to study the formation of cortical plaques in an effort to determine the possible temporal sequence of their pathogenesis.

MATERIALS AND METHODS

Tissue from 195 individuals, specifically, 104 non-demented cCAD subjects (age range 26–93 years) and 91 age-matched, non-demented, non-HD subjects (age range 24–90 years) obtained through either the forensic or hospital pathology service at the University of Kentucky, was used in these studies. The death of each individual was sudden and unexpected. Each cCAD subject had 75% or greater stenosis of at least one of the four major epicardial arteries, and cCAD was either the cause of death or an incidental finding at autopsy in these subjects. For the purposes of this study "critical" stenosis of a coronary artery, defined as 75% or greater stenosis, including occlusion, of the cross-sectional macroscopic and microscopic examination at autopsy (16) was based on forensic protocol. After complete
autopsy and toxicologic analysis, which would rule out other causes of death, critical stenosis of a coronary artery is sufficient to account for a death within a reasonable medical probability (17). Non-HD subjects may have had atherosclerotic plaques of the coronary arteries, but stenosis was less than critical. Non-HD subjects were also devoid of cardiac valvular disease or hypertension. Though generally long-standing, the duration of CAD could not be determined accurately for most of the decedents in that they never sought medical attention or were unaware of their condition.

Each subject was determined to be non-demented by retrospective review of medical and personal records and by direct interviews with closest relatives or significant others (7). This review might not have detected the presence of mild cognitive alterations but did exclude the presence of profound dementia.

Subjects with a postmortem interval exceeding 24 hours were not used. No subject was utilized in which significant cerebral atherosclerosis was present, defined as 25% or greater stenosis of any major cerebral artery. The degree of narrowing of a cerebral artery was determined by serial cross-sectional luminal inspection.

Each brain was examined in the fresh state and sections of parahippocampal gyrus at the level of the lateral geniculate (PHG) and frontal pole (FP)—devoid of gross abnormality—were removed and immersed fixed in 10% buffered formalin; more recent cases were immersion fixed in 4% paraformaldehyde to optimize immunocytochemistry. Prior to embedding in parafin, three 50 μm vibratome sections were taken for processing by ALZ-50 immunocytochemical methods (18). All analyses of stained sections were performed blind to subject age and group designation.

Silver Staining

Paraffin sections (8 μm) of the PHG and FP from each subject (cCAD = 104, non-HD = 91) were stained by the Bielschowsky method. The incidence of plaques for each individual was calculated utilizing Bielschowsky-stained sections. Sections stained by the Bielschowsky method were evaluated for the presence of NFT, although they were not quantified. If arg-P were observed in either the PHG or FP of an individual, that subject was scored as positive for plaques; if no arg-P were found the individual was scored as negative for the presence of plaques. Argyrophilic plaques were quantified (number per mm²; three random fields) in the PHG from 115 of the 195 individuals in the study (cCAD = 62, non-HD = 53). Data for the FP and a portion of the data for PHG have been previously reported (7).

ALZ-50 Staining

ALZ-50 immunocytochemistry (18), utilizing antibody supplied by Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, New York, was performed on vibratome sections of the PHG (N = 99; cCAD = 57, non-HD = 42) and FP (N = 54; cCAD = 35, non-HD = 19) directly adjacent to sections stained by the Bielschowsky method. Control sections for the ALZ-50 studies were processed simultaneously and included: 1) PHG from an individual with AD as a positive control, 2) PHG from an infant as a negative control, and 3) a section of PHG incubated without primary antibody. No ALZ-50 IR was found in any section incubated without primary antibody.

Sections stained by ALZ-50 immunocytochemical methods were evaluated for the presence or absence of reactive neurons and neuritic processes associated with plaques. The numerical density of ALZ-50 IR neurons (number per mm²; three random fields) was determined in the PHG (N = 99) and the FP (N = 54). The number of ALZ-50 IR NT in PHG and FP were semi-quantitated based on the following scoring system: a = no NT, b = questionable presence of NT, c = few, d = moderate, and e = heavy. Anatomic localization of ALZ-50 IR neurons or NT was not performed, but any laminar pattern of NT was noted.

Amyloid Staining

The PHG was immunostained with the A4 amyloid antibody (10D5, Athena Neurosciences, San Francisco, CA) in 8 μm paraffin sections (pretreated with formic acid and pepsin) directly adjacent to those stained by the Bielschowsky method in 71 of the 99 subjects utilized in the ALZ-50 immunocytochemical studies (cCAD = 42, non-HD = 29). The FP was similarly immunostained with A4 in 21 of the 54 subjects utilized in the ALZ-50 immunochemical studies (cCAD = 13, non-HD = 8). The mean number of A4-immunostained plaques (A4-P) in PHG was determined for each individual (number per mm²; three random fields).

Statistical Analysis

The proportion of subjects with plaques present was compared between the cCAD and non-HD groups by using χ² analysis. The mean numerical densities of morphologic features (number/mm²) were calculated for each individual. Group means were compared using two sample t-tests. To determine the difference between the numerical density of plaques as demonstrated by A4 reactivity and Bielschowsky silver method, a two way analysis of variance (ANOVA) (grouped by staining method) was employed. The relationship between age and numerical density, and the relationship between different morphologic features were determined by the significance of a Pearson’s correlation coefficient r.

RESULTS

Incidence of Plaques in Either the FP or PHG

Plaques were assessed in the two different regions of the brain using Bielschowsky-stained sections. In the cCAD group 65% of the 104 subjects were observed to have arg-P while only 24% of the 91 non-HD subjects manifested these lesions. A χ² analysis revealed a significantly greater prevalence of plaques (p < 0.0001) in the cCAD group as compared to the non-HD subjects.

The entire sample of subjects was sorted by age into three different groups: a) below the age of 55; b) between the ages of 55 and 69; and c) over the age of 69. Overall, there is a significant age-related incidence of arg-P (regardless of type) appearing in either the FP or PHG (χ² p = 0.0001). This age-related incidence was further investigated according to group designation (cCAD vs. non-HD). In the cCAD group the greatest incidence was for individuals over the age of 69. Individuals in this age range had a 91% probability of having arg-P as compared to 40% probability in the non-HD subjects (p < 0.001). When comparing all individuals over the age of 55 years
Argyrophilic Plaques in the PHG

Fig. 1. Argyrophilic plaque density in cCAD and non-HD subjects exhibiting plaques plotted according to subject age.

of age with cCAD there is a 77% probability of plaque incidence as compared with 28% for the non-HD subjects.

These differences cannot be accounted for by a disproportionate number of individuals in each of the age ranges. A $\chi^2$ analysis verified that there were no significant differences in the number of individuals assigned to each of the age groups for both the cCAD and non-HD groups ($\chi^2 \, p > 0.1$). In addition, the mean age for the two groups (cCAD 62.5 ± 1.4 sem and non-HD 64.1 ± 1.4 sem) was not significantly different ($t[193] = 0.771, \, p > 0.1$).

Numerical Density of Plaques in the PHG

The density of both arg-P and A4-P was examined for both cCAD and non-HD subjects (Figs. 1, 2). Only those subjects with at least one plaque were employed in this analysis. A $t$-test failed to demonstrate any significant difference in numerical density between the two groups regardless of the type of plaque staining employed ($t[39] = 1.168, \, p > 0.1$ arg-P; $t[32] = 0.187, \, p > 0.1$ A4-P). Although the incidence of plaques is much greater in the cCAD group, when non-HD subjects express plaques they do so at the same numerical density. A repeated measures ANOVA (grouped by staining method) demonstrated a highly significant main effect for the staining method used to detect plaques ($F[1,32] = 21.302, \, p < 0.0001$), indicating that the A4 antibody was much more sensitive in detecting plaques in both cCAD and non-HD subjects.

In every case where plaques are observed with the Bielschowsky method they are also observed with the A4 antibody, but in greater numbers.

A correlation was performed between plaque density and age of each individual at the time of death. The analysis failed to reveal any significant correlation for the cCAD group ($p > 0.1$) but did reveal a significant correlation for the non-HD group ($r = 0.703, \, p < 0.01$) indicating that the numerical density of plaques in the non-HD group is age related.

Plaques in the FP

As in the PHG, in every individual where plaques were observed in the FP with the Bielschowsky method they are also observed with the A4 antibody, but in greater numbers. Every individual with plaques in the FP had plaques in the PHG, but not every individual with plaques in the PHG had them in the FP.

ALZ-50 Reactive Neurons in the PHG and FP

The numerical density of ALZ-50 IR neurons in the PHG (Figs. 3, 4, 5a, d) was significantly greater in the cCAD group than in the non-HD subjects ($t[97] = 2.502, \, p < 0.01$). Both subject groups showed a highly significant correlation between the age of the individual and the numerical density of these immunoreactive cells (cCAD $r = 0.467, \, p < 0.001$; non-HD $r = 0.396, \, p < 0.01$) (Fig. 6). There was also a very interesting relationship between having cCAD and the presence of ALZ-50 IR neurons. In the cCAD group 100% of the subjects demonstrated these ALZ-50 IR neurons in PHG as did 86% of the non-HD subjects. The non-HD subjects which did not show any reactive neurons were primarily under the age of 55.

In the FP there was no difference in the number of ALZ-50 IR neurons between cCAD and non-HD subjects. It is noteworthy that 100% of the cCAD subjects had ALZ-50 IR neurons in FP as did 86% of the non-HD subjects.

Both the cCAD and non-HD groups showed a significant correlation between the numerical density of ALZ-50 IR neurons and arg-P in the PHG (cCAD $r = 0.286, \, p < 0.05$; non-HD $r = 0.452, \, p < 0.005$). A very significant relationship exists between the presence of arg-P...
and ALZ-50 IR neurons. In 100% of the cases where arg-P are present the individual also demonstrates ALZ-50 IR neurons. However, only 41% of the individuals with ALZ-50 IR neurons have arg-P.

A similar relationship occurs in the FP; all individuals with arg-P in the FP demonstrate ALZ-50 IR neurons while only 42% of the individuals with ALZ-50 IR neurons in FP have arg-P.

Neuropil Threads in the PHG

A χ² analysis revealed a significantly greater incidence of NT in the PHG of cCAD subjects (72%) as compared to the non-HD group (50%) (p < 0.05). In the cCAD group, 46% have a moderate (Fig. 5a) to heavy (Fig. 5d) concentration of NT as compared to 19% for the non-HD group (p < 0.001). There is a highly significant correlation between the presence of NT and the number of arg-P for the cCAD group (r = 0.521, p < 0.0001) and only a marginally significant correlation for the non-HD subjects (r = 0.282, p < 0.09). In every case where arg-P were present that individual also had NT. However, 52% of the cCAD subjects without arg-P still had NT and 43% of the non-HD subjects without arg-P had NT (Fig. 4). The presence of NT in FP was never more than questionable.

There is a highly significant correlation between the numerical density of ALZ-50 IR neurons and NT in PHG for both the cCAD subjects (r = 0.423, p < 0.001) and the non-HD group (r = 0.772, p < 0.0001). In every case where NT are present ALZ-50 IR neurons are also observed for both groups. However, in 25% of the cCAD cases and 33% of the non-HD subjects ALZ-50 IR neurons were observed in the absence of NT (Fig. 3).

The FP is similar to the PHG with regard to the presence of ALZ-50 IR neurons and NT. In every case, when NT are present ALZ-50 IR neurons are also observed. However, in 46% of the cCAD subjects and 74% of the non-HD cases ALZ-50 IR neurons were observed in the absence of NT.
Fig. 5. Photomicrograph of matched fields-of-view in adjacent sections stained, in order, with ALZ-50 antibody (a and d), by the Bielschowsky method (b and e), and with A4 antibody (c and f) from a 62 year old (a–c) and a 64 year old (d–f) cCAD subject processed according to the Materials and Methods section. A staining pattern similar to that found in the 62 year old cCAD subject was found in a single 79 year old non-HD subject and three additional cCAD subjects (29, 52 and 80 years of age).

The calibration bar = 50 μm for a–f. a) ALZ-50 IR neurons appear to have relatively few processes while there is a moderate deposition of NT. b) Bielschowsky stained section devoid of arg-P. c) A4 IR plaques are apparent while no A4 IR is found in the nearby blood vessel. d) ALZ-50 IR neurons appear to have almost no processes while there is a heavy deposition of NT. e) Moderate numbers of arg-P are of the diffuse form only. f) Abundant A4-P are apparent while A4 IR occurs within the lumen of a nearby blood vessel.
DISCUSSION

Numerous investigators have reported AD-like pathology in non-demented individuals without attempting to group these subjects based on pre-existing medical condition (19–21); in many of these studies the term “non-demented” seemed to be synonymous with “normal control.” In the present studies we have investigated a large population of non-demented individuals grouped according to the presence or absence of cCAD. Individuals with cCAD are two to three times as likely to have cortical plaques as an individual with less than critical CAD. This does not, however, apply to the formation of NFT; only 5 of 104 cCAD and 2 of 91 non-HD subjects had NFT. The possible cause of the formation of plaques in cCAD has been previously discussed (7).

All current evidence indicates that these plaques in cCAD, and non-HD for that matter, are indistinguishable from SP found in AD. More study will be needed to establish whether they are identical. The progressive and dynamic events leading to the formation of plaques in AD have been extremely difficult to study in end-stage AD patients. The extremely high incidence of plaques in cCAD subjects—coupled with significant morphologic similarities between these plaques and those found in AD—suggests cCAD as an appropriate model to study plaque formation. The discovery of events occurring early in the course of formation of AD-like pathology may benefit efforts to arrest processes eventually leading to dementia. This, of course, is based on the premise that pathologic hallmarks of AD are directly linked to the processes causing dementia in affected individuals. Although there is currently some speculation as to the importance of the relationship between amyloid-containing SP and duration and severity of dementia in AD (22), it is reported that the presence of SP is not a consistent feature of aging (21).

The present data suggest a possible chain of events leading to cortical plaque formation: 1) the presence of ALZ-50 IR neurons precedes the formation of ALZ-50 IR NT in both cCAD and non-HD subjects and, further, that ALZ-50 IR NT may be the residua of ALZ-50 IR neurons; 2) the presence of ALZ-50 IR neurons and NT antedates the formation of A4-P and arg-P in both cCAD and non-HD subjects; and 3) deposits of amyloid reactive with A4 antibodies precede the occurrence of observable arg-P in both cCAD and non-HD subjects. This is similar to observations in Down’s syndrome (13) and may extend to the sequence of SP formation in AD.

Utilizing only those individuals having either arg-P or A4-P in PHG, we found there were no differences in the numerical density of either type of cortical plaque between cCAD and non-HD subjects. There was an age-related increase in the numerical density of arg-P in the non-HD population, an increase which did not occur in the cCAD subjects. These distinctions suggest that within a population of cCAD subjects the number of arg-P is related to the length of time an individual has had cCAD and not to age at death. It is noteworthy that there were consistently higher densities of A4-P compared to the densities of arg-P from individual to individual. Five percent of the subjects with A4-P had no arg-P (Fig. 5b, c), whereas 100% of the individuals with arg-P had A4-P. This could indicate that A4 deposition occurs prior to argyrophilic reactivity. Because of the very low percentage difference between those individuals with A4-P—but without arg-P—and those individuals with both types of plaques, the data may well indicate A4 is possibly more sensitive than traditional silver staining methods for localizing plaques.

Significantly more ALZ-50 IR neurons were found in the PHG in cCAD compared to non-HD. In addition, there was a relationship between increasing age and increasing density of such neurons. This relationship was significant in both non-HD and cCAD. It has been suggested that ALZ-50 IR neurons in cCAD and non-HD subjects merely represent NFT in affected individuals. If this were the case, then NFT should be observable in adjacent Bielschowsky-stained sections; they were not. In fact, NFT were rarely found in any subject investigated and, when they were present, minimal numbers were found only in individuals 65 years of age and older. This does not necessarily imply that if the younger cCAD individuals lived longer, then some ALZ-50 IR neurons may have at a later date been observable by silver stains and interpreted to be NFT.

Neuropil threads were also observed in the PHG with a higher degree of regularity in cCAD compared to non-HD. They often were configured in a laminar pattern in PHG when accompanied by heavy deposits of ALZ-50
IR NT (Fig. 5d). In contrast to previous reports that NT are primarily present in association with NFT (23, 24), this laminar pattern of NT occurred in the absence of NFT but never in the absence of ALZ-50 IR neurons. Every subject in the study with ALZ-50 IR NT had ALZ-50 IR neurons, while 28% of the subjects had ALZ-50 IR neurons alone (Fig. 3). This, along with histopathologic evidence (Fig. 4), tends to support the hypothesis that ALZ-50 IR neurons play a causal role in ALZ-50 IR NT formation, and that ALZ-50 IR neurons, for the most part, leave as a residuum ALZ-50 IR NT in both cCAD and non-HD subjects. We would speculate that this may apply to NT formation in AD. Although there is a reported correlation between NT and severity of dementia in AD (25), a more recent study indicates that NT are not a consistent pathologic feature of the dementing disorders Pick's disease, diffuse Lewy body disease or progressive supranuclear palsy (26). It is of interest that, in contrast to our histopathologic findings in AD, only minimal numbers of NT were found in the FP and they never exhibited a laminar pattern. This could indicate, as previously proposed, that it is the spread of pathology into the association areas of the brain that clinically manifests itself as dementia (20, 27).

We have found that there is a significant correlation between the presence of ALZ-50 IR NT and plaques in the PHG, although plaques are not a necessary prerequisite to having NT. In contrast to one report (28), but in agreement with two others (29, 30), ALZ-50 IR NT were found in the absence of either A4-P or arg-P in 43% of the study population (Fig. 4). In contrast, every subject exhibiting arg-P and, therefore, A4-P had ALZ-50 IR NT (Fig. 5). This tends to support the notion that NT play a role in cortical plaque formation, although there is not yet any conclusive evidence that the two processes are directly linked.

Similarly, we have found a significant correlation between the presence of ALZ-50 IR neurons and the presence of plaques in both cCAD and non-HD subjects. Fifty-nine percent of the study population have ALZ-50 IR neurons in the absence of plaques, while 100% of the subjects with such plaques have ALZ-50 IR neurons. Organic heart disease expressed as cCAD seemingly exaggerates this condition since the affected individuals have a greater density and incidence of ALZ-50 IR neurons, and, consequently, they have a greater incidence of plaques. This would support the hypothesis that the presence of ALZ-50 IR neurons precedes the onset of plaque formation. As noted, there is no conclusive evidence that the nidi for ALZ-50 IR neuron formation and plaque formation constitute identical processes, or are even linked. On the other hand, the data taken as a whole strongly implicate a temporal sequence of plaque formation in non-demented individuals.

We propose a longitudinal process of plaque formation from our cross-sectional data. The proposed temporal sequence in the pathogenesis of these plaques is based on three assumptions: 1) the formation of a senile plaque is a dynamic process; 2) no pathologic alterations or features are observable prior to initiation of the formation of plaques; and 3) the end-point of plaque formation is reached when older forms of plaques (e.g. dense core SP, neuritic SP) are the predominant observable pathologic alteration or feature. Based on these assumptions we postulate five discernable steps comprising the dynamic process of plaque formation. We further speculate, assuming there is some link between early neurodegeneration labeled by ALZ-50 antibody and amyloid deposition, that 1) the first recognizable pathologic feature in subjects known to develop plaques is the presence of ALZ-50 IR neurons (Fig. 3), thus the first step in plaque formation; 2) the subsequent step in the formation of plaques is the deposition of ALZ-50 IR NT in the presence of ALZ-50 IR neurons (Fig. 4); 3) the third step is the deposition of packets of diffuse A4-positive material (preplaques) in the presence of ALZ-50 IR neurons and NT (Fig. 5a–c); 4) the penultimate step is the deposition of sufficient A4-positive material forming argyrophilic plaques of the diffuse form only (Fig. 5d–f); and 5) the final step, a point near the end of the continuum of plaque formation, is the formation of neuritic and dense core arg-P in addition to diffuse form plaques. This last step is consistent with suggestions of other investigators that the initial type of SP is the diffuse form (3, 6). This proposed sequence of plaque formation is observed in both cCAD and "normal aging" (non-HD subjects). It does, however, appear to occur much later in life in the non-HD subjects.

Although none of the subjects in this study were overtly demented, we are unable to state conclusively whether mild cognitive alterations associated with the formation of plaques were apparent in these individuals. In support of this concept, several reports indicate that mild cognitive alterations do occur in non-demented individuals with AD-like pathology (31, 32). We cannot determine if cCAD may be a forerunner to AD. However, if cCAD proves to underlie the development of AD, then we hypothesize that the subjects investigated in this study probably died before manifesting the clinical symptoms of AD. In this vein, as medical therapy improves longevity for cCAD patients, cognitive dysfunction may establish itself as an increasingly obvious clinical consequence.

Forensic Note

The findings reported here have significant medico-legal implications. We opine that the established practice of diagnosing AD without accompanying correlative clinical data (5) should be abandoned in light of these findings. The traditional practice and pathologic diagnostic criteria may incorrectly yield a diagnosis of AD specifi-
cally after the autopsy of a non-demented subject with cCAD. Such a diagnosis indicates that the subject was demented. Dementia denotes critical deterioration or loss of the intellect, of the ability to reason, and of the capacity to form intent. Various legal issues as to whether the decedent had testamentary capacity when completing a will, intended the outcome of his or her acts (e.g. a charge of intentional homicide or suicide), or was able to appreciate dangerous environmental conditions (e.g. a motor vehicle operator) are, in our view, not resolvable exclusively on the basis of examination of the brain at autopsy.

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