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Focal pathology in the Edinger-Westphal nucleus explains pupillary hypersensitivity in Alzheimer's disease

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Abstract Patients who suffer from Alzheimer's disease (AD) and a sub-population of community-dwelling elders show an exaggerated pupillary reaction to dilute tropicamide, a cholinergic antagonist. This finding may serve as an early diagnostic marker of AD. Here we report a likely pathological basis for this hypersensitive pupillary response. Our observations indicate that the Edinger-Westphal nucleus (EW), a known center for the control of pupillary function, is a selective target of Alzheimer pathology early in the course of the disease. In all AD cases examined, the EW contained plaques and tangles. In contrast, the adjacent somatic portion of the oculomotor complex was virtually spared of pathology. Early pathology in the EW is likely to initiate a cascade of events that may give rise to pupillary hypersensitivity.

Key words Edinger-Westphal nucleus · Cranial nerve III · Alzheimer's disease · Pupillary hypersensitivity · Plaques and tangles

Introduction

In late 1994 we demonstrated that clinically diagnosed Alzheimer's disease (AD) patients exhibited a hypersensitive mydriatic response to the topical administration of dilute (0.01%) tropicamide, a cholinergic antagonist [44]. This finding did not fit the known clinical presentation and anatomical distribution of pathology in AD [40]. However, a review of the available literature suggested that the deposition and distribution of AD pathology within the brain stem is more extensive in rostral regions, within which centers for pupillary function are located [3, 18, 20–22].

The parasympathetic efferent output for pupillary response originates in the small-celled autonomic division of the oculomotor complex referred to as the Edinger-Westphal nucleus (EW) [28, 33]. The EW represents the visceral portion of the oculomotor complex and in addition to its role in controlling pupillary constriction may mediate between the sympathetic and parasympathetic innervation of the iris musculature. Because of its rostral location within the brain stem, we suspected that the EW might be a target for pathological changes that could lead to the observed pupillary hypersensitivity of patients with probable AD.

The EW lies medial and dorsal to the main oculomotor nuclei in the midbrain. It is located ventral to the periaqueductal gray matter (PAG) and extends from the level of the caudal pole of the red nucleus to the level of the interstitial nucleus of Cajal, a distance of approximately 5–7 mm. It receives input from the retina via the pretectum [9, 26, 32, 33], and in turn sends its axons through the oculomotor nerve to the ciliary ganglion. In animals, injections of cholinergic agents (e.g., acetylcholine plus physostigmine, or carbachol) into this region have been shown to affect pupil size [45].

Many of the rostrally-situated brain stem structures which show a heavy deposition of pathological lesions in AD are situated within the midbrain [3, 12, 20, 22]. For example, a recent study [50] of the midbrain PAG region showed very strong correlations between the pathological burden in this area and pathology in the entorhinal cortex. The entorhinal cortex is markedly affected by AD pathology early in the course of the disease. Given the location of the EW within the midbrain, these observations suggest that the EW might also be an early target for pathology. There have been contradictory reports in the literature

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with respect to pathology in the oculomotor complex [2, 23]. Little information, however, is available on pathology within the EW itself. The present study was undertaken to fill this gap.

Materials and methods

Eight brains from clinically and pathologically confirmed (CERAD criteria) [37] cases of AD, seven brains from community-dwelling, neurologically and pathologically normal individuals, three brains from neurologically normal community-dwelling elderly with AD pathology in the cortex and one brain from a case of clinically and pathologically confirmed progressive supranuclear palsy were used in this study. All AD brains were obtained from individuals with a clinical diagnosis of progressive dementia characteristic of probable AD. The normal control brains were from individuals with no clinical history of neurological or psychiatric disorders. Neither age nor postmortem interval (PMI) were different among the patient and control groups (age: Jonckheere-Terpstra Test: J-T = –0.083, $P = 0.934$; PMI: J-T = –0.332, $P = 0.740$).

Tissue preparation

The brain stem of each case was separated by a cut placed rostral to substantia nigra into the thalamus, to preserve the oculomotor (and EW) nucleus. All brain tissue was placed in cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24–30 h, then into graded concentrations of sucrose (10–40% in 0.1 M phosphate buffer) for cryoprotection and stored at 4 °C. Fixed tissue was sectioned at 40 μm on a freezing microtome into 0.1 M phosphate buffer containing 0.02% sodium azide. Series of one in four sections from each brain stem was stained for Nissl employing cresyl violet and used for anatomical localization and morphological characterization of the EW and adjacent structures.

Immunohistochemistry

Immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method employing the vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.). Free-floating sections were rinsed three times in 0.1 M phosphate-buffered saline (PBS), at pH 7.4. This rinse was repeated after every incubation step. Sections were treated with 0.4% Triton X-100 in PBS for 30 min at room temperature and then soaked for 1 h in the carrier medium consisting of 10% normal goat serum and 0.1% Triton X-100 in PBS. Tissue was incubated in the primary antibody at appropriate dilutions for 24 h at 4 °C. Sections were then incubated in biotinylated goat secondary IgG (1/500) for 15 h, followed by the ABC complex (1/100) for 2 h. The resultant peroxidase labeling was visualized by incubating the sections in 0.005% diaminobenzidine and 0.01% H_2O_2 in 50 mM TRIS-HCl pH 7.6. Following termination of the reaction by rinsing in the TRIS-HCl buffer, sections were mounted on slides, air-dried, dehydrated in graded alcohols, cleared in xylene and coverslipped under permount. Control sections were processed using nonspecific IgG in place of the primary antibody or by omitting the primary antibody.

Visualization and quantification of plaques and tangles

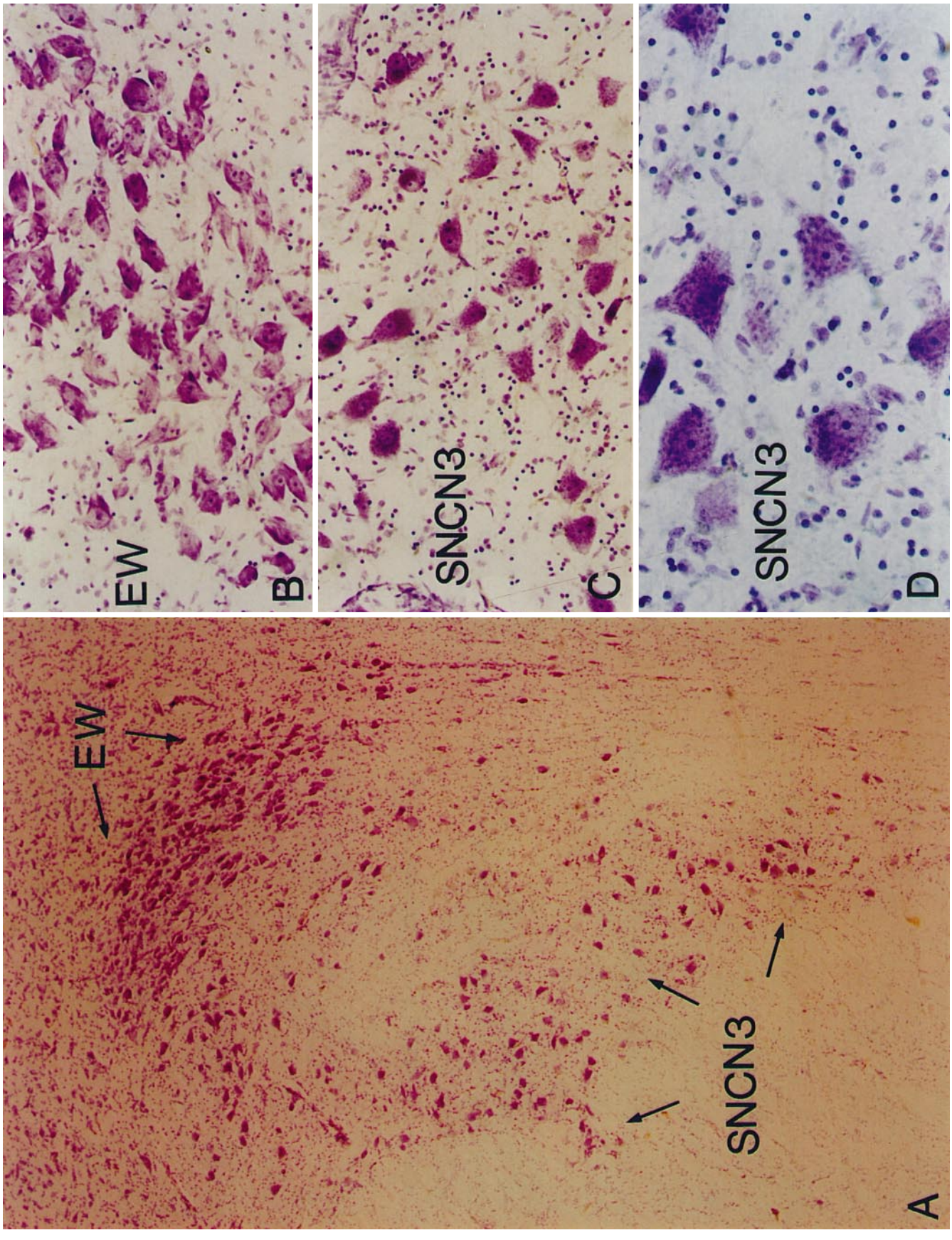
Polyclonal antibody 1282, which recognizes Aβ (kindly supplied by Dr. Dennis Selkoe, Harvard Medical School, Boston, Mass.), a monoclonal antibody (PHF1) which specifically recognizes tau phosphorylated at Ser396/Ser404, and monoclonal antibody Alz-50, which recognizes epitopes associated with the cytoskeletal pathology of AD (generous gift of Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, New York), were used to assess the distribution of AD pathology. Thioflavin-S staining was used to visualize compact plaques, dystrophic neurites, neuropil threads and tangles. All stained sections were subjected to careful microscopic examination and the presence of AD-type lesions in the EW were noted. The specificity of such pathology was ascertained by comparing the EW with the somatic portion of CN3 and other neighboring structures.

To obtain a quantitative measure of the extent of tangle and neuropil thread/dystrophic neurite (NT/DN) formation, we counted the number of PHF1-positive lesions in the EW of four AD cases with qualitatively heaviest pathology, three randomly selected normal controls and the three cases that were clinically silent but exhibited some cortical pathology. Counting was carried out at 40 × magnification using an ocular grid placed in the eye-piece of the microscope. At least one to two sections through the anterior, intermediate and posterior regions of the EW, matched across all cases, were used for counting. The ocular grid was sequentially moved through the EW in each section and the number of pathological lesions within each grid were counted. The mean numbers of tangles and NT/DN per section in the three groups were analyzed for significant effects using non-parametric tests (i.e., Kruskal-Wallis and Kolmogorov-Smirnov).

Fig. 1 A–D Morphological characteristics of neurons within the oculomotor complex in Nissl-stained sections from normal subjects. **A** The neurons of the somatic component of the nucleus of the third cranial nerve *(SNCN3)* are diffusely scattered, while the neurons of the Edinger-Westphal *(EW)* nucleus are grouped together in a compact region dorsal and medial to the SNCN3. **B** Neurons within the EW are fusiform or oval in shape and the Nissl substance is diffusely scattered throughout the cell. **C** Neurons within the SNCN3 are larger than EW neurons, have multipolar morphology and stain darkly for Nissl. **D** At high magnification, the Nissl substance within the SNCN3 has a granular and clumped appearance. **A** × 215; **B, C** × 560; **D** ×1120

Results

The brain stem atlas of Olszewski and Baxter [39] guided the anatomical identification of the EW in this study. The EW could be identified within the oculomotor complex by the morphological characteristics of its cells (Fig. 1 a–c). The neurons of this nucleus were densely arranged, small to medium in size and fusiform, oval or triangular in shape (Fig. 1 b). The Nissl substance was diffusely distributed throughout the cytoplasm. In contrast, neurons of the somatic portion of the nucleus of the third cranial nerve (SNCN3) (Fig. 1 c) were large, multipolar and stained darker than cells in the EW. The Nissl substance in SNCN3 neurons was granular and clumped in appearance (Fig. 1 d). The EW could be divided into three regions, an anterior unpaired portion lying in the midline, a paired intermediate portion lying dorsal and medial to the SNCN3 and a posterior paired portion lying on either side of the midline. The intermediate aspects of the EW also contained a ventral subdivision, separated from the dorsal portion by the SNCN3 (see Fig. 3 a). The distribution and boundaries of EW and SNCN3 neurons were confirmed using the cholinergic markers choline acetyltransferase (ChAT) and acetylcholinesterase (AChE). All of the EW and SNCN3 neurons contained ChAT immunoreactivity (polyclonal antibody, 1/500; gift of Dr. Louise Hersh, University of Kentucky) and AChE activity (according to previously published histochemical methods [11, 49]). The distribu-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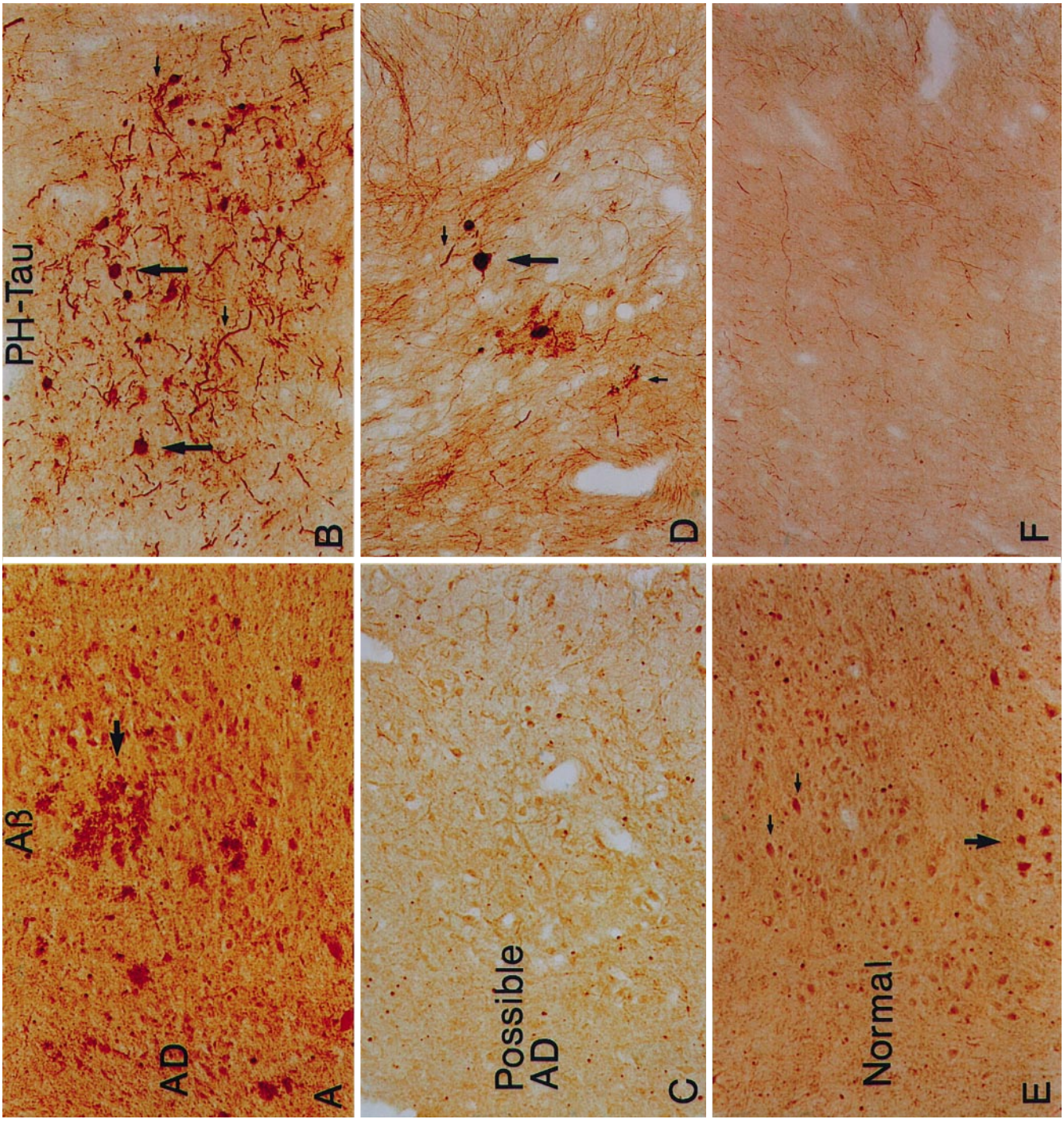

Fig. 2 A–F β-Amyloid *(Aβ)*-stained plaques and hyper-phosphorylated tau (*PH-Tau*, PHF1)-stained tangles and neuropil threads/dystrophic neurites (NT/DN) in the EW of AD and normal brains. **A** Many Aβ-positive plaques *(arrow)* are observed within the EW of AD brains. **B** Staining with the PHF1 antibody visualizes a large number of PH-Tau-positive structures with the morphology of tangles *(large arrows)* and NT/DN *(small arrows)* within the EW of AD brains. Virtually the same results are obtained using the Alz-50 antibody. **C** Staining for Aβ in a clinically non-demented case with the pathological diagnosis of possible AD fails to visualize any plaques in the EW. **D** In the same case as in **C**, a number of structures with the morphology of tangles *(large arrow)* and NT/DN *(small arrows)* are PH-Tau positive. **E** No Aβ-positive plaques are found in the EW of normal cases. In some normal and AD cases, weak Aβ staining is observed within the neurons of the EW *(small arrows)*, and darker staining in the neurons of the SNCN3 *(large arrow)*. This staining is entirely due to recognition of high contents of the APP within these neurons by the Aβ antibodies (Geula and Scinto, unpublished observations). **F** No PH-Tau staining is present within the EW of normal cases. Similar results were obtained using the thioflavin-S stain. (*AD* Alzheimer's disease, *APP* amyloid precursor protein). **A–F** × 280

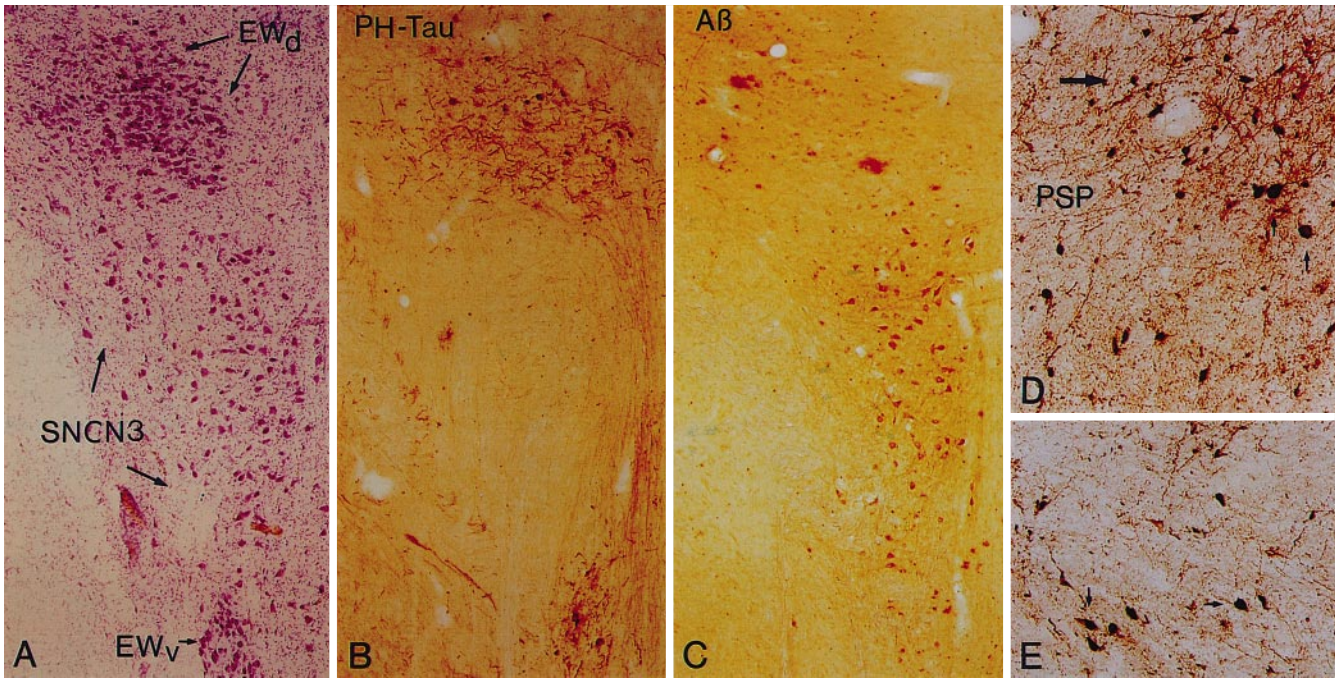

Fig. 3 A–E Regional specificity of AD pathology within the oculomotor complex of AD brains. **A** Nissl-stained sections through the intermediate aspect of the oculomotor complex visualize neurons within the dorsal and ventral portions of EW (*EWd* and *EWv*, respectively) and the SNCN3. **B** A high density of PH-Tau-positive tangles and NT/DN is found within the EW of AD cases. The SNCN3, however, is almost completely free of pathology. **C** Aβ-positive plaques are found within the EW in AD. No plaques are observed within the SNCN3. Aβ staining in neurons within the oculomotor complex is entirely due to high cellular content of APP (see Fig. 2). **D** PHF1 staining in the oculomotor complex from a patient suffering from progressive supranuclear palsy *(PSP)* reveals PH-Tau-positive neurons, tangles and NT/DN within the EW *(large arrow)* as well as the SNCN3 *(small arrows)*. **E** In the PSP case, tangles are found throughout the SNCN3 *(small arrows)*, consistent with clinical oculomotor abnormalities in this disorder. **A–C** × 180; **D, E** × 360

tion of ChAT- and AChE-positive neurons within these structures was virtually identical to the Nissl-stained neurons described above.

We used PHF1 and Alz-50 immunoreactivity to study the distribution of tangles and NT/DN within the oculomotor complex and adjacent regions. In normal control cases, no PHF1- or Alz-50-positive tangles or NT/DN were observed within the oculomotor complex (Fig. 2 f). Unlike the control cases, PHF1 and Alz-50 staining revealed a dense accumulation of structures with the morphology of tangles and NT/DN in the EW of all AD cases (Fig. 2 b). Adjacent sections stained with thioflavin-S confirmed the presence of tangles and NT/DN in the EW. In striking contrast, the SNCN3 was almost completely free of tangles and NT/DN (Fig. 3 b). Some adjacent areas, such as the dorsal raphe nucleus and the PAG, exhibited heavy burdens of pathology (data not shown). Examination of the cerebral cortex revealed that AD cases with the greatest number of cortical tangles and NT/DN, also exhibited the heaviest deposition of pathology in the EW.

Next, we determined the presence of tangles and NT/DN in the EW of the three cases that had been clinically silent, but displayed an accumulation of these lesions within the cerebral cortex. Cortical pathology in one case was relatively mild, while there was sufficient pathology present in the other cases to satisfy a classification of possible AD by CERAD criteria [37]. A moderate density of PHF-1 and Alz-50 positive neurons and NT/DN were observed in the EW (Fig. 2 d) of all three cases. Thioflavin-S-stained sections showed occasional tangles and a moderate density of NT/DN (data not shown). Again, the SNCN3 was completely free of pathology. In all cases, tangles and NT/DN were also observed in the PAG and the dorsal raphe.

The results of the quantitative assessment of tangles and NT/DN are presented in Figs. 4 and 5. The three groups differed significantly in the number of tangles (P = 0.03, two-tailed, K sample Kolmogorov-Smirnov) and NT/DN (P = 0.016, two-tailed) at all levels of the EW studied. The EW of the AD group and the control group with AD pathology had significantly higher numbers of tangles and NT/DN as compared with the normal control group (P = 0.015, two-sample Kolmogorov-Smirnov test). There was also a trend towards significant differences in the numbers of tangles and NT/DN in the EW of the AD sample and controls with cortical pathology (P = 0.065). Tangle counts in posterior sectors of EW did not show such a trend (P = 0.290, two-tailed).

The presence of plaques within the EW was investigated using Aβ immunohistochemistry. In the normal control cases, no Aβ-positive plaques were observed in the EW (Fig. 2 e) or the SNCN3. A relatively small number of diffuse Aβ deposits were observed in the cerebral cortex and PAG of some normal cases. By contrast, many Aβ-positive plaques were distributed throughout the EW of all AD

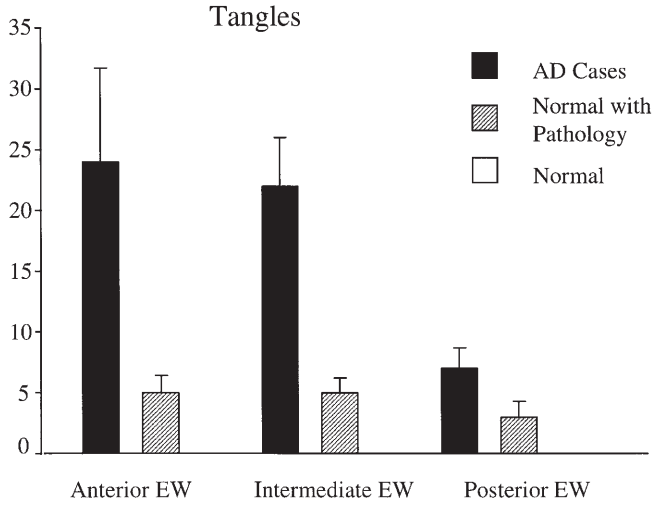

Fig. 4 Bar graph of the mean number of tangles in anterior, intermediate and posterior sectors of the EW of AD cases, controls with cortical pathology, and normal control cases without cortical pathology. AD cases exhibited the highest counts in all sectors. Normal control cases without cortical pathology exhibited no tangles in any sectors

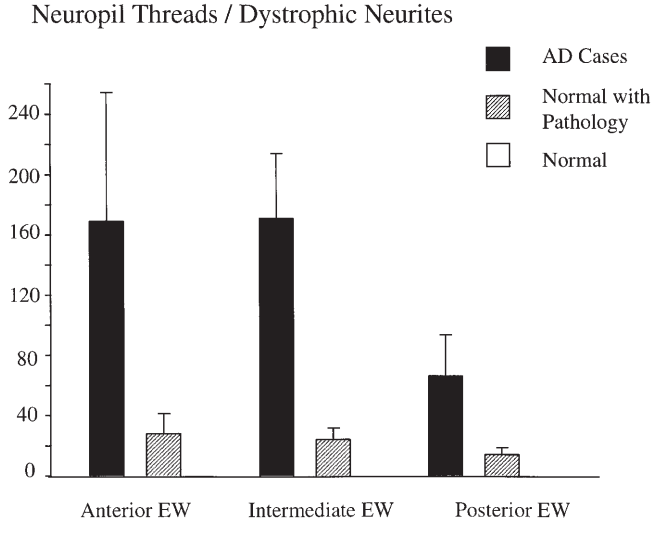

Fig. 5 Bar graph comparing the mean number of NT/DN in the anterior, intermediate and posterior sectors of EW for AD cases, controls with cortical pathology and normal control cases without cortical pathology. NT/DN counts were highest for AD cases in all sectors. Normal controls exhibited no NT/DN in any sectors

cases (Figs. 2a, 3c). Thioflavin-S-stained sections revealed that most of these Aβ-positive plaques were of the diffuse type. Very rare diffuse plaques were observed within the SNCN3 in AD cases. All of the AD brains contained a high density of Aβ- and thioflavin-S-positive plaques within the cerebral cortex and the PAG. The EW of the three clinically silent cases with cortical pathology contained no plaques (Fig. 2c). The cerebral cortex of these cases, however, exhibited a density of plaques between those of normal control and definite cases of AD.

We studied the distribution of AD-type pathology within the oculomotor complex from a patient who suffered from progressive supranuclear palsy (PSP), a disorder known to present with clinical oculomotor abnormalities. Numerous Alz-50- and PHF1-positive tangles, neurons and NT/DN were observed in both the EW and SNCN3 in this case (Fig. 3d, e). This distribution is in sharp contrast to that observed in AD cases in which the SNCN3 was virtually spared (Fig. 3b, c).

Discussion

There is an acknowledged need for early biological markers of AD if we are to develop other than palliative treatments for the disorder. The development of new therapeutic agents, aimed at slowing disease progression, is crucially dependent on our ability to identify individuals who are at the earliest stages of the pathological cascade prior to the onset of clinical symptoms. Linking candidate biological markers to the specific disease cascade of AD is a critical step in assessing the face validity of a proposed marker. The observations reported here confirm our speculation and demonstrate that the EW is a specific target of AD pathology and, thus, a likely link between the clinical observation of pupillary hypersensitivity to dilute tropicamide and the pathological process in AD. An earlier study [18] of two AD cases reported the presence of neurofibrillary tangles in the EW. Our observations extend this report and further suggest that the EW is a specific target of pathology, while the SNCN3 is virtually spared. In contrast, PSP patients appear to exhibit more general pathology throughout the oculomotor complex. The preliminary observation of non-selective pathology in the oculomotor complex of the PSP case, including the EW, may explain the recent report [31] of pupillary hypersensitivity in a sample of PSP patients.

Of particular note is our observation of the presence of pathology in the EW in three cases that were clinically silent but showed AD pathology within the cerebral cortex. The presence of pathology in the EW of these cases suggests that the formation of tangles and NT/DN in this nucleus may constitute a relatively early event in the pathological cascade of AD.

Most studies of pupillary hypersensitivity to dilute tropicamide have found that AD patients have an exaggerated pupil dilation response to dilute tropicamide. However, a number have failed to demonstrate significant group differences when comparing elderly community dwelling individuals to patients with probable AD. Results from most of the 29 published accounts [1, 4–8, 13–17, 19, 24, 25, 27, 29–31, 34–36, 38, 41–44, 46–48] of which we are aware, strongly suggest that the pupil assay is a relatively accurate marker of the underlying pathophysiology of the disease. Despite a bewildering array of measurement techniques, drug formulations and experimental conditions (none of which completely replicated the original procedures), 16 [1, 4, 7, 8, 13–17, 19, 24, 25, 27, 29, 34, 36, 38, 41–44, 48] of 24 [1, 7, 13, 14, 17, 19, 24, 25, 27, 29, 33, 34, 36, 38, 41–44, 46–48] reports comparing probable AD patients with normal controls found

that, as a group, probable AD patients dilated more rapidly and/or to a greater extent than normal controls. Ten of these publications [1, 13, 17, 19, 24, 27, 36, 42, 44, 48] have demonstrated that group differences were statistically significant. Two [34, 41] found statistically significant differences under certain conditions. Two [25, 29] found differences that were not statistically significant and one [14] showed a trend toward significance. The current observations on midbrain pathology suggest a potential explanation for the variations in findings detailed above. The presence of AD pathology in the EW similar to the three elderly cases in our sample, who were clinically normal, could lead to a hypersensitive response to tropicamide in otherwise clinically silent individuals. Thus, based on our findings, a proportion of community dwelling elders should be expected to exhibit a hypersensitive response to dilute tropicamide, giving rise to the varied findings. In conclusion, since AD pathology appears to be present in the EW early in the course of the disease, a hypersensitive pupil response may serve as an early marker for the disease process well before patients have sufficient pathology to exhibit clinical symptoms.

Our qualitative impression from Nissl-stained sections indicate that, in addition to pathological lesions, the EW may also display neuronal loss in AD (unpublished observations). Pathology within the EW, a known center for pupillary control, leading to neuronal loss is likely to initiate a cascade of events leading to the observed hypersensitivity of the pupil response in AD. The results of the present study suggest that the selective vulnerability of cholinergic neurons to AD pathology is not an exclusive feature of the basal forebrain [10], but is also a characteristic of some brain stem cholinergic structures, such as the EW.

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