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Accumulation of Amyloid β and Tau and the Formation of Neurofilament Inclusions Following Diffuse Brain Injury in the Pig

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Abstract. Brain trauma in humans increases the risk for developing Alzheimer disease (AD) and may induce the acute formation of AD-like plaques containing amyloid β (Aβ). To further explore the potential link between brain trauma and neurodegeneration, we conducted neuropathological studies using a pig model of diffuse brain injury. Brain injury was induced in anesthetized animals via nonimpact head rotational acceleration of 110° over 20 ms in the coronal plane (n = 15 injured, n = 3 noninjured). At 1, 3, 7, and 10 days post-trauma, control and injured animals were euthanized and immunohistochemical analysis was performed on brain sections using antibodies specific for Aβ, β-amyloid precursor protein (βPP), tau, and neurofilament (NF) proteins. In addition to diffuse axonal pathology, we detected accumulation of Aβ and tau that colocalized with immunoreactive βPP and NF in damaged axons throughout the white matter in all injured animals at 3–10 days post-trauma. In a subset of brain injured animals, diffuse Aβ-containing plaque-like profiles were found in both the gray and white matter, and accumulations of tau and NF rich inclusions were observed in neuronal perikarya. These results show that this pig model of diffuse brain injury is characterized by accumulations of proteins that also form pathological aggregates in AD and related neurodegenerative diseases.

Key Words: Alzheimer disease; Amyloid precursor protein; Amyloid β; Brain trauma; Neurodegeneration; Neurofilament; Tau.

INTRODUCTION

While traumatic brain injury is one of the leading causes of death and disability (1, 2), mounting evidence also suggests that brain trauma may have prolonged effects and initiate insidiously progressive neurodegenerative processes. Previously, postmortem histopathologic analysis of brains from boxers with *dementia pugilistica* ("punch-drunk syndrome") revealed neurofibrillary tangles (NFTs) and diffuse plaques composed of amyloid β peptides (Aβs) similar to the hallmark lesions of Alzheimer disease (AD) (3, 4). Subsequently, a single incident of brain trauma was shown to induce the formation of Aβ plaques within days following injury (5, 6). In addition, brain trauma patients have been shown to have accelerated cognitive decline during aging (7, 8) and an increased risk of developing AD, even if the injury occurred in the remote past (9–11). Moreover, we and others have recently observed that brain trauma in the rat induces substantially progressive neuron loss, axonal degeneration, and atrophy that proceeds unabated for at least one year following injury (12–13). Taken together, the results from these studies suggest that neurodegenerative changes triggered by brain trauma may follow a remarkably complex and prolonged temporal course. However, the mechanisms underlying the relationship between brain trauma and neurodegenerative processes remain unknown.

It has been observed that brain trauma in humans and experimental animals induces marked accumulations of β-amyloid precursor proteins (βPPs) (14–18), suggesting that ample substrate is available for Aβ production. However, experimental investigations of this relationship have been hampered since accumulation of Aβ has not been observed in standard rodent models of focal brain trauma (14–16, 18, 19). As previously suggested, the post-traumatic absence of Aβ accumulation in rodents may reflect a difference in the processing of βPP compared with humans (15, 19).

To further explore the potential link between brain trauma and neurodegeneration, in the present study, we used a well-characterized and clinically relevant model of diffuse brain injury in the pig (20–22). The most salient feature of this pig model is the production of widespread axonal pathology in the white matter resulting from nonimpact rotational acceleration of the head. This head rotation induces inertial loading to the pig brain that replicates the forces experienced by the human brain during traumatic events such as automotive crashes. The rationale to use this model was based on the observation that many trauma patients who developed Aβ plaques suffered from diffuse brain injury. In addition, this model affords an opportunity to evaluate neurodegenerative changes following diffuse brain trauma in an animal with a relatively high order, gyrencephalic brain.

MATERIALS AND METHODS

In these studies, we carefully adhered to the animal welfare guidelines set forth in the *Guide for the Care and Use of Laboratory Animals,* US Department of Health and Human Services Publication, 85-23. All animal procedures were approved by

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TABLE
Summary of Antibodies Used for Immunohistochemistry

Antibody	Epitope protein/amino acids	Type	Dilution	Reference
369W	βPP/645-694a	P	1:1000	(44, 45)
22C11	βPP/60-100	M	1:5	(46–48)
13335	Aβ/1-42	P	1:1000	(49)
Karen	βPP/N-Terminal	P	1:800	(49)
2332	Aβ/1-17	P	1:4000	(50–52)
BCO5	Aβ/1-42(43)	M	1:200	(53, 54)
4G8	Aβ/17-24	M	1:1000	(55, 56)
6E10	Aβ	M	1:200	(51, 56, 57)
10Δ5	Aβ/1-28	M	1:50	(58, 59)
Tau-2	Tau/192-204	M	1:400	(60–62)
PHF-1	PHF-tau	M	1:200	(63–66)
PHF-6	PHF-tau	M	1:250	(67–69)
PHF-13.5	PHF-tau	M	1:1000	(67) (69)
NR4	NF-L	M	1:400	(70–72)
N52	NF-H	M	1:400	(70, 73, 74)
Alz-50	A68 protein	M	1:5	(75)

the University of Pennsylvania Institutional Animal Care and Use Committee.

Preinjury Preparation

Eighteen miniature young adult swine (4 months of age, Hanford and Hormel strains), both male and female, weighing 17–20 kg, were used for this study (n = 3 noninjured, n = 15 brain injured). The animals were fasted for 12 h, after which anesthesia was induced with an initial injection of midazolam (400–600 mg/kg, i.m.). Once sedated, animals received 2%–4% isoflurane via snout mask until they reached a plane of surgical anesthesia. A venous catheter was then inserted in the ear, and the animals were endotracheally intubated and maintained on 1.5%–2% isoflurane. Physiologic monitoring and apparatus included noninvasive ECG electrode leads affixed to the and chest and extremities, a pulse oximeter placed on the skin of the tail, a rectal thermometer, and sampling tubes for end tidal CO_2 measurement attached to the endotracheal tube. Arterial blood gasses were also periodically evaluated pre- and postinjury. The pigs were continuously monitored and all data from physiologic monitoring were collected on a computer driven storage system. Intracranial pressure monitoring was not performed since previous studies demonstrated only small transient changes using the injury parameters applied in this study (20).

Brain Injury

Brain trauma was induced via head rotational acceleration as previously described in detail (20). Briefly, the head of each animal was secured to a padded snout clamp, which was mounted to the linkage assembly of pneumatic actuator device that converts the linear motion to an angular (rotational) motion. Rotation of the sidearm is triggered by the release of pressurized nitrogen into the actuator. For these experiments, the linkage was adjusted to produce a pure impulsive head rotation 110° in the coronal plane over a period of 20 ms, with the center of rotation close to the brain center of mass. Ten seconds prior to injury isoflurane anesthesia was withdrawn. The injury parameters were set to induce biphasic head rotational acceleration with a predominant deceleration phase. Following injury the animals were released from the device. All animals received buprenorphine (0.1 mg/kg, i.m., q 12 h, p.r.n) for postoperative analgesia. It is important to note that previous studies with these techniques demonstrated that injured animals were awake and ambulatory within 8 hours of injury (20).

Histopathology

At 1–10 days after brain injury the animals received an overdose of pentobarbital (150 mg/kg, i.v.) and were transcardially perfused with saline following by 4% paraformaldehyde (n = 3, sham (no injury); n = 3, 1 day; n = 3, 3 days; n = 6, 7 days; n = 3, 10 days). The brains were removed, postfixed in 4% paraformaldehyde and stored in phosphate buffer saline and cryoprotected with sucrose. Subsequently, the brains were blocked into 0.5 cm coronal sections for gross examination and photography. A series of 40-μm-frozen sections were cut from the front face of each block and mounted on microscope slides. Some blocks were cut into 3–5-mm-thick blocks and processed for paraffin embedding in an automated tissue processor (Shandon Hypercenter XP, Shandon Scientific Instruments, Cheshire, UK). Serial 6 μm sections from these blocks were cut on a rotary microtome and mounted on poly-L-lysine-coated slides. Primary antibodies specific for NF proteins, βPP, Aβ, and normal tau as well as paired helical filament (PHF) tau in AD NFTs used in these studies are outlined in the Table. Immunostaining was performed on free floating and paraffin-embedded sections using an avidin-biotin-immunoperoxidase complex method. The sections were incubated with primary antibody overnight at 4°C and then incubated at room temperature for 1 hour each with the appropriate secondary and tertiary antibodies, followed by enzymatic development with 3,3′-diaminobenzidine. Omission of the primary antibody or application of control serum on adjacent sections provided a negative control. Double labeling was performed with fluorescein isothiocyanate (FITC) and Texas red fluorescent secondary antibodies. Positive controls for NFTs and Aβ plaques were performed on human AD tissue and run in parallel with the pig tissue.

RESULTS

Physiology and Behavior

Consistent with previous reports, immediately following trauma, no substantial changes in arterial blood gases, pulse oximetry, or end tidal CO_2 were observed following injury. All animals began to awaken within 15 min following injury. Although the animals were able to ambulate typically within 1 hour following injury, they appeared to have slightly sluggish responses to sensory stimuli (startle reflex, tactile response) for up to 8 hours post-trauma. However, by 24 hours postinjury, all of the animals appeared completely normal based on gross neurosensory examination (normal startle reflexes, gait, rooting behavior, eating, and drinking).

Axonal and Neuronal Soma Pathology

Consistent with previous findings, axonal bulbs and varicose axonal swellings were observed following trauma at all timepoints evaluated (1 day–10 days) (Fig. 1). These axonal pathologies were identified by antibodies targeting βPP (Karen, 369W, 22C11) and NF proteins (NR4, N52). Axonal pathology was widespread throughout the brain and found in combination with gliosis most commonly in the root of gyri and at the interface of the gray and white matter (data not shown). No tissue tears and almost no vascular disruption were noted in regions of axonal injury. Modest neuronal damage was primarily found in the CA1 and CA3 subfields of the hippocampus as evidenced by pyknotic neurons and a general thinning of the pyramidal cell layers. No overt neuronal damage was observed in the cortex.

Accumulation of Aβ and Tau in Axonal Bulbs

At 3–10 days post-trauma we found that Aβ and tau accumulated in most axonal bulbs found throughout the brains, demonstrated by all specific antibodies utilized (Aβ immunostains: 2332, 13335, BCO5, 4G8, 6E10 and 10Δ5; Tau immunostains: Tau-2, PHF-1, PHF-6 and PHF-13.5) (Fig. 2). Positive staining with antibodies specific for PHF tau suggests that highly phosphorylated tau (like that in AD NFTs) accumulated in these brains. No Aβ or tau accumulations in axons were detected at 1 day post-trauma. Colocalization of NF proteins, βPP, Aβ, and tau was found in most, but not all, axonal bulbs demonstrated by multiple immunostains with fluorescence microscopy (Fig. 3A–H). However, no Aβ or tau was found accumulating in axonal swellings (Fig. 3I, J). Therefore, Aβ and tau accumulation was limited to the terminal ends of disconnected axons.

Aβ in Plaques

At 3–10 days postinjury, we found Aβ containing plaque-like profiles in the pig brain tissue (stained with antibodies 2332, 13335 and BCO5) (Fig. 4). These plaques were predominantly found in white matter with axonal pathology as well as in layer III of the cortex, and these findings were confirmed by positive staining in identical regions of adjacent thin (6 μm) sections. However, the pig Aβ plaques did not stain as robustly as plaques from positive control AD brains, and they were not very numerous (the most plaques found in any section was 10). In addition Aβ containing plaques were only found in approximately one third of the injured animals, which also represented the group with the highest total amount of axonal pathology.

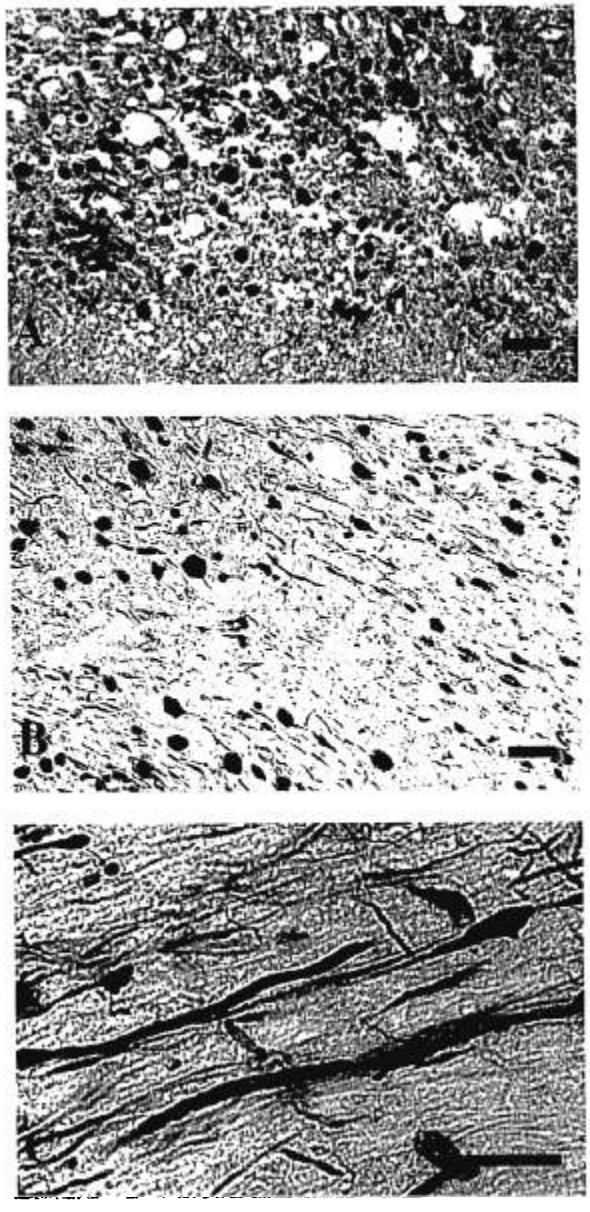

Fig. 1. Representative photomicrographs of pig brain sections demonstrating axonal pathologies following brain trauma. Numerous axonal bulbs were found in the subcortical white matter in sections stained with antibodies against βPP (A) and NF-H (B). NF-H immunostains also revealed varicose swellings of axons in the deep white matter (C). Scale bar = 50 μm.

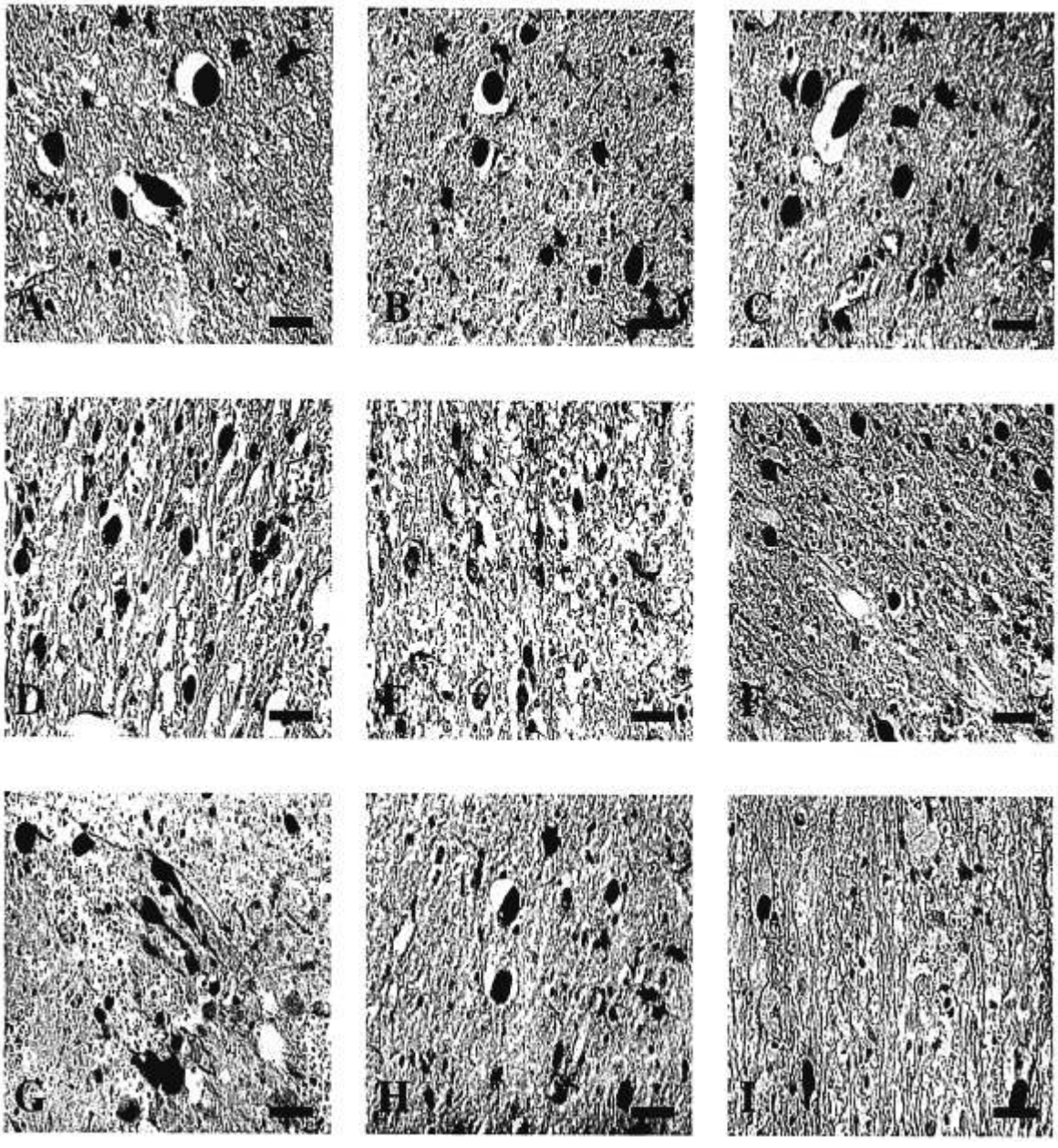

Fig. 2. Representative photomicrographs demonstrating Aβ and tau accumulation in axonal bulbs of brain injured pigs. Aβ was identified with several specific antibodies, including 10Δ5 (A), 13335 (B), 2332 (C), 4G8 (D), 6E10 (E), and BCO5 (F). Tau staining was also found with several specific antibodies including PHF-1 (G), PHF-13.5 (H), and PHF-6 (I). Scale bar = 50 μm.

Tau Accumulation in Neurons

Accumulation of tau was found in the cytoplasm of neurons throughout the frontal, parietal, and temporal corticies at 3–10 days after brain injury (identified with antibodies; Tau-2, PHF-tau, PHF-1, PHF-6, and PHF-13.5) (Fig. 5C–E). The frequency and prevalence of this staining also appeared to correspond with the relative extent of axonal pathology. Our AD brain sections also demonstrated cytoplasmic staining with the same set of antibodies (Fig. 5A, B).

Neurofilament-rich Inclusions in Neurons

At 3–10 days post-trauma we found NF immunoreactive inclusions in the cytoplasm of neurons in the parietal and temporal cortices. These inclusions had a highly dense core surrounded by cytoplasm similar to the Lewy body (LB) inclusions found in human neurodegenerative diseases such as demential with LBs (DLB) and Parkinson disease (PD) (Fig. 6A–G). Strong NF immunostaining of these inclusions was found in adjacent thin sections (6 μm). However, we only found

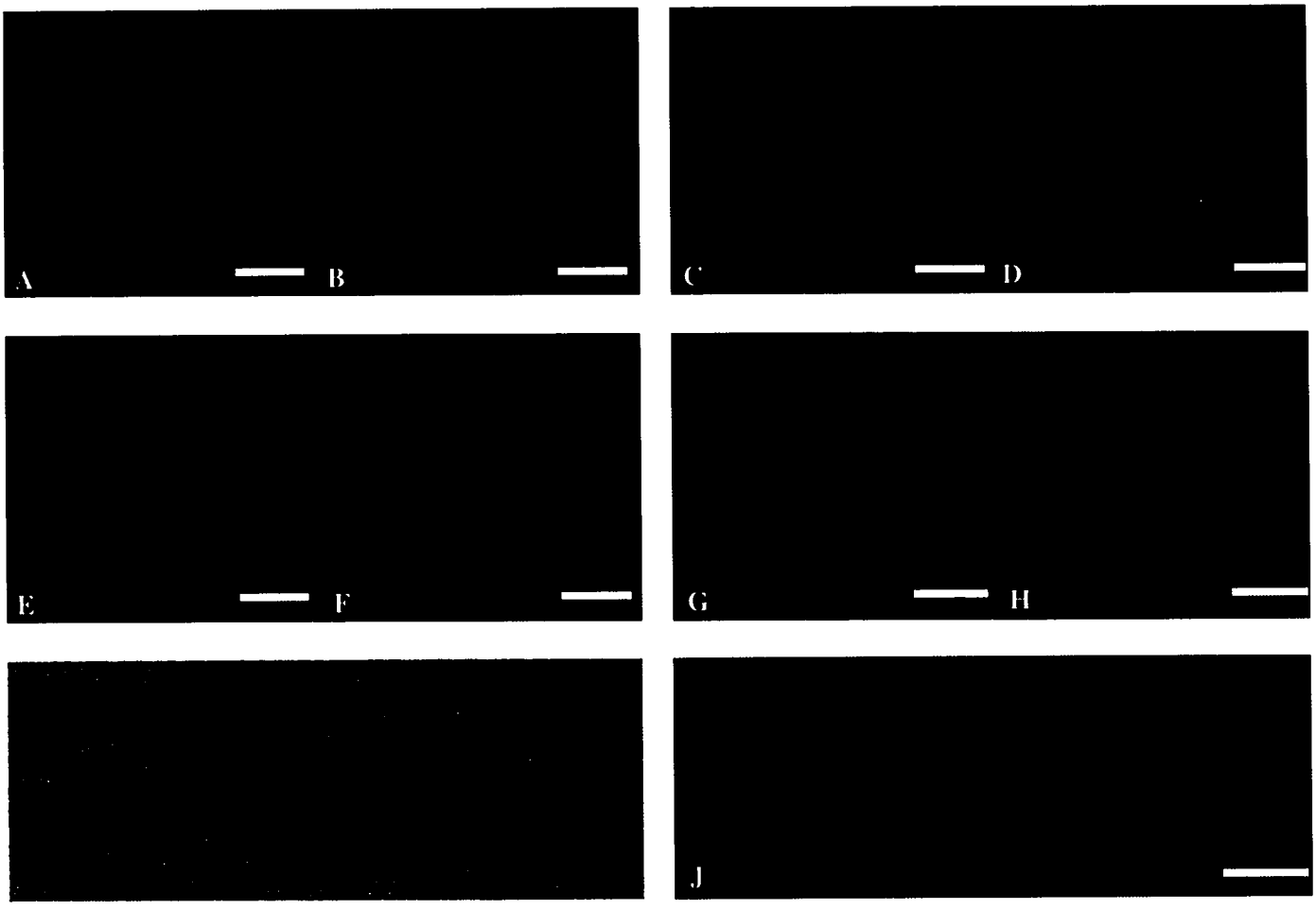

Fig. 3. Representative photomicrographs demonstrating coaccumulation of Aβ, βPP, tau, and neurofilament in axonal bulbs following brain injury in the pig. Double immunolabeling with fluorescent staining demonstrated colocalization of Aβ (A) with NF-H (B), Tau (C) with Aβ (D), Aβ (E) with βPP (F), and βPP (G) with tau (H). In another type of axonal pathology, varicose swellings, typical βPP accumulation was found, demonstrated by light microscopy (I). However, in the same section, Aβ staining was not found in varicose axonal swellings, demonstrated by fluorescence microscopy (J). Scale bar = 50 μm.

this pathology in the same subset of animals that demonstrated Aβ plaque-like profiles (i.e. approximately one third of the injured animals).

DISCUSSION

In this study we found that inertial brain trauma in the pig produced diffuse axonal pathology in combination with several unique pathologic features that may be suggestive of neurodegenerative processes. The most remarkable and consistent finding was extensive Aβ and tau accumulation in damaged axons following trauma. In addition, in a subset of brain injured animals, diffuse Aβ-containing plaque-like profiles were found in both the gray and white matter, and accumulations of tau and NF rich inclusions were observed in neuronal perikarya. To our knowledge, this is the first report of these collective findings in an animal model of brain trauma.

The observation of widespread Aβ accumulation in damaged axons following inertial brain injury in the pig may have important implications. It has previously been suggested that aberrant conversion of βPP to Aβ at synapses may play a critical role in the evolution of AD. This same aberrant processing of βPP has been proposed to be initiated by brain trauma in humans, leading to the formation of diffuse Aβ plaques in the gray matter within days following injury (5, 6). However, the most abundant accumulations of βPP resulting from brain trauma are found in damaged axons in the white matter (6, 17). Nonetheless, previous studies have not identified colocalization of Aβ with the axonal pool of βPP in studies of brain injured humans or in rodent models of brain trauma (14–16, 18). The ability to identify Aβ in damaged axons in the present study may reflect the use of highly specific Aβ antibodies in conjunction with a gyrencephalic animal model of diffuse axonal pathology.

It is important to consider that this axonal pool of Aβ may be released into the surrounding tissue from lysis or

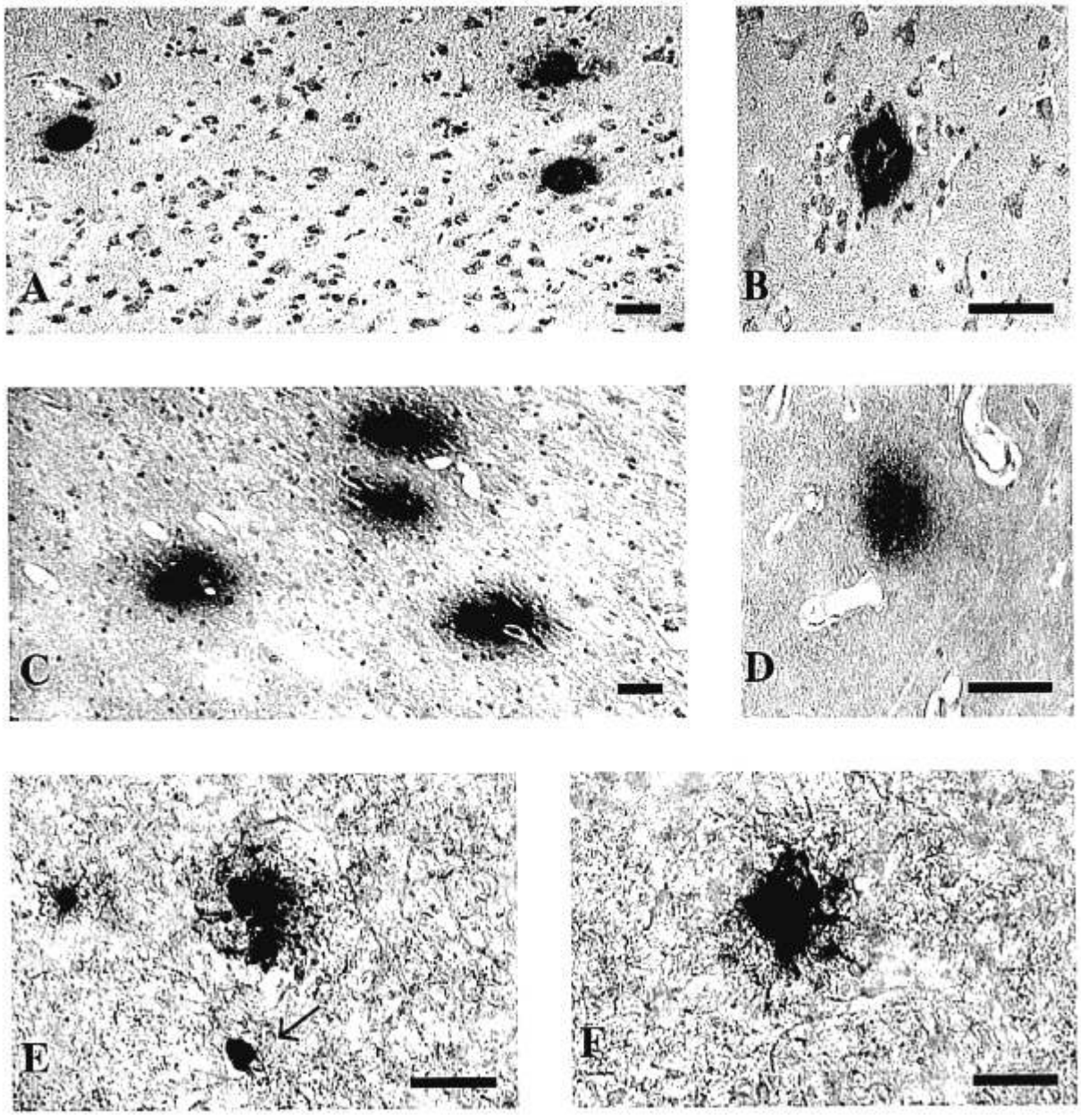

Fig. 4. Representative photomicrographs of tissue sections immunostained for Aβ demonstrating plaque formation. Typical Aβ plaques in the gray matter of an Alzheimer disease brain (A and B). Aβ plaques in pig brain following trauma in the gray matter (C and D), and white matter (E and F). Note the axonal bulb staining for Aβ in proximity to a plaque (arrow, E). Scale bar = 50 μm.

leakage of axonal bulbs. While Aβ alone has not been shown to be substantially toxic in vivo (23, 24), we have previously proposed a "two hit" hypothesis whereby Aβ may potentiate damage when combined with brain injury. Using transgenic mice that overexpress mutant human βPP and eventually develop Aβ plaques (25), we found that brain trauma at an age prior to plaque formation induced massive neuron death accompanied by a marked increases in soluble Aβ peptide levels (19). Moreover, a recent report has also described a large increase in Aβ peptides in the cerebrospinal fluid of brain injured patients (26). Our present results suggest that damaged axons are one potential source for a massive increase in soluble Aβ following brain trauma. Collectively, these data provide corroborative evidence that the production and release of Aβ plays a role in the delayed pathogenesis of brain trauma.

The colocalization of βPP and Aβ in damaged axons in the present study appeared to be limited to a specific morphologic subtype of axonal pathology. The major

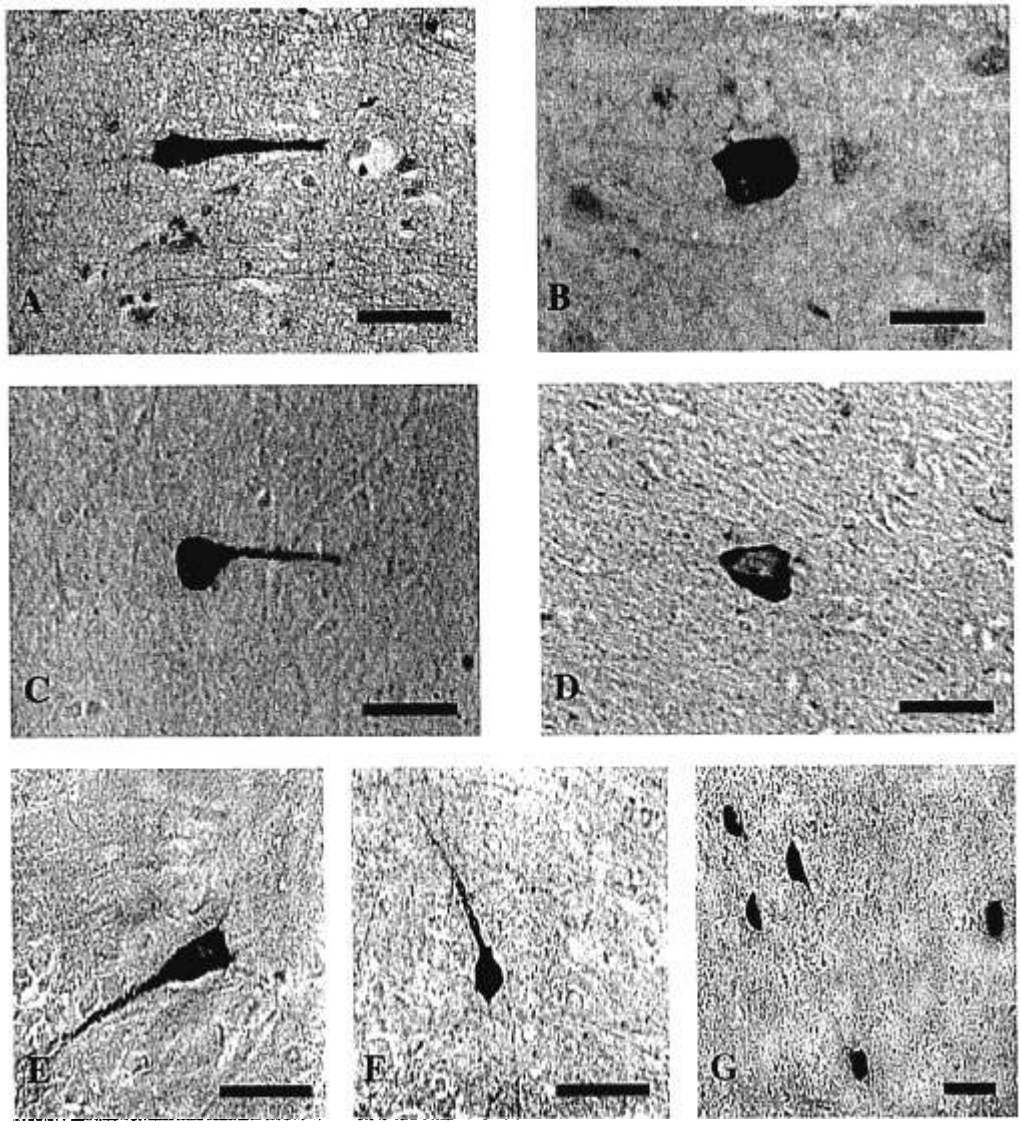

Fig. 5. Representative photomicrographs of tau staining in neurons. Typical pathology of tau staining in neurons in Alzheimer disease brains (A and B). Neuronal staining with various specific antibodies targeting tau is also found in brain injured pigs (C–G). Scale bar = 50 μm.

morphologic characteristics or "phenotypes" of post-traumatic axonal pathology include 1) varicose swellings encompassing long regions of injured axons, and 2) discrete axonal bulbs (also referred to as retraction balls and terminal clubs), characterized by individual rounded swellings at the terminal end of disconnected axons (20, 27). In the present study, βPP accumulated in both discrete axonal bulbs and in elongated varicose axonal swellings consistent with previous observations (15–17). However, Aβ was only observed accumulating in axonal bulbs, i.e. only in axon regions proximal to clearly identified axotomy. These data suggest that axotomy induces a unique intra-axonal proteolytic milieu that favors the production and accumulation of Aβ in axonal bulbs. Conversely, this process does not appear to occur in damaged yet still connected axons, despite substantial swelling and βPP accumulation.

In addition to the widespread accumulation of Aβ in axonal bulbs, we also found a limited number of Aβ containing diffuse plaque-like profiles at 3–10 days following brain injury in the pig. These were found on adjacent sections and were identified with several highly specific anti-Aβ antibodies. Although this finding may appear novel, it should be emphasized that the plaque-like profiles were relatively few in number and were primarily identified in the white matter, a location inconsistent with the distribution of Aβ plaques described in recent studies of brain injured humans (5, 6). Nonetheless, the white matter location of the Aβ plaques in brain injured pigs may reflect the release of Aβ from damaged axons and

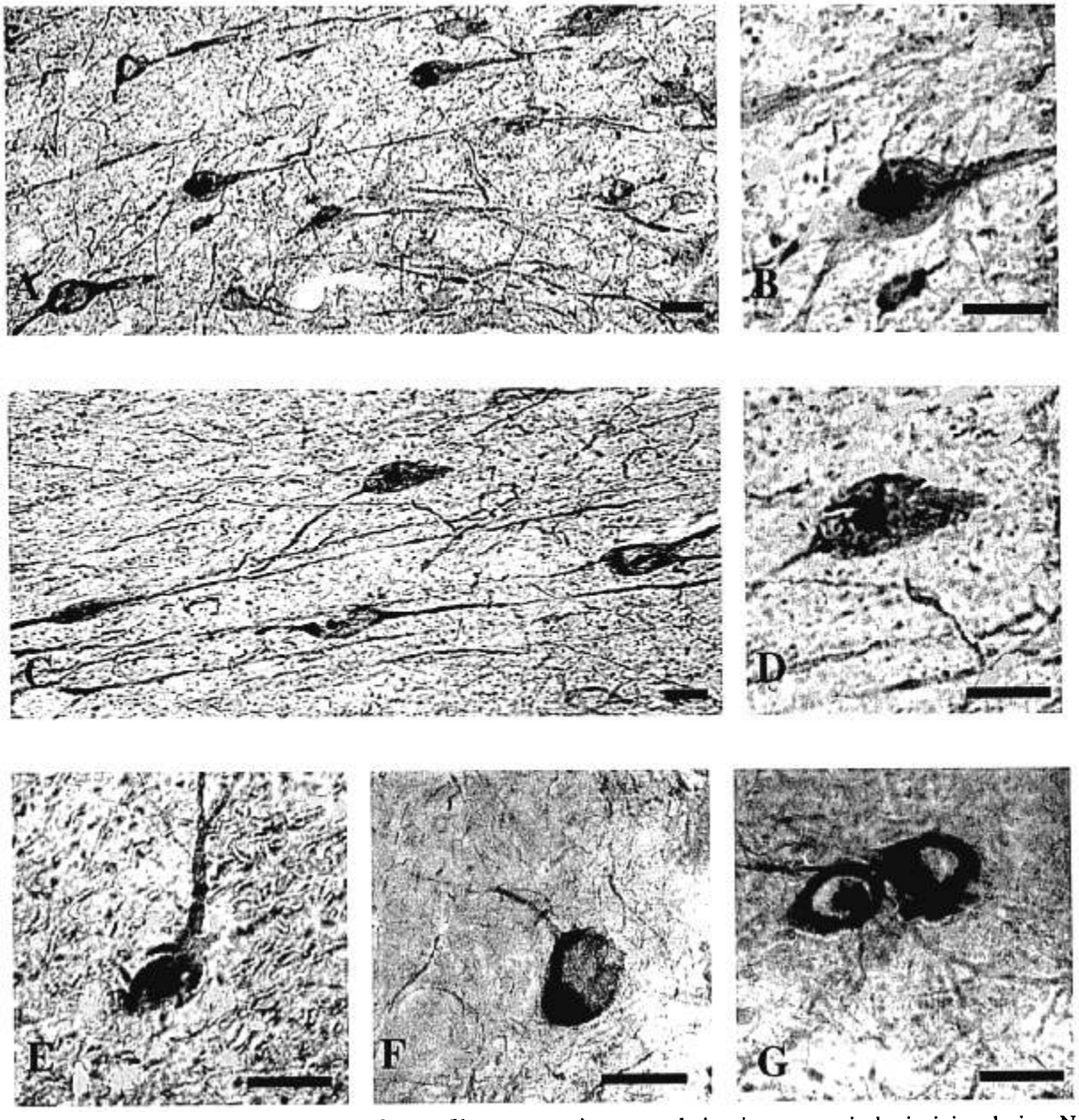

Fig. 6. Representative photomicrographs of neurofilament protein accumulation in neurons in brain injured pigs. Neurofilament immunoreactivity demonstrates neurofilament inclusions in neurons, seen as darkly stained profiles in the cytoplasm. Scale bar = 50 μm.

subsequent extracellular aggregation in the same region. Furthermore, the formation of Aβ plaques appeared to be related to the extent of axonal pathology.

Based on the identification of Aβ plaques in brain injured humans, we and others have previously attempted to elucidate potential Aβ plaque formation in rodent models of focal brain trauma without success (14–16, 18, 19). We did not even find acceleration or augmentation of Aβ plaque formation following brain trauma in transgenic mice that otherwise go on to develop Aβ plaques (19, 28). It is not presently clear whether the inability to replicate the human condition of Aβ plaque formation in rodent models of brain trauma is due to species effect or mechanisms of injury. While results from the present study may suggest that Aβ plaques are produced following inertial diffuse brain injury in the pig, further studies are needed to confirm this potentially important finding.

Another potential link between neurodegenerative changes and diffuse brain injury in the pig is the accumulation of the microtubule-associated protein, tau, in damaged axons. Highly phosphorylated tau is the primary constituent of PHFs that form NFTs, one of the two major pathologic features of AD (29–31). Since tau is an integral structural protein in axons, it has been presumed

that it would accumulate in damaged axons with impaired transport. However, axonal accumulation of tau has not been previously observed following trauma despite several investigative efforts (32, 33). The colocalization of tau with Aβ in damaged axons observed in the present study may have important implications since it is has been suggested that tau potentiates Aβ toxicity in vitro and facilitates the polymerization of Aβ peptides that may lead to Aβ plaque formation (34, 35).

The accumulation of tau was also found in the cytoplasm of neurons of brain injured pigs in the present study. Since these profiles were identified with antibodies that recognize the highly phosphorylated forms of tau that form AD PHFs, these profiles most closely resemble the so-called "pre-tangle" somatodendritic tau lesions seen in AD brains. Indeed, it will be important to perform ultrastructural analyses of these lesions in future studies to determine if tau filaments are present in these perikaryal tau accumulations. Recently, neuronal staining for tau has also been found following brain injury in the rat (36). These findings may have important clinical implications since NFTs have been found in the brains of boxers, but no NFT-like lesions have been detected in the human brain following a single incident of brain trauma (3, 4).

Yet another unexpected finding in this study was that NF proteins, the building blocks of NFs, formed inclusion bodies in neurons following brain injury. While cytoplasmic NF rich inclusions, known as LBs, are signature lesions of DLB and PD, they have not previously been reported following trauma in humans. Nonetheless, accumulations of NFs are well documented in damaged axons following brain trauma in humans and experimental animals (20, 27, 37, 38). NF proteins are components of LBs, but alpha-synuclein may be the major building block of these lesions in PD and DLB (39). In addition, LBs are very common in the AD brain (40, 41). However, the mechanisms of development and the role of NF protein inclusions in neurodegenerative diseases have yet to be elucidated following brain trauma. Recent studies have also shown accumulation of NF protein in neuronal perikarya following trauma in a nontransgenic animal model of brain injury (42) and that LB-like inclusions may render neurons more vulnerable to degenerate following brain trauma in a transgenic mouse that overexpresses a NF hybrid protein (43). Since the predominant pathology in the pig diffuse brain injury model is damage to axons, not cortical neurons, our finding of cytoplasmic NF inclusions suggests that impaired axonal transport may play a role in the perikaryal accumulation of NF proteins.

Taken together, the results from the present study demonstrate that several pathologic characteristics of neurodegenerative diseases may also be found following diffuse brain injury in the pig. Accordingly, our results support the proposed link between brain trauma and the initiation of neurodegenerative processes. It is not clear if the markers of neurodegenerative changes found accompanying diffuse axonal pathology in our pig model are consequences of injury specific mechanisms (i.e. inertial brain injury), or a general response of a gyrencephalic brain to trauma. Nonetheless, mechanisms of trauma-induced neurodegenerative processes may be further explored using this unique model.

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