Nestin-Positive Ependymal Cells Are Increased in the Human Spinal Cord after Traumatic Central Nervous System Injury

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Abstract

Endogenous neural progenitor cell niches have been identified in adult mammalian brain and spinal cord. Few studies have examined human spinal cord tissue for a neural progenitor cell response in disease or after injury. Here, we have compared cervical spinal cord sections from 14 individuals who died as a result of nontraumatic causes (controls) with 27 who died from injury with evidence of trauma to the central nervous system. Nestin immunoreactivity was used as a marker of neural progenitor cell response. There were significant increases in the percentage of ependymal cells that were nestin positive between controls and trauma cases. When sections from lumbar and thoracic spinal cord were available, nestin positivity was seen at all three spinal levels, suggesting that nestin reactivity is not simply a localized reaction to injury. There was a positive correlation between the percentage of ependymal cells that were nestin positive and post-injury survival time but not for age, postmortem delay, or glial fibrillary acidic protein (GFAP) immunoreactivity. No double-labelled nestin and GFAP cells were identified in the ependymal, subependymal, or parenchymal regions of the spinal cord. We need to further characterize this subset of ependymal cells to determine their role after injury, whether they are a population of neural progenitor cells with the potential for proliferation, migration, and differentiation for spinal cord repair, or whether they have other roles more in line with hypothalamic tanycytes, which they closely resemble.

Key words: GFAP; neural progenitor cells; postmortem; tanycyte; trauma

Introduction

THE LONG-HELD BELIEF that neurogenesis could not occur in the adult human central nervous system (CNS) has been challenged by the recent identification of neural progenitor cells (NPC) in the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus.^{1,2} The obvious potential of these cells in treating neurodegenerative conditions or injuries to the brain has led to an increased focus on the characteristics of these cells and how they may be manipulated *in vivo* to proliferate, migrate, and differentiate to repair areas of tissue damage.

Endogenous NPC have now also been located in the mammalian spinal cord, and are thought to reside primarily in the ependymal or subependymal regions of the central canal. Cells taken from these regions can be cultured to form neurospheres and, under the right conditions, differentiate into mature astrocytes, oligodendrocytes, and neurons.^{3–9} Proliferation can be stimulated by epidermal growth factor (EGF) with basic fibroblast growth factor (bFGF),^{6,10} or by injury.^{7,11}

Proliferation of ependymal cells in the normal uninjured rat spinal cord is limited, but appears to follow a rostrocaudal axis with a higher proliferation of cells in the more caudal regions of the spinal cord.¹² Dividing cells express markers of mature astrocytes or oligodendrocytes, but remain *in situ* rather than migrating to the surrounding tissue.¹³ After spinal cord injury in the rat, NPCs proliferate and differentiate into glial cells but not neurons,^{5,14,15} and can migrate toward an injury site.^{11,14,16}

Endogenous NPC present in the adult human spinal cord have been isolated from fresh autopsy tissue, cultured, and shown to differentiate into neurons and glial cells *in vitro*.^{17,18} One of the common markers for progenitor cells, nestin, is increased in the ependyma of human spinal cords from patients with multiple sclerosis,¹⁹ amyotrophic lateral sclerosis (ALS), and spinal tumors,²⁰ and in hydrocephalic infants.²¹

There are discrepancies in the reported antigenicity, development, location, and response of cells purported to be NPC in the spinal cord injury. For example, Meletis and associates⁵ identified three distinct ependymal cell populations in the mouse central canal

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based on morphological differences, but was unable to determine any molecular marker delineating these subpopulations. Alfaro-Cervello and associates²² described biciliated ependymal cells of the central canal that are nestin positive and glial fibrillary acid protein (GFAP) negative, and which proliferate slowly under normal growth conditions. Dromard and associates¹⁷ indicated a cluster of nestin-positive cells located in the ventral subependymal region as being a possible neurogenic niche, whereas Hamilton and associates⁸ reported that cells with stem cell characteristics may be located in the dorsal pole of the central canal and have a tanycyte-like morphology. Rat tanycytes in the ependyma lining the cerebral ventricles and spinal cord have been described in detail,^{23–25} and although they have little regenerative capacity, they do respond to injury and have a stronger response in the spinal cord than in the brain.

In this study, we will examine the central canal of human spinal cords to investigate the response of ependymal cells to CNS injury using nestin and GFAP immunoreactivity. We will also determine whether patient age, survival time, or spinal level correlate to the amount of nestin immunoreactivity exhibited by these cells.

Methods

Human spinal cord samples

Human cervical spinal cords were obtained from 41 subjects (gestational age 18 weeks to 40 years of age), 25 from the Department of Forensic Medicine Sydney, NSW Health Pathology (DoFM) and 16 from the National Institute of Child Health and Human Development, University of Baltimore, Maryland (NICHD) (Table 1). "Trauma" cases were from subjects who had died as a result of a motor vehicle accidents (MVA), non-accidental death, or falls, and were divided into those who had reported survival times of >30 min ($n = 14$) and those whose survival time were either unknown or reported to be <30 min ($n = 13$). The remaining 14 cases were included as "control" cases, and included spinal cords from two embryos and subjects who had died from a variety of causes including drowning, SIDS, asphyxiation, myocarditis, sudden collapse, and pulmonary fibrosis. Additional thoracic and lumbar spinal cord tissue was available for 12 cases. All cases were de-identified, and included information about age, gender, post-mortem (PM) delay, and at least a brief summary of the circumstances surrounding the death. Ethics approval was obtained from the Sydney Local Health District Human Research Ethics Committee and the University of Technology, Sydney, Human Ethics Committees.

Preparation of spinal cords for histology

Spinal cord sections were stored in 10% buffered formalin before processing through changes of graded alcohols and xylene and embedding in paraffin wax. Transverse sections were cut at 5 μ m with a Microm HM 325 rotary microtome (Thermo Fisher Scientific Inc., MA) for all but three samples, for which tissue was supplied as longitudinal cut unstained sections. Sections were then placed onto Flexi microscope slides (Dako, Denmark). One section from each case was stained with Hematoxylin and Eosin (H&E), and adjacent sections were immunoreacted with anti-nestin and anti-GFAP antibodies, or both.

Immunohistochemistry

All sections were de-paraffinized and taken to water. Sections undergoing immunohistochemistry for anti-nestin then underwent antigen retrieval in ethylenediaminetetraacetic acid (EDTA) pH 8.0 solution. Slides were heated in EDTA solution in a microwave until boiling (1 min) and then allowed to cool to room temperature before

being rinsed in distilled water. All slides were then immersed in 0.1M phosphate-buffered saline (PBS), pH 7.4, with 0.1% Triton X-100 (PBS with Tween [PBST]) for 10 min prior to 3% H₂O₂ in PBST for 30 min to block endogenous peroxidase. They were then washed in three changes of PBST before immersion in 5% normal goat or horse serum (NGS/NHS) in PBST for 30 min to block nonspecific binding. Slides were incubated overnight in primary antibody at 4°C; rabbit anti-GFAP (1:1000, Dako, Denmark) or mouse anti-nestin antibody (1:500; Dako, Denmark). Sections were rinsed in three changes of PBST before incubation in biotinylated secondary antibody; Goat anti-Rabbit (1:200, Vector, USA) or Horse anti-Mouse (1:200, Vector, USA) for 1 h at room temperature followed by three rinses in PBST and 1 h incubation in peroxidase-ABC complex (Vector, USA). Finally, the reaction product was visualized using 3,3'-diaminobenzidine (DAB) tetrahydrochloride, and the nuclei were lightly counterstained with Mayer's Hematoxylin before dehydration and cover-slipping with DPX.

For nestin-positive sections, double labeling with anti-GFAP and anti-nestin using fluorescent secondary antibodies was then performed on adjacent sections. These sections underwent antigen retrieval, were incubated in normal serum in PBST for 30 min, and were then incubated overnight in a cocktail of the two primary antibodies described. After rinsing in three changes of PBST, the sections were incubated in AF 488 goat anti-rabbit and AF 568 goat anti-mouse (1:200 Invitrogen, USA) for 1 h at room temperature in the dark. Sections were washed with PBST and all sections were counterstained with Hoechst (33342, Invitrogen, CA) for 10 min to visualize the cell nuclei before cover-slipping with Fluoromount (Dako, Denmark). The primary antibody was omitted from negative control slides.

Analysis of central canal morphology

H&E-stained sections were observed using the Olympus BX51 Light Microscope (Olympus, Japan), and digital images were captured with an Olympus DP70 digital camera. All images were centered on the central canal, and have the dorsal aspect at the top of the image. The lumen area, perimeter, circularity, and epithelial height were measured for each section. The number of ependymal cells was counted, and a measure of ependymal cells/mm was calculated for comparison between the groups. Longitudinal sections were omitted from the lumen measurements, and sections with poor central canal morphology were omitted from all measurements.

Analysis of immunohistochemistry

High power digital images of nestin/DAB-stained sections were captured as described. The total number of ependymal cells identified by their nuclei and the number of nestin-positive ependymal cells were counted in each spinal cord section. This measurement was expressed as the percentage of nestin positive cells (% nestin).

Digital images of GFAP/DAB-stained sections were centered on the central canal such that both gray matter (GM) and white matter (WM) were visible. An additional image was taken of the dorsal roots in each sample. The dorsal root does not contain astrocytes, and, therefore, is negative for GFAP. This image was used as an internal control for each section to control for any variation found in background staining intensity on different slides. The average gray scale value was calculated, from four regions of interest in the WM and the GM adjacent to the central canal and the dorsal root, using ImageJ software to give a measure of GFAP staining intensity. The percentage increase from background (dorsal root) was then calculated as a measure of GFAP-staining intensity (% GFAP) for the WM and GM in each section. Longitudinal sections were omitted from this analysis.

TABLE 1. DETAILS OF CONTROL AND TRAUMA CASES

Case no.	Age (yrs)	Gender	Cause of death	Postmortem delay (h)	Post-injury survival time (h)	Source	% nestin	% GFAP
<i>Non trauma controls</i>								
1	G18w	F	-	1	0	NICHD	0	++
2	G18w	F	-	1	0	NICHD	0	+
3	42.2	F	HASCVD	4	0	NICHD	0	+
4	40.6	F	Pulmonary fibrosis	13	0	NICHD	0	+
5	2	F	Drowning	16	0	DoFM*	0	-
6	2.2	F	Myocarditis	21	0	NICHD	0	+
7	0.1	M	SIDS	23	0	NICHD	0	+
8	0.2	M	SIDS	24	0	NICHD	0	+
9	19.4	F	Collapse/unresponsive	24	-	NICHD	0	++
10	3	M	Drowning	26	0	DoFM	0	+
11	0.2	F	SIDS	27	0	NICHD	0	+
12	2.8	M	Asphyxia/choking	16	-	NICHD	+	++
13	4.5	F	Fit/myocarditis	21	-	NICHD	+	++
14	43.8	F	Possible Myocarditis	34	0	NICHD	+	+
<i>Trauma with no recorded post-injury survival</i>								
15	21.9	M	MVA	13	-	NICHD	0	++
16	36	M	MVA	48	0	DoFM	0	+
17	27.1	M	MVA	15	-	NICHD	0	+
18	14.5	M	MVA	16	-	NICHD	0	+
19	5	F	MVA	16	0	DoFM†	+	++
20	0.4	F	MVA	36	0	DoFM	+	++
21	4	F	MVA	48	0	DoFM†	+	++
22	40	M	MVA/airplane	48	0	DoFM	+	++
23	1.9	F	Non-accidental	48	0	DoFM*	++	-
24	22	M	Collapse/fall	7	-	NICHD	++	+
25	19	M	MVA	48	0	DoFM	++	+
26	9	M	MVA	18	0	DoFM†	++	++
27	4	F	MVA	24	0	DoFM†	++	++
<i>Trauma with recorded post-injury survival</i>								
28	0.8	M	Non-accidental	7	2	DoFM†	0	+
29	0.5	M	Non-accidental	22	24	DoFM	0	+
30	2	M	Non-accidental	15	0.5	DoFM	0	++
31	2	M	MVA	12	1	DoFM	+	+
32	1.3	F	Non-accidental	24	48	DoFM	+	+
33	9	F	MVA	-	1	DoFM*	+	-
34	0.3	M	Non-accidental	15	9	DoFM†	+	++
35	2	M	MVA	37	0.75	DoFM†	+	++
36	18	M	MVA	22	10	DoFM†	++	+
37	2	F	MVA	45	91	DoFM†	++	+
38	0.4	F	Non-accidental	12	3	DoFM†	++	++
39	4	M	MVA	23	1	DoFM	++	++
40	31	M	MVA	45	336	DoFM†	++	++
41	8	F	MVA	9	84	DoFM†	+++	+

G, gestational age; HASCVD, hypertensive arteriosclerotic cardiovascular disease; SIDS, sudden infant death syndrome; MVA, Motor vehicle accident; NICHD, National Institute of Child Health and Human Development, University of Baltimore, Maryland; DoFM, Department of Forensic Medicine, South West Sydney Area Health Service, Australia; -, not reported; *longitudinal sections only; †multiple levels available; +, 0–10%; ++, 10–20%; +++, 20–30%.

Results

Human spinal cord samples

The details for the 41 cases investigated in this study are shown in Table 1. Tissue was obtained from two sources, but there were no differences in the mean age of subjects (8.2 ± 11.5 vs. 15.1 ± 16.3 years), or PM delay (23.2 ± 15.1 vs. 26.9 ± 15.3 h) between these two sources. Gender distribution was different between the two groups, with cases from the DoFM (16M: 9 F) having more males than the NICHD group (4M:12 F) (Fisher's

exact test $p=0.02$). The cases from the NICHD were mostly selected for use as controls, and, therefore, had shorter post-injury survival times (0h compared with an average of 24.4h for the tissue collected from the DoFM). CNS injuries were reported for the DoFM cases, and included one or more of the following: skull or spinal vertebral fractures; subarachnoid hemorrhage (SAH); subdural hemorrhage (SDH); bruising to the scalp, neck, or spinal ligaments; cerebral or spinal cord hemorrhage; or other brain abnormality (Table 2). Detailed PM reports were not available for the NICHD cases. There were no correlations between age and

TABLE 2. DETAILS OF CNS INJURIES FOR THE DoFM TRAUMA CASES

Case no.	Skull fracture	SAH	SDH	Hemorrhage	Spinal cord	Other abnormality
16		+		Large scalp bruise		
19		+		Corpus callosum, parasagittal gliding contusions, pontine hemorrhage		Abrasions to scalp, total transection of inferior medulla, DAI, bruising around fracture, and laceration of vertebral arteries
20	++	+		Bruising to scalp, intraventricular hemorrhage, bilateral internal capsule		
21					Bruising to cord	
22				Scalp bruising		
23						Periventricular leukomalacia, periventricular nodular heterotopias, cavum septum pellucidum (likely all caused by perinatal hypoxia/ischemia resulting from prematurity)
25	++	+		Cortical contusions inferior surface of right temporal lobe, multiple small hemorrhages in splenium		Lacerations of dura
26	++	+	+		Bruising around spinal process T3/5	Laceration of inferior surface of cerebrum
27	++			Extensive abrasion and areas of bruising of scalp, inferior cerebral hemisphere, EDH (small) associated with laceration of sigmoid sinus		Left cerebellar laceration
28				Bruising to scalp, hemorrhage between C3 and C6	Cervical ligament damage	
29	+		+			
30				Upper cervical spinal cord	? SCIWORA	Bruising to neck and back of head
31	++					Laceration of dura, maceration and extrusion of brain tissue
32				Multifocal bruising of the scalp, congestion/hemorrhage around cervical cord roots		Peritonitis caused by blunt force abdominal injury
33	+					
34			+	Dense hemorrhage in cervical spinal cord		
35		+	+		C6/7 fracture	
36	++	+	+	Bruising of back of scalp, scanty EDH at fracture posteriorly, inferior and lateral temporal lobe contusion		Marked brain swelling with herniation, hypoxic brain damage/brain death
37			+			DAI, swelling, necrotic brain tissue lodged under thoracic spinal cord dura
38				Multiple bruises to scalp, EDH high cervical spinal cord	Bruising at ligamentum flavum cervical spine	
39	++			C5/T1		
40	+			Intracerebral hemorrhage		
41	+			Cortical contusions		Cerebral hypoxia, swelling

CNS, central nervous system; DoFM, Department of Forensic Medicine, South West Sydney Area Health Service, Australia; SAH, subarachnoid hemorrhage; SDH, subdural hemorrhage; EDH, extradural hemorrhage; DAI, diffuse axonal injury; SCIWORA, spinal cord injury without radiological abnormality; +, skull fracture; ++, massive or extensive skull fractures.

PM delay, or between age and post-injury survival time for these cases. It is important to note that post-injury survival time is often difficult to interpret accurately from the narrative related to the circumstances surrounding a death, especially in cases of MVA or non-accidental death, in which there may be confusion surrounding the incident. If a post-injury survival time was documented, it

was used as the reported time, including cases in which instant death was indicated; if survival time was not documented, an estimate of post-injury survival time was made from the available information. There were no significant differences among the three groups for age (F [2, 38]=1.832, $p=0.17$), PM delay (F [2, 37]=1.884, $p=0.16$) or gender ($\chi^2=4.385$, df 2, $p=0.11$).

Central canal and lumen measurements

Measurements were taken around the central canal on H&E-stained sections (Fig. 1A) to compare the size and shape of the spinal cord lumen for different ages and injury statuses. There were no differences between the groups for lumen area, lumen circularity, lumen perimeter, and ependymal cell number. A negative

correlation (Spearman's $r = -0.66$) was found between increasing age and epithelial height ($p < 0.05$), and this reflected the developmental change from a pseudostratified epithelium in younger spinal cords to a simple columnar epithelium in older spinal cords. No other lumen measurements were associated with increases in age.

Ten spinal cords showed distortion of the central canal or were in longitudinal section and it was not possible to make these

FIG. 1. Central canal from cervical spinal cord from case 26 stained with (A) Hematoxylin and Eosin (H&E), (B) anti-human nestin, and (C) anti-glial fibrillary acidic protein (GFAP) and (D) with primary antibody omitted. High power views of the ventral region of the central canal in each of the stained sections showing (E) individual ependymal cells, (F) nestin-stained ependymal cells, (G) homogenous staining of astrocytic processes in the gray matter, and (H) absence of immunostaining in the negative control. Color image is available online at www.liebertpub.com/neu

measurements. These 10 cases included 3 controls, 4 trauma-no survival, and 3 trauma-survival cases. These cases had PM delays times ranging from 0 to 48 h, and ages ranging from 6 months to 43 years.

Nestin-positive ependymal cells

Nestin-positive ependymal cells were located predominantly in the dorsal and ventral regions of the central canal, and displayed a long basal process (Fig. 1B), especially extending into the ventral

GM. Nestin-positive ependymal cells were seen surrounding the central canal in 23/41 cervical spinal cords, including 74% of the trauma cases. Neither of the embryonic cases (gestational age 18 weeks) had nestin-positive ependymal cells. There were significant differences (Fig. 2A) in the percentage of ependymal cells that were nestin positive among controls (1.4 ± 0.72), trauma-no survival (6.9 ± 1.7) and trauma-survival cases (8.8 ± 2.1), (ANOVA $F[2,38]=5.7$, $p<0.01$). There was a positive correlation between the percentage of nestin-positive cells and post-injury survival time (Spearman's $r=0.39$, $p<0.01$) (Fig. 3A), but not for age or PM

FIG. 2. Graph showing (A) the mean percentage of nestin-positive ependymal cells ($F[2,38]=5.7$, $p<0.01$) and (B) the mean percentage increase for glial fibrillary acidic protein (GFAP) staining for control and trauma cases ($F[2,38]=0.35$, $p=0.7$). Bars = standard error of the mean (SEM).

Fluorescent labeling of nestin and GFAP primary antibodies resulted in a similar pattern of nestin staining to that seen using ABC/DAB. No double-labelled nestin-positive/GFAP-positive cells were identified in the ependymal, subependymal, or parenchymal regions of the spinal cord in any control or trauma cases. Projections could be identified easily on the apical surface of nestin-positive ependymal cells, and the long basal processes were seen extending into the GM. One case had occasional GFAP-positive ependymal cells that appeared to traverse the thickness of the epithelial layer. Occasional nestin-positive cells were seen in the subependymal region of the spinal cord, particularly in the ventral areas (Fig. 4). Although these cells were seen in both trauma and control cases, they were not present in sufficient numbers to quantify.

Furthermore, two research groups have conducted *in vitro* studies using human spinal cord tissue. Cells removed from the ependymal regions of spinal cords from fresh autopsy tissue (organ transplant donors) differentiated into neurons and glia *in vitro*,^{17,18} suggesting both a multipotent and a self-renewing capacity. Neurospheres formed from these cells expressed high levels of nestin, a marker of neural progenitor cells, and SOX2, a marker of neural stem cells, and displayed a morphology with long nestin-positive

FIG. 4. Central canal from case 37 with double labeling for nestin (red) and glial fibrillary acidic protein (GFAP) (green). Nuclei are counterstained with Hoechst (blue). There are numerous nestin-positive cells with long basal processes located within the ependymal layer of the central canal and occasional nestin positive subependymal cells (arrowheads). Color image is available online at www.liebertpub.com/neu

processes radially emanating from the neurospheres¹⁸ in a manner reminiscent of tanycyte morphology.

Studies from rodents suggest that adult NPCs reside mainly the ependymal layer of the central canal, and under normal conditions will slowly proliferate for self-renewal, but respond to injury by proliferating and migrating towards the lesion site.^{5,7,14} The progeny of dividing neural progenitor cells differentiate into oligodendrocytes and astrocytes *in vivo*, but have the capacity to differentiate into neurons under the right growth conditions, as is shown by *in vitro* studies.^{5,14} Similar to other studies in animals,^{4,8,11,29,30} human nestin-positive cells were predominantly located in the ependyma layer of the central canal. Our results showed an increase in the percentage of nestin-positive ependymal cells in the human cords, but no overall increase in the ependymal cell number. There were occasional nestin-positive cells in the subependymal region, but these were not apparent in very high numbers. It could not be established whether these were migrating progeny of ependymal progenitor cells, or a separate cell population. The subependymal cells did not coexpress GFAP, a marker for astrocytes, nor did they appear to be migrating from the subependymal region into the surrounding GM.

One problem encountered when using human autopsy tissue, especially when cause of death involves traumatic injury, is that there tends to be a large variation in location and severity of injury. Obviously the forces (acceleration and shear) involved in an MVA, compared with a fall, compared with an assault, will differ signif-

icantly, as will the actual sequence of events and the biomechanics of the affected individual. We were also unable to control for the severity of CNS injury in this cohort. This can be seen by the large variety of CNS injuries reported for the trauma cases in Table 2. It could be supposed that factors traveling in the CSF, most likely inflammatory cytokines, are responsible for the nestin increases in ependymal cells in the spinal cord; however, we saw no evidence of a direct association between any one particular type of injury and increases in nestin. There was also no direct association between injuries that involved the spinal cord and those that involved the brain only. Given that a similar nestin response is seen in cases of nontraumatic CNS disease,^{19,20} it is possible the cells are reacting to increased widespread cellular disruption by activating neural progenitor cells in an attempt to repair damaged areas or generate new healthy tissue. Further studies will need to be undertaken in controlled traumatic injury models in animals to determine how signaling to the spinal cord ependymal cells is occurring following CNS damage and to identify exactly what the signaling factors are.

The differences in subject age and the accuracy of determining exact survival times are also worthy of further consideration as factors influencing the nestin-positive cell response. Sakakibara and colleagues investigated age-related difference of nestin expression in the ependyma of the spinal cord, and found that 4/4 preterm neonates and 8/8 infants were nestin positive compared with 2/33 older children and adults.²⁰ There is a clear implication from these findings that nestin is normally expressed in developing

infants but not in older children and adults. We did not find this to be the case. When considering only the control cases in our study, we found two out of two embryos and six out of eight infants (0–2 years of age) negative for nestin. It has been suggested that nestin is transiently expressed during CNS development,³¹ and that nestin expression reduces between the gestational ages of 14 and 20 weeks.²¹ Both of the fetal cases in our study were quite young (gestational age 18 weeks) compared with those in the study by Sakakibara and colleagues (gestational age 26 weeks to 9 months); therefore, it is possible that there are differing nestin expression profiles during CNS development in the fetal stages. However, our results show that nestin protein expression does not appear to be directly associated with development stage after birth or with increasing age.

Establishing the early survival times in this particular cohort was difficult in some instances. We have, therefore, included a third group termed “trauma-no survival.” Cases were included as “trauma” cases if there was evidence of a CNS trauma at autopsy, and a recorded survival time. This information was collected from the autopsy report, which included a brief narrative from the attending police. MVA cases with no reported survival time were included as “trauma-no survival” for this study, as it was thought that in cases of immediate death there would be no time for the accumulation or activation of a cellular response. However, because of the nature of these traumatic incidents, especially in cases of non-accidental deaths, for which the source of information may be unreliable, and in motor vehicle crashes where the first responders may be delayed, it is possible that the actual survival time may not be the same as that recorded. If this has occurred, then it would be expected that the actual survival time would be, in all cases, longer than that reported, and may explain the increase in nestin expression seen in several of these cases. Additionally, in cases in which cause of death was a collapse or myocardial event, there was no indication whether the individual suffered from a brain or spinal cord injury as part of an ensuing fall, possibly confounding the situation and representing a traumatic event. It is of interest to note here that the causes of death from the donor study conducted by Dromard and colleagues were either stroke (ischemia) implicated in eliciting a response from neural progenitor cells in the brain,^{32,33} or unspecified MVA (trauma). Presently, beta amyloid precursor protein (β -APP) staining for swollen axons is a useful technique for commenting on the likely survival time following injury to the CNS, as the earliest signs of diffuse axonal injury occur at least a half hour post-injury.^{34,35} Nestin reactivity may prove valuable as a further modality to determine time between injury and death.

In animals, nestin expression in the central canal increases at 24–48 h after spinal cord injury,^{4,5,8,29} and can persist for up to 13 months.³⁶ GFAP is usually upregulated in the immediate vicinity of a CNS injury as astrocytes become activated and migrate toward the lesion site in the days and weeks following injury. We have demonstrated a positive correlation between percentage of nestin-positive cells and survival time in our human sample, with the longest survival time being 9 months. As most neural progenitor cells are thought to differentiate into glial cells,^{5,11,14,15} presumably this constitutes ongoing and prolonged glial scar formation, or an attempt to replace glial cells *in vivo*. However, in our samples, increases in nestin immunoreactivity did not correspond to increases in GFAP intensity, and, furthermore, nestin-positive ependymal cells were distributed along the length of the spinal cord in 11 positive cases, suggesting that it was not simply a localized response to injury. Nestin reactivity in the ependymal cells along

the neuroaxis has also been reported following a thoracic injury to rat spinal cord.³⁷ Because of the range of CNS injuries in this study, the GFAP levels that are reported here most likely represent the variation in normal background astrocyte activity in these spinal cords rather than a reaction to localized inflammation or astrogenesis driven by progenitor cell activity.

The nestin-positive cells seen in this study have the morphological appearance of tanycytes^{23–25,28} and appear in the proportions described for ependymal cells *in vitro*: cuboidal, 75%; tanycytes, 19%; and secretory, 6%.³⁸ An extensive review²⁶ of hypothalamic tanycytes identifies their role in neuroendocrine function, suggesting that they act as a bridge between the CSF and the portal venous system, and that they are the only radial glia descendants remaining in adulthood, suggesting a neural progenitor role. A detailed study of cellular organization of the normal rat central canal clearly shows the heterogeneous nature of the ependymal cells,⁸ with a niche of nestin-positive cells with tanycyte morphology located at the dorsal pole of the central canal. After traumatic CNS injury in humans, we have identified nestin staining of cells with tanycytic morphology located mainly in the ventral and dorsal regions of the central canal of the spinal cord. The exact role of these cells after injury remains to be determined; that is, whether they are acting as neural progenitor cells, or whether they have a role similar to that in the brain, with multiple functions, including CSF communication.

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Author Disclosure Statement

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