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Satellite Glial Cell Proliferation in the Trigeminal Ganglia After Chronic Constriction Injury of the Infraorbital Nerve

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Abstract

We have examined satellite glial cell (SGC) proliferation in trigeminal ganglia following chronic constriction injury of the infraorbital nerve. Using BrdU labeling combined with immunohistochemistry for SGC specific proteins we positively confirmed proliferating cells to be SGCs. Proliferation peaks at approximately 4 days after injury and dividing SGCs are preferentially located around neurons that are immunopositive for ATF-3, a marker of nerve injury. After nerve injury there is an increase GFAP expression in SGCs associated with both ATF-3 immunopositive and immunonegative neurons throughout the ganglia. SGCs also express the non-glial proteins, CD45 and CD163, which label resident macrophages and circulating leukocytes, respectively. In addition to SGCs, we found some Schwann cells, endothelial cells, resident macrophages, and circulating leukocytes were BrdU immunopositive.

Keywords

BrdU; immune cells; Trigeminal nerve; trigeminal ganglion; neuropathic pain; nerve injury

Introduction

Cell division is observed in sensory ganglia after nerve injury (Nathaniel and Nathaniel, 1973), skin scarification (Elson et al., 2004), and viral infection (Elson et al., 2003) as soon as one day after injury (Friede and Johnstone, 1967), with maximum proliferation occurring during the first week post injury (Lu and Richardson, 1991), and continuing through 28 days post-injury (Elson et al., 2004). These proliferating cells have been identified as satellite glial cells (SGCs) based on their morphology and location, but have not been positively identified as SGCs using immunohistochemical markers. Recently, a number of proteins have become accepted as phenotypic markers of SGCs, such as the calcium activated potassium channel (SK3) and the inwardly rectifying potassium channel (Kir4.1), and the most widely used, glutamine synthetase (GS; Hanani, 2005; Miller et al., 2002). As these markers are not in neurons, they can be used to positively identify SGCs surrounding neurons independent of their morphology or cellular location. This makes it possible to

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confirm that the dividing cells surrounding neurons are phenotypically SGCs. Further the presence or absence of these phenotypic markers might indicate the origin of these proliferating SGCs, which has not been previously determined.

A further question concerns whether the dividing SGCs are specifically associated with injured neurons, or whether the proliferation is independent of neuronal injury. It has been shown that following nerve damage, such as that produced by chronic constriction injury (CCI), there is an upregulation of GFAP in SGCs, which is taken as an indication of glial activation. It does not appear that increased GFAP is correlated with proximity to injured neurons (Xu et al., 2008), nor whether there is a correlation between dividing SGCs and GFAP increase. In addition to increasing GFAP expression, SGCs have also been shown to begin expressing leukocytes antigens such as CD45 and CD163 (also known as ED2) following nerve injury (Jasmin et al., 2010).

To determine the relationship between proliferating SGCs, nerve injured neurons, and changes in SGC phenotype, we examined the trigeminal ganglion during the first 2 weeks after a CCI of the infraorbital nerve (ION). Cell proliferation was examined using bromodeoxyuridine (BrdU) and combined with immunohistochemistry to identify SCGs.

Materials and Methods

Surgery

ANIMALS—Twenty five adult, male, Sprague-Dawley rats (Charles River Laboratories) weighing 270–330 g were housed on a 12 h light–dark cycle and given food and water *ad libitum*. Procedures for the maintenance and use of the experimental animals conformed to the regulations of UCSF Committee on Animal Research and were carried out in accordance with the NIH regulations on animal use and care.

CHRONIC CONSTRICTION INJURY OF THE INFRAORBITAL NERVE—CCI of the ION was carried out as described previously (Kernisant et al., 2008). In brief, a 2-cm skin incision was made along the upper edge of the orbit to expose the skull and nasal bones. Gentle retraction of the orbital content uncovered ~5 mm of the ION and two ligatures (5.0 chromic gut) were tied loosely around the ION to the point where the nerve was slightly constricted. The skin incision was closed using 6.0 silk. For sham surgeries, the infraorbital nerve was exposed in the same manner as describe above, but ligatures were not placed around the nerve.

MECHANICAL (VON FREY HAIR) TESTING—Three von Frey hairs, 2, 10, and 50 g corresponding to log units 4.31, 5.07, and 5.88 respectively, were used in this study. During testing, mechanical stimulation was done with increasing intensities. Each filament was first applied on the side contralateral to the CCI of the ION then to the ipsilateral. The stimulation consisted of 5–6 consecutive applications performed at 5 s intervals in slightly different area at the center and around the vibrissal pad, as well as in the perioral and perinasal territory. The scoring of the rat behavioral response was based on the method of Vos and colleagues (Vos et al., 1994) as follows: 0 = no detection; 1 = detection and exploration of the von Frey hair; 2 = head withdrawal and/or single grabbing movement; 3 =

attack and/or escape and/or multiple grabbing movements; and 4 = active asymmetrical grooming directed toward the stimulated facial area. For each hair, the highest score was recorded and then the highest score obtained from the three different hairs was averaged. With this method, all animals were confirmed to have nerve injury induced hyperalgesia in the V2 dermatome on the side of the CCI.

BRDU INJECTION—For 1 day experiments one intraperitoneal (i.p.) injection of 100 mg kg^{-1} of BrdU [20 mg mL^{-1} in phosphate buffered saline (PBS) with 0.1% NaOH] was given at the time of the CCI surgery followed by a second 12 h before perfusion. For experiments 2 days and longer the injections were given every 12 h for 48 h (i.e., total of four injections) before the animal was perfused.

Tissue Processing

HISTOLOGY—Rats were deeply anesthetized with 100 mg kg^{-1} of pentobarbital (i.p.) and perfused at 1, 2, 4, 11, and 15 days post-CCI. Perfusions were performed transcardially with 400 mL Tyrode's solution followed by 400 mL of 4% paraformaldehyde. Both the left and right trigeminal ganglia, along with the brain, spinal cord, and gut, were dissected and post-fixed in 4% paraformaldehyde for 3–5 h, and then cryoprotected with 30% sucrose in PBS (pH 7.4) for at least 48 h. The left and right trigeminal ganglia from each animal were embedded together and 10 μm thick longitudinal section on a cryostat along with samples of brain, spinal cord, and gut serving as immunohistochemical controls.

IMMUNOHISTOCHEMISTRY—Optimal dilutions and incubation times for all primary and secondary antibodies were determined prior to experimental use. The following primary antibodies were used at the corresponding dilutions: BrdU (US Biologicals B2850-01, 1:500), SK3 (Alomone APC-025, 1:500), glutamine synthetase (Millipore MAB302, 1:2000), p75 (Chemicon AB1554, 1:1000), Activating Transcription Factor 3 (ATF-3, Santa Cruz sc-188, 1:800), ED1 (Abcam ab31630 1:3000), TCR (Serotec MCA453G, 1:500), GFAP (Sigma G3893, 1:4000), CD163 (ED2) (Serotec MCA342R, 1:500), CD45 (Pharmingen 550566, 1:800), and rat endothelial cell antibody-1 (RECA-1, Serotec, 1:500). SK3 and glutamine synthetase are markers for SGCs, GFAP, a marker for astrocytes is upregulated in SGCs after nerve injury, p75 is a marker for non-myelinating Schwann cells (Akassoglou et al., 2002; Awatramani et al., 2002) and ATF-3 is expressed by injured neurons (Hai et al., 1999). ED1 is expressed by activated macrophages, CD163 by resident macrophages, CD45 by leukocytes, and TCR (T cell receptor) by T cells. A Millipore Apoptag kit was used to assess apoptosis and was used per manufacturer's protocol.

For BrdU immunohistochemistry, slides were incubated at 37°C for 30 min in 2 *N* hydrochloric acid to denature the DNA prior to antibody application. The tissue was then exposed to the primary antibody at room temperature overnight. For light microscopy counting of cells, the tissue was then incubated with speciesspecific biotinylated secondary antibodies for 30 min, followed by incubation with an ABC kit (Vector) and visualized with DAB. For immunofluorescence, species-specific secondary antibodies conjugated to FITC, CY3, CY5 (Jackson), or Pacific Blue (Invitrogen) were used at 1:500 dilution and incubated for 30 min in a humidified chamber. The slides were then washed and cover-slipped with

Vectorshield or with Vectorshield plus DAPI for those not using Pacific Blue as a secondary antibody. Slides were analyzed on a conventional fluorescence microscope as well as by confocal microscopy. For triple labeling of SK3, ATF3, and BrdU we used the same secondary (CY3) for ATF4 and SK3 as both of these antibodies were raised in the same species. We are able to differentiate the two labels as SK3 is never found in nuclei and ATF3 is a nucleus only antibody we used the same secondary for ATF3.

Counting of BrdU labeled cells was performed with 400× magnification using Stereo Investigator software (Neurolucida, MicroBrightfield, Vermont). Nine sections per animal were counted at 24 h, 2 days, 4 days, 11 days, and 15 days after CCI. For apoptosis staining, positive controls were obtained using rat mammary gland. Negative controls were done by omitting terminal deoxynucleotidyl transferase in the incubation medium of ipsilateral trigeminal ganglion sections from CCI rats and from sections of mammary gland.

Results

Proliferation of SGCs

BrdU positive nuclei were seen in the trigeminal ganglion 1 day post-CCI and the number of dividing cells increased until 4 days post-CCI. Thereafter the number of BrdU labeled nuclei decreased until 15 days post-CCI, which was the maximum time examined (Fig. 1). For cell count and distribution (Fig. 1), the BrdU labeled nuclei visualized using DAB and were located around neurons and appeared to be SGCs based on their location and morphology (Fig. 2A). To confirm the identity of these cells, BrdU labeled cells were double immunofluorescent-labeled with glutamine synthetase or SK3, which have both been established as markers of SGCs (Hanani, 2005; Vit et al., 2006). Similar data was obtained with SK3 and glutamine synthetase immunostaining. At all experimental days after CCI, a proportion of cells labeled with BrdU were also SK3 immunopositive, positively identifying these proliferating cells as SGCs (Fig. 2B,C). Further analysis showed that 41.5% (n = 146) of the BrdU immunopositive nuclei were in SK3 immunopositive SGCs while the remainder of the BrdU were scattered in regions containing neurons and in adjacent white matter tracts. The immunolabeled BrdU/SK3 cells appeared to be identical to the DAB positive cells in morphology and location around neurons.

Similar to an earlier report (Schaeffer et al., 2010) we did not observe apoptosis of either neurons or glial cells after CCI during the time period of this study (15 days) although apoptosis of SGCs has been observed 30 days after CCI (Schaeffer et al., 2010). No labeling with either the Millipore Apoptag Kit or Caspase 3 was seen in the ganglia at either 2 or 11 days after CCI (data not shown), but labeling was seen in positive control slides for apoptosis (rat mammary gland). This result indicates that apoptotic cell death is not the factor triggering cell proliferation in the trigeminal after CCI.

Proximity of Dividing SGCs to Damaged Neurons

The ION, the nerve injured in this study, contains axons originating in the V2 (maxillary) division of the trigeminal ganglion, therefore we would expect that somata in this division of the ganglion would be more affected than those in adjacent divisions (Figs. 1 and 3A).

ATF-3 immunostaining for neuronal injury (Seijffers et al., 2006) was used confirm this and to determine if there was a relationship between injured neurons and the SGC proliferation. ATF-3 immunopositive neuronal nuclei were present 2 days post-lesion and the number had increased by day 4 post-injury corresponding to the time course of the increase in proliferating SGCs (Tsujino et al., 2000). Proliferating SGCs were seen exclusively surrounding ATF-3 immunolabeled neurons (Fig. 3B). In eight randomly selected fields of the V2 division of the ganglia, approximately 30% of the neurons were ATF3 immunopositive (n = 186 neurons, ATF3 immunopositive = 30.6, ± 4.1%) of these, 24.5% of the ATF3 immunopositive cells were associated with BrdU labeled SGC nuclei while no non-ATF3 immunopositive neurons were associated with BrdU labeled SGCs. No ATF3 immunopositive SGC nuclei were seen.

Change in Phenotype of SGCs

Previous studies have shown that SGCs begin to express GFAP after nerve injury (Fenzi et al., 2001; Siemionow et al., 2009; Stephenson and Byers, 1995; Takeda et al., 2007; Woodham et al., 1989; Xie et al., 2009; Zhang et al., 2009) and we wished to look at the expression of GFAP in SGCs surrounding injured neurons. While only a small amount of GFAP expression was seen 2 days post-CCI, by 4 days GFAP immunoreactive SGCs were more apparent (Fig. 4A1). The time of GFAP expression in SGCs seems to vary depending on the injury model (Gunjigake et al., 2009; Mika et al., 2010; Peters et al., 2007; Xie et al., 2009). As previously reported, GFAP immunoreactivity was not seen in contralateral (control) ganglia but was visible in the trigeminal ganglia ipsilateral to the injured nerve.

Previous studies suggest that following nerve transection GFAP positive SGCs also increase their expression of the low-affinity nerve growth factor receptor, p75 (Obata et al., 2006; Zhou et al., 1999a, b). In our study, at low magnification, the two markers appeared to overlap but when examined at high magnification it was clear that the p75 and GFAP immunolabeled structures were separate (Fig. 4A). To investigate this further and positively identity SGCs, we used glutamine synthetase and p75 double labeling. In Fig. 4B, p75 immunolabeled cells were morphologically similar to SGCs but were not co-labeled with glutamine synthetase (GS). Based on these finding, we suggest that the p75 immunopositive cells are non-myelinating Schwann cells rather than SGCs. We did not see any p75 immunolabeled profiles with BrdU labeled nuclei in close proximity to neurons but we did find double labeled p75/BrdU cell in areas of axons (Fig. 4C).

We examined SGCs for other phenotypic changes and found that BrdU positive SGCs were not immunoreactive for markers of macrophages (ED1), or T cells (TCR), at either 2 days post-CCI or 4 days post-CCI in slides double labeled with SGC markers. As we and others (Hu et al., 2007; Hu and McLachlan, 2003; Jasmin et al., 2010) have reported previously, CD45 (Fig. 5A) which is expressed by leukocytes was expressed by SGCs in the trigeminal ganglion ipsilateral to the CCI.

Other Cell Types that are Proliferating

Not all BrdU labeled nuclei were in cells identified as SGCs by immunohistochemistry, indicating that other cells in the ganglia also proliferate after nerve injury. Some of these

cells were identified as CD163 immunopositive circulating white blood cells (Fig. 5B). Some p75 immunopositive cells were observed to be double labeled with BrdU (Fig. 5C), but were generally in areas of the trigeminal ganglion consisting solely of myelinated axons, rather than in areas close to the neuronal soma and numbered only 1 or 2 cells per five sections. p75/BrdU immunolabeled cells were not observed in control trigeminal ganglia, suggesting that the proliferation of p75 positive cells is related to nerve injury. A few BrdU immunopositive endothelial cells (1 or 2 cells per five sections), identified by RECA-1 immunohistochemistry, were seen at 2 and 4 days post-CCI, in both the ipsilateral and contralateral ganglion (Fig. 5D).

Discussion

A number of studies using morphology and peri-neuronal location to identify SGCs have suggested that SGCs in primary sensory ganglia proliferate after nerve injury. In this study, we used BrdU labeling in conjunction with immunohistochemistry for SGC antigens to confirm the identity of dividing SGCs and add additional data about changes in phenotype of SGCs, and the proliferation of other cells in the ganglia after injury.

Our results suggest that dividing SGCs are not replacing dying SGCs since we saw no SGC apoptosis in the trigeminal ganglia after CCI during the time course of our study. This is similar to the situation for neurons where nerve loose ligation does not result in neuronal apoptosis, although apoptosis does occur after nerve transection (Himes and Tessler, 1989; Tandrup et al., 2000). SGC apoptosis has been seen 30 days after sciatic nerve CCI (Schaeffer et al., 2010) but why there should be such delayed apoptosis is not clear. We also found no evidence of SGC stress as indicated by ATF3 immunoreactivity, a member of the ATF/cAMP family of transcription factors that is upregulated by nerve injury and other stress responses (Tsujino et al., 2000). This corresponds with other studies that have not reported ATF3 in SGCs after tooth extraction (Gunjigake et al., 2009) or CCI of the ION in mice (Xu et al., 2008). However, ATF3 has been reported in SGCs 7 days following paclitaxel induced nerve injury (Peters et al., 2007). In the latter case, the ATF3 immunopositive profiles were in mostly nodules of Nageotte rather than perineuronal SGCs. SGCs metabolically support neurons (Hanani, 2005), and it is known that the number of SGCs surrounding primary sensory neurons increases with increasing soma size both within and across species. Several studies have suggested that sensory neurons increase size (Cirillo et al., 2010) and metabolic activity following injury (Humbertson et al., 1969; Pannese, 1981). The increase in the number of SGCs may be related to these neuronal changes (Hanani, 2005; Pannese, 1981, 2010).

We observed that SGCs proliferate exclusively around neurons expressing ATF-3. The localization of proliferating SGCs around damaged neurons suggests that factors triggering the proliferation of SGCs are restricted to the immediate surroundings of injured neurons. Factors like ATF-3 has also been shown to promote regeneration of neurons in the periphery (Seijffers et al., 2006) but it is unlikely that ATF-3 itself would not have any direct effects on the proliferation of SGCs since it does not diffuse out of neurons. However, it is possible that other growth factors expressed by regenerating neurons also promote proliferation in SGCs to accommodate the increased metabolic demands of injury responding neurons

(Huebner and Strittmatter, 2009). It has also been suggested that these proliferating SGCs may release neurotrophins and express growth supportive cell surface molecules that may lead to sympathetic-sensory coupling (Shinder et al., 1999).

It is known that GFAP expression in astrocytes enhances glial proliferation (Middeldorp and Hol, 2011). Because the expression of GFAP was not confined to SGCs surrounding neurons that expressed ATF3, it suggests that GFAP is involved in wider processes within the ganglion outside the area of damaged neurons. This expression pattern is significant as GFAP has been shown to contribute to the maintenance of neuropathic pain and it has been shown that GFAP−/− mice show significantly reduced neuropathic pain behavior (Kim et al., 2009).

Although almost half of the BrdU labeled cells after CCI are SGCs, we also found that non-myelinating Schwann cells, endothelial cells, leukocytes, resident macrophages, and, rarely, T cell nuclei also were BrdU immunopositive after CCI. Circulating leukocytes play a much larger part in the development and recovery from pain in the ganglia compared with the CNS as immune cells can move much more freely within the ganglia than within the CNS (Thacker et al., 2007). It has been shown previously that there is an increase in the number of macrophages in the ganglia after nerve injury (Lu and Richardson, 1993), and that a portion of these are infiltrating circulating macrophages (Scholz and Woolf, 2007). The number of macrophages in the ganglia starts to increase 3 days post-nerve injury (Komori et al., 2011) and peaks at 8 days (Lu and Richardson, 1993). We did not find circulating macrophages with BrdU labeled nuclei in the ganglia indicating that this population of macrophages is terminally differentiated. We did find resident macrophages with BrdU labeled nuclei suggesting that the increase in the number of macrophages in the ganglia came both from the proliferation of resident macrophages and the infiltration of circulating macrophages.

While it has long been known that SGCs increase the expression of GFAP after nerve injury (Cherkas et al., 2004) and more recently, CD163 and CD45 (Jasmin et al., 2010) which are resident macrophage and circulating leukocyte markers, respectively, the relationship with proliferation of SGCs and change in phenotype has not been explored. We found that this expression of novel immune markers occurs both at 2 days and at 4 days post-injury, and while some are co-localized with proliferating nuclei, others are not. This data generated key questions on the origin of these proliferating SGCs. Do immune cells move to a pericellular position and become SGCs or do SGCs proliferate and at times become immune cells? Since BrdU positive SGCs are only seen around injured neurons, it can be assumed that they divide in place next to neurons. It would also be logical to assume that since some of those SGCs that do express immune cell markers also sometimes divide, that after nerve injury immune cells do not migrate to the neurons and become SGCs, especially since most proliferating SGCs do not begin expressing novel immune cell proteins.

Our results confirm that SGCs in the trigeminal ganglion proliferate following CCI of the ION, and that this proliferation reaches a maximum at 4 days after injury. No SGC apoptosis is seen within the first 15 days, so it can be presumed that SGCs are not proliferating to replace dying SGCs. Proliferating SGCs are seen only around injured neurons, but activated

SGCs are seen throughout the ganglia and can be associated with neurons that are not injured. In addition to upregulating GFAP, SGCs also begin expressing novel immune markers, CD45 and CD163, but this expression appears to be unrelated to proliferation. The ability of SGCs to express characteristics of other cells is an interesting finding that if explored more in depth could be useful in uncovering the role of SGCs in the response to injury in the ganglia. Other cells, including Schwann cells, endothelial cells, resident macrophages, and circulating leukocytes also proliferate after CCI.

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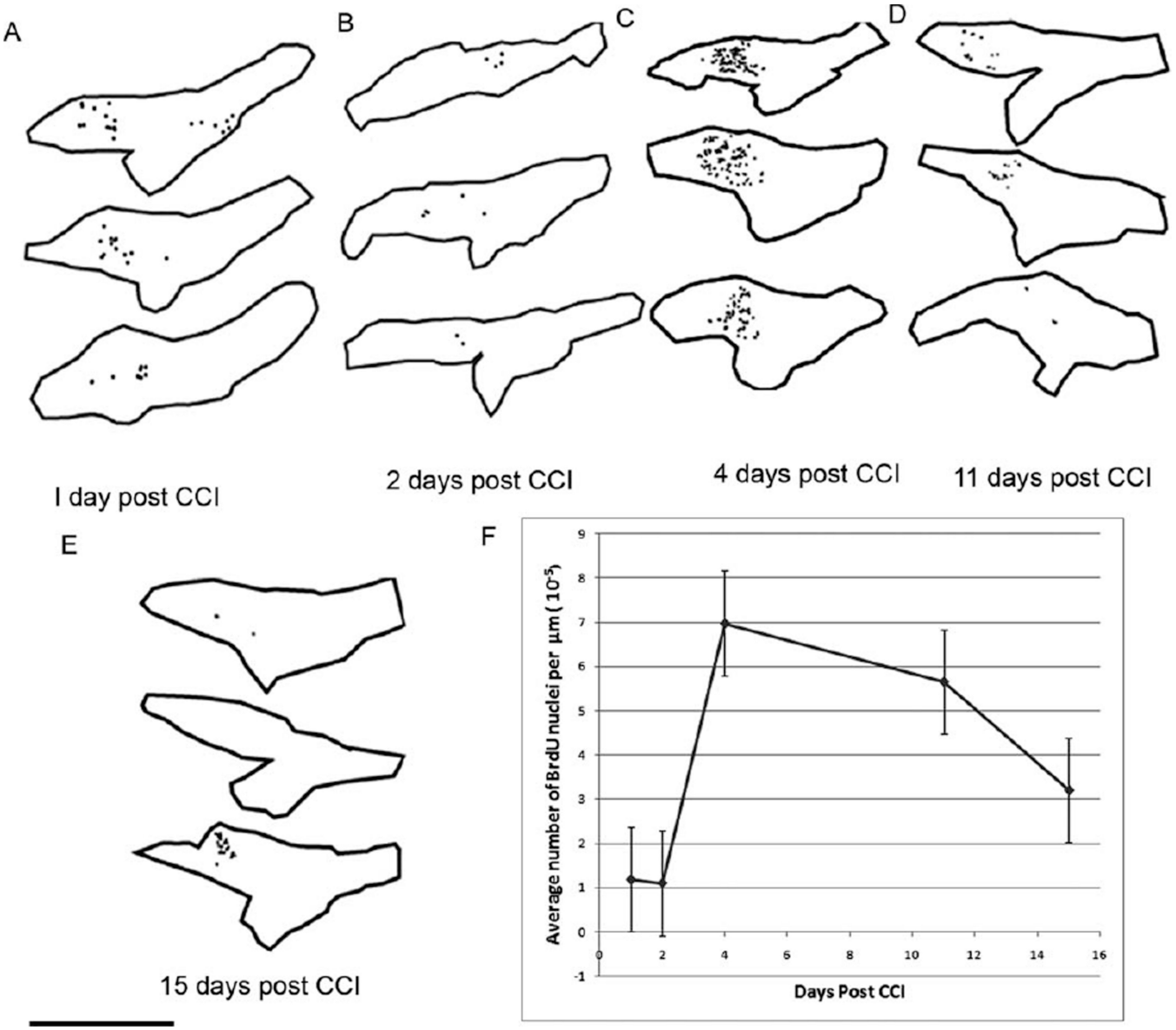

Figure 1.

(A–E) Representative tracings of trigeminal ganglia sections ipsilateral to the CCI of the ION showing the location of BrdU immunolabelled nuclei. Three sections at different depths are shown for each day post-CCI. BrdU nuclei are present 24 h after CCI, and increase until 4 days post-CCI, after which there is a decrease in label. BrdU staining is localized to the V2 area of the trigeminal ganglia which corresponds to the territory of the ION. (F) Numbers of BrdU labeled cells per unit area at different time points after CCI, showing that proliferation is at its maximum 4 days after CCI. Scale bar = 3 mm.

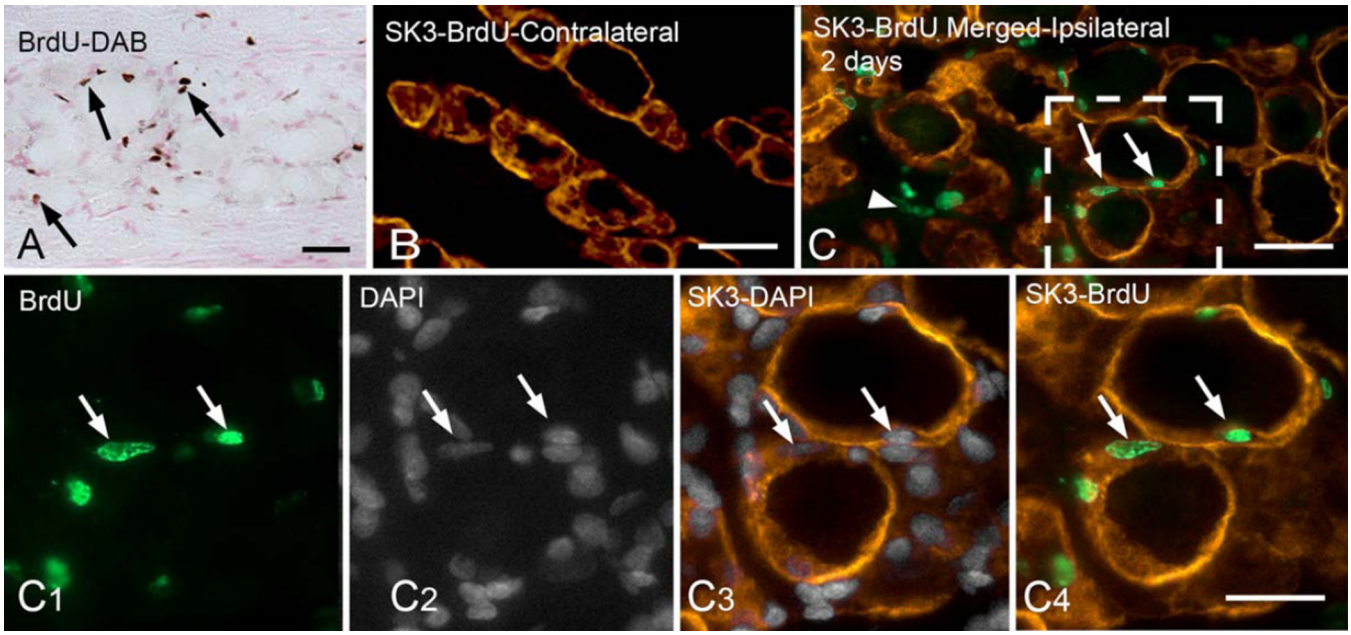

Figure 2.

(A) BrdU immunolabeled nuclei visualized with DAB (arrows) 2 days post-CCI. (B) Trigeminal ganglion contralateral to CCI showing SK3 labeled SGCs and absence of BrdU labeled nuclei. (C) Ipsilateral ganglion flourescent immunostaining showing BrdU immunopositive nuclei (arrows), in SK3 (orange/red) immunopositive SGCs. Note that not all BrdU positive nuclei are associated with SGCs (arrowhead). (C1–C3) High magnification of the region outlined in C. BrdU labeled structures (C1, arrows) are confirmed to be nuclei by DAPI labeling (C2) and are located in SK3 immunopositive SGCs (C3, C4). Scale bars, A = 20 μm, B & C = 30 m, C1–C4 = 40 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

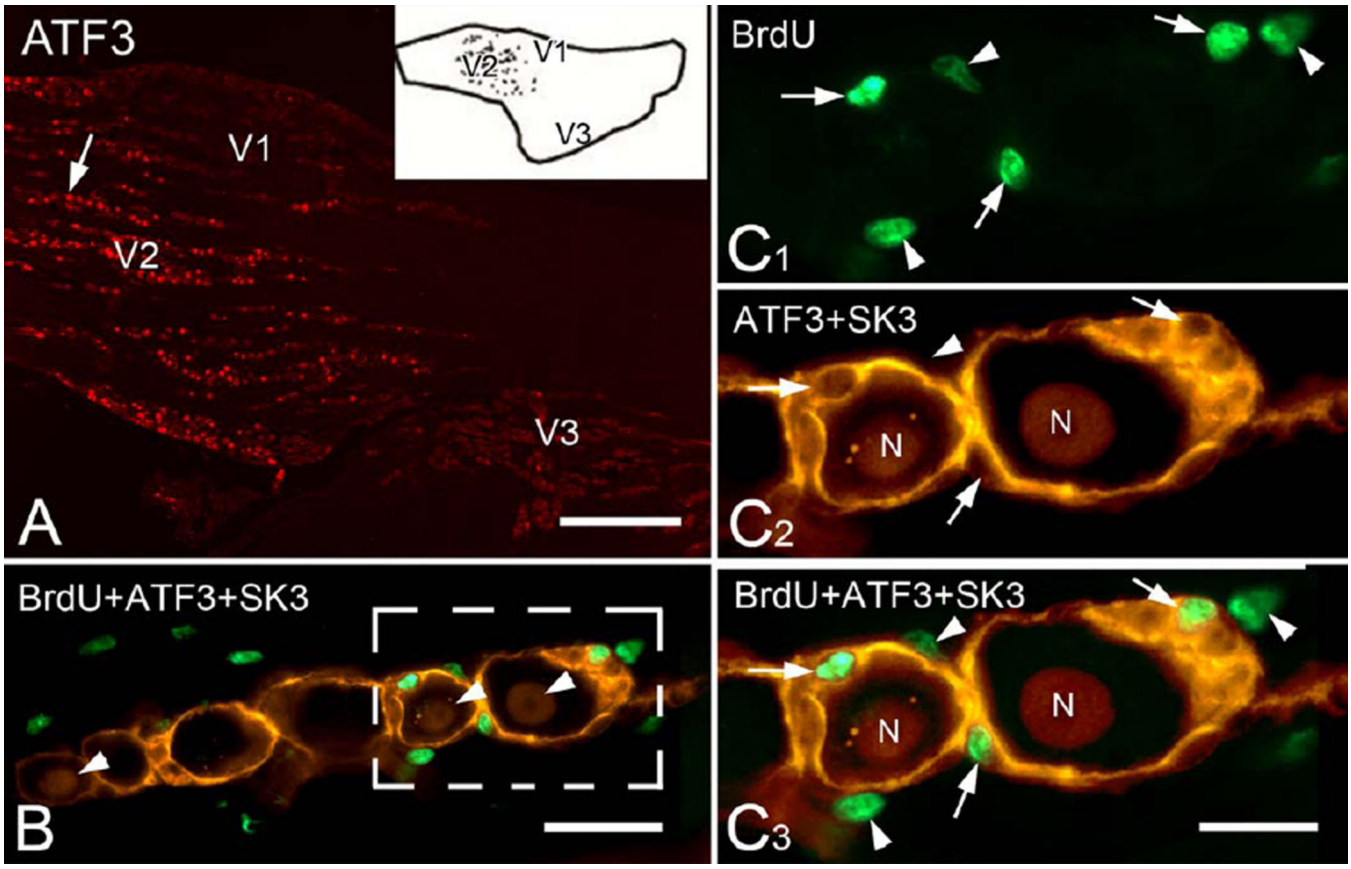

Figure 3.

Proximity of proliferating SGCs to damaged neurons expressing ATF-3. (A) Low magnification of the trigeminal ganglion showing ATF-3 immunopositive neurons (e.g., red dots, arrow) 4 days post-CCI of the ION. Inset is from Figure 1 showing location of BrdU immunopositive nuclei for comparison. (B) Triple labeling for ATF3 (orange/red, arrow heads), SGCs (SK3, orange/red) and BrdU (green). C1–C3 high magnification of the outlined region in B showing SGCs (SK3 immunopositive) with BrdU immunopositive nuclei (Arrows). Arrowheads indicate BrdU immunopositive nuclei that are not in SGCs. N= Nucleus. Scale Bars, A = 500 μm; B = 40 μm, C = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

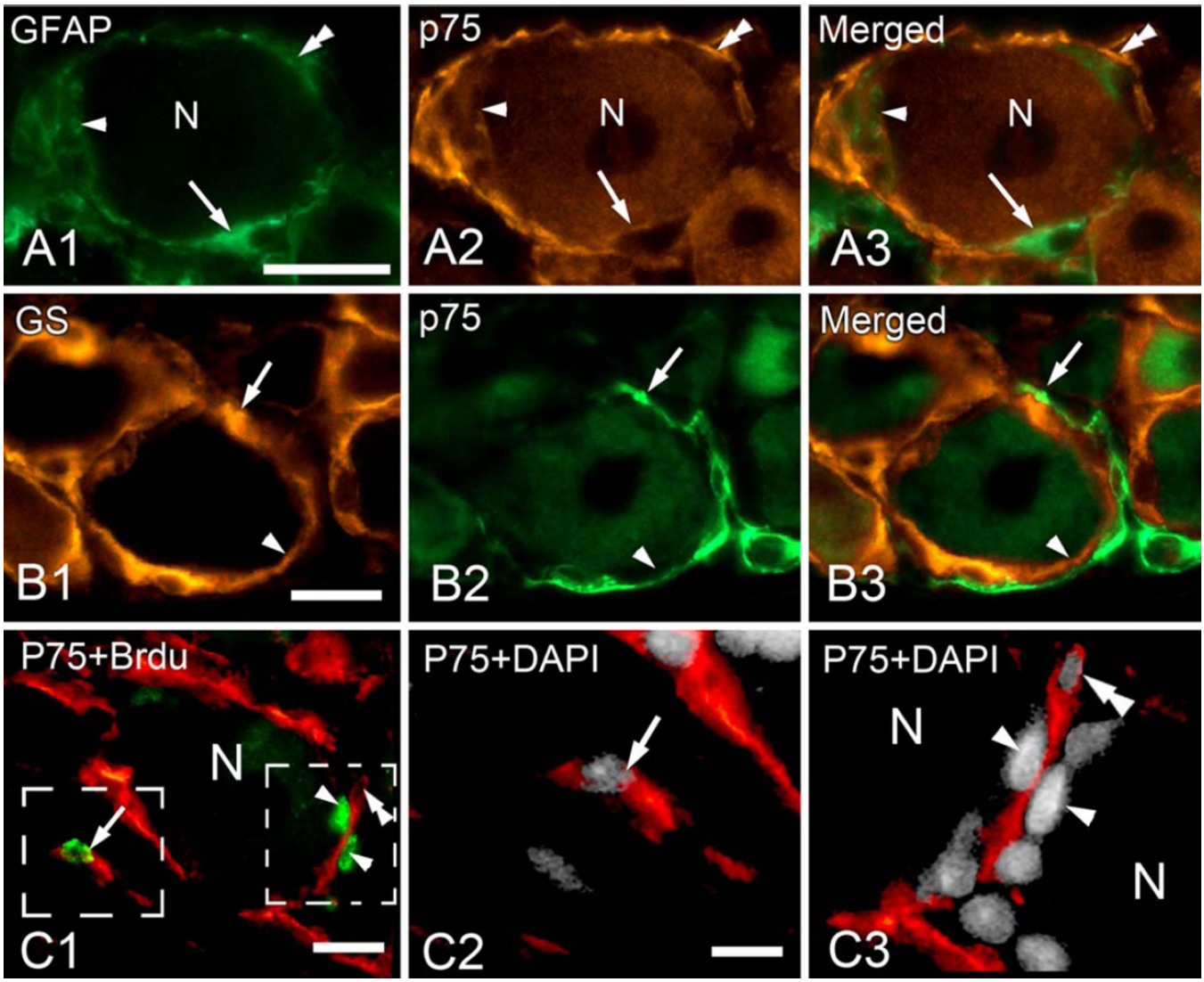

Figure 4.

GFAP and p75 expression 4 days after CCI of the ION. (A) GFAP and p75 expression appears to overlap in SGCs (e.g., arrows and double arrowheads) but the merged image shows areas with highest expression of the two antigens do not overlap. The SGC indicated by the arrowhead has low expression of both GFAP and p75 but neither signal is above background. (B) Double label with glutamine synthetase (GS) and p75 show that although p75 is present in cells that morphologically appear to be SGCs (arrows and arrowheads) in the merged image (B3) it can be seen that the p75 cells are a different population to the SGCs. (C1) Low magnification of BrdU (green) and p75 (orange/red) immunolabeled profiles. A neuron (N) is surrounded by p75 immunostained elements which are associated with BrdU immunopositive nuclei (arrowheads). The outlined regions containing the BrdU immunolabeled nuclei are shown in C2–C3 at higher magnification. (C2) The arrow indicates the DAPI labeled nucleus that corresponds to the BrdU positive nucleus of the p75 immunolabeled cell in a region of white matter shown in C1 (arrow). (C3) The DAPI labeled nuclei (arrowheads) show that the BrdU immunopositive nuclei in C1are not the

nuclei of the p75 immunostained elements but are interposed between the p75 immunopositive elements and the neurons (N). The DAPI labeled nuclei of the p75 immunolabeled cell is indicated by the double arrowhead. Scale Bars, A, B = 20 μm; C1 = 10 μm, C2–C3, = 5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

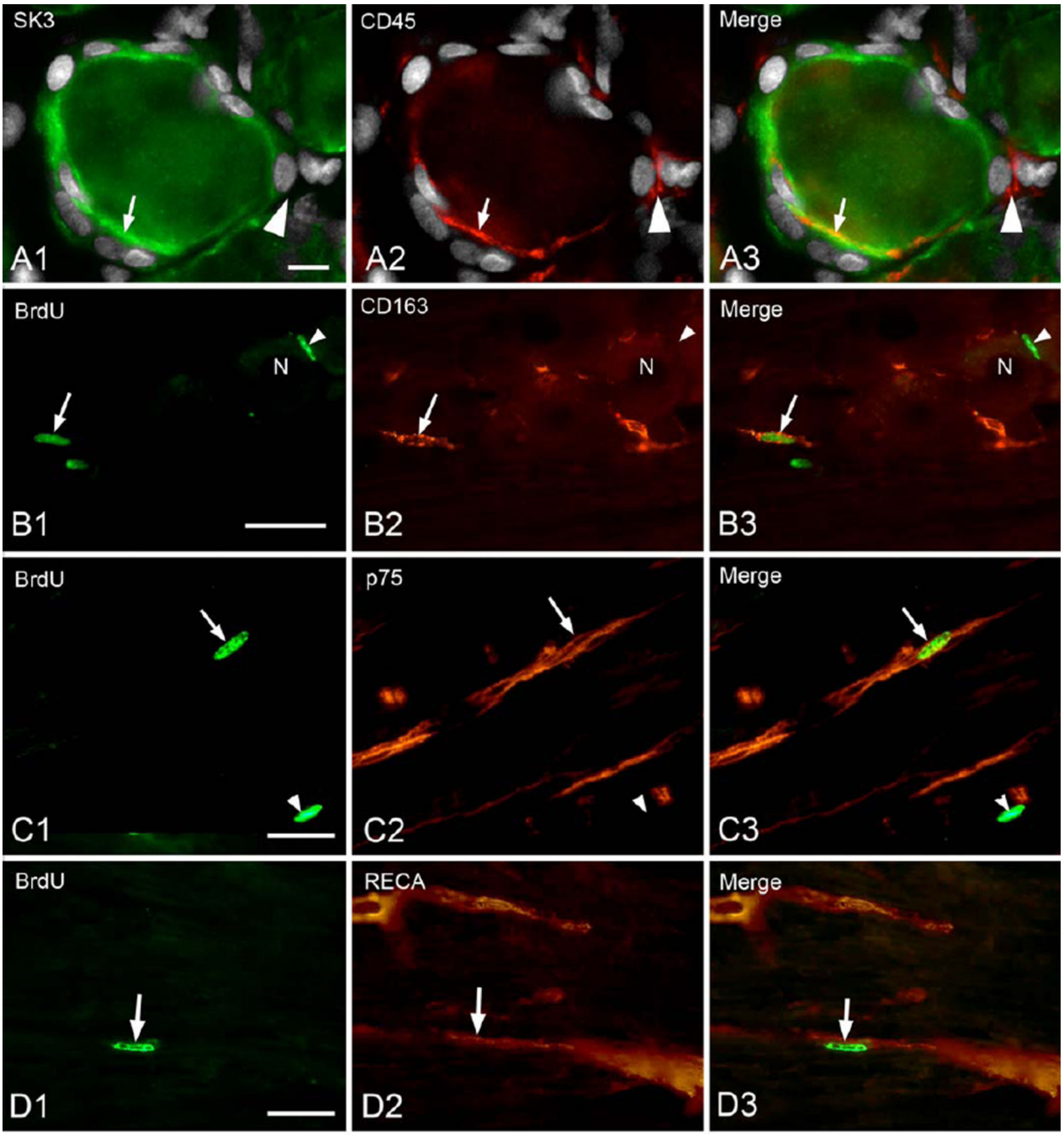

Figure 5.

(A) Seven days following CCI of the ION, CD45 immunostained profiles were present throughout the ganglion. Some SGCs express CD45 immunoreactivity (e.g., A2, arrow) as demonstrated by double labeling with SK3 (A3, arrow). (B) CD163 profiles also increase and some (B2, arrow) and some of these have BrdU immunopositive nuclei (B1, B3). A BrdU immunopositive nucleus in a CD163 immunonegative cells (arrowhead) is also visible adjacent to a neuron (N). (C) In axon rich regions lacking neuronal soma, BrdU immunopositive nuclei (C1, arrow) are seen in elongated p75 immunopositive cells (C2, C3

arrow). BrdU labeled nuclei are also found in the same areas in cells that are not p75 immunopositive (arrowhead). (D) BrdU immunopositive nuclei (D1, arrow) are seen in RECA-1 immunopositive endothelial cells (D2, D3 arrow). Scale Bar: A = 5 μm; B = 30 μm; C, D = 15 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]