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Grafted Lineage-Restricted Precursors Differentiate Exclusively into Neurons in the Adult Spinal Cord

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Multipotent neural stem cells (NSCs) have the potential to differentiate into neuronal and glial cells and are therefore candidates for cell replacement after CNS injury. Their phenotypic fate *in vivo* is dependent on the engraftment site, suggesting that the environment exerts differential effects on neuronal and glial lineages. In particular, when grafted into the adult spinal cord, NSCs are restricted to the glial lineage, indicating that the host spinal cord environment is not permissive for neuronal differentiation. To identify the stage at which neuronal differentiation is inhibited we examined the survival, differentiation, and integration of neuronal restricted precursor (NRP) cells, derived from the embryonic spinal cord of transgenic alkaline phosphatase rats, after transplantation into the adult spinal cord. We found that grafted NRP cells differentiate into mature neurons, survive for at least 1 month, appear to integrate within the host spinal cord, and extend processes in both the gray and white matter. Conversely, grafted glial restricted precursor cells did not differentiate into neurons. We did not observe glial differentiation from the grafted NRP cells, indicating that they retained their neuronal restricted properties *in vivo*. We conclude that the adult nonneurogenic CNS environment does not support the transition of multipotential NSCs to the neuronal commitment stage, but does allow the survival, maturation, and integration of NRP cells. © 2002 Elsevier Science (USA)

Key Words: **neuronal precursors; glial precursors; spinal cord; stem cells; neuronal differentiation; transplantation; transgenic rats; neurogenesis; neuronal replacement.**

INTRODUCTION

Neural stem cells (NSCs) have been isolated and characterized from many regions of the developing and adult CNS (10, 19, 20, 42, 43, 52). NSCs can self-renew under appropriate epigenetic stimulation and have the ability to differentiate into neurons, astrocytes, and oligodendrocytes *in vitro* and *in vivo* after transplantation into the limited neurogenic areas of the adult CNS (12, 51). Multipotent NSCs do not generate differentiated progeny directly. Rather, the process of differentiation involves a stepwise process of lineage restriction (3, 12, 22). Neuronal-restricted precursor (NRP) cells and glial-restricted precursor (GRP) cells differentiate from NSCs in culture. They can also be isolated from the developing spinal cord based on differential cell surface expression of embryonic neural cell adhesion molecule (E-NCAM) and A2B5, respectively (21, 33, 39).

Cultured NSCs and NRP cells, irrespective of which brain region they have been harvested from, have the ability to generate a variety of neuronal types *in vitro* (19, 21, 38, 41–43). However, transplantation of multipotent NSCs into intact nonneurogenic adult CNS regions such as the spinal cord and midbrain leads to either incomplete differentiation or differentiation into predominantly glial phenotypes (5, 6, 44–46, 49, 50). In contrast, neuronal differentiation does occur when NSCs are grafted into one of the few neurogenic areas of the adult brain (15, 45, 48). For example, NSCs present in the adult spinal cord do not generate new neurons *in situ*, normally or after injury (1, 17). Likewise, spinal cord NSCs transplanted back into the adult spinal cord generate only glial cells (45). The same stem cells, however, readily differentiate into neurons *in vitro* and into mature neurons when transplanted into neurogenic regions such as the subventricular zone and hippocampus (45). This failure to obtain neuronal phenotypes after transplanting NSCs into the adult spinal cord is also supported by experiments using embryonic spinal cord NSC grafts (5, 6).

The inability of multipotent NSCs to generate neurons, because of either the absence of supportive signals or the presence of inhibitory factors in the host environment, may occur during one of the following

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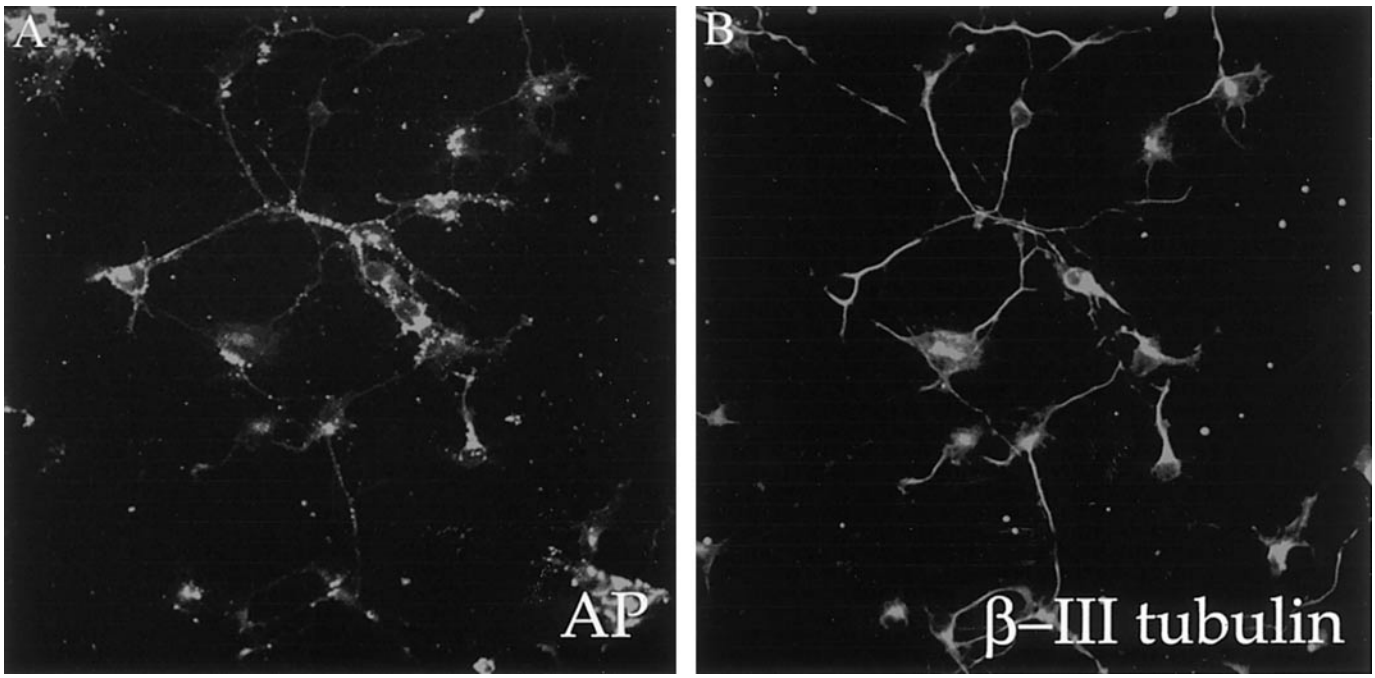


FIG. 1. Cultures of E-NCAM$^+$ embryonic NRP cells derived from an AP transgenic rat coexpress the reporter gene AP (A) and immature neuronal marker βIII-tubulin (B).

steps: (1) the transition of NSCs to the neuronal precursor stage, (2) the failure of neuronal precursors to survive, or (3) the differentiation of neuronal precursors to mature neurons. The available data do not allow us to distinguish between these possibilities, although transplants of dorsal root ganglion neurons into the intact adult spinal cord (9) and transplants of CNS neurons into nonneurogenic regions show that at least postmitotic neurons can survive and extend processes and that CNS white matter may support the survival of postmitotic neurons (7, 8, 14).

To identify the stage at which neuronal differentiation is blocked, we grafted NRP cells that are committed to the neuronal lineage into the intact adult spinal cord and followed their survival, differentiation, and integration using cells derived from an alkaline phosphatase transgenic rat. Our results demonstrate that NRP cells survive, migrate, extend neurites, integrate with the host spinal cord, and generate phenotypically mature neurons but not glia. Conversely, glial-restricted precursor cell transplants do not differentiate into neurons, demonstrating that transdifferentiation in the mature spinal cord is a rare occurrence. Thus, our results suggest that the failure of multipotent NSCs to generate neurons in the adult spinal cord results from an environment that does not support the initial commitment to the neuronal precursor stage. These results imply that NRP cells may be better suited than NSCs for neuronal replacement strategies after CNS injuries.

MATERIALS AND METHODS

Cell Isolation, Purification, and Culture

NRP Cells

Isolation. Alkaline phosphatase transgenic F344 rat embryos were removed at embryonic day 13.5 (E13.5) and placed in dishes containing Hanks' balanced salt solution (HBSS). Spinal cords were mechanically dissected from surrounding connective tissue using sharpened No. 5 forceps. Isolated spinal cords were incubated in 0.05% trypsin/EDTA solution for 15 min at 37°C. The trypsin solution was replaced with fresh medium, spun at 1000 rpm for 5 min, and resuspended in culture medium. The spinal cords were gently triturated with a fire-polished Pasteur pipette to dissociate the cells.

Purification and culture. The E-NCAM$^+$ neuronal-restricted cell population was isolated from the E13.5 dissociated cells using immunopanning or fluorescence-activated cell sorting (FACS). Antibody panning was carried out as previously described (55) with minor modifications. The dissociated cells were plated on E-NCAM antibody (5A5, Developmental Studies Hybridoma Bank, Iowa City, IA)-coated dishes to allow binding of all E-NCAM$^+$ cells to the dish. E-NCAM antibody-coated dishes were prepared by sequentially coating petri dishes with an unlabeled antimouse IgM antibody (10 μg/ml, Southern Biotech, Birmingham, AL) overnight, rinsing dishes with DPBS, followed by

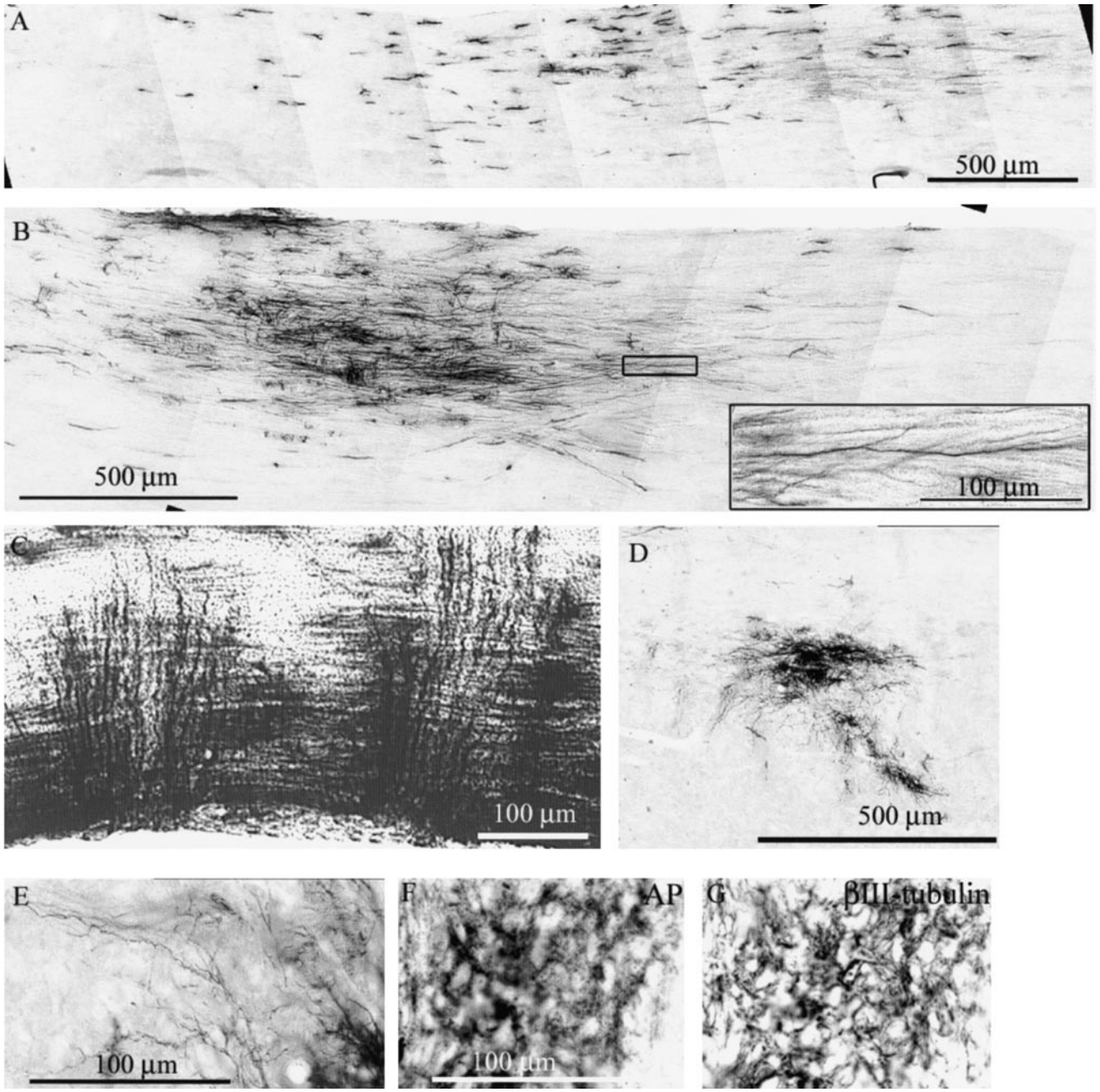


FIG. 2. NRP cells grafted into the intact adult spinal cord survive, migrate, and extend processes. Transplanted cells were examined at 1, 2, and 4 weeks after transplantation in parasagittal sections of the spinal cord with alkaline phosphatase histochemistry. Surviving cells were seen at all time points and all animals grafted ($N = 21$). (A) NRP cells survived and migrated, in both the rostral and caudal directions, long distances in the white matter. (B) NRP cells in the white matter extended long branched processes, typically along the rostral–caudal axis (see high-power inset of boxed area). Processes also extended into the gray matter and toward the spinal roots (C). Cells grafted into the gray matter (D) demonstrated more limited migration and process length. Processes extended diffusely throughout the adjacent gray matter regions but still had the ability to branch. Immunofluorescence staining of grafted AP^+ NRP cells (F) demonstrated the colocalization of βIII-tubulin (G) after transplant.

coating with 5A5 hybridoma supernatant for 1 h at room temperature. Cells were allowed to bind to the plate for 1 h at room temperature. Unbound cells were removed and the plate was rinsed eight times with DPBS. Bound cells were mechanically scraped off and plated on dishes first coated with poly-L-lysine (pLL) (13.3 μg/ml, Sigma, St. Louis, MO) or fibronectin (20 μg/ml, Sigma) and then with laminin (20 μg/ml, Gibco BRL, Rockville, MD) in medium consisting of DMEM-F12 (Life Technologies/BRL) supplemented with bo-

vine serum albumin (1 mg/ml), B27 supplement (20 μl), Pen/Strep (100 IU/ml), L-glutamine (292 μg/ml), N2 supplement (10 μl/ml), basic fibroblast growth factor (bFGF) (20 ng/ml, BD Biosciences, Medford, MA), and NT-3 (20 ng/ml). FACS was also used to purify E-NCAM$^+$ cells as previously described (35). Growth factors were added every other day. In all cases, an aliquot of cells was analyzed the next day to determine the efficiency of the immunopanning. In general, greater than 90% of the bound cells expressed detectable E-NCAM immunoreactivity. Cell populations that did not were repanned or discarded. Cells were maintained in culture for up to three passages (1–2 weeks) and harvested for transplantation.

GRP Cells

Immortalized A2B5$^+$ GRP cells (GRIP-1) (54), derived from E13.5 rat embryos, were used in transplant experiments. The GRIP-1 cells were retrovirally transduced with GFP and cells expressing the GFP reporter were enriched by FACS (54). Cells were expanded in a chemically defined basal medium as previously described (54). For maintaining GRIP-1 cells in an undifferentiated state, cells were passaged onto fibronectin-coated dishes using 0.05% trypsin–EDTA (Gibco BRL). Cells passaged greater than 10 times from initial isolation were used for transplant experiments.

Transplantation of NRP Cells and GRP Cells into Intact Adult Spinal Cord

Preparation of Cells for Grafting

NRP cells and GRP cells maintained in culture were dissociated from tissue culture flasks using 0.05% trypsin/EDTA, washed, resuspended at a concentration of 10,000–50,000 cells/μl in fresh culture media, and placed on ice for the duration of the grafting session. At the end of the grafting session, cell viability of the remaining cells on ice was assessed by trypan blue and typically averaged over 90%.

Animal Surgery and Intraspinal Grafting

Adult female Sprague–Dawley rats (Taconic, Germantown, NY) weighing on average 250–300 g were anesthetized with an ip injection of acepromazine maleate (0.7 mg/kg; Fermenta Animal Health Co., Kansas City, MO), ketamine (95 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA), and xylazine (10 mg/kg, Bayer Co., Shawnee Mission, KS), the back neck musculature was incised, and a laminectomy at C3–C4 was performed. The dura was incised right of midline and a single sample of NRP cells (10,000–50,000 cells in 1 μl of basal media) or GRP-GFP cells was microinjected into the cervical spinal cord using a Hamilton syringe containing a glass pipette pulled to diameters of 50–100 μm. Gray matter and white matter areas were targeted in separate animals. The glass tip was advanced slowly into the spinal cord using a micromanipulator (World Precision Instruments, Sarasota, FL) and cells were then injected over 1 min and the tip slowly withdrawn. The dura was closed with two 9-0 sutures, the musculature was reapposed and sutured with 4-0 sutures, and the skin closed with wound clips. Animals received Buprenex postoperatively. Animals were immunosuppressed with cyclosporin A injection solution (Sandoz Pharmaceuticals, East Hanover, NJ) administered subcutaneously at a dose of 1 mg/100 gm of body weight starting 2 days prior to cell grafting and daily thereafter. Although rat cells were used in the current study, strain differences between graft and host necessitated the use of immune suppression.

Tissue Processing

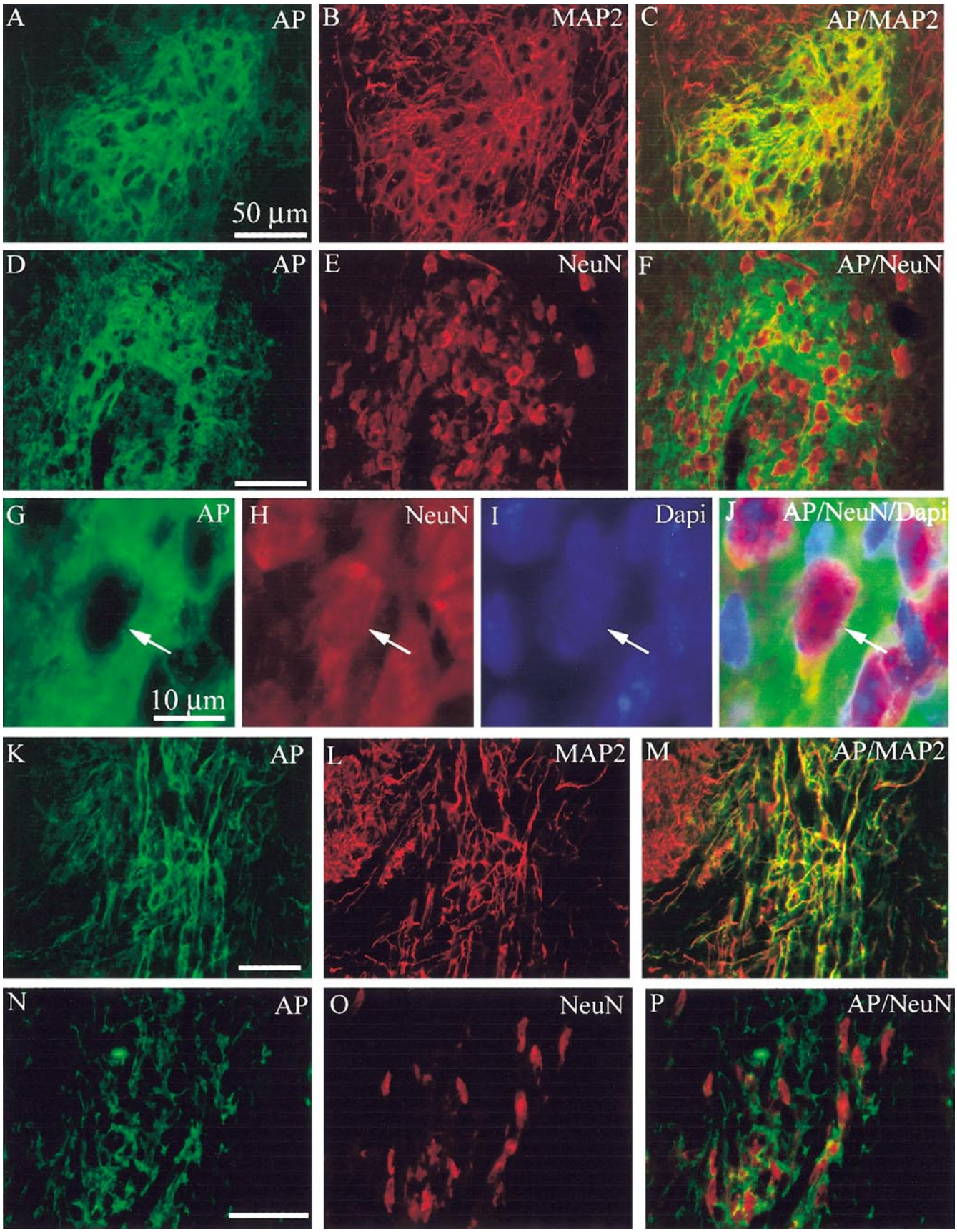
Animals were sacrificed at 1, 2, and 4 weeks after transplantation and transcardially perfused with saline followed by ice-cold 4% paraformaldehyde. The spinal cord was carefully removed and the segments of interest were cut and cryoprotected in 30% sucrose–0.1 M phosphate buffer at 4°C for 2 days. The tissue was blocked in OCT (Fisher Scientific), fast frozen on dry ice, and stored at −80°C until needed. Tissue blocks were sectioned parasagittally or coronally using a cryostat at 20 μm thickness and sections were collected on gelatin/pLL-coated glass slides and stored at 4° or −20°C until further analyzed.

Alkaline Phosphatase Histochemistry

Serial sections were analyzed by alkaline phosphatase histochemistry to determine the location, migration, and neurite extension of grafted NRP cells. Sections were washed three times in PBS, heat-treated at 60°C in PBS for 1 h to inactivate endogenous alkaline phosphatase, briefly washed in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), and incubated at room temperature in the dark with alkaline phosphatase buffer containing 1.0 mg/ml NBT, 0.1 mg/ml BCIP, and 5 mM levamisole (Sigma) in alkaline phosphatase buffer for 1 h to overnight. Slides were coverslipped in aqueous mounting media and visualized using a light microscope.

Immunohistochemistry and Phenotypic Analysis

Sections were washed in PBS, blocked in 10% goat serum for 1 h, and incubated with primary antibodies overnight at 4°C or room temperature. AP-expressing cells were identified with either a monoclonal (Sigma, 1:100) or polyclonal (Serotec, 1:50) antibody to human placental alkaline phosphatase. A panel of primary antibodies was used to determine the phenotypic differentiation of grafted cells. Neuronal differentiation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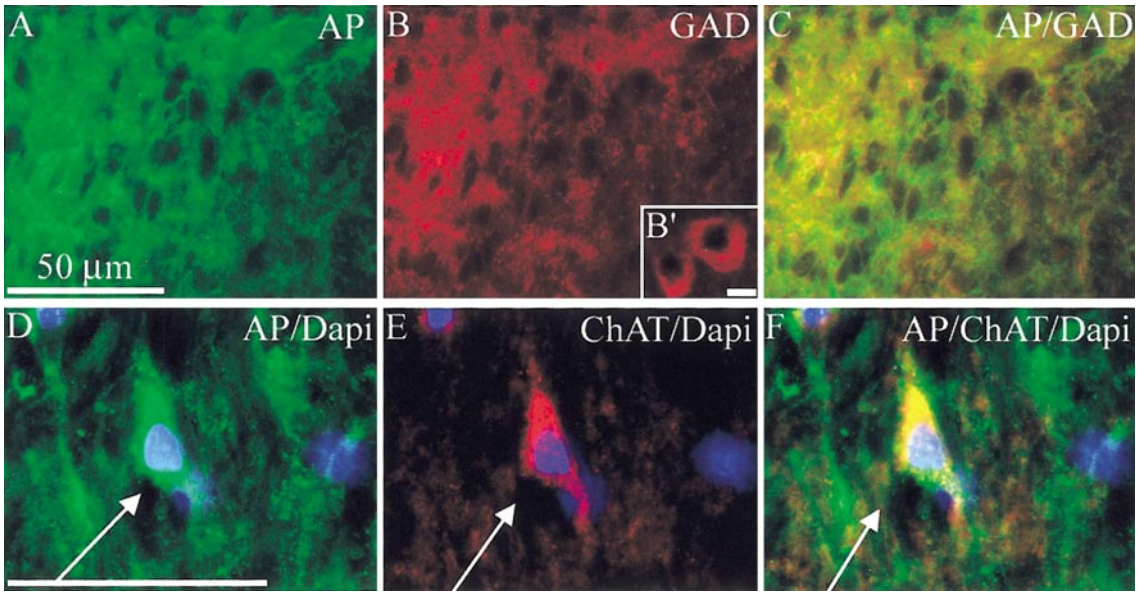


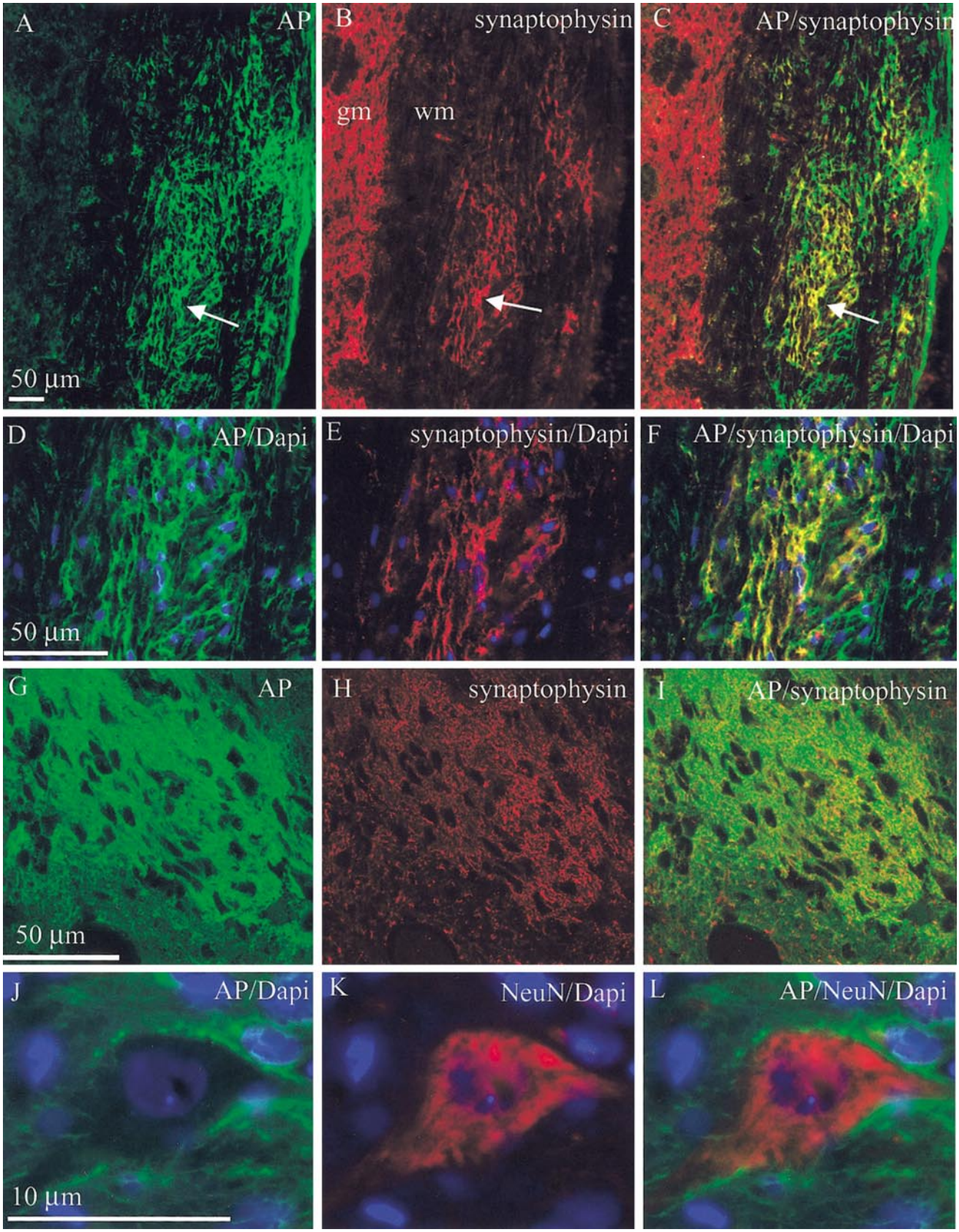
FIG. 4. NRP graft-derived neurons express neurotransmitter phenotypes. NRP cells (A, D) were examined for their ability to exhibit neurotransmitter phenotypes typical of spinal cord neurons (B, gabaergic, and E, cholinergic) using double-fluorescence immunostaining. Many AP^+ cells colocalized with GAD65,67 (A–C), while only a small subset colocalized with ChAT labeling (D–F). Examples of host neurons stained for GAD65,67 are shown in B′. Sections were counterstained with DAPI (blue) to identify all nuclei in D–F. Scale bars: (A, 50 μm), A–C; (B′, 10 μm); (D, 50 μm), D–F.

was assessed by the following antibodies: Tuj1 (Babco 1:500), recognizing the neuronal specific βIII-tubulin (4); MAP2ab (Chemicon 1:100), which recognizes cell bodies and dendrites of mature neurons (32); and NeuN (Chemicon 1:100), which identifies the nuclear antigen A60 in postmitotic neurons (36). Neurotransmitter phenotypes of the graft-derived neurons were assessed by antibodies to ChAT (Sigma, 1:100) to identify cholinergic neurons and GAD 65/67 (Sigma, 1:500) to identify GABAergic neurons. Synaptic integration was examined with an antibody to synaptophysin (Sigma, 1:100), which identifies a protein localized to presynaptic vesicles (53). GFAP (1:100, Boehringer Mannheim, Germany) was used to identify astrocytes and RIP (Chemicon, 1:10,000), CNPase (1:1000, Sternberger, Lutherville, MD), and PLP (Chemicon, 1:100) were used to identify oligodendrocytes. E-NCAM and A2B5 antibodies were obtained from the Developmental Hybridoma Studies Bank (DHSB, University of Iowa) and used at 1:1 dilution.

Species-specific fluorescence-conjugated (rhodamine or FITC) secondary antibodies (1:200, Jackson Immunoresearch, West Grove, PA) were applied for 2 h onto slides and coverslipped with Vectashield mounting media with or without DAPI (Vector Labs, Burlingame, CA). Staining was visualized using a Leica DMRBE fluorescence microscope and images were captured using a Photometric Sensys KAF-1400 CCD camera (Photometric Inc.). Images were processed on a Macintosh G4 using IP Lab (Scanalytics) and figures prepared with Adobe Photoshop 6.0. In some cases, images were acquired with a Zeiss Axiovert 200M confocal microscope.

To get an estimate of the percentage of NRP cells that differentiated into mature neurons, AP^+ cells positive for NeuN were counted in a subset of samples. Photographs of sections triple labeled for AP, NeuN, and DAPI were taken with at 200× magnification as separate black and white images; 100-μm^2 non-overlapping regions were delinated in Photoshop 6.0 and AP^+ $DAPI^+$ cells were marked. The NeuN image was then overlaid onto the marked image and overlapping cells were counted as triple-labeled cells. Cells were classified as AP^+ if they possessed DAPI nuclei and were surrounded by cytoplasmic AP immunostaining. Triple-labeled cells were defined as cells containing a

FIG. 3. NRP cells differentiate into mature neurons upon transplantation into the spinal cord. NRP cells grafted into the spinal gray (A, D) or white (K, N) matter were examined for their expression of neuronal phenotypes at 1 and 2 weeks with double fluorescence immunostaining. The majority of AP^+ cells in the gray matter (A, D) coexpressed MAP2ab (B, C) and NeuN (E, F). (G–J) Higher power views of a region in D–F demonstrates the colocalization of NeuN in a AP^+ cell. Note that AP typically identified the cytoplasm of grafted cells whereas NeuN labels predominantly the nuclear compartment (compare H and I). Arrow points to the nucleus of the AP+/NeuN+/Dapi+ positive cell. Similarly, AP+ cells grafted into the white matter (K, N) coexpressed the mature neuronal markers MAP2ab (L, M) and NeuN (O, P). DAPI (blue) in I stains all nuclei. Scale bars: (A, D, K, N, 50 μm), A–C, D–F, K–M; and N–PI; (G, 10 μm), G–J.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gm
wm
C
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D
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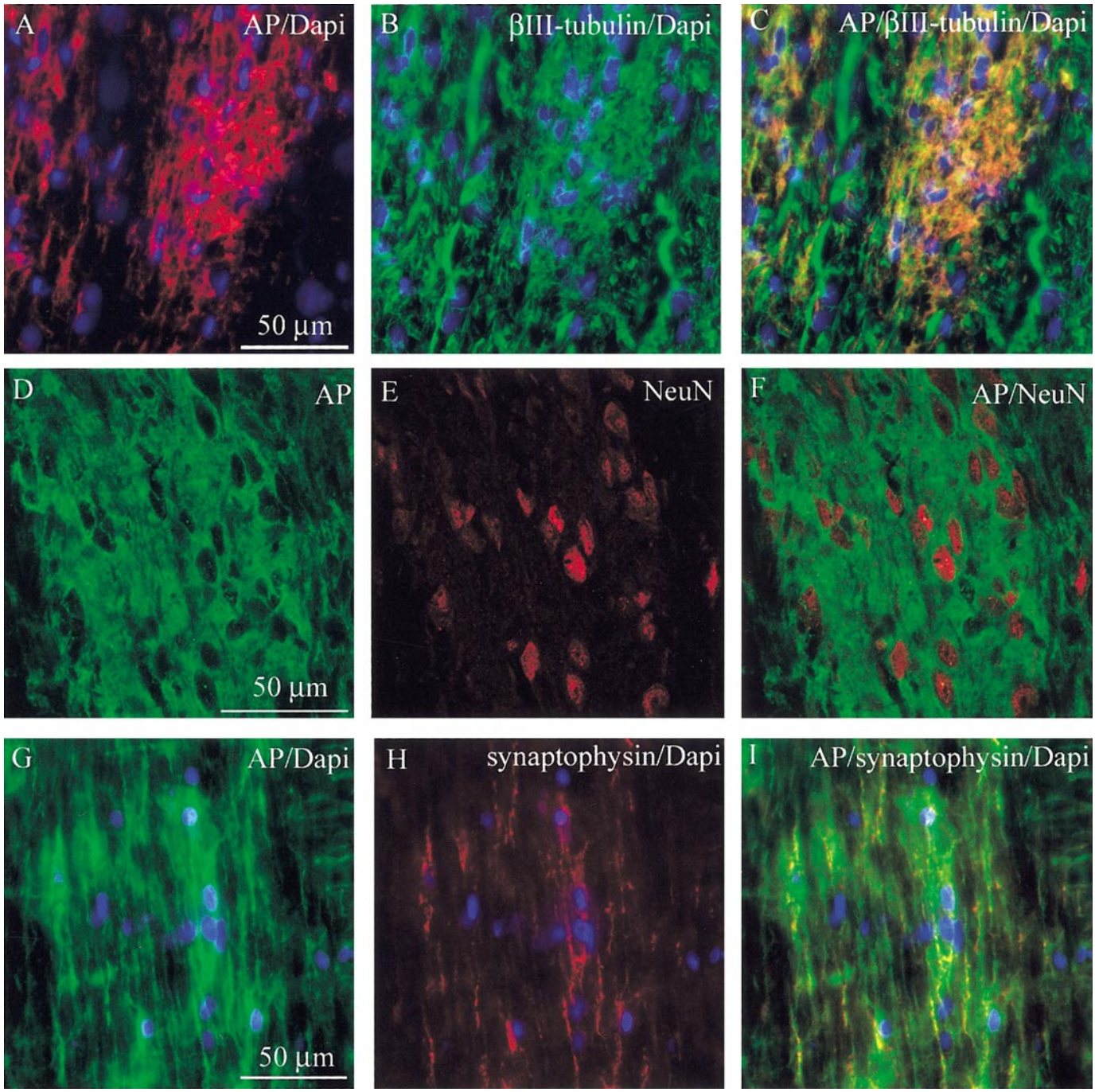


FIG. 6. Differentiated neurons survive for at least 1 month after intraspinal grafting. The persistence of mature neuronal phenotypes of grafted NRP cells was examined at 1 month. AP^+ cells (A, D, G) continued to colocalize with the mature neuronal phenotypes βIII-tubulin (B, C), NeuN (E, F), and synaptophysin (H, I). Sections were counterstained with DAPI (blue) to identify all nuclei in A–C and G–I. Scale bars: (A, 50 μm), A–C; (D, 50 μm), D–F; (G, 50 μm), G–I.

FIG. 5. NRP graft-derived neurons integrate with host spinal cord. NRP cells grafted into the spinal white (A, D) and gray (G) matter were assessed for their expression of synaptophysin (B, E, H), a marker of synaptic vesicles and synapse formation at 1 and 2 weeks using double-fluorescence immunostaining. Note that synaptophysin staining is predominantly localized to the gray matter (gm) in the host spinal cord (B). Most AP^+ cells double stained for synaptophysin in both the white (B, C and E, F) and gray (H, I) matter locations. D and E are higher power photographs of the region indicated with arrows in A–C. AP^+ fibers (J) can be seen terminating on the cell body of a host $NeuN^+$ neuron (K, L). Sections were counterstained with DAPI (blue) to identify all nuclei in D–F and J–L. Scale bars: (A, 50 μm), A–C; (D, 50 μm), D–F; (G, 50 μm), G–I; (J, 10 μm), J–L.

DAPI nucleus, nuclear/perinuclear NeuN staining, and cytoplasmic AP staining (see Figs. 3G–3J). A total of three 100-μm^2 fields from two animals at 4 weeks postgrafting representing a total of 132 cells were counted to give an estimate of $NeuN^+$ differentiation.

RESULTS

Grafted NRP Cells Survive, Migrate, and Extend Neurites

NSCs grafted into the adult spinal cord have resulted in either limited differentiation or differentiation into predominantly glial cells (5, 6, 45). To determine whether the failure to differentiate into neurons was at the transition of multipotential stem cell to neuronal-restricted precursor cell or in the survival and maturation of transplanted neuronal precursor cells, we grafted NRP cells, isolated from rat embryos at E13.5 as previously described (21, 33), into the intact adult spinal cord. To follow the fate of the grafted cells efficiently, we isolated NRP cells from a transgenic rat stably expressing the marker gene human placental AP under the control of the ROSA promoter (26). Cells transplanted into the spinal cord white matter or gray matter were initially analyzed *in vivo* with respect to survival and migration for up to 1 month using AP histochemistry to identify the donor cells.

We found that NRP cells from the AP transgenic rat robustly and stably expressed the reporter gene AP and differentiated into mature neurons *in vitro* (Fig. 1 and (35)). Prior to transplantation, NRP cells, maintained *in vitro* under nondifferentiating conditions, express the early neuronal precursor marker βIII-tubulin (Fig. 1) but do not express any markers of mature neurons (21, 33). Following transplantation, we observed at all time points studied (1, 2, and 4 weeks) and in all animals grafted ($N = 21$) robust survival of NRP cells and expression of the AP transgene. NRP cells survived in both the gray matter and white matter environments but displayed site-specific morphological characteristics (Fig. 2). In grafts into the white matter, NRP cells migrated for long (2–8 mm) distances (Fig. 2A) along the rostral–caudal axis and extended long processes, many over 1 mm, typically parallel to the longitudinal spinal tracts (Fig. 2B). Migration and process outgrowth occurred in both the rostral and caudal directions and elongating processes were seen to branch (Fig 2B, high-power inset). Some cells also extended processes into the gray matter and toward the dorsal and ventral roots (data not shown and Fig. 2C, respectively). Cells in the gray matter demonstrated more limited migration and neurite extension (Fig. 2D). Cells bodies typically remained near the injection site but did extend processes (Fig. 2E) that branched diffusely throughout the adjacent gray matter and into white matter. Immunohistochemical staining revealed that AP-positive NRP cells (Fig. 2F) continued to express βIII-tubulin (Fig. 2G) after transplantation.

These results demonstrate that the adult spinal cord, both gray and white matter, permits the survival of fetal neuronal precursors. NRP cells differentially migrate and extend processes depending on whether they are grafted into white or gray matter. Thus, NRP cells respond to differential molecular cues specific to the local environment that influence migration and process extension. Furthermore, the remarkable length and branching of the grafted NRP cell processes suggest that extensive growth and morphological differentiation occur in this environment.

Grafted NRPs Differentiate into Mature Neurons and Integrate with the Host

While our results demonstrated that grafted NRP cells could survive, acquire neuronal morphologies, and extend neurites, it was possible that signals promoting the acquisition of mature neuronal properties were absent from the adult spinal cord. We therefore examined whether the adult spinal cord allowed the grafted NRP cells to generate mature neurons by double labeling with an antibody against the AP transgene to identify the grafted cells and a panel of neuronal specific antibodies to assess their *in vivo* differentiation.

At 1 and 2 weeks posttransplantation, NRP graft-derived cells expressed markers of mature neurons. Cells located in the spinal cord gray matter were immunoreactive for several neuronal specific markers, including βIII-tubulin (Figs. 2F and 2G), MAP2ab (Figs. 3A–3C), and NeuN (Figs. 3D–3F and 3G–3J). Interestingly, no major differences in βIII-tubulin, MAP2, and NeuN expression were observed between cells located in the white matter and gray matter. Specifically, NRPs differentiated into βIII-tubulin (data not shown), $MAP2ab^+$ (Figs. 3K–3M), and $NeuN^+$ (Figs. 3N–3P) neurons to the same extent in white matter as in gray matter. In addition, NeuN immunoreactivity suggested that transplant-derived neurons were postmitotic. Thus, NRP cells transplanted into the adult spinal cord generated mature neuronal phenotypes in all areas of the spinal cord.

NRP cells in culture, when induced to differentiate, generate neurotransmitter-producing neurons, including GABAergic and cholinergic neurons (21). We tested whether the adult environment would also support the differentiation of NRP cells to neurons expressing neurotransmitter phenotypes using antibodies against the biosynthetic enzymes glutamic acid decarboxylase (GAD65/67), a marker for GABAergic neurons, and choline acetyltransferase (ChAT), a marker for cholinergic neurons. We found that many transplant-derived cells differentiated into GABAergic neurons (Figs. 4A–4C), but only a small subset of cells differentiated into

cholinergic neurons (Figs. 4D–4F). However, NRP cells did not mature into serotonergic neurons (data not shown), a neurotransmitter phenotype restricted to brain stem neurons.

To determine whether grafted NRP cells could differentiate into neurons that may integrate with host neurons, we analyzed synaptophysin expression from the newly generated neurons. Synaptophysin is specific for synaptic vesicles and is associated with functional synapses (47, 53); therefore, synaptophysin immunoreactivity in the host tissue is localized to the gray matter and is sparse in the white matter of the spinal cord (Fig. 5B). NRP cells do not express synaptophysin prior to transplant (21). Typically, the majority of grafted NRP cells in the white matter (Figs. 5A–5C and 5D–5F) and in the gray matter (Figs. 5G–5I) were immunoreactive for synaptophysin. Expression was seen as punctate labeling of cell bodies and processes and was observed as early as 1 week postgrafting. In addition, AP^+ fibers readily decorated the cell bodies of $NeuN^+$ host neurons, suggesting that connections between graft-derived neurons and host neurons occurred (Figs. 5J–5L).

Thus, transplanted NRP cells differentiate into mature neurons in both the white and gray matter of the intact adult spinal cord and express neurotransmitter phenotypes expected of mature spinal cord neurons. Furthermore, differentiated neurons expressed markers suggestive of integration with the host spinal cord.

Differentiated Neurons Survive for Long Periods

While our results suggested that NRP cells grafted into the intact adult spinal cord could survive, differentiate, and integrate as early as 1 week postimplantation in both gray and white matter microenvironments, we also tested whether these newly formed differentiated neurons could persist for longer periods *in vivo*. Animals were allowed to survive to 4 weeks postgrafting and the spinal cords were examined for the persistence of NRP-derived mature neurons using mature neuronal specific markers. AP reporter gene expression could still be robustly detected using both histochemistry (data not shown) and immunohistochemistry (Figs. 6A, 6D, and 6G). Numerous βIII-tubulin and $NeuN^+$ cells colabeling with AP were detected in both gray (data not shown) and white matter environments (Figs. 6A–6C and 6D–6F). Cells at this time also continued to robustly express synaptophysin (Figs. 6G–6I) and GAD65/67 (data not shown). Estimated counts (see Materials and Methods) of AP^+/$DAPI^+$ cells expressing NeuN at 4 weeks revealed an average of 50% colocalizing these markers. Thus, neurons with phenotypically mature characteristics generated from the NRP cell grafts survived for up to 4 weeks, demonstrating that donor-derived neurons could mature and persist for long periods after transplantation.

Grafted NRPs Do Not Differentiate into Astrocytes or Oligodendrocytes or Induce a Glial Scar

NRPs derived from the embryonic neural tube have been shown to generate exclusively neurons *in vitro*, even under culture conditions that favor glial cell survival and differentiation (21). To examine whether grafted NRPs also maintain their neuronal-restricted properties after homotypic transplantation into the adult spinal cord, we assessed their ability to adopt astroglial or oligodendroglial phenotypes. In addition, we also assessed whether NRP transplants induced a gliotic scar.

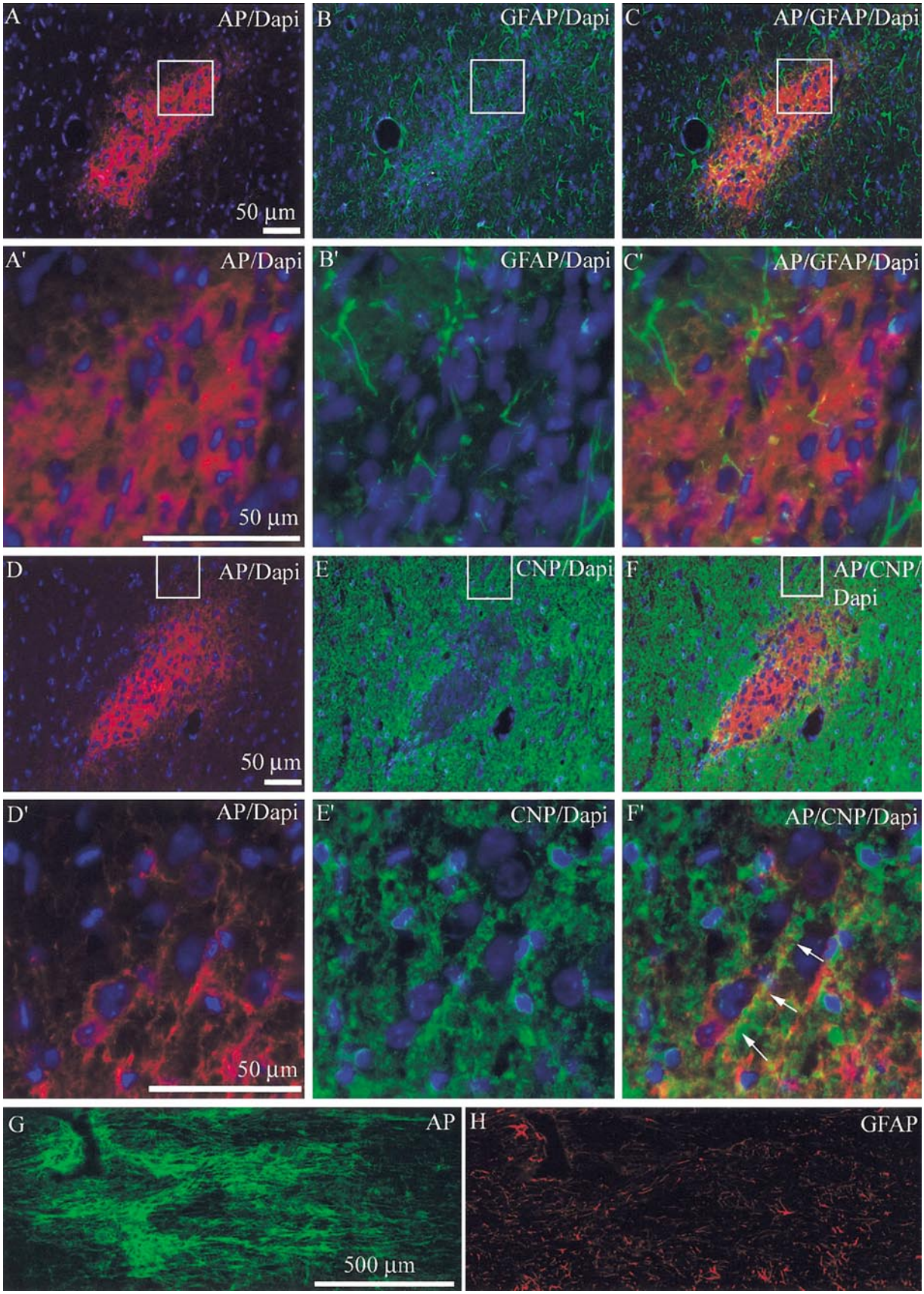
Astrocytic differentiation was evaluated by immunoreactivity to GFAP (Figs. 7A–7C and 7A–7C) and oligodendrocytic differentiation studied with antibodies to CNPase (Figs. 7D–7F and 7D–7F), RIP (data not shown), and PLP (data not shown). AP-labeled NRP cells did not costain with any of the glial markers used (Fig. 7). Interestingly, while the oligodendrocytic markers did not colocalize with the AP immunoreactivity of the grafted cells, the processes extending from the NRP-derived neurons appeared integrated with the myelin staining (Figs. 7D'–7F'), suggesting that newly formed neurites may have been myelinated. While $GFAP^+$ host astrocytes were observed near the grafted cells, there was no coexpression of AP with GFAP (Figs. 7A'–7C'). We examined whether NRP cell transplants induced a glial scar reaction in the spinal cord using an antibody against GFAP. NRP cell grafts located in the white matter (Fig. 7G) did not induce a $GFAP^+$ gliosis (Fig. 7H). Similar results were observed in gray matter transplants (Figs. 7A–7C).

Thus, in contrast to the results with NSC transplants we find that NRP cells did not respond to the gliogenic signals of the adult spinal cord but remained restricted to the neuronal lineage. Moreover, NRP grafts did not induce a major gliotic scar reaction, demonstrating that the injury was very limited.

Glial-Restricted Precursors Do Not Adopt Neuronal Phenotypes upon Grafting

Recent results have suggested that glial precursor cells can transdifferentiate into neurons (11, 27, 28). Transdifferentiation may be induced to follow pathways different from the normal process of differentiation by specific culture conditions and methods of differentiating neurons.

To determine if the spinal cord environment was inhibitory to transdifferentiation as well as neuronal differentiation of NSCs we utilized an immortalized glial restricted precursor cell line (GRIP-1, (54). As these cells can differentiate into MAP-2/ βIII-tubu-


A
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C
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A'
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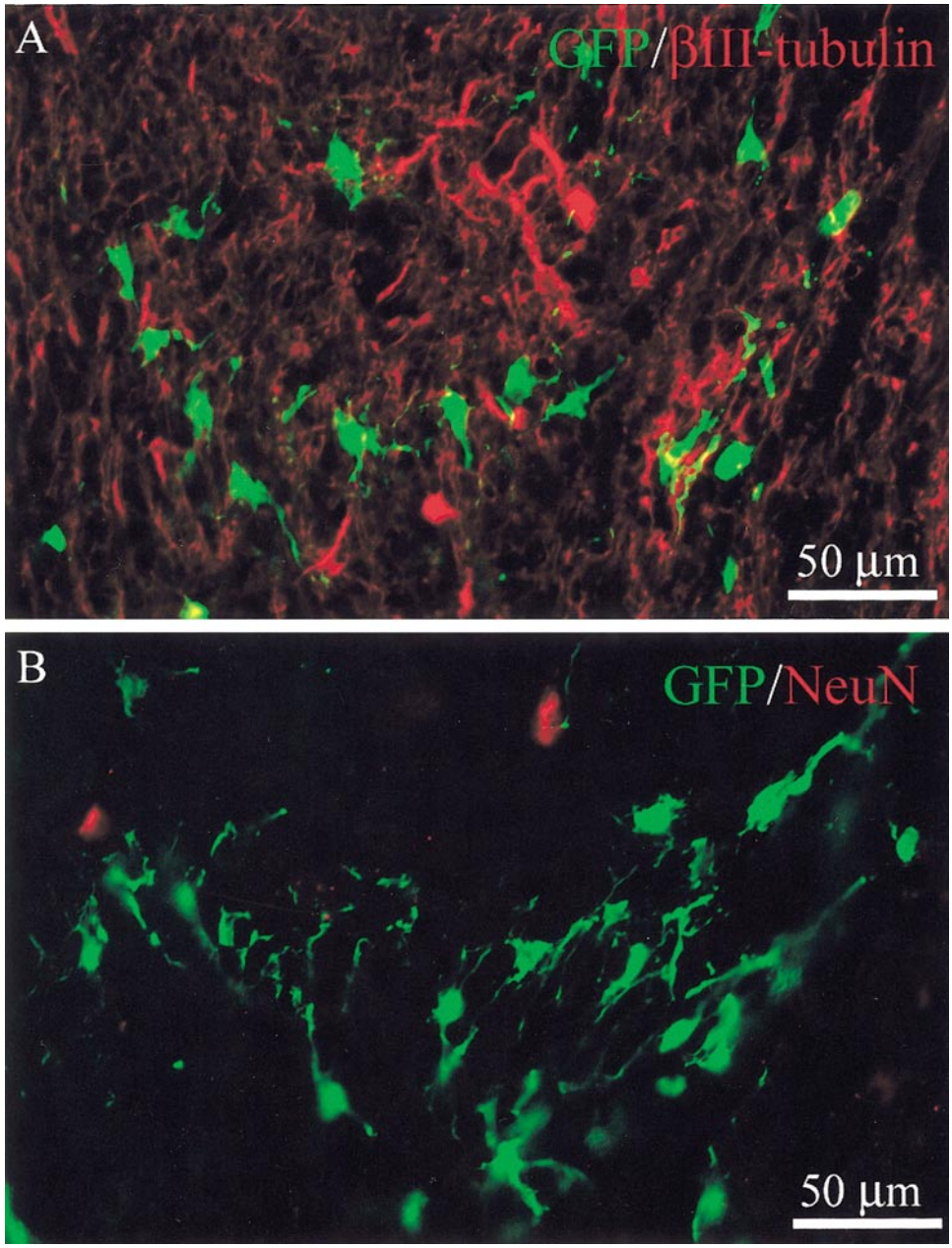

FIG. 8. GRP cells do not transdifferentiate into neurons in the adult spinal cord. GFP-labeled GRP cells grafted into the intact spinal cord were examined at 2 weeks for their ability to differentiate into neurons. GFP+ GRP cells (green) did not express the neuronal markers βIII-tubulin (A, red) or NeuN (B, red) when grafted into the gray matter. Scale bars, 50 μm.

lin/NF immunoreactive neuronal appearing cells in culture, we wanted to find out whether they could also generate neuronal phenotypes *in vivo*. Cells were tested prior to transplantation to prevent the implantation of predifferentiated neurons and long-term (>10) passaged cells were used, as our results indi-

FIG. 7. NRP cells are restricted to a neuronal lineage in the adult spinal cord and do not induce a gliotic scar. NRP cells grafts were examined for their ability to differentiate into astrocytes and oligodendrocytes and whether the cells induced an astrocytic scar at 1 and 2 weeks. AP^+ cells (A, D) did not colocalize with the astrocytic marker GFAP (B, C) or the oligodendrocytic marker CNP (E, F). The boxed regions in A–C are shown at higher power in A′–C′ to further demonstrate that AP^+ cells are not coexpressing GFAP. The boxed regions in D–F are shown at higher magnification in D′–F′. Note that AP^+ processes (D′) appeared to be adjacent to CNP staining (E′, F′ arrows), suggesting that processes emanating from NRP-derived cells are being myelinated. NRP cell grafts (G) do not appear to induce a major gliotic scar reaction (H). Sections were counterstained with DAPI (blue) to identify all nuclei. Scale bars: (A, 50 μm), A–C; (A′, 50 μm), A′–C′; (D, 50 μm), D–F; (D′, 50 μm), D′–F′; (G, 500 μm), G, H.

cated that neuronal differentiation was seen only after prolonged culture.

Animals were maintained for 1–4 weeks after transplantation and sections were processed for immunocytochemistry as described under Material and Methods. When grafted into the normal adult spinal cord, GRP cells can differentiate into astrocytes and oligodendrocytes (54). However, using several markers of neurons, grafted GRP cells did not differentiate into βIII-tubulin$^+$ (Fig. 8A), MAP2ab$^+$ (data not shown), or NeuN$^+$ cells (Fig. 8B) in the gray matter (Fig. 8) and white matter (data not shown). Thus, GRPs remain restricted to a glial fate *in vivo* despite their ability to generate neurons *in vitro*. Neuronal differentiation is therefore specific to NRP cells in the spinal cord environment and cannot be initiated from either NSCs or from glial precursor cells.

DISCUSSION

Our results show that NRP cells, unlike multipotential neural stem cells from the adult and embryonic CNS, will differentiate into neurons after transplantation into the intact spinal cord. Neurons thus formed will survive in both gray and white matter, migrate, extend processes, and acquire distinct neurotransmitter profiles. The early formed neurons appear to integrate into the environment and there is no evidence of a major gliotic scar or any glial differentiation. Moreover, the newly formed neurons persist up to 1 month *in vivo*. The behavior of NRP cells is in contrast to the behavior of glial precursor cells, which do not form neurons but instead differentiate into oligodendrocytes and astrocytes. We interpret these results to indicate that the adult host environment is competent to promote neuronal and glial differentiation and that the failure to observe neuronal differentiation from multipotent NSCs is due to a block at the transition from multipotent to neuronal restricted cells. While our results suggest integration of graft-derived neurons with the host, electrophysiological assessment would be necessary to demonstrate functional synaptic integration.

Neurogenesis in the intact and injured adult spinal cord has not been reported and our results suggest that this failure is due to the absence of NRP-like cells in the adult spinal cord. While stem cells isolated from the adult spinal cord can generate neurons *in vitro*, these same cells *in situ* proliferate in the intact and injured spinal cord but lack the requisite signals to initiate the process of neuronal differentiation (1, 2, 17, 18, 34, 37, 57). Similarly, neural stem cells grafted back to the normal or injured spinal cord do not differentiate into neurons (5, 6, 45) but when grafted into neurogenic regions, such as the adult hippocampus, neuronal phenotypes develop (45). Our results do not distinguish between the presence of an inhibitory signal that actively prevents differentiation into neuronal restricted precursors and the absence of a stimulatory signal that promotes differentiation. Both classes of molecules have been described (25), including ngn1, BDNF, FGF, EGF, and BMP. We favor the possibility that inhibitory influences are present, because postnatal dissociated spinal cord cultures will generate new neurons without the addition of proneuronal factors (23) and overexpression of a dominant negative notch ligand construct will promote neuronal differentiation in cultured adult progenitors (56). However, a careful analysis of candidate molecules needs to be performed *in vivo*.

The factors that control differentiation of transplanted stem cells and precursor cells remain unknown. Interpretations of previous transplant studies are complicated by the fact that the "stem cell" transplanted may consist of a heterogeneous mixture of undifferentiated stem cells, more committed precursors, and differentiated cells. We and others have shown that fetal stem cells will readily generate neurons *in vitro* and in the adult after transplantation, suggesting that there is no intrinsic bias against neuronal differentiation. We and others have also shown that NSCs will survive, integrate, and differentiate in the spinal cord but will not generate neurons (5, 6, 45), ruling out the possibility that a failure to see neuronal differentiation from transplanted stem cells is due to the death and failure of engraftment. It is also unlikely that the failure to observe neuronal differentiation is because the environment is strongly gliogenic and thus stem cells have no fate choice but to differentiate into glial cells. Our present results suggest that the failure to observe mature neurons from NSC transplants is not due to an absence of the required trophic factors. Indeed both the gray matter and white matter support the survival of mature neurons that extend processes.

An important practical conclusion from our results is that transplants into the spinal cord will require predifferentiating multipotential stem cells toward a neuronal lineage (24, 30) or using lineage-restricted precursor cells (13, 40) to achieve control over the phenotype of transplanted cells. More committed precursor cells have been successfully generated from NSCs *in vitro* and directly isolated from the spinal cord (22, 29, 40). Thus, it is conceivable that NSCs can be manipulated *in vitro* so that the desired phenotype is selected prior to transplantation. Modifying the immediate environment into which NSCs are grafted may also encourage neuronal differentiation. Indeed, we have shown that NSCs grafted into the adult spinal cord can generate neurons when grafted along with exogenous delivery of BDNF (6). Also, neuronal differentiation after grafting also resulted when NSCs were genetically engineered to produce NT-3, presumably acting in an autocrine fashion (31). Collectively, these findings and our current results demonstrate that cellular dif-

ferentiation from grafted precursor cells involves a complex interaction of intrinsic and extrinsic factors. Interestingly, our current results show that the mature white matter environment did not inhibit neuronal differentiation. Future studies investigating the cellular replacement potential of NRP cells should give insight into whether ectopic production of neurons may contribute to neurological dysfunction or be exploited to introduce novel neuronal relays and circuits in the damaged CNS.

Our present results demonstrate that neuronal restricted precursors maintain their commitment to the neuronal lineages after implantation into the mature spinal cord. Similar results have been observed from grafts into postnatal brain and in an accompanying manuscript Whittemore and colleagues (5a) report similar findings to those presented in the current paper (16, 58). Glial precursors may have the ability to transdifferentiate into neurons *in vitro* (27, 54). We have characterized an immortalized GRP cell that can be induced to develop neuronal phenotypes after prolonged culture (54). Our present results, however, failed to detect transdifferentiated neurons derived from these cells after transplantation, suggesting that the adult spinal cord environment does not provide the necessary pathways for transdifferentiation of glial precursors. Thus neuronal differentiation is specific to grafted NRP cells.

Our results also demonstrate the utility of using cells derived from an alkaline phosphatase rat for CNS transplant studies. The AP transgene expression is directed from the ubiquitous ROSA26 promoter and therefore detected in numerous cell types (26). We have shown that many precursor cell types can be isolated from the AP transgenic rat and that AP expression persists in both undifferentiated cells and their differentiated progeny (35). Grafted cells expressing AP could be robustly detected for at least 1 month (present results) and have been observed as long as 2.5 months (our unpublished results). While grafted GRP cells expressing GFP driven by the CMV promoter could be detected for at least 2 weeks (present study), downregulation of GFP was noted at later time points (our unpublished results). The present results demonstrate that AP expressing grafted cells and their differentiated derivatives can be efficiently detected *in vivo* and used for long-term analysis.

In summary, we suggest while the adult spinal cord is not conducive for neurogenesis from stem cells *in situ* or after transplantation, the environment does support the survival and neuronal differentiation from neuronal precursor cells. Our transplant results suggest that the failure to generate neurons is at the transition of stem cells to neuronal precursors but that once neuronal precursors have formed, both the gray and white matter can support survival, neurite outgrowth, and differentiation into neurons. Neuronal precursors do not differentiate into glial cells in a non-neurogenic environment and glial precursors do not transdifferentiate into neurons. Thus our results suggest that neuronal replacement in the spinal cord is feasible but requires grafting predifferentiated stem cells or neuronal precursor cells. Moreover, a greater understanding of the factors that promote or inhibit the transition from stem cells to neuronal precursor cells in the adult spinal cord may allow strategies for endogenous neurogenesis after spinal cord injury.

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