THE JOURNAL OF COMPARATIVE NEUROLOGY 511:318–328 (2008)

Adult Dorsal Root Ganglia Sensory Neurons Express the Early Neuronal Fate Marker Doublecortin

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ABSTRACT

It has been widely accepted that doublecortin (DCX) may represent a neuronal fate marker transiently expressed by immature neurons during development of the central and peripheral nervous tissue and in neurogenic areas of the adult brain. Previous work described the presence of DCX in the developing dorsal root ganglia (DRG), structures of the peripheral nervous system originating from the neural crest, but no information is available on its expression in adulthood. To this purpose, we have performed an immunohistochemical and biochemical analysis for DCX expression in DRG from adult male mice and rats. To our surprise, we demonstrated that the majority of DRG neurons do express DCX, both in somata and in fibers. DCX^+ cells have been characterized morphologically and phenotypically with well-established markers of DRG neuronal subpopulations. A large number of DCX^+ cells belong to the small and medium-sized nociceptive neurons. Additionally, DCX immunoreactivity is present in the spinal cord dorsal horns, the projection area of DRG neurons. The novel and unexpected localization for DCX protein opens up new, interesting vistas on the functional role of this protein in mature neurons and in particular in sensory neurons. J. Comp. Neurol. 511:318–328, 2008.

Indexing terms: doublecortin; dorsal root ganglia; sensory neurons; $P2X_3$; isolectin IB4; substance P

Doublecortin (DCX) is a microtubule-associated protein that is widely expressed by immature neurons during central and peripheral nervous system development (Des Portes et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Hannan et al., 1999; Couillard-Despres, 2001) and is involved in the regulation of migration (Gleeson et al., 1999; Corbo et al., 2002; Bai et al., 2003). Neuroblasts migrating along radial glial processes during cortex lamination express indeed high levels of DCX (Francis et al., 1999; Gleeson et al., 1999), and mutations in the DCX gene are associated with disrupted neuroblast migration in a genetically transmitted disease characterized by a smooth and four-layered cortex and known as *lissencephaly* (Berg et al., 1998; Des Portes et al., 1998; Gleeson et al., 1998). In addition to its developmental role, DCX expression in the adult brain is restricted mainly to areas where neurogenesis takes place, such as the subventricular zone (SVZ) and the hippocampal dentate gyrus (Lois and Alvarez- Buylla, 1994; Alvarez-Buylla and Garcia-Verdugo, 2002; Van Praag et al., 2002; Kempermann et al., 2003). Based on these observations, DCX expression is widely utilized as a tool for investigating adult neurogenesis (Brown et al., 2003; Rao and Shetty, 2004; Couillard-Despres et al., 2005).

Nonetheless, DCX-immunoreactive cells have also been detected in areas of the adult CNS in the apparent absence of neurogenesis (Nacher et al., 2001), but their functional significance is largely unknown. It is of interest that, in the adult brain, some DCX^+ cells display morphological features of mature neurons, whereas others resemble migrating neuroblasts. These peculiar features have contributed to the hypothesis that some DCX-expressing cells could represent a reservoir of partially undifferenti-

Grant sponsor: Fondazione Cariplo, Grant number: 2005-0634; Grant sponsor: Ministero della Istruzione della Università e della Ricerca; Grant number: PRIN 2005; Grant sponsor: Regione Piemonte, Grant number: CIPE 2004.

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Received 6 February 2008; Revised 11 June 2008; Accepted 31 July 2008

DOI 10.1002/cne.21845

Published online September 19, 2008 in Wiley InterScience (www.interscience.wiley.com).

ated neurons that under specific circumstances may complete their maturation during adult life (Nacher et al., 2001).

A vast array of experimental works has suggested that new neurons could be added to the dorsal root ganglia (DRG) postnatally (Devor and Govrin-Lippmann, 1985; Devor et al., 1991; Cecchini et al., 1995; Popken and Farel, 1997; Ciaroni et al., 2000; Namaka et al., 2001; Farel, 2002; Li et al., 2007), although adult DRG neurogenesis has not been confirmed in vivo (La Forte 1991; Pover et al., 1994; Geuna et al., 2000; Farel, 2003). Moreover, DRG represent areas subjected to high plasticity, enabling them to respond to and recover from injury (Groves et al., 2003; Kuo et al., 2005; Guseva and Chelyshev, 2006). For these reasons, we have investigated the expression of doublecortin in rodent DRG. To this purpose, we have performed immunohistochemical and biochemical analysis on DRG from 3-month-old male mice and rats. Surprisingly, we demonstrated that the majority of DRG neurons still retain DCX expression during adulthood, both in somata and in fibers. DCX^+ cells have been further characterized morphologically and phenotypically by immunohistochemistry with well-established markers of DRG neuronal subpopulations. Large numbers of DCX^+ cells belong to the small to medium-sized nociceptive neurons. Moreover, we demonstrate that DCX is expressed in the spinal cord (SC) dorsal horns, the projection area of DRG neurons, and that, at this level, DCX expression retains the same pattern of colocalization with the neuronal markers observed in the DRG.

MATERIALS AND METHODS

Animals

Adult (3-month-old) male CD1 mice (n = 6) and Sprague-Dawley rats (n = 3) purchased from Charles River Laboratories (Wilmington, MA) were utilized. All animals were maintained in high-efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging System) at the University of Piemonte Orientale animal facility and, kept in numbers of three or four per cage, had unlimited access to water and food. Animal treatments were performed in accordance with the NIH guidelines and also were reviewed and approved by the local IACUC.

Tissue preparation

Mice and rats were deeply anesthetized with Avertin (400 mg/kg i.p.) and pentobarbital (75 mg/kg i.p.), respectively. The animals were perfused transcardially with saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C. Their brains, spinal cords, and DRG were removed, postfixed in 4% PFA, and then cryoprotected in 30% sucrose/PB 0.1 M. The DRG were embedded in OCT, frozen, and cut longitudinally on a cryostat. Consecutive 10-μm-thick sections were thaw-mounted on Superfrost slides (Fisher, Hampton, NH) and stored at –20°C until use. Brain and thoracic spinal cord were cut coronally as 40-μm-thick sections, collected serially, and stored at –20°C in a cryoprotectant solution (glycerol, ethylene glycol, and 0.2 mM PB, pH 7.4, 1:1:2 by volume) until processing.

Antibodies

The rabbit polyclonal anti-DCX (catalog No. ab18723; lot No. 155505; Abcam, Cambridge, MA) was raised against a 16-amino-acid synthetic peptide (sequence: YLPLSLDDSDSLGDSM; manufacturer's technical information), which corresponds to amino acids 387–402 of human DCX, 351–366 of mouse DCX, and 350–365 of rat DCX. According to the manufacturer, when tested by Western blot, this antiserum detects a 40–45-kDa band in mouse brain lysates. The rabbit polyclonal anti-DCX (catalog No. 4604; lot No. 1; Cell Signaling Technology, Beverly, MA) was raised against two synthetic peptides corrisponding to amino acids 48–69 (sequence GHFDERDKTSRNMRGSRMNGLP) and amino acids 380–402 (sequence LRKHKDLYLPLSLDDSDSLGDSM) of human DCX (manufacturer's technical information). According to the manufacturer, on Western blot, this antiserum recognizes one or two bands at ~45 kDa. Guinea pig polyclonal anti-$P2X_3$ (catalog No. GP10108; Neuromics, Northfield, MN) was raised against the peptide VEKQSTDSGAYSIGH corresponding to residues 383–397 of the carboxy-terminus of rat $P2X_3$. This antiserum recognizes a single band of 57 kDa in Western blot (Fabbretti et al., 2006). Guinea pig polyclonal anti-substance P (catalog No. AB5892; Chemicon, Temecula, CA; 1:1,000) was raised against a peptide corresponding to amino acids 1–11 of rat substance P. Only cells with the classic distribution and morphology are stained with this antibody in the adult DRG (Hwang et al., 2005).

Immunohistochemistry

Cryosections from T10, T11, and T12 DRGs were utilized for this study. After rinsing, sections were blocked for 1 hour with 10% normal goat serum in 0.3% Triton X-100 in 0.05 M TBS, pH 7.4, and then incubated in presence of rabbit polyclonal anti-DCX (1:3,000; Abcam) or rabbit polyclonal anti-DCX (1:200; Cell Signaling Technology) overnight at 4°C. On the next day, after several washes, sections were incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (1:600; Molecular Probes, Eugene, OR). For double labelling with isolectin B4 (IB4), sections were incubated with biotinylated IB4 (1:300; Sigma, St. Louis, MO) overnight at 4°C. Visualization was achieved by incubation in Texas red-labelled streptavidin (1:250; Vector Laboratories, Burlingame, CA). Double immunofluorescence was performed by incubating sections overnight at 4°C with rabbit polyclonal anti-DCX Abcam antibody and one of the following antibodies: guinea pig polyclonal anti-$P2X_3$ (1:600) or guinea pig polyclonal anti-substance P (1:1,000). After washes, sections were incubated in a mixture of Alexa 488-conjugated goat anti-rabbit IgG (1:600; Molecular Probes) and Alexa 633-conjugated goat anti-guinea pig IgG (1:600; Molecular Probes). TBS containing 3% normal goat serum and 0.1% Triton X-100 served as antibody diluent. Double-immunofluorescence labelling of thoracic spinal cord was performed on 40-μm free-floating sections as described above, except that the Abcam anti-DCX antibody was utilized at the diluition of 1:1,500. The sections were then mounted on slides and coverslipped with Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark) as antifading agent. To test the specificity of the immunolabelling, sections were incubated with the anti-DCX antibody previously preadsorbed with 30-fold excess immunizing peptide (Abcam; ab19804) overnight at 4°C. Fluorescent signals were detected by confocal scanning laser microscope (Leica TCS-NT; Leica Lasertechnik, Heidelberg, Germany) with a ×20 objective and ×40 PL

Apo oil objective (1.25 NA), using an argon laser (exciting at 488 nm) or a helium-neon laser (exciting at 543–633 nm). Adobe Photoshop CS (Adobe Systems, Inc., San Jose, CA) was used for digital processing of the images. Only light intensity, brightness, and contrast adjustments were applied to improve information. To exclude false-positive signals from overlying cells, pinhole settings that corresponded to an optical thickness of less than 2 μm were used.

Western blot analysis

Spinal cord, hippocampus, lung, and DRG from adult CD1 mice were used to prepare protein extracts. DRG from three mice were dissected with a stereomicroscope (Leica) and pooled. Tissues were homogenized in 50 mM Tris/HCl pH 8, 100 mM NaCl, 1% Nonidet NP-40, 5 mM dithiothreitol (DTT), 5 mM EGTA, 1 mM sodium orthovanadate, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma). Extracts were clarified by centrifugation, and protein concentration was determined by using the Bradford protein assay (Sigma). Proteins were electrophoresed onto 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to nitrocellulose filters, which were blocked with 6% bovine serum albumin and incubated with the rabbit primary antibodies anti-DCX (1:400; Abcam; 1:1,000, Cell Signaling Technology) overnight at 4°C. Peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Bio-Rad, Hercules, CA) was used as secondary antibody. Signal was detected by The SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer's instructions, and bands were captured digitally by using the Molecular Imager ChemiDoc XRS System (Bio-Rad).

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was extracted from DRG, SC, hippocampus, and lung with the SVTotal RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA with ImProm-II Reverse Transcription System (Promega). Forward (5′-CTTTTGGTTCAGCAGAAGGG-3′) and reverse (5′-CAAATGTTCTGGGAGGCACT-3′) primers corresponding to exon 2 of the mouse DCX gene (Santra et al., 2006) were used for the amplification of a 199-bp product. PCR was carried out in a final volume of 50 μl with 20 ng of cDNA template, 0.4 μM of forward and reverse primers (Sigma Aldrich), and 1.25 units of GoTaq Flexi DNA Polymerase (Promega). Initial denaturation for 4 minutes at 94°C was followed by 30 cycles as follows: 30 seconds at 94°C, 30 seconds at 65°C, 30 seconds at 72°C. The final step consisted of 10 minutes of extension at 72°C. PCR product was run onto 1.2% agarose gels and visualized with ethidium bromide with a 50-bp DNA ladder as standard (Sigma). The identity of the DCX RT-PCR product was confirmed by sequencing.

RESULTS

The protein DCX is expressed in the adult mouse DRG

When the Abcam anti-DCX antibody was utilized for immunohistochemistry, immunoreactivity (IR) was present in most neuronal cells in the adult mouse DRG. The highest level of expression was observed in cells morphologically resembling the dark, small to medium-sized type A cells (Lawson, 1992; Lagares and Avendaño, 2000; Fig. 1A,B). Immunoreactivity was mainly in the cytosol, particularly at the periphery of the cell bodies (Fig. 1B), with a pattern overlapping microtubule distribution (Francis et al., 1999; Gleeson et al., 1999). Weakly stained $DCX^+/IB4^-$ medium-sized to large neurons expressing low levels of DCX were also present. In this case, DCX immunoreactivity was either spread all over the cytosol or mainly confined at the soma periphery (Fig. 1B). DCX IR was also present along the fibers projecting centrally to the SC dorsal horns. At this level, DCX staining was apparent as a broad band of axon terminals extending across the entire mediolateral extent of the superficial dorsal horns (Fig. 1C). We then tested a rabbit polyclonal antibody (Cell Signaling Technology) recognizing a different DCX epitope. Again, DRG immunolabelling was localized mainly in small neurons, whereas large neurons were weakly stained (Fig. 1D,E). When tested in the spinal cord, the immunoreactivity pattern was very similar to that obtained with the Abcam antibody (Fig. 1F). Antibody specificity for DCX was further assessed using adult mouse brain sections as positive control. Staining of sections through the subgranular zone of the hippocampal dentate gyrus (Fig. 2A) and the SVZ in the lateral wall of the lateral ventricules (Fig. 2B) produced a pattern of immunoreactivity that was typical of DCX^+ neuroblasts in neurogenic regions. Moreover, DRG and SC immunostaining was abolished when the Abcam antibody was preadsorbed with the corresponding immunizing peptide (Fig. 2C,D).

To confirm further that the DCX protein and its mRNA were present in the adult mouse DRG and SC, Western blotting and RT-PCR experiments were performed by using protein and RNA from adult mouse hippocampus and from lung as positive and negative controls, respectively. A single 199-bp band corresponding to the DCX cDNA amplification product was detected in the hippocampus as well as in the SC and DRG cDNAs but not in lung cDNA or in RT(–) samples (Fig. 2E). When tested in Western blotting, the Abcam DCX antibody recognized two distinct bands in hippocampus protein extracts (Fig. 2F): a single band at 40 kDa, possibly corresponding to the native form of DCX (Des Portes et al., 1998; Gleeson et al., 1998), and one at 43 kDa, which has been suggested to correspond to the phosphorylated form of DCX (Francis et al., 1999; Gleeson et al., 1999). Bands of 40 and 43 kDa were present also in DRG and SC extracts, whereas no bands were detected in lung protein extracts (Fig. 2F). Moreover, the intensity of both bands detected in hippocampus and DRG extracts was strongly diminished when the primary antibody was preincubated with the immunizing peptide (Fig. 2F).

Phenotypic characterization of DCX^+ cells in the adult mouse DRG

To characterize further the novel DCX-positive cell population, we performed double-labelling experiments with markers of DRG neuronal subpopulations. Small neurons can be classified mainly into two populations, identified by the presence or absence of neuropeptides: the nonpeptidergic $P2X_3^+$ and isolectin $IB4^+$ neurons and the peptidergic calcitonin gene-related peptide (CGRP)- and sub-

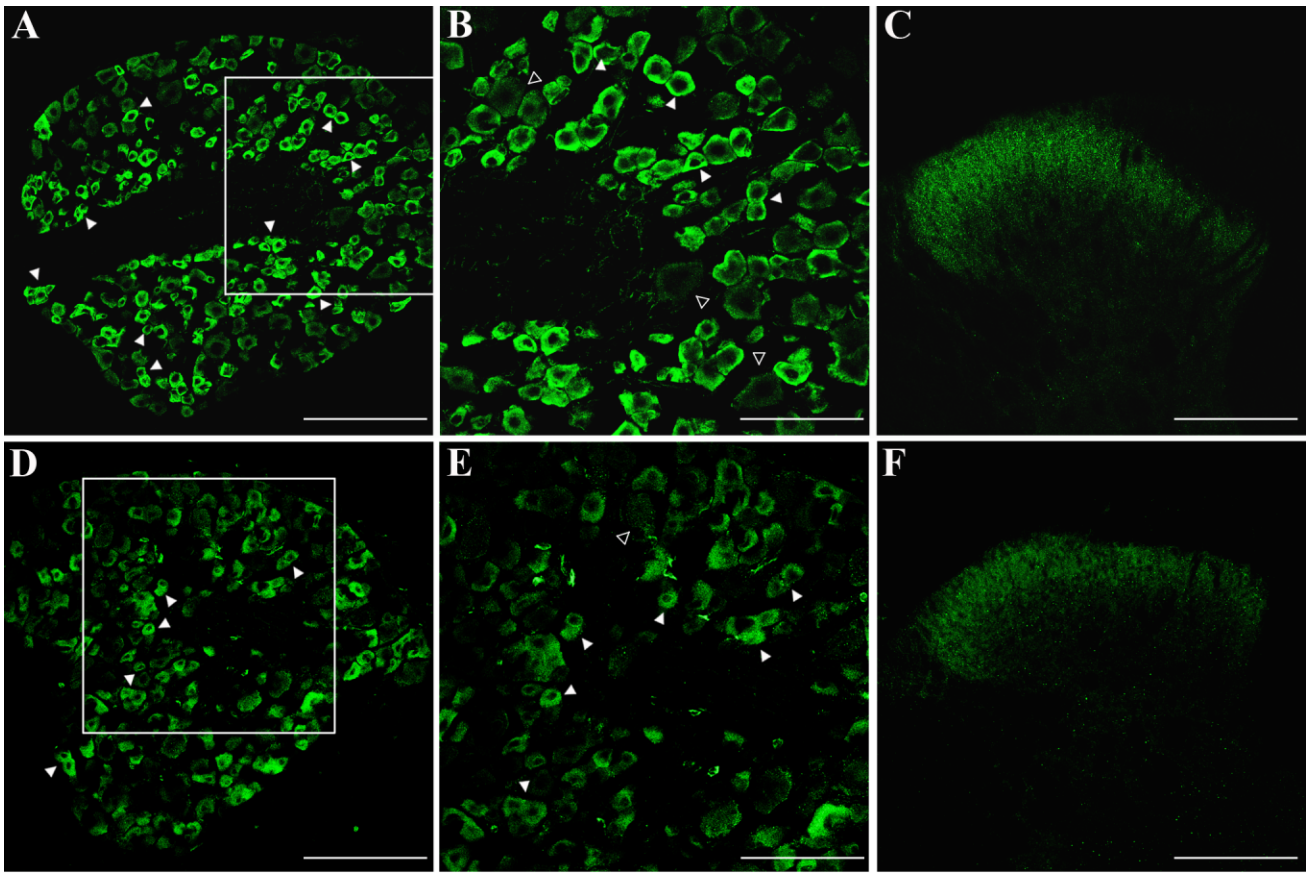

Fig. 1. Doublecortin (DCX) immunoreactivity in adult mouse dorsal root ganglia (DRG) and spinal cord (SC) dorsal horns. Representative immunofluorescent photomicrographs of DRG (A,B,D,E) and SC dorsal horn (C,F) sections from adult mice stained with two antibodies recognizing different epitopes of DCX (Abcam No. ab18723: A–C; Cell Signaling No. 4604: D–F). **A,D:** DCX immunoreactivity is strongly expressed in a subpopulation of small to medium-sized DRG neurons (solid arrowheads). **B,E:** High-power images of the boxed areas in A and D, respectively. Solid arrowheads indicate small to medium-sized DRG neurons stained with DCX antibodies. Large neurons (open arrowheads) are weakly stained. **C,F:** DCX immunoreactivity in the entire mediolateral extension of the superficial dorsal horns. Scale bars = 150 μm in A,C,D,F; 75 μm in B,E.

stance P (SP)-immunoreactive population. Confocal microscopy analysis of DRG sections doubly stained for DCX and $P2X_3$ showed that all $P2X_3$-positive neurons coexpressed DCX (Fig. 3A–C). We also verified whether DCX was expressed in $IB4^+$ cells. IB4 staining identifies a wider number of small nonpeptidergic neurons, comprehensive of the $P2X_3$-expressing population (Bradbury et al., 1998; Vulchanova et al., 1998). Although most IB4-stained neurons were DCX immunoreactive, two populations of cells could be identified in the small DCX^+ cells population: a population of $DCX^+/IB4^+$ neurons and a population of $DCX^+/IB4^-$ cells (Fig. 3D–F). We hypothesized that the small DCX^+ neurons that were negative for $P2X_3$ and IB4 may belong to the subpopulation of peptidergic neurons. To evaluate this possibility, we performed double-immunofluorescence experiments for DCX and SP. Confocal microscopy analysis of DRG sections showed that SP-expressing neurons were also immunolabelled for DCX (Fig. 3G–I). Altogether, these data indicate that DCX is expressed by both the peptidergic and the nonpeptidergic subpopulations of nociceptors of adult mouse DRG, confirming our initial morphological observations.

The DRG fibers centrally projecting to the SC showed the same pattern of colocalization observed in the DRG cell somata, with $IB4^+$ axons also being DCX immunoreactive (Fig. 4A–C). In the dorsal horn, DCX IR was present in IB4-labelled central terminals of lamina II (Fig. 4D–F). Nonetheless, the projection area of DCX-positive fibers in the dorsal horns had a wider extension compared with IB4 labelling, possibly representing the axonal terminals of the $DCX^+/IB4^-$ medium-sized to large DRG neurons.

The DCX protein is expressed in DRG and SC dorsal horns of the adult rat

We have characterized the DCX-positive population in adult rat DRG and confirmed that also in this species most DRG neurons were DCX^+, although staining was generally less intense than in the mouse. DCX immunoreactivity was seen mainly in neuronal cell bodies and in scattered fibers. In most cells, staining was widely distributed throughout the cytosol, although a minor cell population showed labelling restricted to part of the cytosol (Fig. 5A). As observed in the mouse, rat DCX immunoreactivity was present mainly in small to medium-sized type A cells (Fig.

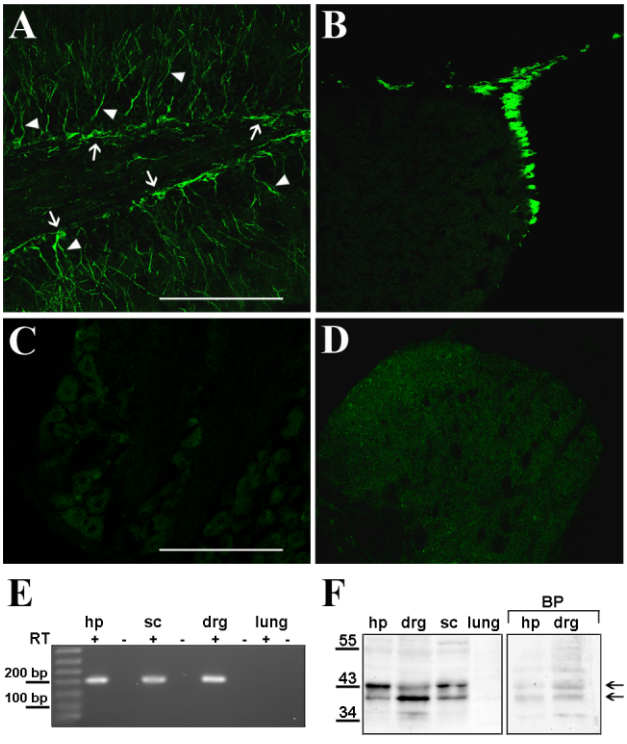

Fig. 2. Doublecortin (DCX) expression in adult mouse dorsal root ganglia (DRG) and spinal cord (SC) is confirmed by immunohistochemistry, by reverse transcription-polymerase chain reaction (RT-PCR), and by Western blot analysis. **A,B:** The specificity of the Abcam anti-DCX was confirmed by immunohistochemical analysis on adult mouse brain sections. A: Immunodecoration of cell bodies located in the subgranular zone of dentate gyrus (arrows) and their dendritic arborization (arrowheads) extending throughout the granular cell layer clearly identifies DCX^+ immature neurons in the well-characterized neurogenic area. B: The Abcam antibody recognizes typical chains of immature neuroblasts in the subventricular zone. **C,D:** Neutralization of Abcam antibody. Incubation of the primary antibody with the blocking peptide results in a complete loss of staining both in DRG (C) and in SC (D) sections. **E:** Primers designed for detection of mouse DCX transcript were used to evaluate DCX gene expression in the adult DRG and SC by RT-PCR. RNA was prepared from adult mouse DRG, SC, and from hippocampus (hp) and lung as positive and negative controls, respectively. For each preparation, 400 ng of RNA was either reverse transcribed [RT(+) samples] or incubated under the same conditions but in absence of the reverse transcriptase enzyme [RT(–) samples]. An amplicon of the expected size (199 bp) was obtained by PCR on RT(+) samples of hp, DRG, and SC and not from lung cDNA or from RT(–) samples. The 50-bp DNA ladder was utilized as molecular weight standard. **F:** Western blot analysis on tissue homogenates from adult mouse hp, DRG, SC, and lung with the Abcam antibody. Electrophoresis was performed by using 60 μg of protein extracts from DRG and SC and 20 μg from hp and lung. The antibody recognizes a lower molecular weight band at 40 kDa and one at 43 kDa. Bands of corresponding molecular weight are present in the DRG and SC protein extracts but absent in lung extracts. Incubation of the primary antibody with the corresponding blocking peptide (BP) results in a loss of signal intensity for both bands in hp and DRG protein extracts. Scale bars = 75 μm in A (applies to A,B); 75 μm in C (applies to C,D).

5A). Confocal microscopic analysis of DCX and $P2X_3$ double-stained sections revealed a partial colocalization of the two proteins in the adult rat, with a subpopulation of $P2X_3$ neurons coexpressing DCX (Fig. 5A–C). Similar results were obtained with IB4 labelling as a marker of small neurons (Fig. 5D–F). Confocal microscopic analysis of DRG sections doubly stained for DCX and SP demonstrated that SP^+ neurons coexpressed DCX (Fig. 5G–I). We concluded that, as in the mouse, also in the rat DCX is expressed in both nonpeptidergic and peptidergic small to medium-sized neurons. We also found a similar pattern of colocalization at the level of the fibers centrally projecting to the SC dorsal horns (Fig. 6A–C). Some IB4-labelled fibers expressed DCX, whereas others were DCX^- (Fig. 6A–C). In the SC dorsal horns, DCX immunoreactivity colocalized with SP in lamina I and in the outer portion of lamina II (Vulchanova et al., 1998) but also extended to the inner part of lamina II (Fig. 6G–I). Colocalization studies with isolectin IB4 showed a complete overlapping of DCX immunoreactivity with IB4 signal, whose labelling identifies lamina II (Vulchanova et al., 1998). Altogether, our results indicate that, in the rat, DCX is expressed in the cells bodies and in the axon terminals of peptidergic and nonpeptidergic nociceptors projecting to laminae I and II of the SC dorsal horns.

DISCUSSION

Here we describe for the first time the presence of the protein DCX in the adult DRG sensory neurons from both mouse and rat. The protein is expressed mainly by small to medium-sized neurons belonging to the peptidergic, SP^+, and nonpeptidergic $P2X_3^+$/IB4-labelled populations.

This novel finding was quite unexpected, insofar as DCX is commonly regarded as a developmentally regulated protein, expressed in immature neurons of both CNS and peripheral nervous system (PNS; Francis et al., 1999; Gleeson et al., 1999) and as an early marker of newly generated neuroblasts in neurogenic areas of the adult brain (Cameron et al., 1993; Doetsch et al., 1997; Seri et al., 2001). In both situations, DCX is attributed mainly a regulatory function in cell migration, based on its role in microtubule reorganization. Now, the presence of DCX in DRG neurons suggests novel functions for this protein in the adult nervous system.

DCX and plasticity

Microtubule reorganization is widely implicated in neuroblast migration, but it may also subserve plasticity events such as synaptogenesis and axonal outgrowth (Lankford et al., 1990; Gordon-Weeks, 1991). DRG are areas of high plasticity in the nervous system, as suggested by their capacity to regenerate sensory fibers in response to injury (Lozeron et al., 2004). In addition, although peripheral nerve lesions such as nerve crush can induce DRG neuronal loss within 2–3 months from injury, at later times the number of DRG neurons is fully restored (Groves et al., 1997, 2003). One possible explanation for DCX being expressed in adult DRG neurons is that the protein may be readily available to contribute, through microtubule reorganization, to plasticity events. Nacher et al. (2001) first described DCX^+ neurons with a mature phenotype in the rat piriform cortex, a cerebral region involved in ongoing structural plasticity events (for review see Brosh and Barkai, 2004; Barkai, 2005) but where neurogenesis has yet to be demonstrated. More recently, the hypothalamic suprachiasmatic nucleus (SCN), an area involved in the control of circadian rhythms, has been identified as an additional CNS region where DCX is expressed through adulthood (Geoghegan and Carter,

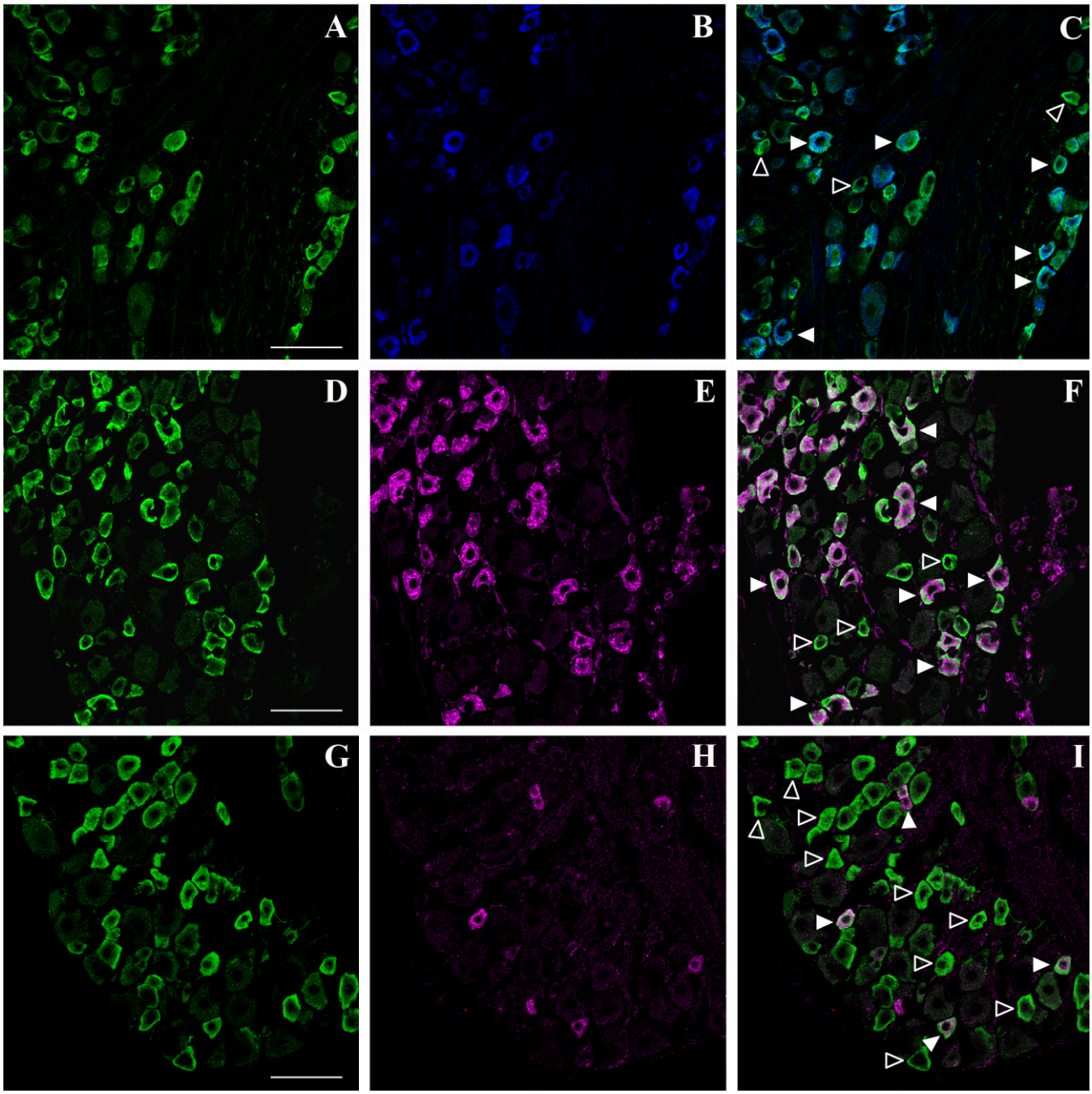

Fig. 3. Phenotypic characterization of doublecortin (DCX)-expressing cells in the adult mouse dorsal root ganglia (DRG) by confocal microscopic analysis. **A,D,G:** DCX (green) is expressed in a subpopulation of DRG neurons morphologically resembling small to medium-sized nociceptors. **B:** $P2X_3$ immunoreactivity (blue, pseudocolor) in small nociceptive neurons. **C:** Overlay of the two channels demonstrates that most $P2X_3^+$ neurons are DCX immunoreactive (solid arrowheads). Open arrowheads indicate $DCX^+/P2X_3^-$ neurons. **E:** IB4-labelled (magenta, pseudocolor) small nociceptive neurons. **F:** Overlay of the two channels shows that IB4-stained neurons are DCX immunoreactive (solid arrowheads). A subpopulation of small DCX^+ neurons is not labelled with IB4 (open arrowheads). **H:** Substance P (SP) immunoreactivity (magenta, pseudocolor) is expressed by a subpopulation of small peptidergic neurons. **I:** Overlay of the two channels shows that most SP^+ neurons are also DCX immunoreactive (solid arrowheads), but many small DCX^+ neurons do not express SP (open arrowheads). Scale bar = 75 μm in A (applies to A–C); 75 μm in D (applies to D–F); 75 μm in G (applies to G–I).

2007). In line with what we observed in the DRG, DCX is expressed by mature, neuropeptide-containing SCN neurons. Both in the piriform cortex and in the SCN, the DCX immunoreactivity pattern is similar to that of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), which is expressed by mature neurons after damage (Rutishauser and Landmesser, 1996), suggesting a common functional role for the two proteins. We also evaluated the presence of PSA-$NCAM^+$ cells in adult DRG, but for this location, unlike the case for the piriform cortex

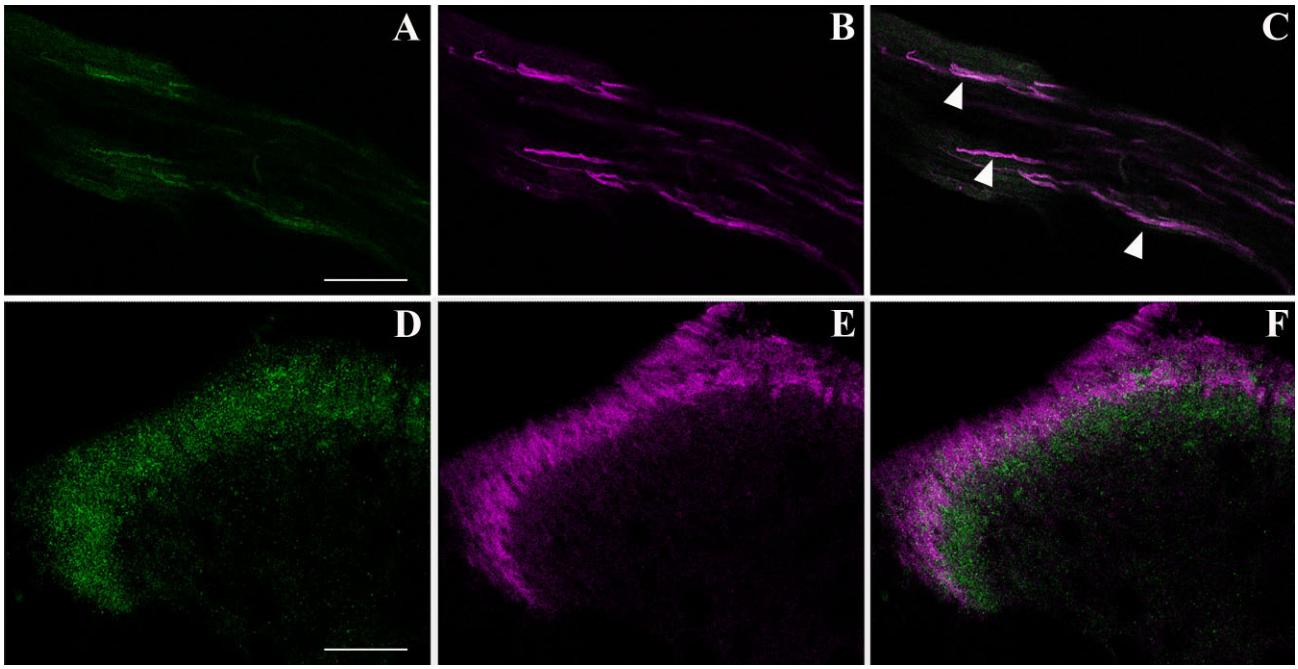

Fig. 4. Phenotypic characterization of doublecortin (DCX)-positive fibers centrally projecting to the mouse spinal cord (SC) dorsal horns by confocal microscopic analysis. **A:** Some nerve fibers extending centrally from the DRG neurons to the SC are DCX immunoreactive (green). **B:** IB4-labelled fibers (magenta, pseudocolor). **C:** DCX colocalizes with IB4 at the fiber level (arrowheads). **D–F:** Confocal microscopic analysis of the mouse SC dorsal horns doubly stained for DCX (green) and IB4 (magenta, pseudocolor). **D:** DCX immunoreactivity is present at the level of the superficial dorsal horns. **E:** IB4 labelling of dorsal horns, the target region of DRG nociceptors. **F:** Overlay of the two channels shows the colocalization of DCX^+ and IB4-stained terminals of DRG nociceptive neurons at the SC level. DCX expression extends to a larger dorsal horn area compared with IB4 staining. Scale bars = 75 μm in A (applies to A–C); 75 μm in D (applies to D–F).

and the SCN, we could not detect any specific signal for the adhesion molecule (data not shown). DCX^+ cells in the piriform cortex and in the SCN coexpress NeuN (neuron-specific nuclear protein), a widely accepted marker of fully differentiated neurons (Mullen et al., 1992). The majority of the DCX^+ neurons in DRG were also expressing NeuN (data not shown), confirming that they share features of mature neurons.

DCX and neuroprotection

A recent finding suggests a direct neuroprotective role for DCX protein (Santra et al., 2006). In vitro, DCX overexpression protects rat SVZ and human glioma cells from oxygen and glucose deprivation, whereas knock down of DCX expression by small interfering RNA increases their vulnerability to damaging insults. Small nociceptors represent a highly vulnerable cellular subpopulation, which rapidly degenerates in response to specific damaging conditions (Lisney, 1989; Tandrup et al., 2000; Lozeron et al., 2004; Guseva and Chelyshev, 2006). The presence of DCX in DRG may possibly underlie a potential protective role against insults, which allows neuronal cells to recover from damage. In this regard, it will be interesting to evaluate the consequences of different damaging conditions for DRG neuronal subpopulations after DCX knock down.

DCX in sensory ganglia and neurogenesis

Although it is still a controversial matter, for decades several groups have collected data suggesting that, in different animal species, DRG may undergo an age-dependent increase in neuron number, leading to the proposal that neurogenesis may occur in this tissue after birth (Devor and Govrin-Lippman, 1985; Devor et al., 1991; Cecchini et al., 1995; Popken and Farel, 1997; Ciaroni et al., 2000; Farel, 2002). Recently, the production of neurospheres that can differentiate into neurons and glia has been demonstrated in vitro from adult rat DRG and trigeminal ganglia explants (Namaka et al., 2001; Lagares et al., 2007; Li et al., 2007), although DRG neurogenesis in vivo awaits confirmation (La Forte et al., 1991; Ciaroni et al., 2000; Farel, 2002). An alternative hypothesis to explain the possible addition of new DRG neurons after birth has been proposed, namely, the ongoing maturation of preexisting postmitotic immature cells to fully differentiated neurons both under physiological conditions and in response to external insults (La Forte et al., 1991; Cecchini et al., 1993; Ciaroni et al., 2000; Farel, 2002, 2003). At this stage, we cannot exclude that the presence of DCX in DRG may indicate ongoing neurogenesis in this location, although the fact that most of the cells express the protein would argue against this possibility. Additionally, we never observed $BrdU^+$ cells in DRG collected from rats that were administered the thymidine analogue (data not shown). In a recent work, Lagares et al. (2007) described the presence of few, sparse DCX^+ cells in the rat trigeminal ganglia (TG), an area of the PNS that shares, at least in part, a common origin with DRG (D'Amico-Martel and Noden, 1983; Fontaine-Perus et al., 1985). Unlike TG, where very few DCX-immunolabelled cells could be ob-

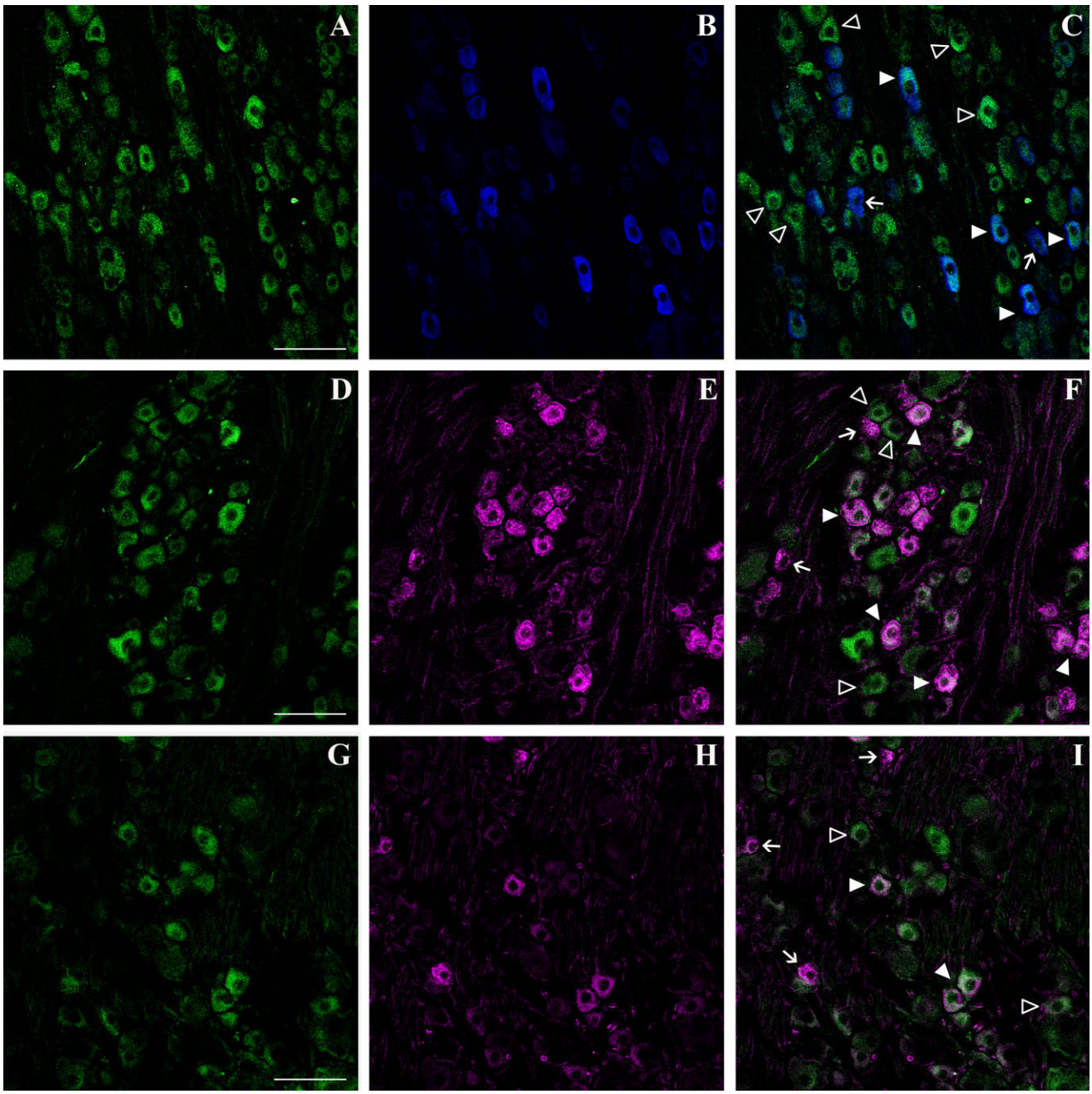

Fig. 5. Phenotypic characterization of doublecortin (DCX)-expressing cells in the adult rat dorsal root ganglia (DRG). A–I: Colocalization of DCX with $P2X_3$ (A–C), IB4 (D–F), and substance P (SP; G–I) analyzed by confocal microscopy. **A,D,G:** DCX (green) immunoreactivity in DRG neurons. Most small to medium-sized neurons are strongly stained. **B:** $P2X_3$ immunoreactivity (blue, pseudocolor) in small DRG nociceptive neurons. **C:** Overlay of the two channels shows a partial colocalization between $P2X_3$- and DCX-immunoreactive neurons (solid arrowheads). Open arrowheads indicate $DCX^+/P2X_3^-$ neurons. Arrows indicate $P2X_3^+$ neurons that do not express DCX. **E:** IB4-labelled (magenta, pseudocolor) small nociceptive DRG neurons. **F:** Overlay of the two channels shows that a subpopulation of small IB4-labelled neurons coexpresses DCX (solid arrowheads). Arrows indicate IB4-labelled neurons that do not express DCX. Small DCX^+ neurons that are not labelled with IB4 are also present (open arrowheads). **H:** SP immunoreactivity (magenta, pseudocolor) in small DRG neurons. **I:** Overlay of the two channels shows SP^+/DCX^+ cells (solid arrowheads). Arrows indicate SP^+ cells that do not express DCX, whereas DCX^+/SP^- neurons are identified by open arrowheads. Scale bars = 75 μm in A (applies to A–C); 75 μm in D (applies to D–F); 75 μm in G (applies to G–I).

served, in DRG most of the neuronal cells express DCX. Additionally, in rat TG, DCX^+ cells apparently do not coexpress protein markers of mature neurons, such as the neuron-specific enolase (NSE; Vega et al., 1990). We did not evaluate NSE immunoreactivity in DRG, but, as previously stated, most DCX^+ cells colocalized with the mature neuronal marker NeuN. Some Nissl-stained cells morphologically resembling mature sensory neurons did

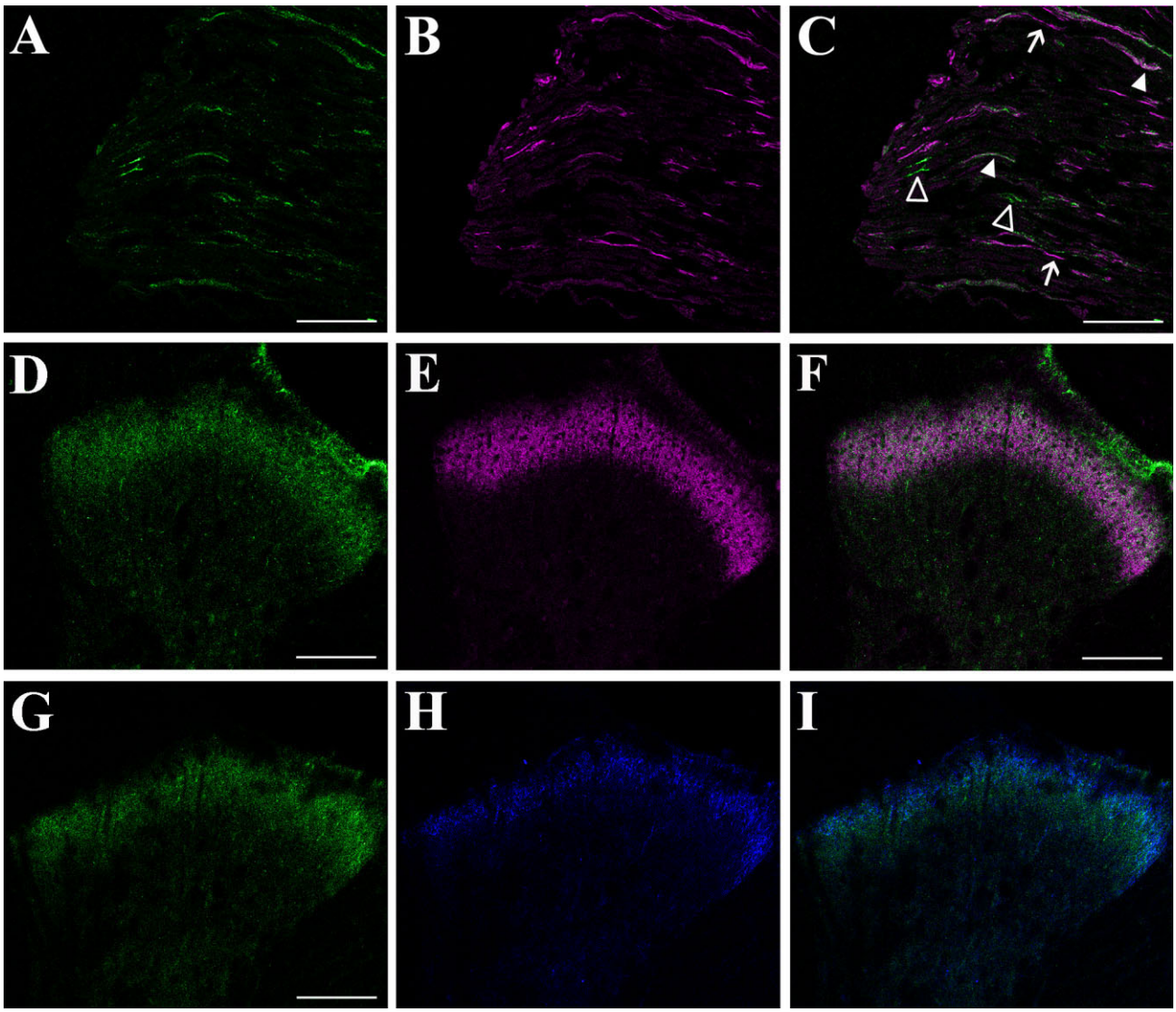

Fig. 6. Phenotypic characterization of doublecortin (DCX)-positive fibers centrally projecting to the rat spinal cord (SC) dorsal horns by confocal microscopic analysis. **A:** Some nerve fibers extending centrally from the DRG neurons to the SC are DCX immunoreactive (green). **B:** IB4-labelled fibers (magenta, pseudocolor). **C:** DCX colocalizes with IB4 in some (solid arrowheads) but not all (open arrowheads) fibers. Arrows identify IB4-labelled fibers that do not express DCX. **D–I:** Confocal analysis of the mouse SC dorsal horns doubly stained for DCX and IB4 (D–F) and for DCX and substance P (SP; G–I). D,G: DCX immunoreactivity is present in the superficial layers of dorsal horns. **E:** IB4 labelling of lamina II in the dorsal horns, the target region of DRG nociceptors. **F:** Overlay of the two channels shows the colocalization of DCX^+- and IB4-stained terminals of DRG nociceptive neurons at the SC level. **H:** SP immunoreactivity in lamina I and the dorsal part of lamina II in the SC dorsal horns (blue, pseudocolor). **I:** DCX^+ axons terminals colocalize with SP immunoreactivity in the SC. DCX expression extends to a larger area in the inner laminae of dorsal horns compared with SP, probably representing the IB4-labelled lamina II. Scale bars = 150 μm in A (applies to A–C); 150 in D (applies to D–F); 150 μm in G (applies to G–I).

not express NeuN, and, as a consequence, few DCX^+/$NeuN^-$ cells were also present in adult DRG (data not shown). The absence of NeuN does not necessarily imply a less well-differentiated neuronal phenotype, insofar as $NeuN^-$ neurons have been described, among them Purkinje cells, olfactory bulb mitral cells, retinal photoreceptor cells (Mullen et al., 1992), and many neurons of the dorsal SCN (Geoghegan and Carter, 2007). However, at this stage, we cannot exclude that DCX^+/$NeuN^+$ and DCX^+/$NeuN^-$ cells may represent functionally different cell populations. Whether or not DCX expression in DRG underlies neurogenesis occurring in this location, the role of DCX as a selective marker of new neurons generated in the adult brain requires reconsideration. As described by Kempermann et al. (2003), some of the DCX-expressing cells in the dentate gyrus coexpress nestin, which is regarded as a stem/precursor cell marker (Lendahl et al., 1990; Reynolds et al., 1992). Moreover Walker et al. (2007) showed that DCX^+ cells have different proliferating and differentiating properties, which correlate with protein expression levels, highly expressing cells being committed to the neuronal lineage and low-expressing ones being

undifferentiated precursors able to proliferate and give rise to neurospheres. Altogether, these data strongly suggest that DCX^+ cells may represent a highly heterogenous cell population.

In conclusion, our data strongly suggest a novel role for DCX in DRG neurons. Additional experimental work must be performed to unravel fully the functional significance of the DCX protein in adult nervous tissues and, in particular, in sensory neurons.

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