The boundary cap: a source of neural crest stem cells that generate multiple sensory neuron subtypes

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Development (vertical sidebar text)

Summary

The boundary cap (BC) is a transient neural crest-derived group of cells located at the dorsal root entry zone (DREZ) that have been shown to differentiate into sensory neurons and glia in vivo. We find that when placed in culture, BC cells self-renew, show multipotency in clonal cultures and express neural crest stem cell (NCSCs) markers. Unlike sciatic nerve NCSCs, the BC-NCSC (bNCSCs) generates sensory neurons upon differentiation. The bNCSCs constitute a common source of cells for functionally diverse types of neurons, as a single bNCSC can give rise to several types of nociceptive and thermoreceptive sensory neurons. Our data suggests that BC cells comprise a source of multipotent sensory specified stem cells that persist throughout embryogenesis.

Key words: Mouse, Peripheral nervous system, Migration, Fkh3, Foxs1

Introduction

There is a marked diversity of neurons and glia that develop in the mammalian nervous system. The development of different cell types is accompanied by lineage restriction of multipotent cells. The neural crest is a transient population of cells that migrates from the dorsal neural tube during development and generates the majority of the neurons and glia of the peripheral nervous system. The thoracolumbar region of the trunk neural crest gives rise to cells of the sensory dorsal root ganglion (Müller and Ingvar, 1923; Pannese, 1974) and the autonomic sympathetic neurons (Le Douarin, 1980). The dorsal root ganglion itself shows further cellular diversity, with many different types of sensory neurons that can be subdivided into different subclasses regarding function, size and receptor expression (Snider, 1994). The different subclasses of dorsal root ganglion neurons are born at different developmental stages with the large diameter mechanoreceptive neurons being born first, showing a peak of birth in the cervical region by E10.5 in the mouse, while the birth of small diameter nociceptive neurons peaks at E11.5 (Lawson and Biscoe, 1979).

More recently, late emigrating trunk neural crest was shown to give rise to the BC cells of the dorsal root entry zone (DREZ) and motor exit points, which appear morphologically in the rat at E13 and in the mouse at E10.5 (Topilko et al., 1994). Unlike dorsal root ganglion progenitor cells that cease proliferation around E12, at which time the programmed cell death commences in the mouse (Lawson and Biscoe, 1979; Pinon et al., 1996), the boundary cap cells proliferate throughout embryogenesis (Altman and Bayer, 1984). Despite continuous

proliferation of these cells, the size of the BC decreases from E17 onwards. Its disappearance at early postnatal stages appears not to be correlated with increased apoptosis (Altman and Bayer, 1984; Golding and Cohen, 1997). The BC cells express monoamine oxidase B (Maob) (Vitalis et al., 2003), the zinc-finger transcription factor Krox20 (Egr2 – Mouse Genome Informatics) (Wilkinson et al., 1989) and the TrkB neurotrophin receptor (Ernfors et al., 1992). In the chick, the late emigrating cranial neural crest cells expressing Cad7 and Krox20 localise specifically to the cranial motor nerve exit points (Niederlander and Lumsden, 1996) and maintain a boundary that is permissible for axons to grow through, but prevents neuronal migration (Vermeren et al., 2003). The BC is important for sensory afferent ingrowth at the DREZ (Golding and Cohen, 1997). Recently, genetic tracing of boundary cap cells using the Krox20 locus has revealed that some trunk sensory neurons are derived from the boundary cap, and that the boundary cap cells also contribute with satellite cells and Schwann cells to the peripheral nervous system (Maro et al., 2004).

The finding that the BC contributes with glial and neuronal cells to the peripheral nervous system suggests that they could be multipotent stem cells. Self-renewing NCSCs isolated from the migrating neural crest or tissues derived from it such as the sciatic nerve and the enteric nervous system in the gut share some common traits with neural crest cells (NCCs), as the ability to form not only neurons but also smooth muscle cells and glia (Anderson, 1997; Bixby et al., 2002; Morrison et al., 1999; Rao and Anderson, 1997). However, it is intriguing that these NCSCs differentiate only into autonomic neurons and

have never been observed to spontaneously differentiate into sensory neurons in vitro or after transplantation in vivo (Anderson, 2000; Morrison et al., 1999; White et al., 2001). Both sensory and autonomic neurons can be generated in cultures of neural tube explants, in which sensory precursor cells constitute about 1% of the cells and divide only during the first 2 days of culture (Greenwood et al., 1999). Application of Wnt1 has been shown to direct these cells towards a sensory lineage, as shown by the expression of the transcription factor Brn3a, a marker for sensory precursor cells (Lee et al., 2004).

In this study, we identify the BC as a source of NCSCs with unique characteristics. We show that the bNCSCs express markers similar to previously isolated NCSCs, are multipotent by forming neurons, glia and smooth muscle-like cells and are able to self-renew but when differentiated spontaneously generate functional sensory neurons of several subclasses.

Materials and methods

Cell culture

Dorsal root ganglia from E11 mice were dissected and dissociated by mechano-enzymatic dissociation using Collagenase/Dispase (1 mg/ml, Roche) and DNase (0.5 mg/ml, Sigma) at room temperature for 30 minutes. The cells were plated in propagation medium: N2 medium containing bFGF and EGF (20 ng/ml, RnD Systems), and B27 supplement (Gibco). 12 hours after plating, all media, together with the high amount of non-attached cells were removed and new media added. For differentiation, the cells were plated on poly-D-lysine (PDL) (50 µg/ml, Sigma) and laminin (20 mg/ml, Invitrogen) for 5 days in differentiation medium: N2 medium containing BDNF, NGF, GDNF, NT3 (10 ng/ml, Promega) and retinoic acid (100 nM, Sigma). In the autonomic induction experiment, 50 ng/ml bone morphogenic protein 2 (BMP2, RnD systems) was added to the differentiation medium. For the BC micro-dissection, the dorsal root ganglion including the dorsal root was divided into two parts (boundary cap and central part according to Fig. 2H). Cultures were established from the boundary cap and central parts separately as described above, clones were counted at the indicated time points. For enrichment experiment cells were either plated in propagation medium onto PDL for 2 hours or onto plastic overnight (10-12 hours) in propagation medium containing cytosine arabinoside (Ara-C, 1 µg/ml, Sigma) or Actinomycin D (Actino D, 10 µg/ml, Sigma).

Generation of *Fkh3*lac-z /+ knock in mice

A 10 kb fragment from the *Fkh3* locus was isolated and an *nls-lacZ* cassette also containing a neomycin-resistance gene, driven by the PGK promoter and polyA was put in frame 18 amino acids downstream of *Fkh3* start codon, replacing a 600 bp part of the *Fkh3* gene. Homologous recombined embryonic stem cell clones were injected into C57/Bl6 blastocysts to generate chimeric mice. After germline transmission, the mice were on a 129SV×C57/Bl6 background.

Immunocytochemistry

Immunofluorescence analyses were performed using a variety of antibodies against neuropeptides, transcription factors, receptors and neuronal filaments (see below).

Cells were fixed in 4% PFA (Merck) at room temperature for 30 minutes, permeabilised and blocked using PBS with 0.1% Triton X-100 (Merck) [or 0.1% Tween-20 (Merck) for filament staining] and 3% BSA (Sigma) or 5% serum (Chemicon) from the same species in which the secondary antibody was generated. The cells were incubated with primary antibody overnight at 4°C. Species and isotype-specific fluorescent antibodies (donkey Cy2- or Cy3-conjugated anti-rabbit, mouse, chick or guinea pig, 1:200, Jackson)

were applied for 1 hour at room temperature. The sections were rinsed four times in PBS for 15 minutes, the second wash including Hoechst 33342 (11 ng/ml, Molecular Probes). Pictures were taken using a Zeiss Axiovert 100M or Zeiss LSM 510 confocal microscope. Antibodies were as follows: βIII-Tubulin (Tuj1, mouse, 1:250, Promega), Brn3a (mouse, 1:200, Covance), calcitonin gene-related peptide (Cgrp; Calca – Mouse Genome Informatics; guinea pig, 1:200, Peninsula Labs), choline acetyltransferase (ChAT, goat, 1:100, Chemicon), glial fibrillary acidic protein (Gfap, rabbit, 1:400, DAKO), GDNF family receptor Ret (goat, 10 µg/ml, RnD systems), low affinity neurotrophin receptor p75 (p75NTR, mouse, 1:200, Chemicon), monoamine oxidase B (Maob, rabbit, 1:1000, a gift from Olivier Cases), Nestin (mouse, 1:500, Hybridomabank), peripherin (Per; Prph1 – Mouse Genome Informatics; rabbit, 1:500, Chemicon), smooth muscle actin (Sma, mouse, 1:400, Sigma) and tyrosine hydroxylase (Th, rabbit, 1:5000, Diasorin).

RT-PCR

Total RNA from 20 stem cell clones, dorsal root ganglia, boundary cap and central part of dorsal root ganglia from E11 mouse embryo were extracted using Absolutely RNA™ Nanoprep kit (Stratagene) following manufacturer's instruction. Reverse transcription (RT) was carried out 10 minutes at 65°C followed by 1 hour at 37°C and 15 minutes at 70°C in 20 µl reactions containing 0.5 mM dNTP each, 10 mM DTT, 0.5 µg oligod(T)$_{15}$ (Promega) and 200 U of M-MLV-RT (Gibco BRL). For stem cell clones, total RNA extract was reverse transcribed. For dorsal root ganglia, boundary cap and central part, 500 ng of total RNA were reverse transcribed. The amount of template was adjusted to equal quantity between samples based on the level of HPRT. PCR was conducted in 25 µl reactions containing 10% RT product, 2 µM each dNTP, 10 pmol of each primer (MWG-Biotech AG), 3 mM MgCl$_2$ and 0.625 U of Ampli Taq Gold™ (Roche Molecular Systems). cDNA was denatured 10 minutes at 95°C and amplified for 35 cycles in a two step program as following: 30 seconds at 95°C and then 30 seconds annealing and polymerisation (55°C to 65°C depending on the primers). PCR products were separated on 2% agarose gels containing ethidium bromide. Primers were as follows: Ret (5′-CTTGGCAGAAATGAAGCTTGTACA-3′ and 5′-GTCC-CTCAGCCACCAAGATGT-3′; nucleotides 2595 to 2618 and 2639 to 2659; 64 bp); Brn3a (Pou4f1 ñ Mouse Genome Informatics) (5′-AG-GCCTATTTTGCCGTACAACC-3′ and 5′-CTCCTCAGTAAGTG-GCAGAGAATTTCAT-3′; nucleotides 1956 to 1977 and 2081 to 2108; 152 bp); Ngn1 (Neurog1 – Mouse Genome Informatics) (5′-CCCTCGGCTTCAGAAGACTTCA-3′ and 5′-CGTCGTGTG-GAGCAGGTCTTT-3′; nucleotides 625 to 646 and 691 to 711; 86 bp), Ngn2 (Neurog2 ñ Mouse Genome Informatics) (5′-TCCAACTC-CACGTCCCCATACC-3′ and 5′-GCTGCCAGTAGTCCACGTCT-GA-3′; nucleotides 658 to 678 and 709 to 730; 72 bp); Krox20 (5′-TG-GATGCCAGTTGTTCTGAGACTT-3′ and 5′-GCTGTCCTCGAT-CAAAGGAATCA-3′; nucleotides 1972 to 1995 and 2020 to 2042; 70 bp), Otx1 (5′-GCAGCCTCCTACCCTATGTCCTAT-3′ and 5′-TGCAGTCTACACCGCCAAAGTA-3′; nucleotides 652 to 675 and 734 to 745; 93 bp), Pax2 (5′-AGTCTTTGAGCGTCCTTCCTATCC-3′ and 5′-CATTCCCCTGTTCTGATTTGATGT-3′; nucleotides 795 to 818 and 842 to 865; 70 bp), Pax5 (5′-AACAGGATCATTCGGA-CAAAAGTA-3′ and 5′-AGCCTGTAGACACTATGCTGTGA-3′; nucleotides 438 to 461 and 497 to 519; 81 bp).

In situ hybridisation

Embryos were collected at different stages, fixed overnight (4% PFA, in PBS), washed in PBS, cryopreserved (30% sucrose in PBS), embedded in OCT and sectioned at 14 µm. Before hybridisation, slides were air dried for 2-3 hours at room temperature. Full-length mouse Krox20 was used to synthesise digoxigenin-labelled antisense riboprobe according to supplier's instructions (Roche) and purified by LiCl precipitation. Sections were hybridised overnight at 70°C with a solution containing 0.19 M NaCl, 10 mM Tris (pH 7.2), 5 mM

$NaH_2PO_4*2H_2O/Na_2HPO4$ (pH 6.8), 50 mM EDTA, 50% formamide, 10% dextran sulphate, 1 mg/ml yeast tRNA, $1\times$Denhardt solution and 100 to 200 ng/ml of probe. Sections were then washed for times for 20 minutes at 65°C in $0.4\times$SSC pH 7.5, 50% formamide, 0.1% Tween 20 and three times for 20 minutes at room temperature in 0.1 M maleic acid, 0.15 M NaCl and 0.1% Tween 20 (pH 7.5). Sections were blocked 1 hour at room temperature in presence of 20% goat serum and 2% blocking agent (Roche) prior to incubation overnight with AP-conjugated anti-DIG-Fab-fragments (Roche, 1:2000). After extensive washing, hybridised riboprobes were revealed by performing a NBT/BCIP reaction in 0.1 M Tris HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ and 0.1% Tween 20.

X-Gal staining

Embryos or cells were fixed in Webster solution for 45 minutes on ice and then stained in coloration solution [3.1 mM $FeK_3(CN)_6$, 3.1 mM $FeK_4(CN)_6$ and 0.4 mg/ml X-gal in phosphate buffer] overnight at 37°C. Embryos or cells were then post fixed in Webster for 48 hours at 4°C.

Calcium imaging

Ratiometric microfluorometric measurements of Ca^{2+} concentrations were performed using Fura-2. Clones differentiated for 5 days or freshly isolated dissociated adult dorsal root ganglion cells were transferred from propagation medium to Hank's balanced salt solution (HBSS; 145 mM NaCl, 5 mM KCl, 10 mM HEPES 10, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose) buffered to pH 7.4 at room temperature and containing 2 μM Fura-2 acetoxymethyl (AM) ester (derived from a stock solution of 2 mM Fura dissolved in DMSO) and 80 μM Pluronic F-127 (both from Molecular Probes) for 30 minutes. Coverslips were subsequently placed in a recording chamber and viewed with $10\times$ Fluoar objectives in a Zeiss Axiovert 200 using DCLP 410 and LP 440 filters. Pairs of images were collected at intervals of 1 or 2 seconds with alternating exposure of 340 and 380 nm each for 50-200 mseconds using a Polychrome IV and an air-cooled CCD Imago Camera (640×480 pixel resolution) that were controlled by TILLviSION 4.0 software (all from Till Photonics, Gräfeling, Germany). This software was also used to compute ratios of fluorescent images on a pixel by pixel basis representing changes of intracellular Ca^{2+} transients. Coverslips were superfused with HBSS and drugs were applied using a gravity driven application system with magnetic valves allowing rapid fluid exchange rates at an uniform speed in each experiment of ~2 ml/minute. We used 1 mM of the TRPM8 agonist menthol for 10 seconds, 1 μM of the TRPV1 agonist capsaicin (both diluted from a 10 M or 20 mM stock solution in 100% ethanol to a final ethanol concentration of 0.01 or 0.005%, respectively) for 4 seconds, 30% hypo-osmolar solution 30 seconds and 100 mM KCl for 4 seconds allowing a washout of 30 or 60 seconds between each stimulus. As cold stimuli, cooled HBSS was applied using a custom made application system in which the temperature close to the cell as measured in the field of analysis as measured by a thermocoupler was lowered to 8°C.

Results

### The dorsal root ganglion contains clone forming cells

E11.5 dorsal root ganglia were dissected, dissociated and grown in propagation medium. After a few days, the formation of small phase bright clusters of ~5-10 cells could be observed. When these had grown to ~100 cells, individual clones were transferred to new culture wells. The cells appeared to divide indefinitely and have been propagated for more than 6 months. The maximal number of stem cell clones formed was 418 ± 62 (n=4) per 100,000 cells initially plated. Immunocytochemistry and RT-PCR were used to characterise expression of neural crest markers by the stem cells. NCSCs have previously been characterised by their expression of the intermediate filament nestin and the low-affinity neurotrophin receptor $p75^{NTR}$. Immunohistochemistry was performed on cells dissociated and grown for 5 hours in propagation medium to avoid differentiation (Stemple and Anderson, 1992) (Fig. 1). All healthy cells, as identified by nuclear Hoechst staining, were found to be positive for these two markers and for Ret [n=1340 (nestin), n=1340 ($p75^{NTR}$), n=756 (Ret) cells, respectively; Fig. 1A-D,F).

In addition to markers previously used to characterise NCSCs, a number of other transcription factors and cell membrane proteins have been described in the neural crest and sensory neurons. RT-PCR analysis showed expression of these neural crest and sensory neuron genes in the stem cells (Ret,

Fig. 1. The stem cells express neural crest and sensory lineage-specific markers. (A-D,F) Undifferentiated dissociated cells were plated 5 hours on PDL and immunocytochemically stained as indicated below. Cells were counterstained with nuclear Hoechst staining. All cells with an intact nucleus expressed the stem cell/progenitor marker nestin (red) and the low-affinity neurotrophin receptor $p75^{NTR}$ (green), as well as the GDNF family receptor Ret (red in F). (E) Expression of indicated transcription factors and receptors in the E11.5 dorsal root ganglion (DRG) and stem cells (SCs) determined by RT-PCR. Stem cells

expressed mRNAs for the neuronal marker Ret as well as the sensory lineage markers Brn3a, Ngn1 and Ngn2. Notably the stem cells expressed the transcription factor Krox20, a transcription factor exclusively expressed by boundary cap cells lining the DREZ. The stem cells did not express mRNAs encoding CNS and kidney specific transcription factors Otx1, Pax2 and Pax5. Abbreviations: DRG, dorsal root ganglia; SCs, stem cells. RT–, reverse transcriptase negative. Scale bars: 25 μm.
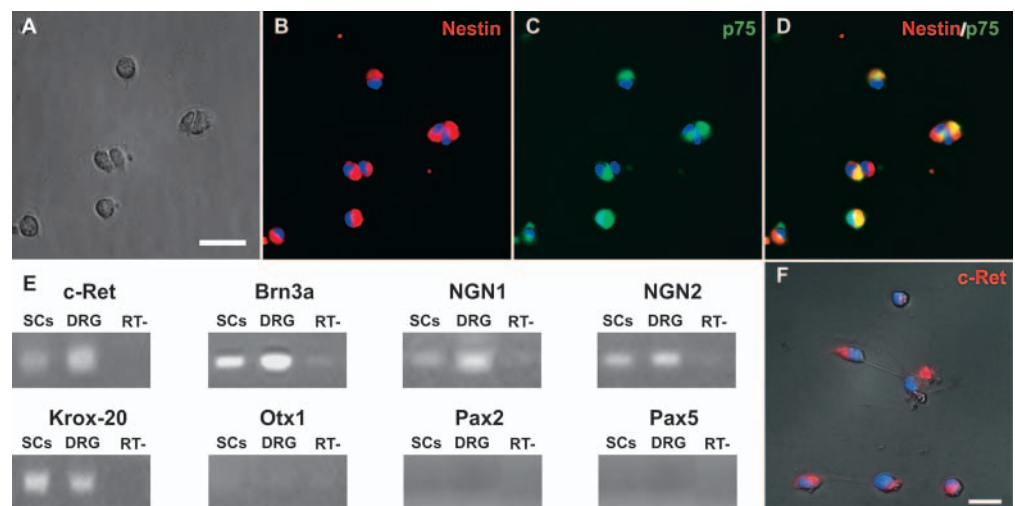
Fig. 2. The boundary cap contains NCSCs. (A) In situ hybridisation for Krox20 on a coronal section of E10.75 mouse embryo showing the dorsal part of the DRG and the boundary cap. (B) Immunocytochemistry for Maob (red) on adjacent section. (C) In situ for Krox20 on a coronal section of E11.75 mouse embryo. (D) Immunocytochemistry for Maob (red) on adjacent section. (E,F) Maob expression in dorsal root ganglion cells 2 hours (E) and 12 hours (F) after plating. (G) Enrichment of stem cells at 12 hours correlated with a several-fold increase of Maob+ cells. The enrichment is not due to transcriptional upregulation or proliferation of the Maob-positive population as it persists in the presence of actinomycin D and cytosine arabinoside (G). Notably, no change was seen in the absolute numbers of Maob-positive cells (data not shown). (H) A coronal section through an E11.5 thoracic dorsal root ganglion stained with Cresyl Violet with BC and dorsal root ganglion indicated. The dorsal root ganglion is outlined. The black lines indicate the parts isolated by micro-dissection of the dorsal root ganglia into a BC part (b) and a central part (c). (I) RT-PCR for Krox20 on dissected tissue with HPRT as internal reference for starting material. Krox20 mRNA is present only in the BC but not in the central part. (J) When cells from the BC and central parts were cultured in propagation medium separately, there was a significant difference in the number of stem cell clones produced from the different samples (***$P<0.001$, Mann-Whitney), showing that the stem cells are located in the BC. Abbreviations: Actino D, Actinomycin D; Ara-C, cytosine arabinoside; BC, boundary cap; c, central part; b, boundary cap part; DRG, dorsal root ganglia; SC, spinal cord. Scale bars: 50 μm.
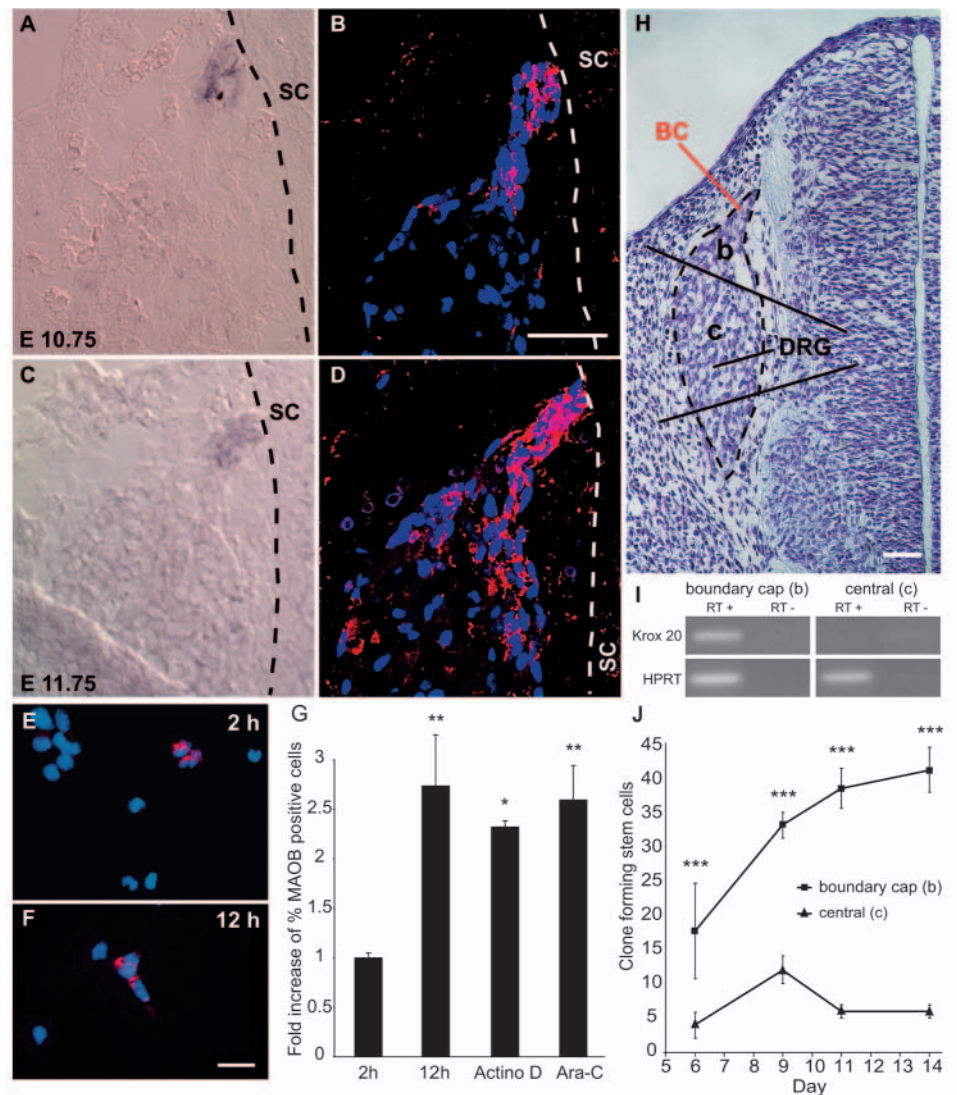
Brn3a, Ngn1, Ngn2 and Krox20; Fig. 1E). Expression of Brn3a, Ngn1, Ngn2 indicates that the cells are of the sensory lineage of the neural crest (Eng et al., 2001; Lin et al., 1998; Perez et al., 1999; Xin et al., 1992). The stem cells and the E11.5 dorsal root ganglia showed a similar expression profile. Otx1, Pax2 and Pax5 were not expressed, showing that the cells have a posterior and peripheral patterning.

The propagating cells are derived from the BC

The BC is located at the DREZ and the motor exit points (Altman and Bayer, 1984). Some BC cells are later in development also scattered medially in the zone between the dorsal root ganglion and the spinal cord (see below). Thus, when dissecting the E11.5 dorsal root ganglion along with the dorsal and ventral roots, two principally different populations of cells are present in the preparation; dorsal root ganglion cells and BC cells. The expression of Krox20 by the stem cells suggested that they originated from the BC. We therefore examined the localisation of BC cells during development.

Monoamine oxidase type B (Maob) is a membrane-bound mitochondrial flavoprotein that oxidatively deaminates biogenic amines. Maob was specifically expressed in the BC cells (Fig. 2B,D), as previously shown (Vitalis et al., 2003). The expression of Maob coincided with the BC marker Krox20 at E10.75 and in both cases, positive cells were in a region located closely associated with the DREZ (Fig. 2A,B). At E11.75, cells positive for both markers remained at the cap region, but in addition Maob-positive cells appeared migrating along the nerve medially into the dorsal root ganglion extending ventrally (Fig. 2C,D), suggesting that BC cells downregulate Krox20 but retain Maob expression as migration commences.

We therefore examined whether Maob was expressed in our culture. As our stem cell protocol includes an enrichment step 12 hours after establishment of the culture, the percentage of Maob-expressing cells was examined before and after this step.

We found a ~2.5-fold increase in the percentage of Maob+ cells at 12 hours (i.e. fold increase of Maob+ cells over total

number of cells at 12 hours compared with at 2 hours) (Fig. 2E-G). Similar results were obtained from cultures inhibiting cell division or transcription with cytosine arabinoside or actinomycin D, respectively (Fig. 2G). As there was no significant change in the absolute number of Maob+ cells in any condition (data not shown), we conclude that the increased proportion of Maob+ cells is due to a loss of other cell types, rather than induction of Maob expression or proliferation. Thus, Maob+ cells survive and are enriched under our culture conditions.

To determine whether the BC cells could be the source of the stem cells, the BC including the dorsal edge of the ganglion (b in Fig. 2H) and the central part of the ganglion (c in Fig. 2H) was micro-dissected, dissociated and plated separately. RT-PCR for Krox20 confirmed the technique of micro-dissecting the BC. Krox20 mRNA was detected in the BC sample but never in the tissue from the central part of the ganglion (Fig. 2I; n=3 dissections). The central part of the dorsal root ganglion was found to contain very few clone-forming stem cells, whereas a significantly higher number of clones formed from the BC at all time points measured (Fig. 2J). Combined, the above results show that the clone forming cells are derived from the BC.

Boundary cap NCSCs (bNCSCs) self-renew and are multipotent

The difference between stem and progenitor cells is the ability of stem cells to self-renew and in the neural crest to give rise to progenies of both neuronal and non-neuronal cell types, including neurons, glia and smooth muscle-like myofibroblasts (Gage, 1998; Shah et al., 1996). Clonal experiments were performed to examine whether individual BC cells were giving rise to all three lineages. Cells were plated at a density well below the previously reported limit for clonal expansion (50 cells/cm^2) and each cell was individually marked and followed over time to exclude contamination from neighbouring cells (Nunes et al., 2003). Cell clusters arising from such cultures were individually transferred into single wells. The clones were differentiated and stained for βIII-tubulin, Gfap and smooth muscle actin (SMA) for identification of neuronal, glia and smooth muscle-like cells (Morrison et al., 1999). Out of 60 clones analysed, all contained SMA+ cells, 88.3% also gave rise to neurons and glia. 8.3% of the clones gave rise to only glia and SMA+ cells, 1.7% neurons and SMA+ cells and 1.7% SMA+ cells only. When we performed repeated subcloning (two additional passages of single cell clones), all out of 127 clones retained the capability of producing both neurons and glia. Thus, the BC neural crest cells are multipotent stem cells.

bNCSCs differentiate into peripheral sensory neurons

To examine if the bNCSCs can differentiate to peripheral neurons, we analysed expression of peripherin, which is a type
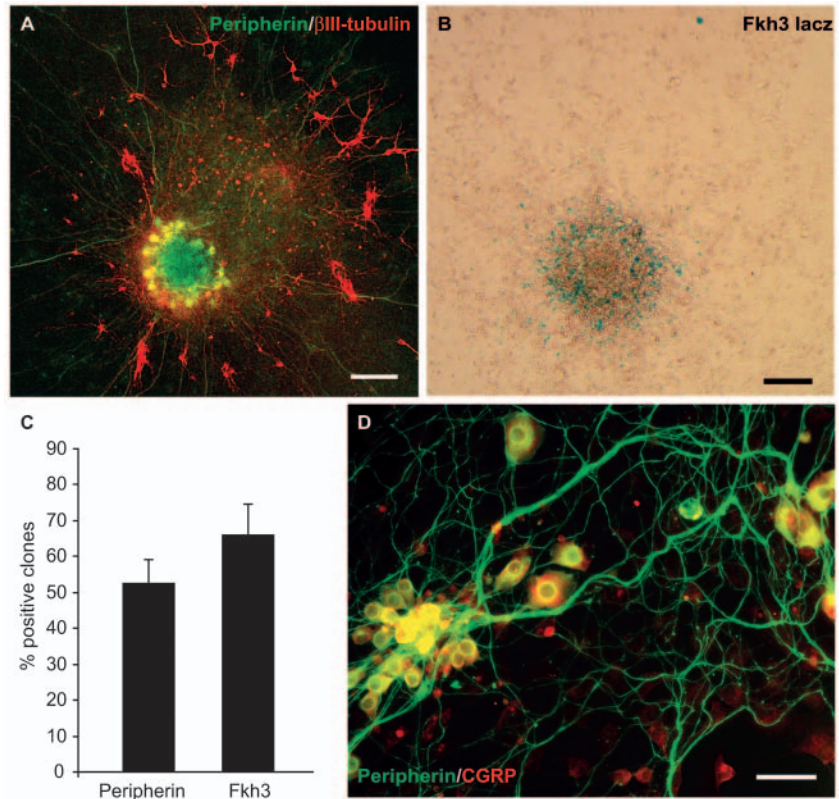
Fig. 3. bNCSCs generate sensory neurons. (A) Example of a differentiated stem cell clone showing two populations of neurons, one double labelled with peripherin and βIII-tubulin (Tuj1, yellow) and one only expressing βIII-tubulin (red). (B) Example of a differentiated stem cell clone produced from the $Fkh3^{lac-z/+}$ mice stained with X-gal, showing that the bNCSCs give rise to Fkh3+ cells of the sensory lineage. (C) Percentage clones positive for peripherin and Fkh3 (n=58 and 25, respectively). (D) Differentiated bNCSCs stained for CGRP (red) and peripherin (green) showing a mature sensory phenotype. Abbreviations: CGRP, calcitonin gene-related peptide Scale bars: in 100 μm in A,B; 25 μm in D.

III intermediate filament expressed in most, but not all, sensory and sympathetic neurons in vivo (Troy et al., 1990). After 5 days in vitro under differentiating conditions, neurons expressing both peripherin and βIII tubulin had long neurites, stretching sometimes throughout the extent of the culture dish (Fig. 3A). The morphology of the cell soma and neuritic processes of the peripherin+/βIII-tubulin+ cells resembled that of cultured primary E11.5 dorsal root ganglion neurons (data not shown). In addition to peripherin+/βIII-tubulin+ neurons, another peripherin−/βIII-tubulin+ population was identified (Fig. 3A). In contrast to peripherin, βIII-tubulin also marks early, not fully differentiated neurons (Moody et al., 1989). Even after 2 weeks of differentiation, these peripherin−/βIII-tubulin+ neurons failed to express any mature neuronal marker (data not shown). The proportion of such cells increased upon multiple passages of the bNCSCs. This property of the bNCSCs is markedly different from the autonomic NCSCs that differentiate independently of cellular cues (Morrison et al., 1999).

As a marker specific for cells of the sensory lineage, we established a forkhead-like transcription factor Fkh3/Foxs1 [the mouse homolog of Fkhl18 (Cederberg et al., 1997)] knock-in ($Fkh3^{lac-z/+}$) mouse. β-Gal was found to be strongly
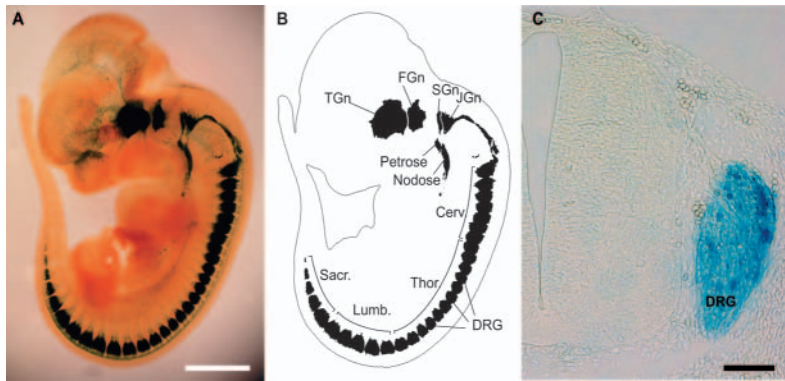
Fig. 4. Fkh3 specifically marks cells in the sensory lineage. (A,B) Whole-mount staining of a *Fkh3*[lac-z/+] mouse for β-gal at E11.5 shows expression of β-gal highly restricted to cranial and spinal sensory ganglia (schematic in B). (C) Coronal section through the lumbar region of the embryo showing that β-gal is highly restricted to the sensory dorsal root ganglia at a cellular resolution. Abbreviations: Cerv, cervical; DRG, dorsal root ganglia; FGn, facial ganglion; JGn, jugular ganglion; Lumb, lumbar; Sacr, sacral; SGn, superior ganglion; TGn, trigeminal ganglion; Thor, thoracic. Scale bars: 1 mm in A; 100 μm in C.

expressed exclusively in cranial and spinal sensory ganglia, including trigeminal, facial, superior, jugular, petrose, nodose and dorsal root ganglion (Fig. 4A,B; weak staining was also observed in blood vessels). Sections through the trunk confirmed expression in the dorsal root ganglion (Fig. 4C) and the other cranial ganglia but not in sympathetic neurons or any other tissue (data not shown). Thus, Fkh3 expression marks cells of the sensory lineage. bNCSC clones were established from these mice. Following in vitro differentiation, close to 70% of the clones generated cells expressing Fkh3 (Fig. 3B,C). In accordance with their identity as peripheral sensory neurons, differentiated peripherin+ cells were found that also expressed calcitonin gene-related peptide (Cgrp) (Fig. 3D). These results show that the bNCSC cells give rise to peripheral sensory neurons. Cells immunoreactive for tyrosine hydroxylase (Th) and choline acetyltransferase were not found in any of the progenies derived from the bNCSCs (0/63 clones).

## bNCSCs show an intrinsic ability to differentiate into the sensory lineage

We next compared the competence of the bNCSCs with sciatic nerve NCSCs (sNCSCs). sNCSCs were derived from the E12.5 sciatic nerve using the same protocol as for the boundary cap. bNCSCs and sNCSCs were spontaneously differentiated in the presence or absence of the inducer of neuronal differentiation,

bone morphogenic protein 2 (Bmp2). BMPs exerted a similar neurogenic effect on both types of NCSCs, (Fig. 5A-C). We also addressed whether the NCSCs were able to differentiate into the autonomic lineage. Cultures were differentiated in the presence of 50 ng/ml of Bmp2 for 5 days. None of the neurons in any of the clones analysed expressed the autonomic marker Th (Fig. 5D,E; bNCSCs *n*=980, sNCSCs *n*=347). When staining for Fkh3 expression, none of the sNCSCs clones was found to give rise to cells of the sensory lineage (*n*=30), while 67% were positive from the bNCSCs (*n*=56; Fig. 5F-H). This shows that the culture condition used in this study is not permissive for a full differentiation of Th+ autonomic neurons. It also shows that bNCSCs and sNCSCs have different intrinsic properties and that the culture condition does not instruct a sensory fate of sNCSCs. We therefore conclude that bNCSCs, independent of extrinsic instructive cues, differentiate into sensory neurons.

Several different classes of functional sensory neurons arise from bNCSCs

We asked if sensory neurons obtained after differentiation were physiologically functional, and whether they were of different sensory subclasses. To this end, we used ratiometric Ca^{2+} imaging. In the first experiment, we looked at the stimuli capsaicin, hypo-osmolarity and KCl and compared the

Fig. 5. bNCSCs show an intrinsic ability to differentiate into the sensory lineage. Comparison between bNCSCs and NCSCs isolated from the sciatic nerve from E12.5 mouse embryos (sNCSCs) established with the same protocol as the bNCSCs. (A) A 50 ng/ml Bmp2 treatment during a 5-day differentiation leads to increased neuronal differentiation in both types of NCSCs. Black bars are Bmp treated; white bars are untreated cultures. (B,C) βIII-tubulin immunocytochemistry of NCSCs treated with 50 ng/ml of Bmp2. sNCSCs are larger in size. (D,E) Same cells as in B and C showing lack of immunoreactivity for tyrosine hydroxylase. (F,G) X-gal staining of cells differentiated for 5 days showing Fkh3-positive cells in the bNCSCs, but not in the sNCSCs. (H) Quantification of experiments in F,G (*n*=25 and *n*=45, respectively). Abbreviations: BC, boundary cap; SN, sciatic nerve; Bmp, bone morphogenic protein. Scale bars: in B, 25 μm for B,D; in C, 25 μm for C,E; 25 μm in F,G.
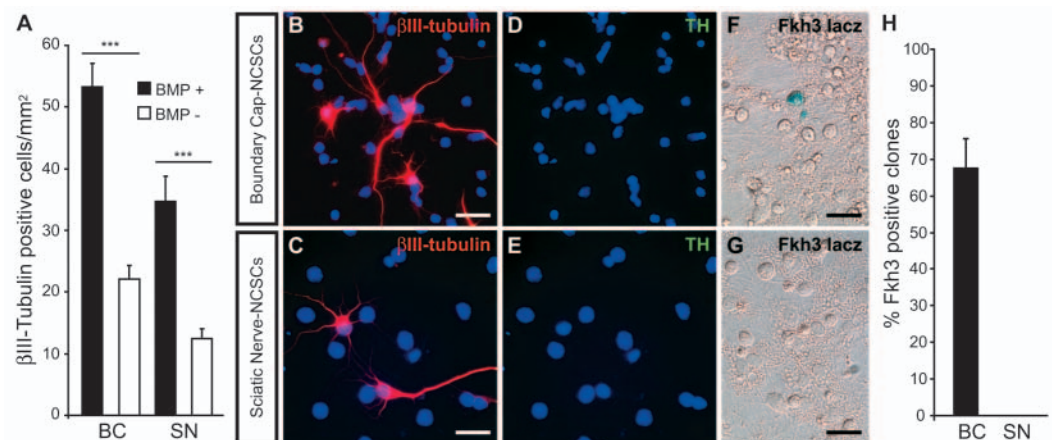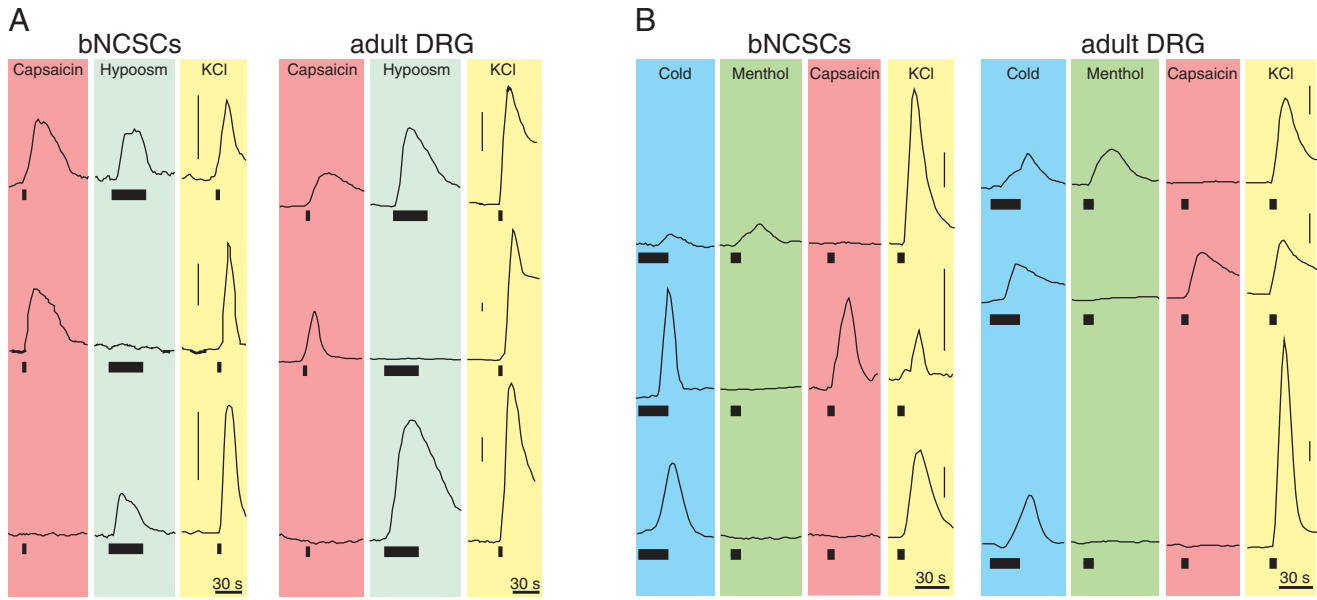
Fig. 6. bNCSC-derived sensory neurons are physiologically functional and of different subclasses. Kinetic profiles of Ca^{2+} transients of single cells in bNCSC cultures, as well as in dissociated adult dorsal root ganglion cells. Each track indicates the change of the ratiometric fluorescence signal (ΔF) from a single neuron during its response to stimuli. The duration of the stimuli is depicted underneath the curve by black horizontal bars. (A) Three different cell types with a differential response to capsaicin and hypo-osmolarity showing the presence of both mechanoinsensitive and mechanosensitive nociceptors. The bottom traces could represent either a non-nociceptive mechanoreceptor or a mechanosensitive capsaicin-insensitive nociceptor. The vertical bars represent a ΔF of 0.1. (B) Three different cell types with a differential response to cold, menthol and capsaicin, showing the presence of both innocuous cool and noxious cold receptors, as well as a cold responsive cell not expressing TRP channels. The vertical bars represent a ΔF of 0.05. Abbreviations: bNCSC, boundary cap neural crest stem cell; DRG, dorsal root ganglion; Hypoosm, 30% hypoosmolarity; KCl, 100 mM potassium chloride.

responses with those of dissociated adult dorsal root ganglion neurons. Potassium chloride challenge is an established way to differentiate between neurons and non-neuronal cells of the dorsal root ganglion and peripheral nerve (Wachtler et al., 1998). As the first stimuli we used capsaicin, an agonist of transient receptor potential ion channel V1 (Trpv1) that, in the peripheral nervous system, can be used as a marker for peptidergic and non-peptidergic nociceptive neurons (Caterina et al., 2000; Guo et al., 1999). We also challenged the cells with a 30% hypo-osmolar solution that causes swelling of the cell body and processes activating the stretch-activated receptors (Viana et al., 2001). Both nociceptive and non-nociceptive mechanoreceptors in the dorsal root ganglion respond to hypo-osmolar stimuli (Viana et al., 2001). We found cells responding to capsaicin or hypo-osmolarity, as well as those responding to both, similar to dissociated adult dorsal root ganglion cells (Fig. 6A). Because cells responding to capsaicin can with some certainty be classified as nociceptive, those also responding to hypo-osmolarity are therefore likely to constitute mechanosensitive nociceptors (Viana et al., 2001). In the case of the non-capsaicin but hypo-osmolarity-responding cells, these could either be non-nociceptive mechanoreceptors (e.g. proprioceptors or cutaneous mechanoreceptors) or capsaicin-insensitive mechanonociceptors.

In another set of recordings, we decided to examine cold-responsive thermoreceptors. The molecular and cellular basis of cold responses is still incompletely understood. Two relatives of Trpv1, Trpm8 and Trpa1 (previously known as Anktm1) have been implicated in mediating the cold response. Trpm8 has been reported to be present in 5-10% of

sensory neurons of adult dorsal root ganglia (Peier et al., 2002). Trpa1 has been shown to be co-expressed by neurons also expressing Trpv1 (Story et al., 2003). Trpm8 is activated by innocuous temperature changes and by the cooling agent menthol, while Trpa1 is insensitive to menthol and is triggered by painful cold stimuli, usually classified as stimuli below 20°C (Harrison and Davis, 1999; McKemy et al., 2002; Peier et al., 2002). When the four stimuli (cold, menthol, capsaicin and potassium chloride) were applied, we could identify three distinct types of cold-sensitive neurons that are also present in adult dissociated dorsal root ganglion cells (Fig. 6B). These were presumptive: (1) innocuous cool receptor responding to cold and menthol; (2) cold-sensitive nociceptors responding to cold and capsaicin, and (3) neurons responding to cold only and not to the application of TRP channel agonists. Interestingly, the fraction of cells responding to cold only was much higher in the bNCSCs (59.7%, n=692) than in the adult dorsal root ganglion neurons (21.3%, n=235). This type of response is the most abundant in migrating neural crest explant cultures (M.K., unpublished), which could indicate that some of the neurons in our culture are not fully differentiated.

We also used Ca^{2+} imaging studies to determine whether bNCSCs develop into a functionally homogenous progeny or whether individual bNCSCs could give rise to heterogeneous offspring. Using the functional test described above, we found that 72% of the clones generated neurons belonging to two or more subtypes. This means that a single bNCSC can give rise to multiple functional subclasses of sensory neurons (Fig. 7) and strongly suggests that the stem cells in the BC have not
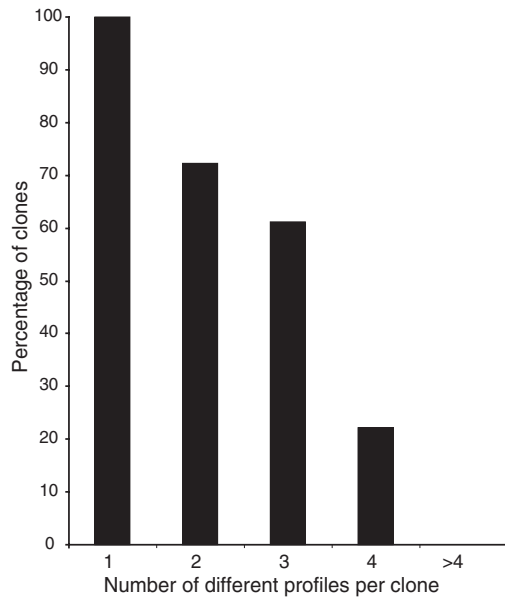
Fig. 7. A single bNCSC clone gives rise to multiple functional subclasses of sensory neurons. Cumulative graph of the percentage of clones producing multiple sensory subtypes. Twenty-five clones were analysed with ratiometric Ca^{2+} imaging and different subtypes (cells with different response profiles) within each clone was counted. The results show that a majority of the clones (72%) produce more than one sensory neuron subclass.

yet undergone a restriction towards a specific sensory neuronal modality.

Discussion

In this study, we have found that the boundary cap consists of multipotent stem cells that are specified to the sensory lineage. A subset of late emigrating neural crest cells migrating in the ventromedial pathway alongside the neural tube contributes to the BC (Le Douarin et al., 1992). These cells migrate specifically to the axonal entry and exit points, and provide an interphase between the central nervous system and the periphery (Niederlander and Lumsden, 1996) and participate in the entry of sensory and exit of motor axons (Golding et al., 1997; Golding and Cohen, 1997; Vermeren et al., 2003). The fate of the BC cell has long been enigmatic. Using the fluorescent lipophilic carbocyanin dye DiI, Sharma et al. traced late migrating cells from the dorsal neural tube in the chick (Sharma et al., 1995). They also found a second wave of cells migrating to the dorsal root ganglion contributing to neurons and satellite cells; however, it has been reported that these were of neuroepithelial origin in the chick. A recent study by Maro et al., used gene targeting to trace the lineage of the Krox20-expressing cells (Maro et al., 2004). In this study, it is shown that ~5% of the dorsal root ganglion neurons are derived from the boundary cap, which also contributes with satellite cells. Our in situ hybridisation/immunocytochemistry experiments show that Maob-positive BC cells migrate into the dorsal root ganglion at E11.5. This result agrees with the study of Maro et al. and indicates that BC cells may continue to invade the ganglion also at later developmental stages. Within the dorsal root ganglion, these cells could essentially give rise to two

populations of cells, satellite glial cells and neurons. The use of tritiated thymidine to determine cell birthdates in the mouse dorsal root ganglion shows that virtually no neurons are born after E13, while the majority of satellite cells are generated after E13 (Lawson and Biscoe, 1979). Thus, BC-derived sensory neurons are probably born in vivo until E13. Based on the tritiated thymidine studies, most dividing BC cells would acquire a glial cell fate during normal development after E13.

bNCSCs are specified to the sensory lineage

Specification and commitment of cell lineages are defined experimentally (Baker and Bronner-Fraser, 2001). A cell lineage is specified to follow a specific pathway if it does so when cultured in a neutral medium in the absence of any instructive signals. A lineage of cells is committed to a pathway of differentiation if they do so regardless of their environment, thus, even in the presence of instructive signals for another cell fate. Specification is best determined in cell culture. Although commitment can be addressed in culture by challenging with different instructive signals, it is best addressed by cell grafting into an ectopic environment of the embryo. A number of instructive signals acting on the neural crest have been identified. BMP potently induces neurogenesis and instructs an autonomic fate in cultured NCCs and NCSCs (Morrison et al., 1999). Although freshly isolated NCCs have the potential to differentiate into sensory neurons following transplantation in the chick or after one passage in culture in the presence of Wnt1/β-catenin signal [shown by the presence of Brn3a-immunoreactivity (Lee et al., 2004)], NCCs passaged more than once fail to do so (White et al., 2001). Furthermore, NCSCs with the ability to self-renew for several passages in culture, fail to differentiate to sensory neurons in vitro even under forced over expression of neurogenins (Lo et al., 2002). We found that the bNCSCs failed to differentiate into autonomic neurons even when challenged with instructive autonomic signals. However, our culture conditions are different from those used in previous studies of sciatic nerve-derived neural crest stem cells (sNCSCs) (Morrison et al., 1999; Shah et al., 1996; Stemple and Anderson, 1992). To test whether the bNCSCs display a different potential when compared with sNCSCs, we compared both NCSC types under identical culture conditions. BMP did not induce any autonomic neurons in any of the stem cells, indicating that our culture conditions are not permissible for the differentiation of Th-positive autonomic neurons. By contrast, BMP potently increased neurogenesis of both bNCSCs and the sNCSCs, indicating that it acts instructively on multipotent progenitor cells to differentiate into neurons, as reported previously (Morrison et al., 1999). As sNCSCs did not differentiate into sensory neurons, we conclude that it is not the defined culture condition used in our study that is instructive towards a sensory fate. Thus, our results show that, unlike sNCSCs, the bNCSCs are specified to differentiate into the sensory lineage. However, it remains to be established whether the bNCSCs are not only specified but also committed to the sensory lineage.

A common progenitor in the BC for nociceptive and thermoreceptive sensory neurons

An important principle distinction of adult dorsal root ganglion neurons is their division into large- and small-diameter neurons. Within each category, there are many functional

phenotypes that subserve such diverse functions as proprioception and touch or pain, itch and thermoreception. The timing and instructive signals determining cell fate between the functional subtypes has remained largely elusive. Because large mechanoreceptive neurons populate the dorsal root ganglion prior to the appearance of small nociceptive neurons, the early arriving mechanoreceptive neurons could provide an instructive scaffold for the later arriving nociceptive neuron progenitor cells (Anderson, 2000). This hypothesis implies that the restriction between different functional types of sensory neurons takes place within the dorsal root ganglion, similar to that described for motoneurons, where the birth of new motoneuron subsets occur in a feed-forward mechanism (Sockanathan and Jessell, 1998; Sockanathan et al., 2003). However, BC cells appear to be among the last cells generated by the neural crest, which would exclude them as scaffolding forming early arriving cells. Expression of sensory lineage transcription factors Ngn1, Ngn2 and Brn3a (Fedtsova and Turner, 1995; Ma et al., 1999) already in the pre-migratory or migratory neural crest argues for a restriction between sensory and autonomic lineages in the migratory neural crest. In agreement, forced expression of Ngn2 in the migrating crest cells in the chick biases these cells to a sensory fate (Perez et al., 1999). Consistently, expression of Ngn2 biases the cells towards the sensory lineage also in the mouse (Zirlinger et al., 2002). Thus, cell-intrinsic differences of migrating neural crest cells together with environmental cues determine cell fate. The instructive signals imposed onto the neural crest populating the dorsal root ganglion during migration or after condensing into a ganglion appear not to fate the stem cells in the BC, as the bNCSCs can generate both nociceptive and thermoreceptive sensory neurons and possibly most or all other types of sensory neurons, as well as glia and smooth muscle cells. This is consistent with the fate of the boundary cap cells, which have been shown to have the potential to generate nociceptive, mechanoreceptive and possibly other neuronal subtypes in vivo, in addition to glia (Maro et al., 2004). By contrast, while generating large quantities of autonomic neurons in vitro, sNCSCs have not been reported to generate anything else than Schwann cells and endoneurial fibroblasts in vivo (Joseph et al., 2004).

Concluding remarks

Dorsal root ganglion neurons are generated from the neural crest shortly after their delamination from the dorsal neural tube, with the cervical ganglia condensing around E9.5. The contribution of this early population of migrating neural crest to most of the dorsal root ganglion neurons has been firmly established in several species, including the mouse. In both the avian and the rodent, the wave of neural crest cells forming the dorsal root ganglion is separated in time from that which populates the BC, probably spaced by neural crest cells migrating dorsolaterally that belong to the melanocyte lineage (Le Douarin et al., 1992; Serbedzija et al., 1989; Serbedzija et al., 1990; Topilko et al., 1994). Thus, the BC cells clearly belong to a different lineage of cells than the early neural crest cells that generates the dorsal root ganglion. We show that the bNCSCs self-renew and can generate neurons, glia and smooth muscle-like cells. Our data suggest that the developmental programs in the dorsal root ganglion, including specification of different neuronal subtypes, cell cycle exit and programmed

cell death are not imposed upon the BC stem cells. Our findings raise a number of important issues such as the nature of the refractory mechanism by which the BC stem cells escape the developmental programs imposed upon the rest of the neural crest, the evolutionary origin and the biological role of this unique population of stem cells.

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