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NELL2 promotes motor and sensory neuron differentiation and stimulates mitogenesis in DRG in vivo

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Abstract

We previously identified a secreted glycoprotein, *n*eural *e*pidermal growth factor-*l*ike *l*ike *2* (NELL2), in a subtraction screen designed to identify molecules regulating sensory neurogenesis and differentiation in the chick dorsal root ganglion (DRG). Characterization of NELL2 expression during embryogenesis revealed that NELL2 was specifically expressed during the peak periods of both sensory and motor neuron differentiation, and within the neural crest was restricted to the sensory lineage. We now provide evidence for a function for NELL2 during neuronal development. We report here that NELL2 acts cell autonomously within CNS and PNS progenitors, in vivo, to promote their differentiation into neurons. Additionally, neuron-secreted NELL2 acts paracrinely to stimulate the mitogenesis of adjacent cells within the nascent DRG. These studies implicate dual functions for NELL2 in both the cell autonomous differentiation of neural progenitor cells while simultaneously exerting paracrine proliferative activity.
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Introduction

Within the developing vertebrate nervous system, an intricate interplay between extrinsic and intrinsic cellular signals ultimately regulates the genesis and differentiation of the neurons that comprise the CNS and PNS. In the process, a variety of neuronal cell types are generated from a population of mitotically active progenitor cells. The question of how such diversity is generated has been a major focus in developmental biology. An experimentally rich system in which to address this issue is the chick dorsal root ganglia (DRG), which derive from a subset of migrating neural crest cells that coalesce laterally to the neural tube (Horstadius, 1950; Lallier and Bronner-Fraser, 1988; Le Douarin, 1982; Teillet et al., 1987; Weston, 1970). To identify genes that regulate this process, we compared cDNA from immature embryonic day E4.5 DRG, during the peak period of neurogenesis and differentiation (Carr and Simpson, 1978), to cDNA from mature E8.5 DRG, which are composed of differentiated postmitotic neurons and glia, using a subtraction/differential screening strategy (Nelson et al., 2004). From this subtraction screen, we identified a candidate cDNA fragment, neural epidermal growth factor-like (NEL, Matsuhashi et al., 1995), by DNA sequencing and BLAST analysis of GenBank (Altschul et al., 1990). Chicken NEL is approximately 40–50% homologous to mammalian NEL-like 1 (NELL1), and approximately 80–90% homologous to mammalian NEL-like 2 (NELL2, Kuroda et al., 1999; Watanabe et al., 1996). Therefore, we refer to chicken NEL as NELL2; chicken NELL1 has not yet been identified.

The NELL genes belong to the (1) laminin G/N-terminal thrombospondin-1 (N-TSP1)/Pentraxin supergene family, which is involved in cell/cell communication and contact (Beckmann et al., 1998); (2) chordin-like domain family; and (3) the epidermal growth factor-like (EGF-like) domain families. NELL contains a cleavable signal peptide

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and N-TSP1 domain, six EGF-like, and five cysteine-rich chordin-like/von Willebrand Factor C (C/vWc) domains, and has been proposed to act as a signaling molecule involved in cell growth and/or differentiation and as a possible trophic factor (Aihara et al., 2003; Garcia Abreu et al., 2002; Kuroda et al., 1999; Matsuhashi et al., 1995; Watanabe et al., 1996). The NELL genes code for large peptides of approximately 90 kDa and are heavily glycosylated with an additional approximately 50 kDa of N-linked carbohydrate moieties for total monomeric size of approximately 140 kDa (Kuroda et al., 1999). The secreted forms of the NELL peptides are processed to approximately 130 kDa monomers and form approximately 400 kDa trimers in solution (Kuroda et al., 1999; see Fig. 1a for schematic of NELL2).

NELL1 and NELL2 gene expression is tightly regulated in several vertebrate tissues both during development and in the adult (Kim et al., 2002; Kuroda et al., 1999; Luce and Burrows, 1999; Matsuhashi et al., 1995; Nelson et al., 2002; Oyasu et al., 2000; Watanabe et al., 1996). NELL2 expression during development is temporally and spatially restricted to periods of differentiation in discrete regions of the nervous system, the pharyngeal arches, and the forelimb and trunk skeletal muscle (Nelson et al., 2002). In the nervous system, NELL2 expression is maximal during the peak period of neurogenesis and differentiation of both spinal cord motor neurons and sensory neurons within the DRG (Nelson et al., 2002). Interestingly, in a manner reminiscent of Notch/Delta interactions, NELL2-positive, postmitotic neurons are of-

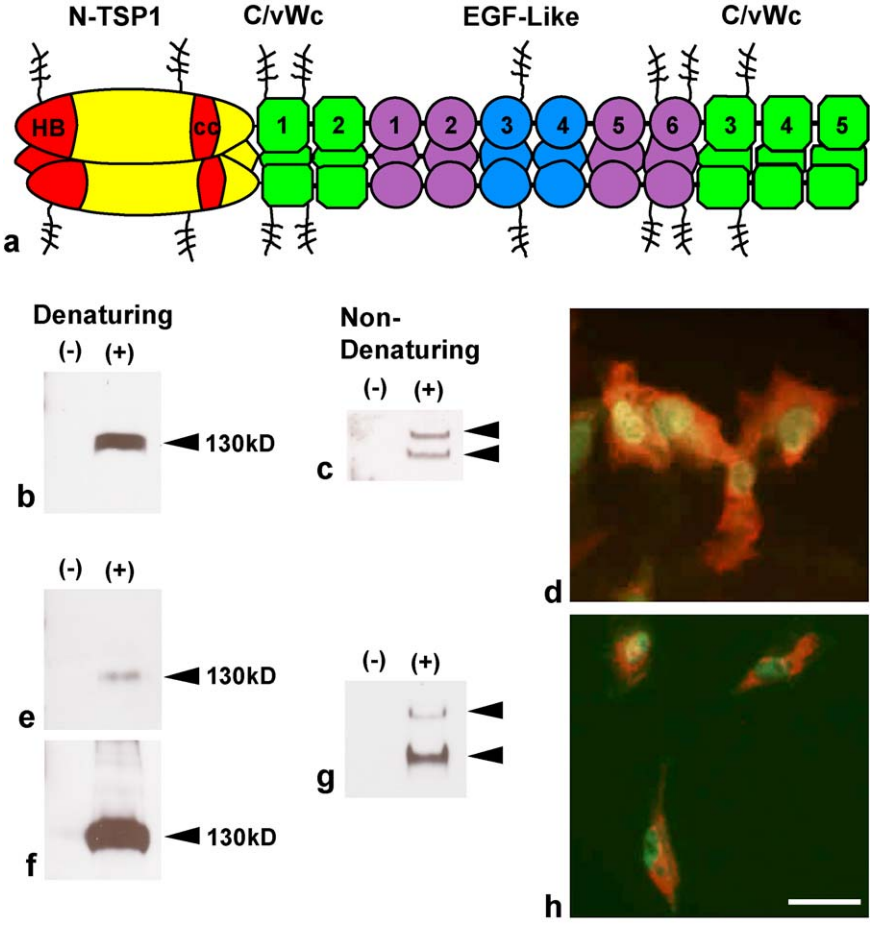

Fig. 1. NELL2 is secreted from in ovo transfected spinal cord. (a) Schematic of NELL2 homotrimer: N-TSP1, N-terminal thrombospondin 1 motif with N-terminal heparin-binding domain (HB) and coiled–coiled (cc) domain; C/vWc, chordin-like/von Willebrand factor c motif; EGF, epidermal growth factor-like motif, blue are Ca^{+2} binding type; predicted N-linked glycosylation sites denoted with hatched lines. Western analysis of supernatants with anti-Myc antibody (9e10, DHSB) demonstrates that NELL2Myc is secreted by transfected COS7 cells (b–d) and chick embryonic spinal cord cultures (e–h), and forms the putative homotrimer in solution from both cell types: (+) conditions are NELL2-transfected; (−) conditions are empty vector control pmiw transfected. (b, e, f) Denaturing SDS-PAGE conditions yield a single band for NELL2 peptide at approximately 130 kDa from both COS7 cells and in ovo transfected embryonic spinal cord cultures; (f) immunoprecipitation of NELL2Myc with anti-Myc antibody (Clontech) from remaining spinal cord supernatant in E. (c, g) Nondenaturing NATIVE-PAGE conditions yields two bands for NELL2, indicating oligomeric complexes, consistent with previous descriptions of homotrimeric NELL2 in solution. COS7 (d) and spinal cord (h) cultures were immunolabeled with anti-Myc antibody (9e10, red) to confirm dual NELL2Myc and GFP expression from pMES (Swartz et al., 2001b). Scale bar: 12 μm.

ten observed directly juxtaposed to mitotically active progenitor cells (Nelson et al., 2002). Although NELL2's expression correlates temporally with the differentiation of both motor and sensory neurons, the functional role of NELL2 in vivo has not been determined.

To determine its role in vivo, we have used in ovo plasmid injection and electroporation to analyze the effect of ectopic NELL2 expression in CNS and PNS progenitor cells (Muramatsu et al., 1997; Swartz et al., 2001a). We demonstrate that NELL2 drives CNS progenitor cells in the spinal cord to exit the cell cycle, promoting their precocious differentiation in a cell autonomous fashion without altering cellular identity, and within motor pools, NELL2 increases the rate of motor neuron differentiation. NELL2 also acts cell autonomously to bias migrating neural crest cells to localize within the DRG anlagen, and to differentiate into sensory neurons rather than peripheral glia, without altering sensory subtype identity. Furthermore, neuron-secreted NELL2 acts paracrinely to induce adjacent cells in the immature DRG to proliferate. These data suggest dual roles for NELL2 in mediating the differentiation of neuronal progenitor cells while simultaneously stimulating neighboring cells to proliferate.

Methods

NELL2 subcloning and expression in vitro

A 6× myc epitope tag was subcloned into the C-terminus of chicken NELL2 by replacing the *Msc*I/*Afl*II fragment in the parent expression vector pmiwC3 (gift of S. Matsuhashi), with a linker designed to remove the stop codon and insert *Eco*RV/*Nsi*I restriction sites to allow cloning of a PCR amplified myc tag containing these sites, pNELL2Myc. Sequencing verified that the NELL2Myc fusion was in-frame. Removing NELL2 from the parent vector with *Hin*dIII, followed with re-ligation, created pmiw empty vector control plasmid.

Expression of rNELL2Myc was verified with transient transfection of 5 μg each of pNELL2Myc and pmiw into 35 mm dishes of COS7 cells with Fugene (Roche) as directed. Supernatants were collected after 48 h, spun, and prepared for polyacrylamide gel electrophoresis (PAGE) under denaturing and nondenaturing conditions. For denaturing SDS-PAGE analysis, 100 μl of supernatants were mixed with 50 μl of 2× sample buffer consisting of SDS and B-mercaptoethanol, and boiled for 5 min to reduce peptides to their primary structure. For nondenaturing NATIVE-PAGE, 100 μl of supernatants was mixed with sample buffer without denaturants and not boiled to maintain protein quaternary structure. Twenty-five microliters of each denatured and nondenatured NELL2Myc and control pmiw supernatants were loaded into 6% SDS-PAGE and 6% NATIVE-PAGE (without SDS) gels, respectively, and ran with SDS and non-SDS running buffers accordingly. Gels were electrotransferred onto PDVF membranes, which were blocked in 6% BSA, 1% casein at room temp for 1 h. Membranes were immunoblotted with the primary anti-myc antibody 9e10 (1:10, DHSB) overnight at 4°C, washed 4×, incubated with secondary goat anti-mouse, biotinylated 1 h at room temp, and washed 4×. Biotinylated complexes were detected with ABC treatment (Vectastain, Vector) and chemiluminescence (SuperSignal, Peirce).

NELL2Myc coding sequence was subcloned into pMES (Swartz et al., 2001b; gift of C. Krull) by sequential digests with *Afl*II, blunt-ending with T4 DNA polymerase, and then with *Xba*I, which was cloned into the *Xba*I/*Sma*I sites of pMES, now called pNELL2. Expression of NELL2Myc from pNELL2 was confirmed with transient transfections of COS7 cells, along with control pGFP plasmid, and analysis of supernatants as described. Dual expression of NELL2Myc and GFP from pNELL2 in the same cell was confirmed by briefly fixing these cultures with 4% paraformaldehyde at room temperature for 30 min. Dishes were washed and blocked with 10% serum for 1 h, immunolabeled with primary 9e10 anti-myc antibody at 1:10 dilution overnight at 4°C, washed 4×, incubated with goat anti-mouse/Texas Red secondary antibody (Jackson Labs) 1:250 dilution at room temp for 1 h, washed, mounted with ProLong Antifade (Molecular Probes), and analyzed by epifluorescence.

Combined in situ hybridization and immunolabeling

Double immunocytochemical detection of BrdU and neurofilament after NELL2 in situ hybridization of E4.5/stage 25 embryos was performed as previously described (Nelson et al., 2002, in press).

In ovo transfections and analysis

In ovo microinjection and electroporation of plasmids were performed as described (Muramatsu et al., 1997; Swartz et al., 2000a). All embryos were treated in accordance with IACUC stipulations. Briefly, plasmid preparations (5 μg/μl) were injected into the neural tubes of windowed chicken embryos at HH stage 10 (Hamburger and Hamilton, 1951), electroporated with gold electrodes (three pulses, 10–20 V, 50 ms) and a square wave electroporater (BTX), resealed, and incubated to desired stages. Expression of NELL2Myc from in ovo transfected cells was confirmed by dissecting pNELL2 and control pGFP-transfected spinal cords incubated to E4.5, dissociating and culturing overnight in DMEM–10% serum. One hundred microliters of each supernatant from these cultures were collected and analyzed under denaturing SDS-PAGE as described. Additionally, 1 ml of the remaining supernatants was used for immunoprecipitation experiments by adding 2 μl of the anti-Myc antibody (Clontech), incubating overnight at 4°C on a rotator, adding 50 μl of equilibrated

Protein A-Sepharose (Pharmacia), and rotating for 4 h, washed 4× with PBS, stripped, and denatured with 25 μl of SDS sample buffer and boiling for 5 min, followed by SDS-PAGE analysis as described. Dual expression of NELL2Myc and GFP in the same cells in spinal cord cultures was detected as described.

For in vivo analysis, transfected embryos at the desired stages were examined for GFP expression with a fluorescent stereo-dissecting microscope (M^2Bio, Zeiss) equipped with eGFP filter set. Embryos with detectable fluorescence in forelimb level DRG were collected, their trunks removed and fixed in 4% paraformaldehyde for 4–6 h at 4°C, and cryoprotected through a sucrose gradient before being embedded in O.C.T. (Tissue-Tek) for cryosectioning. Transverse cryosections of transfected forelimb level trunk regions were analyzed to identify the fate of pNELL2 and control pGFP-transfected cells by immunolabeling with an array of cell type specific markers: MNR2, Isl1, Pax6, Pax7, Nkx2.2, Ben (all DHSB monoclonal antibodies, and all used at 1:20 dilution); rat anti-BrdU used at 1:2000 dilution; rabbit anti-phosphohistone 3 (Upstate Biotechnology) used at 1:500, mouse anti-Tuj1 (Chemicon) used at 1:2000 dilution; rabbit anti-TrkA, TrkB, TrkC (Lefcort et al., 1996; Rifkin et al., 2000) used at 1:2000 dilution. Secondary antibodies were species-specific Cy3, TRITC, or Texas Red conjugated used at 1:500 dilution. Sections were analyzed by epifluorescence for GFP expression and red-secondary immunolabeling with appropriate filter sets, photographed as double exposures, scanned into and compiled with Adobe Photoshop. Embryos were serially sectioned at 10 μm through the forelimbs and into the upper trunk region. All GFP+ cells in all sections were scored for presence or absence of immunolabeling with the respective antibody markers being assessed. Double-counting cells was easily avoided based on morphological distinctions between neighboring cells. Each slide contained ca 16 sections, and at least three slides per embryo were scored for expression of each different antibody marker; at least three embryos were sectioned and labeled and scored per marker, except for the trk labeling of E6.5–E7.5 embryos in which only two embryos per marker were analyzed. Total number of embryos analyzed for each comparison is stated as is the total number of cells scored in each figure legend. Statistical significance was determined with Student's *t* test.

Results

NELL2 is secreted in vivo

Expression of NELL2 was verified by transiently transfecting pNELL2:Myc and empty control parent vector (pmiw) into COS7 cells and analyzing supernatants and/or immunoprecipitations from supernatants for secreted NELL2. We found that like mammalian NELL2 (Kuroda et al., 1999), chicken NELL2 is secreted from COS7 cells and forms the putative homotrimer in solution (Figs. 1b, c). For in ovo microinjections/electroporations, NELL2:Myc was subcloned into the bicistronic eukaryotic expression vector pMES (Swartz et al., 2001b), which contains the CMV IE and chick beta-actin promoters and the IRES2:eGFP sequence for dual expression of NELL2:Myc and eGFP in the same cell, now called pNELL2. Co-expression of both NELL2 and GFP in vitro was confirmed in transient COS7 cells transfections (Fig. 1d). Co-expression in ovo was confirmed by microinjecting and electroporating pNELL2 into HH stage 10 chick neural tubes (Hamburger and Hamilton, 1951), incubating until stage 25, dissecting and culturing dissociated transfected spinal cords overnight. Supernatants and cultures were analyzed as described. NELL2 was secreted from in ovo transfected spinal cord cells and formed the putative homotrimer in solution (Figs. 1e–g) and was co-expressed with GFP in the same cells (Fig. 1h).

An intriguing observation from our previous in situ hybridization analysis was that NELL2 expression in the developing pharyngeal arch target fields was maximal as its expression waned in the respective innervating cranial ganglia (Nelson et al., 2002). Similarly, NELL2 expression in brachial DRG decreased as its expression became restricted to discrete regions within the target forelimb. This raised the question whether NELL2 might function as a target-derived factor that attracted or repelled extending neurites, similar to other glycoproteins that also contain EGF-like domains such as ROBO and its ligand Slit (reviewed in Nguyen-Ba-Charvet and Chedotal, 2002). We tested this hypothesis by coculturing COS7 cell pellets expressing rNELL2 next to explanted E4.5 DRG in collagen gels (Tessier-Lavigne et al., 1988). These studies did not reveal any overt ability for secreted rNELL2 to attract or repel extending DRG neurites (data not shown).

Spinal cord progenitor cells expressing NELL2 withdraw from the cell cycle and translocate to the mantle layer

To determine whether ectopic expression of NELL2 altered the behavior of neural progenitors cells, stage 10 neural tubes were injected with pNELL2 or pGFP control plasmid and electroporated (Muramatsu et al., 1997; Swartz et al., 2001a). Transverse sections of stage 18–20/E3 and stage 25/E4.5 (Figs. 2A, B) control GFP-transfected embryos demonstrated that many progenitor cells located next to the lumen in the ventricular zone continued to express GFP several days after transfection, as did their progeny. In contrast, CNS progenitors transfected with NELL2 translocated from the ventricular zone to the mantle layer where differentiation occurs; this asymmetry in cellular distribution coincided with the onset of ectopic protein expression (Figs. 2C, D; ectopic GFP could be observed approximately 6–12 h post-transfection). These data demonstrate that within CNS progen-

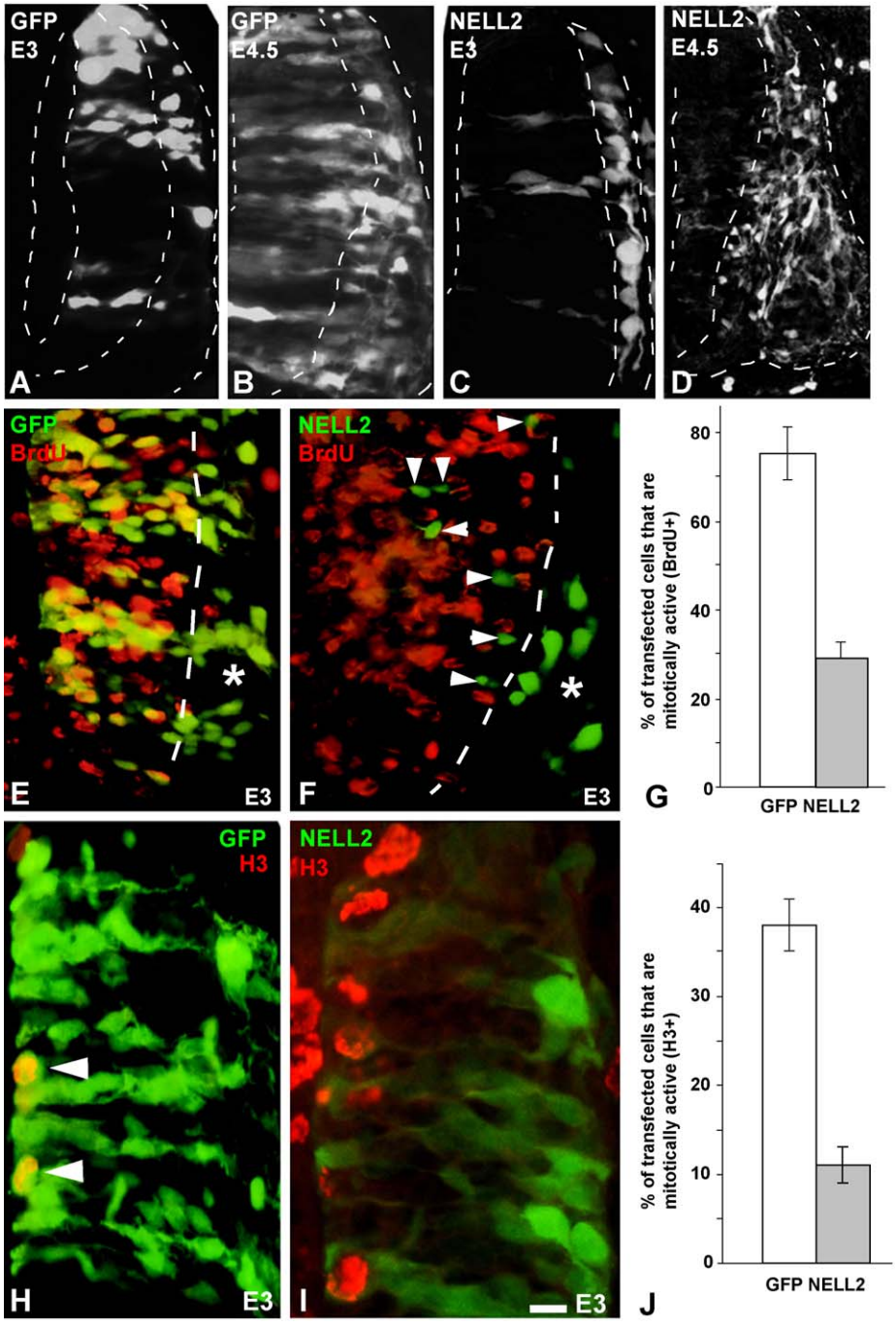

Fig. 2. Spinal cord progenitor cells expressing NELL2 withdraw from the cell cycle and translocate to the mantle layer. All transverse sections of in ovo transfected embryos are oriented with dorsal–ventral as top–bottom and left–right as ventricular surface-mantle layer of the spinal cord, respectively. (A–D) Transverse sections of stage 18–20 (E3) and stage 25 (E4.5) spinal cord demonstrate that control GFP-transfected progenitor cells (A, B, respectively) in the mitotic ventricular zone and their progeny in the postmitotic mantle layer express high levels of ectopic GFP; ventricular zone (left) and mantle layer (right) denoted by hatched lines. In contrast, upon ectopic expression of NELL2, progenitor cells at E3 (C) translocate from the mitotically active ventricular zone to the mantle layer to differentiate, and by E4.5 (D) have become restricted to the mantle layer. (E–G) Transfected control GFP (E) embryos pulsed with BrdU at stage 18, sectioned and processed for BrdU immunocytochemistry (red), demonstrate that the majority of GFP-transfected progenitor cells in the ventricular zone incorporated BrdU; asterisks denote postmitotic cells in the mantle zone. (F) In contrast, NELL2-transfected progenitors still present within the ventricular zone did not incorporate BrdU (arrowheads; asterisks denote postmitotic cells in the mantle zone). (G) Quantification of effects of ectopic NELL2 expression on proliferation measured by BrdU immunolabeling; GFP: $n = 3$ embryos, 90 cells; NELL2: $n = 3$ embryos; 297 cells; $P \leq 0.0001$. (H–J) Similarly, transfected embryos immunolabeled for H3 (red) demonstrate that control GFP (H) transfected progenitor cells in M phase are immunopositive, while NELL2-transfected cells are not; arrowheads denote immunopositive transfected cells. (J) Quantification of effects of ectopic NELL2 expression on proliferation measured by H3 immunolabeling. GFP:68 cells, $n = 2$ embryos; NELL2:187 cells, $n = 2$ embryos; $P \leq 0.004$. Scale bar: A–C, 25 μm; D, 45 μm; E, F, 25 μm; H, I, 20 μm.

itors, NELL2 acts cell autonomously, either intracellularly or autocrinely via cell–surface interactions.

We next analyzed whether ectopic NELL2 expression directly forced CNS progenitors to withdraw from the cell cycle, by combining microinjection/electroporations with BrdU pulses to label CNS progenitors in S phase. Transfected embryos incubated to stage 18–19 were pulsed for 3 h to insure labeling of the majority of mitotically active progenitor cells within the ventricular zone. These studies revealed that only a minority of NELL2+ transfected cells in the ventricular zone were BrdU positive (28.0 ± 1.5%), in contrast to control GFP-transfected embryos in which the majority of transfected cells were BrdU+ (74.0 ± 3.6%; $P \leq 0.0001$; Figs. 2E–G). Similarly, analysis of the subset of mitotically active progenitor cells in M phase identified with anti-phosphohistone 3 (H3) antibody revealed that a greater percentage of control transfected cells in the ventricular zone were H3+ (38.0 ± 3.0%) than were NELL2-transfected (11.0 ± 2.0%; $P \leq 0.004$; Figs. 2H–J). Additional evidence supporting NELL2-induced cell cycle withdrawal is the fact that we consistently observed fewer NELL2-transfected cells than control GFP-transfected cells. A role for NELL2 in neuronal differentiation is consistent with its normal expression pattern in the spinal cord where it is robustly expressed by postmitotic neurons at E4.5 as they emigrate from the ventricular zone and settle in the mantle zone (Fig. 3). Thus, similar to the proneural transcription factor NKL that drives progenitor cells to exit the cell cycle and promotes their differentiation in the spinal cord (Lamar et al., 2001), ectopic NELL2 expression in CNS progenitors promotes their mitotic arrest and translocation to the mantle layer cell autonomously.

NELL2 promotes differentiation of spinal cord progenitors without altering their cellular identity

To determine whether ectopic NELL2 influenced specification of cellular identities, we immunolabeled sections of transfected embryos with antibodies that distinguish subclasses of cell types within the spinal cord. CNS progenitors in the developing spinal cord give rise to distinct classes of cells according to a homeodomain transcription factor code, generated in response to ventralizing and dorsalizing gradients of secreted sonic hedgehog and BMPs, respectively (Briscoe et al., 2000; reviewed by Lee and Pfaff, 2001). Neither transfection of control GFP nor NELL2 induced ectopic motor neuron formation dorsal or ventral to their normal domain, as assessed by immunoexpression of the motor neuron specification factor MNR2 (Fig. 4A). Furthermore, NELL2 transfection did not induce the formation of ectopic Pax6, Pax7, or Nkx2.2 progenitors outside of their normal domains within the ventricular zone of the spinal cord (data not shown).

To further investigate the effect of ectopic-NELL2 expression in CNS progenitors, we used MNR2 expression within developing motor neuron pools as a measure of motor neuron differentiation. MNR2 normally is expressed at high levels by progenitors as they migrate from the ventricular zone to the nascent motor neuron domain in the mantle layer (Novitch et al., 2001; Tanabe et al., 1998). Within developing motor pools, MNR2 is expressed at higher levels medially and at lower levels more laterally; as nascent motor neurons mature in the lateral zone, they increase their expression of more mature motor neuron markers such as Isl1. Analysis of transfected motor progenitor cells approximately 36 h following transfection indicated a relative increase in the number of ectopic-NELL2/MNR2+ immunopositive cells (74.0 ± 8.5%, Figs. 4B, F) compared to GFP/MNR2+ cells from control transfected embryos (56.0 ± 3.4%, Fig. 4F), the result of NELL2's ability to induce differentiation of progenitor cells and their concomitant translocation from the ventricular zone. Furthermore, most NELL2+ cells at this stage were also immunopositive for later motor neuron markers such as Isl1 (Fig. 4D). However, within the next 24 h, a significantly greater percentage of motor neurons ectopically expressing NELL2 had reduced their expression of MNR2 (34.0 ± 2.0%, Fig. 4F) compared to control GFP-

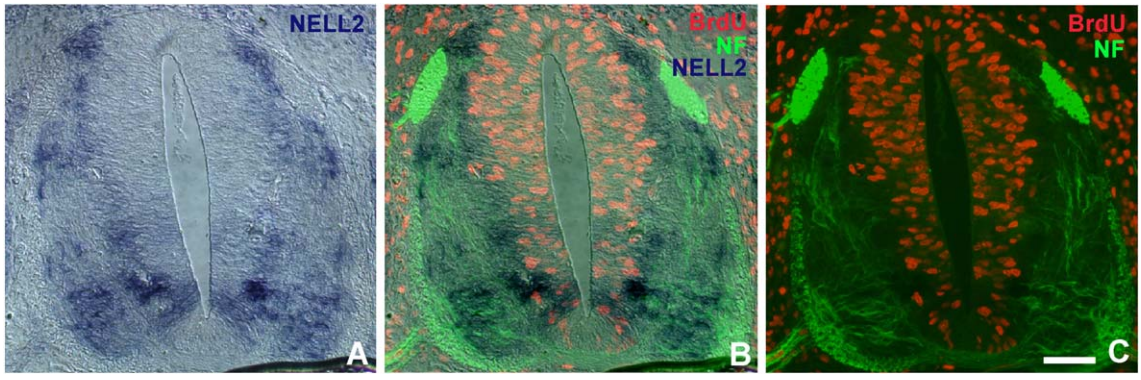

Fig. 3. NELL2 is expressed by nascent, postmitotic CNS neurons as they leave the ventricular zone and coalesce in the mantle zone. A stage 24/25 embryo was injected with BrdU and incubated for 2–3 h, fixed, sectioned, and hybridized with a probe to detect NELL2 mRNA (A, B) then labeled by immunocytochemistry with antibodies to BrdU (B, C) and neurofilament (B, C). Note that by this stage, the vast majority of NELL2+ cells are not in the mitotically active ventricular zone, but rather, migrating toward and residing within the mantle zone of the spinal cord. Scale bar: 60 μm.

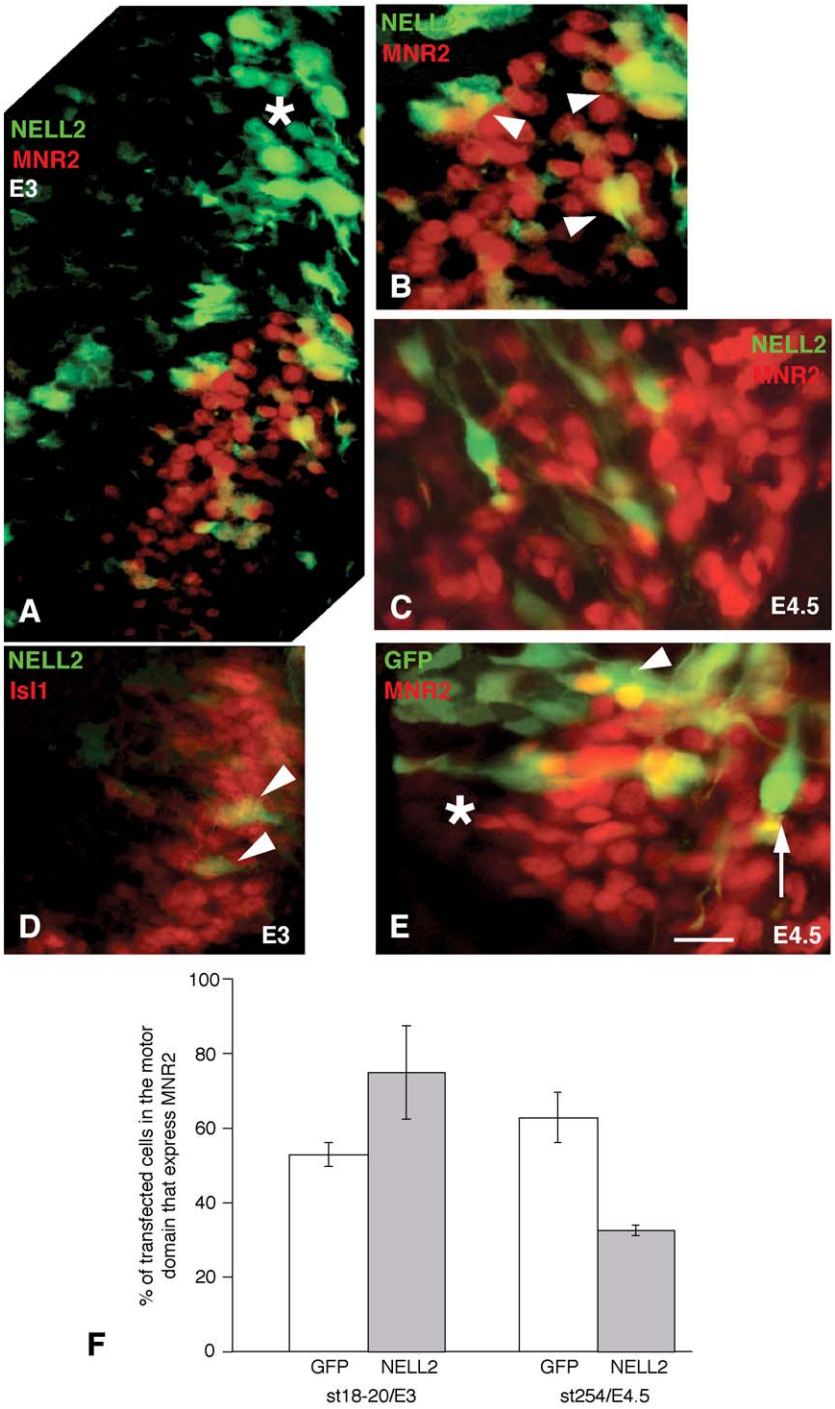

Fig. 4. NELL2 does not alter the identity of transfected CNS progenitor cells, but rather, increases their rate of differentiation. (A–B) NELL2-transfected embryo, stage (st 18–20/E3) immunolabeled with the motor neuron marker MNR2 (red), demonstrates that forced NELL2 expression in progenitor cells does not induce ectopic motor neuron formation dorsal (asterisks) or ventral to their normal domain. (B) A higher magnification view of the motor neuron domain shown in A indicates that many NELL2+ cells are also MNR2+ (arrowheads). (C) However, by stage 25/E4.5, the majority of NELL2-transfected motor neurons have downregulated expression of MNR2. (D) Many NELL2+ cells at E3 have already differentiated into mature motor neurons and express Isl1 (yellow cells; arrowheads). (E) At stage (25/E4.5), control GFP-transfected motor neuron progenitor cells can still be detected in the medial ventricular zone (asterisks), and are immunopositive (arrowhead) for MNR2; cells more laterally situated in the motor neuron domain have matured into MNR2-cells (arrow). (F) Quantification of MNR2 expression in transfected motor neurons. E4.5: GFP, $n = 3$ embryos; 362 cells; NELL2, $n = 3$ embryos, 215 cells; $P \leq 0.015$. Scale bar: A, 50 μm; B, C, E, 30 μm; D, 40 μm.

transfected motor neurons (63.0 ± 6.8%, $P \leq 0.015$; Figs. 4E, F). Furthermore, control GFP-transfected progenitor cells remained in the Olig1/2 domain in the ventricular zone (Fig. 4E), while NELL2-transfected cells migrated out of this proliferative zone into the mantle layer to differentiate (Fig. 4F). Therefore, ectopic NELL2 expression increased the rate at which motor neuron progenitor cells withdrew from the cell cycle, translocated to the mantle layer, and ceased expression of MNR2, indicating that NELL2 promoted their precocious differentiation. Further support for this finding is that by E4.5, while MNR2 expression is decreasing in the NELL2-transfected cells, their expression of Islet-1 is increasing: 85.3 ± 3% (n = 3 embryos, 158 cells) of the NELL2-transfected cells were immunopositive for Islet1. This also indicates that the vast majority (if not all) of NELL2-transfected cells differentiated into neurons.

NELL2 biases migrating neural crest cells to localize to the DRG

Transfection of control GFP plasmid into premigratory neural crest at stage 10 labeled all neural crest derivatives (Fig. 5A), in particular, enteric, sympathetic, sensory, and melanocyte derivatives, as described in previously published lineage-tracing experiments (Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). In contrast, ectopic expression of NELL2 biased the migration of the early wave of neural crest cells such that they preferentially localized in the DRG anlagen (Fig. 5B). Examination of the primary chain of sympathetic ganglia at stage 25 revealed that control GFP-labeled cells contributed to sympathetic ganglia, while ectopic NELL2-labeled cells were absent from sympathetic ganglia (Figs. 5C, D): note that this also demonstrated the existence of non-NELL2-transfected neural crest cells that were capable of generating and populating sympathetic ganglia. Quantification of the position of transfected cells confirmed that ectopic NELL2 expression biased neural crest cells to localize or commit preferentially to the sensory (95.0 ± 5%) rather than sympathetic ganglia (Fig. 5E). In control embryos, 65.0 ± 3% ($P \leq 0.007$) of transfected cells were localized to the DRG (Fig. 5E), consistent with previous studies indicating that the majority of neural crest cells contribute to the DRG because of their larger size at this stage (Perez et al., 1999). This functional bias is consistent with our expression analysis that demonstrated that while NELL2 was strongly expressed by a subset of migrating neural crest and in the DRG anlagen, it was not similarly expressed in the primary nor secondary chain of sympathetic ganglia (Nelson et al., 2002).

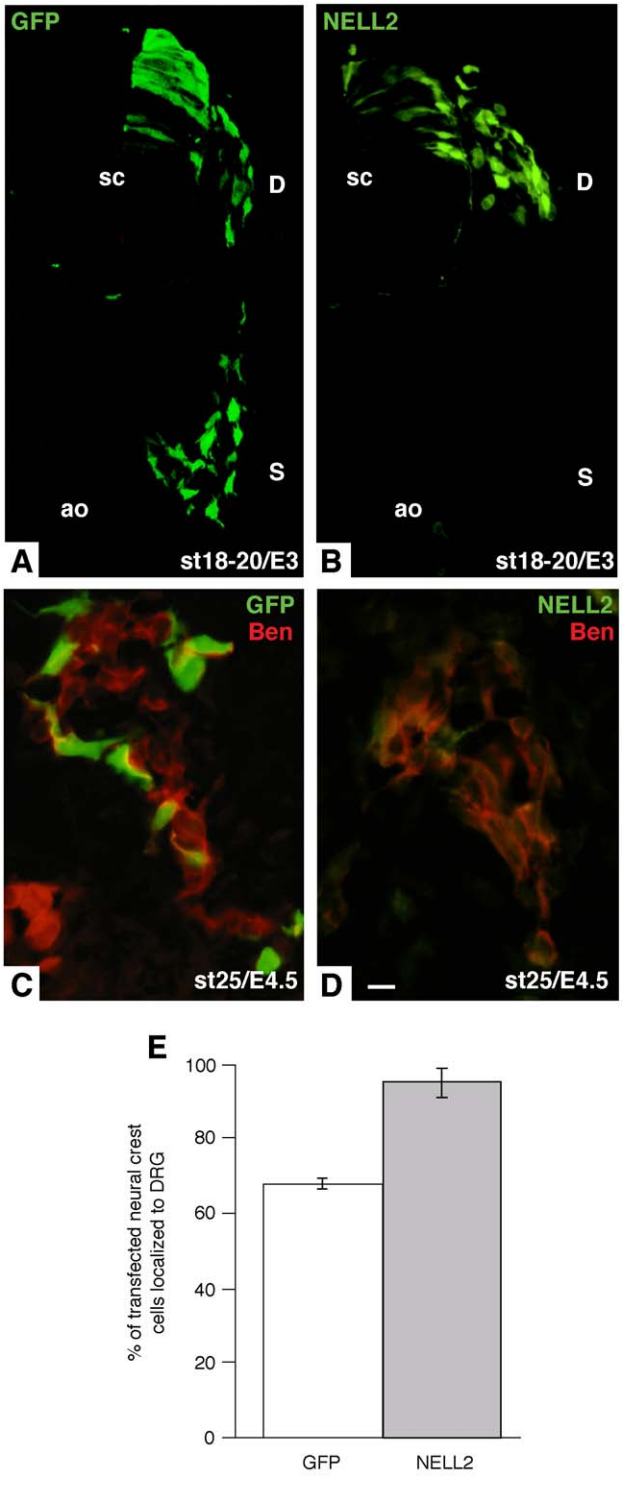

Fig. 5. Ectopic NELL2 expression autocrinely biases transfected neural crest cells to localize in the DRG. (A) GFP control transfected neural crest cells have migrated to their appropriate destinations by stage 18–20/E3 in both the prospective DRG, D, and sympathetic ganglia, S, and continue to contribute to the primary chain of sympathetic ganglia at stage 25/E4.5; ao, aorta; sc, spinal cord. In contrast, ectopic NELL2 (B) expression biased migrating neural crest cells to preferentially localize to the DRG anlagen, D, at stage 18–20/E3 rather than the sympathetic ganglia, S, ao, aorta; sc, spinal cord. (C) Control-GFP-transfected cells contribute to the sympathetic ganglia, identified with the neural marker Ben (red). (D) In contrast, NELL2-transfected cells are absent from sympathetic ganglia. (E) Quantification of the location of transfected cells at stage 18–20/E3. GFP:1117 cells; n = 3 embryos; Nell2: 246 cells, n = 3 embryos; $P \leq 0.007$. Scale bar: A, B, 30 μm; C, D, 10 μm.

NELL2 promotes the differentiation of neural crest cells into sensory neurons

To investigate the fate of NELL2-transfected neural crest cells localized in the DRG, we determined their identity with an array of neuronal markers: Ben, Hu, Tuj1, Isl1, TrkA, TrkB, TrkC. At stage 18–20 (early E3), gangliogenesis has begun and the first neurons have differentiated (Lallier and Bronner-Fraser, 1988; Pannese, 1974; Teillet et al., 1987). However, the majority of cells in the nascent ganglia are progenitor cells that do not express neural markers; rather, they encapsulate the newly differentiating cluster of neurons that aggregate in the inner core of the ganglion (Fig. 6A). Ectopic NELL2 expression resulted in a clear bias, in which NELL2+ cells were significantly more likely to be neurons than were control GFP-transfected cells (Fig. 6I). Even during the earliest stages of ganglion formation when neural markers are first expressed, 64.0 ± 4.7% of NELL2-transfected cells (Figs. 6B, I) already expressed neural markers compared to 15.0% + 0.4% ($P \leq 0.001$) of control GFP-transfected cells (Figs. 6A, I). This is a conservative estimate, as NELL2-transfected cells with clear neuronal morphology not expressing these later neural markers were often observed (Fig. 6B). Furthermore, while axons from transfected NELL2 neurons were detected in peripheral nerve tracts, NELL2-transfected cells (i.e., Schwann cells) were not observed (not shown). During the peak period of sensory neurogenesis and differentiation in control embryos (stage 25/E4.5), GFP expression was detected in nascent neurons and non-neural cells in the core of the ganglion, in progenitor cells in the dorsal pole and in the perimeter, and in non-neural cells in the dorsal root and in the ventral motor tracts (Figs. 6C, I). In contrast, the vast majority of NELL2-transfected cells expressed neuronal markers (87.0 ± 4.3%, $P \leq 0.003$) and were confined to the neural core of the ganglion (Figs. 6D, E, I).

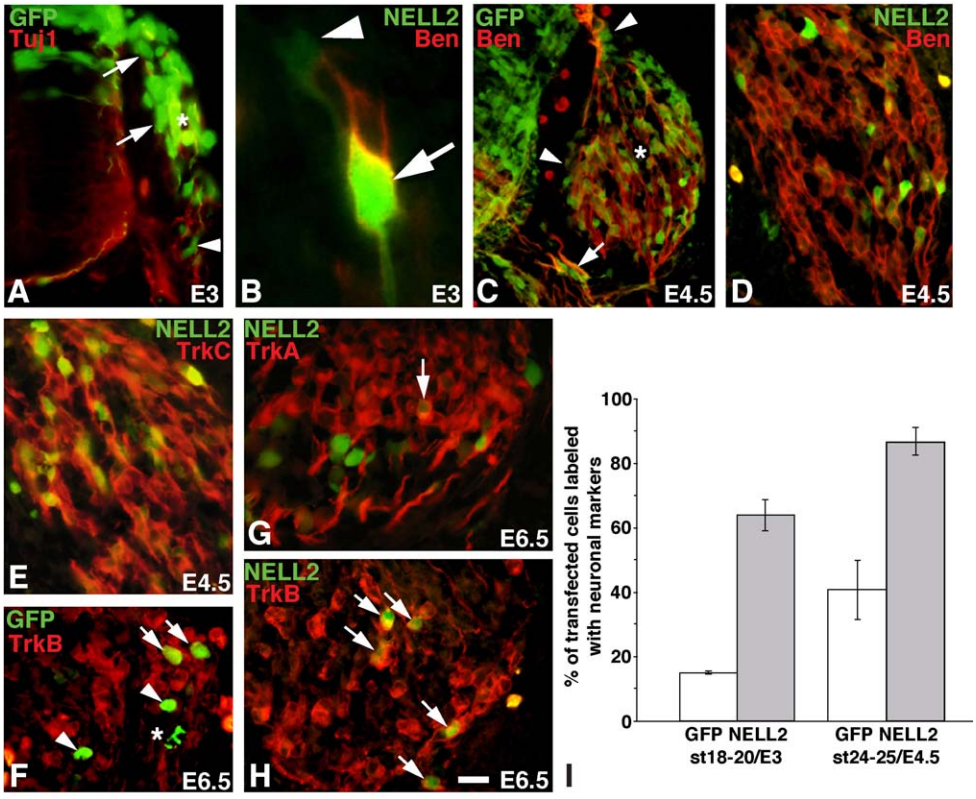

Fig. 6. Ectopic NELL2 expression autocrinely promotes the differentiation of transfected neural crest cells into sensory neurons. (A) Control GFP-transfected embryo at stage 18–20/E3, immunolabeled with a neuronal marker (Tuj1, red), demonstrates that in the DRG anlagen, transfected neurons are in the core (asterisk) encapsulated by transfected progenitor cells (arrows); note transfected cells in the ventral root (arrowhead). (B) By E3, the majority of NELL2-transfected neural crest cells localized to the DRG anlagen express neuronal markers (Ben, red; arrow) or appear neuronal by morphology (arrowhead; Ben, red). (C, D) At E4.5, the peak period of sensory neurogenesis and differentiation, control GFP-transfected cells (C) immunolabeled with neuronal markers (Ben, red) are in the core of the ganglion (asterisk); non-neuronal transfected cells are detected in the progenitor zones in the dorsal pole and ensheathing cell layer (arrowheads), in the ventral motor root (arrow). (D) In contrast, the majority of NELL2-transfected cells are immunolabeled with neuronal markers (Ben, red), and not detected in progenitor zones. (E–H) Ectopic NELL2 does not alter sensory subtype identity. (E) At E4.5, the vast majority of NELL2-transfected cells are TrkC+. (F) E6.5 control GFP-transfected TrkB+ neurons (arrows), TrkB- neurons (arrowheads), and non-neuronal cells (asterisk). (G, H) E6.5 NELL2-transfected TrkA+ neurons (G, arrow), TrkB+ neurons (H, arrows). (I) Quantification of positive expression of the neuronal markers Ben, tuj-1, and trkC was compiled and compared for control and NELL2-transfected embryos: GFP, stage 18–20: 439 cells, $n = 3$ embryos; NELL2, stage 18–20: 224 cells, $n = 6$ embryos; $P \leq 0.001$. GFP, stage 25/E4.5: 659 cells, $n = 3$ embryos; NELL2, stage 25/E4.5: 357 cells, $n = 5$ embryos, $P \leq 0.003$. Scale bar: A, D, 40 μm; B, 12 μm; C, 20 μm; E–H, 50 μm.

To further investigate the fate of ectopic NELL2-transfected cells in the DRG, we analyzed whether ectopic-NELL2 expression influenced the production of neuronal subtypes, which can be loosely categorized as TrkA (nociceptors), TrkB (mechanoreceptors), or TrkC (proprioceptors) neurons (Lefcort et al., 1996; Oakley et al., 1997, 2000). At E4.5, TrkC is expressed by all of the nascent neurons and a subset of progenitors resident in the core of the ganglion (Rifkin et al., 2000). Accordingly at this stage, the vast majority of NELL2-transfected cells were TrkC+ and appeared neuronal (Fig. 6E). At E6.5, ectopic NELL2 expressing cells appeared neuronal and could express any of the three Trk receptors (TrkA 7/30 cells, Fig. 6G; TrkB 53/54 cells, Fig. 6H; TrkC 11/22 cells). Ectopic control GFP-transfected cells at E6.5 included TrkB (+) and (−) neurons and glial cells (TrkB 48/100 cells, Fig. 6F; TrkA 30/92 cells, TrkC 3/15 cells). These results suggest that NELL2 may promote the differentiation of TrkB sensory neurons over other Trk subtypes. However, this apparent bias could be because TrkB neurons normally differentiate during the first (Ngn2-dependent) wave of neurogenesis, and since ectopic-NELL2 expression in transfected progenitors would have driven progenitors out of the cell cycle, the pool of GFP+ progenitor cells remaining to give rise to the second (Ngn-1 dependent) wave of neurogenesis (in which the majority of trkA+ cells are generated) could have been significantly depleted. Interestingly, maximal NELL2 expression does correlate temporally with the first wave of neurogenesis in the DRG, decreasing after approximately E6 as the second Ngn1-dependent wave of neurogenesis begins (Nelson et al.,

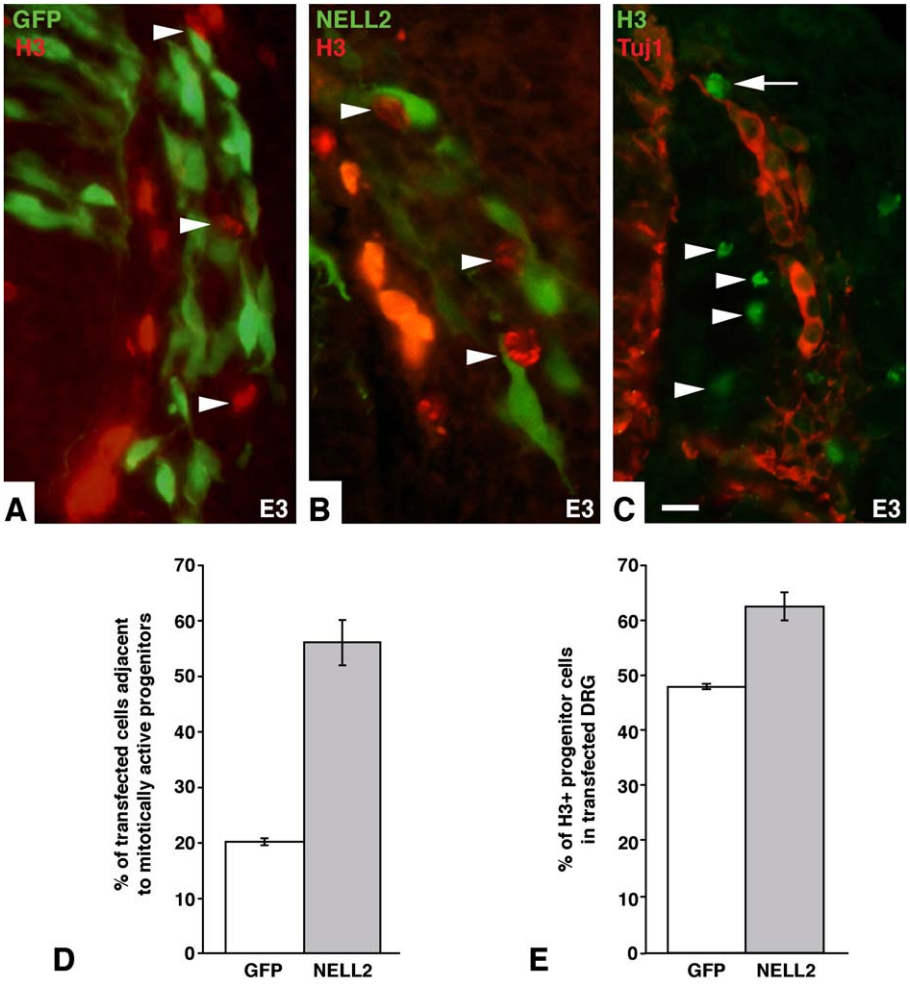

Fig. 7. Neuron-secreted NELL2 paracrinely induces mitogenesis within the nascent DRG. (A) At E3, control GFP-transfected embryo immunolabeled with H3 (red) reveals that the majority of transfected cells are not adjacent to mitotically active progenitor cells (arrowheads). (B) In contrast, ectopic NELL2-transfected cells with a definite neuronal morphology are frequently observed tightly juxtaposed to mitotically active progenitor cells (arrowheads). (C) In nonexperimental embryos of the same stage, nascent neurons (Tuj1+, red) are seldom in contact with mitotically active progenitor cells (H3+, green; arrow denotes contact, arrowheads denote noncontact). (D) Quantification of contacts between NELL2-transfected nascent neurons and mitotically active progenitor cells. GFP: 325 cells, $n = 4$ embryos; NELL2: 127 cells, $n = 3$ embryos; $P \leq 0.0001$. (E) Quantification of mitogenic effect of ectopic NELL2. The mean percentage of H3+ cells that are in both the GFP and NELL2-transfected DRG, rather than in the DRG on the contralateral nontransfected side, is shown (100% = total number of H3+ cells in both experimental side and contralateral control side DRG). GFP: 459 cells, $n = 4$ embryos; NELL2: 131 cells, $n = 2$ embryos; $P \leq 0.028$. Scale bar: 20 μm.

2002). Although the majority of TrkA+ cells are generated during this second wave of neurogenesis, a few TrkA+/NELL2+ transfected neurons were observed (Fig. 6G). Whether these TrkA+/NELL2+ cells were generated from later Ngn1 progenitors is not known, as some TrkA sensory neurons are also generated during the first wave of neurogenesis (Ma et al., 1999; Rifkin et al., 2000). Targeted transfection of progenitors during the second wave of neurogenesis will be required to conclusively determine the effect of NELL2 on sensory neural subtype. Therefore, at this point, we conclude that NELL2 promotes the differentiation of a sensory neuronal fate rather than a glial fate without apparently influencing the specificity of sensory neuronal subtype.

Neuron-secreted NELL2 induces mitogenesis of adjacent cells within the nascent DRG

Since NELL2 is secreted from transfected cells in ovo, we investigated whether secreted NELL2 influenced the fate of adjacent cells. In our in situ hybridization analysis, we noticed that NELL2+ postmitotic cells were often directly juxtaposed to mitotically active progenitor cells in the CNS and PNS (Nelson et al., 2002). To determine whether NELL2 had mitogenic activity on adjacent cells in the nascent DRG in ovo, we immunolabeled sections of transfected embryos with anti-phophoH3, a marker of cells in M phase. In control GFP-transfected embryos, 18.3 ± 1.3% of the transfected cells were in contact with H3 immunopositive cells (Figs. 7A, D). In contrast, 56.0 ± 4.0% ($P \leq 0.0001$) of NELL2-transfected cells were found juxtaposed to H3+ cells (Figs. 7B, D). To determine whether this was an indirect effect of the increased neuronal differentiation of NELL2-transfected cells, we determined the normal percentage of neurons in nontransfected embryos that were adjacent to H3+ progenitor cells in the nascent DRG. We found that in normal, nonexperimental animals, 27.1 ± 2.3% of nascent neurons were in contact with H3+ progenitors (Fig. 7C). This apparent induction of mitogenesis by NELL2-transfected neurons consequently resulted in an increase in the total number of H3+ cells observed in NELL2-transfected DRG (62.5 ± 2.5%) compared to contralateral nontransfected DRG, and to control GFP-transfected and contralateral nontransfected DRG (48.3 ± 0.0%, $P \leq 0.028$; Fig. 7E). Thus, neuron-secreted NELL2 can apparently paracrinely stimulate the mitogenesis of cells within the immature DRG in ovo.

Discussion

These data demonstrate that during the development of the nervous system, NELL2 can alter the behavior of both CNS and PNS progenitor cells. We report here that chicken NELL2 is secreted from in ovo transfected spinal cords and forms a homotrimer in solution. Ectopic expression of NELL2 induces the precocious differentiation of CNS progenitor cells and biases migrating neural crest cells to preferentially localize to the DRG anlagen, promoting their differentiation into sensory neurons in a cell autonomous manner. Furthermore, within the nascent DRG, neuron-secreted NELL2 paracrinely stimulates adjacent cells to proliferate.

These are the first reported in vivo functions for NELL2 and are striking in that molecules typically identified as mediating neuronal differentiation have been members of the *achaete-scute* and *atonal* proneural transcription factor families, that is, Mash, neurogenins, NeuroD (reviewed by Lee, 1997). Misexpression experiments with proneural transcription factors in the CNS have demonstrated their ability to promote neuronal differentiation (Cai et al., 2000; Lamar et al., 2001; Lee, 1997). Some bHLH transcription factors can couple neuronal differentiation with specification of neuronal identity, that is, Mash and Ngn1, resulting in the expansion of particular classes of neurons at the expense of others (Cai et al., 2000; Lo et al., 2002; Parras et al., 2002). In contrast, ectopic NELL2 expression does not specify neuronal subtype within the spinal cord but rather drives CNS progenitor cells out of the cell cycle promoting their differentiation. In this regard, it is interesting that the expression pattern of NELL2 in the spinal cord overlaps with that of NeuroM and NKL, bHLH and zinc-finger transcription factors, respectively (Lamar et al., 2001; Roztocil et al., 1997). Expression of all three genes becomes restricted within the spinal cord by E4.5 to nascent postmitotic neurons (Lamar et al., 2001; Nelson et al., 2002; Roztocil et al., 1997). Ectopic expression of NELL2 or NKL in CNS progenitor cells is sufficient to drive them out of the cell cycle forcing progenitors to emigrate from the ventricular zone and differentiate into neurons (Figs. 2 and 3; Lamar et al., 2001). Based on their restricted expression patterns and activities in the spinal cord, it is likely that NELL2, NeuroM, and NKL act in combination with, or downstream from, other bHLH transcription factors and lateral inhibitory systems to activate or maintain the differentiation state of postmitotic neurons in a cell autonomous manner (Lamar et al., 2001).

In the PNS, we have shown that NELL2 is expressed by a subset of migrating neural crest cells, and maximally expressed in the DRG anlagen through the peak period of sensory neurogenesis and differentiation, while not being similarly expressed in the primary nor secondary chain of sympathetic ganglia (Nelson et al., 2002). This early expression pattern of NELL2 in a subset of migrating neural crest cells parallels the transient expression of Ngn2, which is restricted to the earliest progenitor cells in the DRG anlagen that generate the first wave of TrkB and TrkC sensory neuronal subtypes (Ma et al., 1999; Perez et al., 1999). Ngn1 expression ensues as the DRG matures, restricted to progenitor domains in the immature ganglion—presumably in a separate set of sensory progenitor cells responsible for generating the second wave of later arising TrkA sensory neuronal subtypes (Ma et al., 1999). Within

the immature DRG, NELL2 is expressed by both nascent neurons and a subset of mitotically active progenitor cells (Nelson et al., 2002). The number of these mitotically active (BrdU+) NELL2+ progenitor cells decreases over time, tightly correlated with the differentiation of neuronal progenitor cells (Nelson et al., 2004). The vast majority of NELL2 expressing cells are nascent, postmitotic neurons, often in direct contact with non-NELL2 expressing progenitors cells, reminiscent of the classical lateral inhibition interaction mediated by Notch and Delta (Morrison et al., 2000; Wakamatsu et al., 2000). Finally, NELL2 expression wanes as nascent neurons mature, being restricted temporally to the peak period of neuronal differentiation within the DRG (Nelson et al., 2002).

The precise role of the Ngns in the development of the peripheral sensory nervous system remains complex as recent fate-mapping studies demonstrate that the Ngn2+ sensory-specific neural crest cell subpopulation gives rise to both neurons and glia equally, with no apparent bias in sensory neural subtypes, and that Ngn2 expressing progenitors can migrate to the sympathetic ganglia (Zirlinger et al., 2002). Previous studies have demonstrated that ectopic expression of Ngn2 and Ngn1 biased migrating neural crest cells to localize in the DRG anlagen, and to differentiate into sensory neurons (Perez et al., 1999). We show here that ectopic expression of NELL2 in premigratory neural crest cells also biased them to preferentially localize to the nascent DRG, and to differentiate into sensory neurons rather than glia without apparently altering sensory subtype, in a cell autonomous manner. NELL2 overexpression in migrating neural crest cells could drive them to localize in the DRG anlagen by causing their premature withdrawal from the cell cycle and by default, their differentiation into (DRG) neurons thereby precluding further ventral migration. Alternatively, NELL2 may instruct migrating neural crest cells to specifically localize to the DRG and differentiate into sensory neurons since NELL2 is normally preferentially expressed by this lineage of the neural crest (Nelson et al., 2002).

Electron microscopy in the adult rat brain has demonstrated NELL2 association with the rough ER, and that NELL2 was exclusively expressed by neurons and not detected in any glial derivatives, which further support that NELL2 is secreted by neurons in vivo (Oyasu et al., 2000). We have demonstrated that, in vivo, neuron-secreted NELL2 paracrinely stimulates mitogenesis of adjacent cells in the nascent DRG, inhibiting them from differentiating, reminiscent of the juxtacrine signaling activity of Delta to adjacent Notch-expressing progenitor cells. NELL2 expression normally decreases in sensory neurons as they mature, thereby potentially removing a mitogenic stimulus and facilitating the differentiation of later-born neurons and/or glia. Interestingly, NELL2 and NELL1 have been detected in several transformed cell lines, hyperplasias, and malignancies, indicating that misregulation of NELL genes is correlated with oncogenic potential in certain cancerous pathologies (DiLella et al., 2001; Kuroda et al., 1999; Luce and Burrows, 1999; Maeda et al., 2001).

How NELL2, a secreted glycoprotein, could simultaneously exert both cell autonomous and paracrine activities is not yet understood. NELL2 could induce an intracellular signal during its biosynthesis in the secretory pathway, or activate cells extracellularly via an autocrine cell–surface interaction as has been shown for BDNF in adult sensory neurons (Acheson et al., 1995). Cell–surface interactions between Notch and Delta are known to occur within progenitor cells before committing to Notch-only or Delta-only expression (reviewed by Gaiano and Fishell, 2002). The similarities between the classical lateral inhibition pathway and the cell autonomous and paracrine functions of NELL2 raise the intriguing possibility that NELL2 may directly or indirectly interact with the Notch pathway, a hypothesis currently being tested. Recently, it has been suggested that NELL2 could regulate synaptic vesicle transport or release through a Ca^{+2}-mediated signaling pathway (Kim et al., 2002). Additionally, NELL2 can promote the survival of embryonic rat cortical neurons in culture via a MAP kinase signaling pathway (Aihara et al., 2003). Finally, NELL2 either directly or indirectly could interact with Wnt/β-catenin signaling pathway, which has been shown to be required for sensory neuron differentiation within the DRG (Hari et al., 2002). Secreted NELL2 also binds to heparin sulfate in vitro (Kuroda et al., 1999) suggesting another possible mechanism of direct interaction of NELL2 with cell surface receptors such as heparin sulfate proteoglycans or FGF receptor–heparin complexes, or indirectly with the heparin sulfate-rich extracellular matrix (HS-ECM). Finally, NELL2 could also bind other secreted factors since it contains cysteine-rich chordin-like/von Willebrand factor-C and N-TSP1 motifs, which can interact with TGFβ/BMP family members and a myriad of cell–surface and ECM molecules, respectively (reviewed in Adams and Tucker, 2000; Bornstein and Sage, 2002; Garcia Abreu et al., 2002).

In summary, we have demonstrated that NELL2 has dual cell autonomous and paracrine functions, promoting differentiation of neuronal progenitor cells that express NELL2 while simultaneously stimulating neighboring cells in the DRG to proliferate. It would seem then as if NELL2 activates and/or maintains a neuronal differentiation program within spinal cord and sensory neuron precursors without influencing neuronal subtype decisions. Interestingly, mouse embryonic stem cells upregulate NELL2 expression when induced to differentiate into neurons in response to retinoic acid (Guo et al., 2001). As NELL2 expression wanes in maturing neurons, a loss of its mitogenic stimulus would allow remaining progenitors to differentiate into later-born neurons and/or glia. Recently, it was demonstrated that overexpression of NELL1 in transgenic mice resulted in premature osteoblast differentiation (Zhang et al., 2002), consistent with its misexpression in coronal synostosis (Ting et al., 1999). Although these studies did not

address paracrine functions of secreted NELL1, the cell autonomous effects of NELL1 overexpression and the resulting premature differentiation of osteoblasts suggest that the NELL gene family promotes the differentiation of cells in which they are expressed. A similarly complex role for another secreted glycoprotein Noelin-1 demonstrates that secreted glycoproteins can regulate the behavior of neural progenitors (Barembaum et al., 2000; Morenoi and Bronner-Fraser, 2001). The elucidation of the mechanisms by which a single protein can exert dual cell autonomous and paracrine functions is of great interest and the focus of ongoing studies.

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