Identification and characterization of genes regulating sensory neurogenesis and differentiation
by Branden Ray Nelson

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University
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Abstract:
Vertebrate neurogenesis occurs through the restricted expression of subsets of genes to discrete cell types that organize into the central and peripheral nervous systems during specific developmental periods. A major goal of developmental biology is to understand the molecular and cellular mechanisms underlying this complex process. An experimentally rich system in which to investigate this question is the dorsal root ganglion (DRG), which derives from a population of migrating neural crest cells that aggregate laterally to the neural tube, and contain the cell bodies of the sensory neurons of the peripheral nervous system. In the chick, the peak of sensory neurogenesis and differentiation in the DRG occurs at embryonic day E4.5, at which DRG are considered immature, containing nascent neuroblasts and mitotically active progenitor cells. By E8.5, DRG are considered mature, containing post-mitotic differentiated neurons, resident glia, and Schwann cells. To identify molecules regulating sensory neurogenesis and differentiation, we directly compared immature E4.5 DRG to mature E8.5 DRG through a subtraction/differential screening methodology. Preliminary screenings of candidate cDNAs confirmed that they are differentially expressed. We have extensively characterized the in vivo function of one candidate, neural epidermal growth factor-like like 2 (NELL2), a novel secreted glycoprotein, whose role was previously unknown. In ovo microinjection/electroporation of eukaryotic expression vectors driving ectopic NELL2 expression demonstrate that NELL2 promotes neural differentiation autocrinely and stimulates neighboring cells to proliferate, a novel function for this class of molecule and the first demonstrated in vivo function for NELL2. Furthermore, two receptor tyrosine kinases c-Eyk and c-Rek, members of the same RTK family expressed during sensory neurogenesis, have been investigated as to their roles during DRG development. Preliminary analysis indicates that in particular, Rek loss-of-function exhibits a unique phenotype whereby transfected cells cluster in the dorsal pole of the DRG, as if they were inhibited from differentiating. Further experimentation on these receptors is in progress. Finally we are currently developing new technologies to allow general loss-of-function approaches, including dominant negative, DNAenzyme, and RNAi mediated strategies for protein knockdown in ovo.

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GLOSSARY AND ABBREVIATIONS

14-3-3ζ	Zeta isoform of the 14-3-3 family of cytosolic integrators of signal transduction pathways identified in the subtraction screen.
26S ATPase cS4	One subunit of the regulatory cap complex of the 26S proteosome identified in the subtraction screen.
9e10	Monoclonal antibody specific to the Myc antigen.
BDNF	Brain Derived Neurotrophic Factor
Ben	Monoclonal antibody specific to a cell surface antigen, possibly carbohydrate, expressed by neurons and used as a general neural marker.
bHLH	basic Helix-Loop-Helix family of transcription factors
BMP	Bone Morphogenetic Protein, a family of secreted morphogen that patterns the dorsal neural tube
BrdU	Bromodeoxyuridine, a nucleotide analog that is incorporated into replicating DNA, and then used as a marker of S phase proliferating cells with an anti-BrdU antibody.
CMV IE	Cytomegalovirus immediate early enhancer
CNS	Central Nervous System
COS7	Transformed monkey kidney cell line commonly used for eukaryotic protein expression.
Delta/Serrate/Jagged	Transmembrane ligands for the transmembrane receptor Notch, involved in lateral inhibition (neural versus glial decisions).
DMEM	Dulbecco's modified Eagle's medium, supplemented with fetal bovine/calf serum
DRG	Dorsal root ganglion, spinal ganglia that contains the cell bodies of all trunk peripheral sensory neurons.
DsRedI	Novel red fluorescent protein cloned from the Discostoma coral by virtue of its homology to GFP, used as a reporter.

E4.5	Embryonic day 4.5, that is 4.5 days of incubation of a fertilized chicken egg
ECM	Extra-Cellular Matrix
EGF	Epidermal Growth Factor
ER	Endoplasmic reticulum
EST	Expressed sequence tag
ETS	Family of transcription factors containing ETS-domains
Eyk	East Lansing Tyrosine Kinase, a RTK that belongs to the Axl/Tyro/Mer family of RTKs that is expressed in DRG.
G3PDH	Glyceraldehyde 3-phosphodehydrogenase
GFP	Enhanced green fluorescent protein from the bioluminescent jellyfish Aequoria Victoria, used as a reporter.
GST	Glutathione S-transferase, an affinity fusion tag for protein purification.
H3	Histone 3 marker for M phase mitotically active cells.
HcRedI	Far-red fluorescent protein isolated from the reef coral *Heteractis crispa.*
HSPG	Cell surface heparin sulfate proteoglycans
IP	Immunoprecipitation
IRES2:eGFP	Internal ribosomal entry site 2:enhanced GFP expression cassette that allows independent translation of a coding DNA sequence cloned upstream, and eGFP, used as a reporter.
Isl1	Islet1, a member of the LIM homeodomain transcription factor Family
Mash1	Mammalian acheate-scute homolog-1, a member of the bHLH family of proneural transcription factors, vertebrate homologue of Drosophila acheate-scute

Math1	Mammalian atonal homolog-1, a member of the bHLH family of proneural transcription factors, vertebrate homologue of Drosophila atonal
MNR2	A homeodomain transcription factor that is sufficient to direct somatic motor neuron differentiation.
Mrg	MAS-related genes, G protein-coupled receptors (GPCRs) for noxious stimuli expressed by nociceptors.
MyoD	A bHLH transcription factor that is the terminal muscle determination factor.
NELL1	Neural epidermal growth factor-like like 1, a novel secreted glycoprotein named for its EGF-like domains and expression in neural tissues, which is ~50% homologous to NELL2.
NELL2	Neural epidermal growth factor-like like 2, a novel secreted glycoprotein named for its EGF-like domains and expression in neural tissues, which is ~50% homologous to NELL1.
NeuroD	A bHLH transcription factor that is the terminal neuronal determination factor.
NGF	Nerve Growth Factor
Ngn 1/2	Neurogenin 1 and 2, members of the bHLH family of proneural transcription factors, vertebrate homologs of Drosophila atonal.
NKL	Neural Kruppel Like, transcription factor involved in promoting neuronal differentiation.
Notch	Transmembrane receptor for the transmembrane ligands Delta/Serrate/Jagged, involved in lateral inhibition (neural versus glial decisions).
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
N-TSP1	N-terminus Trombospondin 1

OLIG1/2	bHLH transcription factors involved in motor neurons and then oligodendrocyte development.
$p75^{NTR}$	Low affinity neurotrophin receptor
Pax 3/7	Paired box transcription factors
PKC	Protein Kinase C
pMIW	Parent eukaryotic expression that contained the original NELL2 coding sequence, created by removing NELL2 from pMIWC3 (C3 is the clone name for NELL2) with HindIII, and religating; contains the dual Rous Sarcoma Virus Long Terminal Repeat (RSV LTR) and chicken beta-actin promoters.
PNS	Peripheral nervous system
Rek	Retina expressed tyrosine kinase, a RTK that belongs to the Axl/Tyro/Mer family of RTKs that is expressed in DRG.
RNAi	RNA interference, a method for knocking down protein levels
RTK	Receptor Tyrosine Kinases
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
ShH	Sonic hedgehog, a secreted morphogen that patterns the ventral neural tube.
SSH-PCR	Suppressive Subtractive Hybridization PCR
St	Normal stages of chick development described by Hamburger and Hamilton (HH)
TrkA	High affinity RTK for NGF
TrkB	High affinity RTK for BDNF
TrkC	High affinity RTK for NT3
Tuj1	Beta-III tubulin, a neural specific tubulin used as a neural marker.
vWC	von Willebrand Factor C domain

ABSTRACT

Vertebrate neurogenesis occurs through the restricted expression of subsets of genes to discrete cell types that organize into the central and peripheral nervous systems during specific developmental periods. A major goal of developmental biology is to understand the molecular and cellular mechanisms underlying this complex process. An experimentally rich system in which to investigate this question is the dorsal root ganglion (DRG), which derives from a population of migrating neural crest cells that aggregate laterally to the neural tube, and contain the cell bodies of the sensory neurons of the peripheral nervous system. In the chick, the peak of sensory neurogenesis and differentiation in the DRG occurs at embryonic day E4.5, at which DRG are considered immature, containing nascent neuroblasts and mitotically active progenitor cells. By E8.5, DRG are considered mature, containing post-mitotic differentiated neurons, resident glia, and Schwann cells. To identify molecules regulating sensory neurogenesis and differentiation, we directly compared immature E4.5 DRG to mature E8.5 DRG through a subtraction/differential screening methodology. Preliminary screenings of candidate cDNAs confirmed that they are differentially expressed. We have extensively characterized the *in vivo* function of one candidate, neural epidermal growth factor-like like 2 (NELL2), a novel secreted glycoprotein, whose role was previously unknown. *In ovo* microinjection/electroporation of eukaryotic expression vectors driving ectopic NELL2 expression demonstrate that NELL2 promotes neural differentiation autocrinely and stimulates neighboring cells to proliferate, a novel function for this class of molecule and the first demonstrated *in vivo* function for NELL2. Furthermore, two receptor tyrosine kinases c-Eyk and c-Rek, members of the same RTK family expressed during sensory neurogenesis, have been investigated as to their roles during DRG development. Preliminary analysis indicates that in particular, Rek loss-of-function exhibits a unique phenotype whereby transfected cells cluster in the dorsal pole of the DRG, as if they were inhibited from differentiating. Further experimentation on these receptors is in progress. Finally we are currently developing new technologies to allow general loss-of-function approaches, including dominant negative, DNAenzyme, and RNAi mediated strategies for protein knockdown *in ovo*.

1. INTRODUCTION

Vertebrate neurogenesis occurs through the restricted expression of subsets of genes to discrete cell types that organize into the central and peripheral nervous systems during specific developmental periods. This process occurs through the intricate interplay of extrinsic and intrinsic signals that generate a diverse set of neurons and glia from common progenitor populations (reviewed in Morrison, 2001a; Sommer, 2001). A major goal of developmental biology is to understand the molecular and cellular mechanisms underlying this complex process, and much of what has been learned to date began as studies of simpler systems. Early investigations have predominately focused on identifying intrinsic components of this regulatory system, by identifying transcription factors conserved across phylogeny. In *Drosophila* the two proneural basic Helix-Loop-Helix transcription factors *achaete-scute* and *atonal* have been shown to be responsible for the production of the peripheral nervous system external sensory organs and chordotonal organs respectively, while their vertebrate counterparts *mammalian acheate-scute homolog-1 (Mash1)* and the *neurogenins (Ngn)*, which are related to *atonal*, are necessary and sufficient for the differentiation of peripheral autonomic neurons of the sympathetic ganglia and sensory neurons of the dorsal root ganglia, both derivatives of the neural crest (reviewed in Anderson, 1999). Proneural gene expression is transient in progenitors and upregulates downstream family members, such as NeuroD, which drive these cells to differentiate into neurons, analogous to the pro-muscle transcription factor family whereby upstream factors induce MyoD, the terminal muscle determination factor (Perez et al., 1999; Brunet and Ghysen, 1999).

In the developing CNS, the spinal cord has served as an excellent model system because of its well-defined topology, where mitotically active progenitor cells are located in a single-cell layer in the ventricular zone, surrounded by an intermediate and mantle zone where post-mitotic cells migrate laterally to differentiate (Leber et al., 1990). Mash1, ngn1/2, and other members of the bHLH family of proneural transcription factors such as mammalian atonal homologs Math1 are also expressed in the central nervous system, in largely non-overlapping patterns of gene expression confined to the ventricular zone. Discrete patterns of proneural gene expression within the spinal cord suggest that not only are proneural genes sufficient to permit progenitors to differentiate into neurons, but that they might also play a role in specifying neural identity (Sommer et al., 1996). Indeed different members of the proneural bHLH gene family can contribute to specification of neural identity, for example Math1 specifies dorsal interneuron subpopulations (Johnson, 2002). However genetically swapping bHLH coding sequences has revealed that some of these factors are more generally permissive rather than instructive for the formation of neurons, especially the NGNs (Parras et al., 2002). Furthermore proneural genes induce neural differentiation by simultaneously inducing downstream neural factors and sequestering glial-promoting complexes specifically (reviewed in Morrison, 2001b).

Nevertheless different types of progenitor cells must exist such that some give rise to neurons, some give rise to glia, and some give rise to both neurons and glia (Leber et al., 1990), and regulated so that neurogenesis precedes gliogenesis. Accordingly these proneural progenitor cells differentiate into specific types of neurons as the result of the overlapping pattern of homeodomain transcription factor code, which is generated by graded concentrations of secreted ventralizing sonic hedgehog ShH and dorsalizing BMP

signaling, the major identified extrinsic signals in the spinal cord (Briscoe et al., 2000; Briscoe and Ericson, 2001). These homeodomain transcription factors form precise domains within the ventricular zone through the overlapping expression of pairs of cross-inhibitory class I and class II partners. Within a given domain, the co-expression of particular bHLH proneural genes and homeodomain genes results in the differentiation of discrete types of neurons, which are born at precise times of development (reviewed in Lee and Pfaff, 2001).

This is an elegant story for the production of neurons in the CNS, however it must be more complex than this, as at some point the glial derivatives must also be generated from these progenitor domains. Examination of the progenitor cells in these domains reveals that virtually all cells in a given domain express a given homeodomain transcription factor and a proneural transcription factor. The proneural genes are reciprocally regulated through the classic lateral inhibition pathway mediated by the transmembrane signaling receptor Notch and its transmembrane bound ligands Delta/Serrate/Jagged (reviewed by Gaiano and Fishell, 2002). The process of lateral inhibition allows for the selection of non-equivalent cells from a field of otherwise homogenous cells types. In the presumptive nervous system, the expression of Delta in a given cell results in the upregulation of proneural genes and Delta itself in that cell, which in turn signals through Notch on neighboring cells to downregulate proneural and Delta gene expression in surrounding cells, keeping them in an undifferentiated state. Accordingly Notch is expressed throughout the spinal cord ventricular zone, and its ligands are expressed in largely non-overlapping patterns in this zone, suggesting that different Notch ligands and different subsets of proneural genes are regulated in a dorso-ventral pattern as well, although what

mediates this pattern is not currently known (Ma et al., 1997). Analysis of the expression of these genes within a given domain demonstrates that the majority of progenitor cells must co-express Notch and one of ligands at the same time, as each gene appears to be expressed by the majority of cells in that domain. Co-expression of Notch in inner ear precursors and the differential expression of Delta and Serrate results in the production of hair cells and support cells, demonstrating that progenitor cells can widely express Notch and then utilize its different ligands to sort out cell fates (Eddison et al., 2000). Therefore within a given domain in the spinal cord, progenitor cells differentiate into specific classes of neurons based on the overlapping patterns of Notch and its ligands, and the proneural and homeodomain transcription factors.

A major question then is how does this neural differentiation program relate to glial specific differentiation programs? Recently it was demonstrated that they could be directly coupled. The motor neuron specific progenitor domain in the ventral spinal cord is defined by the coexpression of NGN2 and the transcription factors OLIG1/2 from the homeodomain code, originally named for their expression pattern that overlaps with a domain that generates the glial derivatives oligodendrocytes at later stages (reviewed in Kessaris et al., 2001; Zhou and Anderson, 2002). Early in development, this combination results in the birth of all motor neurons, defined by expression of motor neuron determination transcription factor MNR2, which migrate laterally and ventrally to a region outside of the ventricular zone, giving rise to the nascent motor pools. Later, after motor neurons have been generated, NGN2 is downregulated in this domain through an unknown mechanism, which then allows OLIG1/2+ progenitor cells to switch from generating motor neurons to give rise to oligodendrocytes (Zhou and Anderson, 2002). This suggests that an

as-of-yet unidentified extrinsic/intrinsic signal tells the progenitor domains to stop generating neurons and start generating glial derivatives at precise times during development, and that some progenitors remain in these domains to generate these later arising cell types.

Extrinsic and intrinsic mechanisms are also important for the development of the peripheral sensory nervous system that is generated from a subpopulation of neural crest cells (reviewed in Morrison, 2001a; Sommer, 2001). An experimentally rich system in which to investigate this question as it pertains to vertebrate neurogenesis is the dorsal root ganglion (DRG), and is the system that we have utilized in our studies. Neural crest cells are induced to form at the boundaries between the neural plate and epidermal ectoderm during gastrulation. After neural tube closure, the neural crest migrates out of the dorsal neural tube along well-defined routes whereby subpopulations of crest generate different peripheral derivatives (Lallier and Bronner-Fraser, 1988). Mature DRG contain approximately twenty types of post-mitotic neurons that differ in their sensory modalities, morphologies, and biochemistry, in addition to support glial cells (Scott, 1992). Immature ganglia contain a less well-defined population of cells consisting primarily of nascent, undifferentiated neurons and, importantly, mitotically active progenitor cells that can generate neurons and/or glial cells.

In the nascent DRG, the first-born post-mitotic differentiating neurons aggregate and cluster in the core of the ganglia, ensheathed by mitotically active progenitor cells (Lallier and Bronner-Fraser, 1988). Classic cell-lineage studies of neural crest demonstrate that indeed, these are a multipotent stem cell-like population *in vivo*, with certain crest able to generate all subpopulations of peripheral cell types (Fraser and Bronner-Fraser, 1991;

Frank and Sanes, 1991). However it is becoming clear that subpopulations of crest exist with a more restricted lineage *in vivo*, such that some give rise to sensory rather than sympathetic ganglia, and within a given ganglion, some generate different types of neurons and/or glia (reviewed in Anderson, 1999, 2000; Morrison, 2001a, 2001b; Sommer, 2001). For example high concentrations of the extrinsic signaling molecule BMP promotes neural crest to differentiate into sympathetic specific cells, and is expressed by the dorsal aorta endothelial cells *in vivo,* adjacent to the sympathetic ganglia anlagen. This in turn upregulates the proneural gene Mash1, which is necessary and sufficient to drive crest to differentiate into sympathetic neurons when ectopically expressed. Similarly low BMP concentrations promote neural crest to differentiate into sensory rather than sympathetic cells, and result in the upregulation of the proneural genes NGN1/2, which are necessary and sufficient to promote neural crest to differentiate into sensory ganglia specific cells; DRG fail to form in mice with null mutations in both of these genes (Ma et al., 1999). Single mutations in either NGN2 or NGN1 demonstrate that each proneural gene is expressed by a subpopulation of neural crest sensory precursors, that NGN2 is responsible for the early wave of neurogenesis producing TrkC and TrkB neurons, that NGN1 is responsible for a later wave of neurogenesis producing most if not all TrkA neurons, and that these separate subpopulations can compensate for the loss of each other (Ma et al., 1999). This demonstrates a genetic correlate for a heterogeneic sensory neural crest subpopulation, and accordingly overexpression of either NGN in neural crest cells biases them towards a sensory fate (Perez et al., 1999).

However the precise roles of the NGNs in the development of the peripheral sensory nervous system remains unclear, as revealed by recent fate-mapping studies

demonstrate in which cells transiently expressing Ngn2 are permanently labeled. These studies demonstrate that NGN2+ sensory specific neural crest cell subpopulation gives rise to both neurons and glia with no bias, and no apparent bias in sensory neural subpopulations either (Zirlinger et al., 2002). This seems to contradict these previous studies that have shown that NGN2 gives rise to TrkB and TrkC sensory neural subpopulations early and NGN1 gives rise to the TrkA subpopulation later (Ma et al., 1999), and therefore further experiments are needed to elucidate this apparent discrepancy. It was also shown that while NGN2 could permit Mash1-depependent sympathetic precursors to initiate sympathetic ganglion formation, it could not support their proliferation and further development (Parras et al., 2002), and it normally is not expressed in these cells *in vivo* (Perez et al., 1999; Parras et al., 2002; Zirlinger et al., 2002).

Indeed much of what we have learned about the development of DRG has come from the classic studies of the later effects of neurotrophins and the roles of their cognate Trk receptors during target regulated programmed cell death (Hamburger, at al., 1981; reviewed by Farinas et al., 2002). Mature avian DRG that have undergone retrograde neurotrophin transport and target regulated programmed cell death contain a well-defined organization where the first born large diameter neurons (TrkB+ and TrkC+) are located ventro-laterally, and the later-arising small diameter (TrkA+) neurons are located dorso-medially. Interestingly in the course of investigating the role of NGF in the survival of this dorso-medial population of neurons, Hamburger and colleagues described the location in which these neurons originate as being in the medial dorsal. Small diameter neurons accumulated in this region and displaced the larger neurons towards the ventro-lateral region of the ganglion (Hamburger et al., 1981). This population of small diameter

neurons is TrkA+, and genetically arises from the NGN1 precursor pool which undergoes its peak period of neurogenesis and differentiation later at ~E6 (Ma et al., 1999). Immunoreactivity for TrkA expression at E4.5 demonstrates that indeed a population of nascent neurons in the dorsal region are TrkA+, however these nascent neurons actually co-express other neurotrophin receptors, in particular TrkC, and exhibit very dynamic neurotrophin receptor switching during development (Rifken et al., 2000). NGN1 gene expression is confined to the dorsal pole and the ensheathing cell layer progenitor domains (Perez et al., 1999; Nelson and Lefcort, unpublished). Furthermore the downstream proneural gene NeuroD is also restricted to the progenitor domains at these later stages, and not in nascent neuroblasts in the core of the ganglia that widely express pan-neural markers (Nelson and Lefcort, unpublished). Other studies investigating neuron glial decisions in the ensheathing cell layer propose that, as neurons (and perhaps glia) are born they migrate into the core to differentiate (Wakamatsu et al., 2000). This raises the question then, as where exactly do neurons and glia originate in the DRG, and where do they end up? In other words, do neurons and glia intermingle with other nascent neurons in the core, or do they instead pack around the core in layers or groups in neurons? Although not the specific topic of this dissertation, this intriguing question is currently understudy (Nelson and Lefcort, unpublished).

While much is known about the development of peripheral sensory neurons, the set of identified molecules and their functions cannot fully account for the generation of such diversity nor explain this process. We have utilized this system to identify new molecules involved in regulating sensory neurogenesis and differentiation, by designing a subtraction screen to identify genes specifically expressed in immature E4.5 DRG, at a stage that

represents the peak period of neurogenesis and differentiation. The candidate molecules identified in our preliminary analyses have not been previously identified in this tissue. Extensive characterization of one candidate, neural epidermal growth factor-like like 2 (NELL2) has demonstrated a unique expression pattern and intriguing *in vivo* function, the first demonstrated for this family of secreted glycoproteins. We have also studied the *in vivo* function of two receptor tyrosine kinases during DRG development, c-Eyk and c-Rek, members of the same RTK family of receptors. Finally we have sought to develop techniques for *in ovo* knockdowns, some of which should prove to be very useful and expand the range of the chick model system in experimental embryology.

2. IDENTIFICATION OF GENES REGULATING SENSORY NEUROGENESIS AND DIFFERENTIATION

Abstract

The dorsal root ganglia (DRG) derive from a population of migrating neural crest cells that coalesce laterally to the neural tube. As the DRG matures, discrete cell types emerge from a pool of differentiating progenitor cells. To identify genes that regulate sensory genesis and differentiation, we have screened the transcriptomes of immature and mature DRG. Several differentially expressed genes were identified in these analyses that belong to important regulatory gene families. One molecule we identified is a secreted glycoprotein, neural epidermal growth factor-like like 2 (NELL2), which we found to be exclusively expressed in the immature DRG, in addition to exhibiting a strikingly dynamic expression pattern in the developing spinal cord and hindbrain. Confirmation of the differential expression of NELL2 and other genes identified in our screen demonstrate the usefulness of this approach for isolating key regulatory genes dynamically involved in the genesis and differentiation of discrete cell types and tissues (Nelson et al., 2002a).

Introduction

A central question in biology is how a multicellular organism, consisting of an extremely diverse population of cell types, can arise from a single cell. The restricted expression of subsets of genes to a particular tissue during specific developmental stages ultimately results in the differentiation of discrete cell types. The goal is to identify key

genes that regulate such fundamental events as the genesis and differentiation of these discrete cell types. An experimentally rich system in which to investigate this question as it pertains to vertebrate neurogenesis is the dorsal root ganglion (DRG), which derives from a population of migrating neural crest cells that aggregate laterally to the neural tube. Mature DRG contain approximately twenty types of post-mitotic neurons that differ in their sensory modalities, morphologies, and biochemistry, in addition to support glial cells (Scott, 1992). Immature ganglia contain a less well-defined population of cells consisting primarily of nascent, undifferentiated neurons and importantly, mitotically active progenitor cells that can generate neurons and/or glial cells. Molecular differences between immature and mature ganglia will be reflected in their respective transcriptomes and proteomes. Identification of these molecular differences is key to understanding how such diversity arises, as well as to elucidate how developmental programs can go awry to result in sensory neural pathologies.

Once DRG have formed, sensory neurons mature and innervate discrete central and peripheral targets, followed by an extensive period of target-regulated programmed cell death. However, the intervening cellular and molecular events *prior* to the period of target-mediated cell death, yet *subsequent* to neural crest migration, remain incompletely characterized. An elucidation of these events is required because it is during this time period when all of neurogenesis, and the majority of differentiation of discrete classes of sensory neurons occur. Certainly the neurotrophins (NGF, BDNF, NT-3, NT-4/5) and their receptors (TrkA, TrkB, TrkC and $p75^{NTR}$) play critical roles during sensory neurogenesis (Lindsay, 1996), while the basic-helix-loop-helix transcription factors Neurogenin 1 and Neurogenin 2 are required for the formation of DRG, as mice with null

mutations in both of these genes do not form DRG (Ma et al., 1999). In addition, restricted expression of members of the ETS family of transcription factors can influence the functional identity of subsets of sensory neurons (Lin et al., 1998; Arber et al., 2000), and Notch and Delta-1 interactions can modulate neural/glia fate determination in the developing DRG (Wakamatsu et al., 2000). Though these molecules play important roles during DRG development, they alone cannot fully account for the developmental processes underlying the diversity of sensory neural fate decisions. Thus the goal of this study was to identify genes involved in sensory neurogenesis and differentiation in the nascent DRG by searching for genes with a highly restricted developmental expression pattern.

A powerful experimental strategy to identify genes that regulate specific developmental events is to characterize the transcriptome of a tissue at a particular developmental stage. The identification of differentially expressed genes during development begins by either comparing the ensemble of transcripts or the ensemble of proteins from a particular immature tissue to those from the mature tissue. Three previous transcriptome analyses have been performed on the DRG. Akopian and Woods, (1995) employed a subtraction scheme whereby a mixture of non-neural tissue derived cDNA and neural tissue derived cDNA (driver) was subtracted from postnatal rat DRG cDNA (tester). This resulted in the identification of transcripts specifically or highly expressed in the peripheral sensory nervous system. However because the tester was derived from postnatal DRG, the molecular cues that exclusively govern sensory neurogenesis would not be identified because the DRG had already completed neurogenesis by the time the subtraction was performed. A second experimental

approach utilized RNA fingerprinting to identify genes specific to the NT-3 or NGF dependent subpopulations of mature sensory neurons (Friedel, et al. 1997). This resulted in the identification of transcripts differentially expressed in one of these two subpopulations. However, transcriptomes were derived from mature DRG excised several days after the period of neurogenesis, and then cultured for 4 days, thus reducing the likelihood of identifying genes whose function is to regulate sensory neurogenesis, as well as potentially altering their transcriptome by isolating them from their normal embryonic environment. A third recent experiment utilized suppressive subtractive hybridization PCR (SSH-PCR) to compare neonatal DRG from wild type to Ngn1 -/- mice, in which most TrkA+ neurons, including the nociceptive subclass, fail to generate (Dong, et al. 2001). This resulted in the identification of transcripts known to be expressed in TrkA+ neurons, including a new family of G protein-coupled receptors (GPCRs) termed *MAS-related genes (mrgs)* that are specifically expressed in subpopulations of nociceptors. Although this approach exemplifies the utility of combining SSH-PCR and knockout strategies, it still does not address the problem of identifying molecules that regulate DRG neuron genesis and differentiation.

To this end, our strategy was designed to identify genes that function specifically during sensory neurogenesis and differentiation. We utilized SSH-PCR to directly compare the *in vivo* transcriptome of immature E4.5 chick DRG to the in vivo transcriptome of mature E8.5 DRG (Diatchenko et al., 1996). The genes identified in our screens were not found in the previous DRG transcriptome analyses. Our study has revealed several intriguing molecules whose function during DRG development we are now in the process of determining.

Results

DRG derive from migrating neural crest cells that coalesce laterally to the neural tube beginning at ~ Embryonic Day 2.75-3 in the chick (E2.75-3; Lallier and Bronner-Fraser, 1988). Neurogenesis in the nascent DRG then ensues, peaking at ~ E4.5-5, which is followed by target innervation and programmed cell death of post-mitotic neurons between ~ E5-E12, peaking between E7-E9 (Carr and Simpson, 1978). At E4.5, DRG are immature with ca 30% of the cells being mitotically active progenitor cells (Lefcort, unpublished observations), and the majority of the remaining cells being nascent, post-mitotic neurons (Rifkin et al., 2000: Wakamatsu et al., 2000). Gliogenesis becomes prevalent after E6 (Carr and Simpson, 1978). In order to identify genes that regulate sensory neurogenesis and differentiation we isolated DRG from embryos at E4.5, and compared their cDNA to cDNA derived from mature DRG (E8.5) consisting of post-mitotic, well-differentiated neurons and glia (Nelson, B.R., Anderson, L.W., Kasemeir, J., Lefcort, F., 2002, manuscript in preparation).

Full-length Phage E4.5 chick DRG cDNA Library

We began our investigations by creating a full-length phage E4.5 chick DRG cDNA library (Stratagene) for use as a tool with which to obtain full-length clones identified from our screenings. Results from initial screenings with alpha-tubulin and TrkC probes determined that this library does indeed contain large inserts and a sufficiently high titer and complexity, with appropriate frequencies for a highly abundant transcript (alpha-tubulin and moderately abundant transcripts (TrkC and Brn3A; Table 1). Furthermore, additional cDNAs have been identified through PCR based screening for

members of the proto-cadherin, Brn3A transcription factor, and anaplastic lymphoma kinase (ALK) family of RTKs.

Table 1. Full-length phage E4.5 Chick DRG cDNA Library			
Titer = 10^8 plaque forming units/ml			
cDNA	Method	% Frequency	Insert size
Alpha-tubulin	probe	2.8% 140/5000 clones	1.3-2.3kb
TrkC	probe	0.18% 37/20,000 clones	1-4kb
Brn3A	probe	0.137% 11/8000 clones	
Proto-cadherins	PCR		
Alk	PCR		

Subtraction analysis

The *in vivo* transcriptomes of E4.5 and E8.5 DRG were directly compared with a suppressive subtractive hybridization PCR (SSH-PCR) / differential screening methodology (Lukyanov et al., 1995; Munroe et al., 1995; Diatchenko et al., 1996; von Stein et al., 1997). We have initially screened approximately 600 clones from our E4.5 subtracted library with probes derived from total, unsubtracted cDNAs from both ages, and the subtracted cDNAs from both ages. Figure 1 shows the typical results of our differential screening. The top long arrow points to an amplicon from a clone (identified as neural epidermal growth-factor like like 2, NELL2) having different signal intensities

with these probes, indicating that SSH-PCR has significantly enriched NELL2 at E4.5 and that NELL2 is much less expressed at E8.5. The bottom row is a series of control amplicons, and the lower right arrow points out the subtraction of the glyceraldehyde 3-

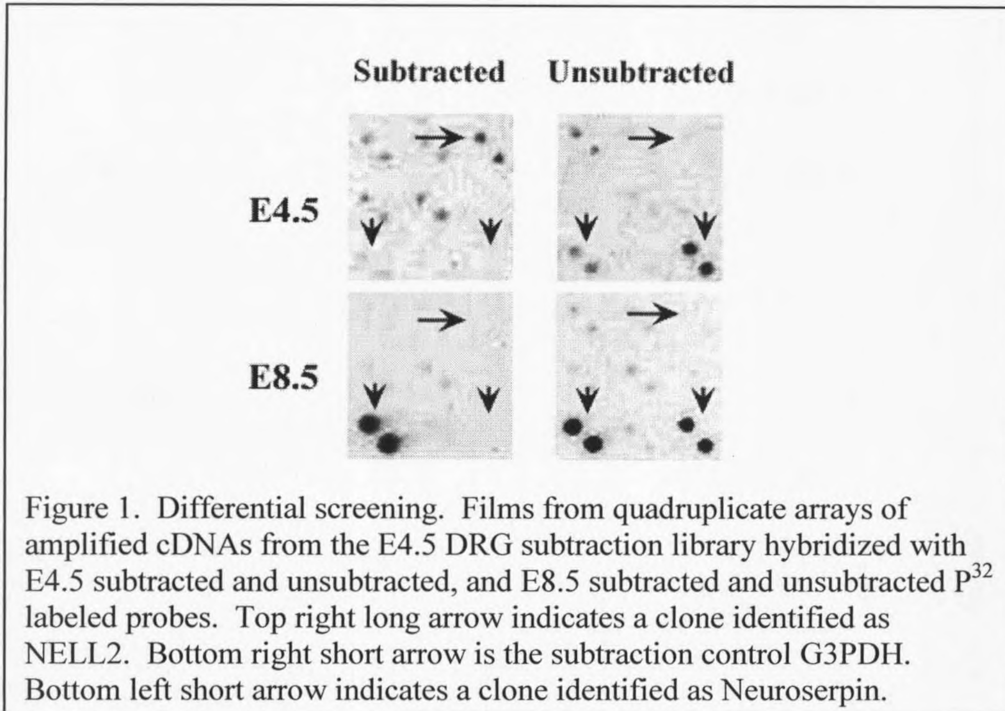

Figure 1. Differential screening. Films from quadruplicate arrays of amplified cDNAs from the E4.5 DRG subtraction library hybridized with E4.5 subtracted and unsubtracted, and E8.5 subtracted and unsubtracted P^{32} labeled probes. Top right long arrow indicates a clone identified as NELL2. Bottom right short arrow is the subtraction control G3PDH. Bottom left short arrow indicates a clone identified as Neuroserpin.

phosphodehydrogenase (G3PDH) amplicon in both subtracted probe sets, while strong signals are seen in both unsubtracted probe sets, indicative of the success of SSH-PCR. The amplicon in the lower left corner (left arrow) is a random clone picked from the E8.5 subtracted library as a reverse control, showing that it was significantly enriched by the reverse experiment and, interestingly, was later identified as Neuroserpin, a gene known to be involved in and expressed in later stages of neural differentiation (Osterwalder et al., 1996; Krueger et al., 1997).

Analysis of the SSH-PCR experiment indicates that 1) G3PDH, a highly abundant house-keeping gene present in both E4.5 and E8.5 transcriptomes was efficiently subtracted out, 2) clones from the E4.5 subtraction library show differential signal

Table 2. Differentially expressed cDNAs identified by SSH-PCR

cDNA	Embryonic Day	# of clones
Neural Epidermal Growth Factor-Like Like 2 (NELL2)	E4.5	41
14-3-3ζ / YWHAZ / *Leonardo*	E4.5	13
26S ATPase complex Subunit 4	E4.5	13
Novel	E4.5	1
Neuroserpin	E8.5	1

intensities indicating that enrichment occurred for a subset of E4.5 specific genes in the forward experiment, and 3) clones from the E8.5 subtraction library show differential signal intensities as well, indicating that the reverse experiment was successful in enriching for E8.5 specific genes. This demonstrates that our transcriptome analysis effectively enriched differentially expressed transcripts and subtracted common, housekeeping transcripts. Table 2 lists the candidate differentially expressed genes chosen on the basis of their specifically enriched signal in the E4.5 subtraction library, ~70 candidates were sequenced. We are continuing to screen this library by probing it with a mixture of probes made by PCR-DIG labeling of previously identified clone fragments, which has already yielded a new novel candidate cDNA. Furthermore we examine the other subtraction library that is enriched for E8.5 specific cDNAs to identify candidates with potential roles in mature ganglia. Finally we will screen a complementary retina subtraction library (T. Reh), enriched for genes expressed in immature retinal progenitor domains rather than mature differentiating retinal domains,

with our DRG subtraction probe sets in a screen designed to identify genes common and unique to these developmental processes in the CNS and PNS.

RT-PCR confirmation of differential gene expression

To verify that the cDNAs isolated in our screens were in fact differentially expressed, we used RT-PCR to compare the relative expression levels of the identified, candidate genes from both differential-screening experiments. Total RNA from E4.5 and E8.5 DRG were quantified by absorption and their concentrations normalized for RT-PCR

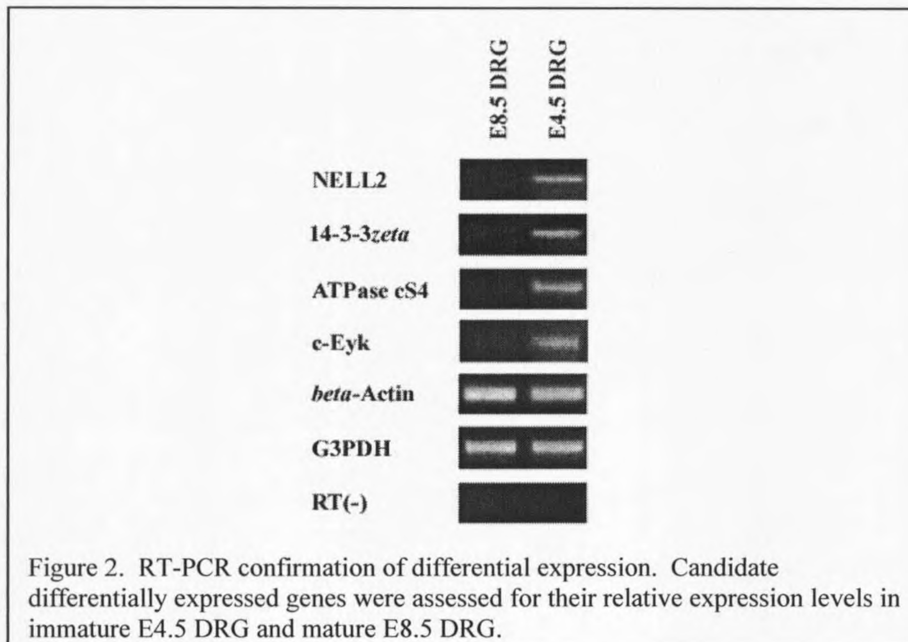

Figure 2. RT-PCR confirmation of differential expression. Candidate differentially expressed genes were assessed for their relative expression levels in immature E4.5 DRG and mature E8.5 DRG.

analysis. Figure 2 demonstrates that the common, housekeeping transcripts beta-actin and G3PDH were present at equal amounts in both samples, and the RT(-) controls demonstrate no genomic DNA contamination in either sample. Indeed RT-PCR of all the identified transcripts from the subtraction analysis confirms their differential expression. NELL2, 14-3-3ζ, and 26S ATPase cS4 were robustly expressed in the immature E4.5

DRG compared to the mature, fully differentiated E8.5 DRG. Furthermore c-Eyk, a candidate RTK identified from a RTK specific differential screen (Anderson and Lefcort unpublished) is also preferentially expressed in the immature E4.5 DRG. These results confirm that the molecules identified in our comparative transcriptome analyses are dynamically expressed during sensory neuron genesis and differentiation.

Discussion

A powerful strategy for understanding the development of specialized cells is to identify the mRNA transcripts that are selectively expressed in defined cell types at discrete stages. Toward this end we have used suppressive subtractive hybridization PCR (SSH-PCR) to identify genes expressed specifically during the peak period of neurogenesis and differentiation in the DRG. Additionally we constructed a full-length phage E4.5 chick DRG cDNA Library for use in obtaining full-length cDNAs from candidate differentially expressed fragments identified in our analysis.

Evidence for the effectiveness of our suppressive subtractive hybridization (SSH) screen includes confirming by RT-PCR that the identified cDNAs from the forward subtraction were in fact differentially expressed in the immature E4.5 DRG compared to mature E8.5 DRG (see Figure 2), and confirming with *in situ* hybridization that NEL is robustly expressed in immature E4.5 DRG while being virtually undetectable in mature E8.5 DRG (Nelson et al., 2002b). The usefulness of this strategy is also supported by the fact that the functions of the identified gene products correlate with mediation of important cellular events during development. For example, neuroserpin, the clone that showed enrichment from the reverse experiment, is an axonally secreted inhibitor of

serine-proteases that is developmentally regulated and important for neuronal migration, neurite outgrowth, and synaptogenesis (Osterwalder et al., 1996; Krueger et al., 1997). ATPase complex subunit 4 (S4) is one of a number of different subunits of the multimeric regulatory complex that combines with the 20S proteolytic core to form the 26S proteasome, which mediates selective protein degradation (Dubiel et al., 1992; Singh et al., 1996). Regulation of cellular proliferation through ubiquitin-mediated proteolytic degradation of cell cycle components is a crucial function of the proteasome, and its levels are particularly high in immature and mitotically active cells (Ichihara and Tanaka, 1995). Furthermore subunits of the proteasome are regulated both spatially and temporally in developing embryos, and also exhibit dynamic subcellular localization during cellular proliferation and differentiation events (Pal et al., 1988; Klein et al., 1990; Kawahara and Yokosawa, 1992; Amsterdam et al., 1993; Hutson et al., 1997). Regulation of protein levels and cellular processes through selective degradation is as essential as the selective transcription/translation events normally associated with these cellular events. Therefore controlling the levels of the various, individual proteasomal subunits themselves would be a highly efficient method for the cell to regulate its degradation machinery.

Another molecule identified in our screen is the zeta isoform of the 14-3-3 family of proteins, which are key regulators and integrators of many different signal transduction pathways, and therefore mediate such diverse cellular processes as proliferation, differentiation, survival and apoptosis (Skoulakis and Davis, 1998; Fu et al., 2000). In fact more than 50 different proteins have been found to associate directly with 14-3-3 family members such as receptor and non-receptor protein kinases and phosphatases,

docking and adaptor proteins, transcription and virulence factors, and other assorted proteins and enzymes including B- and D-Raf and PKC (reviewed by Fu et al., 2000). Interestingly, they are abundantly expressed in the nervous system and disruptions in their function perturb neuronal differentiation (Chang and Rubin, 1997; Kockel et al., 1997; Li et al., 1997; Skoulakis and Davis, 1998). For example, disruption of 14-3-3 binding to the B-Raf kinase domain uncouples NGF induced cellular differentiation of PC12 cells (MacNicol et al., 2000). Furthermore 14-3-3 proteins were first described as activators of tyrosine and tryptophan hydroxylases in the presence of Ca^{+2} calmodulin-dependent protein kinase II, which are the rate-limiting enzymes in the serotonin and catecholamine biosynthesis pathways; interestingly serotonin has been shown to influence neural crest development (Ichimura et al., 1987; Garcia-Arraras and Martinez, 1990; Moiseiwitsch and Lauder, 1995; Hansson et al., 1999). Regulation of 14-3-3 proteins themselves should therefore be under tight control, and it is known that their developmental expression pattern is highly dynamic and complex (Skoulakis and Davis, 1998). Changes in 14-3-3 isoform gene expression during development would also be a highly efficient method of regulating this important class of molecules, and would be expected to alter a cell's ability to integrate various signaling pathways, ultimately influencing cell fate. We have shown that indeed 14-3-3ζ is differentially expressed during DRG development (Figure 2), and therefore it is likely that 14-3-3ζ mediates the integration of signals important for determining neuronal fates during the early stages of ganglion formation. Further experimentation will be necessary to elucidate the exact role 14-3-3ζ plays in this complex process.

Another candidate cDNA fragment of considerable interest identified in our subtraction screen is chicken neural epidermal growth factor-like (NEL, Matsuhashi et al., 1995), by DNA sequencing and BLAST analysis of GenBank (Altschul et al., 1990). Two human and rat cDNAs were later independently identified as NEL-Like 1 and NEL-Like 2; chicken NEL is ~40-50% homologous to NELL1, and ~80% homologous to NELL2 (Watanabe et al., 1996; Kuroda et al., 1999). Therefore we refer to chicken NEL as NELL2; chicken NELL1 has not yet been identified. NELL2 contains six EGF-like domains, which are likely to serve as potential binding sites for novel protein interactions (for schematic see Fig 8 A; Kuroda et al., 1999; Kuroda and Tanizawa, 1999; Oyasu et al., 2000). NELL2 also belongs to the Laminin G / N-terminus Trombospondin 1 (N-TSP1) / Pentraxin gene superfamily (Beckmann et al., 1998). This is an extensive class of multi-domain adhesive proteins in the extracellular matrix that act as molecular bridges between cells and the matrix, and participate in cell-cell communication. NELL2 and TSP1 are structurally similar proteins: both contain Epidermal Growth Factor-Like domains (EGF), von Willebrand Factor C domains (vWC), and a similar N-terminal domain, a heparin binding N-TSP1 motif, which strongly suggest interactions with cell surface heparin sulfate proteoglycans HSPGs. Based on sequence analysis and homologies to other proteins containing these domains, NELL2 has been proposed to act as a signaling ligand in similar key developmental events as function the Notch/DSL, NRG, SLIT, and TSP1 proteins (Shah et al., 1994; Matsuhashi et al., 1995; Watanabe et al., 1996; Kuroda et al., 1999; Yuan et al., 1999).

In summary our transcriptome analysis has successfully identified genes likely to be involved in mediating neurogenesis and differentiation rather than common

housekeeping genes. The known and proposed functions of the identified genes correlate well with the developmental stages of the DRG at which they are expressed. We also obtained a novel candidate differentially expressed cDNA fragment that can be used to screen our full-length library, made into a probe for *in situ* hybridization studies, and even used as a target sequence to specifically knockdown the in vivo gene product through an RNAi-mediated strategy (in progress, see Chapter 5). Investigations into the functions of the identified molecules should provide considerable insight into the cellular and molecular mechanisms regulating sensory neurogenesis and differentiation in the DRG. Currently we have initiated a complementary proteomic analysis of immature and mature DRG by searching for proteins that are differentially regulated during DRG development (Nelson and Lefcort, unpublished).

Experimental Procedures

Construction of full-length phage E4.5 chick DRG cDNA library

Fertilized white leghorn eggs were obtained (Spafas), and grown to appropriate stages (Hamburger and Hamilton, 1951). All embryos were treated in accordance with IACUC stipulations. E4.5 DRG mRNA was isolated using MicroPoly(A)Pure (Ambion, Inc.) as directed. This was converted to full-length double-stranded cDNA and packaged into Lambda phage as directed (Stratagene). PCR-DIG labeled probes generated from a fragment of alpha-tubulin and the extra-cellular domain of TrkC were used to screen the

library (see below), candidates were selected, inserts checked via restriction analysis, sequenced, and identified by blasting GenBank.

Suppressive Subtractive Hybridization PCR (SSH-PCR)

SSH-PCR was used to analyze transcriptome differences between immature, embryonic day 4.5 dorsal root ganglia (E4.5 DRG) and mature, fully differentiated E8.5 DRG (Lukyanov et al., 1995; Diatchenko, et al., 1996). E4.5 and E8.5 DRG mRNAs were isolated using MicroPoly(A)Pure (Ambion, Inc.), as directed. These mRNAs (2ug each) were then prepared for SSH-PCR analysis using PCR-Select (Clontech), as directed. The forward subtraction experiment consisted of subtracting E8.5 DRG cDNA (driver) from E4.5 DRG cDNA (tester), enriching for E4.5 specific cDNAs. The reverse subtraction experiment consisted of subtracting E4.5 DRG cDNA (driver) from E8.5 DRG cDNA (tester), enriching for E8.5 specific cDNAs. E4.5 and E8.5 DRG cDNA subtraction libraries were created from both the forward and reverse subtraction experiments by TA-cloning an aliquot of each secondary, nested PCR reaction into the PCR II vector (Invitrogen). A modified secondary, nested PCR reaction was used to radiolabel ($dCTP^{32}$ 800Ci/mMol, New England Nuclear) or chemically label (PCR DIG dNTP labeling mix, Roche Molecular Biochemicals) E4.5 and E8.5 DRG subtracted and unsubtracted control cDNAs for differential screening. Unincorporated label was removed from all probes with QIAquick PCR Purification spin columns (Qiagen).

DRG cDNA subtraction library differential screening

The E4.5 DRG cDNA subtraction library was differentially screened for candidate differentially expressed clones (Munroe et al., 1995; von Stein et al., 1997). Initially 300 random, recombinant clones were selected for differential screening and used to inoculate 100ul LB-kan/amp cultures grown overnight at 37° C. Inserts were amplified by colony PCR with the nested primer set, and analyzed on large format, 2% Ag/EtBr gels. The remaining cultures were then mixed with glycerol and frozen at –80°C. 0.5ul of each diluted amplicon (1:10) was arrayed in duplicate, onto quadruplicate, positively charged nylon membranes (Osmonics, Inc.). Because the nested primer set was used to create both the amplicons and the probes, oligos corresponding to the sequences of both adaptors were synthesized (MWG Biotech, Inc.) and used to block hybridization signals due to adaptor background. As a control for adaptor background, the 603bp band from the Phi-X174 DNA/HaeIII (Promega) phage subtraction control experiment, which is flanked by both adaptors, was gel extracted (QIAquick Gel Extraction, Qiagen), re-amplified with the nested primer set, and prepared for arraying. As a control for subtraction efficiency, specific primers were used to amplify a segment of G3PDH, which was then prepared for arraying (see RT-PCR methods for sequences). As further controls for subtraction efficiency, 5 random, recombinant clones from the E8.5 DRG cDNA subtraction library were also prepared for arraying. Membranes were denatured in 0.5M NaOH, 1.5M NaCl for 2min, neutralized in 0.5M Tris-Cl, pH 7.5, 1.5M NaCl for 5min, rinsed in 0.2M Tris-Cl/2X SSC for 30 sec, and UV cross-linked (Stratalinker, Stratagene). Membranes were pre-hybridized with the blocking oligos for 1hr in standard hybridization buffer at 72° C in a Mini Oven MKII (Hybaid). Probes were mixed with

another equivalent of blocking oligos and denatured by boiling for 5min, cooled on ice for 2min, added to the membranes and hybridized overnight at 72° C. Membranes were washed twice in 2X SSC/0.1%(w/v) SDS for 15 min at 72° C, and then washed twice again in 0.5X SSC/0.1% (w/v) SDS for 15 min at 72° C. Radiolabeled membranes were then exposed to film (BioMax, Kodak) for 2-7 days at –80° C. DIG labeled membranes were prepared for chemiluminescent detection as directed (Roche Molecular Biochemicals) and exposed to film (BioMax, Kodak) for 5-15min at room temp.. Candidate differentially expressed cDNA clones were grown, plasmids were isolated with the QIAprep mini-prep kit (Qiagen), sequenced on an ABI PRISM Genetic Analyzer with the BIG DYE Terminator kit, and analyzed with Sequencher 3.0 (Gene Codes). Candidates with signals specifically enriched by the subtraction were picked for identification; sequences were aligned to GenBank using the BLAST program. From the first 300 clones arrayed, ~40 were chosen for sequencing. This yielded a large number of cDNA inserts corresponding to NELL2. Therefore a DIG labeled cDNA probed was prepared from one of these clones and used to screen the E4.5 subtraction library to select 300 additional, non-NELL2 recombinant clones. These non-NELL2 clones were prepared and screened as described. Candidates were chosen on the basis of their signal intensities as above, and sequenced. This approach resulted in identification of additional cDNAs, as would additional rounds of screenings and selections in this manner (see Table 2 for results).

Confirmation of differential gene expression

RT-PCR from E4.5 and E8.5 DRG total RNA with primers specific to NELL2, 14-3-3ζ, cS4, and Eyk was used to confirm their differential gene expression. E4.5 and E8.5 DRG were collected as described above, and total RNA isolated with standard techniques. Genomic DNA contamination was removed by digesting with 2U RQ1 RNase-free DNase (Promega) for 30min at 37°C, phenol/chloroform extracting, and isoproponol precipitation. Total RNA was resuspended in 25ul nuclease-free H_2O, quantified by absorption (GeneQuant, Pharmacia Biotech). MacVector 6.5.3 (Oxford Molecular) was used to design specific primers from available sequence data for chicken G3PDH, *Beta*-actin, NELL2, 26S ATPase complex Subunit 4, and Eyk. As the chicken 14-3-3ζ sequence is unknown, the 14-3-3ζ forward primer was designed from a conserved region between human 14-3-3ζ (accession number NM_003406) and mouse 14-3-3ζ (accession numbers D830337 and D87660), upstream from our candidate differentially expressed sequence to yield a specific 800bp amplicon. The ACCESS System (Promega) was used for single-step RT-PCR from total RNA, as directed. Equal concentrations of E4.5 and E8.5 DRG total RNA were used to analyze the relative expression levels of these genes at these developmental stages (150ng for controls and Eyk, 250ng for NELL2, S4, 14-3-3ζ). 5ul aliquots were removed at 20X, 30X, and 40X cycles of PCR and analyzed on 2%Ag/EtBr gels. Single bands corresponding to the correct sizes were detected for all the subtraction clones and controls. Eyk amplicon was confirmed by DNA sequencing. Figure 2 shows results for 30X cycles of PCR.

Specific primer sets

NELL2 (forward 5'-CTCCCACACAGAAGTATTCCC, reverse 5'-TAGGCATTGTCCTCCCGAAG); 14-3-3ζ (forward 5'-GCCTTCCAACTTTTGTCTGCCTC, reverse 5'-TGTTTTTACTGCTGCGCTCG); 26S ATPase complex Subunit 4 (forward 5'-TGGGGAAGAAGAAGAAGAAGAAGACG, reverse 5'-TCATCCATCAGGACTCCAATCAC); G3PDH (forward 5'-ACCACAGTCCATGCCATCAC, reverse 5'-TCCACAACACGGTTGCTGTA); *Beta*-actin (forward 5'-TGGAATCCTGTGGCATCCATGAAAC, reverse 5'-TAAAACGCAGCTCAGTAACAGTCCCG); Eyk (forward 5'-TTGGATTCGGTCATCCCCCAAG, reverse 5'-TCCTGATTCACACCCTGATTTG);

GenBank submission

Chicken 14-3-3ζ partial cDNA sequence has been submitted to GenBank, accession number AF465638. Novel candidate partial cDNA sequence will also be submitted.

3. RESTRICTED NEURAL EPIDERMAL GROWTH FACTOR-LIKE LIKE 2 (NELL2) EXPRESSION DURING MUSCLE AND NEURONAL DIFFERENTIATION

Abstract

We have identified a secreted glycoprotein, neural epidermal growth factor-like like 2 (NELL2), in a screen designed to isolate molecules regulating sensory neuron genesis and differentiation in the dorsal root ganglia (DRG; Nelson et al., 2002a, Chapter 2). In investigating NELL2 expression during embryogenesis, we demonstrate here that NELL2 is highly spatially and temporally regulated, being only transiently expressed in discrete regions of the CNS, PNS, and in a subset of mesodermal derived structures during their peak periods of development. In the CNS and PNS, NELL2 is maximally expressed as motor and sensory neurons differentiate. Interestingly its expression is restricted to sublineages of the neural crest, being strongly expressed throughout the immature DRG, but excluded from sympathetic ganglia. Similarly during muscle development, NELL2 is specifically expressed by hypaxial muscle precursor cells in the differentiating somite and derivatives in the forelimbs and body wall, but not by epaxial muscle precursors. Furthermore, NELL2 is differentially regulated in the CNS and PNS: in the CNS, NELL2 is only expressed by nascent, post-mitotic neurons as they commence their differentiation, yet in the PNS, NELL2 is expressed by subsets of progenitor cells in addition to nascent neurons. Based on this restricted spatial and temporal expression pattern, functional studies are in progress to determine NELL2's role during neuronal differentiation in both the PNS and CNS (Nelson et al., 2002c, Chapter 4)

Results and Discussion

The restricted expression of subsets of genes to a particular tissue during specific developmental stages ultimately results in the differentiation of discrete cell types. The goal is to identify key genes that regulate the genesis and differentiation of these discrete cell types. An experimentally rich system in which to investigate this question as it pertains to vertebrate neurogenesis is the dorsal root ganglion (DRG). DRG derive from a subpopulation of migrating neural crest cells that coalesce laterally to the neural tube (Lallier and Bronner-Fraser, 1988). As the DRG matures, discrete cell types emerge from a pool of differentiating progenitor cells. Mature DRG contain approximately twenty types of post-mitotic neurons that differ in their sensory modalities, morphologies, and biochemistry, in addition to support glial cells (Scott, 1992).

Once DRG have formed, sensory neurons mature and innervate discrete central and peripheral targets, followed by an extensive period of target-regulated programmed cell death (Lindsay 1996). However, the intervening cellular and molecular events that occur *prior* to the period of target-mediated cell death, yet *subsequent* to neural crest migration, remain incompletely characterized. An elucidation of these events is required because it is during this time period when the majority of neurogenesis and differentiation of discrete classes of sensory neurons occurs. Certainly molecules such as the neurotrophins and their cognate Trk receptors, the transcription factors neurogenin 1and 2 and members of the ETS family, and Notch and Delta-1 interactions play critical roles during sensory neurogenesis (Lindsay, 1996; Lin et al., 1998; Ma et al., 1999; Arber et al., 2000; Wakamatsu et al., 2000). However, given the complexity of cellular and molecular interactions mediating DRG development, it is evident that additional

molecular mechanisms must be elucidated. Ultimately an interplay between extrinsic and intrinsic signals is thought to sculpt the formation of the final pattern of cell types within the DRG (Morrison 2001; Sommer 2001).

Neurogenesis in the nascent chick DRG peaks at HH st 25/E4.5-5, which is followed by target innervation and programmed cell death of post-mitotic neurons between E5-E12 (Carr and Simpson, 1978). At E4.5, DRG are immature with ~30% of the cells being mitotically active progenitor cells (Lefcort, unpublished observations), and the majority of the remaining cells being nascent, post-mitotic neurons (Pannese, 1974; Rifkin et al., 2000; Wakamatsu et al., 2000). Gliogenesis becomes prevalent after E6 (Carr and Simpson, 1978). By E8.5 DRG are considered mature in that neurogenesis is complete, neurons express their mature pattern of neurotransmitters, Trk receptors and ETS transcription factors, and the majority of fibers have innervated their targets (Pannese, 1974; Carr and Simpson, 1978; Lindsay 1996; Lin et al., 1998; Arber et al., 2000; Rifkin et al., 2000). Thus in order to identify genes that regulate sensory neurogenesis and differentiation we isolated DRG from immature embryos at E4.5, and compared their cDNA to cDNA derived from mature E8.5 DRG. E4.5 and E8.5 DRG were directly compared with a suppressive subtractive hybridization PCR (SSH-PCR) / differential screening methodology (Lukyanov et al., 1995; Munroe et al., 1995; Diatchenko et al., 1996; von Stein et al., 1997) the results of which will be published separately (see Chapter 2). A candidate cDNA fragment was identified as chicken neural epidermal growth factor-like (NEL, Matsuhashi et al., 1995), by DNA sequencing and BLAST analysis of GenBank (Altschul et al., 1990). Two human and rat cDNAs were later independently identified as NEL-Like 1 and NEL-Like 2; chicken NEL is ~40-50%

homologous to NELL1, and ~80% homologous to NELL2 (Watanabe et al., 1996; Kuroda et al., 1999). Therefore we refer to chicken NEL as NELL2; chicken NELL1 has not yet been identified.

Sequence analysis of NELL2 and initial *in vitro* biochemical characterization indicate that it is a large peptide, ~90kD, with ~50kD of N-linked carbohydrate, contains a cleavable N-terminal signal peptide sequence, and in addition to monomers forms putative homo-trimers in solution (see Fig 8 A for schematic; Matsuhashi et al., 1995; Watanabe et al., 1996; Kuroda et al., 1999). Furthermore NELL2 belongs to an intriguing class of proteins that contain EGF-like domains (Engel, 1989; Davis, 1990), including amongst others, Notch and its ligands Delta/Serrate/Lag (DSL), the Neuregulin (NRG) family of ligands, and the SLIT family of ligands. NELL2 contains six EGF-like domains, which are likely to serve as potential binding sites for novel protein interactions (Kuroda et al., 1999; Kuroda and Tanizawa, 1999; Oyasu et al., 2000). NELL2 also belongs to the Laminin G/N-terminus Thrombospondin 1 (N-TSP1)/Pentraxin gene superfamily (Beckmann et al., 1998), a large class of multi-domain adhesive proteins in the extracellular matrix that act as molecular bridges between cells and the matrix, and participate in cell-cell communication. Based on homologies to the Notch/DSL, NRG, SLIT, and TSP1 proteins, NELL2 has been proposed to act as a signaling ligand in similar key developmental events.

RT-PCR confirmation of differential NELL2 expression

To verify that the candidate NELL2 cDNA isolated in our screen was in fact differentially expressed, we used RT-PCR to independently compare the relative

expression levels of NELL2 in both immature E4.5 and mature E8.5 DRG (Figure 3 A). Figure 3 A demonstrates that the common, house-keeping transcripts β-actin and glyceraldhyde-3 phosphodehydrogenase (G3PDH) were present at equal amounts in both samples, and the RT(-) controls with G3PDH primers show no genomic DNA contamination in either sample. In confirmation of our subtraction and differential screen, the RT-PCR results demonstrate that NELL2 is more robustly expressed in the immature E4.5 DRG (Fig 3 A, 1) compared to the mature, differentiated E8.5 DRG (Fig 3 A, 2).

In situ hybridization analysis of NELL2 expression during embryogenesis

NELL2 expression peaks during sensory and motor neuron differentiation. To further analyze NELL2's differential expression during DRG development, we conducted *in situ* hybridizations on transverse sections of chick embryos at key stages of DRG development (Hamburger and Hamilton, 1951). NELL2 expression commences with the onset of DRG gangliogenesis (data not shown), and is strongly expressed in DRG by st 21 (Fig 3 H). Figure 3 B-D demonstrates that at E4.5, NELL2 is specifically expressed in the DRG, including the dorsal pole (arrow), during the peak period of neurogenesis (Fig. 3 B), in agreement with the initial description of NELL2 in the DRG at this stage (Matsuhashi, et al. 1995). Furthermore, within the subsequent 24hrs (E5.5, data not shown) NELL2's expression in the DRG dramatically decreases, becoming virtually undetectable in maturing E6.5 and mature E8.5 ganglia (Fig 3 C, D

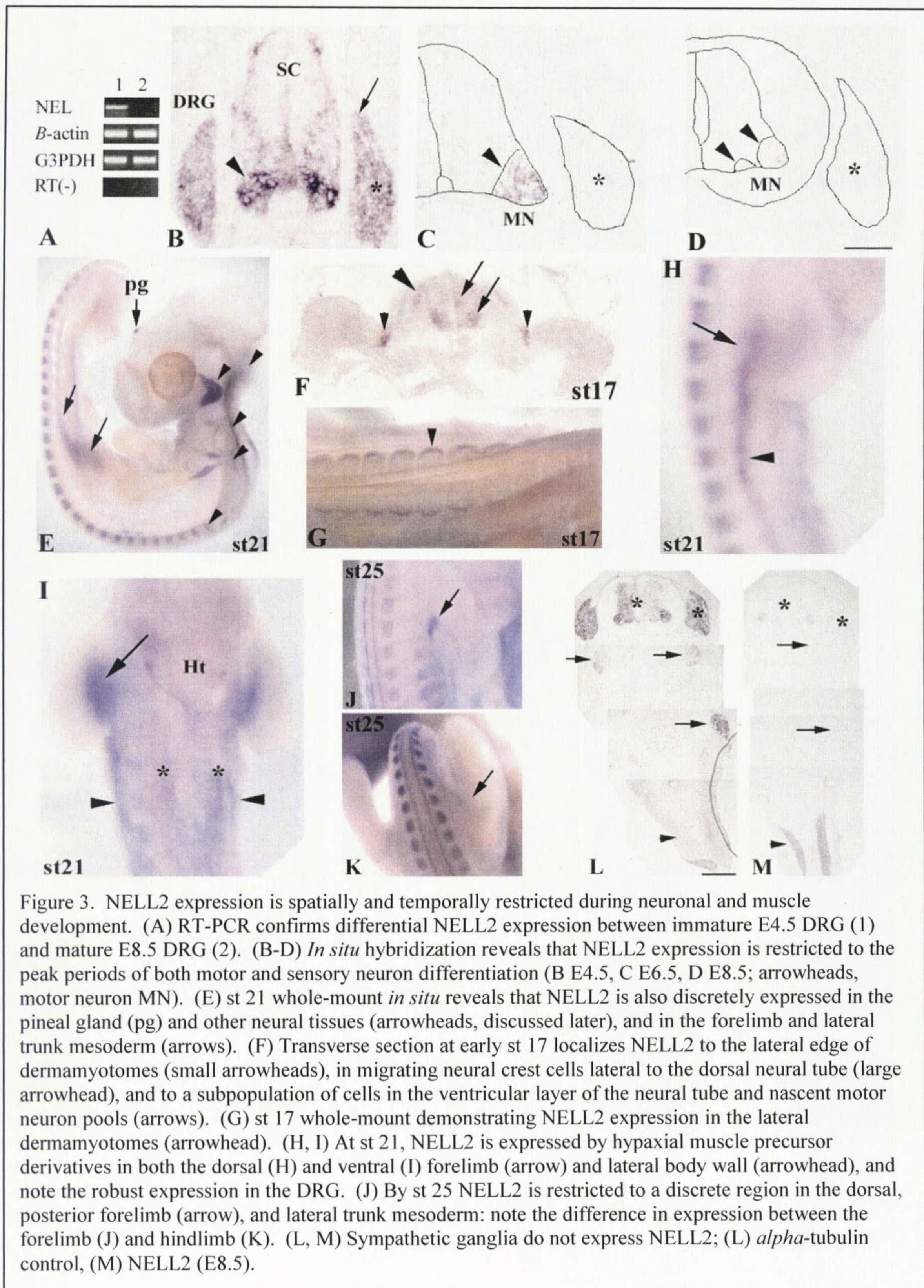

Figure 3. NELL2 expression is spatially and temporally restricted during neuronal and muscle development. (A) RT-PCR confirms differential NELL2 expression between immature E4.5 DRG (1) and mature E8.5 DRG (2). (B-D) *In situ* hybridization reveals that NELL2 expression is restricted to the peak periods of both motor and sensory neuron differentiation (B E4.5, C E6.5, D E8.5; arrowheads, motor neuron MN). (E) st 21 whole-mount *in situ* reveals that NELL2 is also discretely expressed in the pineal gland (pg) and other neural tissues (arrowheads, discussed later), and in the forelimb and lateral trunk mesoderm (arrows). (F) Transverse section at early st 17 localizes NELL2 to the lateral edge of dermamyotomes (small arrowheads), in migrating neural crest cells lateral to the dorsal neural tube (large arrowhead), and to a subpopulation of cells in the ventricular layer of the neural tube and nascent motor neuron pools (arrows). (G) st 17 whole-mount demonstrating NELL2 expression in the lateral dermamyotomes (arrowhead). (H, I) At st 21, NELL2 is expressed by hypaxial muscle precursor derivatives in both the dorsal (H) and ventral (I) forelimb (arrow) and lateral body wall (arrowhead), and note the robust expression in the DRG. (J) By st 25 NELL2 is restricted to a discrete region in the dorsal, posterior forelimb (arrow), and lateral trunk mesoderm: note the difference in expression between the forelimb (J) and hindlimb (K). (L, M) Sympathetic ganglia do not express NELL2; (L) *alpha*-tubulin control, (M) NELL2 (E8.5).

respectively). Interestingly, NELL2 also exhibits a strikingly dynamic pattern of expression in the spinal cord (SC; compare Fig 3 B to C to D). NELL2 is initially highly expressed throughout the mantle layer of the spinal cord, a region undergoing differentiation (Fig 3 B; see also Matsuhashi, et al. 1995), with extremely high levels of expression in motor neuron regions (arrowheads). Within the next few days NELL2 expression is rapidly reduced, becoming restricted to discrete motor neuron pools at later stages (Fig 3 C, D, arrowheads), along the rostral-caudal axis in both lateral and medial motor columns. These results prompted us to investigate additional tissues and stages in which NELL2 might be discretely and dynamically expressed. Whole-mount *in situ* hybridization demonstrates that in addition to expression in the pineal gland (pg), NELL2 is expressed in cranial sensory ganglia and discrete regions within the CNS (Fig 3 E arrowheads, discussed later), and in discrete mesodermal derivatives in the limbs and lateral trunk (Fig 3 E, arrows).

NELL2 is expressed by sublineages of the somite and neural crest. To further characterize NELL2's expression, we examined embryos from earlier and later stages of development. Weak NELL2 expression could first be detected in somites and the neural tube at st 13-15 (data not shown). By st 17 (Fig 3 F, G) NELL2 transcripts are upregulated, and can be detected in the lateral edges of somites encompassing the prospective wing bud and trunk levels. Transverse sections at early st 17 (Fig 3 F; small arrowheads) and whole-mounts at st 17 (Fig 3 G) indicate that this signal is localized to the lateral edge of the dermomyotome, which contain the skeletal/hypaxial muscle precursor cells that migrate into the limbs and trunk to give rise to all skeletal muscle

derivatives (C. Krull, personal communication; Ordhal and Le Douarin, 1992). NELL2 expression also increases during this period in the neural tube, and by st 17 (Fig 3 F arrows) strong expression can be detected in the early spinal cord, especially in nascent motor neuron regions, and in a subset of migrating neural crest cells (Fig 3 F, large arrowhead).

NELL2 is later expressed in discrete regions in the forelimb and lateral trunk mesoderm, by the progeny of the labeled cells detected in the younger somite that have migrated to their target fields. At st 21 NELL2 is expressed in the posterior region of the dorsal, and ventral forelimb (Fig 3 H-I). Also note the faint labeling in the gut (Fig 3 I asterisks), which corresponds to the developing mesonephros and in the heart, corroborating previously reported NELL2 expression in the early kidney and heart (Matsuhashi et al., 1995; Watanabe et al., 1996). Between st 23-25 NELL2 expression in the dorsal forelimb becomes localized to a very discrete posterior region, coinciding with the posterior margin of where the proximal limb extends from the body (Fig 3 J, arrow). By this stage NELL2 expression in the ventral forelimb is strongly reduced (data not shown). NELL2 expression is maintained in a discrete, proximal region through sections of forelimbs at E6.5 and E8.5 (data not shown). Interestingly, this discrete expression pattern in the forelimb is not observed in the hindlimb, even at later stages (Fig 3 K, arrows).

Examination at E4.5 clearly indicated a lack of NELL2 expression in the primary chain of sympathetic ganglia, which have just begun to form at ~E4 and develop into the secondary chain by E8 (Lillie 1908; Lallier and Bronner-Fraser, 1988). Therefore we examined sections from E6.5 and E8.5 embryos to determine whether the secondary chain of sympathetic ganglia

expressed NELL2; NELL2 expression was not detected in sympathetic ganglia, even at this age (Fig 3 L, M).

NELL2 expression is dynamically regulated during cranial sensory ganglia and pharyngeal arch development. NELL2 expression is first weakly detected in the pineal gland anlagen at st 14 (data not shown). By st 17 the pineal gland (Fig 4 A, arrow) and trigeminal ganglia (T) are clearly labeled. By st 19 NELL2 is upregulated and expressed in all cranial sensory ganglia (Fig

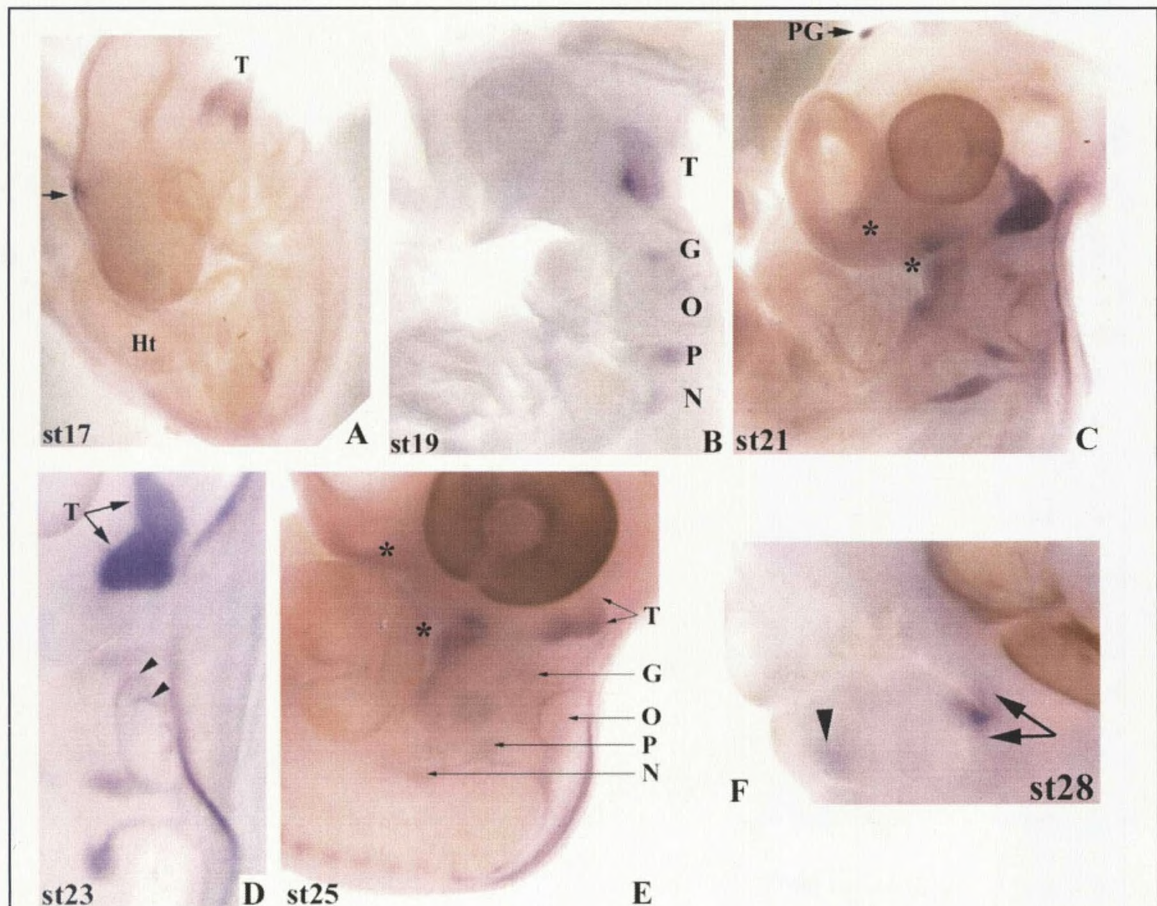

Figure 4. Dynamic NELL2 expression during cranial sensory ganglia and pharyngeal arch development. (A) st17 NELL2 labels the pineal gland (arrow) and trigeminal ganglia (T). (B) By st19 NELL2 is expressed in all cranial sensory ganglia; trigeminal (T), geniculate (G), otic vesicle (O), petrosal (P), nodose (N): note the intense expression in the distal mmV branch of T, and expression in the rostral O. (C, D) NELL2 is maximally expressed in cranial sensory ganglia during st21-23, and labels a region in the face and pharyngeal arches (asterisks): note the staining in the otic vesicle O (D, arrowheads). (E) By st25 NELL2 expression begins to decrease in cranial sensory ganglia, except for the mmV branch of T, while strong expression continues throughout the arches and in the face (asterisks). (F) NELL2 expression becomes restricted in the arches at later stages to discrete regions within the prospective maxilla and mandible.

4 B), and expression peaks during st 21-23 (Fig 4 C, D). By st 25 (Fig 4 E) NELL2 expression decreases in all cranial sensory ganglia, although it is maintained in the mmV branch of the trigeminal ganglia (Fig 4 D, E). Beginning at ~st 19, and by st 21-25 (Fig 4 C, E) NELL2 expression can be detected throughout the pharyngeal arches, during their peak period of development (Lillie, 1908). By st 25 NELL2 becomes increasingly localized around branchial cleft I, from which the maxilla (anterior) and the mandible (posterior) arise, and continues to be strongly expressed in discrete regions in st 28 embryos (Fig 4 F). Transient NELL2 expression in the face is detected between st 21-25, and decreases between st 26-28 (Fig 4 C, E, F). It is interesting to note that NELL2 expression is maximal in the pharyngeal arch target fields as its expression wanes in the respective innervating neural structures.

NELL2 is expressed by differentiating retinal ganglion cells and in the otic vesicle. One of the first tissues to express NELL2 is the developing eye (~st 14, data not shown). At st 18 (data not shown) NELL2 is clearly expressed in the optic cup, with strong expression through the optic stalk. By st 20 (Fig 5 A, arrows) NELL2 is strongly expressed throughout the retinal region circumscribing the lens. This retinal expression becomes localized to ganglion cells in the inner layer by st 25 (Fig 5 B; T. Reh, personal communication), which are interspersed with non-NELL2 expressing cells. NELL2 expression in the otic vesicle can be observed in whole-mount embryos at st 21-25 (see Fig 4). Transverse sections at st 25 indicate that distinct regions within the vestibular-cochlear ganglion (V) express varying levels of NELL2 (Fig 5 C, asterisks), and that NELL2 is also expressed in discrete regions within the otocyst (Fig 5 C, arrowheads).

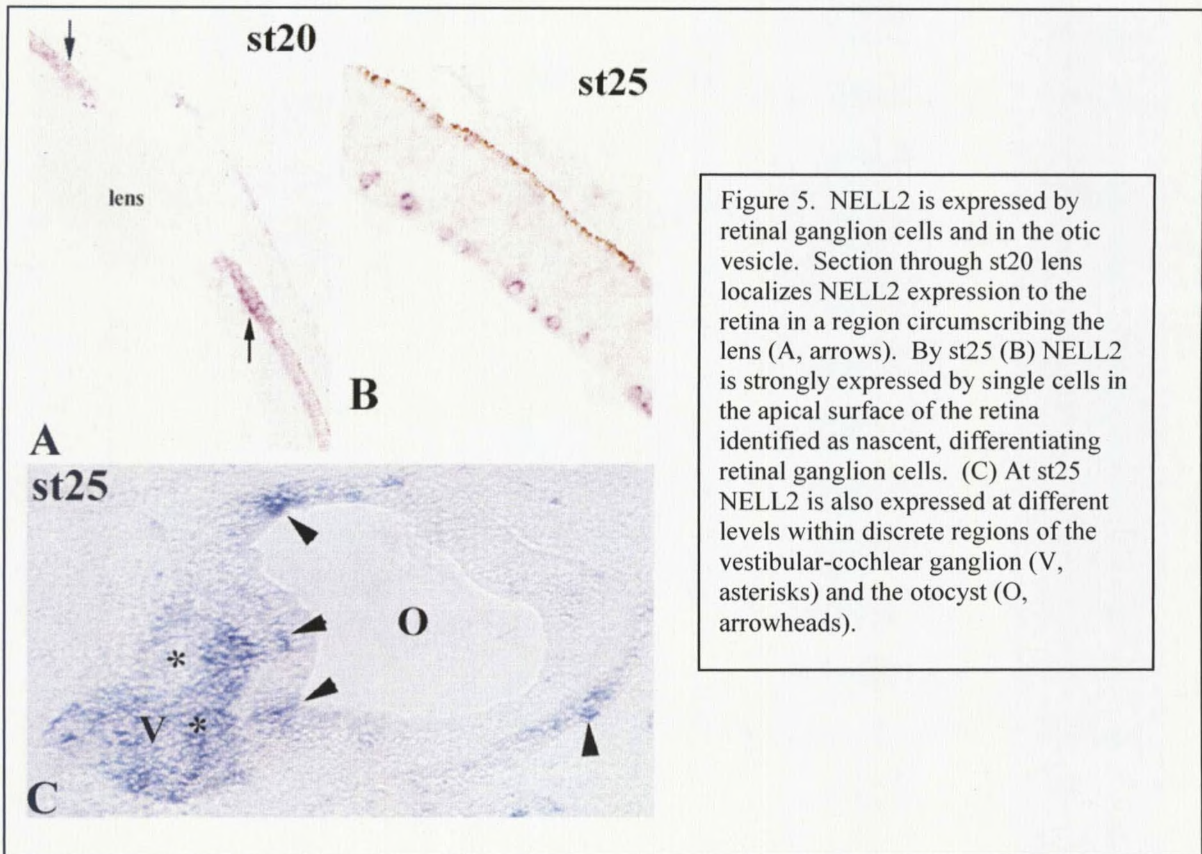

Figure 5. NELL2 is expressed by retinal ganglion cells and in the otic vesicle. Section through st20 lens localizes NELL2 expression to the retina in a region circumscribing the lens (A, arrows). By st25 (B) NELL2 is strongly expressed by single cells in the apical surface of the retina identified as nascent, differentiating retinal ganglion cells. (C) At st25 NELL2 is also expressed at different levels within discrete regions of the vestibular-cochlear ganglion (V, asterisks) and the otocyst (O, arrowheads).

NELL2 expression in the developing CNS. In the CNS, NELL2 is first expressed at ~st 17-18 by a layer of cells in the most lateral region within the neuroepithelium. By st 20 NELL2 is expressed in the wall of the rostral metencephalon (Fig 7 A), and a population of cells in the ventral floor of the mesencephalon (Fig 7 B), diencephalon, and telencephalon (data not shown). NELL2 is also intensely expressed in the midbrain floor in more mature embryos at st 25 and st 28, (Fig 6 A, B arrows). NELL2 expression in the floor of the hindbrain is quite dynamic during these periods. By st 21 NELL2 is strongly expressed in the region of the upper and lower rhombic lips (Fig 6 C, top arrow, bottom arrow, respectively), and in two pairs of stripes in the floor of the hindbrain (Fig 6 C): this ventral expression in the floor extends the entire length of

the hindbrain. At st 21 NELL2 expression is diffuse in anterior rhombomeres (R1-2), while discrete stripes are present in more

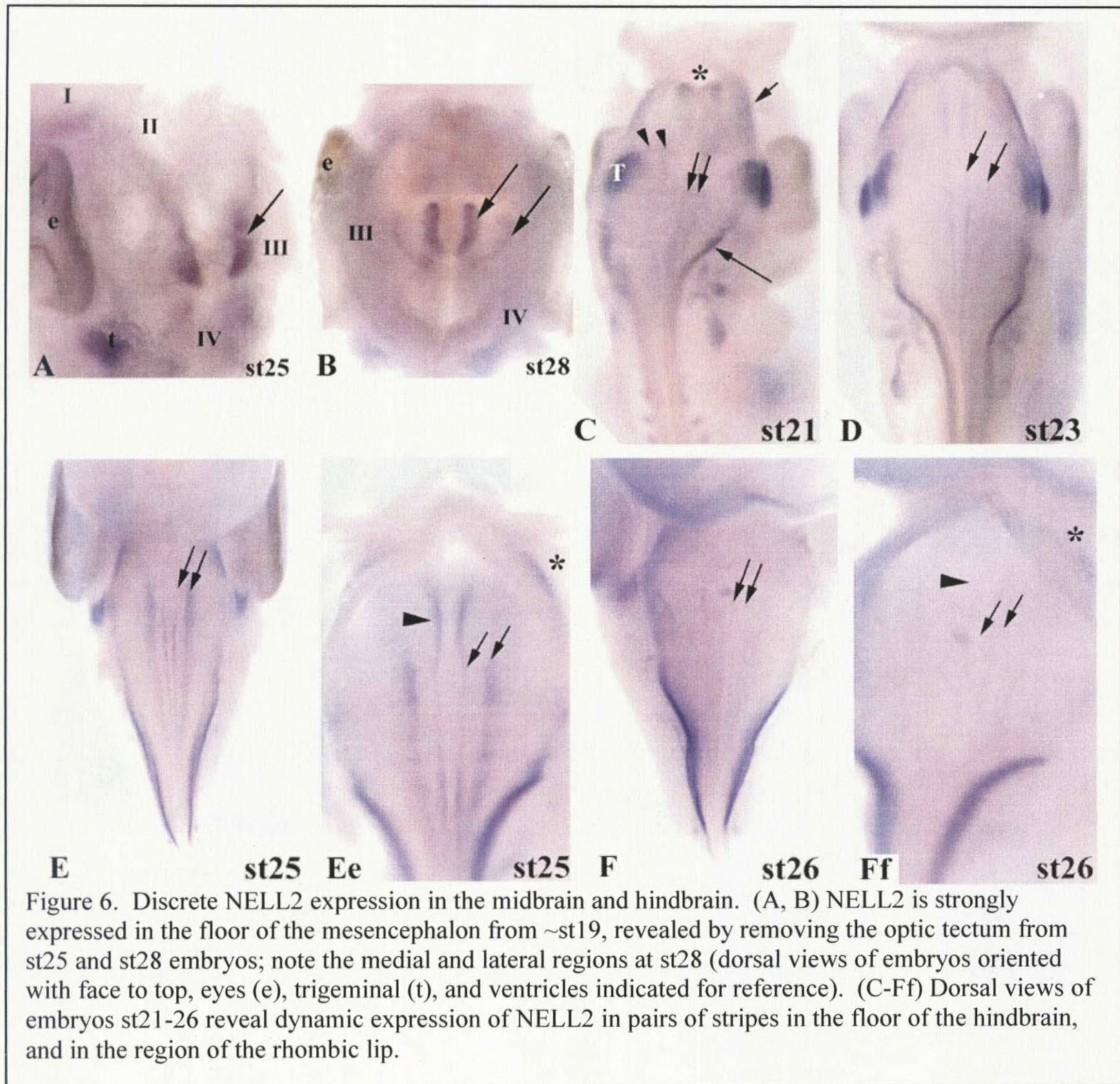

Figure 6. Discrete NELL2 expression in the midbrain and hindbrain. (A, B) NELL2 is strongly expressed in the floor of the mesencephalon from ~st19, revealed by removing the optic tectum from st25 and st28 embryos; note the medial and lateral regions at st28 (dorsal views of embryos oriented with face to top, eyes (e), trigeminal (t), and ventricles indicated for reference). (C-Ff) Dorsal views of embryos st21-26 reveal dynamic expression of NELL2 in pairs of stripes in the floor of the hindbrain, and in the region of the rhombic lip.

posterior rhombomeres, and expression is also detected between the rhombomeric boundaries (Fig 6 C, arrowheads). By st 23 (Fig 6 D) expression in the anterior rhombomeres also becomes restricted to these stripes. At st 25 the medial pair extends the entire length of the hindbrain, through rhombomeres 1-7 (Fig 6 Ee), while the lateral pair of stripes extend caudally from R2 (Fig 6 Ee;small arrowhead; compare anterior regions in Fig 6 C to Fig 6 Ee). NELL2 expression

in these stripes is dramatically reduced after st 25, being barely detectable by st 26 (Fig 6 F, Ff, same embryo rotated forward to view anterior rhombomeres; compare Fig 6 Ee to Ff). Furthermore NELL2 expression in the upper rhombic lip decreases during these stages (compare Fig 6 C top arrow, to Fig 6 Ee, Ff asterisks).

NELL2 is differentially regulated in the CNS and PNS. Except for the very first, weak detection of NELL2 in the spinal cord ventricular zone at the onset of neurogenesis and differentiation, expression of NELL2 in the CNS is restricted exclusively to the differentiating mantle layer of the neuroepithelium, while expression in the PNS (DRG specifically) indicated that NELL2 is located in both differentiating zones and progenitor zones. To determine directly if mitotically active progenitor cells express NELL2, we combined *in situ* hybridizations with BrdU labeling (Bermingham-McDonogh et al., 2001). NELL2 expression throughout the CNS was found not to co-localize with the BrdU+ progenitor zones, but rather was detected in adjacent differentiating layers, in particular the wall of the metencephalon, the floor of the mesencephalon, the retina, and the spinal cord (Fig 7 A, B, D, E respectively). The only exception to this pattern at these stages in the CNS was observed in the pineal gland, where a subpopulation of BrdU labeled cells also expressed NELL2, although most NELL2+ cells were BrdU- (data not shown). This absence of overlap is not the case in the PNS. Sections through both the trigeminal ganglia and DRG at st 20 reveal that indeed mixed populations of Brdu+/NELL2+ (Fig 7 C arrowhead) and BrdU+/NELL2- coexist. At st 25, during the peak period of neurogenesis in the DRG, while NELL2 is predominately expressed in Brdu- cells, there still exists a subpopulation of BrdU+/NELL2+ cells (Fig 7 F). Counts of double-labeled cells in both the trigeminal, and anterior and posterior DRG (indicated by sequential increasing

slide numbering from anterior to posterior respectively) demonstrate that NELL2+/Brdu+ cells decrease along this axes, and over time (Table 3). Interestingly, in both the PNS and retina, NELL2+/BrdU- cells are often observed directly juxtaposed to BrdU+/NELL2- cells, in a pattern reminiscent of Notch/Delta expression.

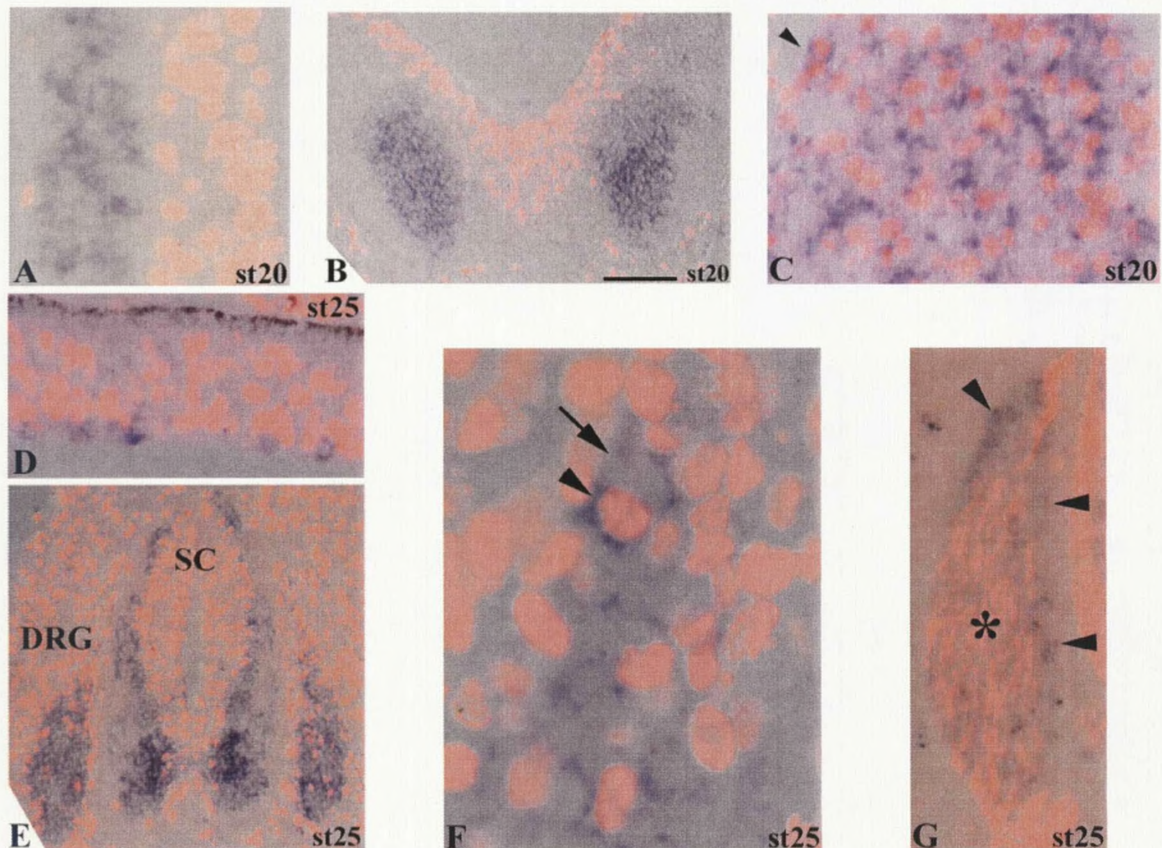

Figure 7. NELL2 is differentially regulated between the CNS and PNS. (A-F) NELL2 *in situ* hybridizations (purple) were combined with BrdU labeling (red). (A) Transverse section through wall of rostral metencephalon at st20 localizes NELL2 to the mantle layer (differentiation zone), while BrdU+ cells are localized to the ventricular layer (progenitor zone). (B) Section through floor of the mesencephalon at st20; note that NELL2 labels a discrete population of cells in the mantle layer of the mesencephalon floor. (C) At st20 NELL2 is expressed in the trigeminal ganglion by a subset of $BrdU^{+}$ cells (arrowhead), however the majority of $NELL2^{+}$ cells are $BrdU^{-}$; also note that BrdU+ cells are often located adjacent to $NELL2^{+}$ cells. (D) The population of NELL2 expressing cells in the apical surface of the retina at st25 is $BrdU^{-}$, yet in contact with proliferating cells. (E, F) Sections through trunk at st25 clearly demonstrates the general pattern of NELL2 labeling mantle layer and BrdU labeling in the ventricular zone in the CNS (spinal cord, SC), while in the PNS mixed populations persist in the immature DRG: note the $NELL2^{+}/BrdU^{+}$ cell (arrowhead) next to the $NELL2^{+}/Brdu^{-}$ cell (arrow) in the dorsal root (F, higher magnification). (G) NELL2 combined with neurofilament; NELL2 (purple), neurofilament (red). At st25, NELL2 is predominately expressed in the neural core of the DRG (asterisks), however NELL2 is also expressed in neurofilament negative cells in the dorsal pole and in the perimeter of the ganglion adjacent to the spinal cord, known zones of progenitor cells (arrowheads).

Table 3. NELL2 expression in DRG precursors decreases during development

Stage	Tissue	slide#	%NELL2+/Brdu+ cells
20	trigeminal	8	17
20	DRG	16	8
20	DRG	22	17.5
20	DRG	26	16.5
25	DRG	9	4.9
25	DRG	11	5
25	DRG	15	4.8

To further confirm that within the DRG NELL2 is expressed by both neurons and progenitor cells, we combined NELL2 in situ hybridization with neurofilament immunocytochemistry (Vargesson et al., 2001). At st 25 neurofilament antibodies label the neural core of the ganglia and the projections to the spinal cord and target fields (Fig 7 G). While the majority of NELL2 expressing cells are located in the neural core of the ganglia, NELL2 is also expressed by non-neural cells in both the dorsal pole and in the medial and lateral perimeter of the ganglion adjacent to the spinal cord (arrowheads, fluorescence signal dampens colorimetric signal slightly), both known zones of proliferation within the DRG (Hamburger et al., 1981).

In summary, these studies demonstrate that NELL2 is highly spatially and temporally regulated in discrete regions within the nervous system, in particular the midbrain, hindbrain, retina, spinal cord, and peripheral sensory ganglia. NELL2 expression is maximal during the peak periods of both sensory and motor neuron differentiation (Carr and Simpson, 1978; Leber et

al., 1990). Furthermore, NELL2 marks specific sublineages of the somite and neural crest. NELL2 expression is restricted to hypaxial muscle precursor cells rather than epaxial muscle precursor cells, during the early specification phase of their differentiation program, and continues to be expressed as derivatives migrate to their target fields in the limbs and body wall, being down-regulated as the terminal differentiation program ensues in a manner analogous to Pax3 (compare with Pax3 expression Williams and Ordhal, 1994; Brand-Saberi, 1999; Stockdale et al., 2000). Thus NELL2 is a new marker for this early phase of hypaxial muscle differentiation. Furthermore NELL2 specifically marks the sensory neural lineage of the neural crest rather than the sympathetic or melanocyte lineage at any stage examined. This restricted expression pattern is similar to that of the neurogenins 1 and 2, which are necessary for DRG formation, regulate distinct waves of neurogenesis within the DRG, and bias neural crest to a sensory neural fate (Sommer et al., 1996; Perez et al., 1999; Ma et al., 1999). Finally NELL2 is differentially regulated between the CNS and PNS in that it is excluded from neural progenitor zones within the CNS, while a subset of mitotically active progenitor cells in the PNS express NELL2. Studies directed towards elucidating the functional significance of this differential expression and NELL2's role during muscle and neuronal development are currently in progress.

Methods

Identification of NELL2 during sensory neurogenesis and differentiation

Fertilized white leghorn eggs were obtained (Spafas), and grown to appropriate stages (Hamburger and Hamilton, 1951). All embryos were treated in accordance with IACUC stipulations. SSH-PCR was used to identify differentially expressed genes

between immature, embryonic day 4.5 dorsal root ganglia (E4.5 DRG) and mature, differentiated E8.5 DRG (Lukyanov et al., 1995; Diatchenko, et al., 1996). The E4.5 DRG cDNA subtraction library was screened for candidate differentially expressed clones (Munroe et al., 1995; von Stein et al., 1997), the details of which will be published separately (see Chapter 2).

RT-PCR confirmation of differential NELL2 expression

E4.5 DRG and E8.5 DRG were collected and total RNA isolated with standard techniques, quantified by measuring absorption at 260nm/280nm, and concentration normalized for RT-PCR analysis. MacVector 6.5.3 (Oxford Molecular) was used to design specific primers from available sequence data for chicken NELL2, and the normalization controls glyceraldhyde-3 phosphodehydrogenase (G3PDH) and *Beta*-actin: NELL2 (forward 5'-CTCCCACACAGAAGTATTCCC, reverse 5'-TAGGCATTGTCCTCCCGAAG); G3PDH (forward 5'-ACCACAGTCCATGCCATCAC, reverse 5'-TCCACAACACGGTTGCTGTA); *Beta*-actin (forward 5'-TGGAATCCTGTGGCATCCATGAAAC, reverse 5'-TAAAACGCAGCTCAGTAACAGTCCCG). The ACCESS System (Promega) was used for single-step RT-PCR from total RNA, as directed. Equal concentrations of E4.5 and E8.5 DRG total RNA were used to analyze the relative expression levels of NELL2 at these developmental stages (150ng for controls, 250ng for NELL2). 5ul aliquots were removed at 20X, 30X, and 40X cycles of PCR and analyzed on 2%Ag/EtBr gels. Single bands corresponding to the correct sizes were detected for NELL2 and controls. RT(-) control was performed with the G3PDH primer set. Results for 30X cycles shown.

In situ hybridizations

A series of chick embryos at selected stages were collected. Whole-mount *in situ* hybridization and section *in situ* hybridization were performed with DIG-labeled ribo-probes as described with slight modifications (Henrique et al., 1995; Chen et al., 1998; Etchevers et al., 2001). DIG-labeled NELL2 probes were generated from the same template as in Matsuhashi et al, 1995, which gave the same results. Sense-control probes gave no signal and are not shown. Detection of differential signal in E4.5, E6.5, and E8.5 DRG and spinal cord was performed by allowing the slides to react at the same time with prepared NBT/BCIP color substrate (Sigma) until optimal signal-to-noise was reached, which always occurred first with the E4.5 slides, all of which were then post-fixed with 4% paraformaldehyde, and coverslipped. Alpha-tubulin DIG-labeled control probe has been described (Miller at al., 1987; Gloster et al., 1994).

Combined in situ hybridizations, BrdU, and neurofilament labeling

BrdU was injected *in ovo* into st20 and st25 embryos, eggs were sealed with plastic tape and incubated for 4hrs, processed for in situ hybridizations (Etchevers et al., 2001; without proteinase K treatment) and Brdu incorporation (Bermingham-McDonogh et al., 2001), or neurofilament detection (Vargesson et al., 2001).

4. NEURAL EPIDERMAL GROWTH FACTOR-LIKE LIKE 2 (NELL2) PROMOTES MOTOR AND SENSORY NEURON DIFFERENTIATION *IN VIVO*

Abstract

We have previously identified a secreted glycoprotein, neural epidermal growth factor-like like 2 (NELL2), through a subtraction screen designed to identify molecules regulating sensory neuron genesis and differentiation in the dorsal root ganglion (DRG, Chapter 2). Characterization of NELL2 expression during embryogenesis demonstrated that NELL2 is specifically expressed during the peak periods of both sensory and motor neuron differentiation, and is restricted to the sensory lineage of the neural crest (Nelson et al., 2002b, Chapter 3). We report here that NELL2 functions autocrinely to promote the differentiation of both CNS and PNS progenitors into neurons, and biases neural crest cells towards a sensory fate *in vivo*. Additionally, neuron-secreted NELL2 acts paracrinely to stimulate the mitogenesis of adjacent cells within the nascent DRG. This is the first demonstration of an *in vivo* function for this molecule.

Introduction

Vertebrate neurogenesis and differentiation results from an intricate interplay between extrinsic and intrinsic cellular signals that generate a variety of different neuronal and glial cell types 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 with which to address this issue is in the chick dorsal root ganglia (DRG), which derive from a subset of migrating neural crest cells that coalesce laterally to the neural tube (Lallier and Bronner-Fraser, 1988). To identify genes that regulate this process we compared cDNA from immature embryonic E4.5 DRG, which contain nascent neuroblasts and mitotically active progenitor cells at the peak period of neurogenesis and differentiation (Carr and Simpson, 1978), to cDNA from mature E8.5 DRG, which contain differentiated post-mitotic neurons and glia through a subtraction/differential screening strategy (see Chapter 2). From this subtraction screen we identified neural epidermal growth factor-like like 2 (NELL2), a large secreted glycoprotein, and confirmed that NELL2 was differentially expressed during DRG development (Nelson et al., 2002b).

NELL1 and NELL2 gene expression is highly regulated during development and in the adult, in a variety of vertebrate systems (Matsuhashi et al., 1995; Watanabe et al., 1996; Luce and Burrows, 1999; Kuroda et al., 1999; Oyasu et al., 2000; Nelson et al., 2002b). These studies have revealed that NELL genes are expressed in discrete tissues, temporally restricted to periods of differentiation in the pharyngeal arches, motor and sensory neurogenesis and differentiation, and the early phase of skeletal muscle differentiation amongst others. NELL2 expression in a subset of PNS progenitors *in vivo*

decreases over time (Nelson et al., 2002b), and mouse embryonic stem cells express NELL2 when induced to differentiate into neurons in response to retinoic acid (Guo et al., 2001). Although these patterns of expression correlate with phases of differentiation in these tissues, suggesting that NELLs may function in this process, nothing at all is known about the actual function of these genes *in vivo.*

The NELL genes belong to the Laminin G/N-terminal TSP1/Pentraxin supergene family, which is involved in cell:cell communication and contact (Beckman et al., 1998), contain a cleavable signal peptide, 6 EGF-like and 5 vWc domains, and have been proposed to act as signaling molecules involved in cell growth and/or differentiation (see schematic in Fig 8 A; Matsuhashi et al., 1995; Watanabe et al., 1996; Kuroda et al 1999). Initial *in vitro* biochemical analyses have demonstrated that NELL1 and NELL2 are secreted from COS7 cells and can bind heparin sulfate (Kuroda et al., 1999; Kuroda and Tanizawa, 1999). Incubation of these cultures with P^{32}-orthophosphate shows that secreted NELL1 and NELL2 are phosphorylated by an as-of-yet unidentified intracellular kinase (Kuroda and Tanizawa, 1999). Secreted rat rNELLs were added to cultures of a cell line that express all isoforms of the epidermal growth factor (EGF) receptor Erb family of receptors, and assayed for receptor phosphorylation (Kuroda et al., 1999). No phosphorylation was observed, suggesting that NELLs do not bind to and activate Erb receptors directly (data not shown, Kuroda et al., 1999). Yeast two-hybrid assays and co-immunoprecipitations of NELL/PKC from COS7 cell lysates have shown that specific isoforms of PKC can bind to the EGF-like domains and phosphorylate NELL1 and NELL2 *in vitro* (Kuroda and Tanizawa, 1999). The significance of this interaction with various PKC isoforms is unclear, as PKC is a cytosolic molecule while NELL contains a

cleavable signal peptide sequence, is routed to the ER for extensive N-linked glycosyaltion events, and subsequently through the Golgi and secreted. Furthermore NELL2 immunocytochemical analysis in the adult rat brain demonstrated that NELL2 immunoreactivity did not completely correspond to immunoreactivity for any of the PKC subunits, and with electron microscopy showed that NELL2 was associated with the rough ER and within the ER, a cellular compartment not readily accessible to cytosolic PKC isoforms (Oyasu et al., 2000). Intriguingly this study also demonstrated that NELL2 was exclusively expressed by neurons and not detected in any glial derivatives, suggesting that NELL2 is secreted by neurons *in vivo*.

To determine directly the function of chicken NELL2 *in vivo*, we have used a gain-of-function approach to analyze the effect of ectopic NELL2 expression in CNS and PNS progenitor cells by transfecting these progenitors *in ovo* and determining their fate at key stages of development. We show that an epitope- tagged NELL2 is secreted from the spinal cord and drives CNS progenitors to exit the cell cycle, promoting their differentiation in an autocrine fashion, without altering their identity. Ectopic expression of NELL2 in neural crest cells acts autocrinely to bias migrating crest towards a sensory fate, and to differentiate into sensory neurons rather than peripheral glia, without altering their sensory sub-type, while paracrinely acts to induce neighboring cells in the immature DRG to proliferate. These are the first such reported results for a biological effect of a NELL gene *in vivo*.

Results

NELL2 is secreted from spinal cord

Expression of pNELL2:Myc was verified by transiently transfecting pNELL2:Myc and empty control parent vector (pmiw) into COS7 cells *in vitro,* analyzing supernatants (and/or immunoprecipitations from remaining supernatants with anti-Myc antibody from Clontech), by both denaturing and non-denaturing PAGE and Western analysis with anti-Myc antibody (9e10 DHSB). Figure 8 shows that NELL2:Myc is secreted from COS7 cells and forms the homo-trimer in solution. For *in ovo* electroporations/microinjections, NELL2:Myc was subcloned into the bicistronic eukaryotic expression vector pMES (Swartz et al., 2001b), which contains the dual CMV IE and chick beta-actin promoters and the IRES2:eGFP sequence for dual NELL2:Myc and eGFP expression in the same cell, referred to now as pNELL2 for simplicity. Co-expression was confirmed by transiently transfecting COS7 cells as above (Fig 8 D). Co-expression *in ovo* was confirmed by microinjecting and electroporating pNELL2 into stage 10 chick neural tubes, incubating until stage 25, dissecting and dissociating transfected spinal cords, and culturing *in vitro* overnight. Supernatants and cultures were analyzed as above. Indeed, NELL2 is secreted from *in ovo* transfected spinal cord cells and forms the putative homo-trimer in solution (Fig 8 H: note that these cells appear to be neuronal).

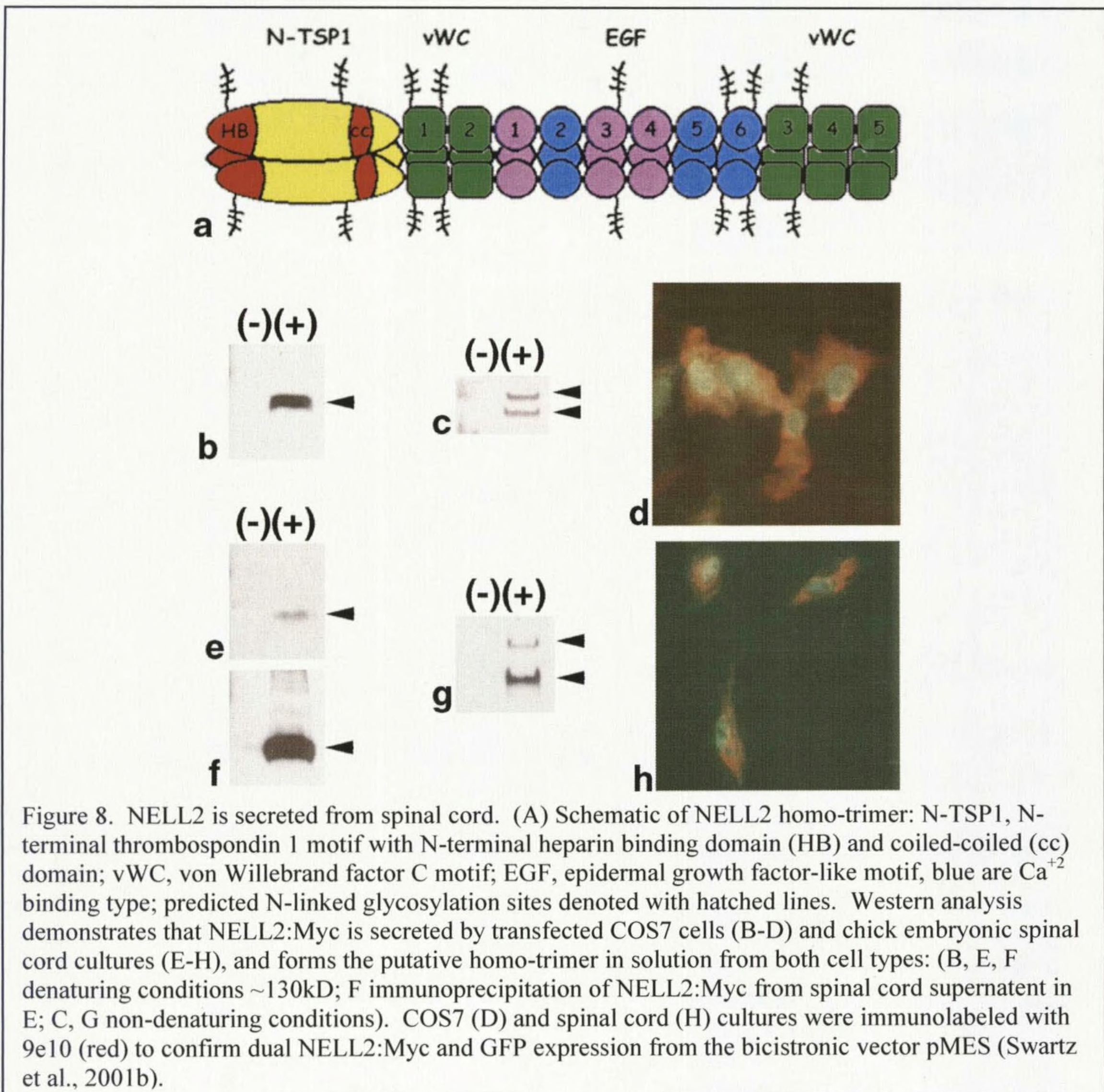

Figure 8. NELL2 is secreted from spinal cord. (A) Schematic of NELL2 homo-trimer: N-TSP1, N-terminal thrombospondin 1 motif with N-terminal heparin binding domain (HB) and coiled-coiled (cc) domain; vWC, von Willebrand factor C motif; EGF, epidermal growth factor-like motif, blue are Ca^{+2} binding type; predicted N-linked glycosylation sites denoted with hatched lines. Western analysis demonstrates that NELL2:Myc is secreted by transfected COS7 cells (B-D) and chick embryonic spinal cord cultures (E-H), and forms the putative homo-trimer in solution from both cell types: (B, E, F denaturing conditions ~130kD; F immunoprecipitation of NELL2:Myc from spinal cord supernatent in E; C, G non-denaturing conditions). COS7 (D) and spinal cord (H) cultures were immunolabeled with 9e10 (red) to confirm dual NELL2:Myc and GFP expression from the bicistronic vector pMES (Swartz et al., 2001b).

NELL2 does not act in a tropic fashion to attract or repel DRG neurites

An intriguing observation from our previous *in situ* hybridization analysis was that NELL2 expression in the developing pharyngeal arch target fields is maximal as its expression wanes in the respective innervating cranial ganglia (Nelson, et al., 2002b). Similarly, NELL2 expression in brachial DRG decreases as its expression becomes 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 secreted glycoproteins that also contain EGF-like domains such as ROBO and its ligand Slit. We directly tested this hypothesis by coculturing COS7 cells expressing rNELL2 next to explanted E4.5 DRG in collagen gels (Tessier-Levine et al., 1988). Initial observations did not reveal any overt ability for secreted rNELL2 to attract or repel extending DRG neurites (data not shown).

CNS progenitor cells ectopically expressing NELL2 translocate to the mantle layer

In ovo microinjection/electroporation allows for the analysis of the effect of transient ectopic DNA transfection into cells (Muramatsu, et al., 1997; Swartz et al., 2001a). Transfected cells express high levels of ectopic DNA in their normal *in vivo* environment. To label pre-migratory neural crest, we injected embryonic neural tube at st10, re-sealed the eggs, and incubated until appropriate developmental stages were reached: Figure 9 A and B demonstrate the methodology and its efficiency at transfecting cells *in vivo*. Transverse sections through the spinal cord of control GFP injected embryos at st20 and st25 (Fig 9 C and D respectively) demonstrate that all populations of cells in the spinal cord are generated from transfected progenitors, which are located next to the lumen in the ventricular zone, and that progenitors continue to express ectopic GFP at these later stages. However, CNS progenitors expressing high levels of ectopic NELL2 translocate from the ventricular zone to the mantle layer where differentiation occurs, and this bias in distribution occurs early after transfection (Fig 9 E, F; ectopic GFP can be observed ~6-12 hrs post-transfection). This result demonstrates that CNS progenitors are competent to respond to NELL2, and that this response occurs through an

autocrine type mechanism, either intracellularly or mediated through cell-surface interactions with secreted NELL2.

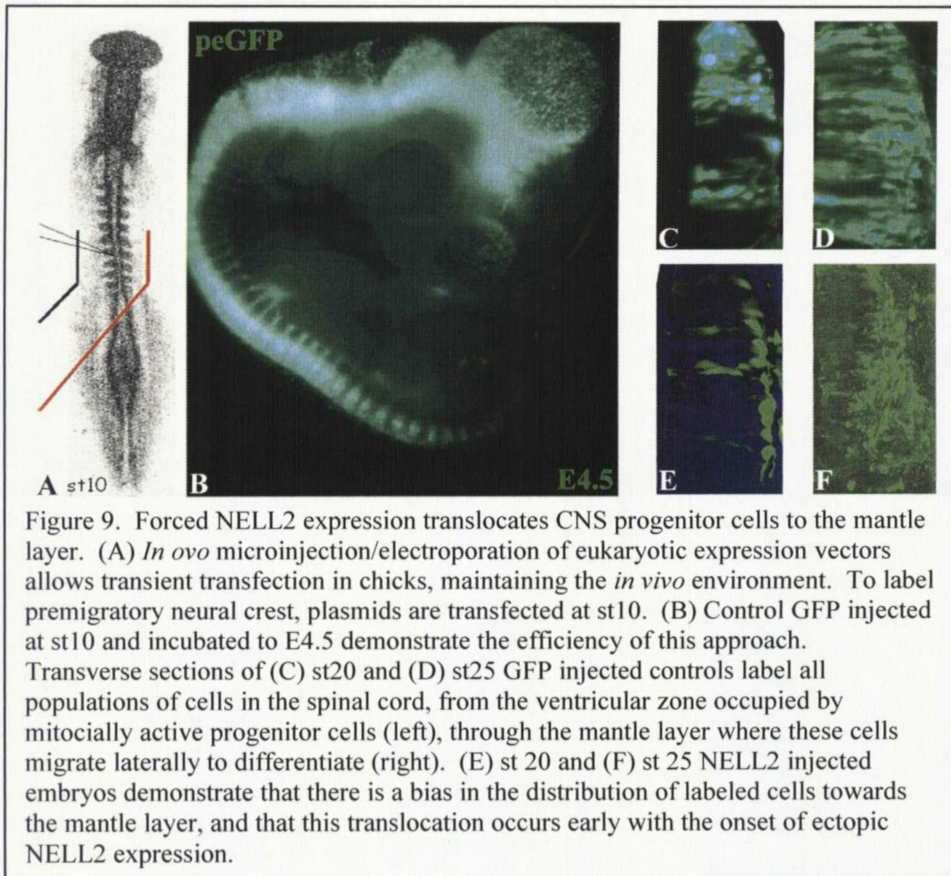

Figure 9. Forced NELL2 expression translocates CNS progenitor cells to the mantle layer. (A) *In ovo* microinjection/electroporation of eukaryotic expression vectors allows transient transfection in chicks, maintaining the *in vivo* environment. To label premigratory neural crest, plasmids are transfected at st10. (B) Control GFP injected at st10 and incubated to E4.5 demonstrate the efficiency of this approach. Transverse sections of (C) st20 and (D) st25 GFP injected controls label all populations of cells in the spinal cord, from the ventricular zone occupied by mitocially active progenitor cells (left), through the mantle layer where these cells migrate laterally to differentiate (right). (E) st 20 and (F) st 25 NELL2 injected embryos demonstrate that there is a bias in the distribution of labeled cells towards the mantle layer, and that this translocation occurs early with the onset of ectopic NELL2 expression.

NELL2 autocrinely promotes differentiation of spinal cord progenitors without altering specification of cell fate

To further investigate this effect in the spinal cord, we combined plasmid injections with antibody staining for different classes of spinal cord progenitors or derivatives to determine if ectopic NELL2 influences specification of cellular identities. CNS progenitors in the developing spinal cord give rise to distinct classes of cells according to a homeodomain transcription factor code, which is generated in response to ventrallizing and dorsallizing gradients of secreted sonic hedgehog and BMP's

respectively (Briscoe et al., 2000). Neither control GFP nor NELL2 ectopic expression was found to induce ectopic motor neuron formation dorsal to their normal ventral domain, as assessed with the motor neuron specification factor MNR2 (Fig 10 A NELL2 st20, B GFP st25; Tanabe et al., 1998). Furthermore, ectopic NELL2 cannot induce the formation of Pax7 progenitors ventral to their normal domain within the spinal cord (Fig 10 C).

To further investigate the ability of ectopic NELL2 to force CNS progenitors to translocate to the mantle layer, we have used the MNR2 to Isl1 transition in motor neuron domains, as a measure of motor neuron differentiation. MNR2 normally is expressed at high levels by Olig1/2 progenitors as they migrate from the ventricular zone to the nascent motor neuron domain (Tanabe et al., 1998; Novitch et al., 2001). Within these developing motor pools, MNR2 is expressed at higher levels medially and lower levels more laterally, where nascent motor neurons increase their expression of later neural markers such as Isl1. Preliminary analysis of transfected cells within the motor neuron domain defined by MNR2 and Isl1 expression indicate that this transition occurs earlier in NELL2 transfected cells, demonstrating that NELL2 promotes differentiation of motor neurons (Fig 10 D-H; Table 4). We have also begun analyzing whether ectopic NELL2 can directly force CNS progenitors to withdraw from the cell cycle, by combining microinjection/electroporations with BrdU pulses, which label CNS progenitors in S phase, whose nuclei are correspondingly located in the lateral region of the ventricular zone (Hays and Nowakowski, 2000). Preliminary observations indicate that NELL2+ progenitors are BrdU-, indicating that like other factors known to promote differentiation in the spinal cord, such as neurogenins and NKL (Lamer et al., 2001), ectopic NELL2

autocrinely promotes mitotic arrest and translocation to the differentiating mantle layer.

Further experimentation to confirm these preliminary observations is in progress.

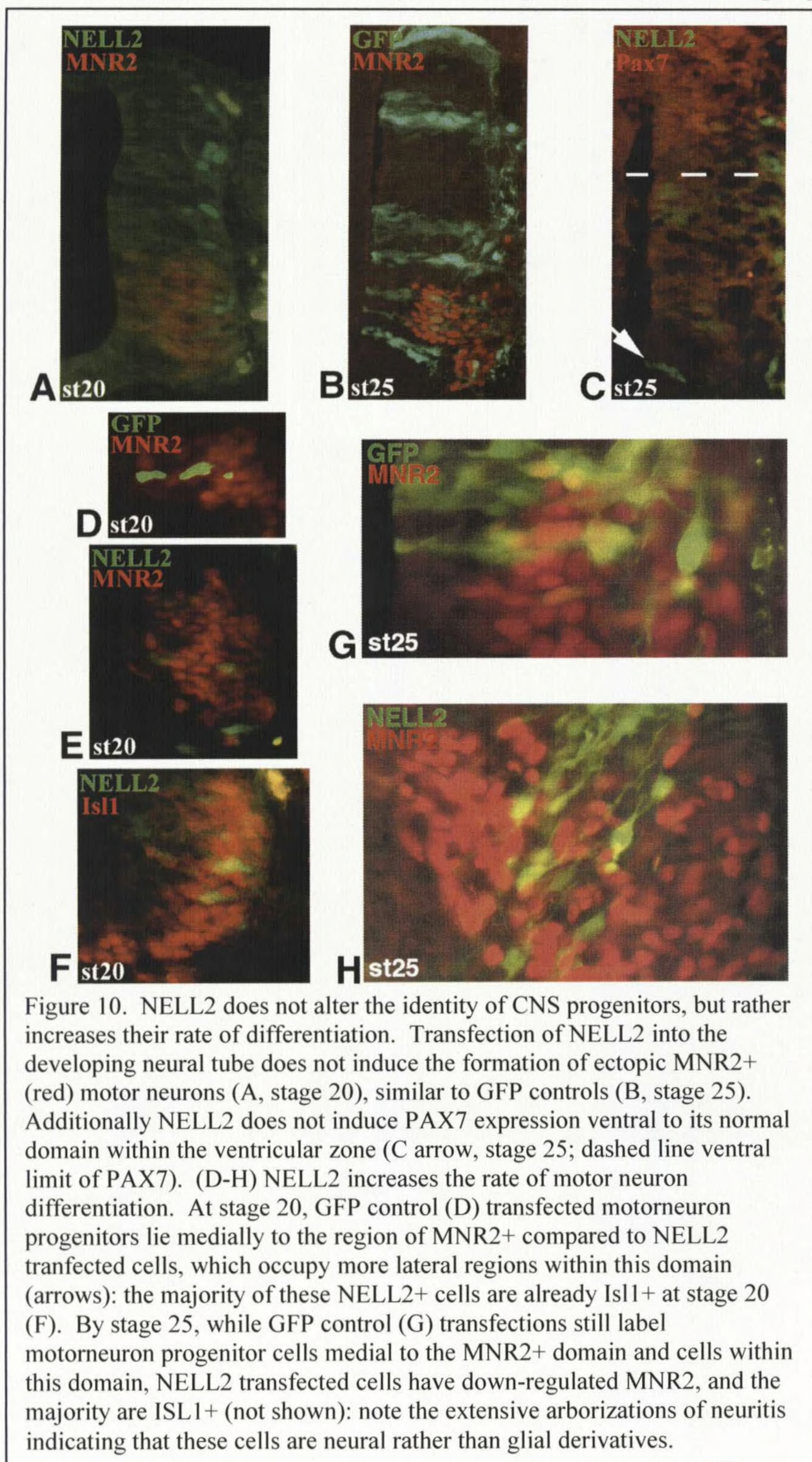

Figure 10. NELL2 does not alter the identity of CNS progenitors, but rather increases their rate of differentiation. Transfection of NELL2 into the developing neural tube does not induce the formation of ectopic MNR2+ (red) motor neurons (A, stage 20), similar to GFP controls (B, stage 25). Additionally NELL2 does not induce PAX7 expression ventral to its normal domain within the ventricular zone (C arrow, stage 25; dashed line ventral limit of PAX7). (D-H) NELL2 increases the rate of motor neuron differentiation. At stage 20, GFP control (D) transfected motorneuron progenitors lie medially to the region of MNR2+ compared to NELL2 tranfected cells, which occupy more lateral regions within this domain (arrows): the majority of these NELL2+ cells are already Isl1+ at stage 20 (F). By stage 25, while GFP control (G) transfections still label motorneuron progenitor cells medial to the MNR2+ domain and cells within this domain, NELL2 transfected cells have down-regulated MNR2, and the majority are ISL1+ (not shown): note the extensive arborizations of neuritis indicating that these cells are neural rather than glial derivatives.

Table 4. MNR2 expression is downregulated in cells ectopically expressing NELL2*

*preliminary results	St18-20	St25
NELL2	69% n=2 embryos 31/45 cells	26% n=4 embryos 60/232 cells
GFP	61% n=3 embryos 27/44 cells	51% n=1 embryo 37/73 cells

NELL2 autocrinely biases neural crest towards a sensory rather than sympathetic fate

Transfection of control GFP into premigratory neural crest at st10 results in the labeling of all neural crest derivatives, in particular enteric, sympathetic, sensory, and melanocyte derivatives, as described in previously published lineage-tracing experiments (Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991). Remarkably, ectopic NELL2 was found to bias the early wave of neural crest migration, which normally contribute to more ventral sympathetic and enteric lineages, to preferentially localize to the DRG anlagen (Fig 11, st20 A GFP, B NELL2). Examination of the primary chain of sympathetic ganglia at st25, identified with staining for the neural marker Ben (red) revealed that control GFP labeled cells continue to contribute to sympathetic ganglia, while ectopic NELL2 labeled cells are absent from sympathetic ganglia (Fig 11 C GFP, D Nell2). Quantification of ectopically labeled cells confirm that NELL2 indeed biases the fate of neural crest cells (95% of cells) to localize or commit preferentially to the

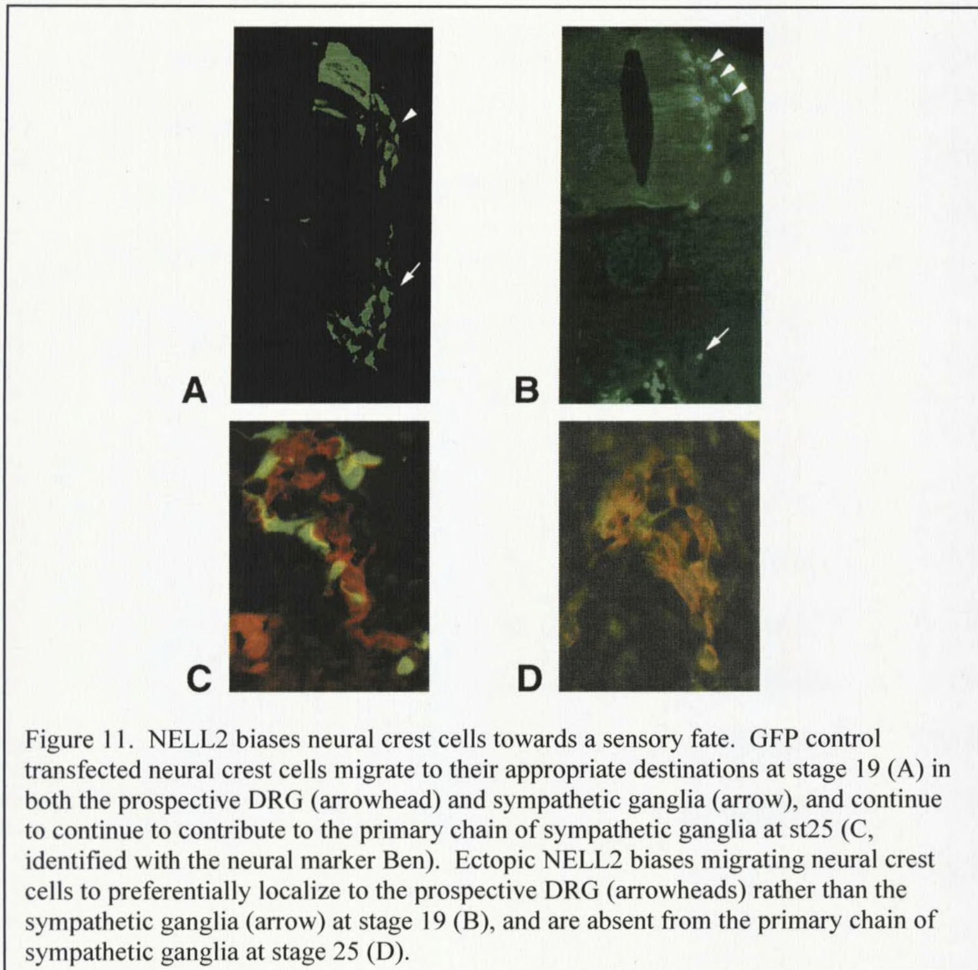

Figure 11. NELL2 biases neural crest cells towards a sensory fate. GFP control transfected neural crest cells migrate to their appropriate destinations at stage 19 (A) in both the prospective DRG (arrowhead) and sympathetic ganglia (arrow), and continue to continue to contribute to the primary chain of sympathetic ganglia at st25 (C, identified with the neural marker Ben). Ectopic NELL2 biases migrating neural crest cells to preferentially localize to the prospective DRG (arrowheads) rather than the sympathetic ganglia (arrow) at stage 19 (B), and are absent from the primary chain of sympathetic ganglia at stage 25 (D).

sensory rather than sympathetic ganglia (Table 5). Control GFP transfection results in ~70% of neural crest cells localizing to the DRG, consistent with control percentages from previous studies of factors influencing neural crest fate choices between sensory and sympathetic lineages, as the DRG normally contains more cells at this stage (Perez et al., 1999). This functional bias is consistent with our previous expression analysis, which demonstrated that while NELL2 is expressed by a subset of migrating neural crest and during DRG gangliogenesis, its expression was not detected in the primary chain early, nor the secondary chain of sympathetic ganglia at later stages (Nelson et al., 2002b).

Table 5. NELL2 biases the fate of Neural Crest Location of labeled cells at st20: % of labeled cells in the DRG versus Sympathetic Ganglia	
NELL2	95-/+5% n=3 embryos 226/246 cells
GFP	71% n=1 embryo 426/594 cells

NELL2 acts autocrinely to promote the
differentiation of neural crest cells into sensory neurons

To investigate the fate of cells expressing ectopic DNA within the DRG, we analyzed transfected embryos grown to key stages of DRG development and determined cell identity with an array of markers. At st18-20 (early E3) gangliogenesis has begun, and the first neurons have just been born and express neural markers (Lallier and Bronner-Fraser, 1988). However the vast majority of cells in the nascent ganglia are progenitor cells that do not express these markers (Table 6); rather they encapsulate the newly born cluster of neurons that are aggregating in the inner core of the ganglia, exemplified by Fig 12 A in which GFP transfected control cells are predominantly non-neural (top arrowhead) surrounding the Tuj1+ neural core (asterisks), and comprise the non- neural cells in the ventral root (bottom arrowhead).

Table 6. Percentage of labeled cells within the DRG expressing neural markers		
	St 18-20	St 25
NELL2	64%+/-4.7 n=6 embryos 224 cells	87%+/-4.3 n=5 embryos 357 cells
GFP	15%+/-0.4 n=2 embryos 375 cells	41%+/-9.3 n=2 embryos 567 cells

In control embryos at st25/E4.5, during the peak period of sensory neurogenesis and differentiation, ectopic GFP DNA expression can be detected in nascent neurons and non-neural cells in the core of the ganglia (Fig 12 B, Ben asterisks), progenitor cells in the dorsal pole and the perimeter (Fig 12 B, top arrowhead), non-neural cells in the dorsal root (Fig 12 B, arrow), and in the ventral motor (Fig 12 B, bottom arrowhead) and sensory tracts (data not shown). Strong ectopic control GFP was still detected as late as examined (E8.5), and labeled derivates at E6.5 include TrkB+ cells (Fig 12 C, arrowhead), other non-TrkB+ cells which appear to have a neuronal type cell body, and cells which appear glial (Fig 12 C, arrow).

Ectopic NELL2 expression however results in a clear bias, in which NELL2 autocrinely promotes the differentiation of neurons rather than glia (Table 6). Even during the earliest stages of ganglion formation, when neural markers are first expressed, 67% of NELL2 cells are neurons, compared to 15% of controls, and this is a conservative