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NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis

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SUMMARY

Genes encoding transcription factors of the helix-loop-helix family are essential for the development of the nervous system in *Drosophila* and vertebrates. Screens of an embryonic chick neural cDNA library have yielded NeuroM, a novel neural-specific helix-loop-helix transcription factor related to the *Drosophila* proneural gene *atonal*. The NeuroM protein most closely resembles the vertebrate NeuroD and Nex1/MATH2 factors, and is capable of transactivating an E-box promoter in vivo. In situ hybridization studies have been conducted, in conjunction with pulse-labeling of S-phase nuclei, to compare *NeuroM* to *NeuroD* expression in the developing nervous system. In spinal cord and optic tectum, *NeuroM* expression precedes that of *NeuroD*. It is transient and restricted to cells lining the ventricular zone that have ceased proliferating but have not yet begun to migrate into the outer layers. In retina, *NeuroM* is also transiently expressed in cells as they withdraw from the mitotic cycle, but persists in horizontal and bipolar neurons until full differentiation, assuming an expression pattern exactly complementary to *NeuroD*. In the peripheral nervous system, *NeuroM* expression closely follows cell proliferation, suggesting that it intervenes at a similar developmental juncture in all parts of the nervous system. We propose that availability of the NeuroM helix-loop-helix factor defines a new stage in neurogenesis, at the transition between undifferentiated, premigratory and differentiating, migratory neural precursors.

Key words: neuronal bHLH, chick nervous system, retina, spinal cord, optic tectum, transcription, neurogenesis

INTRODUCTION

In vertebrates, the nervous system emerges from the ectoderm in response to inductive signals emanating from the underlying mesoderm. This induction is mediated by cell-surface and secreted proteins, and defines the territory from which the neural tube will form. Neuronal precursors proliferate in the ventricular zone (VZ) of the neural tube. Upon their last division, they migrate towards the pial side of the neural tube and differentiate. These steps in neurogenesis are under the control of various regulatory proteins, among which are helix-loop-helix (HLH) transcription factors related to the proneural genes of *Drosophila* (reviewed in Campos-Ortega, 1995). The first such gene identified in vertebrates, *MASH1*, is a homolog of *achaete-scute* (*ac-sc*) (Johnson et al., 1990) expressed by proliferating neuroblasts in many regions of the central and peripheral nervous system. Its targeted disruption results in mice lacking olfactory and autonomic neurons (Guillemot and Joyner, 1993; Guillemot et al., 1993). *XASH3*, another *ac-sc* homolog isolated in *Xenopus*, is also expressed by proliferating neuroblasts and, upon injection into a blastula, its transcript converts epidermal cells into neurons (Ferreiro et al., 1994; Turner and Weintraub, 1994; Zimmerman et al., 1993). Genes related to *atonal*, another *Drosophila* proneural gene, are also found in vertebrates. They can be subdivided into two groups according to their expression patterns. Some are expressed in the proliferative zone (e.g., *Neurogenin1*, *MATH4a*) (Gradwohl et al., 1996; Ma et al., 1996), while others are expressed by postmitotic neurons (e.g., *NeuroD*, *Nex1*/*MATH2*) (Bartholoma and Nave, 1994; Lee et al., 1995; Shimizu et al., 1995). These different expression patterns certainly reflect different functions yet *NeuroD*, like *XASH3*, can change the fate of cells from epidermal to neural in *Xenopus*, probably by bypassing early regulatory stages involving other HLH factors.

Whereas some HLH transcription factors activate the neural differentiation program, others inhibit it. *HES1*, a vertebrate homolog of the *Drosophila hairy* and *enhancer-of-split* genes, negatively regulates neuronal differentiation by two different mechanisms. It represses expression of target genes by binding to the sequence CACNAG in their promoters, and it inhibits activator HLH factors such as MASH1 by heterodimerizing with them and preventing them from binding to E-boxes (CANNTG), their recognition sequence in DNA (Sasai et al., 1992). *HES1* is normally expressed by proliferating neuroblasts in the VZ, and forced expression prevents cells from leaving the mitotic cycle and hence from migrating outside the VZ to differentiate (Ishibashi et al., 1994). Its role in the regulation of neurogenesis is further evidenced in knockout mice, whose neural differentiation takes place prematurely in the absence of *HES1*, leading to severe defects (Ishibashi et al., 1995). Together, these results indicate that, like their

Drosophila homologs, vertebrate HLH factors orchestrate neuronal differentiation.

We report here the isolation and characterization in the chick of *NeuroM*, a novel basic helix-loop-helix (bHLH) gene, which is expressed throughout the developing nervous system and defines a new stage in the cell-differentiation process. Cells expressing *NeuroM* are located at the interface between the proliferative and non-proliferative zones of the neural tube, they have just left the cell cycle but have not yet begun migrating. *NeuroM* is expressed after *ASH1* and *Neurogenin* but before *NeuroD*, suggesting the existence of a HLH gene expression cascade in the developing nervous system.

MATERIALS AND METHODS

Recombinant DNA procedures

Standard protocols were used throughout. PCR amplification was performed on 0.5 μg phage DNA extracted from a home-made E12 chick retina cDNA library, with degenerate primers 5′GGAATTC(C/A)GI(C/A)GIATGAA(A/G)GCIAA(T/C)GC3′ and 5′GCGGATCCACIA(A/G)(A/G)TTIGTIGTIGG(T/C)TG3′, respectively, corresponding to the motifs RRMKANA and QPTTNLV bracketing the bHLH region of mouse atonal homologs. Amplified DNA was cloned in pBluescript and sequenced. The cDNA library was then plated and screened at low stringency using the *NeuroM* PCR fragment as a probe. In addition to *NeuroM*, a full-length cDNA of chicken *NeuroD* and a partial sequence of chicken *Nex1/MATH2* were obtained. The *NeuroM* gene was isolated from a home-made chick erythrocyte DNA library. The complete sequences of chicken *NeuroM* and *NeuroD* are deposited in the GenBank under the respective accession numbers Y09597, Y09596.

Transactivation in freshly dissociated cells

The mouse *MyoD* (Tapscott et al., 1988), chicken *NeuroM* and *NeuroD* cDNAs were cloned at the unique *Eco*RI site downstream of the mouse sarcoma virus LTR in the expression vector pEMSV (pEMSV and pEMSV-MyoD were the gift of Dr Harold Weintraub). The core promoter, 190 bp in length, of the chicken muscle nicotinic acetylcholine receptor α1 subunit was generated by PCR from a larger genomic fragment and cloned upstream of the CAT gene to generate the reporter plasmid pα1-CAT. Dissociated cells from E8 optic tectum were transfected with Lipofectin (Gibco) and assayed for CAT activity as previously described (Matter-Sadzinski et al., 1992).

RNA isolation and northern blots

RNA from different tissues (the gift of Dr Linda Erkman) was isolated as described in Hernandez et al. (1995). 3 μg of total RNA for each tissue or developmental stage were denatured by heating 20 minutes at 65°C in MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0) in presence of 10% formaldehyde. Samples were fractionated by electrophoresis in 1.5% agarose minigels in MOPS buffer containing 6.7% formaldehyde. RNA was then electroblotted and hybridized as described in Khandjian (1986) and Matter et al. (1990). The *NeuroM* probe was a *Pst*I-*Eco*RI cDNA fragment encoding amino acids 175-297.

In situ hybridization

Chick embryos were staged according to Hamburger and Hamilton (1951). For simplicity, the corresponding embryonic days (E) are used: E1.5 (stage 8-9), E2 (stage 12), E2.5 (stage 17), E3.5 (stage 21), E4 (stage 24), E6 (stage 29), E8 (stage 34), E10 (stage 36), E14 (stage 40). In situ hybridizations were performed essentially as described in Nef et al. (1996). Tissues were fixed in 4% paraformaldehyde in PBS, on ice, for 2 to 16 hours depending on size, dehydrated in EtOH and embedded in paraffin wax (Paraplast). 7 μm sections were mounted on TESPA-coated slides. Tissue sections were then dewaxed, rehydrated, treated for 10 minutes with proteinase K at 37°C, fixed again for 15 minutes in 4% formaldehyde at room temperature, acetylated with triethanolamine and finally dehydrated in ethanol. Hybridization was done overnight at 60°C with a solution containing 50% formamide and 5% dextran sulfate in Tris-HCl 20 mM, pH 7.6, EDTA 5 mM, to which either ^{35}S-labeled single-strand RNA probe was added to 30,000 cts/minute/μl or DIG-labeled probe at 1 ng/μl final concentration. Sections were then rinsed in 4× SSC at room temperature, and washed for 30 minutes in 50% formamide at 60°C. They were treated with RNaseA (20 μg/ml) to reduce background, and finally washed for 15 minutes each in 2× SSC and 0.1× SSC at 60°C. Sections labeled with ^{35}S were then dehydrated, dipped in liquid photographic emulsion (Kodak NTB-2) and exposed for approximately 2 weeks. Sections labeled with DIG were processed as described below in the BrdU section. Two antisense *NeuroM* riboprobes were used: a transcript of 366 nucleotides corresponding to amino acids 175-297 and a longer transcript (about 2000 nucleotides) containing 5′ untranslated sequences and ending at the level of amino acid 297. Both probes gave the same hybridization pattern. The antisense *NeuroD* riboprobe was 1300 nucleotides in length, starting at the level of amino acid 72 and ending 350 bp downstream of the stop codon. The antisense *c-Delta-1* riboprobe was 2000 nucleotides in length and encompassed the whole coding sequence (*c-Delta-1* plasmid, the gift of Dr Domingos Henrique).

BrdU labeling

Tissues were dissected, rinsed in HBSS and incubated for 30 minutes in DMEM, 10% FCS containing 100 μM BrdU, followed by a 15-minute chase in culture medium. Explants were either fixed, embedded in paraffin and sectioned or they were incubated for 15 minutes at 37°C in trypsin solution (0.05% for optic tectum and trigeminal ganglion or 0.1% for spinal cord) and single-cell suspension was prepared as described in Matter-Sadzinski et al. (1992). Cells were plated in DMEM-FCS medium onto poly-L-ornithine-coated plastic chamber slides (Lab-Tek) and, after 1 hour of attachment, they were fixed for 15 minutes with 4% paraformaldehyde in PBS and dehydrated in ethanol. Dissociated cells were prehybridized as described in Matter-Sadzinski et al. (1992), and were hybridized and washed under the same conditions as the tissue sections. Sections and dissociated cells were first subjected to the complete in situ hybridization procedure and then processed for BrdU immunodetection.When hybridized with a DIG-labeled probe, slides were treated as described in Biffo et al. (1992) and Myat et al. (1996). When sections or dissociated cells were hybridized with a radioactive probe, BrdU was revealed according to the manufacturer's instructions (Boehringer), except that the DNA denaturation step was omitted. Anti-BrdU labeling was detected using Fast Red substrate. Sections and dissociated cells hybridized with a radioactive probe were then dehydrated and processed for autoradiography. *NeuroM*- and BrdU labelings were examined using bright- and dark-field optics.

RESULTS

Structural analysis of chicken *NeuroM*, an *atonal*-related gene

Degenerate oligonucleotides were designed against conserved motifs in the basic helix-loop-helix domain of the *atonal* gene family, and PCR reactions were performed using template DNA extracted from an E12 chick retina cDNA library (see Materials and Methods). Among the amplified fragments, one corresponded to the bHLH domain of a novel gene that we designated *NeuroM* (M stands for mitosis and migration). This

PCR fragment (270 bp in length) was then used as a probe to screen the E12 retina cDNA library at low stringency. A full-length *NeuroM* cDNA was isolated, containing an open reading frame spanning 990 bases, the conceptual translation of which yielded a protein of calculated molecular mass 36.7 kDa (Fig. 1A). The corresponding gene was isolated, sequenced and found to be intronless within the open reading frame. Sequence comparisons reveal that NeuroM clearly belongs to the group of vertebrate bHLH proteins related to *Drosophila* proneural factor Atonal (Fig. 1B). The chick homolog of NeuroD was also isolated and found to have 77% identity with its mouse counterpart. NeuroD is NeuroM's closest relative but, while their bHLH domains are highly conserved (84% identity), their overall homology is relatively low (56% identity) (Fig. 1C).

To ascertain whether NeuroM is capable of stimulating transcription from a promoter known to be regulated by HLH transcription factors, we cotransfected freshly dissociated E8 optic tectum cells with two DNA constructs. The reporter construct consisted of the E-box-containing, 190 bp core promoter of the α1 subunit of muscle nicotinic acetylcholine receptor (Piette et al., 1990; Wang et al., 1988) driving transcription of the chloramphenicol acetyltransferase (CAT) gene. The trans-activating construct expressed NeuroM from the mouse sarcoma virus LTR. We found that NeuroM considerably increased CAT activity, as compared to a negative control where the transactivating plasmid had no insert (Fig. 2). Likewise, transactivating plasmids expressing mouse MyoD and chicken NeuroD both strongly stimulated CAT synthesis (Fig. 2). Thus, exogenously supplied NeuroM (and NeuroD) are capable of activating an E-box-driven promoter.

Specific neural expression of *NeuroM*

As a first step towards understanding the function of *NeuroM*, its expression in various tissues was assessed by northern blot. On E12, when the chick nervous system has almost acquired its mature structure and is mostly composed of differentiated cells, *NeuroM* mRNA was only detected in the retina. It was not seen in other parts of the nervous system such as the telencephalon, cerebellum and optic tectum, or in non-neural tissues such as muscle, liver and thymus (Fig. 3A). Blots of total RNA prepared from neuroretina in a developmental series extending from E4 to early

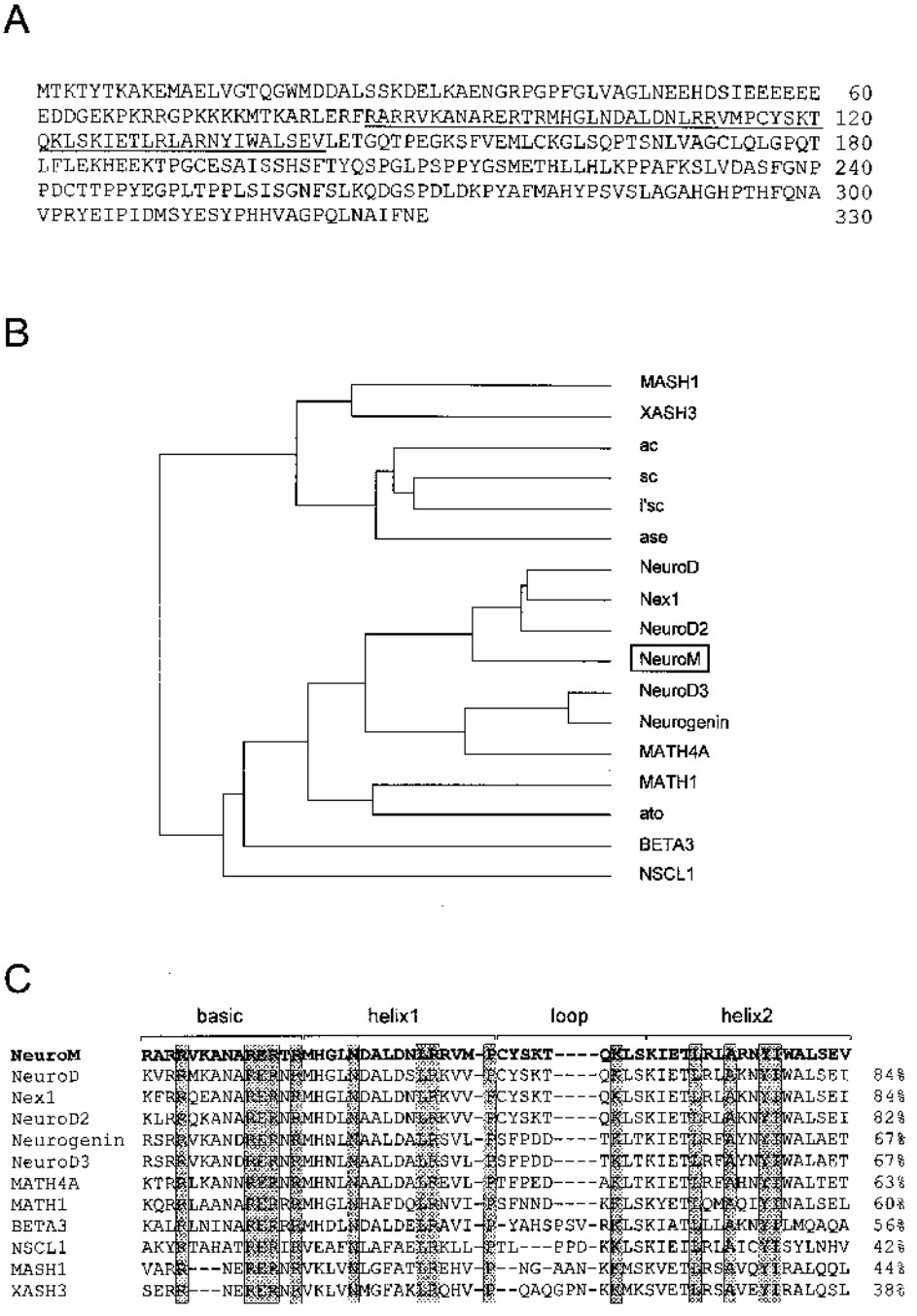

Fig. 1. (A) The *NeuroM* cDNA isolated from a chick E12 retina library encodes a protein of 330 amino acids which belongs to the family of basic helix-loop-helix (bHLH) transcription factors. The bHLH domain is underlined. (B) Dendrogram of the bHLH domains of various neuronal bHLH transcription factors from *Drosophila* and vertebrates (cluster program). NeuroM segregates with the subfamily of atonal-related factors, constituting a separate branch in a group containing NeuroD, Nex-1 and NeuroD2. (C) Multiple alignments of the bHLH domains of vertebrate neuronal bHLH factors. In this domain, identities to NeuroM range from 84% (NeuroD, Nex1/MATH2) to 38% (XASH3). Conserved residues are highlighted. The GenBank accession numbers for *NeuroM* and *NeuroD* areY09597, Y09596, respectively.

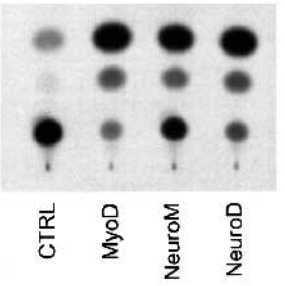

Fig. 2. NeuroM transactivates an E-box-driven promoter. Dissociated E8 optic tectum cells were cotransfected with the reporter plasmid pα1-CAT bearing the core enhancer of the α1 subunit of the nicotinic receptor and the indicated transactivating plasmids encoding bHLH factors. CAT activity was assayed 48 hours later. MyoD is used as a positive control. The detectable CAT activity in the negative control (CTRL) is most likely due to transactivation by endogenous HLH factors. The experiment was repeated several times with very reproducible results.

adulthood (P28) demonstrated that *NeuroM* appears around E4 and is maintained at a high level thereafter (Fig. 3B).

In situ hybridization at an early stage (E4), when undifferentiated neuronal precursors actively proliferate, revealed a much broader expression domain. At this stage, *NeuroM* is expressed in most regions of the CNS and PNS, including the retina, telencephalon, diencephalon, metencephalon, spinal cord, dorsal root and trigeminal ganglia, olfactory epithelium (not shown) and optic tectum. In all examined regions *NeuroM* expression is confined to a layer external to the ventricular zone (Fig. 3C and see below). These initial observations indicated that *NeuroM* is transiently expressed throughout the developing nervous system, several regions of which were analyzed in detail.

Singular expression pattern of *NeuroM* in the spinal cord

The nervous system begins to differentiate after gastrulation, as soon as the three germ layers are established. The neural plate begins as a thickening of the axial dorsal ectoderm, its edges rising dorsally and folding towards the midline until they meet and fuse to form the hollow neural tube. On E1.5, at the neural groove stage, *NeuroM* is detected in a very few individual cells (Fig. 4, arrow). It is a likely marker of primary interneurons, which are the very first cells of the spinal cord to become postmitotic (McConnell and Sechrist, 1980; Sechrist and Bronner-Fraser, 1991). On E2, immediately after neural tube closure, it is detected in the pial zone on the lateral aspects of the neural tube (Fig. 4). No labeling is seen in midline cells, either in the floorplate or the roofplate. On E2.5, *NeuroM* labels a bilateral stripe of cells located between the proliferative ventricular zone and the subpial zone. This becomes even more apparent on E3.5 and at later stages (Fig. 4), the highest level of labeling occurring in the ventral spinal cord, where motor neurons are being generated. *NeuroM* disappears from the ventral spinal cord between E6 and E8, becoming restricted to the dorsal aspect, from where it eventually disappears between E8 and E10 (Fig. 4). This ventral-to-dorsal wave of expression is reminiscent of the pattern of cell proliferation in the spinal cord, which stops ventrally on E6 and goes on dorsally until E8 (Langman and Haden, 1970). The location of *NeuroM*-expressing cells on the external side of the VZ suggests that these cells are postmitotic. However, the fact that *NeuroM* expression temporally follows the ventral-to-dorsal gradient of cell proliferation may suggest that *NeuroM*-expressing cells located at the very edge of the VZ are still proliferating. To resolve this issue, we pulse-labeled isolated E4 spinal cords for 30 minutes with BrdU in order to label S-phase nuclei, and processed the tissue for BrdU incorporation and *NeuroM* expression (Fig. 7). We conclude that the bulk of *NeuroM*-expressing cells have withdrawn from the mitotic cycle as they are adjacent to, but distinct from, the cells labeled with BrdU (Fig. 7A,D). To improve the resolution of this analysis, dissociated cells from E4 spinal cords were stained for BrdU incorporation and hybridized to detect *NeuroM*-expressing cells. Several thousand cells were examined, of which hundreds were labeled with one or the other marker. We did not detect any instance of double labeling (Fig. 7E,F).

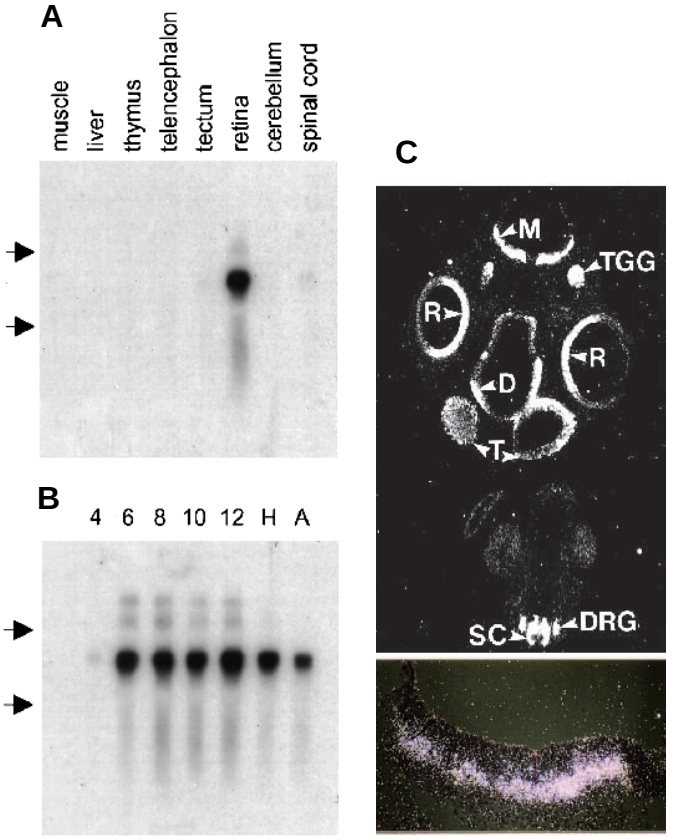

Fig. 3. (A) Northern blot of total RNA (3 μg per lane) from various E12 tissues hybridized with a *NeuroM* probe. A signal is only detectable in the retina. (B) Northern blot of total RNA (3 μg per lane) from retina at various days of development. *NeuroM* is detected at low levels at E4, and at high levels from E6 until adulthood, with a peak around E12 (H, hatchling; A, adult). Arrows indicate positions of 18S and 28S ribosomal RNA. (C) In situ hybridization of a chick embryo at E4 (coronal section). Labeling by *NeuroM* is observed throughout the CNS and PNS. No expression is detected in other tissues. Lower panel: close up of the metencephalon showing that *NeuroM* expression occurs in a layer lining the ventricular zone. (M, metencephalon; TGG, trigeminal ganglion; R, retina; T, telencephalon; D, diencephalon; DRG, dorsal root ganglion; SC, spinal cord).

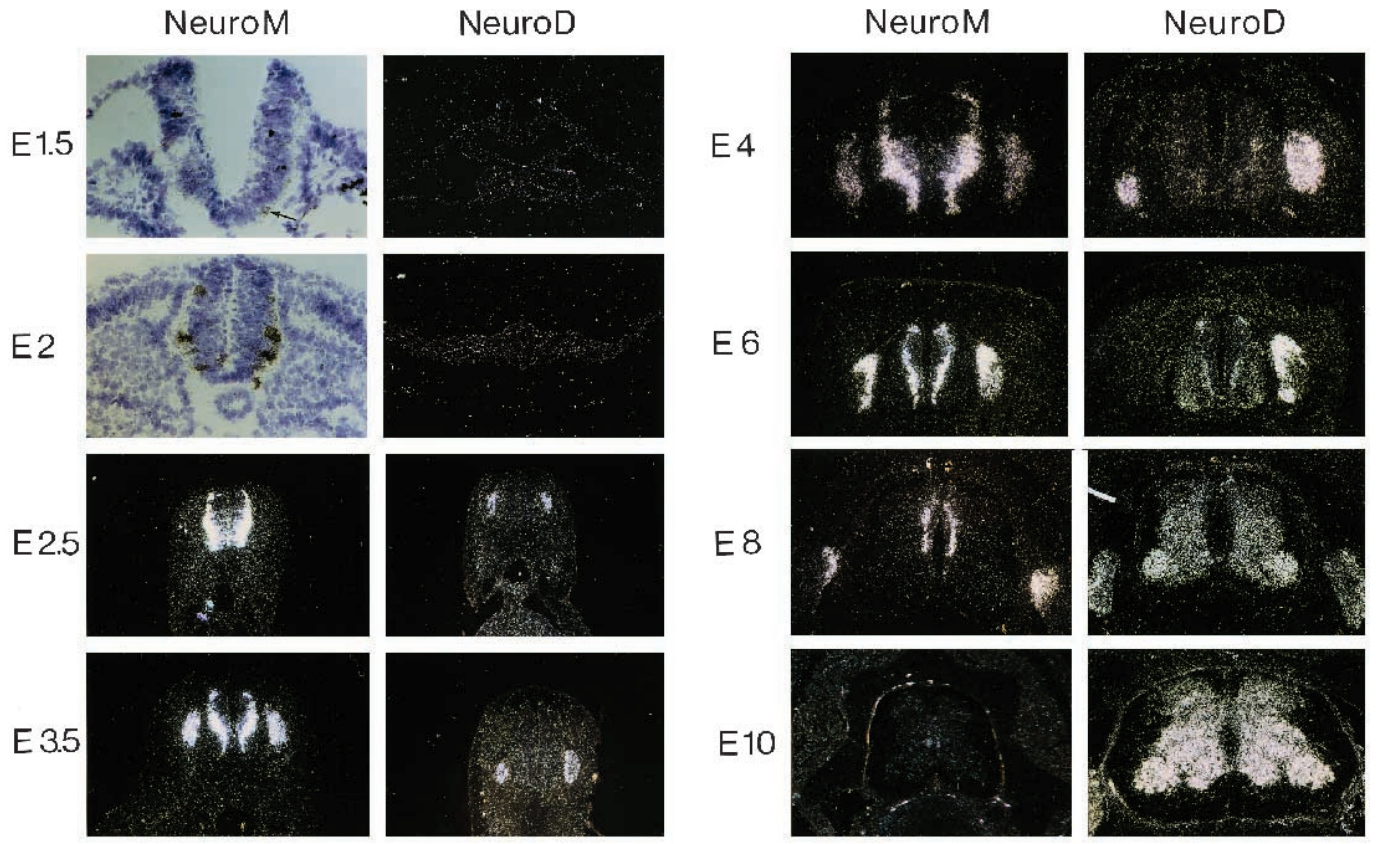

Fig. 4. In situ hybridizations in the developing spinal cord comparing expression of *NeuroM* and *NeuroD* (dark-field optics, except *NeuroM* at E1.5 and E2). Expression of *NeuroM* begins in a small number of cells at E1.5 (arrow), before closure of the neural tube and concomittant with the appearance of primary interneurons, the first postmitotic cells to appear in the spinal cord. At E2, *NeuroM*-positive cells are detected on the pial surface of the spinal cord. From E2.5 on, *NeuroM*-positive cells are observed adjacent to the ventricular zone at all levels of the dorsoventral axis. No expression is detected in either the VZ or the outer mantle zone. As development proceeds, cell proliferation decreases and expression is reduced (E6). On E8, proliferation has stopped in the basal plate and expression is confined to the dorsal aspect of the spinal cord. In the DRG, expression begins around E3 and ends on E8, soon after the last precursors have stopped dividing. The same expression pattern was obtained with two different antisense probes, and a sense probe yielded no detectable signal (not shown). *NeuroD* expression in the spinal cord begins in the mantle zone between E4 and E6. On E6, *NeuroD* is strongest in the region where *NeuroM* is maximally expressed. Expression then increases until E10 in all postmitotic neurons. In the DRG, expression begins on E2.5, two days earlier than in the spinal cord, and is maintained at high levels until E10.

Delta-1, whose product is a lateral inhibition signal, is known to be expressed after the last S-phase in cells located within the VZ (Myat et al., 1996). Hybridization on adjacent sections reveals that while *Delta-1*-expressing cells still reside within the VZ (Fig. 7C), *NeuroM* is solely induced when cells reach the boundary with the mantle, suggesting that *NeuroM* expression (Fig. 7A,B) begins shortly after that of *Delta-1*. Thus, *NeuroM*-positive cells are postmitotic cells that have just left the ventricular zone but have not yet started their migration to outer layers. Whereas there are known instances of genes encoding neuronal transcription factors being specifically expressed either in the ventricular or in the mantle zones, the pattern of expression of *NeuroM* in the spinal cord, at the very edge of the ventricular zone, is completely novel and defines a previously unrecognized transition stage between mitosis and migration.

Compared to *NeuroM*, *NeuroD* has a later onset of expression in the spinal cord, where it is not detected until E4 (Fig. 4). Low levels of labeling are initially observed outside the VZ. On E6, two slightly brighter stripes appear in the ventral spinal cord, in the region of maximum *NeuroM* expression. A similar expression pattern of this gene has been reported in the mouse and in the rat at comparable stages (Lee et al., 1995; Ma et al., 1996). In the chick, two groups of *NeuroD*-expressing cells are also seen in the dorsal-most spinal cord, next to the roofplate. Between E6 and E8, as *NeuroM* begins to recede, there is a burst in *NeuroD* expression. On E8, *NeuroD* labels the whole grey matter in a gradient along the dorsoventral axis, the strongest labeling being associated with the ventral horns (Fig. 4). On E10, expression levels are even higher and the gradient is maintained at least until E18, albeit at somewhat reduced levels (not shown).

In the spinal cord, the spatiotemporal expression patterns of *NeuroM* and *NeuroD* thus define two distinct cell populations. *NeuroM* appears to be associated with non-proliferating, premigratory cells, whereas *NeuroD* labels neurons that are migrating or have reached their final position.

Transient expression of *NeuroM* in postmitotic, premigratory tectal neurons

Whether or not similar developmental mechanisms operate in

the spinal cord and in the rest of the CNS remains poorly documented. We therefore decided to look at *NeuroM* expression in the optic tectum, whose neurogenesis and histology are well characterized (LaVail and Cowan, 1971). On E3.5 and E4, *NeuroM* is undetectable in the VZ and exclusively expressed in the first postmitotic tectal neurons lining the pial aspect of the neuroepithelium. On E6, the cells labeled by *NeuroM* are located in a thin intermediate layer between the VZ and the outer layers (Fig. 5). To localize cells expressing *NeuroM* precisely with respect to the outer VZ, E7 optic tecta were pulse-labeled for 30 minutes with BrdU as floating explants. Cells in S-phase are known to be located in the outer third of the VZ and were labeled by BrdU, as expected. In situ hybridization on BrdU-labeled tecta thus revealed that *NeuroM*-positive cells are postmitotic, and lie adjacent to the outer VZ, where actively dividing precursors are located (Fig. 7G,H). Hybridization on dissociated BrdU-labeled tectal cells confirms that, as in the spinal cord, no expression of *NeuroM* occurs in proliferating cells (not shown). *Delta-1*-expressing cells are localized in the VZ, indicating that, as in the spinal cord, expression of *Delta-1* shortly precedes that of *NeuroM*. Migratory and postmigratory cells in the outer layers do not express *NeuroM* (Fig. 7H,I). On E8, when most tectal cells have stopped dividing and only a few precursors of layers V and VI neurons are still proliferating, *NeuroM* is only detected in a thin layer lining the VZ. On E10, when neurogenesis in the optic tectum has ceased, *NeuroM* expression disappears (Fig. 5). Thus, *NeuroM* expression in the optic tectum resembles that in the spinal cord and is associated with the same stage of the differentiation process, when cells withdraw from the mitotic cycle and are poised to migrate outwards to their final location. In both instances, *NeuroM* expression is transient and stops before the cells begin to migrate.

In the optic tectum as in the spinal cord, *NeuroD* expression has a later onset than *NeuroM*. It begins on E6 and is observable throughout the thickness of the tissue, except for the VZ which shows no labeling (Fig. 5). On E8, *NeuroD* is not only detected in the subventricular layer, like *NeuroM*, but also in more external layers. On E10, when *NeuroM* expression has stopped, *NeuroD* is still expressed throughout the different layers of the optic tectum. Whereas *NeuroM* expression is transient and seems related to the proliferative to postmitotic transition, *NeuroD* expression appears later in development and occurs in differentiated cells.

Transient and persistent expression of *NeuroM* in retina

In the chick retina, neurogenesis begins on E2 and ends on E12. The first neurons to withdraw from the mitotic cycle and to differentiate are the ganglion cells, whereas the last are the bipolar cells (Prada et al., 1991). To investigate whether *NeuroM* and *NeuroD* expressions are associated with particular retinal cell types, in situ hybridizations were performed from E2 (neuroepithelium) to E14 (when the architecture of the mature retina is established). On E2 and E2.5, *NeuroM* is detected in the central region of the retina, at the time and place of appearance of the first postmitotic cells which give rise to ganglion cells (Fig. 6). As development proceeds, the *NeuroM* expression domain expands to the periphery, paralleling retina maturation, and by E4 *NeuroM* is expressed throughout the thickness of the as yet largely undifferentiated neuroepithelium (Fig. 6). Not all cells

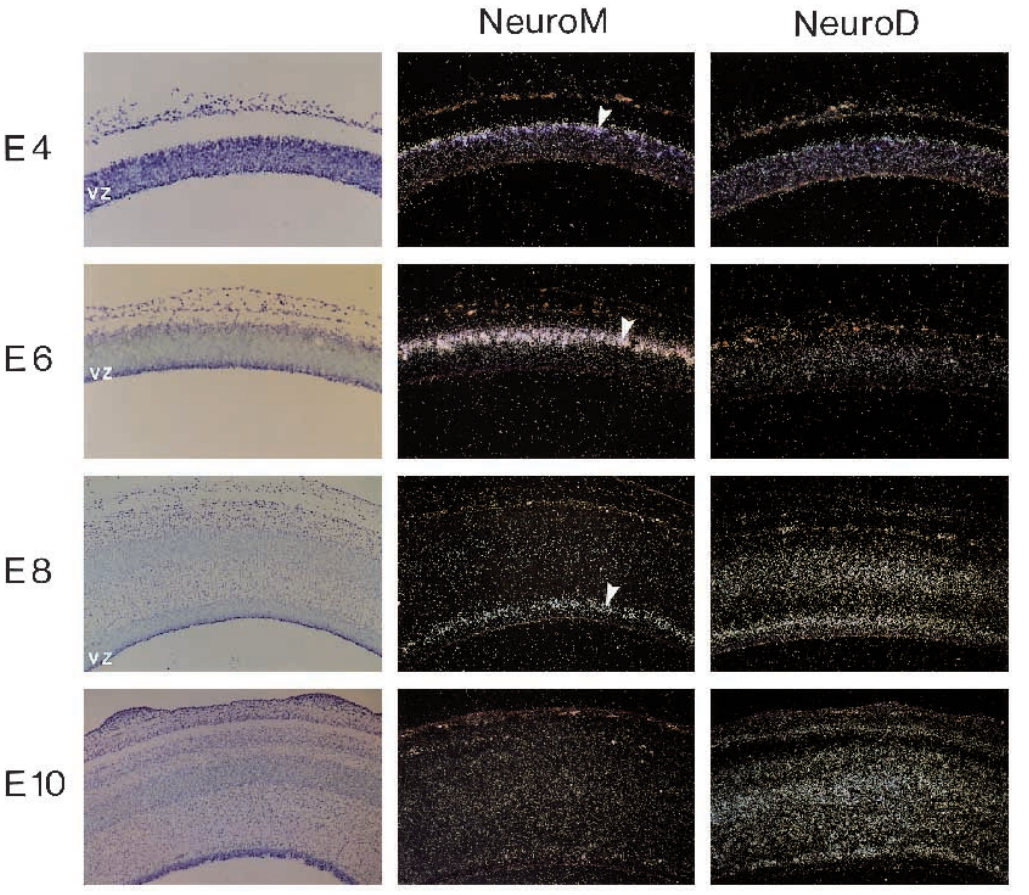

Fig. 5. In situ hybridizations in the developing optic tectum. On E4, *NeuroM*-positive cells are located on the pial side of the neuroepithelium, where postmitotic neural precursors first appear (arrowhead). On E6, expression is stronger and forms a thicker layer above the VZ. Between E6 and E8, the thickness and number of layers rapidly increase in the mantle zone but *NeuroM* expression remains confined to a thin layer of cells lining the VZ. The last precursors stop proliferating around E9 and, on E10, *NeuroM* expression has ceased. *NeuroD* expression begins later, around E8, and labels postmitotic cells in the external layers. In the layer lining the VZ, *NeuroD* expression overlaps that of *NeuroM*. On E10, *NeuroM* has been turned off and *NeuroD* is expressed throughout the optic tectum. Ventricular side is down.

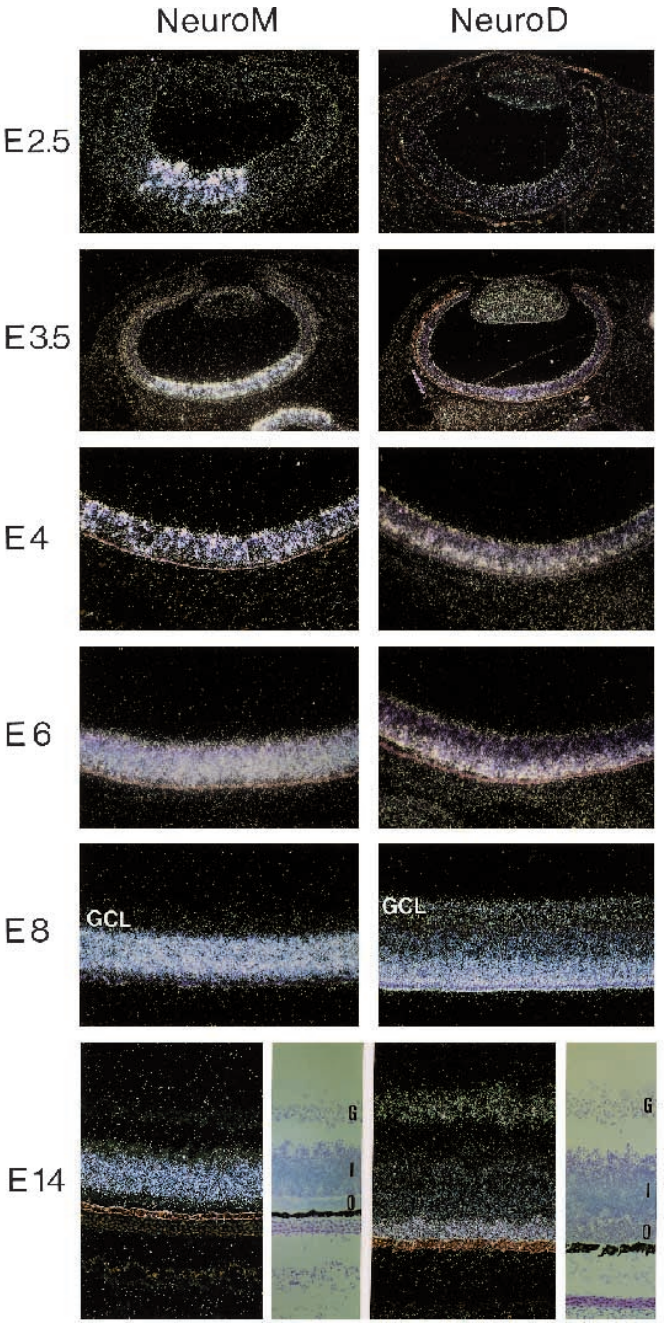

Fig. 6. In situ hybridizations in the developing retina. *NeuroM*-positive cells are detected as early as E2 in the central part of the retina, spreading laterally as the retina matures (E2.5, E3.5). Expression occurs in isolated cells until around E4, and progressively becomes more homogeneous (E6, E8) across the whole neuroepithelium, except in the developing GCL. Homogeneous expression coincides with the time when most retinal cell types are being generated. From E8 on, *NeuroM* expression progressively disappears from the forming ONL where photoreceptors are beginning to differentiate. In the mature retina (E14), *NeuroM* expression is confined to the part of the INL containing horizontal and bipolar cells. *NeuroD* expression begins somewhat later, at E3.5. Thereafter labeling is observed on the ventricular side of the neuroepithelium throughout development and is maintained in the ONL (containing the photoreceptors) in the mature retina (E14). On E8, expression also appears in the ganglion cell layer where it is maintained at later stages (GCL and G, ganglion cell layer; I, inner nuclear layer; O, outer nuclear layer). Ventricular side is down.

are labeled, however, and individual cells expressing *NeuroM* at high levels are interspersed among fields of unlabeled cells**.** At E6, labeling becomes more homogeneous and the majority of retinal cells appear to express *NeuroM*, excepting those in the developing ganglion cell layer (GCL). This supports the idea that, in ganglion cells, as in other central neurons, *NeuroM* is not required during differentiation but rather for a transition state preceding it. From E8 on, *NeuroM* also disappears from the forming photoreceptor layer and becomes restricted to the inner nuclear layer (INL), which contains the amacrine, bipolar, and horizontal cells. When neurogenesis is complete (E14), *NeuroM* expression is confined to the outer part of the INL, containing the bipolar and horizontal neurons, whereas the amacrine cells located in the inner part of the INL are unlabeled (Fig. 5).

In retina, *NeuroD* again has a later expression onset than *NeuroM*. No labeling is detectable at E2 and expression begins at E3.5 in the central part of the retina, progressively expanding to the periphery along the differentiation gradient. On E4, expression is seen throughout the neuroepithelium, being strongest in the zone directly lining the ventricle. At E6 and E8, expression is confined to the VZ, which then contains undifferentiated proliferating neuroblasts and nascent photoreceptors. From E8 on, *NeuroD* is also detected in the ganglion cell layer. In the differentiated retina (E14), *NeuroD* and *NeuroM* are expressed in distinct cell populations, and a strikingly complementary expression pattern becomes apparent, with *NeuroD* being expressed in the ONL and GCL, and *NeuroM* in the INL.

In spite of added complexities, *NeuroM* expression in the retina probably obeys the same principles as in the spinal cord and optic tectum, being associated with the differentiation waves of the various retinal cell populations. However, in contrast to the situation in spinal cord and optic tectum, expression does not stop at the end of neurogenesis, but persists in mature horizontal and bipolar neurons.

Transient expression of *NeuroM* also occurs in the peripheral nervous system

The dorsal root ganglia (DRG) are composed of the proprioceptive neurons innervating muscle and of the cutaneous sensory neurons innervating epidermis. The former are located in the ventrolateral part of the ganglia whereas the latter occupy a dorsomedial position (Lefcort et al., 1996), their neurogenesis extending between E3 and E7.5 (Carr and Simpson, 1978). *NeuroM* is expressed in the DRG from E3.5 (Fig. 4). Labeling is first observed throughout the ganglion, becoming progressively restricted to the dorsomedial aspect where the cutaneous sensory neurons are located. Expression of *NeuroM* stops between E8 and E10, when neurogenesis is complete (Fig. 4).

Whereas in the CNS *NeuroD* consistently appears later than *NeuroM*, the opposite obtains in the DRG. *NeuroD* is present everywhere in the ganglion from E2.5 to E6, whereupon it recedes to the dorsomedial aspect, as *NeuroM* also does. At the time of its expression burst in the spinal cord (E8), *NeuroD* undergoes a switch in the DRG and from then on, it predominantly labels the propioceptive neurons in the ventrolateral region of the ganglion (Fig. 4).

The chick trigeminal ganglion has a dual embryonic origin, being formed by cells derived from the neural crest and from epidermal placodes. Most neurons are generated between E2 and E7, in a distal-to-proximal sequence. By E5 (Fig. 7J), the only mitotically active neuroblasts are found within the

proximal region of each lobe, an area of neural crest origin (D'Amico-Martel and Noden, 1980) and *NeuroM* expression appears to reflect the proliferative wave in the developing ganglion. No labeling is seen in the placode-derived neurons of the distal zone, which withdrew from the mitotic cycle at E2-E3 (Fig. 7J). Neurogenesis ends on E7 and, by E8, *NeuroM* has become undetectable (not shown). Hybridization on dissociated cells from the E5 ganglion shows that *NeuroM*-expressing cells have withdrawn from the mitotic cycle (Fig. 7K,L), suggesting, for *NeuroM*, a similar function in the PNS and in the CNS.

DISCUSSION

In this paper, we characterize the salient expression features of *NeuroM*, a newly isolated gene encoding a neural bHLH transcription factor. *NeuroM* expression is transient throughout the developing nervous system and is associated with the narrow time window when undifferentiated precursors that have stopped proliferating leave the ventricular zone and possibly pause, en route to their final location. Thus, *NeuroM* marks the brief episode in an individual neuron's life between proliferation and migration. *NeuroM* homologs, designated *ATH-3*, have recently been isolated in *Xenopus* and mouse (Takebayashi et al., 1997). Like *NeuroM*, *ATH-3* is expressed in the developing nervous system and persists in adult retina. Its overexpression in *Xenopus* embryos induces ectopic neurogenesis, suggesting an active role in neuronal differentiation. Although circumstantial evidence suggests that *ATH-3* may be a proneural gene in *Xenopus*, this is certainly not the case in the chick, where *NeuroM* expression is confined to postmitotic cells.

In the ventricular zone of the developing CNS, the nuclei of proliferating cells move in and out in the course of the cell cycle, passing through S-phase while they are in the outer part of the ventricular zone, and leaving the ventricular zone after their last

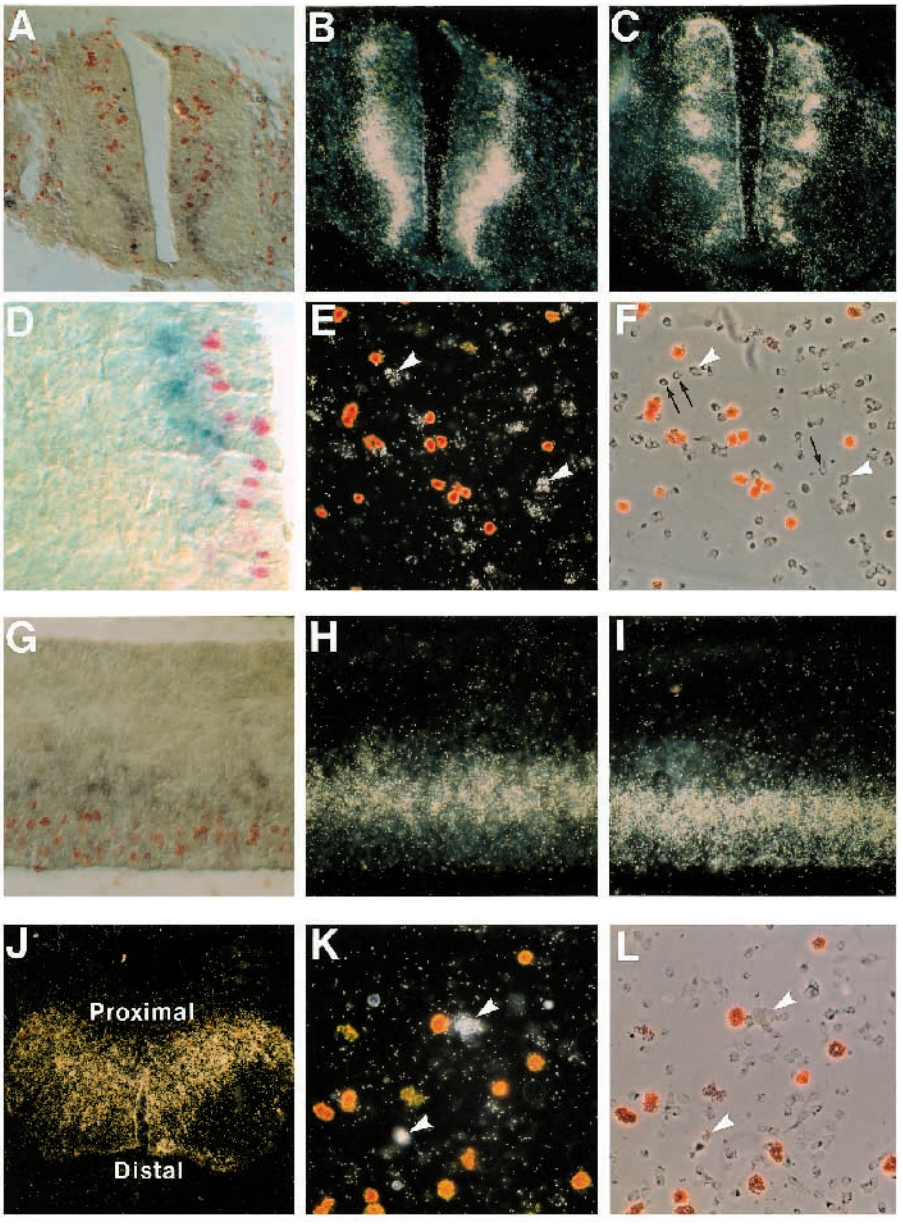

Fig. 7. Double labeling for BrdU incorporation and *NeuroM* expression. Dissected tissues were incubated for 30 minutes in presence of BrdU to label S-phase cells. They were then processed for in situ hybridization with a *NeuroM* riboprobe (DIG- or ^{35}S-labeled) and for immunodetection of incorporated BrdU, either as sections or as dissociated cells. Single labelings were done using ^{35}S-labeled probes. (A-F) Spinal cord at E4. (A) DIG staining (black) for *NeuroM* lies just outside the VZ and does not overlap with BrdU staining (red). This indicates that *NeuroM*-expressing cells are postmitotic. (B) Radioactive *NeuroM* labeling to an adjacent section, no *NeuroM*-positive cells are detected in the VZ. (C) *Delta-1* expression occurs in the VZ (Henrique et al., 1995, Myat et al., 1996) as confirmed by this in situ hybridization done on an adjacent section. (D) High magnification digitally enhanced view of the spinal cord clearly shows that *NeuroM*-positive cells (blue) are postmitotic, although they lie adjacent to proliferating cells (red nuclei). (E) Double-labeling on dissociated cells confirms that *NeuroM*-positive cells (arrowheads) are not proliferating. (F) Bright-field view of E. Unlabeled cells (arrows) are either proliferating cells that did not go through S-phase during the 30-minute BrdU pulse, or postmitotic cells that have ceased expressing *NeuroM*. (G-I) Optic tectum at E7. (G) *NeuroM*-positive cells (black) lie just above the proliferating cells (red nuclei). (H) Adjacent section labeled with a [^{35}S] *NeuroM* probe. No signal is seen in the VZ. (I) Adjacent section labeled with a [^{35}S] *Delta-1* probe. *Delta-1* expression occurs in the VZ. (J-L) Trigeminal ganglion at E5. (J) At this stage, *NeuroM* labeling is restricted to the proximal region of the ganglion where proliferation and differentiation are proceeding, while it is not detected in the distal region where all cells have been postmitotic since E3. (K) Dark-field view of dissociated double-labeled E5 trigeminal ganglion cells. *NeuroM*-positive cells (silver grains, arrowheads) are not proliferating. (L) Bright-field view.

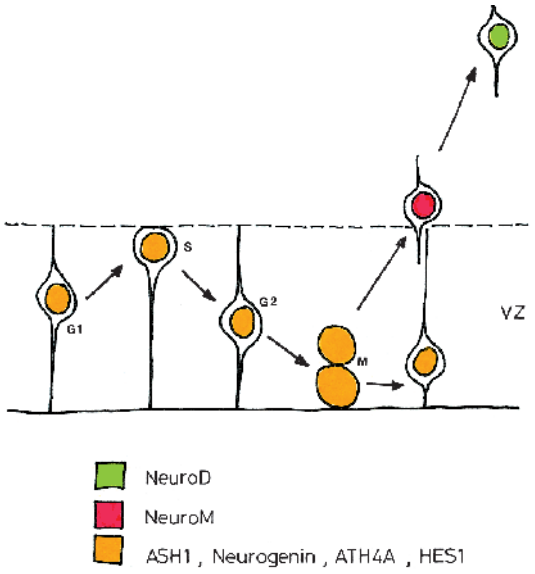

Fig. 8. Sequential expression of HLH genes during neurogenesis. Chenn and McConnell (1995) have shown that when the cleavage plane of a parent cell is oriented parallel to the ventricular surface, it may produce an asymmetric division where one daughter cell loses contact with the lumen and migrates away in the outer layers, while the sibling remains in the proliferative zone. Proliferating progenitors express a number of helix-loop-helix transcription factors. Inhibitory factors such as HES1 keep them in a proliferative and undifferentiated state, whereas activators (e.g., ASH1) promote neuronal differentiation. A change in the balance between these two types of components determines the moment when a cell leaves the mitotic cycle and differentiates. We suggest that, after their last mitosis, precursors exit from the VZ and possibly mark a pause just outside the VZ, during which they express *NeuroM*. *NeuroM* expression may be triggered by activator HLHs in the absence of repression by HES1. NeuroM would then activate *NeuroD* and be turned off as the cells resume their migration.

mitosis. Although cells expressing *NeuroM* lie adjacent to cells in S-phase, they are not themselves labeled with BrdU, suggesting that one of the steps in the conversion of progenitors into nascent neurons is taking place at the edge of the VZ, shortly before neurons embark on migration to the outer layers.

In the spinal cord, inductive signals emanating from the notochord, the floorplate and the overlying ectoderm pattern the tissue along the D-V axis. Differential expression of several transcription factors of the Pax and homeobox families circumscribes domains that correspond to different neuronal populations (reviewed in Tanabe and Jessell, 1996). Motor neurons, for instance, arise in the ventral spinal cord on both sides of the floorplate and they are characterized by the expression of LIM-family homeobox genes. Within the ventral horns, motor neurons are segregated in five columns according to their peripheral targets and each subgroup expresses a specific combination of the LIM genes (*Isl-1*, *Isl-2*, *LIM1* and *LIM3*) (Ericson et al., 1992; Tsuchida et al., 1994). Thus, functionally distinct neurons express different homeobox genes which probably contribute to the establishment of their identities. Likewise, among the neural bHLH genes, *MASH1* and *Neurogenin1* have restricted and distinct expression domains in the developing spinal cord (Guillemot and Joyner, 1993; Ma et al., 1996). In contrast, the bHLH genes *NeuroM* and *NeuroD* do not define functionally distinct populations but rather specific stages of a cell's life, and they are expressed at all levels on the D-V axis, regardless of the cell types being generated.

HLH factors play an essential role in the differentiation of several tissues. In muscle, four related HLH genes are expressed sequentially as undifferentiated precursors undergo several maturation steps to become myotubes. This sequential expression is the result of an activation cascade in which the early genes activate the expression of the late genes. *MyoD* and *myf5* are regarded as early or determination genes that commit precursors to the myogenic lineage by activating *myogenin*, a late or differentiation gene whose expression leads to the final maturation of myoblasts (Weintraub, 1993). Similar mechanisms probably operate in the nervous system, where many HLH factors are expressed in various spatiotemporal patterns. *ASH1*, *ASH3*, *ATH4A* and *Neurogenin1* are expressed in undifferentiated, proliferating cells (Ferreiro et al., 1993, 1994; Gradwohl et al., 1996; Guillemot and Joyner, 1993; Ma et al., 1996), whereas other genes such as *Nex1* and *NeuroD* are expressed in postmitotic, differentiated neurons (Bartholoma and Nave, 1994; Lee et al., 1995). In this context, *NeuroM* defines a new class of HLH factors exclusively expressed at the boundary between the proliferative and the postmitotic regions of the developing nervous tissue. This compartmentalization suggests the existence of HLH gene networks in the nervous system, as in muscle. Indeed, experimental evidence supports this view: *Nex1*/*MATH2* is able to activate its own expression by acting upon E-boxes present in its promoter, and ectopic expression of *Neurogenin1* in *Xenopus* induces *NeuroD* (Bartholoma and Nave, 1994; Ma et al., 1996). However, there is no evidence that the latter induction results from a direct interaction and we suggest that it may be mediated by *NeuroM* which, in view of its spatiotemporal expression pattern, fulfills the requirements for an intermediate between *Neurogenin1* and *NeuroD* in a gene activation cascade (Fig. 8). Consistent with the idea that NeuroM may act as a direct activator of *NeuroD*, instances of overlapping expression between the two genes are observed on E6 in the spinal cord and optic tectum (Figs 3, 4). To determine whether these different bHLH proteins are forming a regulatory cascade, we are currently examining the *NeuroM* and *NeuroD* promoters for the presence of functional E-boxes. The onset of *NeuroM* expression might also result from a relief of the repression exerted by the HLH inhibitor HES1, whose expression in the VZ is instrumental in keeping cells in an undifferentiated state and whose down-regulation allows cells to exit the mitotic cycle, migrate out of the VZ and differentiate (Ishibashi et al., 1995).

Interestingly, expression of *NeuroM* begins shortly after those of *C-Delta-1* and *C-Serrate-1*. In the spinal cord, *C-Delta-1* and *C-Serrate-1* are expressed between E1 and E8 in complementary domains along the D-V axis. These two ligands of C-Notch-1 are transiently expressed in postmitotic, nascent neurons before they migrate out of the proliferative zone (Henrique et al., 1995; Myat et al., 1996). The Delta-Notch signaling pathway mediates a mechanism of lateral inhibition operating in the ventricular zone to maintain a pool of proliferating progenitors throughout the period of neurogenesis, thus regulating the proportion of progenitors that become committed to the neuronal fate (Henrique et al., 1995). In this context, *NeuroM* may mark the population of cells that have just been singled out to leave the mitotically active zone and are about to progress along the migration and differentiation pathway.

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