Mash1 and neurogenin1 Expression Patterns Define Complementary Domains of Neuroepithelium in the Developing CNS and Are Correlated with Regions Expressing Notch Ligands

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Genetic studies in *Drosophila* and in vertebrates have implicated basic helix-loop-helix (bHLH) genes in neuronal fate determination and cell type specification. We have compared directly the expression of *Mash1* and *neurogenin1* (*ngn1*), two bHLH genes that are expressed specifically at early stages of neurogenesis. In the PNS these genes are expressed in complementary autonomic and sensory lineages. In the CNS *in situ* hybridization to serial sections and double-labeling experiments indicate that *Mash1* and *ngn1* are expressed in adjacent and nonoverlapping regions of the neuroepithelium that correspond to future functionally distinct areas of the brain. We also showed that in the PNS several other bHLH genes exhibit similar lineal restriction, as do *ngn1* and *Mash1*, suggesting that complementary cascades of bHLH factors are involved in PNS development. Finally, we found that there is a close association between expression of *ngn1* and *Mash1* and that of two Notch ligands. These observations suggest a basic plan for vertebrate neurogenesis whereby regionalization of the neuroepithelium is followed by activation of a relatively small number of bHLH genes, which are used repeatedly in complementary domains to promote neural determination and differentiation.

Key words: bHLH proteins; Mash1*; neurogenins; Notch ligands; neurogenesis; cell lineage*

Central questions in developmental neurobiology include understanding how the nervous system is patterned into functionally distinct areas and how cellular diversity is generated within these areas. These questions have been addressed, using genetic analysis in the fruit fly *Drosophila*. Several important concepts have emerged from this system. First, proneural genes such as *achaete-scute* (*ac-sc*) and *atonal*, which encode transcription factors of the basic helix-loop-helix (bHLH) type, function as neural determination genes (Jan and Jan, 1993) (for review, see Weintraub, 1993). Second, distinct proneural genes are used in different neuronal sublineages. For example, *ac-sc* is essential for external sensory organ formation, whereas *atonal* is required for that of chordotonal organs (Jan and Jan, 1994). Moreover, ectopic expression of *as-sc* in the wing disk promotes external sensory organ formation (Rodriguez et al., 1990; Brand et al., 1993), whereas that of *atonal* promotes chordotonal but not external organ formation (Jarman et al., 1993). Thus, proneural genes play a critical role not only in the choice of neural fate but also in the choice of neuronal subtype.

The functional characterization of vertebrate homologs of *Drosophila* proneural genes has suggested that the principles described above may be applicable to mammalian neurogenesis as well. For example, the mammalian *ac-sc* homolog 1 (*Mash1*) (Johnson et al., 1990) is expressed in subsets of neuronal precursors in both the PNS and CNS (Lo et al., 1991; Guillemot and Joyner, 1993; Guillemot et al., 1993). Within the PNS *Mash1* expression is restricted to autonomic progenitors (Lo et al., 1991). More recently, we have shown that a novel *atonal*-related gene, *neurogenin* (now called *ngn1*), is, conversely, expressed in sensory but not autonomic ganglia in the PNS (Ma et al., 1996). Thus, just as the proneural genes *ac-sc* and *ato* define distinct neuronal sublineages in *Drosophila*, *Mash1*, and *ngn1* define two major neuronal sublineages within the mammalian PNS.

To obtain further insights into the functions of *Mash1* and *ngn1* in the CNS as well as the PNS, we have compared directly the expression patterns of *ngn1*, *Mash1*, and several other genes by single-color or double-color *in situ* hybridization to adjacent sections. We find that in many embryonic CNS regions *ngn1* and *Mash1* are expressed in complementary domains that exhibit adjacent and nonoverlapping boundaries, suggesting that they are involved in the development of distinct neuronal populations. Within the PNS, moreover, lineages expressing either *ngn1* or *Mash1* subsequently express other bHLH genes, such as *NSCL1* and *eHAND* with similar lineal restrictions, suggesting that different classes of peripheral neurons may be specified by complementary cascades of bHLH proteins. Finally, in the CNS, expression of *ngn1* and *Mash1* is associated closely with that of two vertebrate Notch ligands, *Delta-1* (*Dll-1*) (Bettenhausen et al., 1995) and/or *Jagged* (Lindsell et al., 1995). This overlap is consistent with a potential functional interaction between these two sets of proteins during neuronal fate determination, as demonstrated for their homologs in *Drosophila* and *Xenopus* (Ghysen et al., 1993; Chitnis and Kintner, 1996; Ma et al., 1996).
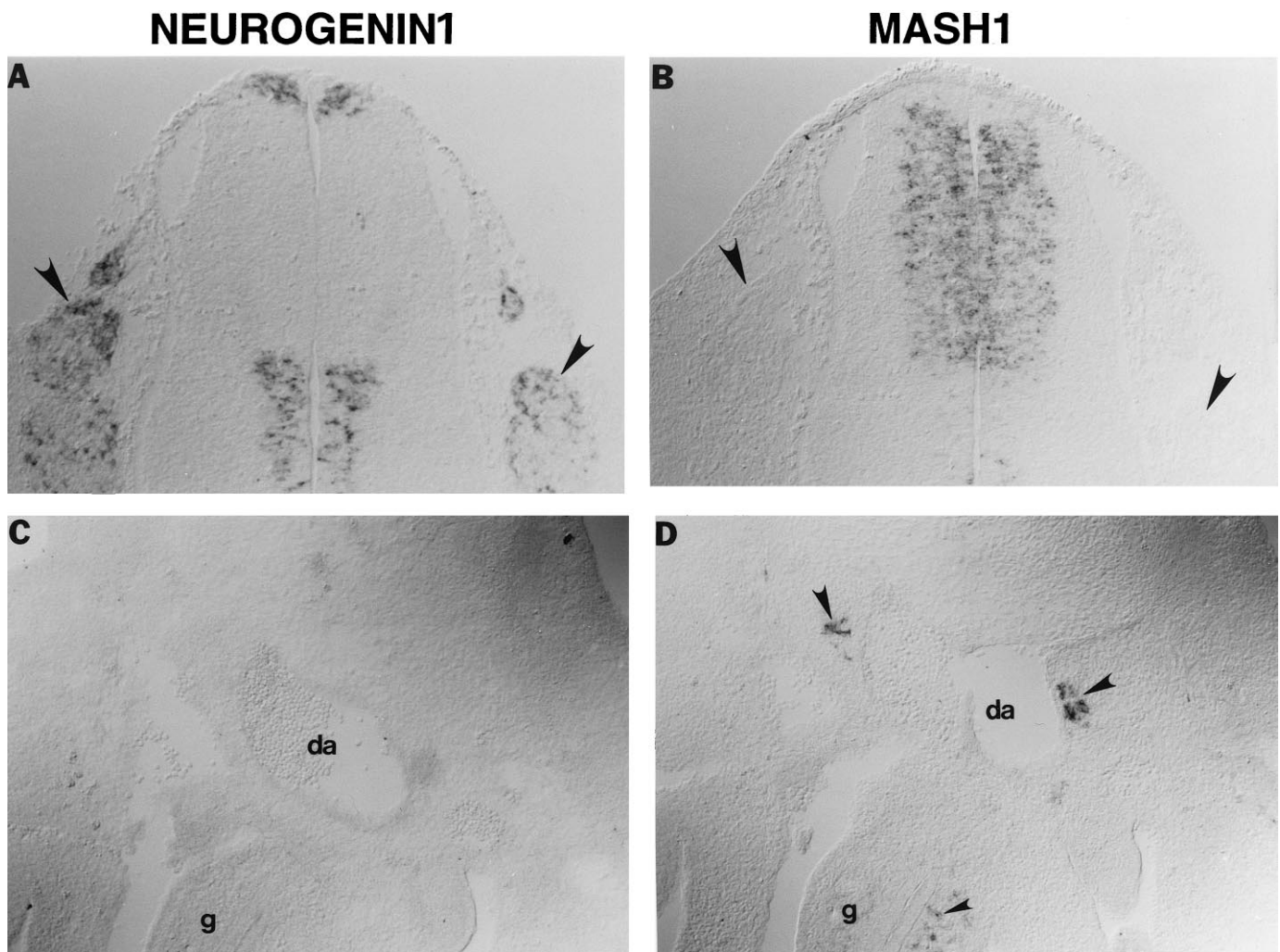
NEUROGENIN1

MASH1

Figure 1. Complementary expression of *ngn1* and *Mash1* during development of the PNS. Shown are adjacent transverse sections through the trunk region of E13.5 (*A*, *B*) or E12.5 (*C*, *D*) rat embryos. *ngn1* is expressed in DRG (*A*, *arrowhead*) in which *Mash1* is not expressed (*B*, *arrowhead*). Conversely, *Mash1* is expressed in sympathetic ganglia adjacent to the dorsal aorta (*da*) (*D*, *large arrowheads*) and enteric ganglia (gut, *g*) (*D*, *small arrowhead*), none of which express *ngn1* (*C*). Mutual exclusivity between *Mash1* and *ngn1* also is observed in the ventricular zone of the spinal cord (*A*, *B*; see also Fig. 4).

MATERIALS AND METHODS

In situ *hybridization.* Digoxigenin-labeled *in situ* hybridization was performed as previously described (Groves et al., 1994) with the following probes: *ngn1* (Ma et al., 1996), *Mash1* (Lo et al., 1991), *Dll-1* (from Domingos Henrique), *Jagged* (Lindsell et al., 1995), *eHAND* (Cserjesi et al., 1995), and *NSCL1* (Begley et al., 1992). For double-color *in situ* hybridization *ngn1* and *Dll-1* were labeled with digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN), whereas *Mash1* and *Jagged* were labeled with fluorescein-UTP (Boehringer Mannheim). A detailed procedure is available on request.

RESULTS

Complementary cascades of bHLH gene expression in the PNS

Previously, we reported in separate studies that MASH1 protein is expressed in peripheral autonomic but not sensory ganglia (Lo et al., 1991) and that *ngn1* mRNA is expressed, conversely, in early sensory but not autonomic ganglia (Ma et al., 1996). To confirm this apparent mutual exclusivity of expression in the PNS, we performed a series of *in situ* hybridization experiments with *Mash1* and *ngn1* probes on adjacent serial sections of rat embryos of various ages. At embryonic day (E) 13.5, *ngn1* mRNA was detected in a subset of cells in the dorsomedial margins of the DRG

(Fig. 1*A*, *arrowheads*), whereas no *Mash1* transcripts were detected in this region (Fig. 1*B*, *arrowheads*). Conversely, at E12.5 *Mash1* mRNA was detected easily in forming sympathetic and enteric ganglia (Fig. 1*D*, *large* and *small arrowheads*, respectively), whereas no *ngn1* mRNA was detectable in this region on adjacent sections (Fig. 1*C*). Similar observations were made in embryos of other ages (data not shown), indicating that this apparent reciprocity is not a function of developmental stage. The mutual exclusivity of expression in the PNS extended to cranial sensory ganglia; *ngn1* mRNA was detected in trigeminal ganglia (Fig. 2*C*, *TG*), the otic epithelium (*small arrow*), and the acoustic component of the facio–acoustic complex (*large arrow*), whereas none of these structures expressed *Mash1* (Fig. 2*D*).

Several other bHLH genes have been isolated that exhibit complementary expression in the PNS similar to that observed for *Mash1* and *ngn1*. For example, *NSCL1* and *NSCL2,* both related to the lymphoid-specific gene *SCL* (Porcher et al., 1996), were reported to be expressed in sensory but not autonomic ganglia (Begley et al., 1992; Göbel et al., 1992), as was *NeuroD* (Lee et al., 1995); *eHAND/Thing1/Hxt* (hereafter referred to as *eHAND*) was, conversely, reported to be expressed in autonomic but not sensory
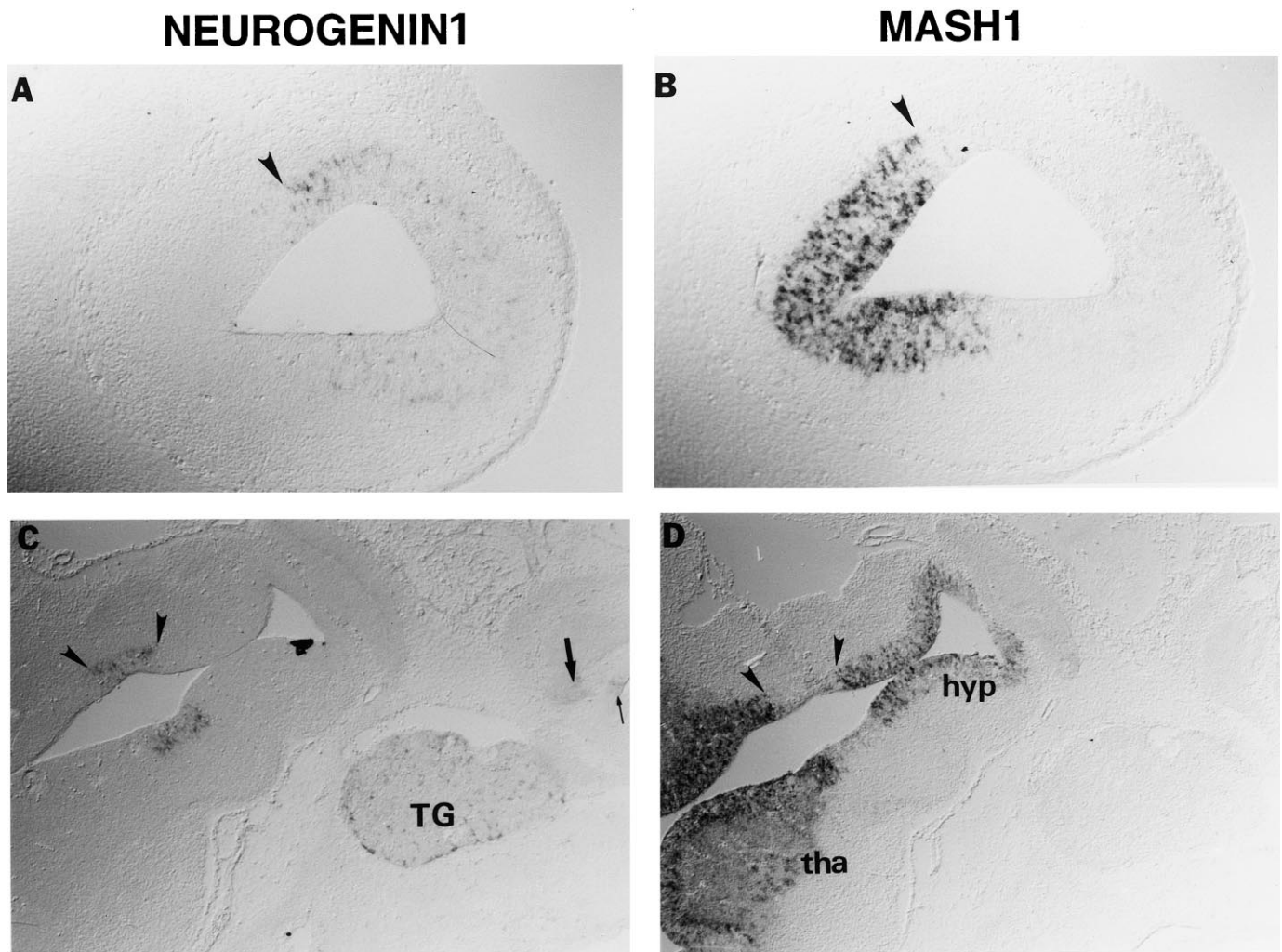
NEUROGENIN1

MASH1

Figure 2. Mutual exclusivity of *ngn1* and *Mash1* expression in forebrain and cranial sensory ganglia. Transverse sections through the developing olfactory lobe (*A, B*, in high magnification) and the diencephalon (*C, D*) of an E14.5 rat embryo. *ngn1* is expressed in the dorsal/rostral side of the olfactory lobe (*A*), whereas *Mash1* is expressed in the ventral/caudal side (*B*). *Mash1* is expressed throughout the hypothalamus (*hyp*) and the thalamus (*tha*) except for a narrow region (*D*) in which *ngn1* is expressed (*C, arrowheads*). *ngn1* is expressed in the trigeminal ganglion (*C, TG*), the acoustic component of the facio–acoustic complex (*C, large arrow*), and the otic epithelium (*C, small arrow*), none of which express *Mash1* (*D*). Expression of *neurogenin1* in these cranial ganglia is much stronger in younger embryos (Sommer et al., 1996).

ganglia (as well as in smooth muscle) (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995). These observations raised the question of the temporal relationship between expression of these other bHLH genes and that of *Mash1* and *ngn1*.

In trunk sensory ganglia early expression of both *ngn1* and *NSCL1* expression is detected at E11.5 (Fig. 3*A,B, arrowheads*). However, at this age, expression of the former gene is stronger and is detected in more cells than that of the latter (compare Fig. 3*A,B*). Conversely, at E12.5 expression of *ngn1* has begun to decline (Fig. 3*C, arrowheads*), whereas that of *NSCL1* has increased (Fig. 3*D, arrowheads*). These data suggest that expression of *NSCL1* follows or persists longer than that of *ngn1*. In support of the former interpretation, *ngn1* mRNA at E12.5 is expressed most strongly in cells at the dorsomedial margins of the ganglia in which precursors are located; conversely, *NSCL1* mRNA is expressed strongly in interior regions of the ganglia in which differentiated neurons are present (compare Fig. 3*D*). These data suggest that *ngn1* expression precedes that of *NSCL1* during sensory neurogenesis. In the CNS, *ngn1* expression also precedes that of *NeuroD* (Ma et al., 1996; Sommer et al., 1996). In the PNS,

however, we did not observe a temporal difference between *ngn1* and *NeuroD* expression during early stages of DRG development (data not shown). Therefore, *NSCL1* seems to be the latest expressed of the three bHLH genes we examined during sensory neurogenesis.

In the case of autonomic ganglia, we wished to know the relationship between the expression of *eHAND* and that of *Mash1*. However, we were unable to detect any temporal separation between the expression of these two bHLH genes in sympathetic ganglia (Fig. 3*E*; data not shown). Therefore, to ask whether expression of *eHAND* is dependent on *Mash1* function, we examined its expression in *Mash1* knock-out mice (Guillemot et al., 1993). No *eHAND* mRNA was detected in the sympathetic anlagen of these mutant embryos (Fig. 3*F*). This does not simply reflect an absence of neural crest-derived cells from these ganglion primordia, however, because we have shown previously that immature sympathetic neuron precursors are present in such mutant embryos (Sommer et al., 1995). These data, therefore, indicate that in autonomic ganglia *eHAND* expression requires that of *Mash1*. This, in turn, suggests that *eHAND* is expressed
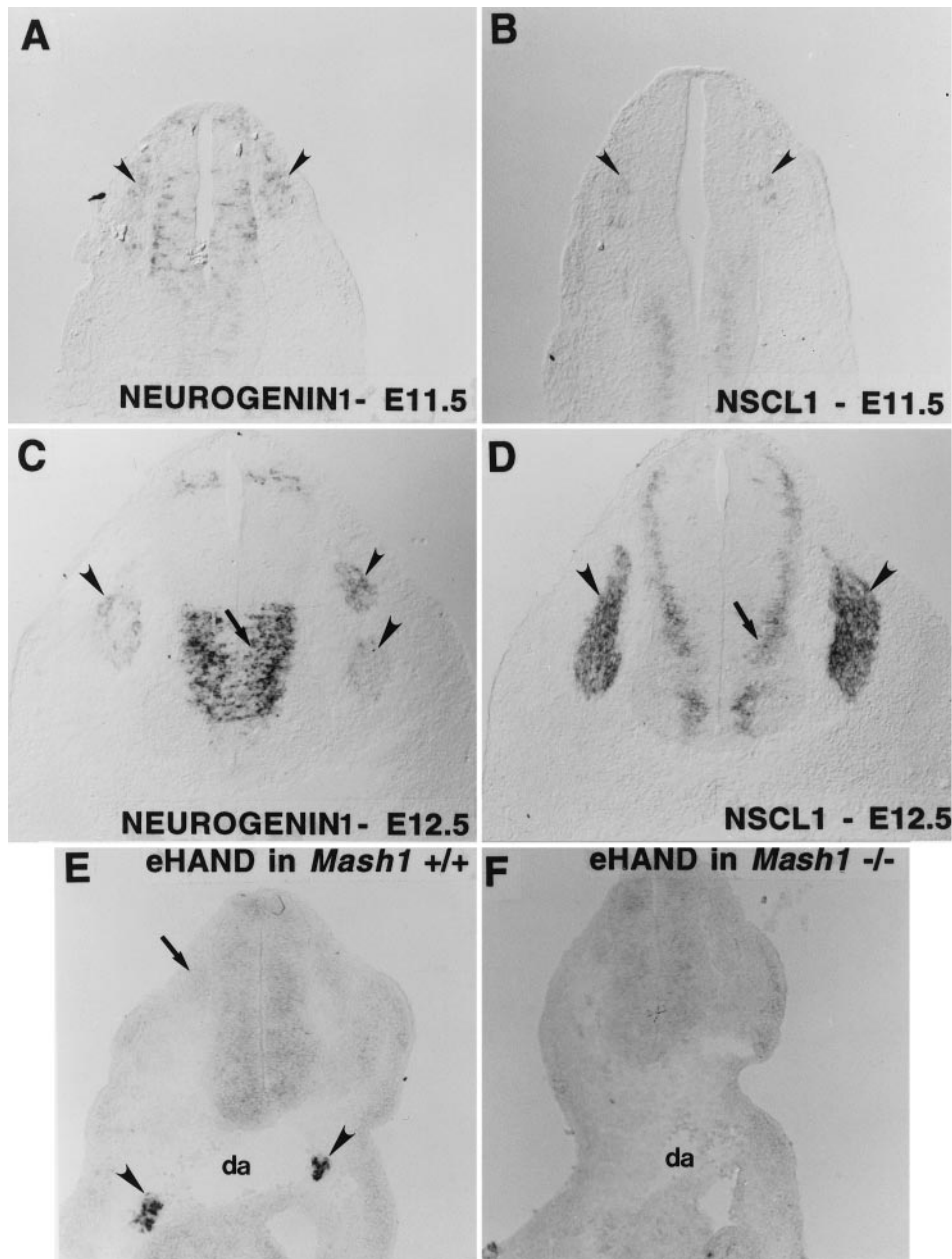
Figure 3. Lineage-specific bHLH cascades within the PNS. Both *ngn1* and *NSCL1* are expressed in rat DRG at E11.5 and E12.5 (*A–D, arrowheads*). *NSCL1* expression is relatively weak at E11.5 (*B, arrowheads*) but strong at E12.5 (*D, arrowheads*). *ngn1* expression at E12.5 has begun to fade in the ventral half of the DRG (*C, arrowheads*). Expression of *NSCL1* is displaced more laterally from the ventricular zone (*D, arrow*) than that of *ngn1* (*C, arrow*). *eHAND* is expressed in the sympathetic ganglia of E10.5 mouse embryo (*E, arrowheads*) but not in the sensory ganglia (*E, arrows*). Expression of *eHAND* in the sympathetic ganglia is undetectable in *Mash1* knock-out mice of similar age (*F*).

either downstream of, or cross-regulated by, MASH1. However, *Mash1* expression is not sufficient for expression of *eHAND*, as seen in the dorsal spinal cord where *Mash1*, but not *eHAND*, is expressed (compare Figs. 1*B*, 3*E*).

Mutually exclusive domains of *Mash1* and *ngn1* expression in the CNS

The foregoing observations of mutually exclusive bHLH gene expression in the PNS raised the question of whether this was true in the CNS as well. As detailed below, the results indicate that expression of *Mash1* and *ngn1* subdivides many regions of the ventricular zone into adjacent and nonoverlapping domains. At E13.5, for example, *ngn1* is expressed in two zones that run the length of the spinal cord, a thin one close to the roof plate and a broad one in the ventral half (Fig. 1*A*). *Mash1*, conversely, appears to be expressed between these two *ngn1* stripes (Fig. 1*B*). Double-label *in situ* hybridization to sagittal sections (passing through the ventricular zone of the spinal cord) indicated that the boundaries of *Mash1* and *ngn1* expression are adjacent and nonoverlapping (Fig. 4*B*). The combined expression of *Mash1* and *ngn1* accounts for essentially the entire ventricular zone of the spinal cord, with the exception of a small dorsal region in which *Math1* is expressed (Akazawa et al., 1995) and the floorplate.

Similarly, in the telencephalon, *Mash1* is expressed in the presumptive basal ganglia (Fig. 4*A, str*), whereas *ngn1* is expressed in the presumptive cortex (*ctx*), as is the related gene *ngn2* (Gradwohl et al., 1996; Sommer et al., 1996). Double labeling again revealed that the boundaries between the telencephalic expression domains of *ngn1* and *Mash1* are adjacent and nonoverlapping (Fig. 4*A, arrowheads*). A similar mutual exclusivity was observed elsewhere in the forebrain. For example, within the prospective olfactory lobes, *ngn1* is expressed in the dorsal part (Fig. 2*A*), whereas *Mash1* mRNA is detected in the ventral regions (Fig. 2*B*). In the diencephalon *Mash1* is expressed throughout the ventricular zone, with the exception of a small region (Fig. 2*D*,
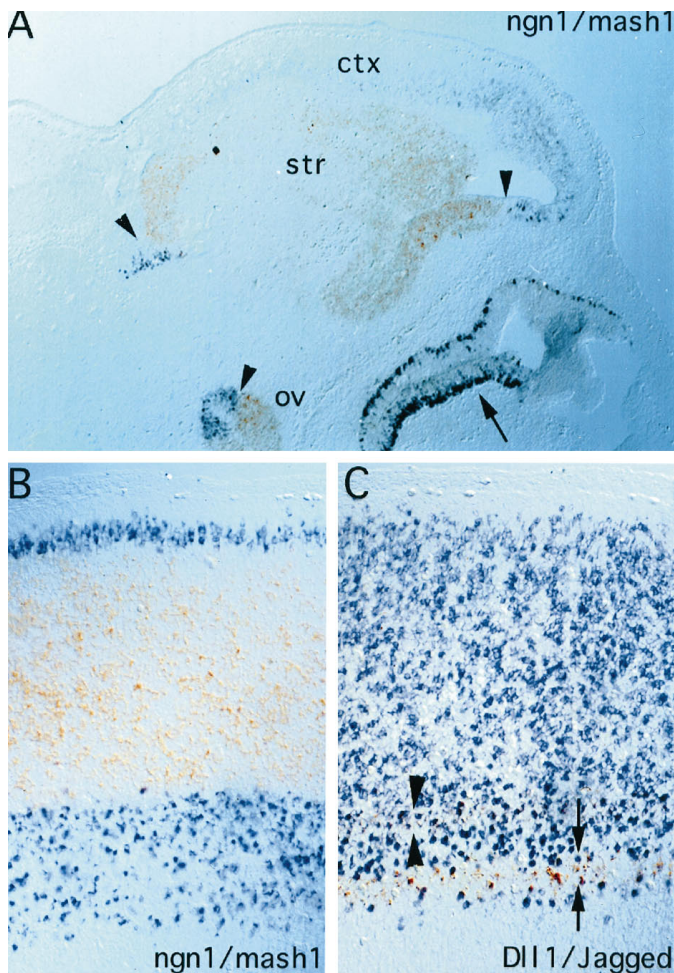
Figure 4. Mutually exclusive expression of *Mash1* and *ngn1* in spinal cord and forebrain of E14.5 rat embryos demonstrated by double-label *in situ* hybridization. Shown are sagittal sections of forebrain (*A*) and spinal cord (*B*); rostral is to the *right*, and dorsal is to the *top* in *A* and *B*. *B*, The section was cut through the ventricular zone, just lateral to the central canal. *ngn1* expression is visualized with the *purple* chromogen and *Mash1* expression with the *brown*. *A*, The borders between *ngn1* and *Mash1* expression are indicated by *arrowheads*. *ngn1* is expressed in the presumptive cortex (*ctx*), and *Mash1* is expressed in the striatum (*str*). Complementarity also is seen in optic vesicle (*ov*). *Mash1* and *ngn1* are expressed in a partially overlapping manner in the olfactory epithelium (*arrow*; see also Fig. 5). *B*, The boundaries between *Mash1* and *ngn1* expression are adjacent and non-overlapping. *C*, Sagittal section through spinal cord adjacent to that shown in *B*, double-labeled with probes for *Jagged* (*brown*) and *Dll-1* (*purple*). *Double arrowheads* demarcate the top stripe of *Jagged* mRNA expression (compare Fig. 6*B*, *arrow*), and *double arrows* demarcate the bottom stripe (compare Fig. 6*B*, *arrowhead*). The top stripe of *Jagged* mRNA (*double arrowheads*) partially overlaps the ventral boundary of the major dorsal *Dll-1* expression domain (compare Fig. 6*D*, *arrow*), although it is not possible to determine at this level of resolution whether individual cells coexpress both genes. The ventral stripe of *Jagged* mRNA (compare Fig. 6*B*, *arrowhead*) appears perfectly interdigitated between the two stripes of *Dll-1* mRNA (compare Fig. 6*D*, *arrow* and *arrowhead*). Note that the boundaries of *Jagged* and *Dll-1* do not correspond to those of *Mash1* and *ngn1*; however, *Jagged* is expressed only in the *ngn1*-expressing region (see also Fig. 6).

arrowheads) in which *ngn1* is expressed instead (Fig. 2*C*). Such mutual exclusivity also is seen within the optic vesicle (Fig. 4*A*, *arrowhead* and *ov*).

In addition to this striking complementarity of *Mash1* and *ngn1* expression, we also observed some CNS regions that express both

genes, including the olfactory epithelium (Fig. 5*A,B*), midbrain (Fig. 5*C,D*), and (at early stages (E11.5–E12.5)) the ventral spinal cord (data not shown). Within these regions of overlap, differences in the expression pattern of the two genes are, nevertheless, evident. For example, in the E14.5 olfactory epithelium, *ngn1* expression is restricted to cells located in the basal layer (Fig. 5*A*), whereas *Mash1* is expressed in cells that span the epithelium (Fig. 5*B*). In the midbrain the domain of highest *ngn1* expression appears displaced laterally from the ventricular wall, whereas that of *Mash1* fills the entire ventricular zone (compare Fig. 5*C,D*). Moreover, expression of *Mash1* appears relatively uniform (Fig. 5*D*), whereas that of *ngn1* is restricted to a subset of cells (Fig. 5*C*).

Relationship of *Mash1* and *ngn1* expression to that of *Delta* and *Jagged*

In *Drosophila* the proneural genes *ac-sc* both activate expression of the lateral inhibitory signal *Delta* and are, in turn, repressed by signaling through Notch, a receptor for *Delta* (Ghysen et al., 1993). Similarly, in *Xenopus* *X-ngnr-1* activates expression of *X-Delta-1* and is negatively regulated by signaling through X-Notch-1 (Ma et al., 1996). Recently, two mammalian Notch ligands, *Dll-1* and *Jagged*, have been identified (Bettenhausen et al., 1995; Lindsell et al., 1995). To determine whether MASH1 and/or Neurogenin1 similarly might activate expression of these Notch ligands in mammals, we compared their expression with that of *Dll-1* and *Jagged*.

In the spinal cord at E13.5, *Jagged* is expressed primarily in the ventral region in several narrow longitudinal (i.e., rostrocaudal) stripes that appear as bands across the ventricular zone in transverse sections (Fig. 6*B*, *arrow*; see also Lindsell et al., 1995). Comparison of adjacent sections indicates that these bands lie within the region in which *ngn1* also is expressed (compare Fig. 6*A,B*; see also Fig. 4*B,C*). Moreover, the top band of *Jagged* mRNA expression (Fig. 6*B*, *arrow*) appears well correlated with the dorsal boundary of the band of *ngn1* expression (Fig. 6*A*, *arrow*). In contrast to *Jagged*, *Dll-1* is expressed primarily in the prospective alar (dorsal) region of the spinal cord (Fig. 6*D*; see also Bettenhausen et al., 1995; Lindsell et al., 1996) in a domain that encompasses the entire band of *Mash1* expression (Figs. 4*C*, 6*C*). Double-label *in situ* hybridization confirms that some boundaries of *Jagged* and *Dll-1* expression, like those of *Mash1* and *ngn1*, are mutually exclusive (Fig. 4*C*, *arrows*), confirming previous observations made in rat (Lindsell et al., 1996) and chick (Myat et al., 1996). However, the dorsal stripe of *Jagged* expression (Fig. 6*B*, *arrow*) overlaps the ventral boundary of *Dll-1* expression (Fig. 6*D*, *arrow*), as confirmed by double-labeling on sagittal sections (Fig. 4*C*, *arrowheads*), so that the complementarity of Notch ligand expression in the spinal cord is not perfect.

Although both *Mash1* and *ngn1* and *Jagged* and *Dll-1* exhibit complementary boundaries of expression in the spinal cord, comparison of adjacent sections indicates that these boundaries are located in distinct regions. For example, *Dll-1* expression (Fig. 6*D*) extends dorsally past the top boundary of the *Mash1* band (Fig. 6*C*) to encompass the thin stripe of *ngn1* expression adjacent to the roof plate (Fig. 6*A*, *arrowhead*; compare Fig. 4*B* vs *C*). Furthermore, the ventral boundary of *Dll-1* expression (Fig. 6*D*, *arrow*) overlaps the top part of the ventral band of *ngn1* expression (Fig. 6*A*, *arrow*). Thus, expression of *Dll-1* is detected in both *Mash1-* and *ngn1*-expressing regions. Interestingly, however, *Jagged* expression is detected only in regions that express *ngn1* (compare Fig. 6*A* vs *B*).

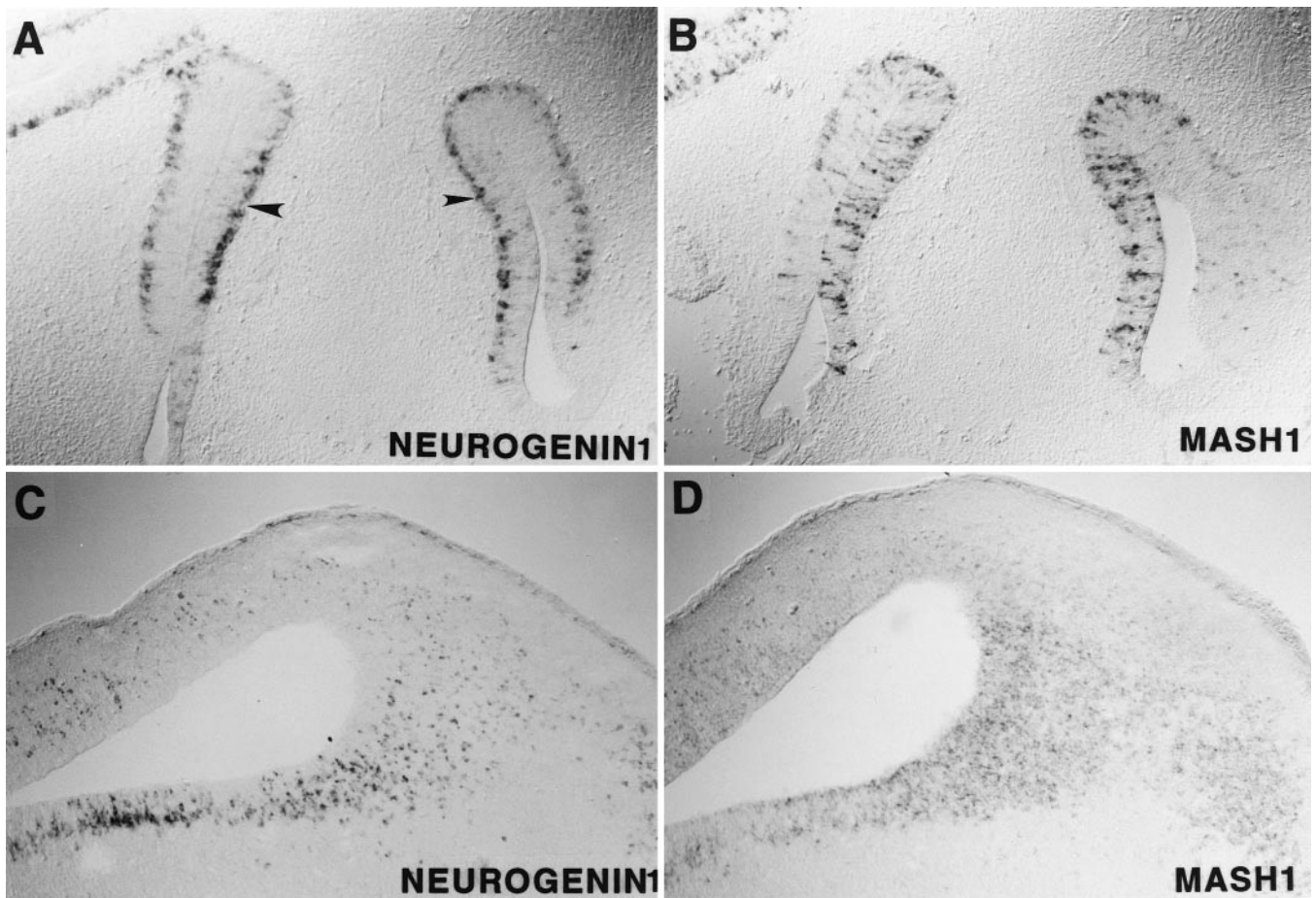The correlation between *Jagged* and *ngn1* expression observed

Figure 5. Overlapping but sequential expression of *ngn1* and *Mash1*. Transverse sections through the olfactory epithelium (*A, B*) and sagittal sections through the midbrain region (*C, D*) of an E14.5 rat embryo are illustrated. *ngn1* is expressed in cells located in the basal layer of the olfactory epithelium (*A, arrowhead*), whereas *Mash1* is expressed in cells spanning the epithelium (*B*). In the midbrain *ngn1* expression is scattered (*C*), whereas that of *Mash1* is much more uniform (*D*). Also, *ngn1* expression (but not that of *Mash1*) is displaced laterally from the ventricular wall (*C, D*).

in the spinal cord seems to extend to other regions of the CNS. For example, in the ventricular zone of the medulla and pons *Jagged* expression is restricted primarily to the region that also expresses high levels of *ngn1* (compare Fig. 6*E* vs *F*). By contrast, in this brainstem region *Dll-1* is expressed in a pattern that overlaps the expression domains of both *Mash1* and *ngn1* (compare Fig. 6*H* vs *E,G*). Similarly, in the telencephalon strong *Dll-1* expression is found in both the striatum, which expresses *Mash1*, and in the cortex, which expresses *ngn1* (Lindsell et al., 1996) (data not shown). By contrast, strong expression of *Jagged* in the brain is observed only in regions in which *ngn1* (or *ngn2*; Gradwohl et al., 1996; Sommer et al., 1996) is strongly expressed, although faint expression can be seen in some regions that only express *Mash1* (such as in the striatum). Thus, a generalization that emerges from these studies is that in the CNS *Jagged* expression is associated primarily with that of *ngn1*, whereas *Dll-1* expression can be associated with that of either *ngn1* or of *Mash1*.

DISCUSSION
Mutual exclusivity of *Mash1* and *neurogenins* expression during mammalian neurogenesis
The most striking finding to emerge from the present studies is that *Mash1* and *ngn1* are expressed in complementary domains, which exhibit adjacent and nonoverlapping boundaries, in many regions of the CNS. In several regions of the CNS the comple-

mentary domains delineated by *Mash1* and *ngn1* expression correspond to future functional subdivisions: in the spinal cord to the alar and basal regions and in the forebrain to the cortex and striatum. This mutual exclusivity mirrors the complementarity of expression of these bHLH genes in the PNS, in which *Mash1* is expressed by autonomic and *ngn1* by sensory progenitors.

Recently, we reported the isolation of two other *ngn1*-related genes, *ngn2* and *ngn3* (Sommer et al., 1996). In the PNS *ngn2* also is restricted to sensory lineages, such as in DRG and several epibranchial placode-derived ganglia in which *Mash1* is not expressed (Sommer et al., 1996). In the CNS, particularly in the forebrain region, expression of *ngn2* (also known as *Math4A*; Gradwohl et al., 1996) shows a similar regional restriction like that of *ngn1* (Sommer et al., 1996) and appears complementary to that of *Mash1* (Gradwohl et al., 1996). By contrast, *ngn3* is expressed only in extremely restricted regions of the CNS. For example, in the spinal cord *ngn3* expression is restricted to the region close to the floorplate (Sommer et al., 1996). Thus, much of the developing CNS can be divided into regions that express either *Mash1* or one or more of the *ngns*.

In the PNS the complementary expression of *Mash1* and the *ngns* appears to be reflected in the expression of several additional bHLH genes that may serve as potential downstream targets. Expression of *NeuroD* (Lee et al., 1995; Ma et al., 1996; Sommer et al., 1996) and *NSCL1* (Begley et al., 1992) (Fig. 3*C,D*) is
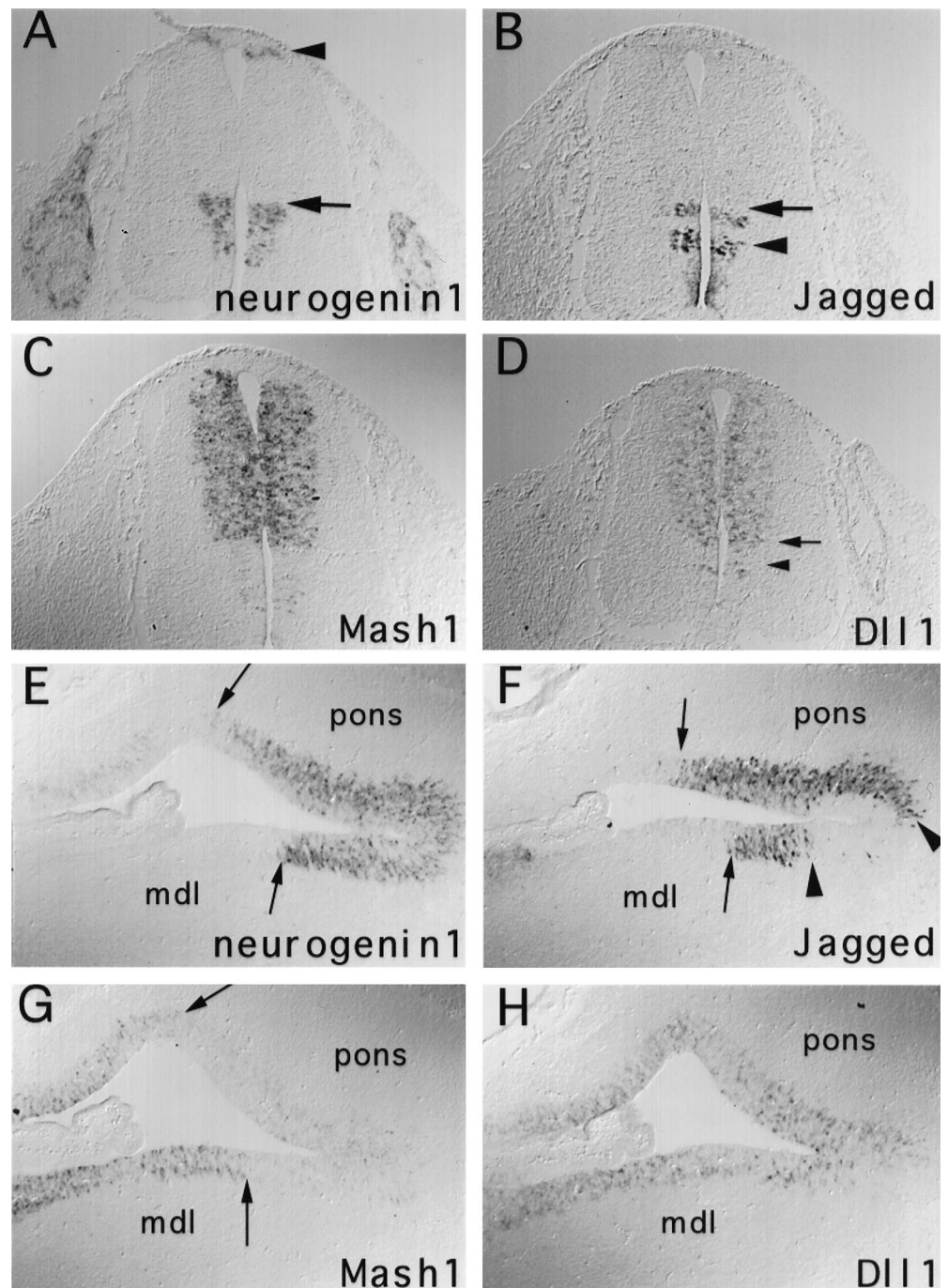
Figure 6. Comparisons of *Mash1* and *ngn1* expression to that of *Dll-1* and *Jagged*. Adjacent transverse trunk sections of E13.5 (*A–D*) and sagittal sections through the medulla and pons of E14.5 (*E–H*) rat embryos are illustrated. The dorsal boundary of the *Jagged* stripe (*B, arrow*) seems to correspond to the dorsal boundary of the ventral *ngn1* expression domain (*A, arrow*). The dorsal *Dll-1* expression domain (*D*) encompasses that of *Mash1* (*C*) and extends both dorsally to the region expressing the dorsal *ngn1* stripe (*A, arrowhead*) and ventrally into the area corresponding to the top part of the ventral *ngn1* stripe (*A*). In the medulla (*mdl*) and pons (*pons*), complementarity between *ngn1* and *Mash1* is indicated by the *arrows* (*E, G*). Expression of *Jagged* is restricted to the region in which *ngn1* is strongly expressed (*F*), whereas *Dll-1* is expressed throughout the ventricular zone (*H*). *Jagged* expression in this region is discontinuous (*F, arrowheads*), as in the spinal cord (*B*).

restricted to the sensory lineage, whereas *eHAND* expression is restricted to autonomic lineages (Cserjesi et al., 1995). The finding that Mash1 function is necessary for expression of *eHAND* in sympathetic ganglia indicates that eHAND is likely a direct or an indirect target of Mash1. In *Xenopus* a homolog of the *ngns*, *X-ngnr-1*, activates expression of *X-NeuroD* in a unidirectional cascade during primary neurogenesis (Ma et al., 1996). Similarly, *X-NeuroD* has been shown to activate expression of *Xenopus NSCL* (J. Lee, personal communication). Our *in situ* data (Fig. 2*A–D*; Sommer et al., 1996) are consistent with the idea that the *ngns*, *neuroD*, and *NSCL1* may function in cascade during sensory neurogenesis in higher vertebrates, as well. Confirmation of this will, however, await loss-of-function analyses in *ngn1* and/or *ngn2* knock-out mice.

Functional significance of complementary bHLH gene expression during neurogenesis

Why are different classes of bHLH genes used to promote neurogenesis in different regions of the CNS and PNS? The answer to this question is likely to relate to differences in the functions of these genes. In the *Drosophila* PNS, *atonal* (which is structurally related to the *ngns*) is required for development of chordotonal organs, whereas *achaete-scute* is required for that of external sensory (es) organs (Jan and Jan, 1994). These genes are not functionally interchangeable, and recent data indicate that the key amino acid residues responsible for these functional differences lie in the basic region and likely mediate protein–protein rather than protein–DNA interactions (Chien et al., 1996). Thus in

Drosophila structural differences among different neural bHLH proteins account for their ability to promote the determination of particular neural cell types, and the same is likely to be true in vertebrates. The noninterchangeable functions of different classes of neural bHLH proteins indicate that the choice of a neural (or neuronal) fate is coupled to the specification of a particular cell identity from the earliest developmental stages. However, a given bHLH gene is not sufficient to specify a particular neural cell type, because in both *Drosophila* and vertebrates individual bHLH genes are required for the development of more than one class of neurons. A single bHLH gene is, therefore, likely to act in combination with other region-specific transcriptional regulators in the pathways that determine neuronal identities. This would explain why, for example, expression of *Mash1* is followed by that of *eHAND* in the PNS, but not the CNS.

Overlapping expression of *Mash1* and *ngns*

Although spatial complementarity between expression of *Mash1* and *ngns* seems to be the rule in most parts of the CNS, there are some regions in which these genes are coexpressed. For example, *Mash1*, *ngn1*, and *ngn2* all are expressed in the midbrain region (Fig. 5C,D; data not shown). Other sites of *ngn1* and *Mash1* coexpression include the olfactory epithelium and midbrain. Interestingly, within these overlapping regions differences in the expression of these genes are clearly seen. For example, in the olfactory epithelium *ngn1* expression is restricted to the basal layer, whereas *Mash1* expression spans the epithelium. Conversely, in the midbrain *Mash1* is expressed in the ventricular zone, whereas both *ngn1* and *ngn2* are displaced slightly lateral to this region (Fig. 5C,D; data not shown). These differences suggest that even in these regions of overlap these genes may function at different developmental stages in the same cells or in distinct but intermingled groups of cells that develop on different schedules.

Relationship of bHLH gene expression to that of Notch ligands

Previously, it has been shown that the Notch ligands *c-Delta-1* and *c-Serrate-1* (a chick homolog of *Jagged*) and *Delta-1* (*Dll-1*) and *Jagged* (Lindsell et al., 1996; Myat et al., 1996) exhibit complementary domains of expression in the spinal cord of chick and rat, respectively. In the chick this complementarity seems perfect. Our data indicate that it is partially lost in rat, however (Fig. 6). Because *Jagged* and *Dll-1* are both ligands for Notch, they may be functionally equivalent. In that case, there would be little selection pressure to maintain such perfect complementarity. The bHLH genes *Mash1* and *ngn1* also exhibit complementary boundaries of expression in the rodent spinal cord. In the fly and the frog, homologs of these genes are demonstrably not functionally equivalent (Chien et al., 1996; Chitnis and Kintner, 1996; Ma et al., 1996). There may, therefore, be more selection pressure to maintain the complementarity of bHLH gene expression within the CNS. Analysis of the expression patterns of the chick homologs of these genes should be informative in this respect.

The side-by-side comparison of bHLH and Notch ligand gene expression presented here indicates that the boundaries of *Jagged* and *Dll-1* expression do not correspond precisely to the boundaries between the domains of *Mash1* and *ngn1* expression (Figs. 4B,C, 6). Specifically, *Dll-1* is expressed in domains expressing either *Mash1* or *ngn1*. On the other hand, high-level *Jagged* expression usually is (but not always) associated with that of *ngn1*. If, indeed, *Jagged* and *Dll-1* are functionally equivalent, it is not clear why expression of *Jagged* should be associated preferentially

with that of *ngn1*. This apparent linkage may reflect regulatory rather than functional constraints on the two genes.

The fact that the expression of the bHLH genes and that of the Notch ligands overlaps in the ventricular zone in both space and time raises the possibility that there is a regulatory interaction between these proteins in higher vertebrates, similar to that which occurs in *Drosophila* and *Xenopus* (Chitnis et al., 1995; Chitnis and Kintner, 1996; Ma et al., 1996) (for review, see Lewis, 1996). Specifically, proneural genes in the fly and *X-ngnr-1* in the frog have been shown to activate expression of *Delta* and its *Xenopus* homolog, *X-Delta-1*, respectively. Our data are compatible with the idea that such an activation occurs in rodents as well, as suggested previously (Kunisch et al., 1994) (for review, see Lewis, 1996). Nevertheless, in *Mash1* knock-out mice, expression of *Dll-1* and *Jagged* in the CNS is not affected (our unpublished results), consistent with the failure to detect any other phenotypic defects in the CNS of these embryos (Guillemot et al., 1993). This may reflect functional redundancy or compensation between *Mash1* and other unrelated bHLH genes; alternatively, Notch ligands may not be regulated by *Mash1* in mice. Similarly, in the case of *ngn1* and *ngn2*, the overlap in their CNS expression may obscure defects in Notch ligand expression in single knock-out mice. Analysis of mice containing targeted mutations in both genes should, however, be informative.

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