Development 124, 1611-1621 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV2133

Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors

Elise Cau, Gérard Gradwohl, Carol Fode and François Guillemot*

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, BP 163, 67404 Illkirch Cédex, CU de Strasbourg, France

*Author for correspondence (e-mail: francois@igbmc.u-strasbg.fr)

SUMMARY

The lineage of olfactory neurons has been relatively well characterized at the cellular level, but the genes that regulate the proliferation and differentiation of their progenitors are currently unknown. In this study, we report the isolation of a novel murine gene, *Math4C/neurogenin1*, which is distantly related to the *Drosophila* proneural gene *atonal*. We show that *Math4C/neurogenin1* and the basic helix-loop-helix gene *Mash1* are expressed in the olfactory epithelium by different dividing progenitor populations, while another basic helix-loop-helix gene, *NeuroD*, is expressed at the onset of neuronal differentiation. These expression patterns suggest that each gene marks a distinct stage of olfactory neuron progenitor development, in the following sequence: *Mash1*>*Math4C/neurogenin1*>*NeuroD*. We have previously reported that inactivation of *Mash1* function leads to a severe reduction in the number of olfactory neurons. We show here that most cells in the olfactory epithelium of *Mash1* mutant embryos fail to express *Math4C/neurogenin1* or *NeuroD*. Strikingly, a subset of progenitor cells in a ventrocaudal domain of *Mash1* mutant olfactory epithelium still express *Math4C/neurogenin1* and *NeuroD* and differentiate into neurons. Cells in this domain also express *Math4A/neurogenin2*, another member of the *Math4/neurogenin* gene family, and not *Mash1*. Our results demonstrate that *Mash1* is required at an early stage in the olfactory neuron lineage to initiate a differentiation program involving *Math4C/neurogenin1* and *NeuroD*. Another gene activates a similar program in a separate population of olfactory neuron progenitors.

Key words: neurogenesis, neural progenitors, olfactory epithelium, olfactory placodes, olfactory receptor neurons, bHLH proteins, *Mash1*, *NeuroD*, *achaete-scute* homolog, *atonal* homolog, mouse

INTRODUCTION

The process of neurogenesis in the nervous system of vertebrates produces a large variety of neuronal cell types. Neurons are generated from populations of neural progenitor cells, which differ in their proliferative characteristics and differentiation potentials (McConnell, 1995). The genes that control the commitment of ectodermal cells to a neural fate, the proliferation of neural progenitor cells and their differentiation into post-mitotic neurons, are still poorly characterized in vertebrates. In contrast, studies in invertebrates, in particular in *Drosophila*, have led to the identification of a number of genes, functioning at successive stages in the developmental pathways, leading to differentiated neurons. Proneural genes control the generation of neural precursors from uncommitted ectodermal cells. The main *Drosophila* proneural genes are the genes of the *achaete-scute* complex (*asc*) and *atonal* (*ato*), which encode transcription factors of the basic helix-loop-helix (bHLH) class (Campuzano and Modolell, 1992; Jarman et al., 1993). Loss-of-function mutations in proneural genes prevent the generation of neural precursors (Ghysen and Dambly-Chaudière, 1988; Jarman et al., 1993) whereas gain-of-function mutations result in the transformation of ectodermal cells into neural precursors at ectopic positions (Rodriguez et al., 1990; Ghysen et al., 1993).

Genes homologous to *Drosophila asc* have been identified in vertebrate species. *ash1* is a vertebrate *asc* homolog expressed in subsets of neural progenitors in the central nervous system (CNS) and the peripheral nervous system (PNS), and it has been identified in all species examined (Johnson et al., 1990; Guillemot and Joyner, 1993; reviewed in Guillemot, 1995). A targeted mutation of *Mash1* in the mouse results in the elimination of most olfactory and autonomic neurons, demonstrating a role for *Mash1* in the development of particular neural lineages (Guillemot et al., 1993; Sommer et al., 1995; Blaugrund et al., 1996).

A number of bHLH genes with similarities to *Drosophila ato* have also been isolated in vertebrates. *Math1*, the closest murine relative of *ato*, is expressed in the dorsal neural tube and the developing cerebellum (Akazawa et al., 1995). *NeuroD/Beta2* (Lee et al., 1995; Naya et al., 1995), *neuroD2/NDRF/kw8* (Kawakami et al., 1996; Kume et al., 1996; McCormick et al., 1996; Yasunami et al., 1996), *Nex1/Math2* (Bartholomä and Nave, 1994; Shimizu et al., 1995) and *Math3* (Takebayashi et al., 1997) constitute a subfamily of genes distantly related to *ato*, and with very similar bHLH domains. These genes are expressed either transiently in differentiating neurons or permanently in terminally differentiated cells. In contrast, *neurogenin1/neuroD3* and

Math4A/neurogenin2, which define a third subfamily of *ato*-related genes, are expressed exclusively in progenitors of the CNS and PNS (Gradwohl et al., 1996; Ma et al., 1996; McCormick et al., 1996; Sommer et al., 1996). The functions of *NeuroD* and a *neurogenin* gene have been studied by RNA injection in *Xenopus* embryos. Overexpression of *NeuroD* accelerates neuronal differentiation in the neural plate and induces neuronal differentiation in ventral ectoderm, demonstrating that *NeuroD* has neuronal differentiation activity (Lee et al., 1995). Overexpression of the *neurogenin* homolog *X-ngnr-1* has similar effects, and in addition it induces *NeuroD* and the neurogenic gene *delta*, which are characteristics of a neural determination gene (Ma et al., 1996).

Most vertebrate neural lineages are still poorly characterized. Development of the lineage of olfactory receptor neurons (ORNs) has been relatively well described, however, owing to the simple structure and accessibility of the olfactory epithelium (OE) in the mouse. This tissue is made up of four cell types that can be distinguished in vivo and in vitro by their morphology, position and antigenic characteristics. They include (1) the ORN themselves, (2) their immediate neuronal precursors (INPs) or globose basal cells, which go through a small number of symmetric divisions before generating post-mitotic neurons, (3) the horizontal basal cells of unknown function and (4) the sustentacular cells, which have supporting and secretory functions (Graziadei and Monti-Graziadei, 1979; Calof and Chikaraishi, 1989; Schwartz Levey et al., 1991; Calof et al., 1996). The OE has the unique ability to maintain neurogenesis into adult stages and to regenerate ORNs when they are destroyed, implying that stem cells of the ORN lineage persist in the OE throughout life (Graziadei and Monti Graziadei, 1978).

Very little is known about the genes controlling the development of the ORN lineage. So far, *Mash1* is the only transcription factor shown to be required for this process. *Mash1* is transiently expressed by ORN progenitors before they reach the INP stage (Guillemot and Joyner, 1993; Gordon et al., 1996), and in mouse embryos mutant for *Mash1*, most ORNs are missing (Guillemot et al., 1993). In this article, we show that *Math4C/neurogenin1* is also expressed in ORN progenitor cells, but after *Mash1*, and that *NeuroD* expression in this lineage overlaps with overt neuronal differentiation. In the absence of *Mash1*, most progenitor cells fail to express *Math4C/neurogenin1* and *NeuroD* and remain undifferentiated in the OE until they die. A subset of progenitor cells present in a ventrocaudal domain of the OE, however, express *Math4C/neurogenin1* and *NeuroD* and differentiate in the absence of *Mash1* function. In contrast to the rest of the OE, cells in this domain express *Math4A/neurogenin2*, another bHLH gene closely related to *Math4C/neurogenin1*, and do not express *Mash1*. These results show that *Mash1* is required at an early stage in a subset of ORN progenitors to activate expression of *Math4C/neurogenin1* and of the neuronal differentiation gene *NeuroD*. Another gene, possibly *Math4A/neurogenin2*, activates a similar differentiation program in a region of the OE unaffected in *Mash1* mutants.

MATERIALS AND METHODS

Isolation of the murine *Math4B* and *Math4C* genes

Genomic DNA and random primed cDNA prepared from E10.5 mouse embryos were subjected to PCR (1 minute, 94°C; 1 minute, 45°C; 30 seconds, 72°C for 5 cycles and 1 minute, 94°C; 1 minute, 60°C; 30 seconds, 72°C for 35 cycles) with the following oligonucleotide primers, corresponding to the amino acid sequences ANNRER and FAHNYI from the basic region and second helix of MATH4A, respectively (Gradwohl et al., 1996): 5′ primer, 5′GAGGATCCGCIAA(T/C)(G/A)(C/A)I(C/A)GNGA(G/A)(C/A)G3′ and 3′ primer, 5′GGGAATTCAT(G/A)TA(G/A)TT(G/A)TGNGC(G/A)AA3′. In addition to sequences corresponding to the bHLH domains of *Math4A* and *NeuroD*, a novel sequence closely related to that of *Math4A* was amplified, that we named *Math4B*. The *Math4B* PCR fragment was used to screen a lambda GEM12 129SV mouse ES cell (D3) genomic library. Four clones were isolated; three of these contained the *Math4B* gene, and one contained a new gene, with a closely related bHLH sequence, which we named *Math4C* (Fig. 2B). *Math4B* and *Math4C* sequences had uninterrupted open reading frames encoding putative proteins of 194 and 244 amino acids, respectively (not shown). A *Math4C* cDNA containing the same open reading frame was subsequently isolated by screening a mouse embryonic cDNA phage library. These results suggest that the protein sequence of MATH4C is encoded in a single exon, as is the case for MATH4A (not shown) and other bHLH proteins like MASH1.

Wild-type and *Mash1* mutant mice

Wild-type and *Mash1* mutant embryos were obtained from intercrosses of *Mash1*+/– mice that have been backcrossed on a CD1 genetic background for more than five generations. For the staging of embryos, midday of the day of the vaginal plug was considered as E0.5. The precise stage of E9.5-10.0 embryos was defined by counting somites.

RNA in situ hybridization and immunocytochemistry

Whole-mount RNA in situ hybridization and immunocytochemistry on E9.5 to E10.5 embryos were performed as described in Wilkinson and Nieto (1993) and Davis et al. (1991), respectively. The same protocols were used for RNA/antibody double-labeling experiments, except that the proteinase K treatment of the RNA hybridization protocol was omitted and replaced by washes in RIPA buffer (Rosen and Beddington, 1993). Following whole-mount antibody or RNA staining, olfactory placodes were dissected with fine needles and flat-mounted for microscopic observation. RNA hybridization and immunostaining were performed on cryosections for embryos older than E10.5. The head of E11.5 and the nasal region of E12.5 and older embryos freshly harvested were fixed in 4% paraformaldehyde in PBS at 4°C for 2 hours, rinsed and equilibrated in 20% sucrose in PBS at 4°C for 4-6 hours, mounted in 7.5% gelatin, 15% sucrose in PBS at 37°C, frozen on dry ice, cut in the cryostat, and processed for ^{35}S- or digoxygenin-labeled RNA in situ hybridization as described (Myat et al., 1996; Gradwohl et al., 1996) or for immunocytochemistry as described (Guillemot et al., 1993; Gradwohl et al., 1996). For BrdU incorporation experiments, 2 mg of BrdU were injected intraperitoneally into pregnant mice 2 hours before they were killed. RNA probes were synthesized from the following cDNA clones: a 1674 bp full-length *Mash1* cDNA clone (Guillemot and Joyner, 1993); a 695 bp *NeuroD* cDNA containing residues V127 to D358 (Lee et al., 1995); a 1412 bp full-length *Math4A* cDNA clone (Gradwohl et al., 1996); a 404 bp *Math4C* clone containing residues M1 to M106 (Fig. 2B) and a 748 bp *Sox2* cDNA (Collignon et al., 1996; kindly provided by Larysa Pevny and Robin Lovell-Badge). Anti-class III β-tubulin and anti-BrdU monoclonal antibodies were from Sigma. Antibody binding was visualized using the Vectastain ABC kit (Vector).

Cell death detection

Apoptotic cell death was detected on sections of OE by the TUNEL procedure (Gavrieli, 1992), modified as follows. Frozen sections of fixed tissue, prepared as described above, were rinsed in PBS,

overlayed with 200 μl of TUNEL buffer (140 mM sodium cacodylate, 30 mM Tris, pH7.2, 1 mM $CoCl_2$, 0.25 mg/ml BSA) containing 40 μm digoxygenin-dUTP and 25 units of terminal transferase (Boehringer Mannheim) and incubated in a humid chamber at 37°C for 1 hour. Slides were then washed and processed for immunostaining with an anti-digoxygenin antibody coupled to peroxidase (1:2500, Boehringer Mannheim).

RESULTS

ORN development in the olfactory placodes proceeds from the periphery to the center

The first aim of this study was to characterize the initial stages of neurogenesis in the olfactory placodes of mouse embryos, using neuronal markers and *Mash1* as a marker of ORN progenitors. The early stages of development of the OE in the mouse have been previously described at the morphological level (Smart, 1971; Cuschieri and Bannister, 1975a,b). Olfactory placodes first appear as a thickening of the ectoderm on the ventrolateral sides of the head at about E9.5. At E10.5, the placodes invaginate, forming the olfactory pits, and ORNs start to grow axons. At E11.5, the olfactory pits deepen and form secondary recesses, and the dendrites of olfactory neurons become apparent. The OE becomes organized in layers around E12.5-13.5, with ORN progenitors progressively localized to the basal side of the epithelium and post-mitotic neurons to an intermediate zone. Sustentacular cells, the supporting cells of the OE, start to differentiate at about E15.5 and have their nuclei localized apically. The thickness of the OE increases at later embryonic stages and the tissue reaches its maximum size after birth.

To define the temporal and spatial aspects of neuronal differentiation during the early, less well-characterized stages of olfactory neurogenesis, we examined the expression of class III β-tubulin by immunocytochemistry and SCG10 by RNA in situ hybridization. These two genes are among the earliest characterized markers of neuronal differentiation (Stein et al., 1988; Easter et al., 1993). β-tubulin and *SCG10* became first detectable in cells localized in the center of the olfactory placode at around the 27-somite stage, just prior to the invagination of the placode (not shown). At E10.5, β-tubulin+/*SCG10*+ ORNs are also found in the center of the olfactory pit (Fig. 1D-F and not shown).

Mash1 marks a subset of ORN progenitors (Guillemot and Joyner, 1993; Gordon et al., 1996) and its activity is required for the generation of most ORNs (Guillemot et al., 1993). We studied the expression of *Mash1* at early stages of OE development, when mutation of this gene probably has its first phenotypic effects. *Mash1* transcripts were first detected at the 24- to 25-somite stage, in clusters of cells scattered in the placode. By the 27- to 28-somite stage, *Mash1*+ cells were grouped in

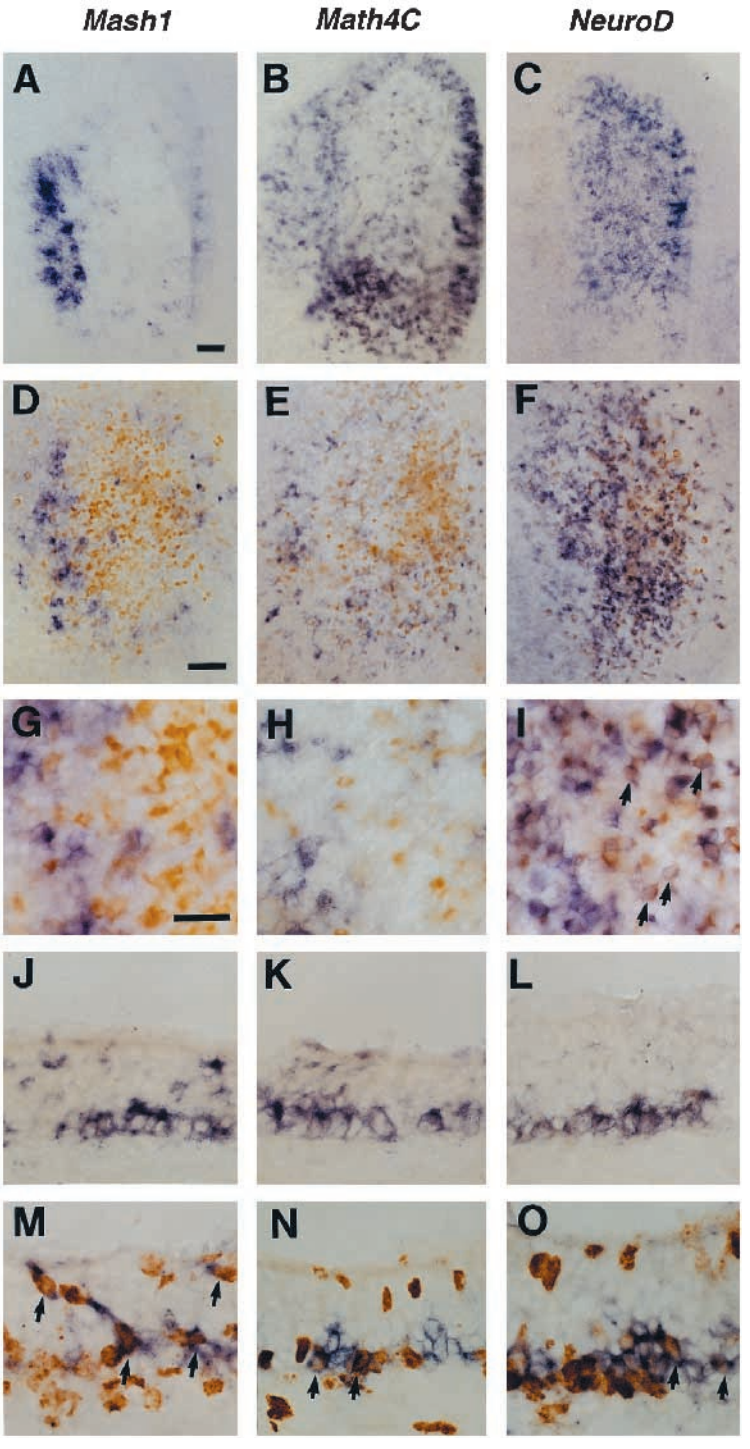

Fig. 1. Distribution of *Mash1*+, *Math4C*+ and *NeuroD*+ cells in the olfactory epithelium (OE). Hybridization of RNA probes for *Mash1* (left panels), *Math4C* (middle panels) and *NeuroD* (right panels) and immunostaining for β-tubulin (D-I) and BrdU (M-O) at E10.5 (A-C), E10.0 (D-I) and E17.5 (J-O). (A-I) Flat mounts of left olfactory placodes: dorsorostral is up, ventrocaudal is down, medial is left and lateral is right. (J-O) Cross sections of OE: apical is up, basal is down. G, H and I show higher magnification photographs of the placodes shown in D, E and F, respectively. Cells hybridized with *Mash1*/*Math4C*/*NeuroD* RNA probes are purple-blue; cells immunostained for β-tubulin expression (D-I) or BrdU incorporation (M-O) are brown. *NeuroD*+, β-tubulin+ double-labeled cells are marked with arrows in I. *Mash1*+/*Math4C*+/*NeuroD*+ cells that have incorporated BrdU during the 2 hours preceding the fixation are marked with arrows in M-O. Bars, 50 μm (A-F); 25 μm (G-O).

cell clusters arranged in a ring surrounding a central area of the placode devoid of labeled cells (not shown) and at E10.5, *Mash1* labeling was restricted to solid cell clusters at the periphery of the olfactory pit (Fig. 1A).

The distribution of *Mash1*+ and β-tubulin+/*SCG10*+ cells in olfactory placodes suggests that olfactory progenitors and differentiated neurons have already segregated at early developmental stages. In double-labeled placodes, *Mash1*+ and β-tubulin+ cells were indeed spatially segregated in a peripheral and a central domain respectively, and very little intermingling was observed (Fig. 1D,G). These results indicate that the differentiation stage of placodal cells is roughly correlated with their position on a periphery-to-center axis, with the less mature cells located at the periphery and the more differentiated cells in the center of the placode. This organization could be due to the addition of immature progenitors at the periphery of the placode by cell division, while cells in the center of the placode would progressively differentiate.

Isolation of a novel *atonal*-related gene, *Math4C* and expression of *Math4C* and *NeuroD* in ORN progenitors

To futher characterize neurogenesis in the olfactory placode and better define the role of *Mash1* in the ORN lineage, we searched for other potential regulators of neural development that would be expressed in ORN progenitors.

We have recently isolated three novel *atonal*-related bHLH genes named *Math4A* (Gradwohl et al., 1996), *Math4B* and *Math4C* (see Materials and Methods and Fig. 2) encoding proteins with high sequence similarity in their bHLH domains and lower but significant similarity with the bHLH domains of other mouse atonal-related proteins such as NeuroD, MATH1, MATH2 and MATH3 (Fig. 2; Gradwohl et al., 1996). A preliminary analysis by RNA in situ hybridization revealed that these three genes are expressed in embryonic CNS and PNS progenitors (Gradwohl et al., 1996; data not shown). In the OE, *Math4C* is expressed in a large cell population, whereas *Math4A* expression is restricted to a limited portion of this tissue (see below) and *Math4B* is not detectably expressed (not shown). These three genes have been independently isolated in other laboratories, and given the names *neurogenin1/NeuroD3* (*Math4C*), *neurogenin2* (*Math4A*) and *neurogenin3* (*Math4B*) (Ma et al., 1996; McCormick et al., 1996; Sommer et al, 1996). The bHLH gene *NeuroD*, which has been shown to promote neuronal differentiation in ectodermal cells of *Xenopus* embryos, is also expressed in olfactory placodes (Lee et al., 1995). We first examined the expression of *Math4C* and *NeuroD* during OE development and compared it with the expression of *Mash1*.

Math4C and *NeuroD* were first expressed in the olfactory placode at the 24- to 25-somite stage and their transcripts were found in many cells throughout the placode at E9.5 (not shown). The expression of the two genes differed markedly from each other and from that of *Mash1* in the olfactory pit at E10.5 (Fig. 1A-C). Because the lateral side of the pit is folded and difficult to observe and *Mash1* expression is absent from its ventrocaudal and dorsorostral sides, we compared the expression patterns of the three genes in the medial side of the pit (left in Fig. 1A-C). *Math4C*+ cells were present both at the periphery and in the center of the pit, while *Mash1*+ cells were found only at the periphery and *NeuroD*+ cells only in the center. Interestingly, *Math4C* was mostly expressed in single cells, whereas *Mash1* was expressed in large cell clusters in the medial side of the pit. Based on the differential distribution of neurons and progenitor cells, which suggest a differentiation gradient in the placode, as described above, *Math4C* appears to mark cells that are at a more advanced stage than those marked by *Mash1*, and *NeuroD* to mark cells more advanced than those marked by *Math4C*. The differentiation state of *Math4C*+ and *NeuroD*+ cells was studied by double-labeling with β-tubulin. In contrast to *Mash1*+ cells, *Math4C*+ and *NeuroD*+ cells were present in the center of the placode where they intermingled with β-tubulin+ cells (Fig. 1E,F). There was no apparent overlap, however, between the populations of *Math4C*+ and β-tubulin+ cells (Fig. 1H), whereas some placodal cells were double-labeled by *NeuroD* and β-tubulin (arrows in Fig. 1I). These results suggest that *Math4C* and *NeuroD* are both expressed in undifferentiated ORN progenitors, with *NeuroD* expression maintained transiently in differentiating neurons.

The simplest interpretation of the above data is that *Mash1*, *Math4C* and *NeuroD* are sequentially expressed in cells of the same lineage, with the following temporal sequence: *Mash1*>*Math4C*>*NeuroD*>β-tubulin. In particular, *Math4C* appears to be turned on and off at earlier stages than *NeuroD*, and continues to be expressed later than *Mash1*. Although the stages of *Mash1* and *Math4C* activation relative to one another could not be deduced from these experiments, a later onset of *Math4C* expression with respect to *Mash1* was supported by the analysis of *Mash1* mutant OE (see below). Whether these genes are transiently co-expressed in ORN progenitors remains to be determined.

We also studied the expression of *Mash1*, *Math4C* and *NeuroD* in the more mature OE, where the distribution of the different cell types has been described in detail. After E11.5, both *Math4C* and *NeuroD* were highly expressed in a 1- to 2-cell-thick layer on the basal side of the OE (Fig. 1K,L at E17.5), where ORN progenitors are located (Graziadei and Monti Graziadei, 1979). To confirm that *Mash1*, *Math4C* and *NeuroD* are expressed in dividing progenitors, double-labeling experiments were performed in E17.5 OE with RNA probes and anti-BrdU antibody after a 2-hour incorporation period (Fig. 1M,O). Many of the basal *Math4C*+ and *NeuroD*+ cells had incorporated BrdU, demonstrating that they were mitotically active. The results of the double-labeling experiments with BrdU and β-tubulin therefore indicate that *Math4C* is transiently expressed in dividing progenitors, while *NeuroD* expression spans the last division of ORN progenitors and the beginning of differentiation of post-mitotic neurons (Fig. 3).

Like *Math4C*+ and *NeuroD*+ cells, the majority of *Mash1*+ cells were localised to the basal side of the OE. Some *Mash1*+ cells, however, were found in intermediate or apical positions at all embryonic stages examined (Fig. 1J). As shown in Fig. 1M, many of the *Mash1*+ cells, in both basal and apical positions of a E17.5 OE, had incorporated BrdU after a 2-hour exposure period, indicating that they are dividing. Both basal and apical *Mash1*+ cells did not express β-tubulin either (not shown), confirming that they are undifferentiated. These results thus suggest that *Mash1* marks two distinct stages of ORN progenitor cells, with different locations in the OE. Based on our analysis of the *Mash1* mutant phenotype and on expression studies (Gordon et al., 1996), *Mash1*+ progenitors precede, and

```
                          basic     X     helix 1    X   loop  X      helix 2          % Identity

                            100        110       120        130        140       150
M-MATH4C/NGN1/NEUROD3  RSRRVKANDRERNR MHNLNAALDALRSVLP SFPDDTKLT KIETLRFAYNYIWALAETLR       100
M-MATH4B/NGN3          ----K--------- -----S------G--- T----A--- --------H------TQ---        86
M-MATH4A/NGN2          KT--L---N----- ------------E--- T--E-A--- --------H------T----        83
M-NEUROD/BETA2/BHF1    KL--M---A----- --G------N--K-V- CYSKTQ--S ------L-K------S-I--        67
M-NDRF/KW8/NEUROD2     KL--Q---A----- --D------N--K-V- CYSKTQ--S ------L-K------S-I--        67
M-MATH3                -A------A---T- --G--D---N--R-M- CYSKTQ--S ------L-R------S-V-E       66
M-MATH2/NEX1           KF--QE--A----- --G--D---N--K-V- CYSKTQ--S ------L-K------S-I--        64
M-MATH1                KQ--LA--A---R- --G--H-F-Q--N-I- --NN-K--S -Y---QM-QI--N--S-L-Q       57
D-atonal               RK--LA--A---R- -Q---Q-F-R--QY-- CLGN-RQ-S -H---QM-QT--S--GDL--       52

CONSENSUS                RR  AN RER R M  LN A D LR   P        L  K ETL  A  YI AL   L

M-MASH1                VA--...-E----- VKLV-LGFAT--EHV- NGAANK-MS -V----S-VQ--R--QQL-D       40
```

Fig. 2. Sequence similarities between the bHLH domain of atonal-related mouse proteins. Alignment of the amino acid sequences of the bHLH domains of murine MATH4A/ngn2 (database accession number Y07621, U76207), MATH4B/ngn3 (YO9167, U76208), MATH4C/ngn1/neuroD3 (YO9166, U67776, U63841), MATH1 (D43694), NeuroD/BETA2/BHF1 (U28068, U24679, D82074), MATH2/NEX1 (D44480, U29086), NDRF/kw8/neuroD2 (D83507, D82868, U58471), MATH3 (MGD-MRK-26094) and *Drosophila* atonal (A40708). A dash indicates amino acid identity at this position. MATH4A, MATH4B and MATH4C constitute a subfamily of atonal-related proteins with very similar bHLH domains, including at position N108 and in the first six residues of the loop, which diverge in the other atonal-related proteins.

are likely precursors of, the basally located INPs/globose cells. Thus, the apically located *Mash1*+ cells may be early ORN progenitors, possibly stem cells, which give rise to more mature *Mash1*+ progenitors migrating to the basal side of the OE (Fig. 3). ORN progenitors are indeed located apically in the first few days of OE development (Smart, 1971). Alternatively, these apically located *Mash1*+ cells could be progenitors for a separate branch of the ORN lineage.

Block of ORN differentiation in absence of *Mash1*

To further characterize the role of *Mash1* in the ORN lineage, we studied the effect of the loss of *Mash1* function on neuronal differentiation and gene expression in ORN progenitors. We have previously generated a mouse strain carrying a null allele of the *Mash1* gene. Animals homozygous for this mutation show a dramatic decrease in number of ORNs at birth (Guillemot et al., 1993). We extended our study of the *Mash1* null mutation by analysing in detail the onset of the mutant phenotype in the OE.

The first β-tubulin+ and *SCG10*+ ORNs appeared as early in mutant as in wild-type placodes, with no clear difference in the number of labeled neurons between mutant and wild-type OE at E9.5 and E10.5 (Fig. 4A,B and not shown). At E11.5, neurons were present in large numbers in a ventrocaudal domain of the mutant OE, but were missing in more rostral regions, which in wild-type OE contained neurons (Fig. 4C,D). At later stages, most regions of the mutant OE were devoid of neurons (Fig. 4E,F), with the exception of the ventrocaudal domain, which retained normal levels of β-tubulin and *SCG10* expression (not shown). Thus ORN progenitors fail to differentiate in the mutant OE, except in a ventrocaudal region, which is not affected by loss of *Mash1* function.

Fate of ORN progenitor cells in absence of *Mash1*

Histological analysis of *Mash1*–/– embryos had previously suggested that cell death was occurring in the mutant OE (Guillemot et al., 1993). To determine if apoptotic cell death was responsible for the lack of differentiated neurons in mutant OE, wild-type and mutant OE were subjected to the TUNEL procedure (Gavrieli, 1992). At E11.5 and earlier stages, very few cells were labeled in both mutant and wild-type OE, indicating that little cell death occurred at these stages even in the absence of *Mash1* function (Fig. 5A,B and not shown). At E12.5, TUNEL-labeling above wild-type levels was observed

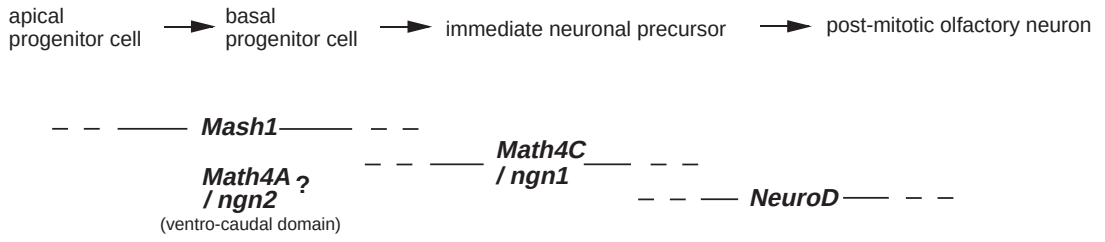

Fig. 3. Model for the expression of bHLH regulators in the olfactory neuron lineage. *Mash1*, *Math4C/ngn1* and *NeuroD* are sequentially expressed, in this order, in progenitor cells of the lineage. *Mash1* is expressed in both apical and basal dividing cells. *Mash1*+ basal cells are transit-amplifying cells that precede, and probaby give rise to, immediate neuronal precursors (Gordon et al., 1996). In this model, *Mash1*+ apical cells represent an earlier stage of the lineage, possibly a stem cell (see Discussion). The model also proposes that *Math4C/ngn1* expression marks the population of immediate neuronal precursors or globose basal cells. *NeuroD* expression spans the last division of the precursors and the beginning of the differentiation of post-mitotic neurons. The continuous lines mark the period of expression of each gene during development of the lineage. The dashed lines illustrate the fact that the precise timings of activation and extinction of gene expression, and the possibility of temporal overlaps between these expressions, have not been established. The position of the ventrocaudal *Math4A*+ cells in the olfactory neuron lineage has not been defined.

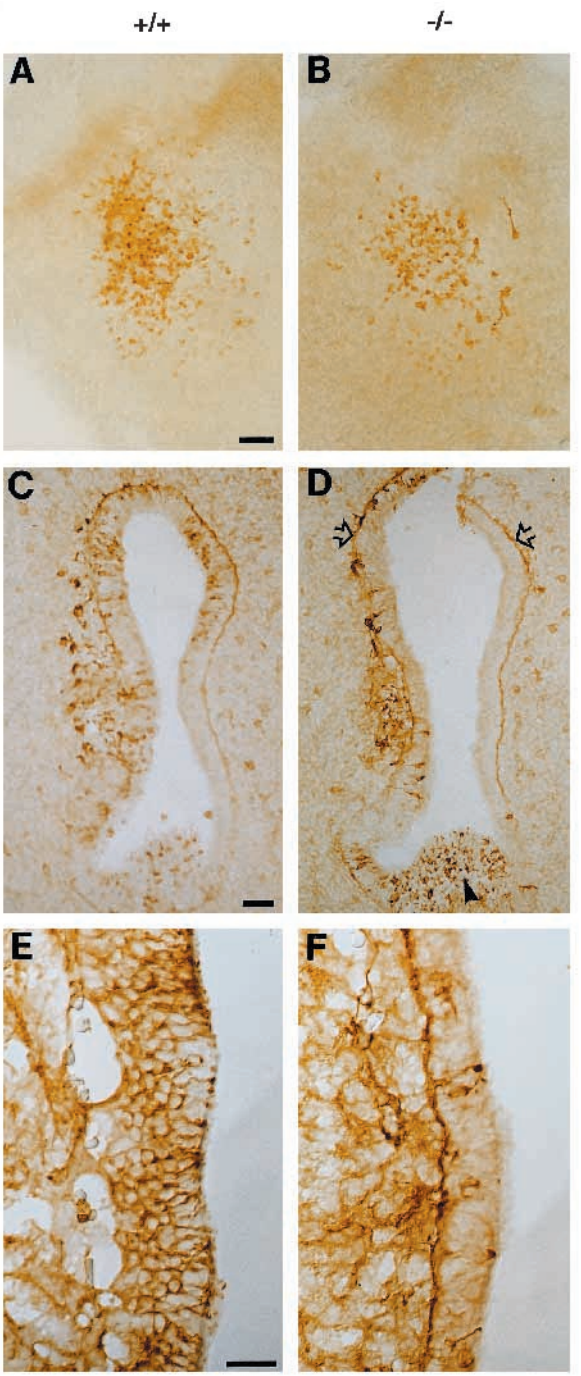

Fig. 4. Block of neuronal differentiation in *Mash1* mutant OE. β-tubulin immunostaining of wild type (A,C,E) and *Mash1* mutant (B,D,F) OE at E9.5 (A,B), E11.5 (C,D) and E13.5 (E,F). (A,B) Flat mounts of placodes. (C-F) Horizontal sections of OE; rostral is up, lateral is right. ORNs are present in apparently normal numbers in mutant placodes at E9.5. At E11.5, they are found in a ventrocaudal (bottom) region of mutant OE (arrowhead in D), but are absent in other regions (open arrows in D) that contain neurons in wild-type OE. Most regions of mutant OE are devoid of neurons at E13.5. Thus, most early born ORNs are *Mash1*-independent, whereas later born ORNs fail to differentiate in *Mash1* mutant OE. Bars, 50 μm (A-D); 25 μm (E,F).

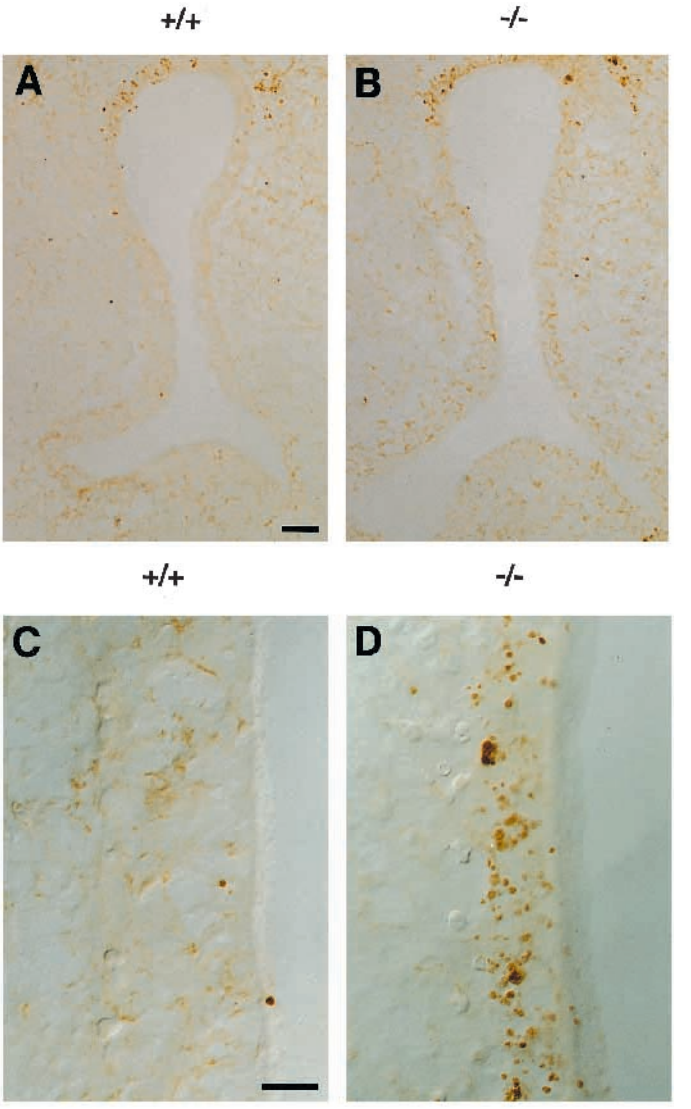

Fig. 5. Death of *Mash1* mutant OE cells. TUNEL-labeling of wild-type (A,C) and *Mash1* mutant (B,D) OE, horizontally sectioned at E11.5 (A,B) and E13.5 (C,D); rostral is up, lateral is right. There is no detectable apoptotic cell death in mutant OE at E11.5 (B). Cell death is abundant throughout the mutant OE at E13.5 (D). Bars, 50 μm (A,B); 25 μm (C,D).

in the dorsorostral extremity of the mutant OE (data not shown). At E13.5, dying cells were present in large numbers in most regions of the mutant OE, whereas TUNEL-labeling remained very low in the wild-type OE (Fig. 5C,D). Thus, cell death is abundant only after E12.5, i.e. after the initial differentiation arrest of *Mash1* mutant cells, indicating that death of progenitors is not a direct cause of the lack of ORNs observed in mutant OE at early stages.

To confirm that mutant progenitor cells were present in the OE before the onset of cell death, we studied the expression of molecular markers in the mutant OE. Using a *Mash1* RNA probe, we detected in *Mash1*–/– embryos a mutant *Mash1* transcript with a distribution similar to the wild-type transcript. In particular, *Mash1*+ cells were present in the same positions in *Mash1* mutant and in wild-type OE at E11.5 and E12.5 (Fig.

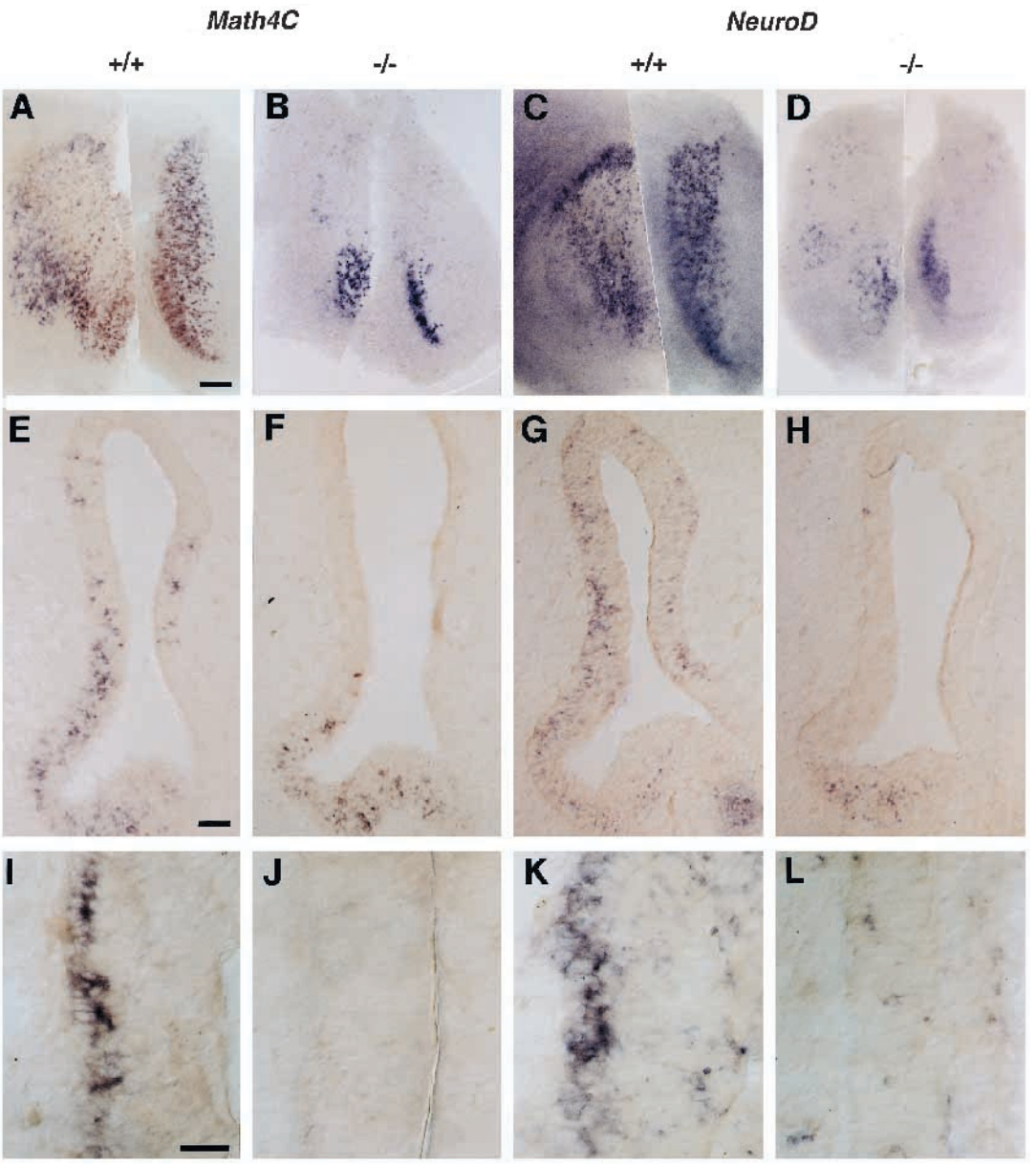

Fig. 6. Regulation of *Math4C* and *NeuroD* expression in *Mash1* mutant OE. Hybridization with RNA probes for *Math4C* (A,B,E,F,I,J) and *NeuroD* (C,D,G,H,K,L) on wild type (A,C,E,G,I,K) and *Mash1* mutant (B,D,F,H,J,L). (A-D) Olfactory pits at E10.5 have been unfolded before mounting to show staining on both sides; rostrodorsal is up, lateral is right. (E-H) OE sectioned at E11.5; rostral is up, medial is right. (I-L) OE at E13.5. Expression of *Math4C* and *NeuroD* is absent in most olfactory progenitor cells from E10.5 onwards. Their expression is maintained in *Mash1*-independent progenitor cells, which constitute a large proportion of the OE at E9.5 (not shown) and are restricted to a ventrocaudal domain of the OE at E10.5 (B,D) and E11.5 (F,H). Bars, 100 μm (A-D); 50 μm (E-H); 25 μm (I-L).

7A,E). We also examined expression of *Sox2*, a marker of neural progenitors in the CNS and in olfactory placodes (Uwanogho et al., 1995; Collignon et al., 1996). We detected similar expression of *Sox2* in wild-type and *Mash1* mutant embryos between E9.5 and E12.5, including in the areas of mutant OE devoid of differentiated neurons (not shown). Thus, until at least E12.5, *Mash1*-expressing cells are present in the mutant OE but fail to differentiate.

Mash1 is required for *Math4C* and *NeuroD* expression in most ORN progenitors

Expression studies suggest that *Math4C* and *NeuroD* are expressed after *Mash1* in ORN progenitors (see above). As ORN progenitors are present in *Mash1* mutant OE until E12.5, it was possible to study transcription of *Math4C* and *NeuroD* in these cells before their elimination by apoptosis.

Both *Math4C* and *NeuroD* expression appeared at similar stages in *Mash1* mutant and in wild-type placodes. Expression of *NeuroD* and *Math4C* was reduced in mutant placodes, however, and confined to the center of the placode, suggesting that some cells of the placode failed to transcribe *Math4C* and *NeuroD* (not shown). At E10.5 and later stages, expression of both *Math4C* and *NeuroD* was absent in most cells of the mutant OE, except in a ventrocaudal domain (Fig. 6). This loss of *Math4C* and *NeuroD* expression around E10.0 is the first defect observed in mutant OE cells.

Mash1 is not required for the expression of *Math4C* and *NeuroD* in the ventrocaudal OE and the brain

Normal levels of *Math4C* and *NeuroD* expression were maintained in a ventrocaudal domain of mutant olfactory pits (Fig. 6A-H). A comparison of *Mash1* and *Math4C* expression patterns in wild-type OE (Fig. 1A,B at E10.5; Fig. 7A,C at E11.5; Fig. 7I,K at E12.5) showed that, in contrast to other regions of the OE where *Mash1* and *Math4C* are co-expressed, only *Math4C* is expressed in this ventrocaudal domain. The

lack of expression of *Mash1* is a clear indication that *Math4C* expression must be regulated differently in this domain from in the rest of the OE. Interestingly, *Math4A*, a gene closely related to *Math4C* (Gradwohl et al., 1996; Fig. 2), is expressed in a region of the OE that overlaps with the *Mash1*-independent domain of *Math4C* expression and neurogenesis (Fig. 7B). This *Math4A* expression domain persists in *Mash1* mutant OE (Fig. 7F). *Math4A* may be activated in parallel with *Math4C* in this ventrocaudal domain of the OE, or it may be involved itself in the activation of *Math4C* expression in *Mash1*-independent ORN progenitor cells.

DISCUSSION

In this article, we describe the expression of four bHLH transcriptional regulators, *Mash1*, *NeuroD*, *Math4A/neurogenin2* and the novel gene *Math4C/neurogenin1*, at early stages of olfactory neurogenesis, and we characterize the consequences of the loss of *Mash1* activity on gene expression and differentiation in the ORN lineage.

Mash1 mutant phenotypes in the OE

The OE of *Mash1*–/– embryos presents three defects which appear sequentially: (1) loss of *Math4C* and *NeuroD* expression, (2) block of neuronal differentiation, and (3) cell death. What are the causal links between loss of *Mash1* and these different phenotypes?

Lack of *Math4C* and *NeuroD* expression are the earliest defects observed in *Mash1* mutant ORN progenitors. This, added to the temporal and spatial distribution of gene expression in the placode, points to the following developmental pathway in the OE: *Mash1*>*Math4C*>*NeuroD*>β-tubulin. The precise timing and the degree of overlap of expression of these genes at the single cell level remain to be defined, but the current data raise the possibility that in the ORN lineage, *Mash1* directly activates the transcription of *Math4C*, and that *Math4C* activates the transcription of *NeuroD*. An indirect activation or even a non-cell autonomous effect of *Mash1* on *Math4C* and *NeuroD* expression cannot, however, be excluded. *X-ngnr-1*, a *Xenopus* gene related to mouse *Math4C/neurogenin1*, has been demonstrated to have a role in inducing *NeuroD* expression and neurogenesis by RNA injection in *Xenopus* embryos (Ma et al., 1996). Thus there is evidence for an interaction between members of these two gene families in other tissues.

Expression of *Mash1*, *Math4C* and *NeuroD* precedes neuronal differentiation in the ORN lineage. In the most parsimonious hypothesis, the main function of *Mash1* and/or *Math4C* could be to activate *NeuroD*, which would alone trigger neuronal differentiation. Experiments in *Xenopus* embryos have demonstrated that ectopic expression of *NeuroD* is sufficient to promote neuronal differentiation in ectodermal cells (Lee et al., 1995). Alternatively, each of these genes could control the expression of a distinct subset of genes contributing to the neuronal phenotype. The generation of loss-of-function and gain-of-function mutations in these genes should help to distinguish between these possibilities.

Cell death in *Mash1* mutant OE is first detected at E12.5, more than 2 days after the initial block in *Math4C* and *NeuroD* expression. This delay suggests that loss of *Mash1* only indirectly leads to cell death in the OE. Thus, *Mash1* could regulate genes that mediate the survival of ORN progenitors, for example by controlling their response to trophic factors. Experiments of bulbectomy in adult mice have provided evidence that ORN progenitors are dependent on extrinsic signals for their survival (Holcomb et al., 1995). Therefore, *Mash1* could both promote neuronal differentiation and regulate certain characteristics of progenitor cells, such as their trophic dependence or proliferative properties.

A cascade of bHLH regulators in the ORN lineage

Analysis of the ORN lineage in mice and in cell cultures has provided evidence that ORN progenitors pass through a succession of developmental stages, which can be distinguished in particular by their proliferative properties (Graziadei and Monti-Graziadei, 1979; Calof and Chikaraishi, 1989). A population of rapidly dividing cells, called globose cells, has been described at the basal side of the OE (Graziadei and Monti Graziadei, 1979; Mackay-Sim and Kittel, 1991; Schwartz Levey et al., 1991). Explant cultures (Calof et al., 1996), lineage tracing (Caggiano et al., 1994) and ^{3}H-thymidine labeling experiments (Graziadei and Monti Graziadei, 1979) have demonstrated that globose cells constitute a population of ORN progenitors with characteristics of immediate neuronal precursors.

Expression studies in OE explants and in regenerating OE have shown that *Mash1* is expressed at an early stage of the ORN lineage by progenitors that are distinct from, and are likely to give rise to, INPs/globose cells (Gordon et al., 1996). Since the expression of *Math4C* follows that of *Mash1* and precedes neuronal differentiation, it could mark the main population of INPs/globose cells. It has been proposed that each stage of ORN progenitor development responds to a distinct set of mitogens, survival factors and differentiation factors, and thus represents a checkpoint at which neuron production can be controlled (Gordon et al, 1996; Calof et al., 1996). Thus *Mash1* and *Math4C* could regulate distinct responses to extrinsic factors in the successive stages of the ORN progenitor cells in which they are expressed.

Mash1 has distinct functions in different neuronal lineages

Analysis of the *Mash1* mutant phenotype has revealed that different genetic mechanisms are used to activate neurogenesis in two domains of the OE. *Mash1* is required to activate *Math4C* transcription and promote neuronal differentiation in most of the OE. In contrast, another bHLH gene, *Math4A*, is expressed exclusively in a ventrocaudal domain of the OE where *Mash1* is not expressed. Whether *Math4A* activates *Math4C* expression in this domain, or whether the two genes are activated in parallel, remains to be determined. It will be important to ascertain whether the genetically distinct populations of *Mash1*-dependent and -independent ORNs also have distinct functions. Each ORN expresses one or a few odorant receptor genes from a family of about 1000 genes (Ressler et al., 1993; Vassar et al., 1993). In rodents, the OE is divided into spatial zones in which different sets of odorant receptor genes are expressed (Ressler et al., 1993; Vassar et al., 1993). It is an intriguing possibility that the *Math4A*+ ventrocaudal domain of the OE represents such a zone where *Mash1*-independent ORNs express a distinct set of odorant receptor genes.

Math4A, *Math4C* and *NeuroD* have similar expression

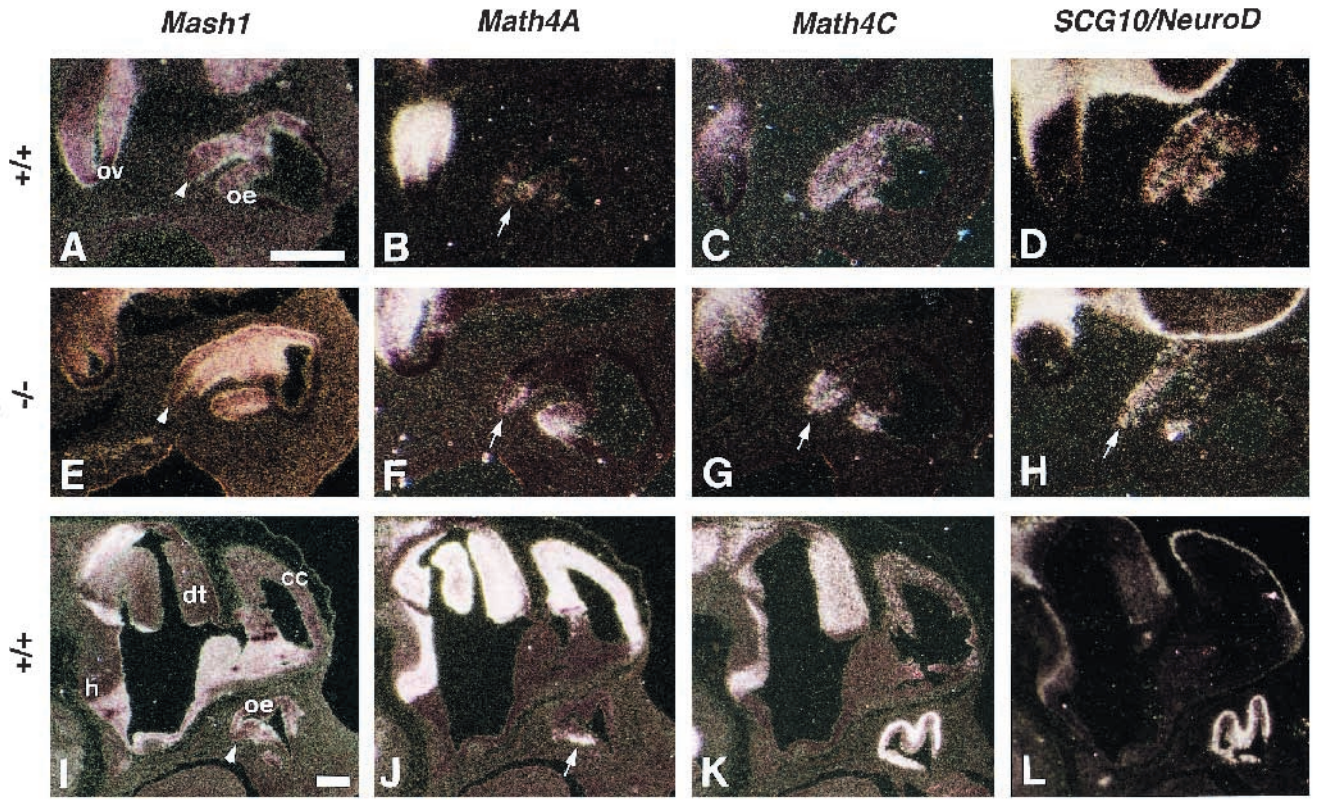

Fig. 7. *Math4A* expression is restricted to a *Mash1*-independent ventrocaudal domain of the OE. In situ hybridization with RNA probes for *Mash1* (A,E,I), *Math4A* (B,F,J), *Math4C* (C,G,K), *SCG10* (D,H) and *NeuroD* (L), on adjacent sagittal sections of the anterior head region of wild-type (A-D,I-L) and *Mash1* mutant (E-H) embryos at E11.5 (A-H) and E12.5 (I-L); dorsal is up, rostral is right. *Mash1* (A,I) is expressed in a large rostrodorsal domain of the OE (arrowheads), and *Math4A* (B,J) in a smaller ventrocaudal domain (arrows) where *Mash1* expression is missing. In *Mash1* mutant OE, most ORN progenitor cells, marked by expression of a mutant *Mash1* transcript (E), fail to express *Math4C* (G) and to differentiate (H), whereas *Mash1*-independent progenitor cells in the ventrocaudal domain express *Math4A*, *Math4C* and differentiate (arrows in F, G and H, respectively). cc, cerebral cortex; dt, dorsal thalamus; h, hypothalamus; oe, olfactory epithelium; ov, optic vesicle. Bar, 500 μm.

patterns in most of the CNS and PNS, with *NeuroD* consistently expressed in more differentiated cells than *Math4A* and *Math4C* (Fig. 7J,K,L; Sommer et al., 1996; our unpublished data), suggesting that *Math4A* and/or *Math4C* may be activating *NeuroD* in a number of neuronal lineages. In contrast, the domain of expression of *Mash1* and those of *Math4A* and *Math4C* are distinct in most regions of the CNS and mutually exclusive in the PNS, where *Mash1* is expressed in autonomic precursors and the *Math4* genes in sensory precursors (Fig. 7I,K,L and unpublished results). The lack of co-expression of *Mash1* and *Math4C* in most neural tissues indicates that *Mash1* must have distinct target genes in the OE and in other neuronal lineages. The comparison of the *Mash1* mutant phenotypes in the ORN and sympathetic neuron lineages strongly supports this conclusion. The analysis of primary cultures and immortalized cell lines derived from *Mash1* mutant neural crest cells have demonstrated that *Mash1* is required at a stage of sympathetic precursor development that already expresses class III β-tubulin and other neuronal markers, for further differentiation into sympathetic neurons (Sommer et al., 1995). In contrast to this late differentiation function, *Mash1* is expressed early in the olfactory placode, where it activates the expression of *Math4C* and of the neuronal differentiation gene *NeuroD*. Furthermore, *Mash1* is expressed in large cell clusters in the olfactory placode, whereas the downstream gene *Math4C* is mainly expressed in isolated cells, suggesting that a process of lateral inhibition (Artavanis-Tsakonas and Simpson, 1991) may restrict *Mash1* activity and neurogenesis to a subset of placodal cells. Altogether, these results point to a function of *Mash1* in the initiation of a differentiation program in ORN progenitors, or even in the determination of the ORN lineage. The early expression of *Sox2* by placodal cells (Uwanogho et al., 1995) suggests a degree of commitment to a neural fate, which precedes *Mash1* activity. *Mash1* expression may thus be necessary for placodal cells to make a further decision towards neuronal development, for example in a bipotential progenitor cell common to ORNs and epithelial sustentacular cells.

In summary, *Mash1* is required at different stages, and serves distinct functions, in the ORN and sympathetic lineages. As previously suggested by ectopic expression experiments with *Drosophila* proneural genes and vertebrate myogenic genes (Brand et al., 1993; Wang et al., 1996), the specific function of a bHLH protein in a cell lineage appears to depend on the particular developmental context in which it is expressed.

We are grateful to Larysa Pevny and Robin Lovell-Badge for the generous gift of the *Sox2* cDNA clone, Valérie Meyer for histologi-

cal preparations, Serge Vicaire for DNA sequencing, Adrien Staub for oligonucleotide synthesis, and to Siew Lan Ang, Pat Simpson and Uwe Strahle for comments on the manuscript. This work was supported by grants from the Fondation pour la Recherche Médicale, the Ministère de l'Enseignement et de la Recherche and the Association pour la Recherche sur le Cancer, and funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique and the Centre Hospitalier Universitaire Régional. C.F. was supported by a long term fellowship from the Human Frontier Science Program. The *Math4B* and *Math4C* DNA sequences have been deposited in the EMBL Nucleotide Sequence Database, with accession numbers Y09166 and Y09167, respectively.

REFERENCES

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S. and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene *atonal* is a positive transcriptional regulator expressed in the developing nervous system. *J. Biol. Chem.* **270**, 8730-8738.

Artavanis-Tsakonas, S. and Simpson, P. (1991). Choosing a cell fate: a view from the *Notch* locus. *Trends. Genet.* **7**, 403-408.

Bartholomä, A. and Nave, K.-A. (1994). NEX-1: a novel brain-specific helix-loop-helix protein with autoregulation and sustained expression in mature cortical neurons. *Mech. Dev.* **48**, 217-228.

Blaugrund, E., Pham, T. D., Tennyson, V. M., Lo, L., Sommer, L., Anderson, D. J. and Gershon, M. D. (1996). Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence. *Development* **122**, 309-320.

Brand, M., Jarman, A. P., Jan, L. Y. and Jan, Y. N. (1993). *asense* is a *Drosophila* neural precursor gene and is capable of initiating sense organ formation. *Development* **119**, 1-17.

Caggiano, M., Kauer, J. S. and Hunter, D. D. (1994). Globose basal cells are neuronal progenitors in the olfactory epithelium: a lineage analysis using a replication-incompetent retrovirus. *Neuron* **13**, 339-352.

Calof, A. L. and Chikaraishi, D. M. (1989). Analysis of neurogenesis in a mammalian neuroepithelium: Proliferation and differentiation of an olfactory neuron precursor in vitro. *Neuron* **3**, 115-127.

Calof, A. L., Hagiwara, N., Holcomb, J. D., Mumm, J. S. and Shou, J. Y. (1996). Neurogenesis and cell death in olfactory epithelium. *J. Neurobiol.* **30**, 67-81.

Campuzano, S. and Modolell, J. (1992). Patterning of the *Drosophila* nervous system: the *achaete-scute* gene complex. *Trends Genet.* **8**, 202-208.

Collignon, J., Sockanathan, S., Hacker, A., Cohen-Tannoudji, M., Norris, D., Rastan, S., Stevanovic, M., Goodfellow, P. N. and Lovell-Badge, R. (1996). A comparison of the properties of Sox-3 with Sry and two related genes, Sox-1 and Sox-2. *Development* **122**, 509-520.

Cuschieri, A. and Bannister, L. H. (1975a). The development of the olfactory mucosa in the mouse: electron microscopy. *J. Anat.* **119**, 471-498.

Cuschieri, A. and Bannister, L. H. (1975b). The development of the olfactory mucosa in the mouse: light microscopy. *J. Anat.* **119**, 277-286.

Davis, C. A., Holmyard, D. P., Millen, K. J. and Joyner, A. L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an *En*-specific antiserum. *Development* **111**, 287-298.

Easter, S. S. Jr., Ross, L. S. and Frankfurter, A. (1993). Initial tract formation in the mouse brain. *J. Neurosci.* **13**, 285-299.

Gavrieli, Y. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493-501.

Ghysen, A. and Dambly-Chaudière, C. (1988). From DNA to form: the *achaete-scute* complex. *Genes Dev.* **2**, 495-501.

Ghysen, A., Dambly-Chaudiere, C., Jan, L. Y. and Jan, Y. N. (1993). Cell interactions and gene interactions in peripheral neurogenesis. *Genes Dev.* **7**, 723-733.

Gordon, M. K., Mumm, J. S., Davis, R. A., Holcomb, J. D. and Calof, A. L. (1996). Dynamics of MASH1 expression *in vitro* and *in vivo* suggest a non-stem-cell site of MASH1 action in the olfactory receptor neuron lineage. *Mol. Cell. Neurosci.* **6**, 363-379.

Gradwohl, G., Fode, C. and Guillemot, F. (1996). Restricted expression of a novel murine *atonal*-related bHLH protein to undifferentiated neural precursors. *Dev. Biol* **180**, 227-241.

Graziadei, P. P. C. and Monti Graziadei, A. G. (1978). Continuous nerve cell renewal in the olfactory system. In: *Handbook of Sensory Physiology,* Vol IX (ed. M. Jacobson), pp. 55-82. Berlin: Springer.

Graziadei, P. P. C. and Monti Graziadei, G. A. (1979). Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. *J. Neurocytol.* **8**, 1-18.

Guillemot, F. (1995). Analysis of the role of basic-helix-loop-helix transcription factors in the development of neural lineages in the mouse. *Biol. Cell.* **84**, 3-6.

Guillemot, F. and Joyner, A. L. (1993). Dynamic expression of the murine *achaete-scute* homologue *Mash-1* in the developing nervous system. *Mech. Dev.* **42**, 171-185.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993). Mammalian a*chaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463-476.

Holcomb, J. D., Mumm, J. S., and Calof, A. L. (1995). Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation in vivo and in vitro. *Dev. Biol.* **172**, 307-323.

Jarman, A. P., Grau, Y., Jan, L. Y. and Jan, Y. N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. *Cell* **73**, 1307-1321.

Johnson, J. E., Birren, S. J. and Anderson, D. J. (1990). Two rat homologues of D*rosophila achaete-scute* specifically expressed in neuronal precursors. *Nature* **346**, 858-861.

Kawakami, H., Maruyama, H., Yasunami, M., Ohkubo, H., Hara, H., Saida, T., Nakanishi, S. and Nakamura, S. (1996). Cloning and expression of a rat brain basic helix-loop-helix factor. *Biochem. Biophys. Res. Commun.* **221**, 199-204.

Kume, H., Maruyama, K., Tomita, T., Iwatsubo, T., Saido, T. C. and Obata, K. (1996). Molecular cloning of a novel basic helix-loop-helix protein from the rat brain. *Biochem. Biophys. Res. Commun.* **219**, 526-530.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. and Weintraub, H. (1995). Conversion of *Xenopus* ectoderm into neurons by neuroD, a basic helix-loop-helix protein. *Science* **268**, 836-844.

Ma, Q., Kintner, C., and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.

Mackay-Sim, A. and Kittel, P. (1991). Cell dynamics in the adult mouse olfactory epithelium: a quantitative autoradiographic study. *J. Neurosci.* **11**, 979-984.

McConnell, S. K. (1995). Strategies for the generation of neuronal diversity in the developing central nervous system. *J. Neurosci.* **15**, 6987-6998.

McCormick, M. B., Tamimi, R. M., Snider, L., Asakura, A., Bergstrom, D., and Tapscott, S. J. (1996). *neuroD2* and *neuroD3*: distinct expression patterns and transcriptional activation potentials within the *neuroD* gene family. *Mol. Cell. Biol.* **16**, 5792-5800.

Myat, A., Henrique, D., Ishhorowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.

Naya, F. J., Stellrecht, C. M. M. and Tsai, M. J. (1995). Tissue-specific regulation of the insulin gene by a novel basic-helix-loop-helix transcription factor. *Genes Dev.* **9**, 1009-1019.

Ressler, K. J., Sullivan, S. L., and Buck, L. (1993). A zonal organization of odorant receptor gene expression in the olfactory epithelium. *Cell* **73**, 597-609.

Rodriguez, I., Hernandez, R., Modolell, J. and Ruiz-Gomez, M. (1990). Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J.* **9**, 3583-3592.

Rosen, B. and Beddington, R. S. P. (1993). Whole-mount *in situ* hybridization in the mouse embryo: gene expression in three dimensions. *Trends Genet.* **9**, 162-167.

Schwartz Levey, M. A., Chikaraishi, D. M. and Kauer, J. S. (1991). Characterization of potential precursor populations in the mouse olfactory epithelium using immunocytochemistry and autoradiography. *J. Neurosci.* **11**, 3556-3564.

Shimizu, C., Akazawa, C., Nakanishi, S. and Kageyama, R. (1995). MATH-2, a mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal, is specifically expressed in the nervous system. *Eur. J. Biochem.* **229**, 239-248.

Smart, I. H. M. (1971). Location and orientation of mitotic figures in the developing mouse olfactory epithelium. *J. Anat.* **109**, 243-251.

Sommer, L., Shah, N., Rao, M. and Anderson, D. J. (1995). The cellular function of MASH1 in autonomic neurogenesis. *Neuron* **15**, 1245-1258.

Sommer, L., Ma, Q., and Anderson, D. J. (1996). *neurogenins*, a novel family of *atonal*-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8**, 221-247.

Stein, R., Mori, N., Matthews, K., Lo, L. C. and Anderson, D. J. (1988). The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. *Neuron* **1**, 463-476.

Takebayashi, K., Takahashi, S., Yokota, C., Tsuda, H., Nakanishi, S., Asashima, M., and Kageyama, R. (1997). Conversion of ectoderm into neural fate by *ATH-3*, a vertebrate basic helix-loop-helix gene homologous to *Drosophila* proneural gene *atonal*. *EMBO J.* (in press).

Uwanogho, D., Rex, M., Cartwright, E. J., Pearl, G., Healy, C., Scotting, P. J. and Sharpe, P. T. (1995). Embryonic expression of the chicken *Sox2*, *Sox3* and *Sox11* genes suggests an interactive role in neuronal development. *Mech. Dev.* **49**, 23-36.

Vassar, R., Ngai, J., and Axel, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell* **74**, 309-318.

Wang, Y. K., Schnegelsberg, P. N. J., Dausman, J. and Jaenisch, R. (1996). Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. *Nature* **379**, 823-825.

Wilkinson, D. G. and Nieto, A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Meth. Enzymol.* **225**, 361-373.

Yasunami, M., Suzuki, K., Maruyama, H., Kawakami, H., Nagai, Y., Hagiwara, M. and Ohkubo, H. (1996). Molecular cloning and characterization of a cDNA encoding a novel basic helix-loop-helix protein structurally related to NeuroD/BHF1. *Biochem. Biophys. Res. Commun.* **220**, 754-758.

(Accepted 6 February 1997)