Mammalian *achaete–scute* and *atonal* homologs regulate neuronal versus glial fate determination in the central nervous system

**Koichi Tomita, Koki Moriyoshi**<sup>1,2</sup>, **Shigetada Nakanishi**<sup>1</sup>, **François Guillemot**<sup>3</sup> and **Ryoichiro Kageyama**<sup>4</sup>

Institute for Virus Research, Kyoto University, Shogoin-Kawahara, Sakyo-ku, Kyoto 606-8507, <sup>1</sup>Department of Biological Sciences, Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan and <sup>2</sup>Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur/Collège de France, 67404 Illkirch, CU de Strasbourg, France

<sup>3</sup>Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92039-0357, USA

<sup>4</sup>Corresponding author
e-mail: rkageyam@virus.kyoto-u.ac.jp

Whereas vertebrate *achaete–scute* complex (as-c) and *atonal* (ato) homologs are required for neurogenesis, their neuronal determination activities in the central nervous system (CNS) are not yet supported by loss-of-function studies, probably because of genetic redundancy. Here, to address this problem, we generated mice double mutant for the as-c homolog *Mash1* and the ato homolog *Math3*. Whereas in *Mash1* or *Math3* single mutants neurons are only weakly affected, in the double mutants tectal neurons, two longitudinal columns of hindbrain neurons and retinal bipolar cells were missing and, instead, those cells that normally differentiate into neurons adopted the glial fate. These results indicate that *Mash1* and *Math3* direct neuronal versus glial fate determination in the CNS and raised the possibility that downregulation of these bHLH genes is one of the mechanisms to initiate gliogenesis.

**Keywords:** bHLH/gliogenesis/Mash1/Math3/ neurogenesis

---

**Introduction**

Neurons and glial cells differentiate from common precursor cells, and development of each cell type is regulated by many transcription factors. Among them, basic helix–loop–helix (bHLH) factors have been shown to play an important role in neuronal determination and differentiation of both invertebrates and vertebrates (Jan and Jan, 1994; Guillemot, 1995; Anderson and Jan, 1997; Kageyama and Nakanishi, 1997; Lee, 1997; Anderson, 1999).

In *Drosophila*, there are at least two classes of proneural bHLH genes, the *achaete–scute* complex (as-c) and *atonal* (ato), which not only determine the neural fate but also specify the identities of the external sensory organ and the chordotonal organ, respectively (Jan and Jan, 1994; Anderson and Jan, 1997). Thus, neural fate determination seems to be coupled to specification of the neuronal subtype identities, and these two steps are regulated by proneural bHLH genes. Similarly, in vertebrates, the as-c homolog *Mash1* and the distant ato homologs *ngn* are expressed in a complementary manner and involved in generation of different subsets of neurons (Guillemot and Joyner, 1993; Gradwohl et al., 1996; Sommer et al., 1996; Ma et al., 1997). In the central nervous system (CNS), *Mash1* and *ngn* are involved in specification of distinct subtypes of neurons such as ventral and dorsal telencephalic neurons (Blader et al., 1997; Casarosa et al., 1999; Torii et al., 1999; Fode et al., 2000). In the peripheral nervous system (PNS), where neural crest cells give rise to autonomic and sensory neurons, *Mash1* is involved in generation of the former neurons while *ngn* are involved in generation of the latter (Guillemot et al., 1993; Sommer et al., 1995; Blaugrund et al., 1996; Hirsch et al., 1998; Ma et al., 1999; Perron et al., 1999). Misexpression studies demonstrated that *Mash1* and *ngn* can induce both pan-neuronal properties and subtype-specific identities in precursor cells (Lo et al., 1998; Perez et al., 1999). Moreover, transient expression of proneural bHLH genes is sufficient to convert P19 embryonal carcinoma cells into neurons (Farah et al., 2000). These gain-of-function studies indicate that vertebrate bHLH genes are involved in both neuronal commitment and specification of particular subtypes such as *Drosophila* proneural genes. However, in *Mash1*- or *ngn*-deficient mice, although subsets of neurons are missing, it is not clear whether the block of generation of neurons occurs at the neuronal versus glial fate determination or just after commitment to the neuronal fate (Sommer et al., 1995; Fode et al., 1998; Hirsch et al., 1998; Ma et al., 1998, 1999). Particularly, in the CNS of *Mash1*- or *ngn*-deficient mice there are still many neurons generated (Casarosa et al., 1999; Fode et al., 2000) and thus the neuronal commitment activity suggested by a gain-of-function study (Lo et al., 1998; Perez et al., 1999; Farah et al., 2000) is not yet fully supported by a loss-of-function study in the CNS, probably because of genetic redundancy.

The loss-of-function evidence that a bHLH gene is important for neuronal versus glial fate choice is shown in the retina. In mice mutant for *Mash1* or the distant ato homolog *NeuroD*, retinal neurons decrease whereas glial cells increase, suggesting that bHLH genes are involved in the process of neuronal versus glial fate determination (Tomita et al., 1996b; Morrow et al., 1999). However, the defects observed in the mutant retina are only partial and still all the neuronal types are generated, suggesting that the bHLH genes could be redundant or not be a key molecule but only influence the differentiation or survival of committed cells. In addition, even if genetic redundancy is the case, *Mash1* and *ngn* are expressed in a complementary manner while other bHLH genes such as *NeuroD*...
function at later stages of development (Lee et al., 1995; Miyata et al., 1999; Liu et al., 2000; Schwab et al., 2000) and, therefore, it is not clear which bHLH genes are actually redundant at the neuronal commitment process.

Here, to address the question about the determination activity of vertebrate bHLH genes in the CNS, we generated mice double mutant for two classes of bHLH genes, Mash1 and Math3, another distant a0 homolog belonging to a different subclass from ngn3 (Takebayashi et al., 1997). Unlike other a0 homologs, Math3 is coexpressed with Mash1 in various regions of the developing CNS and therefore it is possible that these two genes could be functionally redundant. Although the previous misexpression study indicated that the Xenopus counterpart of Math3 has a neuronal determination activity (Takebayashi et al., 1997), we found that Math3(-/-) mice exhibited only a partial loss of cerebellar granule neurons due to apoptosis of their committed precursors, suggesting that Math3 is not essential for neuronal commitment. However, in Math3(-/-)-Mash1(-/-) double-homozygous mice, generation of neurons is blocked at the neural precursor stage in the regions where the two genes are coexpressed. Strikingly, the cells that normally differentiate into neurons adopted the glial fate, indicating a fate switch from neurons to glial cells. These loss-of-function data demonstrated that the vertebrate a0-c and a0 homologs are key molecules in neuronal versus glial fate determination.

Results

Spatio-temporal expression patterns of Math3

The spatio-temporal expression patterns of Math3 were determined by in situ hybridization. In the cerebellum, Math3 expression was observed in the external granular layer (EGL) at embryonic day 17.5 (E17.5) (Figure 1A). The EGL contains dividing neuronal precursors, which are derived from the rhombic lip and committed to the granule cell fate. The EGL cells then stop cell division, migrate inwardly through the Purkinje cell layer and differentiate into mature granule cells, which form the internal granular layer (IGL). Those dividing precursors and postmitotic premigratory cells are present in the outer and inner regions of the EGL, respectively. Math3 was expressed mainly in the outer region of the EGL (Figure 1D and F), and this expression pattern is very similar to the a0 homolog Mash1 (Akazawa et al., 1995) (Figure 1E). The Math3 expression region is different from but slightly overlapped with that of another a0 homolog, NeuroD, which is expressed mainly in the inner region (Miyata et al., 1999) (Figure 1F). Math3 expression continued postnatally (Figure 1B and C) but was not detectable in the adult (data not shown). In the forebrain, Math3 expression was restricted to the ventricular zone, which contains neural precursor cells (Figure 1G). Math3 was expressed mainly in the dorsal forebrain but not in the ventral forebrain (Figure 1G). This expression pattern is very similar to ngn3 but different from Mash1 (Guillenot and Joyner, 1993; Gradwohl et al., 1996; Sommer et al., 1996; Ma et al., 1997). Thus, in the forebrain and cerebellum Math3 is coexpressed with other a0 homologs (Mash1 and ngn3) but not with Mash1.

In the midbrain, Math3 was expressed at a high level in the ventricular zone of the anterior two thirds and at a low level in the posterior region at E12.5 (Figure 1H). However, Math3 was expressed at a high level in the posterior part of the midbrain at E15.5 (Figure 1I). In the hindbrain, Math3 was expressed in two longitudinal columns at E10.5 and E12.5 (Figure 1J and K, asterisks). Interestingly, in these midbrain and hindbrain regions Mash1 was also expressed (Figure 1L–O), suggesting that Math3 and Mash1 may cooperatively regulate neural development in these regions.

Generation and characterization of Math3 knockout mice

The Math3 function in neural development was next determined by generating knockout mice. Math3 gene consists of two exons, and the whole protein-coding region is present in the second exon (Tsuda et al., 1998). In ES cells, the majority of the coding region of the Math3 gene was replaced with the neo gene by homologous recombination (Figure 2A and B). Heterozygous mice generated from the mutant ES cells were fertile and looked normal. Homozygous mice, which lost Math3 expression (Figure 2B), were born according to the Mendelian rule and initially the body size was normal (Figure 2C). However, their growth was progressively retarded (Figure 2C and D) and, by 3 weeks after birth, >40% of them died partly because they did not drink milk well. In addition, the body weight of those that survived was still ~40% smaller than the wild type (Figure 2C). Approximately 50% of the homozygous mutants survived beyond several months after birth but most of them died within 1 year.

Strikingly, Math3-deficient mice became ataxic 1 week after birth. Adult mutant mice also exhibited ataxic gait (Figure 2E) and, when put on a rod (0 = 8 mm), they stayed only for 9.8 ± 0.7 s (n = 5), whereas the wild-type mice remained balanced for 33.9 ± 2.8 s (n = 5), indicating that Math3 mutation causes severe motor defects.

At E17.5, the gross morphology of the nervous system of Math3-deficient mice appeared normal. Histological analysis of the homozygous mutants showed that the cerebellar anlage had the EGL and was normal in size at E17.5 (Figure 3A and B). Thus, Math3 may not be involved in the initial specification of granule cell fate in the rhombic lip. However, at postnatal day 7 (P7) and in adults, the mutant cerebellum was smaller in size than the wild type (Figure 3C–F). The lobule formation was poor and some of them were missing (Figure 3F). Both the IGL and the molecular layer, which contains parallel fibers of granule cells, were thinner in Math3-deficient mice than in the wild type (Figure 3K, L, Q and R), suggesting that the granule cell number in the IGL was reduced in Math3-deficient mice. In contrast, the Purkinje cell layer (calbindin*) looked normal (Figure 3S and T). The observed reduction of the granule cell number in the IGL could be due to a decrease in dividing cells and/or an increase in apoptosis. We thus monitored cell proliferation by Ki-67, a nuclear antigen expressed by proliferating cells, and cell death by the TUNEL assay. Whereas a normal number of Ki-67* proliferating cells were present in the EGL (Figure 3M and N), TUNEL* dying cells were
Fig. 1. Spatio-temporal expression pattern of Math3 and other bHLH genes. Distribution of bHLH genes on parasagittal sections was examined by in situ hybridization. In all sections, anterior is to the left and dorsal is up. (A–C) Math3 expression in the developing cerebellum at E17.5 (A), P3 (B) and P5 (C). Math3 is expressed in the EGL. (D) At E17.5, Math3 is expressed at the outer region of the EGL, which contains dividing precursors of cerebellar granule cells. (E) Math1 is also expressed in the outer region of the EGL. (F) NeuroD (brown) is expressed mainly in the inner region of the EGL, which contains postmitotic premigratory cells, whereas Math3 (purple) is expressed mainly in the outer region. (G) At E15.5, Math3 is expressed in the ventricular zone of the dorsal telencephalon but not of the ventral telencephalon. (H) At E12.5, Math3 is expressed at a high level in the ventricular zone of the anterior two thirds of the midbrain. (I) At E15.5, Math3 expression is shifted to the ventricular zone of the posterior midbrain. (J) At E10.5, Math3 is expressed in two longitudinal columns of the hindbrain (asterisks). (K) At E12.5, Math3 expression is observed in two longitudinal columns of the hindbrain (asterisks). (L) At E12.5, Mash1 is expressed in the midbrain. The expression level is higher in the ventricular zone of the anterior two thirds of the midbrain. (M) At E15.5, Mash1 is expressed in the developing midbrain. (N and O) At E10.5 (N) and E12.5 (O), Mash1 is expressed in the ventricular zone of the hindbrain. GE, ganglionic eminence; IV, the fourth ventricle; LV, lateral ventricle; Th, thalamus. Scale bar, 300 μm (A–C and G–O); 30 μm (D–F).

increased in the EGL of Math3(−/−) mutants (Figure 3O and P), suggesting that Math3 is critical for survival but not proliferation of the EGL cells. These cerebellar defects may be a primary cause of ataxia.

It has been shown that cerebellar granule cell development is regulated by two sequentially expressed aro homologs, Math1 and NeuroD (Akazawa et al., 1995; Ben-Arie et al., 1997; Miyata et al., 1999). Math1 is essential for the initial specification of granule cells while NeuroD is required for development of postmitotic cells. In Math3(−/−) cerebellum, both Math1 and NeuroD expression was not affected (Figure 3G–J), indicating that Math3 is required for granule cell development even though Math1 and NeuroD are properly expressed.

Besides the cerebellum, the histology of the postnatal Math3(−/−) nervous system appeared normal (data not shown). Thus, from examination of the mutant mice, Math3 was shown to be important for the survival of differentiating cerebellar neurons, but there was no direct evidence that Math3 is involved in neuronal fate determination. We assumed that these rather weak phenotypes of Math3-deficient mice may be due to compensation by other bHLH genes. Since Math3 is coexpressed with Mash1 in various regions (Figure 1), we next generated Math3(−/−)-Mash1(−/−) double-mutant mice to explore the possible neuronal determination function. By crossing Math3(−/−)-Mash1(+/−) male and Math3(+/−)-Mash1(+/−) female mice, we obtained 15 Math3(−/−)-Mash1(−/−) embryos out of 123 embryos (12.2%) at E15.5. Since this ratio is almost the same as expected (12.5%), most double mutants survived until E15.5. However, we currently failed to obtain double mutants at E17.5, suggesting that they died between E15.5 and E17.5. The double mutants exhibited more severe defects in the
midbrain, hindbrain and retina than Math3(−/−) or Mash1(−/−) single mutants, as shown below, suggesting that the two genes cooperatively regulate neural development.

**Block of neurogenesis and ectopic gliogenesis in Math3(−/−)-Mash1(−/−) midbrain**

Staining in whole mount with antibody to neurofilament (NF) revealed that both cranial and dorsal root ganglia looked normal in Math3(−/−), Mash1(−/−) and Math3(−/−)-Mash1(−/−) embryos at E10.5 (Figure 4A–D). However, compared with the wild-type and Math3(−/−) embryos, neurite extension was slightly reduced in the midbrain and hindbrain of Mash1(−/−) embryos (Figure 4C, arrowhead) and more severely reduced in those of Math3(−/−)-Mash1(−/−) embryos (Figure 4D, arrowheads), suggesting that development of the double-mutant brain was more severely affected. Histological
Fig. 3. The cerebellar defects of Math3-deficient mice. (A–F) The wild-type (A, C and E) and Math3(−/−) (B, D and F) cerebellum at E17.5 (A and B), P7 (C and D) and adult (E and F). The Math3(−/−) cerebellar anlage has the EGL and appears normal at E17.5. However, at P7 and adult the Math3(−/−) cerebellum is smaller and the lobule formation is poor. The posterior region is more severely affected in Math3(−/−) cerebellum. (G and H) In situ hybridization of Math1 in E17.5 wild-type (G) and Math3(−/−) (H) cerebellum. Math1 expression appears normal in Math3(−/−) cerebellum. (I and J) In situ hybridization of NeuroD in E17.5 wild-type (I) and Math3(−/−) (J) cerebellum. NeuroD expression appears normal in Math3(−/−) cerebellum. (K and L) Histology of wild-type (K) and Math3(−/−) (L) cerebellum at P14. The molecular layer (ML) and IGL of Math3(−/−) cerebellum are smaller, suggesting that granule cell number is reduced. The Purkinje cell layer (PCL) appears normal in Math3(−/−) cerebellum. (M and N) Staining with anti-Ki-67 antibody. Cells in the outer region of the EGL are mitotic in both wild-type (M) and Math3(−/−) (N) cerebellum at P14. (O and P) TUNEL assay. Only some cells are TUNEL+ in the wild-type EGL (O) whereas many cells are TUNEL+ in the Math3(−/−) EGL (P), indicating that many Math3(−/−) precursors are dying in the EGL. (Q and R) Histology of wild-type (Q) and Math3(−/−) (R) cerebellum at P30. The ML and IGL of Math3(−/−) cerebellum are still smaller, suggesting that granule cell number is reduced. (S and T) Staining with anti-calbindin antibody of wild-type (S) and Math3(−/−) (T) cerebellum at P30. The Purkinje cell layer appears normal in Math3(−/−) cerebellum. In all sections, anterior is to the left and dorsal is up. Scale bar, 200 μm (A and B); 500 μm (C and D); 800 μm (E and F); 100 μm (G–J); 50 μm (K–T).

analysis indicated that whereas many NF* neurons differentiatied in the midbrain of the wild-type and Math3(−/−) embryos (Figure 4I and J), there were fewer neurons in Mash1(−/−) (Figure 4K) and virtually no neurons in the Math3(−/−)-Mash1(−/−) mutants at E11.5 (Figure 4L), suggesting that neurogenesis is blocked in the double-mutant midbrain.

At E15.5, the tectum region of wild-type, Math3(−/−) and Mash1(−/−) midbrain consisted of two layers, the ventricular zone and the mantle layer (Figure 5A–C). Although the size of the Math3(−/−) and Mash1(−/−) tectum was slightly reduced compared with the wild type (Figure 5A–C), their mantle layer contained massive neurons (MAP2*) (Figure 5E–G). In contrast, the double-mutant tectum was much thinner and neurons were still completely missing (Figure 5D and H). The double-mutant tectum region consisted of only the ventricular zone (Nestin*) (Figure 5P). Surprisingly, the majority of the cells in the double-mutant ventricular zone expressed S100β, an early astrocyte-specific marker (Figure 5L).
whereas the wild-type and Math3(−/−) cells did not (Figure 5I and J). These results indicate that the ventricular cells that normally differentiate into neurons at E15.5 were blocked from neuronal differentiation and instead adopted the glial fate in the absence of Math3 and Mash1. Interestingly, in Mash1(−/−) tectum, subsets of cells also ectopically expressed S100B (Figure 5K), suggesting that gliogenesis is slightly enhanced in the absence of Mash1. A later glial marker, glial fibrillary acidic protein (GFAP), was not expressed in the mutant tectum (data not shown), suggesting that the ectopic glial cells were still immature. The TUNEL assay showed that apoptosis was significantly enhanced in the Math3(−/−)-Mash1(−/−) tectum (Figure 5T) whereas it was normal in Math3(−/−) and slightly enhanced in the Mash1(−/−) tectum (Figure 5Q–S). Since there was no difference in the number of TUNEL+ cells at E11.5 (data not shown), when neurogenesis is already blocked (Figure 4L), the cell death observed at E15.5 in the double mutants is likely to be a secondary effect due to the block of neurogenesis.

Block of neurogenesis and ectopic gliogenesis in the Math3(−/−)-Mash1(−/−) hindbrain

We next examined defects of the hindbrain, which normally expresses both Math3 and Mash1 in two longitudinal columns. In the wild-type, Math3(−/−) and Mash1(−/−) hindbrain, many MAP2+ or NFβ+ neurons differentiated at E11.5 but only a few neurons were generated in the Math3(−/−)-Mash1(−/−) hindbrain (Figure 6Q), suggesting that neurogenesis is blocked. At E15.5, neuronal differentiation was almost completed in the wild-type, Math3(−/−) and Mash1(−/−) hindbrain (Figure 6E–G). However, in the Math3(−/−)-Mash1(−/−) hindbrain, there were still very few neurons in the two longitudinal columns (Figure 6H, asterisks, and R) whereas the other neurons were generated. Strikingly, in the adjacent sections, the regions devoid of neurons contained ectopic glial cells (GFAP+) (Figure 6L and R). The majority (>70%) of the cells expressed GFAP in the double-mutant longitudinal columns, in contrast to the wild type, Math3(−/−) and Mash1(−/−) where only ~10% of the cells expressed GFAP at this stage (Figure 6I–K and R). This defect was likely a fate switch from neurons to glial cells, but it was also possible that neurons died while glial cells proliferated. To distinguish between these possibilities, cell proliferation and apoptosis were examined by Ki-67 staining and TUNEL assay. No difference in Ki-67 and TUNEL staining was detected at E11.5 and E15.5 (Figure 6M–R), indicating that cell proliferation and death are not involved in these defects. These results indicate that the cells that normally differentiate into neurons adopted the glial fate in the double mutants.

Lack of bipolar cells and ectopic generation of glial cells in the Math3(−/−)-Mash1(−/−) retina

To examine further the possible fate switch in the double mutants, we next examined the retina. The retina is an
ideal system to analyze the cell fate because it has only six types of neuron and one type of glial cells, which can be clearly identified by position, cell morphology and specific markers (Cepko et al., 1996). The mature retina consists of three cellular layers, the ganglion cell layer (GCL), the inner nuclear layer (INL), and the outer nuclear layer (ONL). The INL contains three types of interneuron (amacrine, bipolar and horizontal cells) and one type of glia (Müller glial cells). It has been shown that both Math3 and Mash1 are expressed by differentiating bipolar cells (Jasini and Reh, 1996; Roztocil et al., 1997; Takebayashi et al., 1997). Because most retinal cells including glial cells differentiate postnatally, we used the retinal explant culture, which mimics in vivo development well. Retinal explants were prepared from E15.5 embryos and cultured for 2 weeks, during which period the majority of retinal cells finish differentiation. By this method, it was possible to monitor the later stage of cell differentiation well after the mutant hosts died. After 2 weeks of culture, the wild-type and mutant retina consisted of three cellular layers (Figure 7A, E, I and M). Hematoxylin–eosin (HE) staining indicated that the cell number of each layer was normal in Math3(-/-), Mash1(-/-) and Math3(-/-)-Mash1(-/-) retina (Figure 7A, E, I, M and Y). However, whereas...
bipolar cells (PKC⁺, mGlurR6⁺, L7⁺) were normally present in Math3(-/-) (Figure 7F and Z), they were reduced in number in Mash1(-/-) retina (Figure 7J and Z) and completely missing in the Math3(-/-)-Mash1(-/-) retina (Figure 7N, U, V and Z). Instead, Müller glial cells (vimentin⁺, glutamine synthetase⁺) were slightly increased in Mash1(-/-) (Figure 7K and Z) and significantly increased in the double-mutant retina (Figure 7O, W and Z). The other cell types such as rods (rhodopsin⁺) and amacrine cells (HPC-1⁺) were not affected in the double-mutant retina (Figure 7P, X and Y). To determine whether any changes of birth date, which is important for cell type specification, are involved in the defects, the retinal explants were examined at days 5, 7 and 10 of culture. At
day 5, no glial cells were detectable whereas at days 7 and 10 there were differentiating glial cells in both the wild type and the double mutants (data not shown), indicating that the time course of gliogenesis is not affected in the double mutants. In addition, there were already more glial cells in the double mutants at day 7, suggesting that more cells initially adopt the glial fate. In contrast, bipolar cells were not detectable in the double mutants at any time points, excluding the possibility that once borne bipolar cells die in the double mutants. Furthermore, there was no significant difference in the Ki-67 staining and TUNEL assay between the wild type and double mutants (data not shown). These results indicated that cell proliferation and apoptosis are not involved in the loss of bipolar cells and concomitant increase of Müller glial cells, supporting the idea that there is a fate switch from neurons to glial cells in the absence of Math3 and Mash1.

Discussion

**Math3 and Mash1 are neuronal determination genes**

In this study, we examined the neuronal fate determination activities of mammalian as-c and ato homologs. Whereas the previous misexpression studies indicated that vertebrate proneural genes can convert uncommitted cells into the neuronal fate (Ma et al., 1996; Blader et al., 1997; Takebayashi et al., 1997; Lo et al., 1998; Perez et al., 1999), disruption of each gene alone failed to demonstrate that they function at the neuronal versus glial fate determination (Guillemot et al., 1993; Sommer et al., 1995; Hirsch et al., 1998). Or rather, some of these loss-of-function studies indicated that vertebrate proneural genes only regulate the late stage development of committed neuronal cells. This apparent discrepancy between the gain- and loss-of-function studies may be due to genetic redundancy. Here, we showed that Math3 and Mash1 are coexpressed in various regions of the CNS and that mice double mutant for the two bHLH genes exhibit the block of the neuronal versus glial fate choice. These results support the conclusion that the vertebrate as-c and ato homologs function as neuronal determination genes in the CNS.

**Fate switch from neurons to glia**

The double mutants displayed loss of neurons and concomitant gliogenesis in the tectum, hindbrain and retina. Since neuronal apoptosis and glial proliferation cannot account for these defects, it is most likely that the cells that normally differentiate into neurons adopted the glial fate in the absence of Math3 and Mash1, indicating that Math3 and Mash1 not only specify the neuronal fate but also prevent ectopic gliogenesis. These data suggest that, whereas most neural precursor cells initially differentiate into neurons and only later into glial cells, they already have the potential to differentiate into glial cells from the early stages of development. Thus, the apparent sequence of events first and glial cells afterwards during development may not be the change of competence but the result of early expression of the proneural bHLH genes, which direct neurogenesis and inhibit gliogenesis. These data also raise the possibility that at least one mechanism to initiate gliogenesis is downregulation of neuronal determination genes. Recently, we and others have found that misexpression of Notch or its effectors, Hes1 and Hes5, which can functionally antagonize proneural bHLH genes (Kageyama and Nakashibesti, 1997; Ohtsuka et al., 1999), induces gliogenesis at the expense of the neuronal fate (Furukawa et al., 2000; Gaiano et al., 2000; Hojo et al., 2000; Morrison et al., 2000). The mechanism for Notch-induced gliogenesis is not yet understood, but it is likely that Notch signaling suppresses proneural bHLH genes such as Math3 and Mash1, thereby promoting gliogenesis, although it remains to be determined whether this is the only mechanism for Notch-induced gliogenesis.

Is gliogenesis a default pathway after inactivation of proneural bHLH genes? Recent studies indicate that oligodendrocyte development is promoted by two related bHLH genes, Olig1 and Olig2 (Lu et al., 2000; Zhou et al., 2000). These results suggest that, like neurogenesis, gliogenesis in general is positively regulated by bHLH genes. Thus, glial development may not be a simple default pathway but may require glia-specific bHLH genes. It is likely that proneural bHLH genes inhibit gliogenesis by suppressing such gliogenic genes, although the precise mechanism for the possible suppression by proneural bHLH genes remains to be determined.

A similar fate switch was reported in the inner ear of mice mutant for Mathl, a positive regulator of hair cell development (Bermingham et al., 1999; Zheng and Gao, 2000). In the inner ear, common precursors give rise to hair cells (sensory neurons) and support cells. Null mutation of Mathl leads to agenesis of hair cells and an increase in support cells, suggesting a fate switch from hair cells to support cells (Bermingham et al., 1999). Thus, vertebrate as-c and ato homologs specify the neuronal versus non-neuronal cell fate in various regions. Interestingly, the opposite effect is observed in mutations for the Notch ligands, which exhibit extra hair cells at the expense of support cells (Haddon et al., 1998; Lanford et al., 1999). Since the Notch ligand Jagged2 is expressed by hair cells in mice, it is likely that Jagged2 suppresses...
the neighboring cells from adopting the hair cell fate by activation of Notch signaling.

**Redundant functions of mammalian as-c and ato homologs**

We showed that mice double mutant for Math3 and Mash1 exhibited severe defects in the regions that normally express both genes. This redundant function of as-c and ato homologs in various parts of the CNS is interesting since, in *Drosophila*, the two groups of proneural bHLH genes as-c and ato direct generation of different subsets of neurons and thus their redundant functions are not known. These two classes of proneural genes seem to have intrinsically different functions since ato mutation cannot be rescued by scute (Chien et al., 1996). Domain-swapping analysis indicated that the basic region of the proneural proteins encodes the neuronal subtype-specific information (Chien et al., 1996). It has been shown that vertebrate bHLH genes also have subtype-specific activities (Lo et al., 1998; Perez et al., 1999; Fode et al., 2000). Although our experiments suggest redundant functions of Math3 and Mash1, they do not formally prove whether Math3 and Mash1 compensate each other in the same precursor cells or whether Math3-dependent precursors substitute Mash1-dependent precursors and vice versa in single mutants. Since no significant change of cell proliferation or death was observed in the hindbrain and retina of single mutants, we prefer the idea that Math3 and Mash1 function in the same precursors and that, only when neither is expressed do precursor cells choose the glial fate. The subtype specificity of the as-c homolog Mash1 and the ato homolog Math3 may not be so strict and the two bHLH genes could be functionally interchangeable to some extent.

**Cooperative functions of bHLH and homeobox genes**

Retinal explant assays demonstrated that Math3 and Mash1 are essential for bipolar cell development. It has been shown that the homeobox gene Chx10 is also essential for bipolar cell development: Chx10-null mice lack bipolar cells (Burmeister et al., 1996). Thus, at least three genes, Math3, Mash1 and Chx10, are involved in bipolar cell development although it is not clear at which stage Chx10 functions. Interestingly, in contrast to Math3(−/−)-Mash1(−/−) double-mutant retina, Müller glial cells are not increased and therefore a fate switch does not occur in Chx10-null mice, suggesting that the cells that normally differentiate into bipolar cells are likely to result in apoptosis in the absence of Chx10 rather than differentiate into glial cells. These results strongly suggest that the bHLH genes and Chx10 have distinct functions in bipolar cell development. Chx10 may not only confer the cell type-specific identities, as suggested by other homeobox genes (Tanabe and Jessell, 1996), but also regulate cell survival. In contrast, the main function of Math3 and Mash1 is determination of the neuronal fate but not cell survival. It remains to be analyzed whether the two bHLH genes can also directly confer some of the bipolar cell-specific identities. Further characterization of bHLH and homeobox genes will be necessary to decipher the roles of each factor in specification of particular neuronal subtypes.

**Materials and methods**

**Generation of Math3 mutant mice**

TT2 ES cells (1 × 10⁶) (Gibco) were electroporated with 30 μg of the linearized targeting vector in 0.8 ml of phosphate-buffered saline (PBS) and selected with 250 μg/ml G418 (Gibco). ES cell lines with Math3 mutation were identified by Southern blot analysis using 5' and 3' probes shown in Figure 2. Chimeric mice were generated by aggregating the Math3-mutant ES cells with CD1 morula and then by implanting them into the uteri of pseudopregnant foster mothers. The resulting chimeric males were bred with CD1 females. Homozygous mice generated from two independent mutated ES cell lines showed identical phenotypes.

**In situ hybridization**

In situ hybridization on frozen sections was performed, as described previously (Akazawa et al., 1992). The digoxigenin- or FITC-labeled probes used in this study were as follows: Math3 (Takebayashi et al., 1997), Mash1 (Guillenot and Joyner, 1993), Math1 (Akazawa et al., 1995), NeuroD (Lee et al., 1995), L7 (Oberdick et al., 1990) and mGluR6 (Nakajima et al., 1993).

**Whole-mount immunohistochemistry**

Whole-mount immunohistochemistry was performed as described previously (Mark et al., 1993). In brief, E10.5 embryos were fixed in 4% paraformaldehyde in PBS at 4°C for 3 h and were bleached with 0.1% H₂O₂ overnight at 4°C. Then the embryos were incubated with anti-NF antibody (2H3, Hybridoma Bank) for 3 days at 4°C and next with peroxidase-conjugated secondary antibody (Chemicon) overnight at 4°C. The peroxidase deposits were visualized by 4-chloro-1-naphthol.

**Histological analysis**

For examination of adult brain, mice were deeply anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). The brain was dissected rapidly, incubated in 25% sucrose in PB overnight at 4°C and frozen sections were made.

For immunohistochemistry, sections were fixed with 4% paraformaldehyde in PBS for 10 min, preincubated in PBS containing 5% normal goat serum, 1% bovine serum albumin, 0.1% Triton X-100 and 0.02% sodium azide for 30 min and then incubated with the following antibodies: monoclonal antibody against Ki-67 (Pharmingen), calbindin-D (Sigma), 160 kDa NF (Amersham), MAP2 (Sigma), PKC (Amersham), Nestin (Pharmingen), glutamate synthetase (Chemicon), HPC-1 (Sigma), S100β (Sigma) and vimentin (Histofine), and rabbit polyclonal antibody against GFAP (DAKO) and rhodopsin (LSL). As a secondary antibody, biotinylated goat antibodies against mouse and rabbit IgG (Vector) were used. The antibody complex was visualized by avidin-labeled fluorescein or ABC kit (Vector). TUNEL assay was performed as described previously (Tomita et al., 1996a).

**Retinal explant culture**

The explant of neural retina was placed onto a Millicell chamber filter (Millipore) with the ganglion cell layer upward. The chamber was transferred to a six-well culture plate. Explants were cultured with 50% MEM with HEPES, 25% Hanks' solution, 25% heat-inactivated horse serum, 200 μM L-glutamine and 6.75 mg/ml glucose at 34°C in 5% CO₂ (Tomita et al., 1996a).

**Acknowledgements**

We thank Drs S.Takada, D.Watanabe and J.Kitano for technical advice and Dr H.Ohkubo for NeuroD cDNA. Monoclonal antibody 2H3 was obtained from the Developmental Studies Hybridoma Bank. This work was supported by Grant-in-Aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, Sports, and Culture of Japan and Japan Society for the Promotion of Science.

**References**


Akazawa, C., Ishibashi, M., Shimizu, C., Nakaniishi, S. and Kageyama, R.


*Received July 17, 2000; revised August 15, 2000; accepted August 18, 2000*