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Conversion of ectoderm into a neural fate by *ATH-3*, a vertebrate basic helix–loop–helix gene homologous to *Drosophila* proneural gene *atonal*

Koichi Takebayashi, Shuji Takahashi[1], Chika Yokota[1], Hiroshi Tsuda, Shigetada Nakanishi, Makoto Asashima[1] and Ryoichiro Kageyama[2]

Department of Biological Sciences, Kyoto University Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606 and [1]Department of Biology, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153, Japan

[2]Corresponding author

K.Takebayashi, S.Takahashi and C.Yokota contributed equally to this work

We have isolated a novel basic helix–loop–helix (bHLH) gene homologous to the *Drosophila* proneural gene *atonal*, termed *ATH-3*, from *Xenopus* and mouse. *ATH-3* is expressed in the developing nervous system, with high levels of expression in the brain, retina and cranial ganglions. Injection of *ATH-3* RNA into *Xenopus* embryos dramatically expands the neural tube and induces ectopic neural tissues in the epidermis but inhibits non-neural development. This *ATH-3*-induced neural hyperplasia does not require cell division, indicating that surrounding cells which are normally non-neural types adopt a neural fate. In a *Xenopus* animal cap assay, *ATH-3* is able to convert ectodermal cells into neurons expressing anterior markers without inducing mesoderm. Interestingly, a single amino acid change from Ser to Asp in the basic region, which mimics phosphorylation of Ser, severely impairs the anterior marker-inducing ability without affecting general neurogenic activities. These results provide evidence that *ATH-3* can directly convert non-neural or undetermined cells into a neural fate, and suggest that the Ser residue in the basic region may be critical for the regulation of ATH-3 activity by phosphorylation.

Keywords: *atonal*/helix–loop–helix factor/mouse neurogenesis/*Xenopus*

Introduction

In *Drosophila*, neural development is controlled positively or negatively by multiple basic helix–loop–helix (bHLH) genes (Campos-Ortega and Jan, 1991; Jan and Jan, 1993; Jarman *et al.*, 1993, 1994). For example, *achaete–scute* complex (AS-C) and *atonal* are proneural genes, and the former is required for external sensory organ development while the latter is required for photoreceptor and chordotonal organ development. In contrast, *hairy* and *Enhancer of split* [*E(spl)*] inhibit neural development by antagonizing the proneural genes.

As in *Drosophila*, vertebrate neurogenesis is also controlled positively or negatively by multiple bHLH genes (Guillemot *et al.*, 1993; Ferreiro *et al.*, 1994; Ishibashi *et al.*, 1994, 1995; Turner and Weintraub, 1994; Kageyama *et al.*, 1995; Lee *et al.*, 1995; Tomita *et al.*, 1996). *XASH-3*, a *Xenopus* bHLH gene homologous to the *Drosophila* proneural gene complex AS-C (Zimmerman *et al.*, 1993), converts ectodermal cells to a neural fate and therefore acts as a proneural gene (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). *Mash-1*, a mammalian bHLH gene homologous to AS-C, regulates early stages of neuronal differentiation, and *NeuroD*, another vertebrate bHLH gene, functions in terminal differentiation of neurons (Johnson *et al.*, 1990; Guillemot *et al.*, 1993; Lee *et al.*, 1995). Thus, multiple bHLH genes positively regulate vertebrate neurogenesis at different stages. In contrast, *HES-1*, a mammalian bHLH gene homologous to *Drosophila hairy* and *E(spl)*, is expressed in neural precursor cells and acts as a negative regulator of neurogenesis (Akazawa *et al.*, 1992; Sasai *et al.*, 1992). Forced expression of *HES-1* blocks neuronal differentiation in the brain (Ishibashi *et al.*, 1994) and retina (Tomita *et al.*, 1996). Conversely, a *HES-1*-null mutation accelerates neuronal differentiation and results in severe anomalies of the brain (Ishibashi *et al.*, 1995) and eye (Tomita *et al.*, 1996). Thus, *HES-1* prevents premature neurogenesis and regulates brain and eye morphogenesis.

The exact mechanism by which *HES-1* prevents premature neurogenesis is unclear, but it is likely that *HES-1* antagonizes bHLH genes that positively regulate neurogenesis, as in the case of *Drosophila*. Interestingly, expression of *Mash-1* is up-regulated in *HES-1*-null mice (Ishibashi *et al.*, 1995), suggesting that *Mash-1* up-regulation may contribute to premature neurogenesis. However, null mutation of *Mash-1* does not cause any apparent abnormalities in the central nervous system (CNS) (Guillemot *et al.*, 1993) and, based upon the expression patterns, *XASH-3* or its mammalian equivalent and *NeuroD* are unlikely to compensate for *Mash-1*. Therefore, additional bHLH genes may be required for the CNS development.

We have isolated from *Xenopus* and mouse a novel bHLH gene, termed *ATH-3*, which is homologous to the *Drosophila* proneural gene *atonal* (Jarman *et al.*, 1993, 1994). In *Xenopus*, *ATH-3* expression starts in the presumptive CNS during neural induction and persists at a high level in the brain and retina. In addition, *ATH-3* is able to promote neural development at the expense of adjacent non-neural tissues. In the animal cap assay, *ATH-3* can directly convert ectodermal cells into neurons expressing anterior markers. Interestingly, a Ser→Asp mutation in the basic region, which mimics phosphorylation of Ser, impairs anterior marker-inducing abilities without losing the general neurogenic activity. These results indicate that *ATH-3* can act as a vertebrate proneural

Fig. 1. Primary structure of *Xenopus* and mouse *ATH-3*. (**A**) Nucleotide and deduced amino acid sequences of the *Xenopus ATH-3* cDNA. The putative bHLH domain and the in-frame stop codons in the 5′-non-coding region are underlined. (**B**) Nucleotide and deduced amino acid sequences of the mouse *ATH-3* cDNA. The putative bHLH domain and the in-frame stop codon in the 5′-non-coding region are underlined.

gene and that ATH-3 activity may be regulated by phosphorylation of Ser in the basic region.

Results

Structural analysis of Xenopus and mouse ATH-3

PCR with degenerate oligonucleotide primers was carried out to search for a novel bHLH gene expressed in the developing nervous system. We obtained a PCR fragment from a bHLH gene, which was termed *ATH-3* because of structural similarity to the *Drosophila* proneural gene *atonal*. This PCR fragment was used to screen a mouse genomic library to obtain the mouse *ATH-3* gene (Isaka *et al.*, 1996). By using this *ATH-3* genomic clone as a probe, *Xenopus* and mouse cDNA libraries were screened to determine the full-length coding sequences.

Xenopus and mouse ATH-3 consisted of 315 (Figure 1A) and 330 amino acid residues (Figure 1B), respectively, and shared 93% identity in the bHLH domain (Figure 1, underlined, and Figure 2B). The structural similarity extended to the upstream and downstream regions of the bHLH domain, with an overall identity of 67% (Figure 2A). In addition, ATH-3 showed significant sequence homology to MATH-1 (Akazawa *et al.*, 1995), MATH-2/NEX-1 (Bartholomä and Nave, 1994; Shimizu *et al.*, 1995), NeuroD/BETA2 (Lee *et al.*, 1995; Naya *et al.*, 1995), NDRF/KW8 (Kume *et al.*, 1996; Yasunami *et al.*, 1996) and Atonal (Jarman *et al.*, 1993) in the bHLH domain (Figure 2B), suggesting that these factors share a

A

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Mouse      MAKMYMKSKDMVELVNTQSWMDKGLSSQNEMKEQERRPGSYGMLGTLTEE    50
                         | |*||   ||* *|||| ||||    *   *  | * ||
Xenopus    ----------MSEMVNVHGWMEEALSSQDEMKERNQSAYDIIS-GLCHEE    39

Mouse      HDSI--EEDEEEEEDGDKPKRRGPKKKKMTKARLERFRARRVKANARERTRM 100
           * ||  |||*|||||||*|||*|||||||||||||*||||*||||||||||*||
Xenopus    RGSIDGEEDDEEEEDGEKPKKRGPKKKKMTKARVERFRVRRVKANARERSRM  91

Mouse      HGLNDALDNLRRVMPCYSKTQKLSKIETLRLARNYIWALSEVLETGQTLE   150
           |||||||*|||||||||||||||||||||||||||||||**||*||**|
Xenopus    HGLNDALENLRRVMPCYSKTQKLSKIETLRLARNYIWALSDILEQGQNAE   141

Mouse      GKGFVEMLCKGLSQPTSNLVAGCLQLGPQSTLLEKHEEKSSICDSTISVH   200
           ||||*|*|||||||||||||||||||  *|*|||||| ||||****|
Xenopus    GKGFLEILCKGLSQPTSNLVAGCLQLGPQAMFLDKHEEKSHICDSSLTGH   191

Mouse      SFNYQSPGLPSPPYGHMETHSLHLKPQPFKSLGD-SFGSHPPDCSTPPYEG  250
           * ||||||||||||| ** | |||||* || **| |***| * |*||||||
Xenopus    TYNYQSPGLPSPPYGNIDVHHLHLKPSSFKPVMDPSVVTHTLNCTTPPYEG  242

Mouse      PLTPPLSISGNFSLKQDGSPDLEKSYNFMPHYTSASLSSGHVHSTPFQTG   300
           *|||||||  ||||||||  |||**||| |   |  * |   |*| * | |
Xenopus    ALTPPLSIGGNFSLKQDSSPDMDKSYAFRSPYPALGLGGSHGHGSHFHTS   292

Mouse      TPRYDVPVDLSYDSYSHHSIGTQLNTIFSD                       330
            |||**|*|* |* | ||         ||**
Xenopus    VPRYELPIDMAYEPYPHHA-------IFTE                       315
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B

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           <  BASIC   ><    HELIX-1   ><    LOOP    ><     HELIX-2     > Identity
MATH-3     RRVKANARERTRMHGLNDALDNLRRVMPCY--SK---TQKLSKIETLRLARNYIWALSEVL   100%
XATH-3     RRVKANARERSRMHGLNDALENLRRVMPCY--SK---TQKLSKIETLRLARNYIWALSDIL    93%

MATH-1     RRLAANARERRRMHGLNHAFDQLRNVIPSF--NN---DKKLSKYETLQMAQIYINALSELL    63%
MATH-2     RRQEANARERNRMHGLNDALDNLRKVVPCY--SK---TQKLSKIETLRLAKNYIWALSEIL    88%
NeuroD     RRMKANARERNRMHGLNAALDNLRKVVPCY--SK---TQKLSKIETLRLAKNYIWALSEIL    88%
NDRF       RRQKANARERNRMHDLNAALDNLRKVVPCY--SK---TQKLSKIETLRLAKNYIWALSEIL    86%
Atonal     RRLAANARERRRMQNLNQAFDRLRQYLPCL--GN---DRQLSKHETLQMAQTYISALGDLL    54%

Mash-1     RR---NERERNRVKLVNLGFATLREHVPNG-AAN----KKMSKVETLRSAVEYIRALQQLL    44%
Mash-2     RR---NERERNRVKLVNLGFQALRQHVPHG-GAN----KKLSKVETLRSAVEYIRALQRLL    45%
Xash-3     RR---NERERNRVKLVNMGFAKLRQHVPQAQGPN----KKMSKVETLRSAVEYIRALQSLL    44%

HES-1      RKSSKPIMEKRRRARINESLSQLKTLILDALKKDSSRHSKLEKADILEMTVKHLRNLQRAQ    19%
E47        RRMANNARERVRVRDINEAFRELGRMCQMHL--K--SDKAQTKLLILQQAVQVILGLEQQV    32%
MyoD       RRKAATMRERRRLSKVNEAFETLKRCTSSNP--N----QRLPKVEILRNAIRYIEGLQALL    39%
Id-1       PALLDEQQVNVLLYDMNGCYSRLKELVPTLP-----QNRKVSKVEILQHVIDYIRDLQLEL    21%
N-myc      RRRNHNILERQRRNDLRSSFLTLRDHVPELV--K---NEKAAKVVILKKATEYVHSLQAEE    30%
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Fig. 2. Structural comparison of *Xenopus* and mouse ATH-3 and other HLH factors. (**A**) Amino acid sequence comparison of mouse and *Xenopus* ATH-3. Identical residues between the two are indicated by a bar (|), and conservative changes are shown by an asterisk. The putative bHLH domain is underlined. (**B**) Sequence comparison of mouse ATH-3 (MATH-3), *Xenopus* ATH-3 (XATH-3) and other HLH factors. The positions of the basic region, helices 1 and 2 and the loop are indicated above. The conserved residues in comparison with ATH-3 are shown in bold. The identity (%) to MATH-3 is indicated on the right. Sources for sequences are as follows: mouse MATH-1 (Akazawa *et al.*, 1995); mouse MATH-2 (Bartholomä and Nave, 1994; Shimizu *et al.*, 1995); mouse NeuroD (Lee *et al.*, 1995); mouse NDRF (Yasunami *et al.*, 1996); *Drosophila* Atonal (Jarman *et al.*, 1993); rat Mash-1 (Johnson *et al.*, 1990); rat Mash-2 (Johnson *et al.*, 1990); *Xenopus* Xash-3 (Zimmerman *et al.*, 1993); rat HES-1 (Sasai *et al.*, 1992); human E47 (Murre *et al.*, 1989); mouse MyoD (Davis *et al.*, 1987); mouse Id-1 (Benezra *et al.*, 1990); and human N-myc (Kohl *et al.*, 1986).

recent common ancestral gene. However, unlike the latter factors, ATH-3 contained serine (amino acid residue 89 in *Xenopus*) or threonine (98 in mouse) in the basic region, which forms a potential phosphorylation site. It has been shown that myogenic bHLH factors contain a threonine residue in the basic region and that its phosphorylation inactivates the myogenic activity (Li *et al.*, 1992). Thus, the activity of ATH-3 could also be regulated by phosphorylation and dephosphorylation of the basic region. Another structural feature common to *Xenopus* and mouse

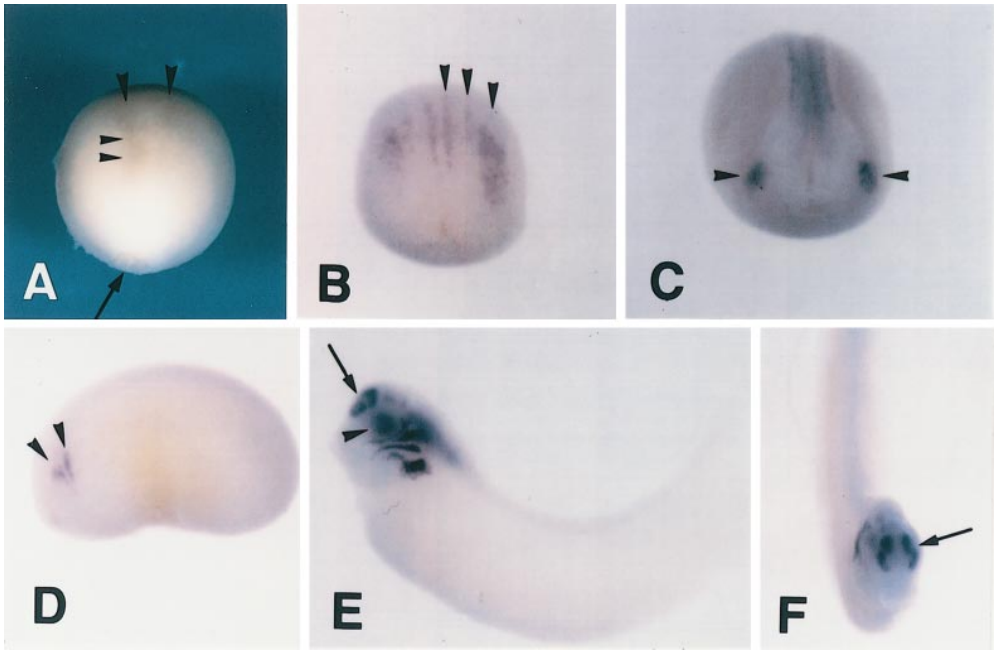

Fig. 3. *In situ* hybridization of *Xenopus ATH-3*. (**A**) Posterio-dorsal view of a stage 12 embryo. *ATH-3* is weakly expressed in the presumptive neural plate (arrowheads). The blastopore is shown by an arrow. (**B**) Posterior view of a stage 14 embryo. *ATH-3* is expressed in three stripes on either side of the midline of the neural plate (arrowheads). (**C**) Anterior view of a stage 18 embryo. Strong *ATH-3* expression occurs in the cranial ganglions (arrowheads). Weaker *ATH-3* expression remains in the neural plate. (**D**) Lateral view of a stage 21 embryo. Anterior is toward the left. *ATH-3* is expressed in the cranial ganglions (arrowheads). (**E**) Lateral view of a stage 33 tadpole. Anterior is toward the left. *ATH-3* is expressed in the forebrain (arrow), eye (arrowhead) and cranial ganglions. (**F**) Anterior view of a stage 33 tadpole. A high level of the *ATH-3* transcript is present in the forebrain (arrow). Dorsal is towards the top in all figures.

ATH-3 is the acidic region (Glu–Asp stretch; amino acid residues 38–56 of *Xenopus* and 49–65 of mouse) and the basic region (Lys–Arg stretch; amino acid residues 57–72 of *Xenopus* and 66–81 of mouse), which are located upstream of the bHLH domain. These acidic and basic regions are also present in MATH-2, NeuroD and NDRF, and could be involved in transcriptional activity.

Spatial and temporal distribution of ATH-3

The spatiotemporal expression patterns of *ATH-3* were determined by *in situ* hybridization. In *Xenopus*, *ATH-3* expression was first detected weakly at stage 12 in two stripes within the presumptive neural plate (Figure 3A, arrowheads). At stage 14, *ATH-3* was expressed in three stripes on both sides of the midline of the neural plate (Figure 3B, arrowheads). Assessed by their positions, cells in these stripes may correspond to primary neuronal precursors, which differentiate into motor and sensory neurons and interneurons. At stages 18 and 21, strong *ATH-3* expression appeared in the cranial ganglions (Figure 3C and D, arrowheads) while weaker expression remained in the spinal cord. Later, *ATH-3* was expressed strongly in the eye, forebrain and cranial ganglions, where active neuronal differentiation occurs (Figure 3E and F). Thus, *ATH-3* initially may be expressed in neural progenitor cells and later in differentiating post-mitotic neurons of the anterior nervous system in *Xenopus* embryos.

In mice, weak expression of *ATH-3* initially was detected widely in the neural tube around embryonic day (E) 8.5, before the process of turning (Figure 4A). By E9.5, *ATH-3* expression had become prominent in the ventral part of the brain and spinal cord (Figure 4B) and initiated weakly in the retina (data not shown). At E10.5, *ATH-3* was expressed at high levels in the trigeminal (Figure 4C, arrow) and dorsal root ganglions (Figure 4D) and the ventral part of the midbrain and hindbrain (data not shown). At E12.5, *ATH-3* expression was detected in the mantle layer of the brain and spinal cord, trigeminal ganglion, Rathke's pouch, dorsal root ganglion and the ventricular zone of the neural retina (Figure 4F and H). At E16.5, the expression persisted at a high level in the diencephalon (Figure 4J) and the neural retina (Figure 4L) but decreased to quite low levels in other regions. Thus, *ATH-3* expression initially occurred widely in the nervous system but then became restricted to the anterior region in mouse embryos, indicating that *Xenopus* and mice show somewhat similar patterns of spatiotemporal expression.

Northern blot analysis demonstrated that a high level of *ATH-3* expression continued after birth to adulthood in mouse retina (Figure 5A). *In situ* hybridization analysis showed that *ATH-3* was expressed in the outer half of the ventricular zone at postnatal day (P) 0 (Figure 5B). During P3–P5, the inner (INL) and outer nuclear layers (ONL) develop in the retina and, after this stage, *ATH-3* expression continued in the INL (Figure 5B), indicating that mature retinal neurons also expressed *ATH-3*. In adults, *ATH-3* was not expressed in any other tissues that we examined (Figure 5A and data not shown). Thus, *ATH-3* expression persisted in the neural retina from E9.5 onwards, suggesting that *ATH-3* may be involved not only in the development but also in the maintenance of retinal cells.

Neural hyperplasia by injection of ATH-3 RNA

To assess the function of *ATH-3* in neural development, *in vitro* generated *Xenopus ATH-3* RNA was injected into *Xenopus* embryos. When 50 or 100 pg of *ATH-3* RNA

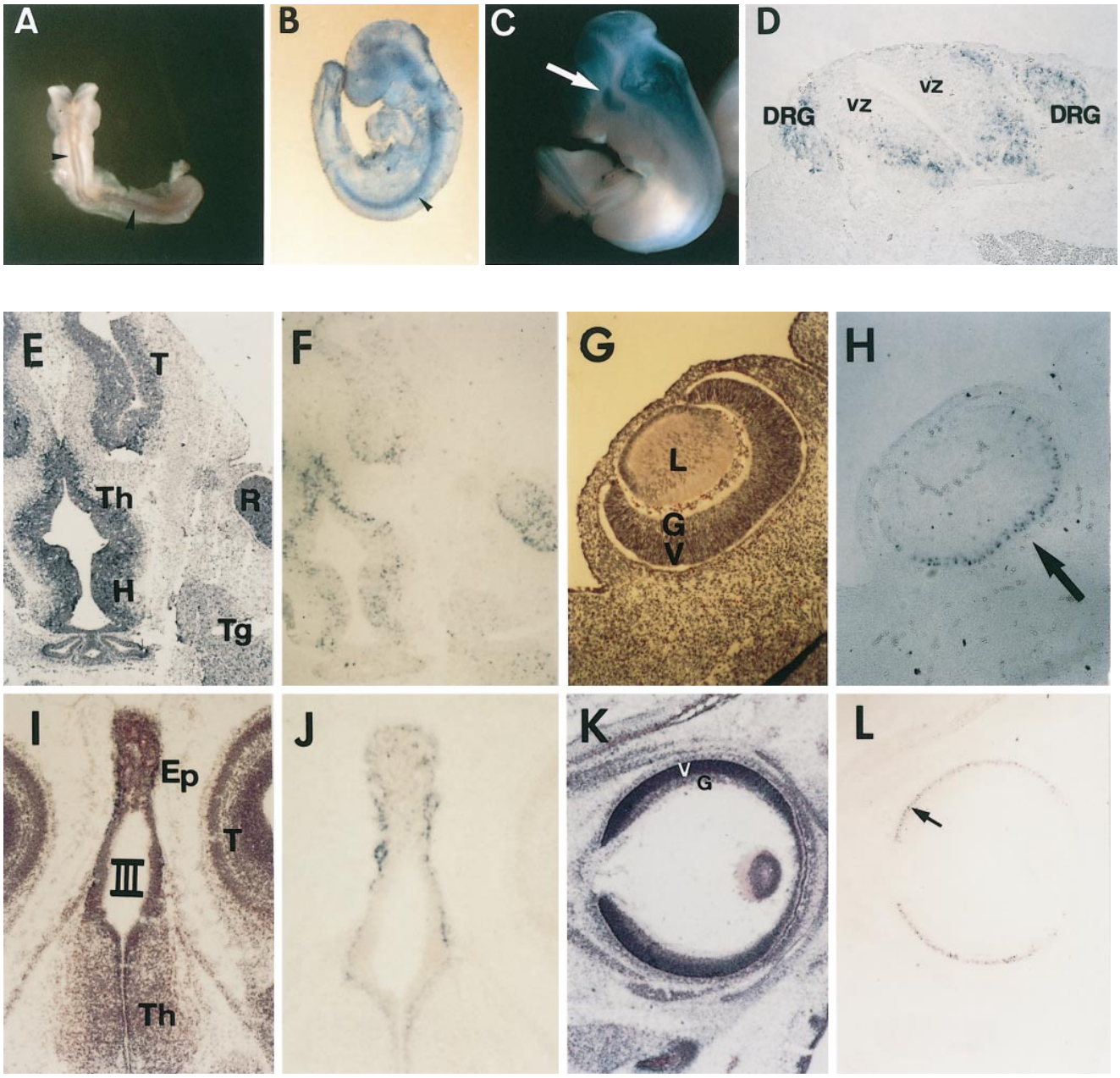

Fig. 4. *In situ* hybridization of *ATH-3* in mouse embryos. (**A**) Dorsal view of an E8.5 embryo. *ATH-3* is weakly expressed in the neural tube (arrowheads). (**B**) Lateral view of an E9.5 embryo. *ATH-3* is expressed in the ventral region of the CNS (arrowhead). (**C**) Dorso-lateral view of an E10.5 embryo. *ATH-3* is expressed in the trigeminal (arrow) and dorsal root ganglions and the CNS. (**D**) Frontal section of an E10.5 embryo. *ATH-3* is expressed in the mantle layer of the spinal cord and in the dorsal root ganglion (DRG). (**E**) HE staining of the frontal section of E12.5 brain. (**F**) The same section as (E). *ATH-3* is expressed in the mantle layer of the CNS and in the trigeminal ganglion. (**G**) HE staining of E12.5 eye. (**H**) The same section as (G). *ATH-3* is expressed in the outer half of the ventricular zone (arrow). (**I**) HE staining of the frontal section of E16.5 brain. (**J**) The same section as (I). *ATH-3* expression remains in the diencephalon including the epithalamus and thalamus but not in the telencephalon. (**K**) HE staining of E16.5 eye. (**L**) The same section as (K). *ATH-3* is expressed in the outer half of the ventricular zone (arrow). Ep, epithalamus; G, ganglion cell layer; H, hypothalamus; III, third ventricle; L, lens; R, retina; T, telencephalon; Tg, trigeminal ganglion; Th, thalamus; V, ventricular zone.

was injected into one cell of two-cell stage *Xenopus* embryos, the most notable and frequent phenotype was expansion of the CNS on the injected side (Table I). Co-injection of β-galactosidase RNA allowed us to confirm the injected side by X-gal staining (data not shown).

Immunological analyses with the pan-neural marker NEU-1 (Itoh and Kubota, 1989) showed that the neural tube was significantly enlarged laterally at stages 25 and 30 (Figure 6A and B, arrowheads). The optic cup was deformed and displaced on the injected side (Figure 6B, arrow). This *ATH-3* phenotype of neural tube enlargement is different from that of *NeuroD*, which does not cause neural tube hyperplasia (Lee *et al.*, 1995), but seems quite similar to that of *XASH-3*, which expands the neural tube (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). In addition to the expanded neural tube, ectopic NEU-1 or neural cell adhesion molecule (N-CAM) staining was detected in the epidermis on the injected side (Figure 6B, G and H). Ectopic neurogenesis was also induced in the epidermis when *ATH-3* RNA was injected into a ventral

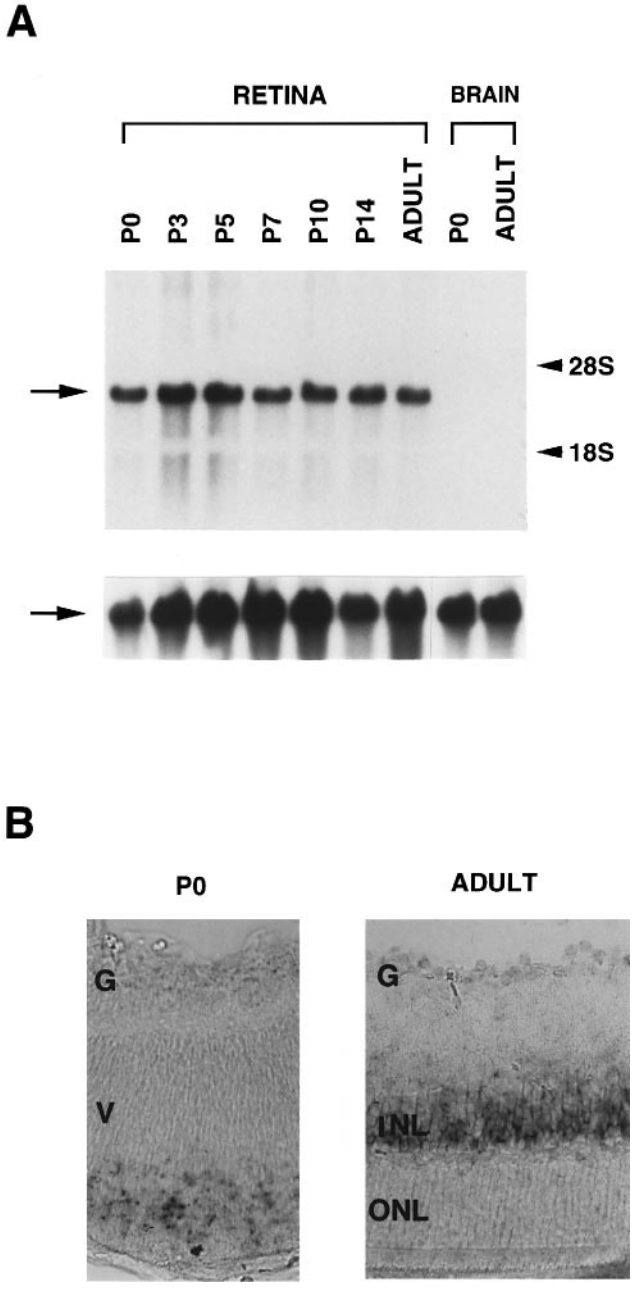

Fig. 5. Expression of *ATH-3* in postnatal mice. (**A**) Northern blot analysis. Retina and brain RNAs from mice at various stages were electrophoresed, as indicated above each lane. *ATH-3* RNA (top arrow) is present in the postnatal and adult retina but not in the postnatal brain. EF1α (bottom arrow) is used as a control. (**B**) *In situ* hybridization of P0 and adult retina. At P0, *ATH-3* is expressed in the outer half of the ventricular zone (V). In adult, *ATH-3* is expressed mainly in the inner nuclear layer. G, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

cell of four-cell stage embryos (Figure 6E). Ectopic NEU-1-positive cells in the epidermis showed the morphology of neurons with multiple processes (Figure 6E). This *ATH-3* phenotype is quite similar to that of *NeuroD*, which ectopically converts neural crest and epidermal cells into neurons (Lee *et al.*, 1995). These results suggest that *ATH-3* cannot only expand the neural tube like *XASH-3* but can also induce ectopic neurogenesis in the epidermis like *NeuroD*.

At the tadpole stage, the brain was significantly enlarged

Table I. Phenotypes of *ATH-3* RNA-injected *Xenopus* embryos[a]

	Control injection[b]	*Xenopus ATH-3*	
		50 pg	100 pg
Tested embryos	21	36	30
No effects	21 (100%)	1 (3%)	0 (0%)
Expansion of forebrain[c]	0 (0%)	34 (94%)	25 (83%)
Anterior anomalies[d]	0 (0%)	1 (3%)	5 (17%)

[a]*ATH-3* RNA was injected into one cell of two-cell stage *Xenopus* embryos, and the phenotypes on the injected side were examined. The numbers and ratios (%) of tested embryos are indicated.
[b]Globin RNA (100 pg) (Krieg and Melton, 1984) was injected.
[c]The forebrain was expanded laterally towards the eye region, and the eye became deformed and displaced. No apparent abnormalities were detected in other regions.
[d]The anterior neural tube was expanded and the adjacent tissues in addition to eyes were severely deformed.

(Figure 6I, arrowhead), and the eye was deformed and displaced ventrally on the injected side. Histological analyses revealed that the forebrain dramatically expanded laterally and anteriorly on the injected side (Figure 6J, arrowheads). As a result, the development of the surrounding regions was disturbed. The hindbrain was also expanded by overexpression of *ATH-3* (Figure 6K, arrowheads). Because the injected *ATH-3* RNA was likely to have disappeared by this time, the above results indicate that neural hyperplasia remained permanently by transient expression of *ATH-3*.

The observed neural hyperplasia could be due to either cell proliferation or conversion of surrounding non-neural cells to a neural fate. To distinguish between these possibilities, cell division of *ATH-3* RNA-injected embryos was blocked by hydroxyurea/aphidicolin (HUA) treatment at mid-gastrulation. It has been shown that embryos treated with HUA at mid-gastrulation stop cell division but develop almost normally until tailbud stages (Harris and Hartenstein, 1991; Turner and Weintraub, 1994). As shown in Figure 6C, *ATH-3* overexpression expanded the CNS and induced ectopic neurogenesis in the epidermis on the injected side even in the presence of HUA. Thus, cell proliferation is not necessary for neural hyperplasia induced by *ATH-3*, suggesting that overexpression of *ATH-3* converts adjacent non-neural cells to a neural fate.

Suppression of non-neural tissue development by ATH-3

If the surrounding non-neural cells are converted into a neural fate, development of non-neural tissues should be reduced in *ATH-3* RNA-injected embryos. To test this possibility, we next determined expression of non-neural markers in the injected embryos. On the *ATH-3* RNA-injected side, expression of twist, a marker for non-neural types of neural crest cells (Hopwood *et al.*, 1989; Turner and Weintraub, 1994), was severely reduced (Figure 7B and C), suggesting that ectopic *ATH-3* expression decreased the population of neural crest cells with the potential to differentiate into non-neural cells. These cells may instead become ectopic neurons, which were observed in the epidermis of *ATH-3* RNA-injected embryos (see Figure 6E, G and H).

In the injected embryos, expression of keratin, an

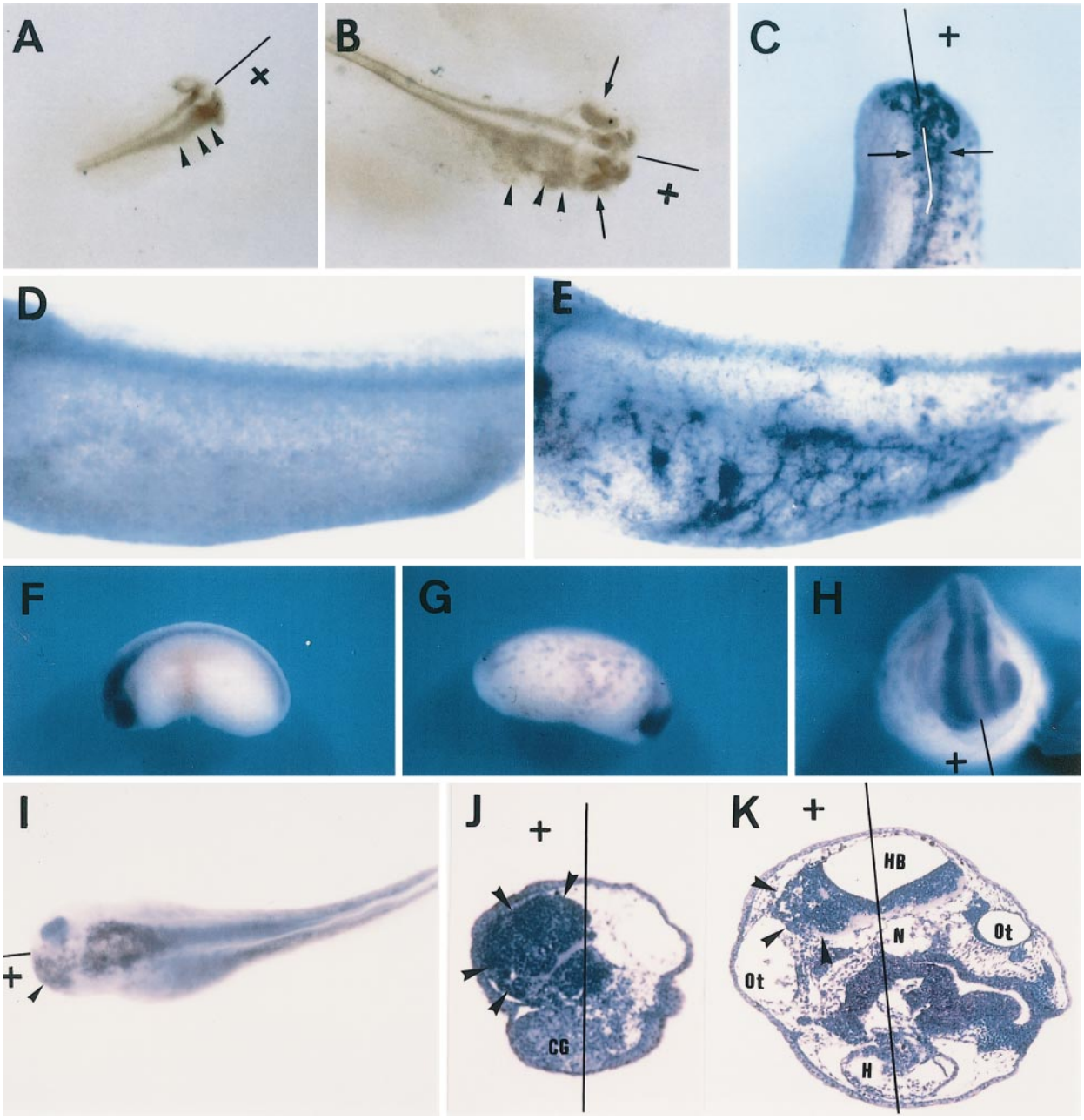

Fig. 6. Hyperplasia of the neural tube and ectopic neural tissues by injection of *ATH-3* RNA. The *in vitro* synthesized *Xenopus ATH-3* RNA was injected into one cell of two-cell stage embryos (A–C and F–K) or a ventral animal cell of four-cell stage embryos (D and E). Injected embryos were examined at stage 25 (A, C and F–H), stage 30 (B) and the tadpole stage (D, E and I–K). (A–E) Whole-mount immunological staining with NEU-1 monoclonal antibody. Some embryos were cleared to enhance the signals (A and B). (**A**) Dorsal view. NEU-1-positive CNS (brown) is expanded laterally in the anterior region (arrowheads) on the injected side (+). (**B**) Dorsal view. The anterior CNS is expanded significantly (brown, arrowheads) on the injected side (+). The optic cup (arrow) is deformed and displaced on the injected side (+). (**C**) Dorsal view. Expanded CNS and ectopic neurogenesis in the epidermis (blue staining) are observed on the injected side of an embryo treated with HUA. (**D**) Lateral view of the uninjected side. No apparent NEU-1 staining is detected. (**E**) Lateral view of the injected side. Ectopic NEU-1 staining (blue) exhibits morphology of neurons with processes. (F–H) Whole-mount *in situ* hybridization of N-CAM. (**F**) Lateral view of the uninjected side. (**G**) Lateral view of the injected side. Ectopic N-CAM staining is observed in the epidermis. (**H**) Anterior view. The forebrain is expanded and ectopic neurogenesis occurs in the epidermis on the injected side (+). (**I**) Dorsal view. The forebrain (arrowhead) is expanded on the injected side (+). (**J**) Frontal section anterior to the eye. The forebrain dramatically expands anteriorly (arrowheads) on the injected side (+). (**K**) Frontal section at the level of hindbrain. The hindbrain is expanded laterally (arrowheads) on the injected side (+). CG, cement gland; H, heart; HB, hindbrain; N, notochord; Ot, otic vesicle.

epidermal marker (Jonas *et al.*, 1985), was also significantly reduced (Figure 7E and F). In addition, somite formation was also severely blocked, as revealed by impaired myosin expression (Figure 7H, arrows). These results support the hypothesis that *ATH-3* expands the neural tissues at the expense of adjacent non-neural cells.

Activation of neural gene expression in animal caps by ATH-3

To assess more clearly the *ATH-3* function in neurogenesis, additional markers were examined in the injected embryos. The *ATH-3* RNA was injected into both cells of two-cell stage *Xenopus* embryos, and subsequently the animal caps

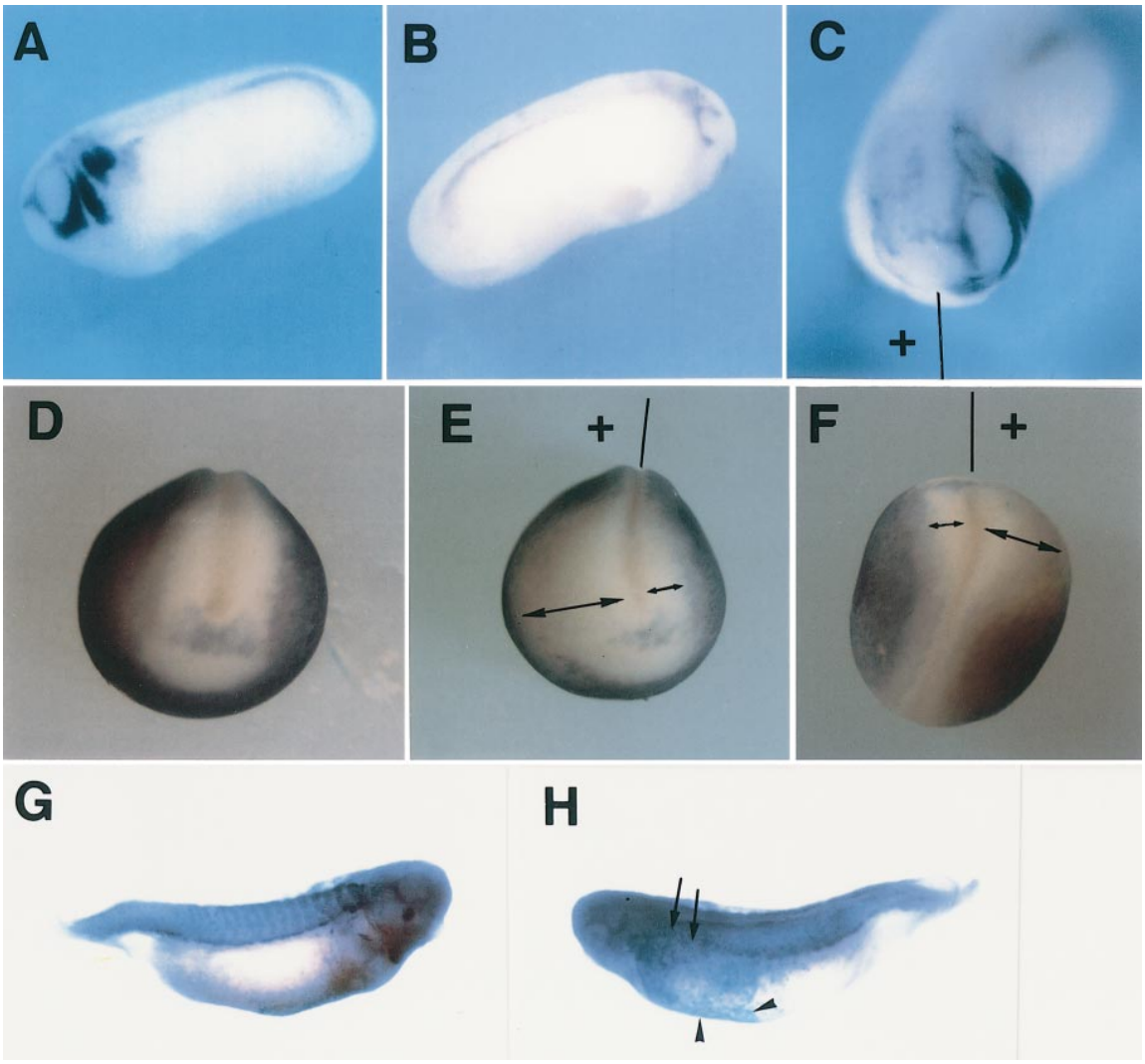

Fig. 7. Suppression of non-neural tissue development by injection of *ATH-3* RNA. The *in vitro* synthesized *Xenopus ATH-3* RNA was injected into one cell of two-cell stage embryos and the phenotypes were examined at stages 19 (D–F) and 25 (A–C) and the tadpole stage (G and H). (A–C) *In situ* hybridization of twist. (**A**) Lateral view of the uninjected side. Anterior is toward the left. (**B**) Lateral view of the injected side. Twist expression is severely reduced. Anterior is toward the right. (**C**) Anterior view. On the injected side (+), twist expression is reduced or almost absent. (D–F) *In situ* hybridization of keratin. (**D**) Anterior view of an uninjected embryo. Keratin is expressed outside the neural tube. (E and F) Anterior (**E**) and dorsal (**F**) views. Keratin expression is reduced and the unstained forebrain is expanded on the injected side. In (F), anterior is toward the top. (G and H) Whole-mount immunological staining with anti-myosin monoclonal antibody. (**G**) Lateral view of the uninjected side. Somites are visible. (**H**) Lateral view of the injected side. Formation of some somites is blocked (arrows). The injected side is confirmed by blue staining with X-gal solution (arrowheads).

were isolated from the injected embryos at stage 9. These explants were cultured for an additional 3 h (equivalent to stage 11), 1 day (stages 25–30) or 3 days (stages 35–40). Uninjected animal caps are known to become atypical epidermis and, therefore, neural and mesodermal markers were not expressed (Figure 8, lane 2). In contrast, injection of *ATH-3* RNA induced significant expression of the pan-neural marker N-CAM (Kintner and Melton, 1987) and the neuronal markers type-II β-tubulin (Good *et al.*, 1989) and neurofilament-M (NF-M) (Sharpe, 1988) in stage 25–30 animal caps (Figure 8, lane 3), indicating that *ATH-3* can induce neuronal differentiation. In addition, expression of the anterior neural markers XANF-1 (Zaraisky *et al.*, 1992) and XIF-3 (Sharpe *et al.*, 1989) and the retinal marker opsin (Saha and Grainger, 1993) was also significantly induced in *ATH-3* RNA-injected animal caps (Figure 8, lane 3). However, other markers such as the posterior neural marker XlHbox6 (Wright *et al.*, 1990) and the floor-plate marker F-spondin (Ruiz i Altaba *et al.*, 1993) were not induced by *ATH-3* (Figure 8, lane 3). Thus, *ATH-3* can promote development of neurons with anterior features, suggesting that *ATH-3* may have a role in specification of anterior neuronal types. This neural induction by *ATH-3* was also observed when the culture was continued for 3 days (equivalent to stages 35–40) (data not shown), indicating that the activation of neural gene expression by *ATH-3* is stable. This is in sharp contrast to the action of *XASH-3*, which only transiently induces neural gene expression in animal caps (Ferreiro *et al.*, 1994).

In *ATH-3* RNA-injected animal caps, expression of mesodermal markers was not detected; *Xbra* (Smith *et al.*, 1991) and *goosecoid* (Blumberg *et al.*, 1991) were not expressed in the animal cap explants that had been cultured

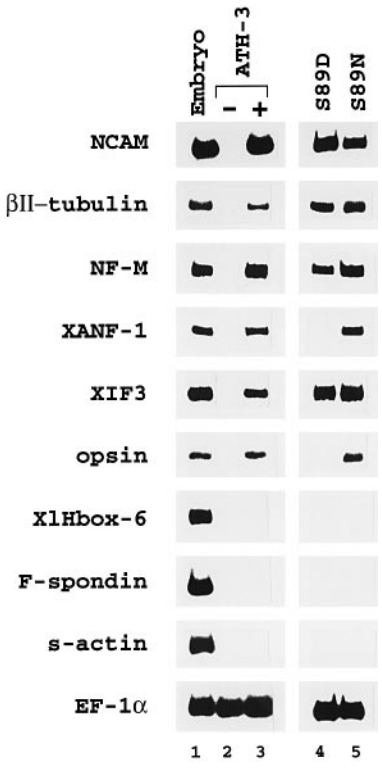

Fig. 8. *Xenopus* animal cap assay. The *in vitro* synthesized wild-type *Xenopus ATH-3*, S89D or S89N RNA was injected into both cells of two-cell stage *Xenopus* embryos, and subsequently the animal caps were isolated from the injected or uninjected embryos at stage 9. These explants were cultured for an additional day (equivalent to stages 25–30), and then RNA was extracted and subjected to RT–PCR analysis, as indicated on the left. Lane 1, control RT–PCR experiment using RNA from whole embryos; lane 2, uninjected animal caps; lane 3, *ATH-3* RNA-injected animal caps; lane 4, S89D RNA-injected animal caps; lane 5, S89N RNA-injected animal caps. We performed at least two independent experiments and obtained the same results.

for 3 h (data not shown). In addition, S-actin (Stutz and Spohr, 1986) was not expressed in the injected animal caps that had been cultured for 1 day (equivalent to stages 25–30) (Figure 8 lane 3). Thus, *ATH-3* promoted neuronal differentiation without inducing mesoderm, suggesting that *ATH-3* may directly convert ectodermal cells into neurons.

Modification of ATH-3 activities by a single amino acid change in the basic region

Among the vertebrate neural bHLH factors that have been characterized, ATH-3 has a unique structural feature; a serine or threonine residue in the basic region (Figure 2B), which forms a potential phosphorylation site. As an initial step in relating the possible phosphorylation to the neurogenic activity of ATH-3, a Ser89→Asp mutation was introduced into *Xenopus* ATH-3 (S89D), which mimics the phosphorylation of Ser. Another mutation was also introduced to change Ser89 to Asn (S89N), which could represent a non-phosphorylated form. On the animal cap analysis, S89D was able to induce N-CAM, type II β-tubulin and NF-M expression (Figure 8, lane 4), indicating that S89D keeps the general neurogenic activities. However, it failed to induce the anterior neural markers XANF-1 and opsin (Figure 8, lane 4). In contrast, S89N induced anterior neural gene expression as well as general neural markers, like wild type *ATH-3* (Figure 8, lane 5). These results indicate that Ser89 is critical for the regulation of ATH-3 activity by phosphorylation and that modification of a single amino acid residue in the basic region can regulate some of the neurogenic activities of a bHLH factor.

Discussion

ATH-3 may function in both determination and differentiation steps of Xenopus neural development

In this study, we showed that overexpression of *ATH-3* can induce ectopic neurons and hyperplasia of the CNS but suppress development of non-neural tissues in *Xenopus* embryos. Interestingly, *ATH-3*-induced neural hyperplasia does not require cell division, indicating that the adjacent non-neural lineage cells adopt a neural fate. In addition, *ATH-3* can induce neurons without inducing mesoderm in the animal cap assay. These results provide evidence that *ATH-3* can directly convert non-neural or undetermined cells into neurons.

It has been proposed that there are at least two separate developmental choices for generation of neurons in vertebrates; first, whether or not to adopt a neural lineage, and second, whether or not to differentiate as a neuron (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994; Lee *et al.*, 1995). The first step involves the initial decision between neural and epidermal fates in the ectoderm by a proneural gene, while the second step is the subsequent process of neuronal differentiation. It has been shown that the *Xenopus* bHLH gene *XASH-3* can expand the neural tube at the expense of adjacent non-neural ectoderm and therefore acts as a proneural gene (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). We showed that *ATH-3* can induce similar effects in *Xenopus* embryos, i.e. expansion of the neural tube and disturbance of development of the surrounding non-neural tissues. These results suggest that, like *XASH-3*, *ATH-3* can function as a proneural gene in *Xenopus* embryos. The early onset of *ATH-3* expression before the neural plate appears is also consistent with the notion that *ATH-3* is a proneural gene. Interestingly, whereas in animal cap assays *XASH-3* can only transiently induce neural gene expressions and requires the neural inducer noggin for stable induction (Ferreiro *et al.*, 1994), *ATH-3* can stably induce neural gene expression without noggin. Thus, ATH-3 seems to have a stronger neurogenic activity than XASH-3, or ATH-3 may be less susceptible to inhibitory signals such as Notch and HES-1.

Another bHLH gene, *NeuroD*, regulates the terminal differentiation step of neural development. It has been shown that *NeuroD* can induce ectopic neural tissues in the epidermis of *Xenopus* embryos. *ATH-3* also induces ectopic neural tissues in the epidermis, like *NeuroD*, suggesting that *ATH-3* and *NeuroD* have similar neurogenic functions. Furthermore, *ATH-3* is expressed at a high level in the nervous system during neuronal differentiation stages, suggesting that *ATH-3* can also act as a *NeuroD*-like differentiation gene. Thus, *ATH-3* may function in both proneural and neuronal differentiation stages in *Xenopus* embryos.

In the case of mice, the *ATH-3* function remains to be determined. Because *ATH-3* expression starts around E8.5 in the neural tube, after the neural lineage is mainly

determined, *ATH-3* may function primarily in neuronal differentiation stages.

ATH-3 activity may be regulated by phosphorylation of Ser (or Thr) in the basic region

ATH-3 has a serine or threonine residue in the basic region, which could serve as a phosphorylation site. Other neural bHLH factors contain asparagine or arginine in the corresponding position, thus indicating that the amino acid residue in this position is not conserved among the neural bHLH factors (Figure 2B). Interestingly, the myogenic bHLH factors contain a threonine residue in the basic region, and it has been shown that its phosphorylation inactivates the myogenic activities (Li *et al.*, 1992). Change from Thr to Asp, which mimics the phosphorylation in the basic region of myogenin, causes loss of DNA binding and myogenic activities (Brennan *et al.*, 1991). In the case of *Xenopus* ATH-3, mutation of Ser89 to Asp (S89D) maintains a general neurogenic activity but severely impairs anterior marker-inducing abilities in the *Xenopus* animal cap assay. In contrast, S89N, which could represent a non-phosphorylated form, can induce both general and anterior neural markers. These results thus point to the importance of the possible phosphorylation of Ser/Thr in the basic region of ATH-3, which can modify the anterior-specific neurogenic activities, although it remains to be determined whether or not the basic region of ATH-3 is phosphorylated *in vivo*.

The mechanism by which S89D, which loses anterior neurogenic activity, can induce general neural markers is quite interesting, and we can offer two possibilities. One is that S89D could lose the DNA binding activity and titrate negative regulators by forming a non-DNA binding heterodimer complex, thereby activating gene expression. The other possibility is that the DNA binding specificity of S89D could be changed by addition of a negative charge in the basic region; it could bind to the promoter of general neural genes but not of anterior genes. Determination of ATH-3 binding sequences will be necessary to address these problems.

ATH-3 in retinal development

It is striking that *ATH-3*, expressed at a high level in the retina, can induce *opsin* expression in the animal cap assay, raising the possibility that *ATH-3* may be involved in retinal specification. However, induction of anterior neural markers by *ATH-3* could reflect a general property of *Xenopus* ectoderm, since neural inducers like noggin, chordin and follistatin also induce a similar spectrum of anterior markers (Lamb *et al.*, 1993; Hemmati-Brivanlou and Melton, 1994; Sasai *et al.*, 1995). Nevertheless, we speculate that the opsin-inducing activity may be an intrinsic property of ATH-3, because S89D loses the opsin-inducing ability but maintains general neurogenic activities in the animal cap assay.

Eye morphogenesis is known to be regulated by the eye master control gene *Pax-6* (Halder *et al.*, 1995), and it is possible, therefore, that *ATH-3* expression is regulated by *Pax-6*. However, *ATH-3* was similarly expressed in the optic vesicles of wild-type and *Small eye* mutant mice, which have a *Pax-6* mutation (Hill *et al.*, 1991; Walther and Gruss, 1991), indicating that *ATH-3* expression does not depend upon *Pax-6* (our unpublished data). Thus, *ATH-3* and *Pax-6* may constitute different genetic pathways for retinal development. Because only a part of the *Pax-6* expression domains becomes retina, it has been suggested that another factor not regulated by *Pax-6* may be required for retinal specification (Macdonald and Wilson, 1996). Thus, our findings that *ATH-3* can induce opsin expression suggest that *ATH-3* may have an independent function in the process of retinal specification within *Pax-6*-expressing regions.

Materials and methods

Isolation of the mouse ATH-3 gene and cDNA

Oligo(dT)-primed cDNA was prepared from E9.5 mouse CNS and subjected to PCR with fully degenerate oligonucleotide primers corresponding to the amino acid sequences NARER and TLQMA of Atonal, as previously described (Akazawa *et al.*, 1995; Shimizu *et al.*, 1995). We subcloned and sequenced the amplified fragments, and obtained the *ATH-3* sequence. The PCR clone of *ATH-3* was labelled with [α-^{32}P]dCTP by a random primer method and used as a probe for screening a mouse genomic library (Stratagene) (Takebayashi *et al.*, 1994). Sequence analysis indicated that an ~7.5 kb *Bgl*II fragment of the *ATH-3* gene contained the open reading frame (Isaka *et al.*, 1996). Subsequently, a 685 bp *Hin*cII–*Pst*I fragment from the mouse *ATH-3* gene which contained most of the coding region was used as a probe for screening the P0–P10 mouse retina cDNA library. Approximately one million plaques were screened, and 200 positive clones were obtained. Thirty clones were sequenced, and all contained the *ATH-3* sequence.

Isolation of Xenopus ATH-3 cDNA

The 685 bp *Hin*cII–*Pst*I fragment of the mouse *ATH-3* gene was used as a probe for screening the *Xenopus* stage 30 embryo cDNA library (Stratagene). Four positive clones were isolated and sequenced, and all contained *ATH-3* cDNA.

In situ hybridization and Northern blot analysis of ATH-3

In situ hybridization experiments were performed, essentially as described previously (Harland, 1991; Hatada *et al.*, 1995; Takebayashi *et al.*, 1995). Digoxigenin-labelled antisense RNAs corresponding to the 658 bp *Pvu*II–*Hin*dIII mouse *ATH-3* genomic fragment and the 634 bp *Xba*I–*Eco*RI *Xenopus ATH-3* cDNA fragment were synthesized *in vitro*. These probes were hybridized to whole mouse embryos, 10 mm cryostat sections or *Xenopus* albino embryos.

For Northern blot analysis, 15 μg of total RNA was electrophoresed on a formamide/1.2% agarose gel and transferred to a nylon membrane. The filter was hybridized at 42°C with the ^{32}P-labelled *Hin*cII–*Eco*RI fragment of mouse *ATH-3* cDNA and washed, as previously described (Shimizu *et al.*, 1995).

Analysis of RNA-injected Xenopus embryos

The full-length coding region of the *Xenopus ATH-3* cDNA was subcloned into pSP64T vector, and capped *ATH-3* RNA was produced *in vitro* as described before (Krieg and Melton, 1984). The *ATH-3* and β-galactosidase RNAs were injected into one cell of two-cell or four-cell stage *Xenopus* embryos. At various stages, the injected embryos were subjected to histological study with haematoxylin-eosin (HE) staining, immunological study with NEU-1 monoclonal antibody (Itoh and Kubota, 1989) and anti-myosin monoclonal antibody MF 20 (Bader *et al.*, 1982) or *in situ* hybridization of N-CAM, twist and keratin. The probe regions for N-CAM, twist and keratin are nucleotide residues 1466–2020 (Accession nos. M76710), 155–652 (M27730) and 615–1171 (M11940), respectively. To identify the injected side, X-gal staining was performed.

For HUA treatment, injected embryos were transferred to 20 mM hydroxyurea–0.15 mM aphidicolin at stage 10.5, as previously described (Harris and Hartenstein, 1991).

Xenopus animal cap assay

Mutations were introduced into *ATH-3* cDNA by hybridizing oligonucleotides 5′-AAGTCCATGCATTCTATCACGCTCCCTGGCATTG GC-3′ and 5′-AAGTCCATGCATTCTGTTACGCTCCCTGGCATTG-GC-3′ with heat-denatured *ATH-3* cDNA for S89D and S89N, respectively. Each mutant was cloned into pSP64T and sequenced.

In vitro synthesized *ATH-3*, S89D or S89N RNA was injected into

both cells of two-cell stage *Xenopus* embryos (1 ng/embryo). Animal caps that had been either injected or uninjected with RNA were explanted at the late blastula stage (stage 9) and cultured in Steinberg's solution for 3 h, 1 day or 3 days. Total RNA was extracted from animal caps by using the acidic guanidine thiocyanate method. Reverse transcription–PCR (RT–PCR) analysis for XANF-1 (Zaraisky *et al.*, 1992), EF-1α (Krieg *et al.*, 1989), N-CAM (Kintner and Melton, 1987), XlHbox6 (Wright *et al.*, 1990), NF-M, F-spondin (Ruiz i Altaba *et al.*, 1993), XIF3 (Sharpe *et al.*, 1989) and S-actin (Stutz and Spohr, 1986) was done, as previously described (Hemmati-Brivanlou and Melton, 1994; Hatada *et al.*, 1995; Sasai *et al.*, 1995). In addition, we performed RT–PCR by using the following primers; opsin (Saha and Grainger, 1993), 5′-TTCGGATGGTCCAGATACATCC-3′ and 5′-GGTGGTAAGAGATTCCTGTTGC-3′ (36 cycles); and type II β-tubulin (Good *et al.*, 1989), 5′-ATTAACAAGTCGTGGCAGCC-3′ and 5′-TCTGGACATTGCATCTACC-3′ (36 cycles).

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Note added in proof

Nucleotide sequences of *Xenopus* (*XATH-3*) and mouse *ATH-3* (*MATH-3*) have been deposited in DDBJ/EMBL/GenBank databases with accession numbers D85188 and D85845.