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A homeobox gene, *pnx*, is involved in the formation of posterior neurons in zebrafish

Young-Ki Bae[1,2], Takashi Shimizu[1,2], Taijiro Yabe[2], Cheol-Hee Kim[4], Tsutomu Hirata[2], Hideaki Nojima[2], Osamu Muraoka[1], Toshio Hirano[2,3] and Masahiko Hibi[1,2,*]

[1]Laboratory for Vertebrate Axis Formation, Center for Developmental Biology, RIKEN, Kobe, Hyogo 650-0047, Japan
[2]Department of Molecular Oncology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan
[3]Department of Frontier Biosciences, Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka 565-0871, Japan
[4]Department of Biology, Chungnam National University, Daejeon 305-764, Korea
*Author for correspondence (e-mail: hibi@cdb.riken.go.jp)

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SUMMARY

A homeobox gene, *pnx*, is expressed in prospective posterior neurogenic regions and later in primary neurons. *pnx* expression was regulated by a signal from the non-axial mesendoderm and by Notch signaling. Pnx contains an Eh1 repressor domain, which interacted with Groucho and acted as a transcriptional repressor. Misexpression of *pnx* increased neural precursor cells and postmitotic neurons, which express *neurogenin1* and *elavl3/HuC*, respectively. Expression of an antimorphic Pnx (VP16Pnx) or inhibition of Pnx by antisense morpholino oligonucleotide led to the reduction in the number of a subset of primary neurons. Misexpression of *pnx* promoted neurogenesis independent of Notch signaling. Epistatic analyses showed that Pnx also functions downstream of the Notch signal. These data indicate that *pnx* is a novel repressor-type homeobox gene that regulates posterior neurogenesis.

Key words: Homeobox, Proneural gene, Neurogenic gene, Zebrafish

INTRODUCTION

Neuronal tissues are generated through a series of steps in vertebrates. These steps include neural induction, anteroposterior (AP) patterning and neurogenesis. In amphibian and teleost embryos, neuroectoderm is initially induced by bone morphogenetic protein (BMP) inhibitors, such as Noggin, Chordin, Follistatin and Cerberus, which are generated from the dorsal organizer (the Spemann-Mangold organizer). Induced neuroectoderm is characteristically anterior and subjected to posteriorization during gastrulation (Nieuwkoop's two-signal model) (reviewed by Gamse and Sive, 2000; Sasai and De Robertis, 1997). In zebrafish, transplantation of non-axial mesendoderm into the forebrain region at the early gastrula stage transforms the forebrain progenitors to hindbrain-like structures (Koshida et al., 1998; Woo and Fraser, 1997), indicating that posterior neuroectoderm is specified by posteriorizing signals emanating from the non-axial mesendoderm. Fate-map studies in zebrafish have revealed that progenitors of the hindbrain and spinal cord are located near the prospective mesendoderm that occupies the lateral marginal blastoderm at the early gastrula stages and that these progenitors move to the posterior dorsal midline through convergent-extension movement (Woo and Fraser, 1995). These reports show that the progenitors of the posterior neuroectoderm are initially located close to the blastoderm margin and receive posteriorizing signals from the marginal blastoderm. Gain- and loss-of-function studies suggest that fibroblast growth factors, Wnts, retinoic acid and Nodal-related molecules function as posteriorizing signals (Blumberg et al., 1997; Cox and Hemmati-Brivanlou, 1995; Doniach, 1995; Durston et al., 1989; Erter et al., 1998; Kengaku and Okamoto, 1995; Kudoh et al., 2002; Lamb and Harland, 1995; McGrew et al., 1995; Papalopulu et al., 1991; Piccolo et al., 1999; Sharpe, 1991; Thisse et al., 2000). Transplantation analysis has shown that the commitment of these progenitors to become hindbrain (and likely spinal cord) takes place at the late gastrula stage (Woo and Fraser, 1998).

After neural induction and patterning, neurogenic regions, the domains in which neurogenesis takes place, are established at the gastrula or neurula stages in amphibia and teleosts. The neurogenic regions are prefigured by the expression of proneural genes, which function to promote the formation of neurons. Many of these genes are homologues of the *Drosophila achaete-scute* and *atonal* genes and encode basic helix-loop-helix (bHLH) proteins. In zebrafish as well as in *Xenopus*, the proneural gene *neurogenin1* (*ngn1*; *X-ngnr-1* in *Xenopus*) is expressed in three longitudinal stripes on each side, in which primary motor-, inter- and Rohon Beard (sensory-) neurons arise, at the late-gastrula to early-segmentation (neurula) stage (Blader et al., 1997; Kim et al., 1997; Ma et al., 1996). Proneural genes, such as *neurogenin*, further induce downstream bHLH genes, such as *neurod*, to elicit the transition from proliferative neural precursor cells to postmitotic neurons, which express neuron-specific markers, N-tubulin, and *elav*-related genes (including *elavl3/HuC*)

(Blader et al., 1997; Kim et al., 1997; Lee et al., 1995; Ma et al., 1996). In addition to neural specification and determination, proneural genes also trigger the process of lateral inhibition. Cells that highly express proneural genes become neurons and simultaneously express the Notch ligands Delta or Serrate, which activate Notch signaling in neighboring cells (Chitnis and Kintner, 1996; Ma et al., 1996). As a consequence of the Notch signal activation, these neighboring cells express repressors of neuronal differentiation that belong to the enhancer of split-hairy (Hes/Her) family transcription factors and cease differentiating to neurons, as is also proposed for *Drosophila* neurogenesis (Chitnis et al., 1995; Wettstein et al., 1997). In zebrafish, the *delta* genes (*deltaA*, *deltaB* and *deltaD*) are expressed in neurogenic regions and later in primary neurons from the late gastrula stage (Appel and Eisen, 1998; Haddon et al., 1998b). The Hes/Her family genes *her4* and *her9* are expressed in the neural plate, and *her4* has been shown to be regulated by the Notch signal and to inhibit neurogenesis in zebrafish (Leve et al., 2001; Takke et al., 1999). Mutations in components of the Notch pathway in zebrafish, including *deltaA* and *notch1* (*notch1a* – Zebrafish Information Network) (*deadly seven*), lead to an increase in the numbers of *ngn1*-expressing neural cells and an expansion of the primary neurons within the three stripes (Appel et al., 2001; Gray et al., 2001). Furthermore, primary neurons are produced in very excessive numbers in the mutant embryos of *mind bomb* (*mib*; previously known as *white tail*), as observed in embryos expressing dominant-negative Delta and Su(H) protein, which also functions downstream of Notch (Appel and Eisen, 1998; Haddon et al., 1998a; Jiang et al., 1996; Schier et al., 1996). It has recently been reported that *mib* encodes a RING ubiquitin ligase that is required for efficient activation of the Delta-mediated Notch signaling (Itoh et al., 2003). These reports show that proneural genes activate a lateral inhibition program in which Notch signaling is involved, which restricts the numbers of neurons in zebrafish.

There are several genes that are reported to function downstream of neural inducers and upstream of proneural genes in *Xenopus* and zebrafish. These include the *Xenopus* Sox-related genes *SoxD* and *Sox2* (Kishi et al., 2000; Mizuseki et al., 1998b), *Zic*-related genes *Zic-r1* and *Zic3* (Mizuseki et al., 1998a; Nakata et al., 1997), and Iroquois genes (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998), all of which can be regulated by the organizer-derived BMP inhibitors. However, these genes are expressed homogenously in subdivisions or the entire region of neuroectoderm, but not in the 'neurogenic regions'. None of them has been shown to be involved in the formation of posterior neurons.

Using an expression cloning strategy, we isolated a zebrafish homeobox gene, *pnx*, which is expressed in posterior neurogenic regions earlier than *neurogenin1* and *delta* genes. Expression of *pnx* is initially regulated by the neural inducers and a posteriorizing signal from the non-axial mesendoderm; latterly, *pnx* expression in primary neurons is regulated by the Notch signaling. Gain- and loss-of-function studies of Pnx indicated that it is a transcriptional repressor that functions upstream of proneural genes to define posterior neurogenic regions. We found that Pnx functions downstream of the Notch signal. These data indicate that *pnx* is a novel repressor-type homeobox gene that functions in neurogenesis.

MATERIALS AND METHODS

Fish embryos

The wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses of fish with the Oregon AB or AB/India genetic background. *chordino*, *swirl* and *mind bomb* mutant embryos were obtained by crossing heterozygous *chordino*^{tt250}, *swr*^{ta72a} and *mib*^{m178} fish, respectively. The obtained embryos were grown at 28.5°C in 1/3 Ringer's solution (39 mM NaCl, 0.97 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM HEPES at pH 7.2), and the developmental stage of the embryos was determined by the time after fertilization (hpf, hours post-fertilization) at 28.5°C and by morphological criteria (Kimmel et al., 1995).

cDNA library construction and screening

Procedures for the cDNA library construction and screening were previously published (Yamanaka et al., 1998). Briefly, a cDNA library was constructed from LiCl-treated (dorsalized) early gastrula embryos and inserted into a modified version of pCS2+ (Turner and Weintraub, 1994) (pCS2+SfiI). The bacteria transformants containing 200-300 cDNA clones each were pooled and 5′-capped RNAs were synthesized in vitro from each pool. One to two nanograms of 5′-capped RNA was injected into one- or two-cell stage embryos and the effects of the RNA injection were evaluated by morphological inspection and in situ hybridization of the early segmentation-stage embryos with the markers, *six3* (a marker for forebrain), *engrailed3* (for the mid-hindbrain boundary), *krox20* (for rhombomeres 3 and 5) and *deltaB* (for primary neurons). Zebrafish *sax1* cDNA was isolated from a zebrafish early gastrula cDNA library by low stringency hybridization using the *pnx* cDNA as a probe. The nucleotide sequences for *pnx* and *sax1* were deposited in the GenBank database under Accession Numbers AB067731 and AB067732, respectively.

Transplantation

Transplantation of the ventrolateral marginal tissues of the zebrafish early gastrula was performed as described (Koshida et al., 1998; Woo and Fraser, 1997). The yolk of one- or two-cell stage embryos was injected with 0.5% lysine-fixable tetramethylrhodamine-dextran (2,000 kDa, Molecular Probes). When the embryonic shield became apparent (shield stage, 6.5 hpf), the ventrolateral marginal blastomeres were excised with a tungsten needle and then transplanted through a glass micropipette into the animal pole of a sibling shield-stage embryo.

Plasmid construction and RNA and morpholino injections

To construct the expression vector for EnR-fusion proteins (pCS2+EnR), the repressor domain of *Drosophila* Engrailed (amino acid residues 1-226) was amplified from pTB-En (Fan and Sokol, 1997) by PCR and inserted into the *Bam*HI and *Eco*RI sites of pCS2+. To construct expression vectors for Pnx-ΔN, VP16-Pnx and EnR-Pnx, the cDNA fragment containing the amino acid residues 35-182 of Pnx was amplified from pCS2+*Sfi*I-Pnx by PCR and subcloned into pCS2+, pCS2+NLSVP16AD (containing amino acid residues 412-490 of Herpes simplex virus protein I VP16) and pCS2+EnR.

To construct a plasmid for N-Pnx, the cDNA fragment of amino acid residues 1-34 of Pnx was amplified by PCR. To construct the expression plasmids for GAL4DB (the DNA-binding domain of the yeast transcription factor Gal4)-fusion proteins, the cDNA fragment of Pnx, Pnx-ΔN, N-Pnx, EnR or VP16 was subcloned into pSG424 (Sadowski and Ptashne, 1989). The cDNA fragments of the GAL4DB-Pnx fusion protein were amplified from the pSG424 plasmids by PCR and inserted into pEGFP-C1 (Clonetech). pCS3+MT zGroucho2 was constructed by inserting zebrafish *groucho2* (Takke and Campos-Ortega, 1999) into pCS3+MT.

To construct a plasmid for Myc-tagged green fluorescent protein (MTGFP), the *Nco*I and *Eco*RI fragment of pEGFP-C1 (Clonetech) was inserted into the pCS2+MT plasmid. The expression vector for

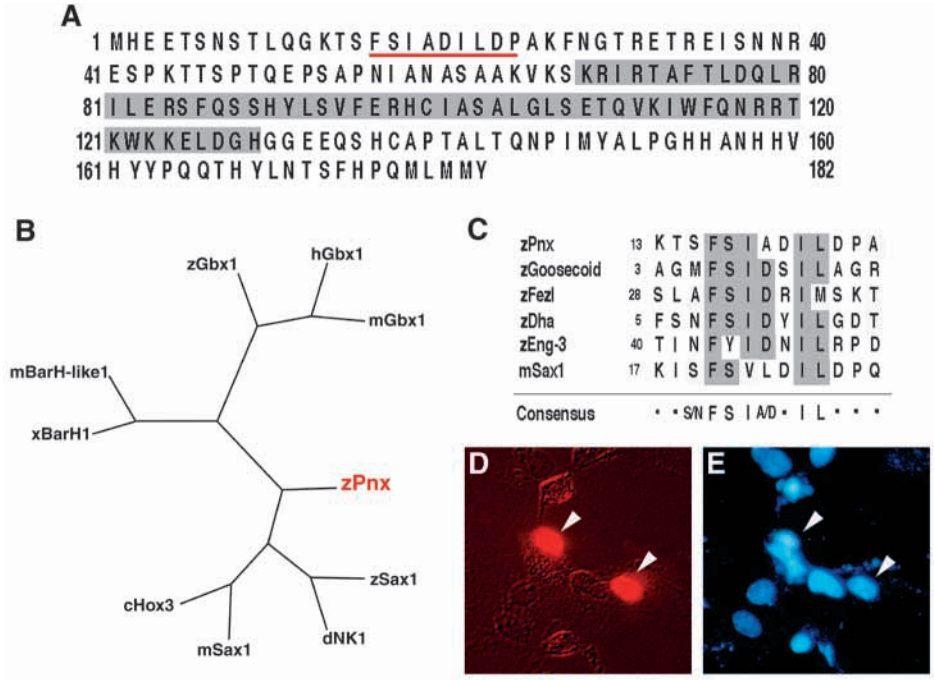

Fig. 1. Structure of *pnx*. Pnx is a novel homeodomain-containing protein. (A) Amino acid sequence of Pnx. Pnx contains an Eh1 (Engrailed homology 1) repressor domain in its N-terminal region (underlined) and a homeodomain in its C-terminal region (shaded box). (B) Phylogenetic tree for comparison between Pnx, Nkx1/Sax-family homeodomain-containing proteins and Gbx. The phylogenetic tree was constructed using the ClustalW program provided by DDBJ and the full-length amino acid sequences of the proteins (except for chick Hox3, which has only a C-terminal partial sequence). Accession Numbers of the proteins used for the phylogenetic tree are AB067731 (zebrafish Pnx), AB067732 (zebrafish Sax1), NP033149 (mouse Sax1), JU0069 (chicken Hox3), A33976 (*Drosophila* NK-1), AAG14450 (*Xenopus* BarH1), XP123759 (mouse BarH-like1), XP069853 (human Gbx1), XP144233 (mouse Gbx1) and AAK83070 (zebrafish Gbx1). (C) Alignment of the Eh1 repressor domains of zebrafish Pnx, Goosecoid, Fez-like, Dharma/Bozozok, and Engrailed3, and mouse Sax1. The consensus sequence for the Eh1 domain is also shown. (D,E) Nuclear localization of Pnx protein. COS7 cells were transfected with an expression vector for Myc epitope-tagged Pnx. Myc-Pnx was immunostained with an anti-Myc antibody. Pnx was detected in the nuclei of the transfected cells (indicated by arrowheads) (D). (E) A DAPI image of D. DAPI marks nuclei.

Pnx-GFP was constructed by inserting the PCR fragment containing the 5′UTR and the coding region of *pnx* into the *Cla*I and *Nco*I site of the MTGFP plasmid.

Synthetic capped RNAs for Pnx, EnRPnx, VP16Pnx, Squint (Rebagliati et al., 1998), Fgf8/Ace (Furthauer et al., 1997), Dkk1 (Hashimoto et al., 2000), Antivin (Thisse et al., 2000), a dominant negative form of *Xenopus* Delta-1 (*XDl*^{stu}) (Chitnis et al., 1995) and zebrafish Notch5ICD (Itoh et al., 2003) were transcribed in vitro using the linearized plasmid DNA as a template, then dissolved in 0.2 M KCl with 0.2% Phenol Red as a tracking dye, and injected into one-cell-stage embryos using a PV830 Pneumatic PicoPump (WPI).

The antisense morpholino oligonucleotides were generated by Gene Tools (LLC, Corvallis, Oregon). *pnx* MO for 5′UTR, 5′-CCTGtCGGTcACTTCaGAGAcGAGT-3′; control MO for 5′UTR, 5′-GAtTTGgTCGTTTCTTCcTGCtTCC-3′ (lower case letters indicate mispaired bases); *pnx* MO for the translational initiation site (TIS), 5′-GAATTGCTCGTTTCTTCGTGCATCC-3′; control MO for TIS, 5′-GAtTTGgTCGTTTCTTCcTGCtTCC-3′. The MOs were dissolved in 1× Danieau's buffer (Nasevicius and Ekker, 2000) for the stock (10 mg/ml). For injection, the MO was diluted in 1× Danieau's buffer to 0.4-2 mg/ml.

Whole-mount in situ hybridization, β-galactosidase detection and immunohistochemistry

Whole-mount in situ hybridization was performed principally as described previously (Jowett and Yan, 1996). BM purple AP substrate (Roche) and Fast Red tablets (Sigma) were used as a substrate for alkaline phosphatase. To prepare an antisense *pnx* riboprobe, the *Eco*RI-*Xba*I fragment of *pnx* cDNA was subcloned into pBluescriptSK+ (Stratagene) (pBSK+zPnx). pBSK+zPnx was digested by *Eco*RI and transcribed with T3 RNA polymerase. Antisense riboprobes in this study were generated as described: *ngn1* (Kim et al., 1997), *krox20* (Jowett and Yan, 1996), *hoxb1b* (Alexandre et al., 1996), *elavl3/HuC* (Kim et al., 1996), *olig2* (Park et al., 2002), *islet1* (Inoue et al., 1994) and *islet2* (Tokumoto et al., 1995). *sox19* cDNA (Vriz and Lovell-Badge, 1995) was amplified by PCR and used to generate a riboprobe. Immunohistochemistry was performed as described previously (Fujii et al., 2000). Monoclonal antibodies, znp1, zn5 and zn12 (Trevarrow et al., 1990) were used at the concentrations 1:500, 1:200, and 1:500, respectively. Immune complex was detected using the Vectastain ABC kit (Vector Laboratories), and visualized in 0.7 mg/ml diaminobenzidine (Sigma) and 0.003% H_2O_2. For the detection of β-galactosidase activity, embryos were fixed in 2.5% paraformaldehyde in PBS overnight at 4°C, rinsed three times for 10 minutes in PBS with 0.02% NP-40, and one for 10 minutes with X-gal staining buffer (100 mM phosphate buffer at pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide) at 37°C for 1 hour, and fixed again in 4% paraformaldehyde in PBS overnight at 4°C before in situ hybridization. Photographs were taken using an AxioPlan-2 microscope (Carl Zeiss) and HC-2500 3CCD camera (Fuji Film). Figures were assembled using Adobe Photoshop, V6.0.

Reporter assay and immunostaining

For the luciferase reporter assay, human embryonic kidney (HEK) 293T cells were transfected with the expression vectors for the Gal4-Pnx fusion proteins (0.2 μg), pFR-Luc plasmid (1 μg, 5× GAL4 Binding Element Luciferase, Stratagene) and pCS2+nβgal (0.2 μg,) (Turner and Weintraub, 1994) in the presence or absence of pCS3+MT zGroucho2 (2 μg). After a 36 hour incubation, the cells were harvested and the luciferase activities were measured. The β-galactosidase activities were used for normalization of the transfection efficiency.

For the immunostaining of cells, COS7 cells were transfected with pCS2+MT-Pnx. After a 24 hour incubation, the cells were fixed in 4% paraformaldehyde for 15 minutes, labeled with anti-Myc-epitope monoclonal antibody (1:200 dilution, 9E10, Sigma) and visualized using an Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution, Molecular Probes).

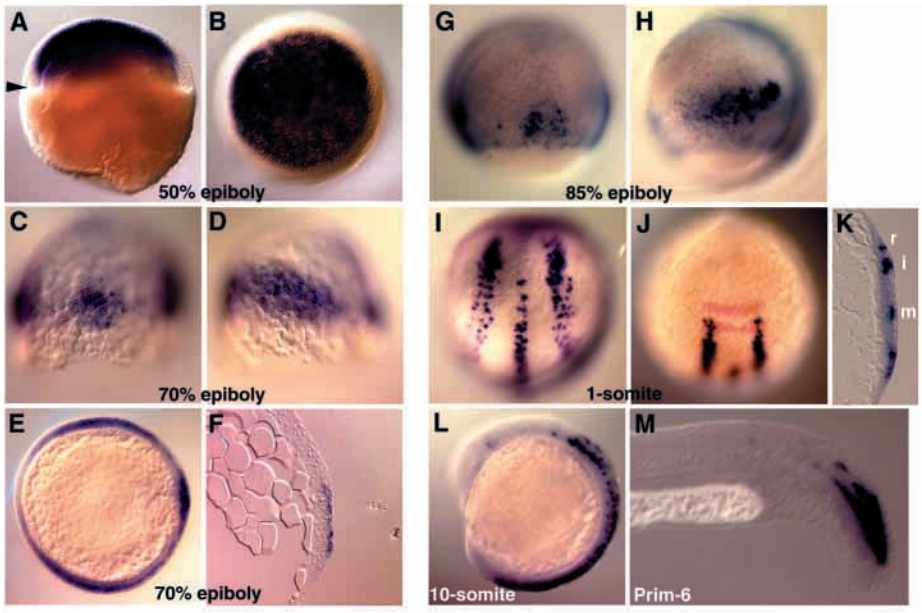

Fig. 2. Expression of *pnx*. Expression of *pnx* was detected by whole-mount in situ hybridization. (A,B) 50% epiboly stage. Lateral view (A) and animal pole view (B). Dorsal is towards the right. *pnx* was expressed in the entire ectoderm exclusive of the marginal blastoderm (arrowhead in A). (C-F) 70%-epiboly stage. Dorsal view (C), lateral view (D), animal pole view (E) and sagittal section of the dorsal marginal region (F). (D-F) Dorsal is towards the right. The *pnx* transcripts were detected in the ventrolateral and dorsomedial regions of the ectoderm at the mid-gastrula stage. (G,H) 85% epiboly stage. Dorsal view (G) and lateral view (H). (I-K) One-somite stage. (I) Dorsal view. (J) Two-color staining with *pnx* (purple) and *krox20* (red). (K) Horizontal section of the *pnx*-stained tail-bud state embryos (m, primary motoneurons; i, primary inter neurons; r, Rohon-Beard neurons). From the late gastrula to early segmentation stages, the *pnx* transcripts became restricted to three stripes of primary neurons posterior to rhombomere 4 (the *krox20* expression indicates rhombomeres 3 and 5). (L) Ten-somite stage (14 hpf), lateral view. (M) Prim-6 stage (25 hpf), lateral view. The expression of *pnx* gradually disappeared from the anterior region from the late segmentation stages and it was only detected in the caudal neural tube at the early pharyngula stage.

Co-immunoprecipitation and western blot analysis

The various Pnx mutant constructs and the expression vector for Myc-tagged zebrafish Groucho2 were co-transfected into 293T cells. Thirty-six hours after transfection, the cells were lysed on ice in lysis buffer (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 4 mM PMSF, 10 mg/ml aprotinin). The lysates were immunoprecipitated with anti-GFP rabbit polyclonal antibodies (MBL), separated by electrophoresis on a 4-20% polyacrylamide gel, and transferred to a PVDF membrane. The membrane was immunoblotted with an anti-Myc-epitope monoclonal antibody (1:5000 dilution, 9E10, Sigma). The immune complexes were visualized by a chemiluminescence system (Renaissance; Dupont NEN Products).

RESULTS

pnx, an *nkx-1* family homeobox gene, is expressed in posterior neurogenic regions

Using an expression cloning strategy, we isolated a novel homeobox gene that elicited the expansion of posterior primary neurons and the posteriorization of neuroectoderm when it was misexpressed. We named it *pnx* (posterior neuron-specific homeobox). The *pnx* gene encodes a 182 amino acid protein that contains a homeodomain in its middle region (Fig. 1A). The homeodomain of Pnx displayed sequence similarities with the homeodomains of *Drosophila* NK-1, and mouse and zebrafish Sax1 (Fig. 1B), suggesting that Pnx is a novel member of the Nkx1/Sax-family of homeodomain-containing proteins. Pnx also contains a domain that is homologous to the repressor motif (Engrailed homology; Eh1) of *Drosophila* Engrailed and other homeodomain-containing transcription factors that function as transcriptional repressors (Fig. 1C) (Muhr et al., 2001; Smith and Jaynes, 1996). Radiation hybrid mapping revealed that the *pnx* gene was localized close to the telomere of linkage group 10. Myc-tagged Pnx protein and PnxGFP (green fluorescence protein) localized to the nuclei of the COS7 cells (Fig. 1D) and the blastomeres of the zebrafish embryos (see Fig. 6D), respectively.

pnx expression was first detected exclusively in the prospective ectoderm and not in the blastoderm margin, which gives rise to the mesoderm and endoderm, at the late blastula stage (Fig. 2A,B). From the early to mid gastrula stages, the *pnx* transcripts became localized to two domains: the ventrolateral ectoderm and the dorsal-midline ectoderm, in which progenitors of the hindbrain and spinal cord are located (Woo and Fraser, 1995) (Fig. 2C-F). The *pnx* expression domains became spotty at the late gastrula stage and the pattern of transcripts appeared as dots forming three stripes on each side of the embryo at the early segmentation stage, which correspond to the primary neurons (motoneurons, interneurons and Rohon-Beard neurons) (Fig. 2G-K). Compared with other neuronal markers, such as *elavl3/HuC*, *pnx* was expressed more strongly in primary motoneurons and interneurons than in Rohon-Beard neurons. The anterior limit of the *pnx* expression was between the two *krox20*-expressing domains, marking rhombomeres 3 and 5 (Fig. 2J), indicating that *pnx* is expressed posterior to rhombomere 4. The *pnx* expression disappeared in an anterior to posterior direction during the segmentation and pharyngula stages (Fig. 2L,M), and it was rarely detected at all after 48 hours post fertilization (data not shown). These data indicate that the expression domains of *pnx* correspond to posterior neurogenic regions and later to posterior primary neurons. *pnx* was expressed earlier than the *ngn1* and Delta genes (Blader et al., 1997; Haddon et al., 1998b; Kim et al., 1997), suggesting that *pnx* is one of the earliest genes to be expressed in the posterior neurogenic regions.

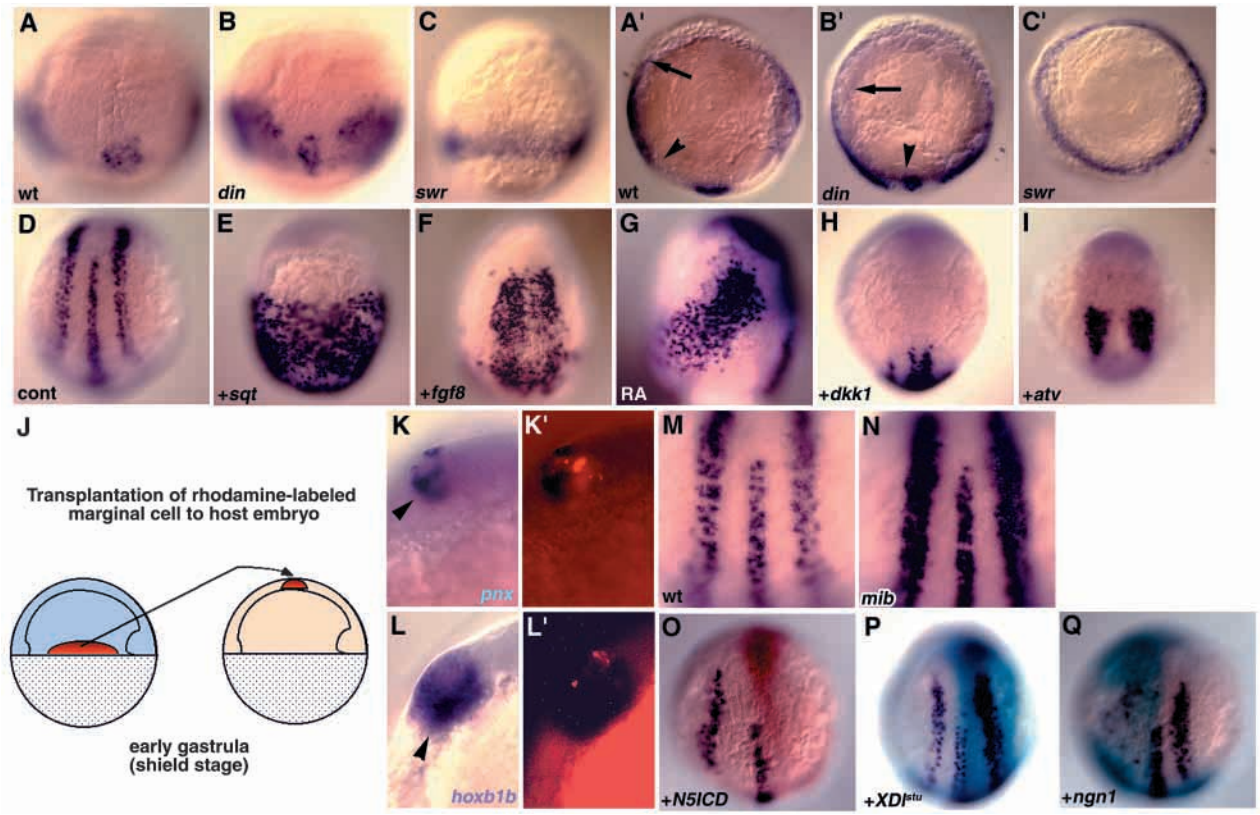

Fig. 3. Regulation of *pnx* expression. (A-C′) Expression of *pnx* in wild-type (A,A′), *chordino* (B,B′) and *swirl* (C,C′) mutant embryos at the mid-gastrula stage (85% epiboly). (A-C) Dorsal views. (A′-C′) Vegetal pole views, with dorsal towards the bottom. Arrows and arrowheads indicate the ventral and dorsal limit of lateral expression domains of *pnx*, respectively. Lateral expression domains of *pnx*, which correspond to the primary inter neurons and Rohon-Beard (RB) neurons, became closer to the dorsal midline in *chordino* mutant embryos. By contrast, the *pnx* transcripts were detected in the entire ectoderm margin at the mid gastrula stage in *swirl* mutant embryos. (D-I) Reciprocal regulation of *pnx* by prospective posteriorizing and anteriorizing signals. The expression of *pnx* was expanded or ectopically induced at the three-somite stage in the embryos injected with 0.5 pg of *sqt* RNA (E) and 10 pg of *fgf8/ace* RNA (F), or treated with 1 μM retinoic acid at the gastrula stage (G) (D, control). (D-F) Dorsal views and (G) lateral view. (H,I) *pnx* expression was reduced and the expression domain was posteriorly shifted in embryos injected with 20 pg of *dkk1* RNA (H) and 1 pg of *antivin* (I) RNA at the three-somite stage. Dorsal views. (J-L′) Non-axial mesendoderm induced the ectopic expression of *pnx* and *hoxb1b* in the forebrain region. (J) Procedure of the experiment. Marginal blastomere cells from the rhodamine-dextran-injected embryos were transplanted into the animal-pole region of host embryos at the shield stage, which were then stained at the three-somite-stage for *pnx* (K,K′) or *hoxb1b* (L,L′). Lateral views, with dorsal towards the right (K-L′). (K′,L′) Epifluorescence images of K,L, respectively. Ectopic expression of *pnx* and *hoxb1b* is indicated by arrowheads. (M-Q) Regulation of *pnx* by the Delta-Notch signal. The number of *pnx*-expressing cells at the three-somite stage in *mind bomb* (*mib*) mutant embryos (N) and embryos injected with RNA of 100 pg of *XDl*^{stu} RNA (P) was increased in comparison with the wild-type non-injected control embryos (M). By contrast, the *pnx*-expressing cells were absent or severely reduced in number in the embryos injected with RNA of zebrafish *notch5ICD* (50 pg, O) or *neurogenin1* (50 pg, Q). Dorsal views.

Regulation of *pnx* expression

We next examined the regulation of *pnx* expression by neural inducers (BMP antagonists), neuroectoderm-anteriorizing and posteriorizing factors, and the Notch signal. In *chordino* mutants, which harbor a mutation in the *chordin* gene, *pnx* expression in the dorsal midline was not affected, but its ventrolateral expression domain was closer to the midline at the mid-gastrula stage (Fig. 3B,B′). By contrast, the *pnx* transcripts were detected in circular domains of the vegetal side of the ectoderm in *swirl* mutant embryos, which harbor a mutation in the *bmp2b* gene (Fig. 3C,C′). These data indicate that the expression of *pnx* is regulated by the BMP and BMP antagonistic neural inducers.

We examined whether *pnx* could be induced by a signal from the marginal blastomeres of shield-stage embryos, which are considered to be a source of posteriorizing signals in the zebrafish (Woo and Fraser, 1997). When marginal blastoderm cells in the ventral or lateral positions of shield-stage embryos were transplanted into the animal pole of sibling embryos, which corresponds to the prospective forebrain region, ectopic expression of *pnx* as well as of *hoxb1b* was detected in the cells residing near the transplanted cells (Fig. 3K,K′), indicating that *pnx* could be induced by a signal from the non-axial mesendoderm. The expression domain of *pnx* was prominently expanded in embryos overexpressing the putative posteriorizing factors, Nodal (Squint) and Fgf8/Ace, and in embryos treated with retinoic acid (Fig. 3E-G). By contrast, the *pnx* expression domain was reduced in embryos overexpressing the Wnt inhibitor Dkk1 and the Nodal/Activin inhibitor Antivin (Fig. 3H,I), the misexpression of which

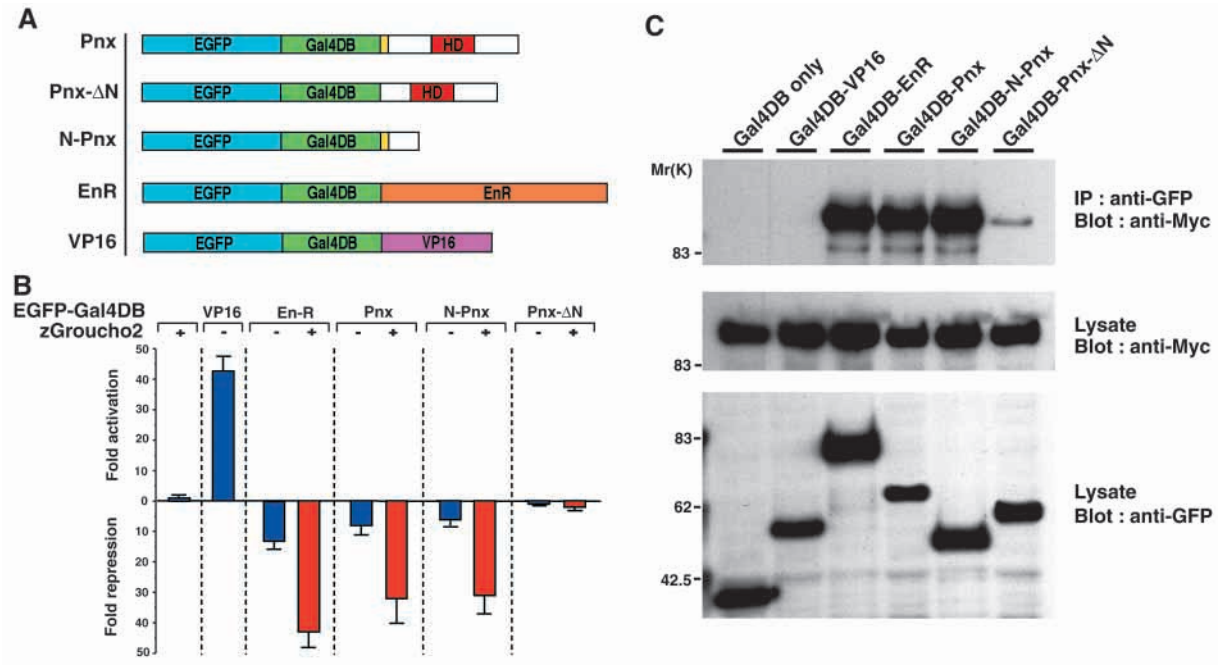

Fig. 4. Pnx protein acts as a transcriptional repressor in cells. (A) Schematic representation of Pnx mutants. All the Pnx mutants were constructed to be expressed as fusion proteins with the DNA-binding domain (Gal4DB) of the yeast transcription factor GAL4 and GFP. 'Pnx' is the wild-type full-length Pnx. In Pnx-ΔN the N-terminal domain of Pnx, which contains the Eh1 repressor domain, is deleted. N-Pnx consists of only the N-terminal domain of Pnx. EnR and VP16 are fusion proteins in which the DNA-binding domain (Gal4DB) of the yeast transcription factor GAL4 is fused to the repressor domain of Engrailed and the trans-activation domain (TAD) of VP16, respectively. (B) Repressor activities of Pnx. Human 293T cells were transfected with 5×GAL4BS-luciferase and expression vectors for the Pnx variants in the presence or absence of an expression vector for zebrafish Groucho2. Luciferase activities were determined at 36 hours after transfection and are indicated as fold activation/repression compared with the activity obtained from pSG424 (expressing Gal4DB alone)-transfected cells. Deletion of the N-terminal Eh1 domain abolished the repressor activity of the Pnx construct. Data points represent the average of at least three transfections. Error bars indicate the standard deviation of three independent experiments. The repressor activities of the Pnx, N-Pnx and EnR constructs were enhanced up to 3.3~5.1 fold by the co-expression of zebrafish Groucho2. Zebrafish Groucho2 alone did not reduce the luciferase reporter activity. Error bars indicate the standard deviation of three independent experiments. (C) The Eh1 domain of Pnx interacts with zebrafish Groucho2. The Gal4DB-Pnx proteins and Myc epitope-tagged Groucho2 were co-expressed in 293T cells. After 36 hours, the Pnx proteins were immunoprecipitated with anti-GFP antibodies and immunoblotted with anti-Myc epitope antibodies. Expression of the Pnx fusion proteins and the Myc-tagged Groucho2 was also detected by immunoblotting the cell lysates with anti-GFP and anti-Myc antibodies, respectively.

induces an enlarged anterior neuroectoderm in zebrafish (Hashimoto et al., 2000; Thisse et al., 2000). These findings indicated that the *pnx* expression could be controlled positively by the posteriorizing factors and negatively by the anteriorizing factors, at least when the factors were ectopically activated.

The number of *pnx*-expressing cells was increased and almost homogenous within the three stripes in *mind bomb* (*mib*) mutant embryos, in which the Notch signaling is perturbed (Haddon et al., 1998a; Itoh et al., 2003; Riley et al., 1999), at the early segmentation (neurula) stage (Fig. 3N), although the homogenous expression in the posterior neurogenic regions at the mid-gastrula stage was not affected in the *mib* mutant embryos (data not shown). A similar expansion of *pnx*-expressing cells was detected in embryos expressing a dominant-negative (DN) form of *Xenopus* Delta-1 (*XDl*^{stu}, Fig. 3P). By contrast, the *pnx*-expressing cells were severely reduced in number or absent in the embryos expressing an activated form (cytoplasmic domain) of zebrafish Notch5 (Fig. 3O). Similarly, misexpression of *ngn1*, which elicits an increased or ectopic expression of *deltaA*, *deltaD* and *her4* (Takke et al., 1999) (data not shown), attenuated the *pnx*-expressing cells (Fig. 3Q). All of these data indicate that the initial induction of *pnx* expression is not regulated by the Notch signal, but the spotty expression within the neurogenic regions at the segmentation (neurula) stages is controlled by the Notch signal.

Pnx acts as a transcriptional repressor

Pnx contains an Eh1 repressor domain. To address whether Pnx functions as a transcriptional repressor, we constructed a series of Pnx mutant proteins that could be expressed as fusion proteins with GFP and the DNA-binding domain of the yeast transcription factor Gal4 (Fig. 4A). In Pnx-ΔN, the N-terminal region containing the Eh1 domain was deleted. N-Pnx contained only the N-terminal region. EnR and VP16 were constructed with the repressor domain of *Drosophila* Engrailed and the transcriptional activation domain of Herpes Simplex Virus type I VP16 (Sadowski et al., 1988) for positive and negative controls, respectively. Human 293T cells were transfected with expression vectors for the Gal4-Pnx fusion proteins and the luciferase reporter gene containing 5× Gal4-binding sites, and the levels of Gal4-dependent transactivation and repression were determined (Fig. 4B). Expression of the wild-type Pnx and the EnR proteins repressed the basal

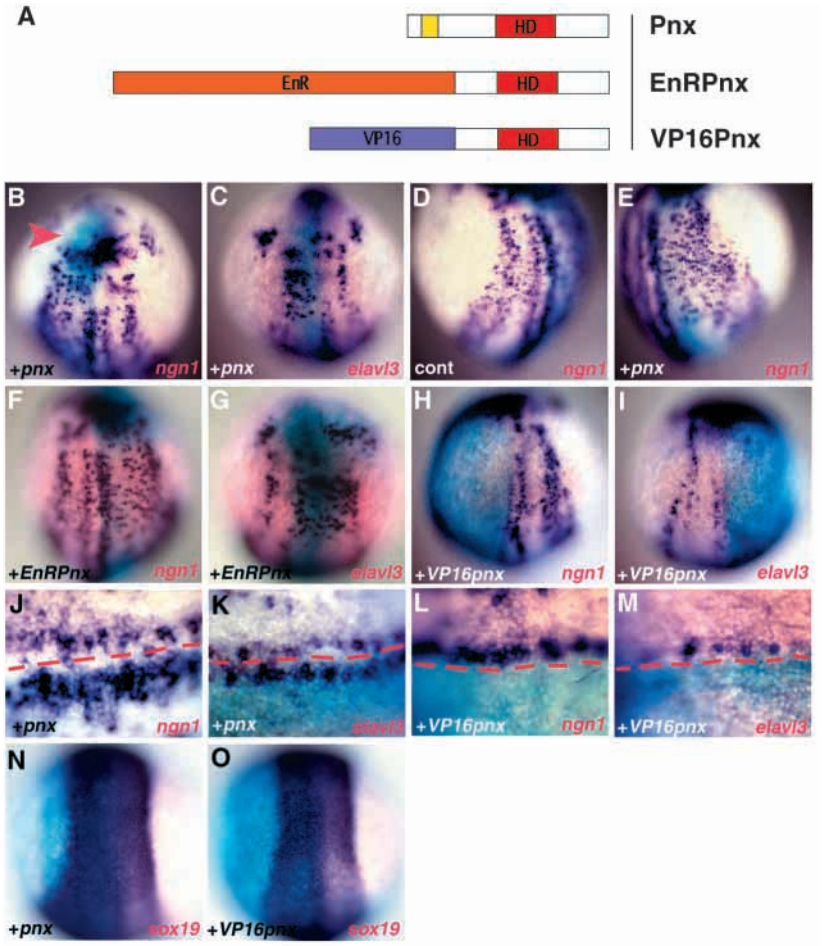

Fig. 5. Repressor activity of Pnx was required for the formation of posterior neurons. Schematic representation of Pnx constructs. Ten picograms of *pnx*, 5 pg of *EnRpnx* or 20 pg of *VP16pnx* RNA were co-injected with 50 pg of β-galactosidase RNA into one blastomere of two- to four-cell stage zebrafish embryos. The embryos were fixed at the three-somite stage, and the expression of β-galactosidase was stained by X-gal. (B-E) Overexpression of *pnx* in posterior neuroectoderm increased the numbers of cells expressing *ngn1* (B) and *elavl3* (C), whereas the overexpression of *pnx* in anterior neuroectoderm inhibited their expression (indicated by an arrowhead in B). Dorsal (B,C) and dorsolateral (D,E) views. (D,E) *ngn1*-expressing cells were increased in the right side (injected side, E) of the embryos, in which *pnx* and β-galactosidase RNA were localized, compared with the control side (left side, D). Expression of EnR-Pnx also elicited an increased expression of *ngn1* (right side, F) and *elavl3* (G). By contrast, expression of VP16-Pnx strongly inhibited the expression of *ngn1* (left side, H) and *elavl3* (right side, I). Highly magnified views of the *pnx* RNA (J,K) and *VP16pnx* RNA (L,M). The borderlines between the injected (lower) and the non-injected (upper) sides are indicated by broken lines. (N,O) Overexpression of *pnx* slightly expanded neuroectoderm expressing *sox19* (N), but expression of *VP16pnx* did not significantly affect the formation of neural plate (O). The left sides are injected sides.

transcriptional activities. The deletion of the Eh1 domain (Pnx-ΔN) suppressed the repressor activity of Pnx, while the N-terminal domain alone (N-Pnx) was sufficient for the repressor activity. By contrast, the expression of the VP16 activation domain activated transcription. These data suggest that Pnx acts as a transcriptional repressor and that the Eh1 domain in the N-terminal region is essential and sufficient for the repressor activity.

The Eh1 domains of various homeodomain-containing proteins have been shown to interact with the Groucho family of transcriptional co-repressors (Kobayashi et al., 2001; Muhr et al., 2001). We thus examined the interaction between Pnx and zebrafish Groucho2 (Fig. 4C). Myc-tagged Groucho2 was co-expressed with GFP-tagged Pnx in 293T cells. Groucho2 was co-immunoprecipitated with Pnx but very little was associated with Pnx-ΔN, indicating that Pnx interacted with Groucho2 through the Eh1 domain in its N-terminal region, at least in these cells. Co-expression of Groucho2 with wild-type Pnx, EnR or N-Pn, enhanced the repressor activity of the Pnx proteins (Fig. 4B), indicating a cooperation between Pnx and Groucho for the transcriptional repression. These data further suggest that a Groucho mediates the repressor activity of Pnx.

Pnx is involved in the formation of posterior neurons

Injection of *pnx* RNA into one-cell stage embryos elicited the expansion of primary neurons (data not shown), as observed when the RNA was injected into two- or four-cell stage embryos. In addition to the neurogenic phenotype, the ubiquitous *pnx* expression posteriorized the neuroectoderm (the expression of forebrain-specific genes was abrogated and the anterior hindbrain region was shifted to the anterior side) and often induced abnormalities in the formation of axial mesendoderm (data not shown). However, the reverse phenotypes of these non-neurogenic phenotypes were not observed in the embryos in which the Pnx function was perturbed (described below). These phenotypes might be artifacts associated with the misexpression in non-ectoderm (see Discussion). Therefore, to analyze the specific effects of *pnx* expression in the ectoderm, we injected *pnx* RNA together with β-galactosidase RNA into one blastomere of two- or four-cell stage embryos, and examined the effects of *pnx* overexpression by in situ hybridization with various genetic markers, when the β-galactosidase RNA was expressed in the ectoderm.

The misexpression of *pnx* in the posterior ectoderm elicited an expansion and/or ectopic expression of the proneural gene *ngn1* and postmitotic neuronal marker *elavl3/HuC* within the domain expressing β-galactosidase [84% (95/112) for *ngn1*, 72% (78/108) for *elavl3*, Fig. 5C,E,J,K]. The increased *ngn1*- and *elavl3*-expressing cells were scattered within the neural plate and never exhibited a homogenous expression pattern. The expression of the Engrailed repressor domain (EnR) fusion protein of Pnx (EnRPnx) exhibited similar effects on *ngn1* and

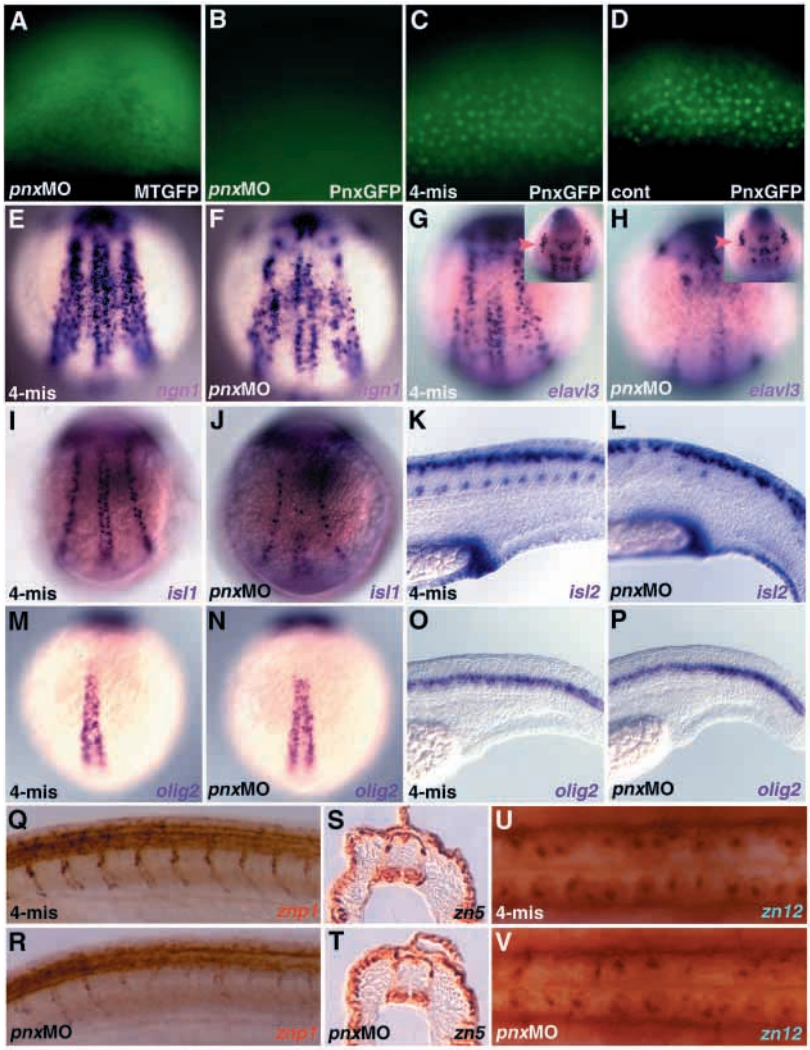

Fig. 6. Pnx is involved in primary neurogenesis. (A-D) Specific inhibition of the translation of *pnx* RNA by antisense morpholino oligonucleotide (MO). Two nanograms of the MO recognizing the 5′ untranslated region of *pnx* (*pnx* MO; A,B) or the control MO (4-mis; C), which contains four mispaired bases, were co-injected with RNA for Pnx-GFP (B,C) or Myc-tagged GFP (MTGFP; A) into one-cell-stage embryos. (D) Pnx-GFP-expressing control embryo. The expression of Pnx-GFP and MTGFP was detected by fluorescence microscopy. *pnx* MO (F,H,J,L,N,P,R,T,V) and 4-mis MO (E,G,I,K,M,O,Q,S,U)-injected embryos were stained with markers for neural precursors and primary and secondary neurons. (F,H) *ngn1*- and *elavl3*-expressing cells were diminished in the posterior neuroectoderm of *pnx* MO-injected embryos at the one- to three-somite stage. (G,H) *elavl3* expression in trigeminal ganglions was not significantly affected in the *pnx* MO-injected embryos (marked by arrowheads in insets). (I,J) At the five-somite stage, *pnx* MO-injected embryos displayed a strong reduction in *islet1*-expressing primary motoneurons and a less efficient reduction in *islet1*-positive Rohon-Beard neurons. (K,L) At 24 hpf, the *pnx* MO-injected embryos (L) displayed a reduction in *islet2*-expressing primary motoneurons and defects in axon outgrowth from motoneurons (stained with the znp1 monoclonal antibody, R). (M-P) *olig2* expression was not affected in the *pnx* MO-injected embryos at the one-somite stage (N) and at 24 hpf (P). (S,T) zn5-immunoreactive secondary motoneurons (48 hpf) and (U,V) zn12-immunoreactive Rohon-Beard neurons (25 hpf) appeared to be normal or only marginally affected in the *pnx* MO-injected embryos (T,V). (E-J,M,N) Dorsal views. (K,L,O-R) Lateral views. (S,T) Horizontal sections in the spinal cord region. (U,V) Dorsal views in the spinal cord region.

elavl3 expression to those seen with Pnx [88% (37/42) for *ngn1*, 77% (34/44) for *elavl3*; Fig. 5F,G]. The misexpression of *pnx* in anterior neural plate reduced the *ngn1* expression (Fig. 5B, arrowhead). The expression of the VP16-Pnx fusion protein strongly inhibited the expression of *ngn1* and *elavl3* [89% (113/127) for *ngn1*, 94% (86/92) for *elavl3*; Fig. 5H,I,L,M], supporting the repressor function of Pnx in neurogenesis. These data suggest that *pnx* or *pnx*-related genes are required for promoting *ngn1*- and *elavl3*-expressing cells in the posterior neural plate. The misexpression of Pnx induced a slight expansion of neural plate, which is marked by *sox19* expression (Vriz et al., 1996), but the VP16-Pnx expression did not significantly affect the formation of neural plate (Fig. 5O), suggesting that Pnx does not have a strong neuroectoderm-inducing activity. The effect of the *pnx* misexpression on the neural plate (Fig. 5N) may be a secondary effect accompanied by the expansion of primary neurons.

To examine the specific function of Pnx, we inhibited its translation by injecting antisense morpholino oligonucleotide (MO) into one-cell stage embryos. We used two different MOs against *pnx* [one recognizes the 5′ untranslated region (UTR) and the other recognizes the sequence surrounding the translation initiation site]. The results from these two MOs were essentially the same, and we show the results from the *pnx* MO of the 5′UTR in Fig. 6 and from that of the translational initiation site in Fig. 7. Injection of the *pnx* MO inhibited translation from the PnxGFP RNA containing the 5′UTR and coding sequence of Pnx [98% (43/44)], and GFP, but not from the control RNA for Myc-tagged GFP [100% (39/39)] (Fig. 6A,B). The control *pnx* MO (4mis), which contains four mispaired bases, did not inhibit the translation of PnxGFP [90% (35/39)], suggesting that the effect of the *pnx* MO was specific for Pnx (Fig. 6C). Injection of the *pnx* MO led to a reduction in *ngn1*-expressing neural precursor cells and *elavl3*-expressing neurons in the posterior neuroectoderm but not in the anterior neuroectoderm at the early segmentation (neurula) stage [73% (107/147) for *ngn1*, 82% (110/134) for *elavl3*, Fig. 6F,H]. In these embryos (Pnx morphant embryos), the expression of *islet1* was reduced strongly in primary motoneurons and less efficiently in Rohon-Beard neurons [86% (63/73), Fig. 6J]. We also examined the expression of *islet2*, which is normally expressed in a set of primary motoneurons and Rohon-Beard neurons (Segawa et al., 2001), during the pharyngula period (24 hpf), in the Pnx morphant embryos (Fig. 6K). *islet2* expression was prominently reduced in the posterior primary motoneurons of the Pnx morphant

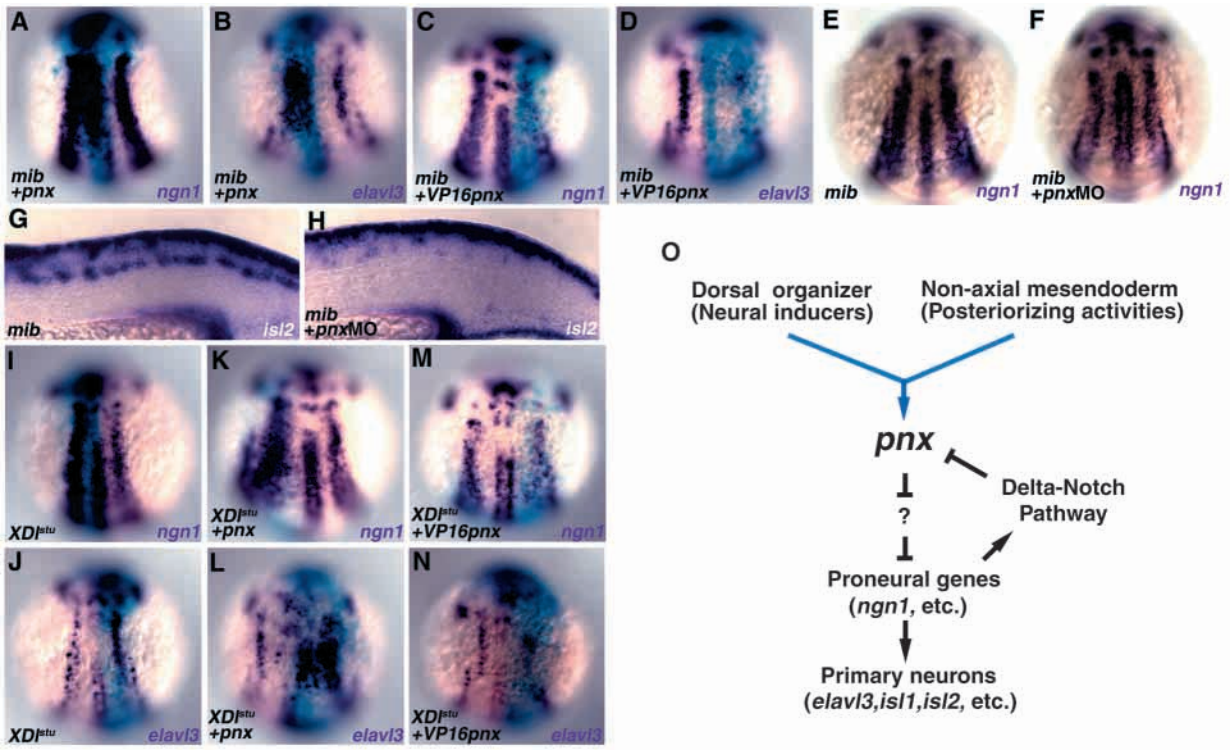

Fig. 7. Pnx can promote primary neurogenesis independently of *Delta-Notch* signaling. (A-H) Ten picograms of *pnx* and β-galactosidase RNA, 20 pg of *VP16pnx* and β-galactosidase RNA, or 2 ng of *pnx* MO (recognizing the translational initiation site) were injected into embryos of crossed *mib* mutants, fixed at the three-somite stage, and stained with *ngn1*, *elavl3* and *islet2* (A-H). The blue region stained with X-gal contained the injected RNA (A-D). The *mib* homozygous mutant embryos were detected by an increase in Rohon-Beard neurons. Misexpression of *pnx* in *mib* expanded the *ngn1*- (A) and *elavl3*-expressing stripes (B) but did not change the density of those cells within the stripes. Expression of VP16-Pnx (C,D) or injection of *pnx* MO (E,F) into the *mib* mutant embryos reduced the numbers of *ngn1* (C,E,F) and *elavl3*-expressing cells (D) at the three-somite stage. *islet2*-expressing primary motoneurons were reduced in the *pnx* MO-injected *mib* mutant embryos (H). (I-N) One hundred picograms of *XDl*^{stu} RNA and β-galactosidase RNA were co-injected with 10 pg of *pnx* RNA or 20 pg *VP16pnx* RNA into the wild-type embryos, and the expression of *ngn1* and *elavl3* at the three-somite stage was detected. Expression of XDl^{stu} increased the density of *ngn1*- (I) and *elavl3*-expressing cells (J), and co-expression of Pnx expanded the *ngn1*- (K) and *elavl3*-expressing (L) stripes without affecting the densities of *ngn1*- and *elavl3*-positive cells. Co-expression of VP16-Pnx reduced the numbers of *ngn1*- (M) and *elavl3*-expressing cells (N). (A-F,I-N) Dorsal views. (G,H) Lateral views in the spinal cord region. (O) Model for the role of *pnx* in posterior neurogenesis.

embryos, whereas the expression in Rohon-Beard neurons was relatively normal [92% (71/77); Fig. 6L]. Consistent with this, the axonal outgrowth from primary motoneurons (stained with znp1) was perturbed [54% (14/26); Fig. 6R] but Rohon-Beard neurons (stained with zn12) were relatively normal at 24 hpf in the Pnx morphant embryos (Fig. 6V). We further examined the expression of *olig2* in the Pnx morphant embryos, as *olig2* is required for the formation of primary motoneurons in zebrafish (Park et al., 2002). However, the expression of *olig2* in the ventral spinal cord was not affected at the early segmentation and pharyngula stages in the Pnx morphant embryos (Fig. 6M-P). Secondary neurons stained with zn5 were not significantly affected in the Pnx morphant embryos (Fig. 6T). These data indicate that inhibition of Pnx function led to a reduction in primary motoneurons. The formation of Rohon-Beard neurons was initially perturbed but it was recovered by the early pharyngula period in the Pnx morphant embryos. The data also indicate that Pnx functions upstream of *ngn1* but not of *olig2*.

Interaction between Pnx and Notch signaling

ngn1 is negatively regulated by Notch signaling, and the loss of Notch signaling leads to an increase in *ngn1*-expressing cells (Blader et al., 1997; Takke et al., 1999). We considered two possible mechanisms for the Pnx-mediated *ngn1* expression: one dependent on the Notch signal (Pnx inhibits Notch-mediated signaling) and one independent of the Notch signal. To address this issue, we misexpressed Pnx with β-galactosidase in *mib* mutant embryos, or Pnx and DN-Delta in wild-type embryos. As reported previously (Blader et al., 1997; Cornell and Eisen, 2002), the expression of *ngn1* as well as *elavl3* was strongly increased and homogenous within the three stripes (Fig. 7A,B,I,J). Misexpression of *pnx* did not affect the density of *ngn1*-expressing cells within the three stripes in these Notch signal-perturbed embryos, but rather elicited an expansion of the *ngn1*-expressing stripes [Fig. 7A, 92% (35/38); B, 79%, (27/34); K, 88% (27/33); and L. 87% (29/33)], suggesting that the Pnx-dependent *ngn1* induction was not mediated by inhibition of the Notch signaling. Thus, Pnx could expand *ngn1*-expressing cells independent of the Notch signal.

We further performed an epistatic analysis of *pnx* and the Notch signal. We injected VP16Pnx or Pnx MO into *mib* embryos or DN-Delta-expressing embryos. Expression of both

ngn1 and *elavl3* was decreased in these embryos, as in theVP16Pnx-exprssing embryos and Pnx morphant embryos [Fig. 7C, 65% (17/26); D, 74% (19/25); F, 97% (35/36); M, 71% (29/41); and N, 69% (30/43)], indicating that *pnx* is epistatic to the Notch signal.

DISCUSSION

pnx is a novel homeobox gene expressed in posterior neurogenic regions

We isolated a novel homeobox gene, *pnx*, and showed that its expression domain was located within the precursors of the hindbrain and spinal cord at the mid-gastrula stage (Fig. 2). *pnx* transcripts were detected in two separate regions (medial and lateral domains) that later gave rise to primary motor, inter and Rohon-Beard neurons, indicating that *pnx* does not simply mark the posterior neuroectoderm but rather defines the posterior neurogenic regions. *pnx* expression was regulated by a signal from the non-axial mesendoderm and could be upregulated by putative posteriorizing signals (Fig. 3), indicating that *pnx* functions downstream of posteriorizing signals derived from non-axial mesendoderm (Fig. 7O). It remains to be elucidated how *pnx* expression is restricted to the neurogenic regions and does not occur between them. In *Xenopus*, the *Zic*-related gene *Zic2* is expressed in the inter-neurogenic regions and suppresses neurogenesis, suggesting that *Zic2* functions as a pre-pattern gene (Brewster et al., 1998). However, in zebrafish, *zic1*, *zic2* and *zic3* do not display an expression profile similar to the *Xenopus Zic2* gene (Grinblat and Sive, 2001). Intriguingly, BMP activity has been shown to provide positional information for the posterior primary neurons (Barth et al., 1999; Nguyen et al., 2000). These studies showed that Rohon-Beard neurons are absent and primary interneurons are expanded laterally in *swirl/bmp2b* mutant embryos, whereas Rohon-Beard neurons are expanded in *somitabun/smad5* mutant embryos, which retain a residual BMP activity, suggesting that a balance between BMP and BMP antagonists may establish neurogenic regions in zebrafish. In the present study, the *swirl* mutant embryos displayed a circular expression of *pnx* and did not have intermediate regions that lacked *pnx* expression in the posterior neuroectoderm (Fig. 3), suggesting that the neurogenic region-specific expression of *pnx* is also regulated by the balance between BMPs and the organizer-derived BMP antagonists (neural inducers, Fig. 7O). Expression of *pnx* in medial neurogenic regions and in primary motoneurons was diminished or absent in *antivin* RNA-injected embryos (Fig. 3I) and *squint* mutant embryos (data not shown), which lacked (a part of) axial mesendoderm. This is consistent with that signals from axial mesendoderm, such as notochord and floor plate, are required for development of motoneurons (Jessell, 2000).

Roles of Pnx in the formation of posterior neurons

Injection of *pnx* RNA into one-cell stage embryos led not only to the expansion of *ngn1*- and *elavl3*-expressing cells but also to posteriorization of the neuroectoderm. We first thought that Pnx also functions in the regionalization of the neuroectoderm. However, a loss of the Pnx function, either by MO injection or by expression of VP16Pnx, which is likely to inhibit the function of Pnx-related protein, did not anteriorize the neuroectoderm (data not shown). Therefore, Pnx functions specifically in neurogenesis but not in neural patterning, although premature neural determination by ubiquitous Pnx expression might affect neural patterning.

Misexpression of *pnx* led to an expansion of *ngn1*- and *elavl3*-expressing cells (Fig. 5). Loss of the Pnx function either by VP16Pnx expression or the Pnx MO led to a reduction in *ngn1*- and *elavl3*-expressing cells (Figs 5, 6). Because *ngn1* functions upstream of the formation of *elavl3*-expressing postmitotic neurons (Kim et al., 1997), Pnx is likely to control the formation of primary neurons by upregulating the proneural genes, such as *ngn1*. However, it was recently reported that the inhibition of *ngn1* function by MO leads to a reduction in Rohon-Beard neurons but not primary motoneurons in zebrafish (Cornell and Eisen, 2002). Similarly, targeted disruption of the *ngn1* gene in mice does not significantly affect the formation of motoneurons in the spinal cord (Ma et al., 1998). This could be explained by the redundant functioning of Neurogenin-family genes. Disruption of both the *ngn1* and *ngn2* genes in mice leads to defects in the formation of motoneurons (Scardigli et al., 2001). Another bHLH gene, *olig2*, which is specifically expressed in the ventral spinal cord and is required for the formation of motoneurons in mice and zebrafish (Mizuguchi et al., 2001; Novitch et al., 2001; Park et al., 2002; Sun et al., 2001), is a possible candidate that functions downstream of *pnx*. However, *olig2* expression was not affected in the *pnx* MO-injected embryos (Fig. 6), suggesting Olig2 may function in parallel to Pnx. In *Drosophila*, the homeobox gene *vnd* (ventral nervous system defective) is involved in the development of ventral neuroblasts in the central nervous system. *vnd* controls expression of the AS-C proneural genes (Skeath et al., 1994), and also provides positional information for ventral neuroblasts (Chu et al., 1998; McDonald et al., 1998), Although Vnd is close to Nkx2 but not Nkx1, the regulation of proneural genes by homeobox genes is conserved between invertebrates and vertebrates.

Although *pnx* is expressed in three neurogenic regions (stripes), VP16-Pnx-expressing embryos and Pnx morphant embryos displayed only a mild reduction in Rohon-Beard neurons at the later stages (24 hpf embryo), compared with the strong reduction in primary motoneurons. Furthermore, the effects of Pnx MOs on the *ngn1* and *elavl3* expression were milder that those of the VP16Pnx expression. These results suggest the existence of molecules that function redundantly with Pnx. Pnx is a member of the Nkx1 gene family, and displays homology with the Nkx1-family proteins Sax1 and Sax2 within their homeodomains. *sax1* is expressed initially in the posterior neuroectoderm in chick and mouse (Schubert et al., 1995; Spann et al., 1994), like *pnx* in zebrafish. Sax1, Sax2 or other Nkx1-related transcription factors may work cooperatively with Pnx in the formation of motoneurons. As described above, Pnx may also cooperate with Olig2, which functions downstream of Hedgehog signals (Park et al., 2002), to promote the development of primary motoneurons. Intriguingly, Olig2 is also reported to function as a transcriptional repressor (Novitch et al., 2001; Zhou et al., 2001). Combinational repressions by Pnx and Olig2 may be required for the primary motoneuron development. It remains to be elucidated how Pnx cooperates with other factors to elicit the formation of interneurons and Rohon-Beard sensory neurons.

Pnx promotes primary neurogenesis through activation of proneural genes

As proposed for neurogenesis in *Drosophila*, in *Xenopus* and zebrafish, proneural genes, such as Neurogenin genes and *XASH3*, promote the expression of Delta genes, which activate Notch signaling in neighboring cells and repress neural differentiation at least partly through Hes/Her-family transcriptional repressors (Chitnis and Kintner, 1996; Ma et al., 1996; Takke et al., 1999). The Notch signaling and Hes/Her protein(s) further repress the expression of *ngn1* and its downstream genes that are required for neuronal differentiation (Takke et al., 1999). This lateral inhibitory mechanism is implicated in generating a restricted number of neurons in the neurogenic region. In this report, we demonstrated that *pnx* also functions in the lateral inhibition mechanism. First, gain and loss of Pnx function showed that *pnx* regulates the expression of the proneural gene *ngn1* (Fig. 4). Second, misexpression of *ngn1*, which elicits an increased or ectopic expression of *deltaA*, *deltaD* and *her4* (Takke et al., 1999) (data not shown), led to a reduction in *pnx*-expressing cells (Fig. 3). Finally, *pnx*-expressing cells increased in number within the neurogenic regions in the *mib* mutant embryos and DN-Delta-expressing embryos at the segmentation (neurula) stages (Fig. 3), indicating that *pnx* expression is negatively regulated by the Notch signal. These data support the idea that Pnx activates proneural gene (such as *ngn1*)-dependent lateral inhibition machinery that suppresses the expression of *pnx* in non-neuronal cells and restricts the numbers of neurons (Fig. 7O).

How does Pnx promote neurogenesis?

Pnx contains an Eh1 repressor domain and interacts with the transcriptional co-repressor Groucho2, at least in 293T cells. Reporter analysis revealed that Pnx acts as a transcriptional repressor and that the Eh1-mediated interaction with Groucho(s) is involved in this repressor activity (Fig. 4). Furthermore, VP16-Pnx functions as an antimorphic molecule in the formation of primary neurons. These data indicate that Pnx functions as a transcriptional repressor and should repress genes that have the ability to repress the proneural genes. Candidates that are repressed by Pnx could include downstream components of the Notch signal, such as the *hes/her*-family genes. However, this is not the case. Misexpression of Pnx still increased the *ngn1*- and *elavl3*-expressing cells in embryos in which Notch signaling was suppressed. Furthermore, Pnx did not inhibit the expression of either *her4* or *her9*, which are the only Hes/Her-family members reported to be expressed in the neural plate (data not shown). Furthermore, inhibition of the Notch signal leads to an increase in the density of neuronal cells 'within the neurogenic region', but does not lead to the expansion of neurogenic regions (Appel et al., 2001; Chitnis and Kintner, 1996). By contrast, the misexpression of Pnx in either wild-type or *mib* mutant embryos elicited an 'expansion' of the *ngn1*-expressing neurogenic regions (Figs 5, 7). Furthermore, *pnx* is epistatic to the Notch signaling in the formation of primary neurons (Fig. 7), providing genetic evidence that the *pnx*-mediated neurogenesis does not require the Notch signal. These data indicate that Pnx can promote neurogenesis not by inhibiting the Notch signal (lateral inhibition mechanisms), but rather by expanding neurogenic regions within the neuroectoderm. To promote neurogenesis, Pnx represses the expression of certain transcriptional repressor(s), other than those downstream of the Notch signal, which inhibit the proneural gene expression and neurogenesis (Fig. 7O). The identification of targets for Pnx will shed light on the mechanisms by which the neurogenic regions are established and proneural genes are regulated.

In this study, we have demonstrated that Pnx is a novel transcriptional repressor that links posteriorizing signals to the initiation of a program for the posterior neurogenesis. The requirement of the repressor activity of Pnx provides a novel mechanism for neurogenesis.

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