Cell, Vol. 87, 43–52, October 4, 1996, Copyright ©1996 by Cell Press

Identification of *neurogenin*, a Vertebrate Neuronal Determination Gene

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Summary

Several bHLH proteins are involved in vertebrate neurogenesis, but those controlling early stages of neuronal determination have not yet been identified. Here we describe a novel, NeuroD-related bHLH protein, NEUROGENIN, whose expression precedes that of NeuroD in both mouse and Xenopus. Expression of Xenopus NEUROGENIN-related-1 (X-NGNR-1) defines the three prospective territories of primary neurogenesis. Overexpression of X-NGNR-1 (or NEUROGENIN) induces ectopic neurogenesis and ectopic expression of *XNeuroD* mRNA. Endogenous *X-ngnr-1* expression becomes restricted to subsets of cells by lateral inhibition, mediated by X-Delta-1 and X-Notch. The properties of X-NGNR-1 are thus analogous to those of the Drosophila proneural genes, suggesting that it functions as a vertebrate neuronal determination factor.

Introduction

Transcription factors in the basic-helix-loop-helix (bHLH) family play a central role in cell type determination in several tissues and organisms (for reviews, see Weintraub, 1993; Jan and Jan, 1994). Moreover, within a given lineage multiple, functionally interchangeable bHLH proteins often act in cascades (Jan and Jan, 1993). For example, at least four different bHLH proteins are sequentially expressed during murine muscle development: MyoD/myf5, myogenin, and MRF4 (Olson and Klein, 1994). Similarly, in Drosophila peripheral neurogenesis expression of the proneural genes *achaete-scute* is followed by that of *asense* (Brand et al., 1993; Domínguez and Campuzano, 1993; Jarman et al., 1993a). It has been suggested that early-acting bHLH proteins control determination, while later-acting ones control differentiation (Weintraub, 1993). Although numerous bHLH proteins expressed during vertebrate neurogenesis have been identified (Johnson et al., 1990; Akazawa et al., 1992; Ferreiro et al., 1992; Sasai et al., 1992; Ishibashi et al., 1993; Turner and Weintraub, 1994; Akazawa et al., 1995; Lee et al., 1995; Shimizu et al., 1995), most of these are expressed at relatively late stages of differentiation; none so far exhibits the properties expected of a neural determination factor (Guillemot et al., 1993; Sommer et al., 1995; and see below).

One feature that characterizes neural determination genes in Drosophila is their interaction with the genetic circuitry underlying lateral inhibition. Lateral inhibitory interactions between neuroectodermal cells, mediated by the products of the neurogenic genes *Notch* and *Delta*, result in the selection of a single sensory organ precursor (SOP) cell from an "equivalence" group of undetermined cells called a "proneural cluster" (Ghysen et al., 1993). All cells in the proneural cluster initially express *achaete-scute*, but during the selection process proneural gene expression becomes restricted at high levels to the SOP (Cubas et al., 1991). This restriction occurs because the proneural genes promote expression of *Delta* (Hinz et al., 1994; Kunisch et al., 1994), and their expression and function are in turn inhibited by signaling through NOTCH (for review, see Ghysen et al., 1993). Thus, cells that express sufficient *achaete-scute*, and hence *Delta*, to inhibit proneural activity in their neighbors adopt an SOP fate (for discussion, see Chitnis, 1995). In this way, the proneural genes both promote a neural fate cell-autonomously and inhibit this fate nonautonomously.

Lateral inhibition mediated by vertebrate homologs of *Notch* and *Delta* has recently been demonstrated to regulate primary neurogenesis in Xenopus (Chitnis et al., 1995). Ectopic expression of a dominant negative form of X-Delta-1 (X-Delta-1^{Stu}) increases the density of neurons that differentiate within each of the three territories of primary neurogenesis (medial, intermediate, and lateral), but does not increase the overall area of the neural plate (Chitnis et al., 1995). Conversely, expression of constitutively active forms of X-Notch-1 suppresses primary neurogenesis (Coffman et al., 1990, 1993; Chitnis, 1995). These data further suggest that the prospective territories of primary neurogenesis are analogous to proneural clusters in Drosophila. This in turn implies the existence of one or more bHLH proteins whose expression defines these prospective neurogenic territories.

Several bHLH proteins expressed during Xenopus neurogenesis have been identified. One such protein, NeuroD, can exert a neuronal determination function when ectopically expressed, but the timing of its expression in vivo suggests it is more likely to function in differentiation (Lee et al., 1995). Several Xenopus homologs of *achaete-scute* have also been identified (Ferreiro et al., 1992; Zimmerman et al., 1993; Turner and Weintraub, 1994). Ectopic expression of one of these, XASH-3, can induce neural plate expansion (Ferreiro et al., 1994; Turner and Weintraub, 1994) or ectopic neurogenesis within the neural plate (Chitnis and Kintner, 1996), depending on the dose of injected RNA. Unlike NeuroD, however, XASH-3 is incapable of converting epidermal cells to neurons. Moreover, XASH-3 is expressed in a very restricted region of the neural plate, corresponding to the future sulcus limitans (Zimmerman et al., 1993). Thus, there must be other bHLH genes whose expression pattern and function are more consistent with a determination function.

In this paper we report the identification of such a gene, called *neurogenin* (*ngn*). In both mouse and Xenopus, expression of NEUROGENIN precedes and overlaps that of NeuroD. In Xenopus, moreover, its expression defines the three territories of prospective primary

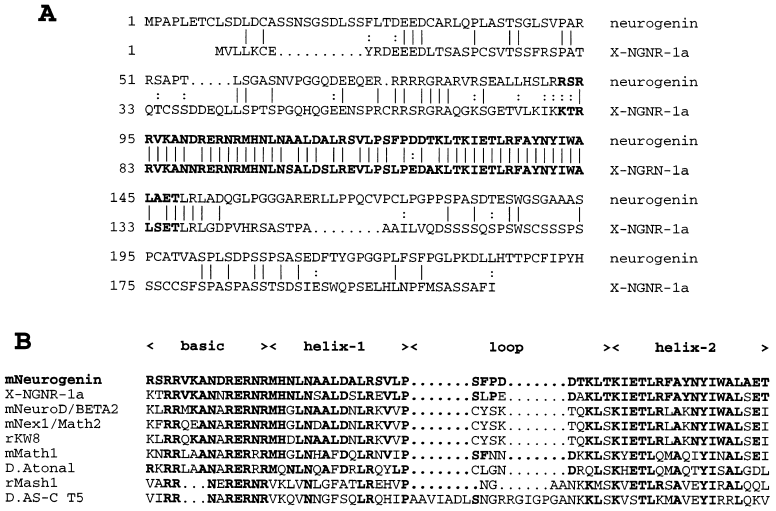

Figure 1. Sequences of Rat and Xenopus NEUROGENIN

(A) Alignment of the entire predicted amino acid sequences of rat NEUROGENIN and a Xenopus NEUROGENIN-related protein, X-NGNR-1.a. The bHLH region is marked in bold type. Solid lines indicate amino acid identity; the dots, conservative substitutions. The initiator methionine was selected based on Kozak's rules (Kozak, 1984) and identification of in-frame up-stream termination codons (data not shown).

(B) Alignment of the NEUROGENIN bHLH domain with other bHLH domains. Identity is shown by bold type. References for the compared sequences are as follows: NeuroD (Lee et al., 1995) /BETA2 (Naya et al., 1995), MATH-2/Nex-1 (Bartholomä and Nave, 1994; Shimizu et al., 1995), MATH-1 (Akazawa et al., 1995), KW8 (Kume et al., 1996), Drosophila atonal (Jarman et al., 1993b), MASH1 (Johnson et al., 1990), AS-C T5 (Villares and Cabrera, 1987).

neurogenesis. Ectopic expression of Xenopus NEUROGENIN-related-1 (X-NGNR-1) induces ectopic primary neurogenesis and ectopic expression of endogenous *XNeuroD* (but not vice-versa), suggesting that these two bHLH proteins function in a unidirectional cascade. Furthermore, the density of cells expressing endogenous *X-ngnr-1* within each of the three primary neurogenic territories appears to be controlled by the Notch–Delta lateral inhibitory circuitry. Thus *X-ngnr-1* appears to be a leading candidate for a vertebrate neuronal determination gene.

Results

Isolation of Murine and Xenopus *neurogenin* cDNA Clones

Previous work has identified MASH1 as a bHLH protein expressed in autonomic but not sensory ganglia of the mammalian PNS (Johnson et al., 1990; Lo et al., 1991; Guillemot and Joyner, 1993; Guillemot et al., 1993). We sought to isolate cDNAs encoding bHLH proteins expressed, conversely, in sensory but not autonomic ganglia. Degenerate reverse transcriptase polymerase chain reaction (RT–PCR) was performed using cDNA prepared from embryonic day 13.5 (E13.5) rat dorsal root ganglia (DRG), using oligonucleotide primers derived from conserved regions of bHLH subfamilies including MASH1 and NeuroD (see Experimental Procedures). Characterization of the PCR products derived from this experiment revealed a cDNA encoding a novel bHLH domain related to that of NeuroD (Figure 1B). This fragment was then used to isolate longer clones from an E13.5 rat DRG cDNA library. A 1.7 kb cDNA was obtained, encoding a predicted protein of 244 amino acid residues (Figure 1A). We named this gene *neurogenin* (*ngn*), based on its gain-of-function phenotype (see below). Within the bHLH domain, NEUROGENIN shows 67% identity to NeuroD and is closely related to other mammalian bHLH proteins including MATH2/Nex-1 (Bartholomä and Nave, 1994; Shimizu et al., 1995), KW8 (Kume et al., 1996), and MATH1 (Akazawa et al., 1995), as well as distantly related to Drosophila atonal (Figure 1B) (Jarman et al., 1993b).

Preliminary experiments indicated that mouse *ngn* mRNA caused ectopic neurogenesis when microinjected into Xenopus embryos (data not shown). To determine whether this phenotype reflected the existence of a Xenopus gene with similar functional characteristics, we screened a stage 17 (St. 17) Xenopus cDNA library at low stringency with a murine *ngn* cDNA probe. Several *ngn*-related cDNAs were obtained. This cDNA, which we have named Xenopus *ngn-related-1* (*X-ngnr-1*), encodes a polypeptide of 215 amino acids displaying 82% sequence identity to rat NEUROGENIN within the bHLH domain (Figure 1B). Although homology was more limited in the regions flanking the bHLH domain (Figure 1A), X-NGNR-1 appears most closely related to murine NEUROGENIN of all the cDNAs we isolated (based on the relative strength of its hybridization signal to the mouse probe [data not shown]).

Sequential Expression of *neurogenin* and *NeuroD* during Mouse and Xenopus Neurogenesis

A preliminary analysis of *ngn* mRNA expression in mouse embryos by in situ hybridization revealed that expression of this gene is apparently restricted to the nervous system (Figure 2, and data not shown). Within the nervous system, the expression of *neurogenin* is spatially or lineally restricted; for example it is expressed in the ventral half of the spinal cord, except for a narrow domain just below the roofplate (Figure 2A). In the peripheral nervous system, *ngn* mRNA is expressed in developing sensory but not in autonomic ganglia (Figure 2A, arrow, and data not shown). A more detailed characterization of *ngn* expression during murine neurogenesis will be reported elsewhere. Interestingly, a comparison of the expression of *ngn* and *NeuroD* expression on adjacent serial sections revealed that the two genes appear to be sequentially expressed in overlapping regions. In the ventral spinal cord, for example, *ngn* mRNA is expressed throughout the ventricular zone, in regions

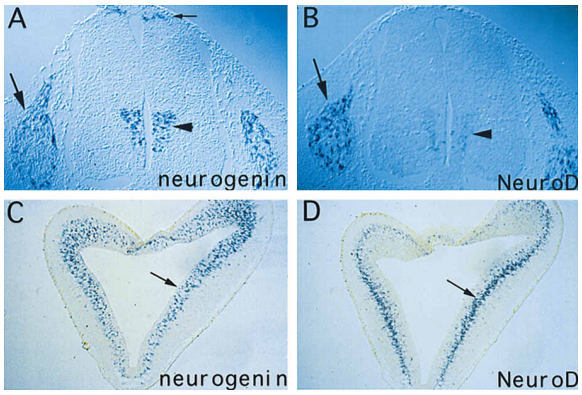

Figure 2. Sequential Expression of *neurogenin* and *NeuroD* in Rat Embryos

Adjacent transverse sections of E13.5 rat trunk spinal cord (A and B) and tangential sections of E14.5 forebrain (top part) (C and D). Arrows in (A) and (B) indicate dorsal root sensory ganglia; no signal was detected in sympathetic or other autonomic ganglia (data not shown). Note that *neurogenin* and *NeuroD* are expressed in similar regions (arrowheads, A and B), but that *NeuroD* is displaced lateral to the ventricular zone where NEUROGENIN is expressed (arrows, C and D).

where uncommitted progenitors are located, while *NeuroD* transcripts are expressed at the lateral border of the ventricular zone that contains migrating neuroblasts (Ramón y Cajal, 1995) (Figures 2A and 2B, arrowheads). However both genes show a similar dorso-ventral restriction in their domains of expression within the spinal cord (except that *NeuroD* is not expressed below the roof plate). A similar spatial segregation is seen in the mesencephalic-diencephalic region (Figures 2C and 2D, arrows). The lateral displacement of *ngn* and *NeuroD* mRNAs is also observed at E10.5–E11.5, the earliest stage at which expression of either gene can be detected; expression of both genes declines around E16 (data not shown). These data suggest that NEUROGENIN and NeuroD may function sequentially in common regions of the murine nervous system.

A similar spatial overlap but temporal displacement was found for the expression of *X-ngnr-1* and *XNeuroD* in Xenopus. For example, at St. 12, *X-ngnr-1* expression is observed in three broad patches within the neural plate (Figure 3A), that demarcate the medial, intermediate, and lateral territories where primary motorneurons, interneurons and sensory neurons, respectively, will later differentiate (Figure 3F, m, i, and l). Expression of *X-ngnr-1* within these patches is scattered. In contrast, no *XNeuroD* expression is yet detected at this stage (Figure 3B), nor have any primary neurons yet differentiated (Figure 3C). *XNeuroD* mRNA can be detected at St. 13.5, in narrow rows of cells (Figure 3E, m, i, and l) located within the three domains of primary neurogenesis that are apparent at St. 14 (Figure 3F, m, i, and l). At St.13–13.5, *X-ngnr-1* is expressed in a similar region of the neural plate but in many more cells than *XNeuroD* (Figure 3D). A similar sequential expression of *X-ngnr-1* and *XNeuroD* is seen in the trigeminal placode (Figures 3A and 3D–3F, arrows). Thus in Xenopus as in mouse, expression of *neurogenin/X-ngnr-1* preceeds but spatially overlaps that of *NeuroD/XNeuroD*.

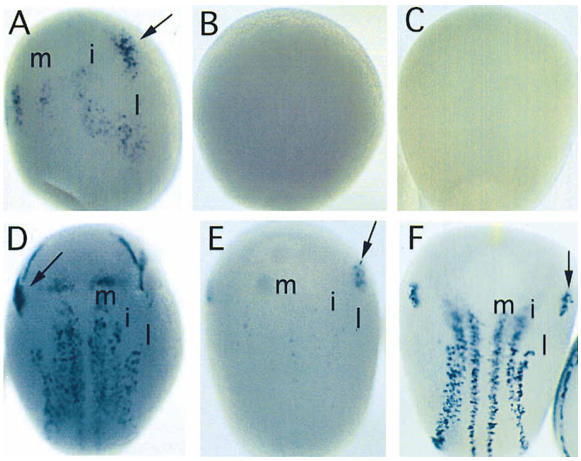

Figure 3. Sequential Expression of *X-ngnr-1* and *XNeuroD* mRNAs in Xenopus

A series of embryos at St. 12 (A–C) and St. 13–13.5 (D and E) and St. 14 (F) are shown, hybridized with probes for *X-ngnr-1* (A and D), *XNeuroD* (B and E) and *N-tubulin* (C and F). Note that *X-ngnr-1* is expressed prior to both *XNeuroD* and *N-tubulin*, in patches that define the three prospective territories of primary neurogenesis (A, m, i, and l). Arrows in (A) and (D)–(F) indicate trigeminal placode (A) or ganglia (D–F). Note also that at St. 13.5, the domain of *X-ngnr-1* expression within the m, i, and l regions of the neural plate is larger than the domain of either *XNeuroD* or *N-tubulin* expression.

Ectopic X-NGNR-1 Expression Induces Ectopic Neurogenesis and Endogenous NeuroD, but Not Vice-Versa

The observation that expression of *X-ngnr-1* temporally and spatially prefigures the expression of *XNeuroD*, taken together with the sequence homology between the two genes, led us to test whether expression of X-NGNR-1 like that of XNeuroD is sufficient to induce premature and/or ectopic primary neurogenesis. We therefore injected *X-ngnr-1* mRNA into one blastomere of two-cell stage embryos and examined the pattern of neurogenesis after further development by whole mount in situ hybridization using a probe for N-tubulin, a neuron-specific marker in Xenopus (Chitnis et al., 1995). The distribution of β-galactosidase activity translated from a coinjected *lacZ* mRNA was used to assess the overall distribution of the injected mRNAs in each individual embryo. Two types of negative controls were used: the uninjected side of the same embryo, and separate embryos injected only with *lacZ* mRNA.

Overexpression of *X-ngnr-1* mRNA caused extensive ectopic neurogenesis within the neural plate (100% of embryos examined; ≥ 100 embryos injected). At St. 13.5, for example, nearly every cell on the injected side of the embryo appeared to express N-tubulin, whereas on the uninjected side the three stripes of primary neurogenesis were clearly distinguishable (Figure 4A, cf. con versus inj sides); moreover within these stripes the distribution of neurons was more scattered than on the injected side. In addition to the increased extent of neurogenesis, the timing of neuronal differentiation was accelerated on the injected side, so that N-tubulin$^+$ cells were seen on the injected side at St. 12.5, a time at which no expression of this marker was detected on the contralateral control side (data not shown). Moreover, *X-ngnr-1*

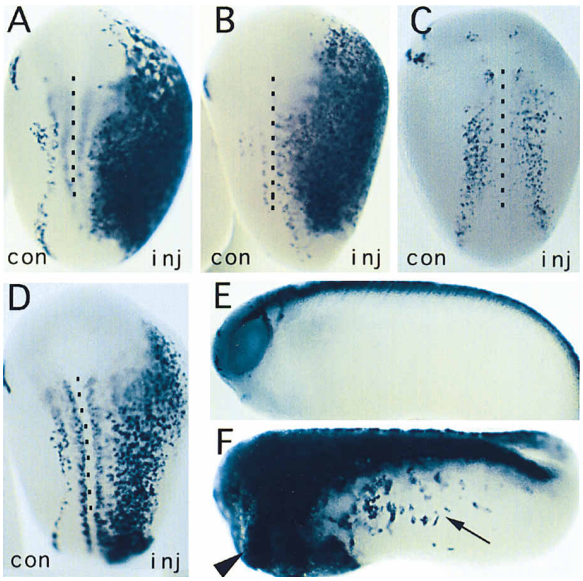

Figure 4. Induction of Endogenous *XNeuroD* Expression and Neurogenesis by Injection of *X-ngnr-1* mRNA

Ectopic neurogenesis was visualized in St. 13.5 (neural plate stage) embryos by whole mount in situ hybridization with an *N-tubulin* probe (A and D). Embryos were injected on one side (inj) with either *X-ngnr-1* (A and B) or *XNeuroD* (C and D) synthetic RNA. X-NGNR-1 induced ectopic neurogenesis (A) as well as expression of endogenous *XNeuroD* (B); XNeuroD also induced ectopic neurogenesis (D) but did not induce *X-ngnr-1* (C). No ectopic expression of N-tubulin was observed in *lacZ* RNA-injected embryos (E). At the tail bud stage (F), ectopic neurogenesis is induced by X-NGNR-1 in the skin (arrow) and the entire anterior region (arrowhead). A similar phenotype is seen in XNeuroD-injected embryos at this stage (Lee et al., 1995; and data not shown).

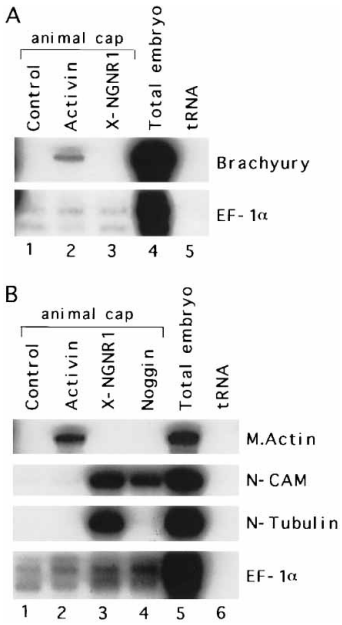

Figure 5. X-NGNR-1 Induces Expression of Neural and Neuronal Markers in the Absence of Mesodermal Markers, in Animal Caps

Neither of two mesodermal markers, Xenopus *brachyury* (A) and muscle-specific *actin* (*M.Actin*) (B), is induced in animal caps cultured from embryos injected with *X-ngnr-1* RNA (A and B, lanes 3), whereas both are induced in positive control caps cultured with activin (A and B, lanes 2). In contrast, X-NGNR-1 induces expression of both *NCAM* and *N-tubulin* (B, lane 3). For comparison, caps injected with *noggin* RNA express *NCAM* but not *N-tubulin* (B, lane 4). Elongation factor-1α (EF-1α) serves as a control for RNA loading (Ferreiro et al., 1994). "Total embryo" indicates total RNA from embryos of the equivalent stage (St. 11 for [A], St. 15–16 for [B]); "tRNA" indicates carrier tRNA control, while "Control" animal caps represents RNA from animals caps cultured from uninjected embryos.

also caused ectopic neuronal differentiation in regions of nonneurogenic ectoderm that flank the neural plate. The consequence of this can be most easily observed at St. 24–26 (tail bud stage), where supernumerary neurons are observed within the epidermis (Figure 4F, arrow). Extensive ectopic neurogenesis was also observed in the most anterior part of the embryo, where for example the eye was missing and replaced by amorphous neural tissue (Figure 4F, arrowhead). In these respects, the phenotype of *X-ngnr-1* mRNA-injected embryos appeared similar or identical to that previously reported for *XNeuroD* (Lee et al., 1995) (see also Figure 4D).

The fact that overexpression of *X-ngnr-1* yielded an *XNeuroD*-like phenotype, coupled with the fact that endogenous *X-ngnr-1* expression temporally precedes and overlaps that of *XNeuroD*, suggested that the latter might be a target of transcriptional activation by the former. To test this, embryos injected with *X-ngnr-1* mRNA were hybridized with an *XNeuroD* probe. A massive, ectopic induction of endogenous *XNeuroD* mRNA was observed in *X-ngnr-1* mRNA-injected embryos (100% of embryos examined; > 50 embryos injected) (Figure 4B, inj side). The extent of ectopic expression was similar to that observed with an N-tubulin probe (Figure 4A). By contrast, injection of *XNeuroD* mRNA did not increase the expression of endogenous *X-ngnr-1* mRNA (Figure 4C; cf. con versus inj sides), although it did induce ectopic neurogenesis as previously reported (Lee et al., 1995) (Figure 4D). These data suggest that the neurogenic phenotype of *X-ngnr-1* mRNA–injected embryos may reflect an induction of endogenous *XNeuroD* and suggest that the sequential expression of these two genes during Xenopus neurogenesis reflects a unidirectional cascade in which the former induces transcription of the latter.

To ensure that the ectopic neurogenesis promoted by injection of *X-ngnr-1* RNA was not a secondary consequence of induction of mesodermal tissue, we performed animal cap experiments. Animal caps from embryos injected in both blastomeres with various RNAs at the 2-cell stage were dissected and allowed to develop in vitro, after which time they were assayed for expression of various marker mRNAs by RNase protection (Ferreiro et al., 1994). As expected from the whole mount in situ analysis, injection of *X-ngnr-1* RNA caused induction of expression of *N-tubulin* mRNA (Figure 5B, lane 3). No mesodermal induction was detected under these conditions, by criteria of expression of either muscle-specific *actin* mRNA (Figure 5B, lane 2) (Ferreiro et al., 1994) or Xenopus *brachyury* (Figure 5A, lane 3), both of which markers were induced by culturing the caps in activin as a positive control (Figures 5A and 5B, lanes 2). These data indicate that the promotion of neurogenesis by X-NGNR-1 is not an indirect result of mesoderm

induction. Moreover they demonstrate that X-NGNR-1 is able to directly convert naive nonneurogenic ectoderm to neural tissue, since in animal caps cultured on their own no neural induction occurs (Figure 5B, lane 1) and the tissue instead develops into epidermis (Gurdon, 1987).

The effect of X-NGNR-1 was also compared to that of noggin (Smith and Harland, 1992) in the animal cap experiments. Noggin, which promotes neural induction (Lamb et al., 1993) induced expression of *NCAM* (Figure 5B, lane 4), a marker of undifferentiated neural tissue (Kintner and Melton, 1987), but not of *N-tubulin*, a marker of differentiated neurons (Oschwald et al., 1991; Chitnis et al., 1995). X-NGNR-1, by contrast, induced expression of both *NCAM* and *N-tubulin* mRNAs (Figure 5B, lane 3). These data are consistent with the idea that noggin promotes neuralization but is insufficient for neuronal differentiation (Ferreiro et al., 1994), while X-NGNR-1 promotes both neuralization and consequent neuronal differentiation.

X-NGNR-1 Expression Precedes, and Can Activate Expression of, X-Delta-1

The foregoing data indicated that *X-ngnr-1* is expressed earlier than *XNeuroD* and is capable of inducing expression of *XNeuroD* as well as of promoting ectopic neurogenesis. Thus, like NeuroD, X-NGNR-1 can exert a neuronal determination function when overexpressed. But can X-NGNR-1 normally play this role in vivo? To address this question, we examined the timing of *X-ngnr-1* expression relative to that of *X-Delta-1*. In Xenopus as in Drosophila, *X-Delta-1* encodes a lateral inhibitory ligand that controls a choice between neuronal and nonneuronal fates (Chitnis et al., 1995). By definition, therefore, at the time Delta is first expressed this choice has not yet been made.

During early gastrulation (St. 10.5), *X-ngnr-1* mRNA can be detected at the lateral margins of the prosepctive neural plate (Figure 6A, arrow). At this stage, *X-Delta-1* mRNA is not yet expressed in this region, although it is detected in an area adjacent to the blastopore (Figure 6B, arrowhead). By midgastrulation (St. 11.5), both *X-ngnr-1* (Figure 6C) and *X-Delta-1* (Figure 6D) mRNAs can be detected in three distinct patches within the neural plate (Figures 6C and 6D, m, i, and l), prefiguring the regions where primary neurogenesis will occur. Within these regions, the domain of *X-ngnr-1* expression appears to encompass that of *X-Delta-1*. At the same stage, *X-ngnr-1* expression can be observed in the presumptive trigeminal placode (Figure 6C, arrow), where *X-Delta-1* mRNA is not yet detectable (Figure 6D, arrow). At neither of these stages is expression of *XNeuroD* detected (data not shown). These data indicate that expression of *X-ngnr-1* precedes that of *X-Delta-1* in both the CNS (neural plate) and the PNS (trigeminal placode), whereas *XNeuroD* is not expressed until after *X-Delta-1*.

In Drosophila, the proneural genes (*achaete-scute*) activate expression of *Delta* (Hinz et al., 1994; Kunisch et al., 1994). The fact that expression of *X-ngnr-1* precedes but spatially overlaps that of *X-Delta-1* suggested, therefore, that the former might be capable of activating expression of the latter. In support of this

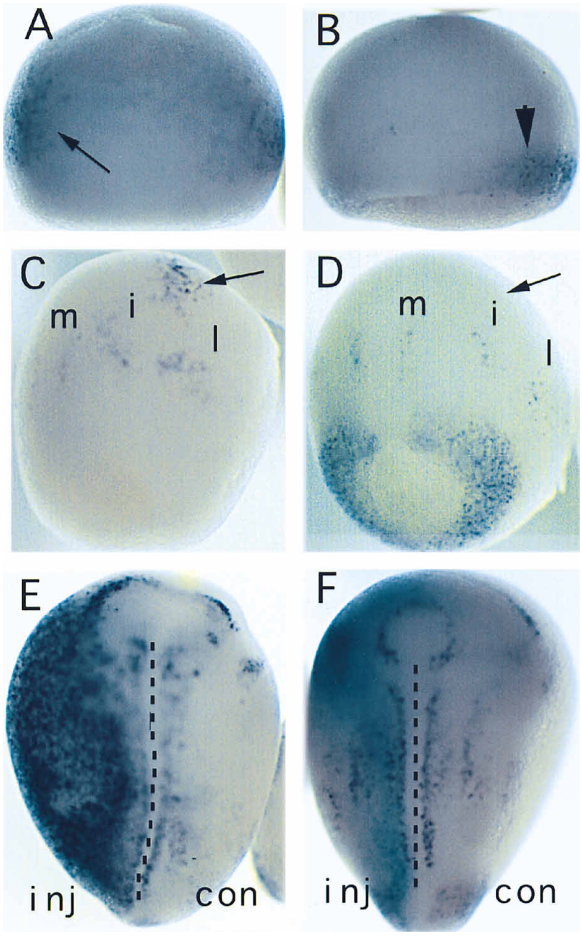

Figure 6. Expression of *X-ngnr-1* Preceeds and Induces That of *X-Delta-1*

(A–D) in situ hybridization of embryos at St. 10.5–11.0 (A and B) and St. 11.5 (C and D) showing that expression of *X-ngnr-1* (A and C) precedes that of *X-Delta-1* (B and D), in both the prospective neural plate (A, arrow) and trigeminal placode (C and D, arrows). At St. 11.5, the *X-Delta-1*-expressing cells within the medial, intermediate, and lateral (m, i, and l) regions of the neural plate (D) overlap the domains of *X-ngnr-1*-expressing cells (C).

(E) injection of *X-ngnr-1* mRNA induces ectopic expression of endogenous *X-Delta-1* mRNA on the injected (inj) side of a St. 13.5 embryo.

(F) control injection of *lacZ* mRNA alone has no effect on *X-Delta-1* expression.

idea, injection of synthetic *X-ngnr-1* RNA induced ectopic expression of endogenous *X-Delta-1* mRNA (100% of embryos examined; $\geq$ 50 embryos injected) (Figure 6E, inj side), whereas control injections of *lacZ* mRNA had no such effect (Figure 6F, inj side). Thus, like the proneural genes in Drosophila, *X-ngnr-1* can activate expression of a lateral inhibitory ligand that controls a choice between neuronal and nonneuronal fates, within a group of developmentally equivalent cells.

XNotch1^{ICD} Inhibits Both the Expression and Function of X-NGNR-1

In Drosophila, the expression of *achaete-scute* is restricted to sensory organ precursor cells by lateral inhibitory interactions mediated by Notch and Delta (Ghysen

et al., 1993). This suggested by analogy that the scattered expression of *X-ngnr-1* might reflect its restriction to subsets of neural precursors by lateral inhibition. Three different experiments support this idea. First, injection of a dominant-active form of Notch (Struhl et al., 1993) (the intracellular domain, or ICD), which inhibits primary neurogenesis (Figure 7C; cf. arrowhead [inj side] versus arrow [control side]), also repressed the expression of endogenous *X-ngnr-1* mRNA (18/18 embryos tested) (Figure 7A; cf. arrowhead versus arrow); in contrast control injections of *lacZ* mRNA had no such effect (data not shown). Conversely, blocking lateral inhibition by injection of a dominant-negative form of *X-Delta-1* (*X-Delta-1*^{Stu}; Figure 7D, arrowhead) (Chitnis et al., 1995) caused an apparent increase in the density of strongly *X-ngnr-1*-positive cells, as well as a slight expansion of the *X-ngnr-1*-positive domain in 60% of injected embryos (31/50 embryos tested) (Figure 7B; cf. arrowhead versus arrow). In contrast such an effect was not seen in control *lacZ*-injected embryos (except in one isolated case out of 39 embryos examined). This second result suggested that the density of *X-ngnr-1*-expressing cells within each domain of primary neurogenesis is normally limited by lateral inhibition. The fact that exogenous Notch^{ICD} is, moreover, able to strongly suppress endogenous *X-ngnr-1* expression (Figure 7A) supports the idea that this lateral inhibition is mediated, at least in part, by endogenous *X-Notch* genes.

To determine whether Notch-mediated signaling can inhibit the function as well as the expression of *X-ngnr-1*, exogenous *X-ngnr-1* mRNA was coinjected with either *lacZ* mRNA or *lacZ* mRNA plus *Notch*^{ICD} mRNA. An inhibition of *X-ngnr-1*-promoted ectopic neurogenesis was observed with high penetrance (27/29 embryos tested) (cf. Figures 7E and 7F, arrowheads). However, within the injected side of the experimental embryos, the inhibition of neurogenesis showed variable expressivity and appeared most complete in those regions that received the highest amount of the coinjected mRNAs (as determined by X-Gal staining; Figure 7F, cf. arrowhead versus arrow on inj side). These data indicate that the function, as well as the expression, of X-NGNR-1 is sensitive to inhibition by Notch^{ICD}, at least above a certain threshhold level of the latter (see below).

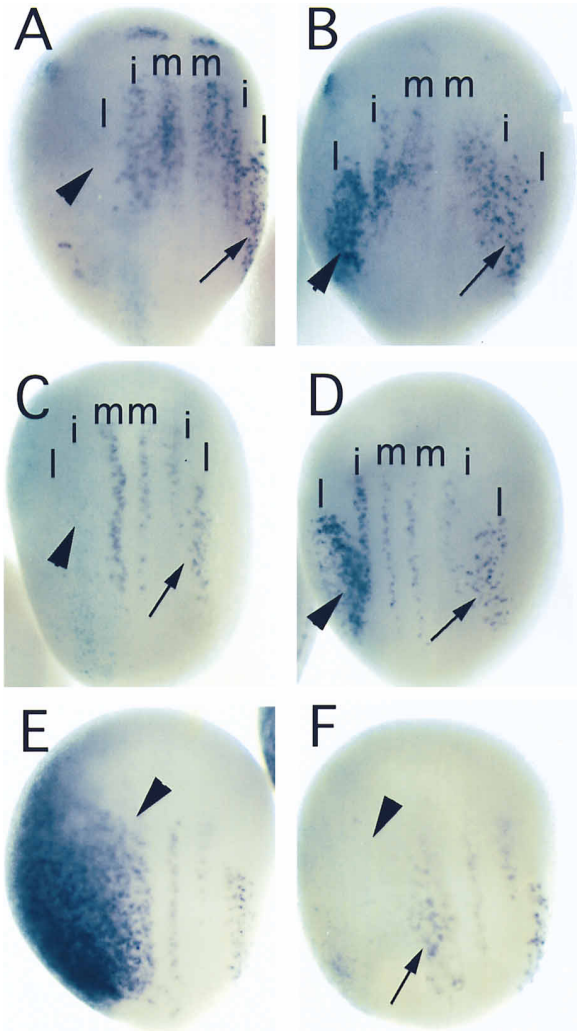

Figure 7. Expression and Function of *X-ngnr-1* Are Restricted to Subsets of Cells by Lateral Inhibition Mediated by X-Notch and X-Delta-1

(A and B) opposite effects of injection of constitutively active *X-Notch*^{ICD} RNA (A) and dominant negative *X-Delta-1*^{Stu} RNA (B) on expression of endogenous *X-ngnr-1* mRNA. In both cases, only the lateral stripe of *X-ngnr-1* expression is affected (arrowhead, A and B; compare to arrow indicating uninjected side), due to restricted distribution of the injected RNA as defined by the lacZ tracer (light blue staining). *X-Notch*^{ICD} RNA represses expression of *X-ngnr-1* (A, arrowhead), while *X-Delta-1*^{Stu} RNA increases the density of *X-ngnr-1*-expressing cells (B, arrowhead). A similar effect on *N-tubulin* expression is seen in animals expressing *X-Notch*^{ICD} (C) or *X-Delta-1*^{Stu} RNA (D) in the lateral stripe (light blue stain, C). In other embryos exhibiting a different distribution of the injected RNAs, the development of the medial or lateral stripes was similarly affected (data not shown). (E and F) suppression of X-NGNR-1 neurogenic function by Notch^{ICD}. Blastomeres were coinjected either with *X-ngnr-1* plus *lacZ* RNAs, (E) or in addition with *X-Notch*^{ICD} RNA (F), and hybridized with *N-tubulin* probes at St. 13.5. Note that ectopic neurogenesis is inhibited by Notch^{ICD} (F, arrowhead), in regions receiving the highest levels of injected RNAs (light blue staining), but that some ectopic neurogenesis is still seen in other parts of the injected side (F, arrow). By contrast ectopic neurogenesis on the injected side of embryos receiving X-NGNR-1 plus lacZ is relatively uniform (E, arrowhead).

Discussion

We have described the isolation of a novel bHLH gene, *neurogenin*, which displays many of the characteristics expected of a vertebrate neuronal determination gene. Xenopus NEUROGENIN-RELATED-1 (X-NGNR-1) can cause nonneurogenic ectodermal cells to differentiate to neurons when ectopically expressed. Endogenous *X-ngnr-1* mRNA is expressed before and during the time that the choice of the neuronal fate is being made, in the prospective territory of primary neurogenesis. *X-ngnr-1* expression within this territory is, moreover, scattered, and our data suggest that this reflects a restriction of its expression to subsets of neuronal precursors by lateral inhibition. Specifically, we have shown that X-NGNR-1 activates expression of *X-Delta-1*; that its expression and function can be inhibited by Notch-mediated signaling; and that dominant-negative X-Delta-1 can increase

NEUROGENIN BOTH ACTIVATES, AND IS INHIBITED BY, THE LATERAL INHIBITION MACHINERY

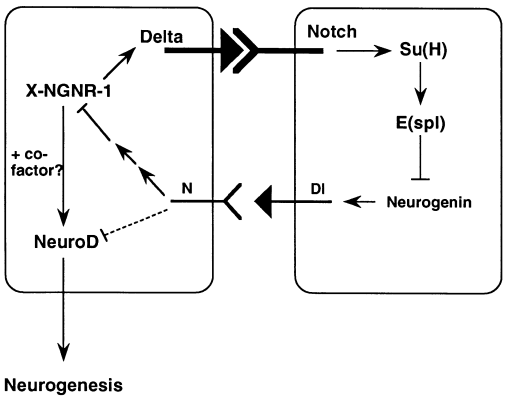

Figure 8. Model for the Role of X-NGNR-1 and XNeuroD in Lateral Inhibition and Neuronal Determination

The model draws heavily on analogies to Drosophila (Ghysen et al., 1993). X-NGNR-1 initially activates expression of X-Delta-1 and is inhibited by signaling through X-Notch (left side of diagram). The inhibition of *X-ngnr-1* expression may be mediated by Suppressor of Hairless [Su(H)] and enhancer of split [E(spl)] proteins, by analogy to Drosophila (Artavanis-Tsakonas et al., 1995). Expression of X-NGNR-1 above a certain threshhold, or in the presence of a hypothetical cofactor (see text), leads to expression of XNeuroD and neuronal differentiation in a manner insensitive to inhibition by Notch signaling (dashed blunt arrow) (Chitnis and Kintner, 1996).

the density of X-NGNR-1-expressing cells. We have also shown that X-NGNR-1 activates expression of a downstream bHLH gene, *XNeuroD*, which in turn promotes neuronal differentiation. Together, these data suggest that X-NGNR-1 functions at an early stage in neuronal determination to control the choice of a neuronal fate by uncommitted cells of the neural plate, perhaps analagously to the proneural genes in Drosophila (Figure 8).

X-NGNR-1 Interacts Genetically with the Lateral Inhibition Machinery of Xenopus

The initial expression of *X-ngnr-1* occurs in three territories (medial, intermediate, and lateral) that demarcate the domains in which primary neurogenesis will eventually occur (Chitnis et al., 1995). Our data suggest that within these territories, lateral inhibition restricts *X-ngnr-1* expression to a limited number of neuronal precursor cells. As X-NGNR-1 approaches a threshhold level in some precursors, it activates expression of X-Delta-1; X-Delta-1 in turn represses expression of X-NGNR-1 in neighboring cells, preventing them from acquiring a neuronal fate (Figure 8). As X-NGNR-1 expression becomes restricted to presumptive neuronal precursors, it leads (directly or indirectly) to expression of XNeuroD (Figure 8) and execution of the neuronal differentation program. Thus the genetic circuitry linking *X-ngnr-1* and the lateral inhibition machinery is very similar to that which links the proneural and neurogenic genes in Drosophila (Ghysen et al., 1993). One apparent difference, however, is that *X-ngnr-1* expression appears scattered from the very earliest stages, whereas *achaete-scute* expression within the proneural clusters initially appears relatively uniform (Cubas et al., 1991); the reason for this difference is not yet clear.

The observation that *X-ngnr-1* both activates, and is inhibited by, the lateral inhibitory circuitry raises the paradox of how a neurogenic phenotype can nevertheless be obtained by overexpressing this gene. The simplest answer is that the injected *X-ngnr-1* RNA bypasses X-Notch-mediated transcriptional repression (Figure 7B) of the endogenous *X-ngnr-1* gene. However, our data suggest that Notch is also able to inhibit the function of X-NGNR-1 translated from exogenous RNA (Figure 7F), either by a posttranscriptional mechanism or by inhibiting expression of X-NGNR-1 target genes. Nevertheless, this inhibition appears to require high levels of X-Notch^{ICD} expression, being strongest in those regions that contain the highest level of coinjected RNAs (Figure 7F). In the situation where *X-ngnr-1* RNA alone is injected (Figure 7E), the level of endogenous X-Notch signaling may be simply insufficient to override the large amounts of exogenous X-NGNR-1 protein.

Relationship of XASH-3 and X-NGNR-1

Xash3 is the only other neural bHLH gene that is known to be expressed as early as *X-ngnr-1* in the neural plate (Zimmerman et al., 1993). The available evidence, however, more clearly identifies *X-ngnr-1* as a vertebrate analog of the Drosophila proneural genes during primary neurogenesis. First, while the expression of *X-ngnr-1* correlates extremely well with the three domains of the neural plate where primary neurons form, *Xash-3* is expressed in an "intermediate" zone of the neural plate that may in fact correspond to the sulcus limitans (Zimmerman et al., 1993). Second, the activity of XASH3 in ectopic expression studies appears to be different from that of X-NGNR-1. For instance, ectopic expression of XASH-3 at high levels causes an expansion of neural tissue (Ferreiro et al., 1994; Turner and Weintraub, 1994), a phenotype never observed with X-NGNR-1. While ectopic expression of XASH-3 can also induce ectopic neuronal differentiation, it does so only when lateral inhibition is also blocked using the dominant-negative X-Delta-1, and only then within the posterior neural plate (Chitnis and Kintner, 1996). X-NGNR-1 does not have similar restrictions in its activity and can promote neurogenesis anteriorly and outside the neural plate. Finally, although exogenous XASH-3 is sensitive to lateral inhibition mediated by X-Notch-1 and X-Delta-1 (Chitnis and Kintner, 1996), there is no evidence that endogenous *Xash-3* expression is normally regulated by such inhibition. Thus, *X-ngnr-1* fulfills more of the criteria expected for a gene whose activity defines the "proneural" domains wherein primary neurons arise in the neural plate. Whether XASH-3 also contributes to primary or secondary neurogenesis, but in different cells or at a different step in the pathway as X-NGNR-1, remains to be determined.

X-NGNR-1 Performs Two Distinct and Temporally Separated Functions

In our experiments, injection of *X-ngnr-1* mRNA results in the induction of both *X-Delta-1* and *XNeuroD*. During

normal development, however, expression of *XNeuroD* is delayed relative to that of *X-Delta-1*. How is the sequential expression of these two putative target genes of X-NGNR-1 normally achieved? One possibility is that *X-Delta-1* requires a lower threshhold of X-NGNR-1 activity than *XNeuroD* to be activated and that it takes time for X-NGNR-1 to accumulate to levels sufficient to induce *XNeuroD*. Another explanation is that a cofactor is required together with X-NGNR-1 to activate *XNeuroD* and that expression of this cofactor is delayed (Figure 8). Precedent for such a temporal separation of transcription factor functions is found during mother–daughter segregation in yeast, where the Swi5 protein acts first to activate *ASH1* expression (which in turn blocks Swi5p function in daughter cells; Bobola et al., 1996; Sil and Herskowitz, 1996), and later to activate *HO* expression in mother cells. The delay in *HO* activation by Swi5p reflects an induction of the necessary coactivators Swi4p and Swi6p (Amon, 1996). Interestingly, in both Xenopus neurogenesis and yeast the temporal separation provides a time window for these determinative factors (Swi5p or X-NGNR-1) to provide an inhibitory signal to neighboring or daughter cells, while allowing them to later promote an alternative fate cell-autonomously.

Determination versus Differentiation Genes

We have documented a sequential expression of *ngn* and *NeuroD* mRNAs during both murine and Xenopus neurogenesis and, in the latter system, have further demonstrated a unidirectional functional cascade for these genes. Thus in Xenopus neurogenesis, as in mammalian skeletal myogenesis and Drosophila neurogenesis, structurally related bHLH proteins function in cascades (Jan and Jan, 1993). The timing and location of *NeuroD* expression have previously been suggested to reflect a function for this gene in neuronal differentiation (Lee et al., 1995), perhaps analogous to that of *myogenin* during muscle development (for review, see Weintraub, 1993). Our data indicate that *X-ngnr-1* functions in Xenopus neuronal determination, analogous to the roles of *MyoD* and *myf5* during murine myogenesis. (The fact that the name *NeuroD* has already been assigned to a neurogenic differentiation gene [Lee et al., 1995] makes it difficult to maintain a strict analogy between the nomenclature for neurogenic and myogenic bHLH proteins; furthermore, since the naming of myogenic bHLH factors reflects historical rather than functional criteria we feel such an analogy is best avoided.) Whether mouse *neurogenin* similarly functions in neuronal determination is not yet clear and awaits the results of gene-targeting experiments, which are currently in progress.

What is different about determination and differentiation bHLH factors? The similar actions of these proteins in gain-of-function experiments suggest that they may differ only in the time and place of their expression or in the downstream genes they regulate (Jan and Jan, 1993). Indeed, *myogenin* can functionally replace *myf5* during murine myogenesis in vivo (Wang et al., 1996). On the other hand, these genes may possess intrinsic functional differences that have so far escaped detection. For example, it has been proposed that muscle differentiation factors are less sensitive to inhibitors than are determination factors (Weintraub, 1993). However, such a differential sensitivity to inhibitors has not been demonstrated in myogenesis, although MyoD function can be inhibited by Notch^{ICD} (Kopan et al., 1994). X-NGNR-1 and XNeuroD do not appear differentially sensitive to inhibition by coinjected X-Notch^{ICD}, in side-by-side comparisons (Ma et al., unpublished data). It is nevertheless possible that these genes are differentially sensitive to direct inhibition by Notch signaling, but at the transcriptional level; however, this is currently difficult to test since inhibition of X-NGNR-1 expression by Notch^{ICD} indirectly prevents expression of XNeuroD. A differential sensitivity of XASH-3 and XNeuroD to lateral inhibition has been demonstrated (Chitnis and Kintner, 1996), but as mentioned earlier it is not clear how XASH-3 fits into the X-NGNR-1–XNeuroD cascade.

The determination function proposed for *X-ngnr-1* may, therefore, primarily reflect the developmental context in which this gene is expressed. Expression of *X-ngnr-1* in neuroectodermal cells confers competence to participate in the process of lateral inhibition, via induction of *X-Delta-1* expression. However, this state of competence is insufficient to allow neuronal differentiation, precisely because the expression and function of X-NGNR-1 are sensitive to lateral inhibition. While increased expression of X-NGNR-1 imposes a bias towards the neuronal state, this state is unstable until subsequent events render the cell insensitive to further inhibition. The nature of the events that stabilize the neuronal state and commit the cell irreversibly to neuronal differentiation remains to be established.

Experimental Procedures

Isolation of *neurogenin* and *X-ngnr-1* cDNA Clones

Random-primed cDNA template prepared from E13.5 rat embryonic DRGs was subjected to 40 cycles of PCR (1′ at 94°C; 2′ at 45°C; 2′ at 65°C). The 5′ primers are an equimolar mixture of 5′CGCGGATCC(A/C)GNAA(C/T)GA(A/G)(A/C)G(G/C/T)GA(A /G)(A/C)G3′ and 5′CGCG GATCCGCNAA(C/T)GC(A/C/T)(A/C)G(G/CT)GA(A/G)(A/C)G3′, which are derived from RNERER (sequence based on the MASH1 subfamily of bHLH proteins) and ANARER (sequence based on the NeuroD subfamily), respectively, and contain a BamH1 site at the 5′ end. The 3′ primers are 5′CCGGAATTCGT(T/C)TC(A/G/C)A(T/C)(T/C)T T(A/G)CT(A/C /G)A(A/G/T)(T/C)TT3′ and 5′CCGGAATTCGT(T/C)T C(A/G/C)A(T/C)(T/C)TT(A/G/T)GA(A /C/G)A(A/G/T)(T/C)TT3′, both of which are the reverse translation of K(L/M)SK(V/I)ET and contain a EcoRI site at the 5′ end [the alternative Ser codons TCN and AG(C/T) are synthesized as two separate oligos and then mixed, yielding 6-fold degeneracy at this position rather than 16-fold degeneracy for the single sequence (A/T)(G/C)N]. The 130 bp PCR product was purified from a polyacrylamide gel, cloned into M13mp19 (New England Bio-labs), and sequenced. The *ngn* PCR product was then used to screen a lambda ZAP cDNA library prepared from rat E13.5 DRG (Saito et al., 1995). The isolated 1.2 kb and 1.7 kb cDNA clones were sequenced on both strands by Caltech sequencing core facility. The 1.7kb cDNA fragment was then used to screen a mouse lambda-2 129 genomic library (a gift of Z. Chen). From a 17 kb positive clone, a 2.0 kb fragment hybridizing to rat *ngn* cDNA was isolated and sequenced, which contains an open reading frame (ORF) differing from the rat one at six positions (data not shown). The 2.2kb-*X-ngnr-1a* cDNA was isolated by screening a Xenopus St. 17 cDNA library (Kintner and Melton, 1987) at low stringency using the rat cDNA as probe. An isoform of *X-ngnr-1a* called *X-ngnr1b* was also isolated from this screen (data not shown). *X-ngnr-1a* and *X-ngnr-1b* show the same expression patterns and

phenotypes in mRNA injection (data not shown) and are referred to collectively as X-NGNR-1 in the text.

In Situ Hybridization

Nonradioactive in situ hybridization to frozen sections of mouse embryos was performed as previously described (Birren et al., 1993). Whole-mount in situ hybridization was performed essentially as described (Chitnis et al., 1995) using digoxigenin-labeled antisense probes for *X-ngnr-1*, *XNeuroD* (Lee et al., 1995), *X-Delta-1*, and *N-tubulin* (Chitnis et al., 1995), with the following modification. At the step of developing the alkaline phosphatase reaction using NBT/BCIP substrates, 0.45 μl rather than 4.5 μl of NBT stock solution (75 mg/ml in 70% dimethyl formamide) was added to each 1 ml of staining buffer. This change reduced background staining and improved visualization of low abundance mRNAs.

Ectopic Expression in Xenopus Embryos

The *X-ngnr-1* open reading frame was cloned in-frame into the EcoR1 site of the vector pMT-CS2 (Turner and Weintraub, 1994). Capped *X-ngnr-1* mRNA was transcribed using SP6 RNA polymerase as decribed (Kintner and Melton, 1987). *X-ngnr-1* mRNA (0.4 pg) was coinjected with tracer *lacZ* mRNA (0.4 pg) into one blastomere of two-celled embryos (Coffman et al., 1993). *Notch*^{ICD} (0.4 pg) and *X-Delta-1*^{Stu} (1.0 pg) RNAs were prepared as described (Chitnis et al., 1995). Injection of *LacZ* mRNA alone (0.4 pg) was done as a control. Histochemical staining for β-galactosidase was performed to visualize the distribution of injected mRNAs. Embryos were collected at the neural plate stage (St. 13–14) or tail-bud stage (St. 26) and subjected to in situ hybridization using probes as indicated in the figure legends. Animal cap assays were performed as described previously (Ferreiro et al., 1994); 0.4 pg/embryo of *X-ngnr-1* or *noggin* RNAs were injected for the animal cap assays. For caps collected at St. 11, 100 pM activin was used; for caps collected at St. 16, 200 pM activin was used.

Acknowledgments

We are grateful to Andres Collazo and Suzanne Cohen-Cory (Fraser laboratory, Caltech) for providing Xenopus embryos for in situ analysis and to Wenying Shou (Dunphy lab) for providing St. 12 and 22 Xenopus embryo cDNAs. We thank Amy Greenwood and Alice Paquette for assistance in dissecting embryonic DRGs, Andy Groves for performing pilot in situ hybridization experiments, Steve Padilla and Ling Wang for technical assistance, and Jacqueline Lee for providing the Xenopus *NeuroD* cDNA probe. We thank S. Fraser, B. Wold, R. Axel, and K. Zinn for their comments on the manuscript, and the Seaver Institute for support. D. J. A. is an Associate Investigator of the Howard Hughes Medical Institute. C. K. was supported by a grant from the NIH.

Received June 24, 1996; revised August 16, 1996.

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GenBank Accession Numbers

Nucleotide sequences of the rat, mouse, and two isoforms of the Xenopus *neurogenin* have been deposited in GenBank database, with accession numbers U67777, U67776, U67778, and U67779.