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Dlx proteins position the neural plate border and determine adjacent cell fates

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Summary

The lateral border of the neural plate is a major source of signals that induce primary neurons, neural crest cells and cranial placodes as well as provide patterning cues to mesodermal structures such as somites and heart. Whereas secreted BMP, FGF and Wnt proteins influence the differentiation of neural and non-neural ectoderm, we show here that members of the Dlx family of transcription factors position the border between neural and non-neural ectoderm and are required for the specification of adjacent cell fates. Inhibition of endogenous Dlx activity in *Xenopus* embryos with an EnR-Dlx homeodomain fusion protein expands the neural plate into non-neural ectoderm tissue whereas ectopic activation of Dlx target genes inhibits neural plate differentiation. Importantly, the stereotypic pattern of border cell fates in the adjacent ectoderm is re-established only under conditions where the expanded neural plate abuts Dlx-positive non-neural ectoderm. Experiments in which presumptive neural plate was grafted to ventral ectoderm reiterate induction of neural crest and placodal lineages and also demonstrate that Dlx activity is required in non-neural ectoderm for the production of signals needed for induction of these cells. We propose that Dlx proteins regulate intercellular signaling across the interface between neural and non-neural ectoderm that is critical for inducing and patterning adjacent cell fates.

Keywords

Dlx; Neural crest; Neural induction; *Xenopus*; *hairy2a*; *slug*; *snail*; *msx*

Introduction

The juxtaposition of presumptive neural plate and epidermis forms a signaling center responsible for the stereotypic pattern of dorsal neurons in the neural plate (including lateral primary neurons in amphibians and fish), roofplate, neural crest cells and cranial placodes (Baker and Bronner-Fraser, 2001; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). In addition, signals from this region influence mesodermal structures such as somites

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(Lassar and Munsterberg, 1996; Pourquie, 2000) and heart (Raffin et al., 2000; Sarasa and Climent, 1987). Although considerable progress has been made recently in the molecular characterization of neural-inducing factors, relatively little is known about the molecules that determine the site of the border between neural plate and epidermis.

Diffusible proteins such as BMP, Wnt and FGF isoforms play an important role in patterning neural and non-neural ectoderm along the mediolateral axis in *Xenopus* and zebrafish. BMP antagonists, such as chordin and noggin, initiate neural induction in the dorsal ectoderm while BMP signaling in the ventral ectoderm represses neural fates and promotes epidermal differentiation (Brewster et al., 1998; Hemmati-Brivanlou and Melton, 1997; Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 1997; Sasai et al., 1994; Smith et al., 1993; Wilson and Hemmati-Brivanlou, 1995). One model for neural plate border formation suggests that the ectoderm is differentially patterned by threshold levels of BMP signaling: high levels induce epidermal fates, low or absent signaling permits neural differentiation while intermediate levels induce border fates (Marchant et al., 1998; Morgan and Sargent, 1997; Nguyen et al., 1998; Wilson et al., 1997). However, studies showing that not all aspects of neural and neural crest induction are recapitulated by modulating BMP levels in non-neural tissues (e.g. LaBonne and Bronner-Fraser, 1998) have prompted an examination of additional factors that might synergize with BMP to control cell fate at the border region of late blastula embryos (Bachiller et al., 2000; Klingensmith et al., 1999; Streit and Stern, 1999). In particular, Wnt and FGF isoforms appear critical for induction of neural crest and cranial placodal cells (Adamska et al., 2000; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Phillips et al., 2001; Saint-Jeannet et al., 1997; Streit and Stern, 1999; Vallin et al., 2001; Wilson et al., 2001).

Several transcription factors have been proposed to influence neural/non-neural ectodermal patterning. For example, Xiro, Xash-3 and Zic family members are induced by pre- to early gastrula stage dorsalizing and neuralizing signals, such as noggin, and with time their expression becomes localized to the future neural plate (Gomez-Skarmeta et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 1997; Turner and Weintraub, 1994). Overexpression of these factors in whole embryos expands the neural plate. Where examined, however, either an abnormally broad domain of neural crest marker expression overlaps the ectopic neural plate region or the neural crest markers are lost (Gomez-Skarmeta et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 1997; Turner and Weintraub, 1994), indicating that normal border signaling has not occurred. In contrast, the Dlx family of transcription factors represent a class of proteins that, along with Msx-1 and PV.1, inhibit neural plate differentiation when overexpressed in *Xenopus* (Ault et al., 1997; Feledy et al., 1999; Pera and Kessel, 1999; Suzuki et al., 1997). Prior to gastrulation, transcripts encoding these factors are expressed diffusely in the ectoderm but subsequently become excluded from the developing neural plate (Luo et al., 2001a). At least one Dlx family member, Dlx3, has been shown to be regulated by BMP and canonical Wnt/ β -catenin signaling that provide ventralizing signals in pre-gastrula stage *Xenopus* embryos (Beanan et al., 2000). These data suggest that *Dlx* genes might reinforce or refine the early ectodermal pattern established by BMP and Wnt signaling. Interestingly, the medial expression borders,

adjacent to the neural plate, differ among Dlx family members, leading Luo et al., to propose that distinct cell fates might arise through differential action of Dlx family members (Luo et al., 2001b). These studies suggest a model in which Dlx activity may regulate the position of the neural plate border. A direct test of this model by loss-of-function has not been done nor have the consequences of shifting the endogenous spatial expression pattern of Dlx expression on adjacent cell fates been examined. Moreover, it is not certain whether the normal role of Dlx proteins is solely inhibitory. We originally postulated a positive role based on our observation that Dlx3 transcripts were downregulated in *narrowminded*, a zebrafish mutant that is deficient in Rohon-Beard neurons and that exhibits delayed appearance of neural crest cells (Artinger et al., 1999).

In this paper, we describe loss- and gain-of-function studies to investigate the role of Dlx genes in positioning the lateral edge of the neural plate and specifying adjacent cell fates. Dlx3 or Dlx5 homeodomains, which are highly conserved among Dlx family members, were fused to the Engrailed transcriptional repressor or the VP16 transcriptional activator domains to modulate transcription of genes regulated by Dlx. These fusion proteins were misexpressed in localized regions within *Xenopus* ectoderm to modify endogenous Dlx function and subsequent alterations in cell fates were analyzed. Whereas we envisaged that pre-gastrula stage BMP, Wnt and FGF signaling biases ectodermal cells towards neural or epidermal fates, our results indicate that Dlx-dependent transcription positions of the lateral border of the neural plate and, importantly, is required in non-neural ectoderm to induce signals that specify the stereotypic pattern of neural crest cells and cranial placodes.

Materials and Methods

Plasmid construction

The VP16-Dlx3hd, EnR-Dlx3hd, VP16-Dlx3hd-GR and EnR-Dlx3hd-GR constructs were made by subcloning the homeodomain coding region of the zebrafish *Dlx3* by PCR using the following oligonucleotide primers: 5'CCCCATCGATATGGTAAACGGAAA-ACCC (for the EnR constructs) or 5'CCCCATGTCTATGGTAAAC-GGAAAACCC (for the VP16 constructs) and 5'CCCCGGCGC-GCCCTTCTTGAACCTTGGATCTC. The amplified fragments were then digested with *ClaI* and *AscI* (for the EnR constructs) or *BglII* and *AscI* (for the VP16 constructs) and ligated into the polylinker of vectors containing either the EnR repressing domain or VP16 activating domain and the GR ligand binding domain described by Kolm and Sive (Kolm and Sive, 1995), separated by a polylinker in a pCS2+ (Turner and Weintraub, 1994) backbone that contains an SP6 promoter used to generate synthetic mRNA for microinjection. Enr-Dlx5hd-GR and VP16-Dlx5hd-GR plasmids were constructed in an identical manner by subcloning the *Xenopus Dlx5* homeodomain.

Embryos and microinjections

Xenopus laevis embryos were fertilized in vitro, dejellied in 2% cysteine-HCl (pH 7.8), and maintained in 0.1× MMR. Embryos were reared at 14–22°C and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Capped mRNA was synthesized using the mMessage Machine kit (Ambion). mRNA was injected into one dorsoanimal blastomere of four to eight cell stage embryos in 3% Ficoll in 1× MMR. For all VP16 constructs,

100-200 pg of mRNA was injected while for the EnR constructs, 50-80 pg of mRNA was injected. All injections included 250-300 pg of β -galactosidase mRNA to provide a lineage tracer. Dexamethasone (10 μ M) was added at either stage 5/6, stage 8/9, stage 10 or stage 11.5-12 to induce the GR fusion proteins.

Transplants and explants

EnR-Dlx3hd mRNA along with β -galactosidase mRNA and GFP mRNA as lineage tracers were injected into two ventral animal blastomeres of four to eight cell stage host embryos. Control host embryos were uninjected or were injected with the lineage tracers alone. Donor embryos were first injected with rhodamine dextran ($10 \times 10^3 M_r$; Molecular Probes) into both blastomeres of a two-cell stage embryos. Embryos were then cultured until stage 12 when a portion of the neural plate and subjacent mesoderm was transplanted into the ventral ectoderm of recipient stage 12 embryos. The fluorescent signal was acquired in monochrome and pseudo-colored green for clarity.

In situ hybridization and probes

Embryos that were co-stained for β -galactosidase were fixed for 40 minutes at room temperature in MEMFA (0.1 M Mops pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde), rinsed in 1 \times PBS with 2 mM MgCl₂ and incubated in staining solution at 37°C with Magenta Gal (Biosynth) before processing for in situ hybridization as described previously (Harland, 1991). The following plasmids were used to generate digoxigenin-labeled probes (RNA polymerase was used for linearization): pSp72-*Xslug* (*BgIII*, SP6) (Mayor et al., 1995), pSp72-*XSnail* (*BgIII*, Sp6) (Essex et al., 1993), pKS-*XSox2* (*XbaI*, T7) (Mizuseki et al., 1998), pKS-*Msx-1* (*Hox7.1*) (*EcoRI*, T7) (Su et al., 1991), pKS-*D112* (*Dlx3*) (*BgIII*, T7) (Papalopulu and Kintner, 1993), pKS-*XHairy2a* (*BamHI*, T7) (J. W.), pGEM3-*keratin* (*BamHI*, SP6) (Jonas et al., 1989), pBSII-*Xsix1* (*NotI*, T7) (Pandur and Moody, 2000), sp70-NCAM (*EcoRV*, SP6) and *N-tubulin* (*BamHI*, T3) (Oschwald et al., 1991; Richter et al., 1988). Stained embryos were postfixed in MEMFA and embedded in JB4 according to the manufacturer's directions (Polysciences) for histological examination.

Immunohistochemistry

Embryos were fixed in MEMFA and processed for immunohistochemistry (Hemmati Brivanlou and Harland, 1989) using an EpA antibody (Jones, 1985) detected with an alkaline-phosphatase-conjugated secondary antibody.

Results

Alteration of Dlx function regulates the size of the neural plate but cannot initiate epidermal differentiation

The progressive refinement of the *Dlx3* expression pattern in late cleavage to early gastrula stage *Xenopus* embryos (Fig. 1A-C) suggests that it mediates or responds to cues involved in neural plate border formation. During late blastula stages, *Dlx3* mRNA is detectable throughout the ectoderm (Fig. 1A). By the onset of gastrulation, *Dlx3* mRNA is reduced in the dorsal ectoderm and becomes localized ventrally to non-neural ectoderm as gastrulation proceeds (Fig. 1B). Expression is restricted exclusively to the non-neural ectoderm by the

beginning of neurulation (Fig. 1C). Other *Dlx* family members have overlapping expression patterns at these stages (Luo et al., 2001a). To examine the role played by endogenous *Dlx*, we engineered transcriptional activating and repressing constructs using the homeodomain of *Dlx3* or *Dlx5* (Fig. 1D). The homeodomains were fused to either the VP16 activation domain (VP16-*Dlx3hd*) or the engrailed repressor (EnR) domain (EnR-*Dlx3hd*). The amino acid sequences of the homeodomains are highly conserved among *Dlx* family members, sharing 80-88% positional identity. Critically, all 4 of the residues in helices three to four responsible for basepair-specific contacts between homeodomain proteins and the DNA major groove are identical (Dave et al., 2000; Laughon, 1991; Mathias et al., 2001). Therefore, these constructs are predicted to modulate target genes of all known *Dlx* family members, which is advantageous given their overlapping expression. Conditionally inducible versions of these constructs were made by fusion to the glucocorticoid receptor ligand binding domain. Cytoplasmic and nuclear factors are commonly expressed as GR fusion proteins to maintain transcription factors in an inactive heat shock complex until the addition of the dexamethasone (Kolm and Sive, 1995). By manipulating the timing of dexamethasone addition, it is possible to investigate the temporal requirements of *Dlx* activity.

Ectopic activation of target genes by injection of VP16-*Dlx3hd* mRNA resulted in a loss of the early neural plate marker, *Xsox2* (Kishi et al., 2000) (91% of embryos showed a loss, $n=69$; Fig. 2B). This confirms that the anti-neural plate potential of the intact *Dlx3* protein (Feledy et al., 1999) (Fig. 3C) depends on transcriptional activator function. In this and all subsequent experiments, β -galactosidase mRNA was co-injected to mark the progeny of injected blastomeres (magenta stain). We then investigated whether repression of downstream targets of endogenous *Dlx* proteins would have a reciprocal effect on neural plate formation. As shown in Fig. 2C, injection of EnR-*Dlx3hd* mRNA expanded *Xsox2* expression laterally on the injected side of the embryo (92% expanded, $n=79$). Control embryos injected with β -galactosidase mRNA alone showed no change in *Xsox2* expression (0% expanded, $n=136$; Fig. 2A). Similar results were seen when embryos were injected with VP16-*Dlx5hd*-GR (83% showed a loss, $n=30$) (not shown) and EnR-*Dlx5hd*-Gr (61% expanded *Xsox2*, $n=31$) (Fig. 2D) when dexamethasone was added immediately (stage 5/6).

To ensure that the effects on the neural plate were not limited to *Xsox2* or to early stages of neural induction, we examined the effects of the *Dlx* constructs on NCAM expression. NCAM was lost after VP16-*Dlx3hd* injection (93% lost, $n=42$) but was expanded laterally by EnR-*Dlx3hd* (89% expanded, $n=57$; Fig. 2E-G). Transverse sections of older stage (stage 27-30) embryos revealed that the neural tube had grossly normal morphology although the region marked by β -galactosidase had expanded (Fig. 2I) as compared to the contralateral side or comparable region in Fig. 2H.

Co-injection with full-length *Dlx3* mRNA rescued the EnR-*Dlx3hd* effects in a dose-dependent manner (Fig. 3) indicating that the EnR-*Dlx3hd* phenotype reflects repression of normal targets of *Dlx* proteins. As would be expected, the highest doses of full-length *Dlx3* caused the overexpression phenotype (Fig. 3C). Thus, we conclude that the phenotypic effects of the *Dlx* fusion proteins reflect a modulation of natural *Dlx* target genes. Taken together, these data suggests that *Dlx* activity delimits the neural plate and prevents its

expansion, consistent with conclusions drawn from overexpression studies (Feledy et al., 1999; Luo et al., 2001b).

We then asked if suppression or expansion of the neural plate is accompanied by compensatory changes in epidermal specification, which is visualized by expression of epidermal *keratin* and the epidermal-specific antibody, EpA (Jones, 1985). VP16-Dlx3hd did not affect *keratin* or EpA levels when expressed in the neural plate region (*keratin*: 76% of embryos exhibited no change, $n=84$, Fig. 2K; EpA: 86% no change, $n=59$ Fig. 2O) despite its marked ability to inhibit neural plate differentiation. In contrast, EnR-Dlx3hd suppressed *keratin* and EpA expression where injected (*keratin*: 95% suppressed, $n=138$ Fig. 2L,M; EpA: 85%, $n=73$, Fig. 2P), consistent with expansion of the neural plate (Fig. 2C,G). Control embryos injected with β -galactosidase mRNA showed no change in *keratin* or EpA expression (*keratin*: 0% affected, $n=129$ Fig. 2J; EpA: 4%, $n=81$, Fig. 2N). We conclude that although Dlx activity inhibits neural plate differentiation, it is not sufficient to redirect presumptive neural plate cells to adopt an epidermal cell fate.

The neural plate-epidermal border region is patterned normally but displaced laterally in EnR-Dlx3hd-expressing tissue

The preceding experiments show that EnR-Dlx3hd can expand the neural plate. Expansion of the neural plate is also seen upon overexpression of *Zic*, *Xash3* and *Xiro* family members, but the neural crest domain is either expanded diffusely or missing and lateral neurons occur ectopically (Gomez-Skarmeta et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 1997; Turner and Weintraub, 1994), most likely reflecting a disturbance of the normal patterning mechanism. We therefore examined a panel of markers to ask whether patterning of these cell lineages were similarly disordered or occurred normally in EnR-Dlx3hd-injected tissues. *Xh2a* and *Xmsx-1* mark the neural plate border at the end of gastrulation (stage 12). *Xsnail* and *Xslug* are expressed in the neural crest, which appears during late gastrula/early neural plate stages (stages 12-14). *N-tubulin* marks the medial, intermediate and lateral rows of primary neurons in neurula and neural tube stage (stages 13-16) embryos (Chitnis et al., 1995). Cranial placodes arise from thickenings in the ectoderm immediately lateral to the neural plate and give rise to a wide variety of derivatives, including paired sense organs (reviewed by Baker and Bronner-Fraser, 2001). *Xsix1*, a homeobox-containing transcription factor, is expressed in the cranial placodal thickenings present in an anterior and lateral band of early neurulae, marking the prospective olfactory anlagen and persists in the late neurulae, where it marks the olfactory, otic, trigeminal and dorsolateral placodes (Pandur and Moody, 2000).

We frequently observed that each marker was displaced to the lateral margin of the areas that contained the injected EnR-Dlx3hd and β -galactosidase mRNAs (Fig. 4; Table 1). Notably, the size of the *Xh2a* and *Xmsx* expression domains were not changed when displaced laterally (Fig. 4C,F) nor was the premigratory neural crest field, marked by *Xslug* and *Xsnail*, altered (compare the injected and uninjected sides in Fig. 4I,N,P). Similarly, the characteristic three rows of primary neurons, marked by *N-tubulin*, arose in the expanded neural plate in *EnR-Dlx3hd*-injected embryos, but their pattern was shifted outwards such that the lateral neurons were now positioned along the new neural plate border (Fig. 4S). An

identical result was observed in EnR-Dlx3hd-injected zebrafish as well (Fig. 4T,U) suggesting that a Dlx-dependent mechanism positions lateral neurons in both species. The neurons remained tightly organized in rows and ectopic neurons were not observed. The placodal marker *Xsix1* was also shifted outwards in embryos expressing EnR-Dlx3hd (compare Fig. 4V with 4X). Interestingly, *Xsix1* was displaced to a region just lateral to the domain of EnR-Dlx3 expression, consistent with expression outside the expanded neural plate. Lateral displacement of trigeminal placodes was also observed in embryos stained with *N-tubulin*, which also marks these placodes (not shown). The ability of EnR-Dlx3hd to displace the stereotypic pattern of marker expression laterally by late gastrula stages suggests that endogenous Dlx activity is upstream of neural crest, lateral primary neuron and cranial placode precursor specification.

While the cell fate markers were often displaced laterally to the border of the EnR-Dlx3hd region, we also observed a loss of markers in some embryos (Table 1, Fig. 4Y and see below). Loss was correlated with large domains of injected EnR-Dlx3hd that extended to the ventral side of the embryo. Thus, depletion of endogenous Dlx activity permits the neural plate to expand maximally to about twice its normal size, regardless of whether or not a larger area expresses EnR-Dlx3hd. The loss of markers, however, raised the possibility that Dlx proteins are required in the non-neural ectoderm to provide neural crest inducing signals. This hypothesis is tested by the transplant experiments below.

In contrast to the loss-of-function studies, injection of VP16-Dlx3hd or wild-type Dlx3 did not shift marker expression medially, but rather abolished expression of both border and neural crest markers (Table 1 and Fig. 4B,E,H,M,R). The sole exception was *Xsix1*, which lies outside the neural plate normally (Fig. 4W). Medial displacement occurred when only a small area of the neural plate contained the injected VP16-Dlx3hd mRNA; large patches of VP16-Dlx3hd-injected tissue resulted in a loss of *Xsix1*. The general inability to shift these markers medially suggests that ectopic Dlx activity in presumptive neural plate cannot induce factors needed for correct patterning of the cell lineages that arise at the neural plate border. However, the medial displacement of *Xsix1* that occurs when only a minimal gap separates the neural plate from non-neural ectoderm suggests that short-range communication between neural and non-neural ectoderm is critical for placode formation.

Dlx proteins regulate neural plate border formation during gastrulation

To investigate the temporal requirement for Dlx function in neural plate border formation, embryos were injected as above but with mRNAs encoding the conditional proteins EnR-Dlx3hd-GR or VP16-Dlx3hd-GR. Dexamethasone was added at either stage 6, 8, 10 or stage 11.5-12 and embryos were examined for *Xsox2* and *Xslug* expression. Injected embryos cultured in the absence of dexamethasone exhibited no change in any of the markers tested (not shown). When dexamethasone was added at stage 6, embryos injected with EnR-Dlx3hd-GR (70% expanded, $n=34$) or VP16-Dlx3hd-GR (80% repressed, $n=25$) showed the expansion or repression of *Xsox2*, respectively (Fig. 5A,B), typical of the preceding results with the constitutive constructs. Similarly, the neural crest marker *Xslug* was either lost (48% lost, $n=39$) or shifted laterally (52% shifted laterally, $n=39$; Fig. 5C) when EnR-Dlx3hd-GR-injected embryos were treated with dexamethasone at stage 6. The incidence of

expanded *Xsox2* expression and laterally shifted *Xslug* (Fig. 5I) decreased as dexamethasone was added at later stages. Addition at stage 11.5-12 did not affect *Xsox2* expression (wild-type pattern was observed in 86% of EnR-Dlx3-GR embryos, $n=66$ and in 90% of the VP16-Dlx3hd-GR-injected embryos, $n=93$; Fig. 5E,F). Similarly, EnR-Dlx3-GR did not alter *Xslug* expression when dexamethasone was added at stage 11.5-12 (65% were unaffected, $n=126$; Fig. 5G). Therefore, specification of the neural plate border and induction of neural crest requires Dlx function before the end of gastrulation. We did notice, however, that VP16-Dlx3hd-GR eliminated or reduced *Xslug* expression regardless of whether dexamethasone was added at stage 6 (88% eliminated or reduced, $n=20$; Fig. 5D) or stage 11.5-12 (72% eliminated or reduced, $n=114$; Fig. 5H). Thus, neural crest, but not neural plate, remains sensitive to Dlx inhibition after late gastrulation.

Neural crest and cranial placode induction requires Dlx function in the non-neural ectoderm

The preceding results showed that local depletion of Dlx function during gastrulation causes an expansion of the neural plate and the lateral displacement of the normal pattern of lateral primary neuron, neural crest and cranial placode cell fates. This could occur by a repressive mechanism whereby Dlx genes simply prevent neural plate formation. However, when the area of EnR-Dlx3hd expression extended to the most ventral ectoderm, we noticed that the neural plate expanded maximally to only about twice its normal distance from the dorsal midline and that all of the cell lineage markers examined were absent (Fig. 6A-D; identical results obtained with EnR-Dlx5hd, not shown). This suggests that Dlx proteins not only repress cell fates, but might also be necessary in non-neural ectoderm for the production of factors that are critical for specification of lateral primary neuron, neural crest, and cranial placode cells.

To determine if Dlx function is required in non-neural ectoderm, we took advantage of previous studies showing that grafting neural plate tissue to the non-neural ectoderm causes neural crest cells to be induced in both host and donor tissues where juxtaposed (Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 2000). Regions of neural plate from fluorescent dextran-injected donors were grafted to the ventral ectoderm of host embryos that had been injected with nuclear-localized β -galactosidase mRNA, either alone or with EnR-Dlx3hd mRNA (Fig. 6E). We then assayed the graft region for the induction of *Xslug* and *Xsix1* to determine whether the induction of neural crest and cranial placodes is inhibited when Dlx activity is downregulated. As expected, control grafts showed *Xslug* expression induced at the graft-host tissue interface (blue stain, Fig. 6E inset). Higher magnification views (Fig. 6J-N) show induction of both *Xslug* and *Xsix1* (blue stain) at the interface of the graft (green fluorescent label) and host (magenta β -galactosidase stain) tissues, confirming that juxtaposition of competent neural plate and non-neural ectoderm induces neural crest and cranial placodes. In contrast, *Xslug* and *Xsix1* were not induced when neural plate was transplanted into ventral ectoderm expressing EnR-Dlx3hd (Fig. 6O-S; Table 2). In cases where donor neural ectoderm spanned EnR-Dlx3hd-expressing and -non-expressing host tissue (Fig. 6P), *Xslug* was induced only at the site where the host tissue lacked injected mRNAs (black arrowhead) but not in or near cells with the injected mRNA (red arrowhead). No or minimal *Xslug* and *Xsix1* transcripts were detected in donor neural

ectoderm cultured alone (Fig. 6F,G) whereas similar explants taken from a more lateral position that spanned the border region expressed *Xslug* and *Xsix1* (Fig. 6H,I), indicating that expression in the grafts reflects induction and not contamination with donor border region tissue. Taken together, these studies demonstrate that Dlx-dependent transcription is required in non-neural ectoderm to produce factors that act at over short range to induce neural crest and cranial placode fates.

Discussion

Dlx activity delimits the neural plate and is involved in patterning adjacent cell fates

Gain-of-function experiments showed that Dlx genes position the interface between the neural plate and non-neural ectoderm by locally inhibiting neural plate differentiation (Fig. 2), confirming previous observations that full-length Dlx3 and Dlx5 antagonizes neural plate formation (Feledy et al., 1999; Luo et al., 2001a). Loss-of-function experiments also support this conclusion (Fig. 2) and can be rescued with full-length Dlx3 (Fig. 3), suggesting that our constructs specifically affect natural targets of the full-length Dlx family of proteins. Because of the high degree of conservation among Dlx homeodomains and overlapping expression during late blastula and gastrula stages, our results reflect perturbation of endogenous targets of Dlx proteins in aggregate and we cannot ascribe activities to individual family members or address questions of functional redundancies. Further studies will be required to determine the precise function of individual family members.

A central finding of our study is that local inhibition of Dlx function shifts the stereotypic pattern of lateral primary neurons, neural crest and cranial placodes laterally (Figs 2-4). Thus, Dlx family members act upstream of the specification of cell fates that arise near the lateral border of the neural plate. Furthermore, Dlx genes are required for the induction of these cell fates, as revealed by the lack of marker expression when neural plate is grafted to EnR-Dlx3hd-expressing ectoderm (Fig. 6). Fig. 7 shows a schematic model of Dlx function at the lateral margin of the neural plate. Normally, Dlx activity in non-neural ectoderm (yellow) delimits the mediolateral position of the neural plate/non-neural ectoderm border and lateral neurons, neural crest and cranial placode cells (Fig. 7B). Inhibition of Dlx function in a localized region results in both an expansion of the neural plate and a lateral displacement of border region cell fates to a position abutting Dlx-positive ectoderm (Fig. 7C). Inhibition of a broader region of Dlx activity, however, causes the neural plate to expand maximally to about twice its normal distance from the dorsal midline, but normal border region cell lineages do not arise (Fig. 7D). The absence of stereotypic border region markers in these cases suggests that Dlx activity functions in the prospective epidermis to induce short range signals that specify border cell fates. In this model, the short-range signals are unable to traverse the interposed tissue (Fig. 7D). Ectopic Dlx activity generally fails to displace border cell fates medially (Fig. 7E). In these cases, as with broad regions of Dlx underexpression, we presume that signals needed to induce and pattern lateral neurons, neural crest and cranial placode cells cannot traverse the interposing tissue, which does not express neural or epidermal markers (Fig. 2).

Stereotypic displacement of border fates was not observed in previous studies in which the neural plate was expanded by overexpression of Zic family members, XBF-2, and Xash-3

(Kuo et al., 1998; Mariani and Harland, 1998; Mizuseki et al., 1998; Nakata et al., 1997; Turner and Weintraub, 1994). In these cases, the domains of neural crest and lateral neurons were either expanded or eliminated, indicating that these genes control differentiation of neural plate cells and their derivatives rather than regulate formation of a normal border region per se.

We did not see any consistent effects on the forebrain marker *Otx2* when embryos were injected with either the activating or inhibiting constructs (not shown). We also did not see a shift in the anterior portion of the *Xsix1* domain (not shown). Previous studies have shown that overexpression of *Dlx3* does not affect *Otx2* expression (Feledy et al., 1999). These results suggest that *Dlx* genes function primarily posterior to the forebrain. Positioning the mediolateral and anteroposterior positions of forebrain neuroectoderm might rely on a separate mechanism, possibly involving Wnt, FGF or retinoic acid signaling (Villanueva et al., 2002).

Dlx activity regulates cell fates during gastrulation

The modulation of late gastrula stage markers (Fig. 4A-I) and the timecourse experiment of EnR-Dlx3hd-GR activation by dexamethasone addition (Fig. 5) both indicated that *Dlx* activity patterns cell fates at the medial neural/non-neural ectodermal interface by stages 11.5-12. Because this time precedes the morphological appearance and expression of molecular markers of neural folds and lateral primary neurons, neural crest cells and cranial placodes, *Dlx* activity is needed upstream of the process that induces these cell fates. Classical graft and extirpation experiments suggested that the anterior neural plate border is no longer susceptible to signals that reposition the border by late blastula stage (stage 9+) (Zhang and Jacobson, 1993). While the mediolateral border was not examined specifically, it is possible that these studies revealed the action of diffusible signals, such as BMP and Wnt, that bias ectodermal fates. Our data indicates that endogenous *Dlx* activity is required somewhat later, through stage 11.5-12 (Fig. 5), consistent with *Dlx* genes being regulated by BMP and Wnt signaling. Interestingly, the stages when *Dlx* activity is required corresponds to the time when the transcripts encoding *Dlx*, *Xmsx1* and *Xiro* become spatially localized to respect the neural plate border. The potential involvement of *Dlx* proteins with these and other proteins to regulate their own expression and influence neural, epidermal and border region cell fates is discussed below.

Complex interactions among proteins involved in patterning the neural plate border and adjacent cell fates

Current models for partitioning ectoderm into neural plate and epidermis involve initiating Wnt, BMP and FGF signals (Baker et al., 1999; Barth et al., 1999; Ikeya et al., 1997; Launay et al., 1996; Streit and Stern, 1999; Wilson and Hemmati-Brivanlou, 1995; Wilson et al., 2001). Wnt signaling through the canonical β -catenin pathway provides dorsal cues during cleavage stages (e.g. Heasman et al., 1994; Larabell et al., 1997). By pre-gastrula stages, ectopic activation of BMP and Wnt/ β -catenin signaling pathways elicit a ventralizing effect on embryos suggesting that these proteins might normally antagonize induction of the neural plate at these stages (Baker et al., 1999; Christian and Moon, 1993; Hawley et al., 1995; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995; Wilson et al., 2001). At

least *Dlx3* is negatively regulated by the pre-MBT Wnt/ β -catenin signaling (Beanan et al., 2000), and induced by pre-gastrula BMP signaling (Feledy et al., 1999); thus, *Dlx* genes probably act downstream of these proteins. Whether *Dlx* activity feeds back to influence gastrula-stage BMP and/or Wnt signaling is unclear.

Dlx proteins might regulate border cell fates by cooperating with other transcription factors that are induced by early BMP, Wnt and/or FGF signaling. Injection of VP-*Dlx3*hd prevents induction of markers of lateral primary neurons, neural crest and cranial placodes (Fig. 4). Moreover, epidermal markers were not induced (Fig. 2), suggesting that *Dlx* activity alone is insufficient to respecify neural plate to non-neural ectoderm (see diagram in Fig. 7E) and implicating an additional factor. One candidate is *Msx1*, which is an immediate downstream target of BMP signaling localized to the ventral ectoderm in early gastrula stage *Xenopus* embryos (Feledy et al., 1999; Suzuki et al., 1997). Overexpression of *Msx1* represses neural plate and border cell fates, but unlike *Dlx3*, also converts prospective neural plate into epidermis (Suzuki et al., 1997). Neural inhibition by *Msx-1*, unlike *Dlx* factors, appears to act via transcriptional repression (Yamamoto et al., 2000), suggesting that *Msx-1* and *Dlx* factors regulate distinct target genes. Since *Msx* and *Dlx* proteins can heterodimerize (Zhang et al., 1997), regulatory interaction between the factors is also likely. *Msx-1* loss-of-function experiments are needed to address its role in cell fate specification at the neural plate border. Defects of *Msx1*-deficient mice are not known to involve an expanded neural plate, but potential redundancy or compensation complicates interpretation (Jumlongras et al., 2001; Satokata and Maas, 1994). Nonetheless, it seems possible that induction of neural crest, lateral neurons and cranial placodes might require both *Dlx* activity and the epidermal promoting activity of *Msx1*.

The lateral displacement of border cell fates by *Dlx* genes could indicate a genetic interaction with prospective neural plate factors (see diagram in Fig. 7A). One candidate is *Xiro1*, which is initially expressed broadly in the dorsal ectoderm at the onset of gastrulation but becomes restricted to the prospective anterior neural plate as gastrulation proceeds (Gomez-Skarmeta et al., 1998). Overexpression of *Xiro1* expands the neural plate and, in some instances, can displace *Xslug* expression outward (Gomez-Skarmeta et al., 1998). Furthermore, ectopic *Xiro1* downregulates endogenous *Bmp4* expression whereas ectopic *Xmsx1* downregulates endogenous *Xiro1* (Gomez-Skarmeta et al., 2001). These data combined with our results raise the intriguing possibility that *Dlx* genes, like *Xmsx1*, are involved in a mutual repression circuit with *Xiro1*.

In summary, our results support a model in which diffusible BMP, Wnt, and FGF signaling establishes graded expression patterns of transcription factors involved in positioning the neural plate border and determining adjacent cell fates. The neural plate antagonizing activities of *Dlx* proteins probably function in a reciprocal (inhibitory) circuit with transcription factors that promote neural plate, such as the *Xiro-1* protein. The consequence of this interaction is to sharpen the border between the neural and non-neural ectoderm. In addition, we showed that *Dlx* genes also play a positive role in non-neural ectoderm for the production of factors that are required for the induction and stereotypic mediolateral patterning of lateral neurons, neural crest and placode cells.

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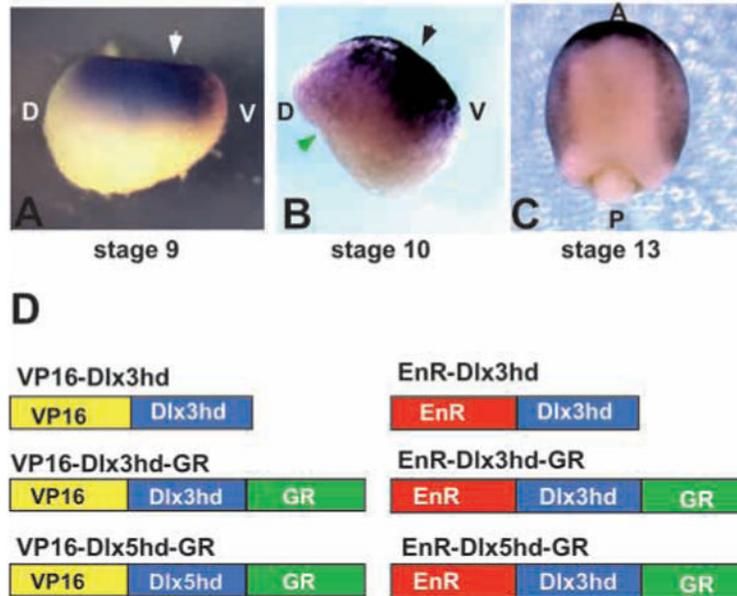
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**Fig. 1.**

Dlx gene expression becomes restricted to the ventral ectoderm. (A) At blastula stage (stage 9), *Xdlx3* is expressed broadly throughout the ectoderm. Animal pole is oriented up. (B) By early gastrula stage (stage 10), *Xdlx3* expression is restricted to the more ventral ectoderm (black arrowhead). Lateral view with dorsal oriented to the left. Dorsal lip is on the left (green arrowhead). (C) By the beginning of neurulation (stage 13), *Xdlx3* expression is completely absent from the neural plate and is expressed throughout the non-neural ectoderm (dorsal view with anterior to the top). (D) Schematic of Dlx homeodomain constructs. The activating Dlx construct was made by ligating regions encoding the Dlx3 homeodomain (blue) to the VP16 activation domain (yellow). A conditional version was generated by fusion to the ligand-binding domain of the human glucocorticoid receptor (GR; green). Inhibitory constructs were made similarly using the Engrailed repressor domain (EnR; red). Identical constructs were made with the Dlx5 homeodomain. The homeodomains are highly conserved among Dlx family members; thus the fusion proteins are envisaged to regulate target genes of all family members comparably. See Materials and Methods for details of construct preparation.

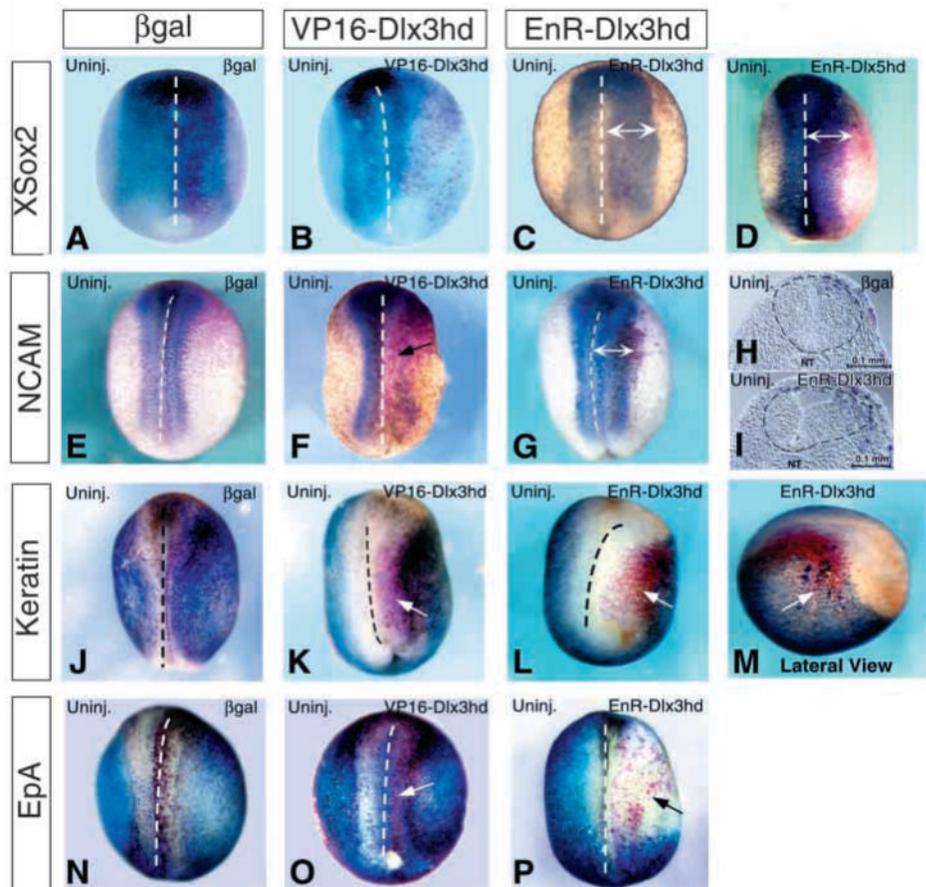


Fig. 2.

Dlx activity restricts neural plate expansion but does not induce epidermal differentiation. β -galactosidase and either EnR-Dlx3hd, EnR-Dlx5hd or VP16-Dlx3hd mRNAs were injected into one dorsal animal blastomere of 4-cell stage embryos. The embryos were then stained for the β -galactosidase (as a lineage label; magenta stain) and assayed for *Xsox2* (stage 13), NCAM (stage 17-18) or *keratin* (stage 17-18) expression by whole-mount in situ hybridization (blue stain), or by EpA immunostaining (stage 17-18) to reveal neural plate or epidermal differentiation. All views are dorsal with anterior to the top except M, which is lateral with anterior to the right and H and I, which are transverse histological sections through the neural tube. Dashed lines indicate the dorsal midline. (A) *Xsox2* expression is the same on the injected and uninjected sides of control embryos injected with β -galactosidase mRNA alone. (B) Injection of VP16-Dlx3hd mRNA reduces the *Xsox2* domain on the injected side. (C,D) In contrast, embryos injected with EnR-Dlx3hd (C) or EnR-Dlx5hd (D) mRNA shows expanded *Xsox2* expression on the injected side. (E-G) The NCAM domain was similarly reduced by or expanded by VP16-Dlx3hd and EnR-Dlx3hd, respectively. (H,I) Transverse sections through stage 25 embryos (H) the control embryo has a symmetrical, closed neural tube. (I) An embryo expressing EnR-Dlx3hd illustrates that the neural tube closed properly but was expanded on the injected side where β -gal-positive cells populate the neural tube. Dorsal is to the top. (J-M) *keratin* expression; a marker expressed throughout the non-neural ectoderm. (J) Normal expression of epidermal *keratin* in a control

embryo injected with β -galactosidase mRNA alone. (K) Injection of VP16-Dlx3hd mRNA did not expand epidermal *keratin* expression. (L) Injection of EnR-Dlx3hd mRNA inhibited epidermal *keratin* expression. White arrows mark the loss of *keratin* expression in the injected region. (M) A lateral view illustrates the loss of *keratin* (blue) in the injected region (magenta β -galactosidase stain). (N-P) VP16-Dlx3hd did not affect the epidermal epitope EpA (O), whereas EnR-Dlx3hd (P) prevented normal expression (N).

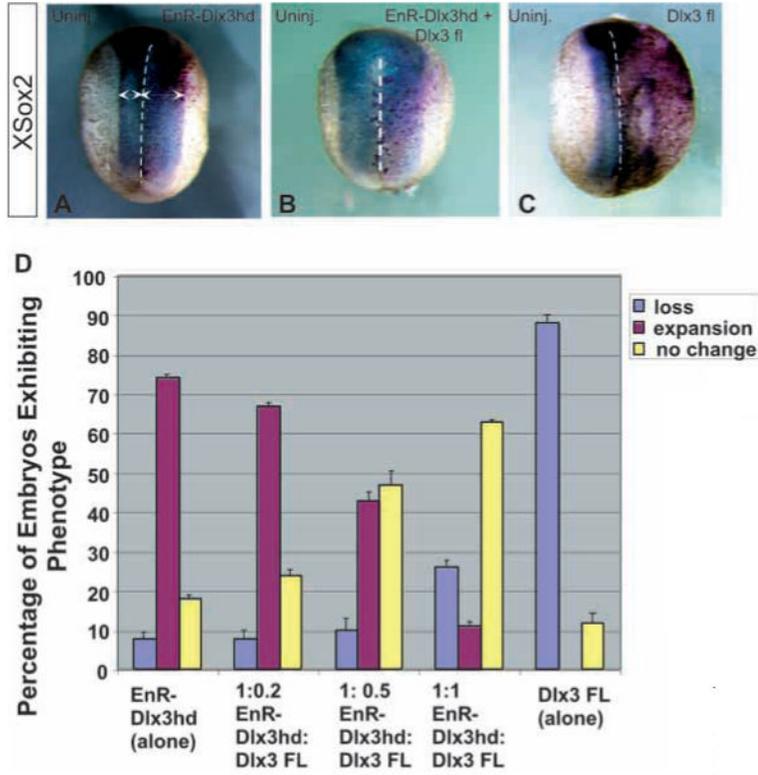


Fig. 3. Rescue of EnR-Dlx3hd activity by full-length Dlx3. A constant amount β -galactosidase and 50 pg of EnR-Dlx3hd mRNAs were injected unilaterally as in Fig. 2 along with increasing doses of mRNA encoding full-length Dlx3 (Dlx3 FL). (AC) Embryos stained for *Xsox2* show the typical expansion of the neural plate obtained with EnR-Dlx3hd (A), the rescue achieved in combination with Dlx3 FL (B), and the overexpression phenotype typical of Dlx3 FL-injected embryos (C). (D) Incidence of each of the phenotypes in A-C as a function of the molar ratio of mRNAs injected. Note that the incidence of each phenotype depends on the relative dose of Dlx3 FL mRNA injected.

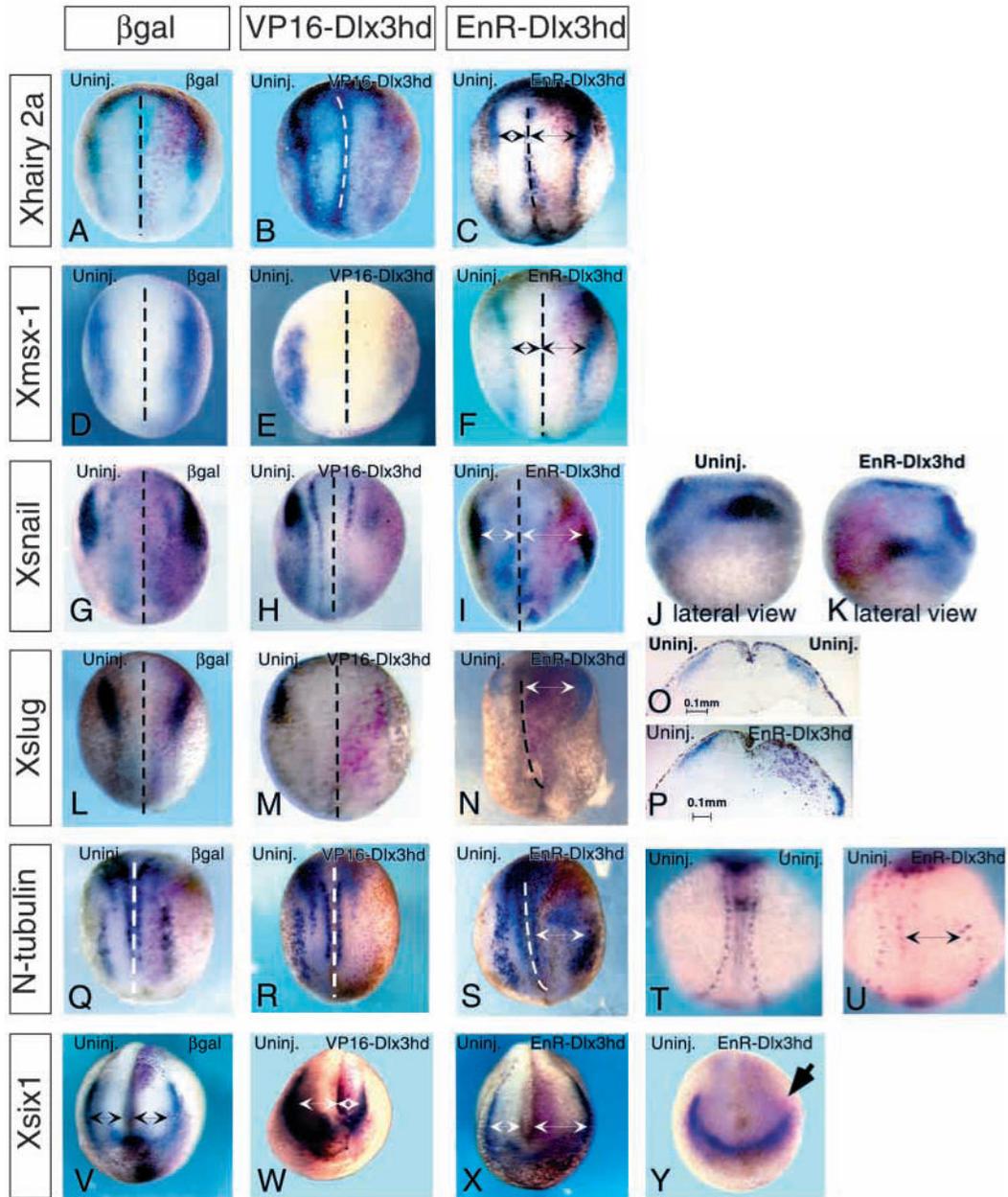
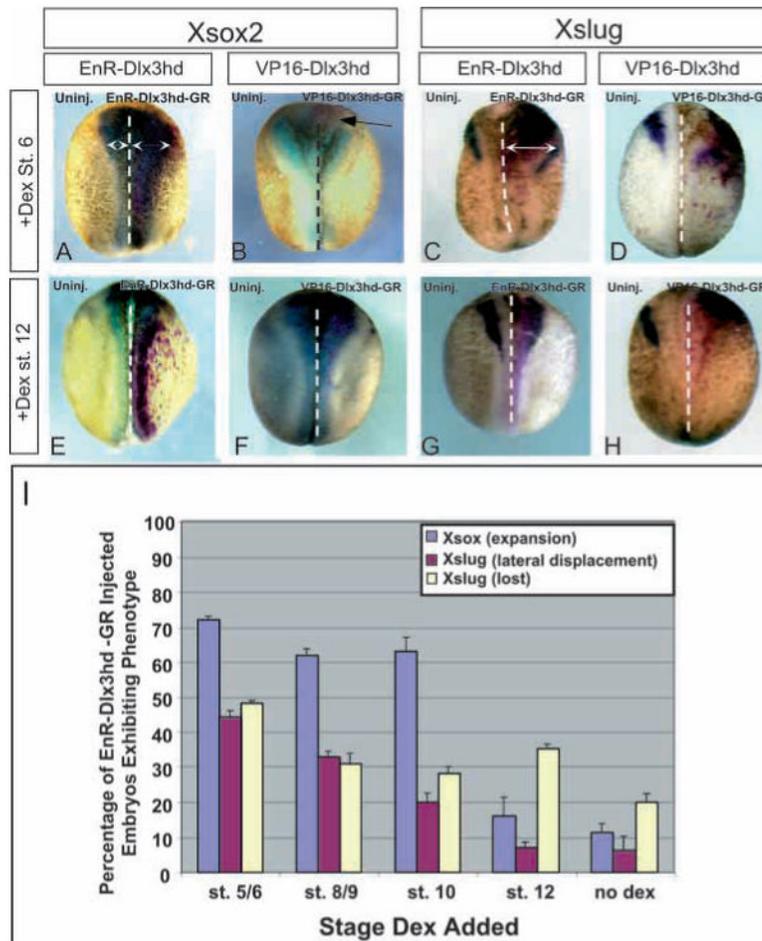


Fig. 4.

Alterations in cell lineages that border the neural plate in embryos with localized expression of VP16-Dlx3hd or EnR-Dlx3hd. Embryos were injected unilaterally with EnR-Dlx3hd or VP16-Dlx3hd at the 8-16 cell stage, cultured until the appropriate developmental stage and then examined by *in situ* hybridization (blue stain). β -galactosidase stain (magenta) indicates progeny of injected blastomeres. All embryos are shown as dorsal views with anterior to the top except for J and K which are lateral views, anterior to the right, and V-Y, anterior views, dorsal to the top. (A-F) Stage 13 embryos showing that markers of cells at the border of the neural plate (*Xhairy2A* and *Xmsx-1*) are ablated by VP16-Dlx3hd and shifted laterally by EnR-Dlx3hd. Arrows mark displacement caused by EnR-Dlx3hd relative to uninjected side

of the embryo. (G-P) Stage 13 embryos showing identical effects on markers of neural crest precursors (*Xsnail* and *Xslug*). Lateral views of *Xsnail* expression illustrate the extent of the shift (J,K) seen in dorsal view (I, arrows). Transverse sections (O,P) showing *Xslug* expression (blue) and β -galactosidase (magenta) illustrate that the size of the *Xslug* expression domain is unaltered but occurs at the lateral margin of the cells expressing the injected mRNA. (Q-U) Primary neurons (marked by *N-tubulin*) are also ablated by VP16-Dlx3hd and displaced laterally by EnR-Dlx3hd in stage 14 *Xenopus* (Q-S) and 2-somite stage zebrafish (T,U). (V-Y) Stage 18 embryos showing cranial placode precursors (marked by *Xsix1*) shifted medially or laterally by localized expression of VP16-Dlx3hd and EnR-Dlx3hd, respectively (W,X). Note that the anterior domain of *Xsix1* is unaffected, even where widespread expression of EnR-Dlx3hd ablates *Xsix1* more laterally (Y).

**Fig. 5.**

Dlx activity is required before the end of gastrulation to position the neural plate border and specify adjacent cell fates. Embryos were injected unilaterally with either VP16-Dlx3hd-GR or EnR-Dlx3hd-GR mRNAs as above and dexamethasone was then added at various times to examine the temporal requirements for Dlx activity. Embryos were assayed for *Xsox2* (stage 15) and *Xslug* (stage 17) by in situ hybridization (blue stain). β -galactosidase staining (magenta stain) marks the progeny of the injected blastomeres. In all cases, control embryos injected with the fusion protein constructs but cultured without dexamethasone remained unaffected (not shown). (A-D) Addition of dexamethasone at stage 6 expanded *Xsox2* (A) or outwardly shifted *Xslug* (C) in embryos expressing EnR-Dlx3hd and ablated both markers in embryos expressing VP16-Dlx3hd (arrows in B and D). (E,F) In contrast, addition of dexamethasone at stage 11.5-12 to either EnR-Dlx3hd- or VP16-Dlx3hd-injected embryos did not affect *Xsox2*. (G) Similarly, stage 11.5-12 dexamethasone addition to EnR-Dlx3hd-injected embryos caused no change in *Xslug*. (H) However, stage 11.5-12 dexamethasone addition to VP16-Dlx3hd-injected embryos ablated *Xslug*, indicating that the neural crest remains sensitive to Dlx activity after gastrulation. (I) The time course of the dexamethasone effect on EnR-Dlx3hd-expressing embryos suggests that endogenous Dlx activity affects neural crest patterning before the end of gastrulation.

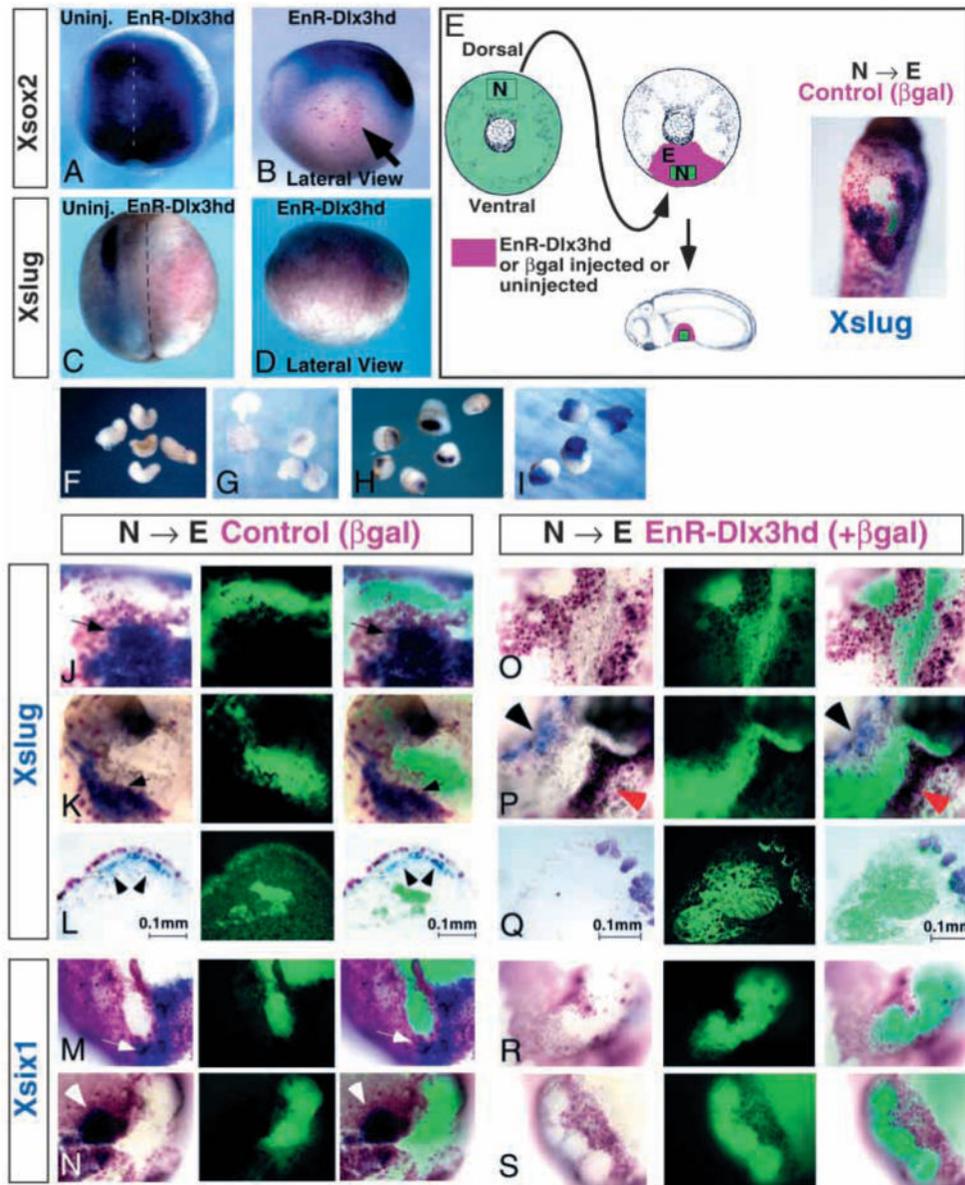
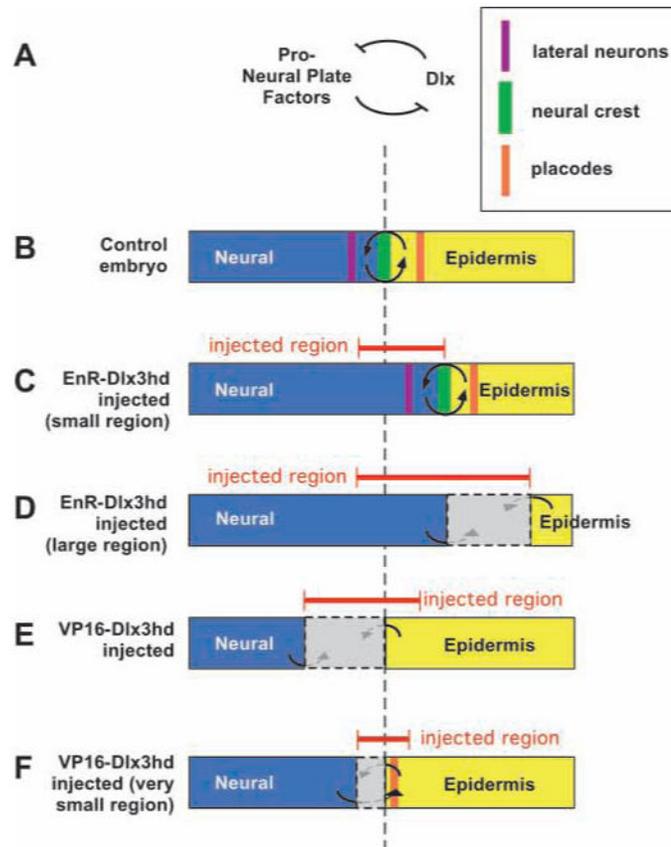


Fig. 6.

A positive role for Dlx function in non-neural ectoderm. (A-D) Embryos were injected unilaterally with EnR-Dlx3hd and β -galactosidase (magenta stain). Broad domains of EnR-Dlx3hd expression dorsoventrally show maximal expansion of the neural plate is limited to approximately twice its normal width, as visualized by *Sox2* expression (A,B blue stain). Arrows in B show staining for β -gal indicative of injected mRNA outside of the *Sox2* domain. Note that in these cases of broad EnR-Dlx3hd expression, *Xslug* expression was not reactivated at the border of the expanded neural plate (C,D), similar to ablation of *Ssix1* (Fig. 4Y) and other markers (not shown). (E) Schematic showing isochronic stage 12 transplant of fluorescent dextran (pseudo colored green stain) injected donor neural plate tissue (N) to ventral ectoderm of host embryos. Host embryos were injected ventrally to express β -galactosidase either alone or with EnR-Dlx3hd. Control grafts showed *Xslug*

induction at stage 25 (photo; ventral view, anterior to top). (F-I) Controls show that donor neural plate explants cultured alone express no or only minimal levels of *Xslug* (F) or *Xsix1* (G), unless taken from a more lateral region that included prospective epidermis (H, *Xslug*; I, *Xsix1*). Therefore, marker expression is indicative of neural crest (*Xslug*) or placode (*Xsix1*) induction. (J-N) High magnification views of grafts into control hosts that had been injected with β -gal mRNA alone. The three panels shown are light field, fluorescent and merged images. Induction (blue stain) of *Xslug* (J-L) and *Xsix1* (M,N) was observed in areas adjacent to and overlapping both donor (green) and host tissue. In particular, note expression (arrows in J,K,M, arrowheads in L,N) within and adjacent to host tissue expressing the injected β -galactosidase (magenta stain). Sections (L) show *Xslug* expression (blue) adjacent to donor tissue (green). (O-S) High magnification views of grafts into experimental hosts that had been injected with EnR-Dlx3hd and β -gal mRNAs. Unlike controls (J-N), *Xslug* and *Xsix1* are not induced adjacent to host tissue that had been injected with EnR-Dlx3hd and β -galactosidase mRNAs (magenta). P shows a graft inserted adjacent to both injected and uninjected host tissue. Note that induction was prevented near cells that expressed EnR-Dlx3hd (red arrowhead) but occurred where host tissue lacked injected mRNAs (black arrowhead).

**Fig. 7.**

Model for role of Dlx in positioning the neural plate border and patterning adjacent cell fates. (A) We postulate that a reciprocal (inhibitory) interaction between Dlx and neural plate factors refines an initial neural plate: non-neural ectoderm bias along the mediolateral axis. The initial bias is established earlier, possibly by BMP, Wnt and/or FGF signaling. This reciprocal interaction leads to a sharpening of the border between the neural plate and non-neural ectoderm and specifies the precise position along the mediolateral axis. (B-F) Schematics of ectoderm along the mediolateral axis illustrate the function of Dlx factors under normal and manipulated conditions. (B) Under normal conditions, Dlx activity is required in non-neural ectoderm (yellow) for short-range communication (arrows) that leads to induction of lateral primary neuron, neural crest and cranial placode precursors. (C) Local inhibition of Dlx activity within the region expressing injected EnR-Dlx3hd (red bars) causes the neural plate to expand. Border region cell lineages are induced laterally where the expanded neural plate contacts Dlx-positive non-neural ectoderm. (D) When Dlx3 activity is inhibited beyond the intrinsic limit of neural plate expansion, the neural plate expands maximally but is separated from Dlx-positive epidermis. Short-range communication is attenuated or not initiated, resulting in absence of neural crest, cranial placodes and lateral primary neurons. (E) Overexpression of Dlx activity (with VP16-Dlx3hd or full-length Dlx3) inhibits neural plate induction but cannot initiate epidermal differentiation (Fig. 2); thus, the neural plate narrows but short-range signaling and normal cell fate specification does not occur in the border region. (F) Cranial placodes can be shifted medially in cases

where a small region of ectopic *Dlx* activity separates competent neural plate and non-neural ectoderm, suggesting that placode-inducing signals can traverse a short region of interposing tissue.

Table 1
Effect of activating and inhibiting Dlx constructs on border formation

	Embryos exhibiting phenotype (%±s.e.m.)		
	EnR-Dlx3hd	VP16-Dlx3hd	β-gal alone
<i>Xhair2a</i>			
Shift (lateral)	35±3.8	0	0
Loss	45±2.2	89±0.2	1±1.2
No change	20±2.2	11±0.6	99±0.1
Number examined	75	47	118
<i>Xmsx1</i>			
Shift (lateral)	40±0.6	0	0
Loss	42±1.8	77±2.1	0
No change	19±2.4	23±3.9	100
Number examined	64	61	75
<i>Xsnail</i>			
Shift (lateral)	51±0.5	0	0
Loss	40±2.7	86±0.8	0
No change	9±4.7	14±2.1	100
Number examined	70	45	107
<i>Xslug</i>			
Shift (lateral)	52±3.3	0	9±2.2
Loss	39±4.6	83±0.7	7±3.9
No change	9±2.7	17±1.5	84±2.4
Number examined	109	93	58
<i>N-tubulin</i>			
Shift (lateral)	83±1.1	0	1±1.5
Loss	16±2.5	75±1.5	5±2.4
No change	1±1.1	25±2.5	94±0.7
Number examined	154	77	137
<i>Xsix1</i>			
Shift (lateral)	25±0.9	0	0
Shift (medial)	n/a	33±2.1	0
Loss	68±1.9	46±3.8	0
No change	6±4.4	21±3.3	100
Number examined	67	106	130

Table 2
Dlx activity is required in the non-neural ectoderm for neural crest and placode progenitor induction

	Induced (%±s.e.m.)	Number examined
<i>Xslug</i>		
N→E (β-gal alone)	79±3.1	62
N→E (EnR-Dlx3hd)	4±4.8	47
Neural plate alone	0	18
Neural plate + border	60±0.4	27
<i>Xsix</i>		
N→E (β-gal alone)	93±1.1	28
N→E (Enr-Dlx3hd)	13±2.4	31
Neural plate alone	17±3.3	17
Neural plate + border	100	13