Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm

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SUMMARY

In *Xenopus* development, dorsal mesoderm is thought to play a key role in both induction and patterning of the nervous system. Previously, we identified a secreted factor, noggin, which is expressed in dorsal mesoderm and which can mimic that tissue's neural-inducing activity, without inducing mesoderm. Here the neural tissue induced in ectodermal explants by noggin is further characterized using four neural-specific genes: two putative RNA-binding proteins, *nrp-1* and *etr-1*; the synaptobrevin *sybII*; and the lipocalin *cpl-1*. First we determine the expression domain of each gene during embryogenesis. Then we analyze expression of these genes in noggin-treated explants. All markers, including the differentiated marker *sybII*, are expressed in noggin-induced neural tissue. Furthermore, *cpl-1*, a marker of dorsal brain, and *etr-1*, a marker absent in much of the dorsal forebrain, are expressed in non-overlapping territories within these explants. We conclude that the despite the absence of mesoderm, noggin-induced neural tissue shows considerable differentiation and organization, which may represent dorsal-ventral patterning of the forebrain.

Key words: noggin, neural induction, dorsal-ventral patterning, neuronal differentiation, RNA-binding protein, synaptobrevin, lipocalin

INTRODUCTION

During gastrulation in amphibian embryos, the mesoderm of the dorsal blastopore lip, or Spemann organizer, induces overlying ectoderm to form neural tissue. Subsequently, this neural tissue becomes organized into a nervous system, with characteristic anterior-posterior and dorsal-ventral patterns. Although these phenomena were described by Spemann and his school in the first half of this century (reviewed by Hamburger, 1988), only in recent years have developmental biologists begun to understand these processes on a molecular level. Specifically, a wide range of useful neural marker genes have been identified and, recently, three neural-inducing factors have been described: noggin (Lamb et al., 1993), follistatin (Hemmati-Brivanlou et al., 1994) and FGF (Kengaku and Okamoto, 1993). These molecules can be used to induce neural tissue in ectodermal explants, and the exact type of neural tissue induced can then be described using a variety of neural marker genes. Since the explants are isolated free of mesoderm and other potential sources of patterning information in the intact embryo, this system offers the opportunity to dissect apart the otherwise interconnected processes of neural induction and neural patterning.

We have used four new neural-specific genes with useful expression patterns to examine the type of neural tissue induced by noggin. Noggin is a novel secreted glycoprotein of about 30×10^3 M_r (Smith and Harland, 1992; Smith et al., 1993). It has been shown to fulfill several criteria for the neural-inducing signal emanating from the Spemann organizer: noggin is expressed in the organizer; it can induce neural tissue in explanted ectoderm, without first inducing mesoderm; and it can do so during gastrulation (Smith and Harland, 1992; Lamb et al., 1993). Interestingly, the tissue induced by noggin seems to be anterior in nature, expressing the genes *otx2* (formerly *otxA*, GenBank accession number L26509; expressed in the forebrain, midbrain and eyes) and *XAG-1* (expressed in the cement gland, an anterior ectodermal structure; Sive et al., 1989) but no marker genes that are more posteriorly restricted (Lamb et al., 1993). Noggin's activity is thus consistent with that of the 'activator' in Nieuwkoop's theory of neural patterning by activation/transformation (reviewed by Hamburger, 1988; Doniach, 1993; Slack and Tannahill, 1992). Nieuwkoop suggested that the neuralizing signal initially 'activates' all of the neural tissue to an anterior state, which is subsequently posteriorized by graded activity of a 'transforming factor.' Furthermore, such transforming factors might also be necessary for the organization and differentiation of noggin-induced neural tissue. This idea could explain the preliminary observations that noggin-induced neural tissue appears uniform in composition and generally lacks differen-

tiated neurons, as tested by staining with a variety of antibodies (Lamb et al., 1993, and F. Mariani, unpublished results).

To address further the organization and differentiation of noggin-induced neural tissue, we examined the expression of four neural-specific genes, previously isolated from *Xenopus* embryos: *nrp-1* (original clone designation 24-39), *etr-1* (17-5), *sybII* (17-30) and *cpl-1* (13-8) (Richter et al., 1988; Good et al., 1990a). The proteins encoded by these genes represent three distinct functional classes. *nrp-1* (Richter et al., 1990) and *etr-1* both encode putative RNA-binding proteins, though they are not closely related to each other. *sybII* is the *Xenopus* homolog of rat *sybII*, a synaptobrevin; synaptobrevins are major components of synaptic vesicles and are thought to be involved in vesicle trafficking and fusion (Bennett and Scheller, 1993; reviewed by Elferink and Scheller, 1993). Lastly, *cpl-1* (Richter et al., unpublished data) encodes a member of the lipocalin superfamily of proteins, which share a common structural motif and a probable function of binding small hydrophobic molecules (reviewed by Godovac-Zimmerman, 1988). A homologous lipocalin identified in *Bufo marinus* is the predominant protein secreted by the choroid plexus (Achen et al., 1992).

Sequences and initial characterizations of *nrp-1* and *cpl-1* are described elsewhere (Richter et al., 1990, and Richter et al., unpublished data; accession no. X84414); here we provide sequences for *etr-1* and *sybII*, and, for all four genes, we describe the normal expression patterns through neurula, tailbud and tadpole stages. Having established which areas of the nervous system are marked by these genes, we then proceed to examine their expression in noggin-induced neural tissue. In this way, we address whether this neural tissue is capable of differentiation and whether it possesses a dorsal-ventral pattern.

MATERIALS AND METHODS

Sequencing, sequence analysis and preparation of antisense probes

The sequence of cDNAs 17-5 and 17-30 was determined as described in Good et al. (1990a). The sequences were analyzed by the GCG computer package (Genetics Computer Group, 1991). The nucleic acid and predicted protein sequences were searched against GenBank (release 84) and using BLAST service of the National Library of Medicine (Altschul et al., 1990), while the predicted proteins were analyzed for motifs with the BLOCKS database using the BLOCKS service (Henikoff and Henikoff, 1991).

*Eco*RI fragments from clones 24-39, 17-30, 17-5 and 13-6 (Richter et al., 1988) were each subcloned into pBS KS- (Stratagene); templates for antisense probes were prepared by digesting with *Xba*I, for 17-5, or *Bam*HI, for all others. Antisense probes were transcribed with T3 RNA polymerase, as described previously (Harland, 1991).

Preparation of embryos and tissue explants

Albino *Xenopus* eggs were obtained and fertilized in vitro, as described previously (Condie and Harland, 1987). Embryos were cultured to a variety of stages, as determined from the normal table of Nieuwkoop and Faber (1967). Cranial ganglia and nerves were identified using Fig. 6 of Hemmati-Brivanlou et al. (1992) as a guide. Ventralized embryos were produced by irradiating the vegetal hemisphere at 30 minutes after fertilization with u.v. light, using a Stratalinker (Stratagene) set to 80 mJoules/cm^2 (Smith and Harland, 1991).

Animal cap ectoderm was dissected from stage 9 embryos, as shown in Lamb et al. (1993). Explants were washed in low calcium, low magnesium Ringer solution (LCMR) (Stewart and Gerhart, 1990), then incubated for 1-2 hours in either LCMR+0.5% bovine serum albumin (BSA), or the same +1 μg/ml purified human noggin protein (Lamb et al., 1993). As positive controls for *sybII* expression, some explants were instead treated with a 1:50 dilution of supernatant from COS cells transfected with an activin cDNA (provided by Regeneron). These explants were allowed to heal partially before treatment to prevent excessive mesoderm induction. After treatment, all explants were washed as before and cultured in LCMR until sibling embryos reached the desired stage.

Fixation and whole-mount in situ hybridization

The method of Harland (1991) was used, with the following modifications. All steps between the initial fixation and the final staining were performed with embryos and explants in baskets (Stachel and Grunwald, 1993), made by removing the tips of Eppendorf tubes and melting on a fine (100-200 mm) plastic mesh (Nitex no. 3-100-47, Tetko). After overnight probe hybridization, samples were washed for 10 minutes in hybridization buffer, then three times, 20 minutes each wash, in 2× SSC at 60°C. Samples were subsequently RNase-treated and washed as described previously, except that CHAPS was not used in buffers. 2% Boehringer Mannheim blocking reagent (BMBR) in maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl, pH 7.5; Lamb et al., 1993) was used instead of PBT+lamb serum in blocking and antibody incubation steps (Lamb et al., 1993); samples

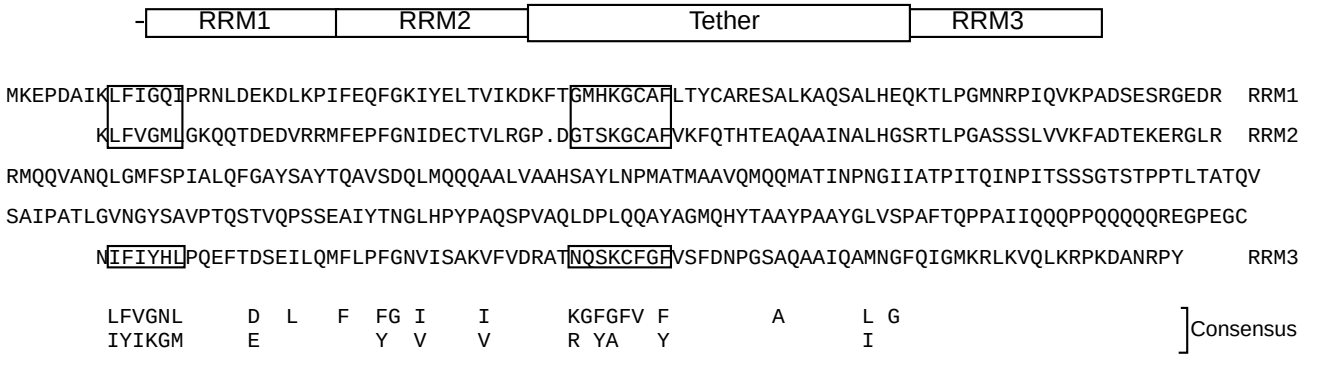

Fig. 1. Sequence of the *etr-1* protein encoded by cDNA 17-5. The diagram above shows the domains of the protein as described in the text. The identification of the RRMs is indicated at the left; each RRM is presented so that the conserved RNP1 and RNP2 (boxed sequences) are aligned. The consensus sequence at the bottom is from Burd and Dreyfuss (1994) and Birney et al. (1993). The nucleotide sequence of the *etr-1* cDNA is deposited in GenBank under accession number U16800.

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                          60                  80                 100                 120
                    .         .         .         .         .         .         .         .
17-30    QAQVDEVVDIMRVNVDKVLERDTKLSELDDRADALQAGASQFETSAAKLKRKYWWKNMKMMIIMGVICAIILIIIIVYFST
R SybII *......................Q.................................L.....L................

R SybI  *....E.....I...........Q................V..S.............C....ML A.....VVV.VI.IF.
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Fig. 2. Alignment of the *Xenopus* sybII predicted protein, encoded by the 17-30 cDNA, with the rat SYBI and SYBII sequences, with dots representing identity. The first Q of the sequence corresponds to the start of the cDNA, while the asterisk in the rat sequences represents amino terminal sequences omitted here. The nucleotide sequence for *Xenopus sybII* is deposited in GenBank under accession number U16801.

were subsequently washed in MAB instead of PBT. Either NBT/BCIP or Boehringer Mannheim purple AP-substrate was used as the substrate for single stainings.

Double staining was performed following a method devised by Doniach (Doniach and Musci, personal communication; see also Jowett and Lettice, 1994). Briefly, fluorescein-labelled probes (*etr-1* and *XAG-1*) were synthesized using fluorescinated-UTP (Boehringer Mannheim) in place of digoxigenin-UTP. Samples were simultaneously hybridized with a fluorescinated probe and a digoxigenin *cpl-1* probe. For *etr-1/cpl-1* double staining, a 1:10,000 dilution of alkaline phosphatase-conjugated anti-fluorescein antibody (Boehringer Mannheim) was used first and samples were subsequently stained with 0.175 mg/ml magenta-phos (Biosynth AG) for 1-3 days, beginning with 12-24 hours at 37°C. After sufficient staining, two steps were used to kill the activity of the first phosphatase: a 10 minute incubation at 65°C in MAB+10 mM EDTA and then dehydration in methanol. Samples were rehydrated into MAB, reblocked with MAB+BMBR for 30 minutes, incubated with anti-digoxigenin antibody, washed, and finally stained with 0.175 mg/ml BCIP (alone) in alkaline phosphatase buffer (50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 5 mM levamisol, 100 mM Tris, pH 9.5) for 8-12 hours at 37°C. For *XAG-1*/*cpl-1* double staining, the same general procedure was used, except that the anti-digoxigenin antibody was used first and samples were stained first with NBT/BCIP, as described. For the second stain, the alkaline phosphatase substrate kit II (Vector Laboratories) was used, requiring 1-3 hours at room temperature.

Histology

General histological techniques for *Xenopus* embryos are described by Kelly et al. (1991). Embryos previously stained by in situ hybridization and refixed in Bouin's fixative were washed in ethanol, then 50% ethanol, 50% AmeriClear (American Scientific Products), then 100% Americlear, for 10 minutes each wash. Next embryos were infiltrated with molten paraffin, using 3 changes, 30 minutes each. Vacuum was used during the last change to remove any residual solvent. Embryos were oriented manually in molds and paraffin was allowed to harden at −20°C. 20 μm transverse sections were made using a standard microtome. Sections were mounted on subbed slides, dewaxed in xylene, washed in ethanol and lastly cleared in BBBA (2:1 benzyl benzoate/benzyl alcohol).

RESULTS AND DISCUSSION

Identities and sequences of neural markers

We have previously isolated cloned cDNAs that are expressed in the embryonic and adult nervous system (Richter et al., 1988). Here we report the sequences and identification of the protein products encoded by cDNAs 17-5 and 17-30. The 17-5 cDNA encodes a predicted protein of 462 amino acids that is a member of a large family of genes that contain RNA recognition motifs (RRM; Fig. 1). The RRM represents a region of 80-90 residues and includes two highly conserved segments, referred to as RNP1 and RNP2 (Bandziulis et al., 1989). Each of the three RRMs of the 17-5 protein matches a consensus sequence for these regions (Birney et al., 1993), but the 17-5 RRMs are distinct from sequences in other identified RNA-binding proteins. The arrangement of RRMs in 17-5 is most similar to proteins in the RRM-containing subfamily named the *elav*-like gene family whose members are mostly expressed in the nervous system (Yao et al., 1993; Good, 1995). In proteins of this family, two consecutive RRMs occupy the amino-terminal half and are connected by a tether region to a third RRM at the carboxy terminus. Sequences similar to 17-5 have been identified by random isolation of localized cDNAs in the mouse brain (Kato, 1992; accession no. X61451) and by random cloning of a human expressed sequence tag isolated from a brain cDNA library (Adams et al., 1993; accession no. T08930); thus, the 17-5 gene is highly conserved through

Table 1. Summary of neural markers and their expression patterns

Clone	Name	Putative gene family	Expression pattern		
			Neurula stages	Tailbud stages	Tadpole stages
23-49	*nrp-1*	RNP	Neural plate, but not future floorplate Weak non-localized maternal component at early stages	Throughout CNS, including eyes, otic placodes, and cranial ganglia and nerves	Throughout brain, eyes, epiphysis, and nasal pits Declining in spinal cord
17-30	*sybII*	Synaptobrevin	Not detectable	Hindbrain, ventral spinal cord, and cranial ganglia and nerves	Throughout CNS, including eyes and epiphysis
17-5	*etr-1*	RNP	Neural plate, stronger anteriorly	Punctate staining in spinal cord and complex pattern in brain, mainly ventral Cranial ganglia and nerves	Throughout CNS, including eyes, epiphysis, nasal pits, and cranial ganglia and nerves
13-6	*cpl-1*	Lipocalin	Anterior edge of neural plate	Strongest in dorsal forebrain, trailing off posteriorly along the dorsal midline of the brain	Not detectable

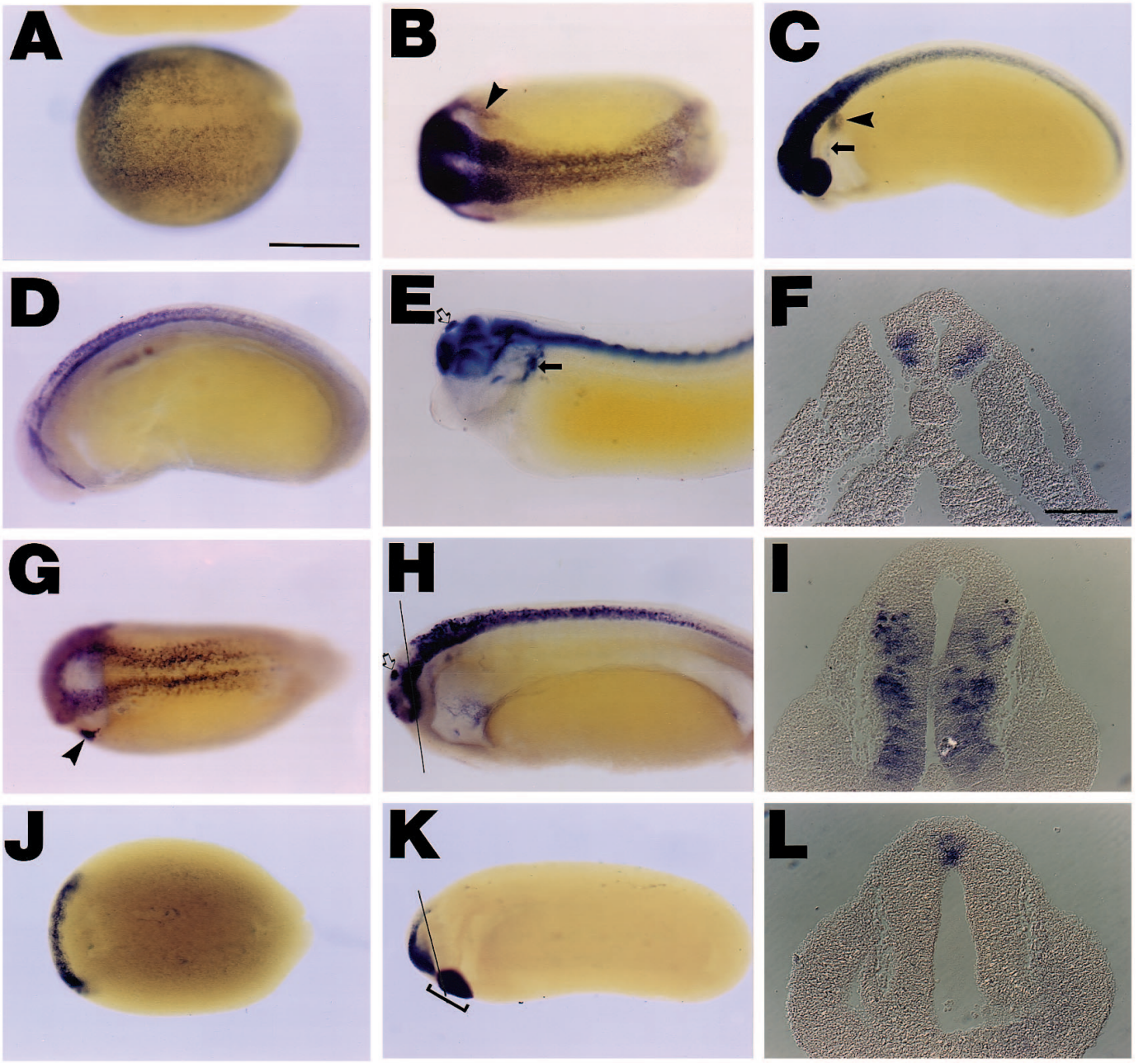

Fig. 3. Whole-mount in situ hybridizations, showing expression patterns of *nrp-1* (A-C), *sybII* (D-F), *etr-1* (G-I) and *cpl-1* (J-L) at various stages of *Xenopus* development. All embryos are oriented with anterior to the left; sections are shown with dorsal pointing up. (A) Dorsal view of stage 13 (early neurula) embryo, showing *nrp-1* expression throughout the neural plate, except for the prospective floorplate. (B) Stage 18 (late neurula), dorsal view, showing *nrp-1* expression in neural folds, around the blastopore, and in lateral stripes (indicated by arrowhead) surrounding the brain. (C) Side view of *nrp-1* expression at stage 26 (tailbud), in the CNS, otic vesicle (arrowhead) and facial nerve (arrow). (D) Side view of stage 24 embryo, stained for *sybII* expression in the spinal cord, hindbrain and trigeminal ganglion but not the anterior brain. (E) *sybII* is expressed throughout the CNS, including eyes, epiphysis (future pineal gland, indicated by open arrow) and cranial ganglia and nerves (vagus nerve indicated by solid arrow), by stage 37 (swimming tadpole). (F) Transverse section of *sybII* staining in the ventral, outer spinal cord of a stage 24 embryo. (G) Dorsal view of *etr-1*'s punctate staining in the neural folds and trigeminal placodes (arrowhead) of a stage 18 embryo. (H) Side view of stage 24 embryo, showing strong *etr-1* expression in spinal cord, hindbrain, ventral mid- and forebrain, and epiphysis (open arrow). The line drawn through the head indicates the level of the section shown in I. (I) Expression of *etr-1* in a transverse section through the ventral forebrain of a stage 24 embryo. (J) Dorsal view of *cpl-1* expression in the anterior neural ridge of a stage 13 embryo. (K) *cpl-1* expression (blue stain) in the dorsal brain at stage 23 (side view); *XAG-1* expression (brown stain, indicated by bracket) marks the cement gland. The line represents the level of the section shown in L. (L) Transverse section through the ventral forebrain and eyes of a stage 24 embryo, showing *cpl-1* expression only at the dorsal midline of the forebrain. A-E, G, H, J and K are the same magnification; scale bar in A represents 0.5 mm. Scale bar in F, for F, I and L, represents 0.1 mm.

evolution. We name the corresponding gene *elav-type ribonucleoprotein 1, etr-1*.

The cDNA 17-30 is a partial cDNA, which predicts a protein that is a member of the synaptobrevin gene family (McMahon et al., 1993). The 81 amino acid stretch encoded by the 17-30 cDNA is 96% identical to the corresponding region of the rat SYBII protein (also referred to as VAMP2 for vesicle associated membrane protein 2), indicating that 17-30 is the *Xenopus* homolog of SYBII (Fig. 2). Given the ubiquitous localization of this protein to synaptic vesicles and its expression throughout the rat brain (Trimble et al., 1990), *Xenopus sybII* should be a marker of differentiated neurons throughout the nervous system.

Normal expression patterns of *nrp-1*, *syb11*, *etr-1* and *cpl-1*

The expression patterns of these genes between stage 13 (late gastrula) and stage 38 (tadpole) were assayed by whole-mount in situ hybridization. A summary of the results is presented in Table 1. *nrp-1*, at stage 13, is expressed throughout the neural plate except for the future floor plate region (Fig. 3A). At this stage, but not later, there is some weak expression throughout the embryo, which may represent the considerable maternal component observed by Richter et al. (1988). As neurulation proceeds, *nrp-1* continues to be expressed in the neural folds, with strongest expression anterior; it is more weakly expressed in broad stripes surrounding the brain and in a ring around the blastopore (Fig. 3B). During tailbud stages (Fig. 3C) staining includes the entire central nervous system (CNS), including eyes and otic placodes, with weak staining in the tissue just behind the cement gland. The developing cranial ganglia and nerves express *nrp-1*

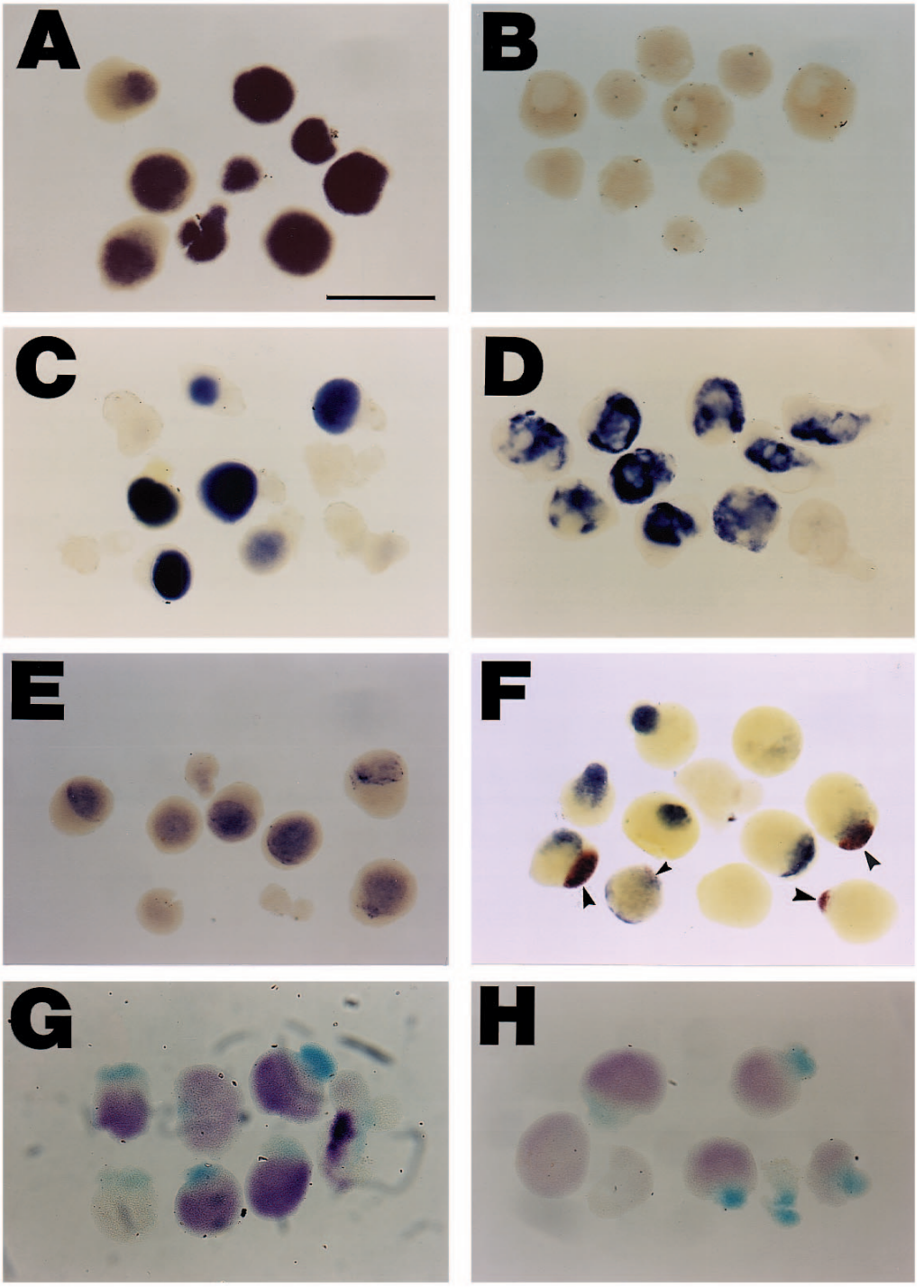

Fig. 4. Expression of *nrp-1* (A,B), *sybII* (C, D), *etr-1* (E, G, H) and *cpl-1* (F-H) in noggin-treated animal caps (except control caps in B and activin caps in D). Explants were cultured until initial tailbud stages (23-26), except for *sybII*-stained explants (C,D), which were cultured until stage 35. (A) Strong and uniform *nrp-1* expression in noggin-treated explants; uninduced caps (B) show no expression. (C) At stage 35, *sybII* is expressed diffusely throughout most noggin-treated explants and in extensive patches in activin-treated explants (D). (E) *etr-1* expression in noggin-treated animal caps is restricted to regions of the explants. (F) *cpl-1* is expressed in noggin-treated animal caps in distinct patches, which are separate from cement glands (marked by *XAG-1* expression, stained brown and indicated by arrowheads). (G) Double staining, showing non-overlapping expression of *etr-1* (purple) and *cpl-1* (turquoise) in noggin-treated animal caps. (H) Double staining of u.v.-ventralized animal caps treated with noggin, showing the same pattern of *etr-1* and *cpl-1* expression as shown in G. Scale bar represents 0.5 mm.

consecutively and transiently, such that it is expressed in the future trigeminal ganglion (cranial ganglion V) with associated ophthalmic and mandibular nerves at stage 20, then in the geniculate ganglion and facial nerve (VII) at stage 24, and finally in the glossopharyngeal (IX) and vagus (X) nerves and ganglia at stage 32. By stage 38, *nrp-1* is no longer expressed in cranial nerves or otic vesicles, and spinal cord expression is weakened, but it is expressed strongly in the brain, eyes, epiphysis (future pineal gland) and nasal pits. Thus *nrp-1* represents a general neural marker during tailbud and tadpole stages, except for minor, non-neural expression near the cement gland which can be distinguished by its reduced intensity. Furthermore, because of its strong expression, *nrp-1* may be preferable to the general neural marker *NCAM* (Kintner and Melton, 1987) in many applications.

sybII also has a maternal component (Richter et al., 1988), but expression of the gene is not detected by in situ hybridization until stage 20, when RNA levels begin to increase (Richter et al., 1988). At this stage, *sybII* is expressed weakly in the spinal cord and future trigeminal ganglion. Subsequent expression in cranial ganglia and nerves follows a similar sequence as for *nrp-1*, but with delayed timing; *sybII* appears first in the trigeminal ganglion and nerves (stage 20-26), then the geniculate ganglion and facial nerve (stage 32), and last vagus and glossopharyngeal nerves and ganglia (stage 38). Spinal cord expression intensifies through the tailbud stages (Fig. 3D); within the spinal cord, staining is restricted to the outer layers on the ventral side (Fig. 3F), where differentiating motoneurons are present (Hughes, 1959; Roberts and Clarke, 1982; Nordlander, 1986). By stage 32, expression is beginning to extend into the hindbrain, midbrain and epiphysis, with very weak expression in the forebrain and dorsal eye. Finally, by stage 38, *sybII* is strongly expressed throughout the CNS (Fig. 3E). The late onset of *sybII* expression, the delayed expression in cranial nerves (relative to *nrp-1*), the expression in ventral outer layers of the neural tube, and the very late expression in the anterior brain (a structure thought to differentiate quite late; Hartenstein, 1993; N. Papalopulu, personal communication; and Hemmati-Brivanlou and Harland, unpublished data) all suggest that *sybII* is only expressed by differentiated neurons, as is consistent with a role in synaptic vesicles.

Unlike *nrp-1* and *sybII*, *etr-1* is not expressed maternally; expression is detected weakly at stage 11 (Richter et al., 1988). At stage 13, *etr-1* is expressed throughout the neural plate, with strongest expression in the anterior two-thirds. Later, salt-and-pepper-type expression continues in the neural folds and small patches of expression lateral to the brain appear (Fig. 3G). These patches probably represent trigeminal placodes, as *etr-1* is strongly expressed in trigeminal ganglia by stage 20. In the early tailbud stages (Fig. 3H), *etr-1* is expressed in the spinal cord, hindbrain and ventral floor of the fore- and midbrain (Fig. 3I); it is absent or very weak in the dorsal fore- and midbrain, except for the epiphysis (future pineal gland) and a small patch in the dorsal forebrain. Expression levels increase during the late tailbud stages and the dorsal forebrain patch expands, such that by stage 38 *etr-1* is expressed strongly throughout the CNS, including eyes, epiphysis, nasal pits, and cranial ganglia and nerves, but excluding a region between the midbrain and hindbrain. Thus *etr-1* is specific to neural tissue, but not all neural tissue; particularly during tailbud stages it marks a complex pattern in the fore- and midbrain, including mainly the ventral floor of these regions.

Lastly, *cpl-1* RNA is undetectable in the egg but detected weakly at stage 11 (Richter et al., 1988). At stage 13, it is expressed strongly in a stripe at the anterior edge of the neural plate (Fig. 3J). This staining persists as the neural folds close, with expression ending up mainly in the dorsal forebrain and trailing off posteriorly down the dorsal midline of the brain (Fig. 3K,L). The level of expression peaks around stage 23 and afterward declines with no change in pattern. Sections reveal that *cpl-1* is strictly brain specific, with no expression in spinal cord, cement gland (marked by brown *XAG-1* staining in Fig. 3K), or non-neural ectoderm (Fig. 3L). Thus *cpl-1* marks only dorsal brain and mainly dorsal forebrain.

Expression of neural markers in noggin-induced neural tissue

Ectoderm dissected from the blastocoel roof (animal cap) of a blastula embryo develops in culture into undifferentiated epidermis; however, animal caps treated with purified noggin protein form neural tissue, in the absence of any detectable mesoderm (Lamb et al., 1993). We wished to characterize this noggin-induced neural tissue further by assaying the expression of the genes described above. By using whole-mount in situ hybridization, we addressed whether these markers are expressed by all or only a subset of cells and whether any pattern develops in the explants.

Since *nrp-1* is a general neural marker at tailbud stages, it is not surprising that *nrp-1* is strongly expressed by nearly all (56/57) of the noggin-treated animal caps assayed (Fig. 4A). Furthermore, in these explants, *nrp-1* is expressed through almost the entire explant, as is *N-CAM* or *otx2* (Lamb et al., 1993). None of the untreated animal caps express *nrp-1* (Fig. 4B). These results confirm that noggin is inducing a significant amount of neural tissue in these experiments.

Noggin-treated animal caps fixed at initial tailbud stages 23-26 do not express the differentiated marker *sybII* (data not shown). However, at these stages, *sybII* is not expressed in the anterior brain regions that noggin is thought to induce; expression in the fore- and midbrain only arises around stage 35. When noggin-treated explants are cultured to stage 35 before fixation, a majority (16/28) do express *sybII* (Fig. 4C). Since *sybII* appears to be expressed by differentiated neurons only, these results suggest that noggin-treated explants attain an advanced level of differentiation. Moreover, noggin action appears to initiate an entire cascade of differentiation without the requirement for additional inducing or transforming factors. For comparison, *sybII* expression was also examined in animal caps treated with activin, an inducer of dorsal mesoderm that then secondarily induces neural tissue, including mainly hindbrain (Bolce et al., 1992) and spinal cord (Cho and De Robertis, 1990). Nearly all of these explants (18/20) express *sybII* extensively but in distinct patches (Fig. 4D), unlike the diffuse expression throughout noggin-treated explants. This more complex pattern of expression could be attributed to interaction with ‘transforming factors’ arising from mesoderm present in activin-treated explants but lacking in noggin-treated explants. Alternatively, the patchy expression of *sybII* may reflect the different type of neural tissue induced in activin-treated explants (more posterior than in noggin-treated caps), or the overall increased complexity of

activin-treated caps (containing both neural and mesodermal tissue).

etr-1 is expressed in nearly all noggin-treated explants (40/42), grown to initial tailbud stages 23-26 (Fig. 4E). However, this expression in many cases is restricted to about two-thirds of the explant, as shown in Fig. 4E. Since explants treated similarly express *nrp-1* throughout, there must be neural tissue present that is not expressing *etr-1*. According to the normal expression of *etr-1* at this stage, this non-expressing tissue could be dorsal forebrain or midbrain, which would be marked by *cpl-1*. In agreement with this idea, noggin-treated explants (64/81) do express *cpl-1* in distinct patches (blue stain in Fig. 4F). In some explants, *cpl-1* is expressed in small protrusions, superficially resembling cement glands; however, *cpl-1* patches are separate from cement gland as marked by *XAG-1* (brown stain in Fig. 4F). To test whether *etr-1*-expressing cells and *cpl-1*-expressing cells were separate populations, we assayed the same explants for both markers, using a new double in situ hybridization technique (Doniach and Musci, personal communication). The reaction products from staining with Magenta-Phos and BCIP are each sufficiently transparent that any overlap can be seen. However, for all explants that express both *etr-1* and *cpl-1* (14/16), these genes are expressed in two separate territories of the explant (Fig. 4G).

This result provides the first evidence that noggin-induced neural tissue is not a uniform mass of anterior-type neural tissue, but an organized structure with at least two cell types. Furthermore, if expression of *cpl-1* and *etr-1* does truly represent establishment of dorsal and ventral brain regions within these explants, we must conclude that such early patterning of the brain can occur in the absence of polarizing signals (other than noggin) from other tissues. While we do not yet understand how the anterior brain is patterned, it is surprising that mesodermal signals, which are implicated both in anterior-posterior patterning of the CNS (reviewed by Doniach, 1993) as well as dorsal-ventral patterning of the spinal cord (Echelard et al., 1993; Krauss et al., 1993; and Roelink et al., 1994), may not be required for initial regionalization.

Explants from ventralized embryos

Given the absence of exogenous patterning signals (other than noggin) in our simplified explant system, there are a limited number of explanations for the origin of the observed pattern. One possibility is that, at the time of dissection, the animal caps already possessed a cryptic prepattern, which was revealed by the induction. There is evidence that animal cap ectoderm is not entirely naive; a variety of experiments have shown that the dorsal side of the ectoderm is more easily induced to form neural tissue than the ventral side (Sharpe et al., 1987; Otte et al., 1991; London et al., 1988). Furthermore, this bias can be influenced by the presence of dorsal mesoderm (Savage and Phillips, 1989; Otte and Moon, 1992), suggesting that dorsal mesoderm may play a role in organizing the ectoderm even before gastrulation begins.

To test whether the pattern in noggin-induced explants was dependent upon prepatterning of the animal cap, we produced ventralized embryos by early u.v. irradiation of the vegetal hemisphere. This manipulation disrupts cortical rotation, the first step in establishment of dorsal structures, and consequently little or no dorsal mesoderm is produced (reviewed by Gerhart et al., 1989). The degree of ventralization can be described by the dorsoanterior index (DAI) scale of Kao and Elinson (1988), where 0 indicates a completely ventralized embryo and DAI 5 is normal. In each of two experiments, the mean DAI of u.v.-treated embryos was 0.4, indicating that most embryos were completely ventralized. Animal caps dissected from these embryos were treated with noggin and assayed for *etr-1* and *cpl-1* expression as described above. The u.v. treatment has no discernible effect upon the double expression pattern (Fig 3H). The expression of *nrp-1*, *sybII* and *otx2* is also unaffected (data not shown). Thus the putative prepatterning of ectoderm by early dorsal-ventral patterning of the embryo has no detectable effect upon neural induction by noggin.

There are a number of other possible explanations for how noggin-treated explants become patterned. Animal caps may possess a cryptic prepattern that is established independently from cortical rotation and subsequent dorsal-ventral patterning. Ectoderm may also be capable of self-organization, by regulative cell-cell interactions within a neural field (Jacobson and Sater, 1988; Saha and Grainger, 1992). Lastly, the pattern may develop from unequal exposure to noggin. Since inducing factors are thought not to penetrate the epidermal layer on explants (Cooke et al., 1987), cells closer to the cut surface have more access to noggin than internal cells and this problem worsens as the explant heals into an impenetrable sphere. If, for example, a very long exposure to noggin is required to induce expression of *cpl-1*, while a shorter treatment induces *etr-1*, then *cpl-1* would be induced near the surface, at the last place to heal closed, while *etr-1* would be induced more internally.

CONCLUDING REMARKS

We have described the expression patterns of four neural-specific genes that are useful for analysis of neural induction and neural patterning. Because of its abundance and widespread expression throughout nervous tissue, *nrp-1* is an excellent general neural marker. *sybII*, in contrast, appears to only mark differentiated neurons. *etr-1* is expressed throughout the CNS, except for the dorsal fore- and midbrain; it is especially useful, then, in combination with *cpl-1*, which is expressed exclusively in the dorsal brain. By assaying the expression of these genes in noggin-treated explants, we have further characterized the induced tissue. Not only does noggin induce extensive neural tissue, but this tissue achieves an advanced level of differentiation and possesses a characteristic pattern of non-overlapping *etr-1* and *cpl-1* expression, which may represent dorsal-ventral organization of the forebrain. Thus the noggin-induced tissue is more complex than we had previously supposed, despite the absence of 'transforming factors' or organizing signals from other tissues. The source of this complexity is as yet unknown, except that it does not involve early prepatterning of the ectoderm by dorsal mesoderm. Future experiments investigating the patterning and differentiation of noggin-induced neural tissue may provide clues about the signals that are required for normal development of the central nervous system.

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