The Wnt/β-catenin signalling pathway and anterior/posterior patterning in the vertebrate central nervous system

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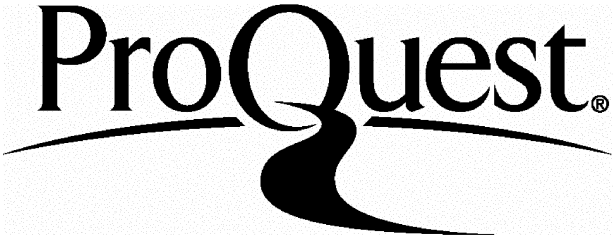

Abstract

The classical Nieuwkoop's "activation/transformation" model proposes two phases of signalling for the generation of anterior/posterior (A/P) pattern in the vertebrate central nervous system (CNS). In an initial "activation" phase a neuralising signal induces neural tissue with an anterior identity (forebrain). This is followed by a "transformation" phase, where subsequent signals confer a more posterior character (midbrain, hindbrain, and spinal cord) to the neuralised tissue. In order to identify factors that may be involved in the process of posteriorisation, a functional screen in *Xenopus* animal cap explants was performed. By injecting *noggin* RNA, which induces anterior neural tissue, together with pools of RNA from a chick somite cDNA library, I searched for clones encoding factors capable of posteriorising the *noggin* injected animal caps. This strategy led to the isolation of clones encoding truncated forms of β-catenin. This molecule induced posterior neural and dorsal mesodermal markers in animal caps neuralised with Noggin. Similar results were obtained with members of the Wnt family, including Xwnt-8 and Xwnt-3a.

To investigate whether the induction of posterior neural markers by the activation of the Wnt/β-catenin signalling pathway is dependent of mesoderm induction, I performed experiments using a chimeric and inducible form of β-catenin (GR-LEFΔNβCTA). Activation of this protein during blastula stages resulted in the induction of posterior neural and mesodermal markers. In contrast, during gastrula stages the activated form of the protein induced posterior neural markers in the absence of mesoderm, indicating that posteriorisation of neural tissue by β-catenin is independent of mesoderm formation. I further investigated the mechanism of induction of posterior neural markers by the Wnt/β-catenin signalling pathway. The results point to an indirect and non-cell autonomous mechanism, where cell-to-cell contact is required. Finally, I show evidence suggesting that the induction of posterior neural markers by the Wnt/β-catenin signalling pathway requires FGF signalling.

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Chapter 1

Introduction

In vertebrates, the central nervous system (CNS) is a structure that co-ordinates essential activities of the organism. The sensory organs transmit information about what surrounds the organism to the CNS. There, this information is processed, decisions are made and transmitted to effector organs. In humans, the CNS is the source of thoughts and emotions; the determinants of the individuality of each man or woman.

The CNS of the adult organism has its origin in the initial steps of embryonic life. During the earlier stages of the development of the embryo, in a process called gastrulation, dramatic cell movements transform the organisation of an apparently uniform mass of cells into the definitive body plan of the embryo. The precursor of the CNS, the neural tube, forms in consequence of the events that happen during gastrulation. In vertebrates, a substantial part of these early events of embryo development are similar even in distantly related species. Studying how the embryo develops in a particular species can give information that may be of general relevance.

Xenopus leavis, the African claw-toed frog, has been widely used as a model system to study early embryonic development. These animals are easily maintained in the laboratory and can produce eggs throughout all year. Injection of the female with human hormone chorionic gonadotropin induces the release of thousands of eggs in few hours. Furthermore, *Xenopus* embryos are large and develop externally of the mother, which facilitates the observation and experimental manipulation.

The next sections will cover critical events of early *Xenopus* embryonic development. Firstly, I will briefly describe the major morphological events during the first day of embryonic development. After that, I will review important classical work and more modern literature concerning the identification of the molecular players responsible for the events that proceed neural tube formation. I will finally focus on the current understanding of the molecular mechanisms responsible for the formation of the neural tube.

1.1 The first day of *Xenopus* embryo development

The development of *Xenopus* embryos starts with fertilisation. The entry of one sperm cell into the egg causes the rotation of the egg inside the vitelline membrane, so that the darker half (animal pole) turns upwards and the pale half (vegetal pole - yolk containing part) turns downwards. Sperm entry also causes completion of egg meiosis and fusion of the nuclei of the two gametes to form the zygote nucleus of the embryo. About one hour after fertilisation, the superficial layer of the egg rotates in relation to the inner part of the egg. This movement, called cortical rotation, displaces a maternal signalling centre that is initially localised in the vegetal pole and will determine where the position of the anterior/posterior (A/P) axis forms. The first cleavage occurs 90 minutes after fertilisation and divides the embryo along the animal/vegetal axis. The second division is perpendicular to the first, along the same axis and the third divides the embryo at the equator. These and subsequent cell divisions happen every 30 minutes and the deriving cells are called blastomeres. Around 5 to 6 hours after fertilisation, the embryo reaches the blastula stage and consists of a few thousand cells. The cells in the vegetal pole are bigger than the ones in the animal pole and the blastocoel, a cavity inside the animal pole, is formed. At this stage the so-called mid-blastula transition occurs. This denominates a group of modifications in the developing embryo that include the initiation of transcription. All proteins and mRNA, before this stage, were of maternal origin. Also, cell divisions, that were synchronous, now become asynchronous.

The blastula stage finishes when it is possible to observe an accumulation of pigmentation in the dorsal/vegetal side of the embryo. This area will involute inside the embryo, forming the dorsal lip of the blastopore. These are the first signs of gastrulation, a complex process of cell movements and rearrangements that internally starts 9 hours after fertilisation. At the end of gastrulation, 14 hours after fertilisation, it is possible to distinguish in the embryo three layers of cells, the germ layers (ectoderm, mesoderm and endoderm). The external layer of the embryo, the ectoderm, will form epidermis and neural tissue. Inside the embryo, the mesoderm and the endoderm will form the internal organs of the embryo. Fate mapping shows that the ectoderm originates from the animal pole, the endoderm from the vegetal pole and the mesoderm from the equatorial region of the pre-gastrula embryo. Fig. 1.1 shows the cell movements that occur during

gastrulation, with the progressive involution of the mesoderm and endoderm into the embryo and the spreading of the ectoderm to cover the embryo. During these stages a new internal cavity that will become the gut, called archenteron, starts to form and the blastocoel is displaced anteriorly.

The neural tube starts to form soon after gastrulation. This process is called neurulation and characteristic of the neurula stages of development. The first visible sign of neurulation is the formation of the neural folds, small elevations at the edges of the neural plate. The neural folds continue to rise and eventually fuse, transforming the neural plate into the neural tube. During this process the embryo grows and changes from a spherical shape into a more elongated form. Simultaneously, other structures in the embryo are formed. The notochord, a rod of cells of mesodermal origin, develops under the neural tube and confers flexibility and resistance to the developing embryo. The somites, also of mesodermal origin, develop on both sides of the notochord/neural tube axis, and are the precursors of muscle tissue.

Twenty four hours after fertilisation, the embryo presents visible signs of structure and differentiation. Tissues such as epidermis, eyes, brain, heart and muscle have already started to develop. The embryo is now at tailbud stage and the process of organogenesis will continue.

In the next section I will review literature concerning two important events that happen in the first 24 hours of *Xenopus* embryonic life: germ layer formation and the induction of the Spemann's organiser.

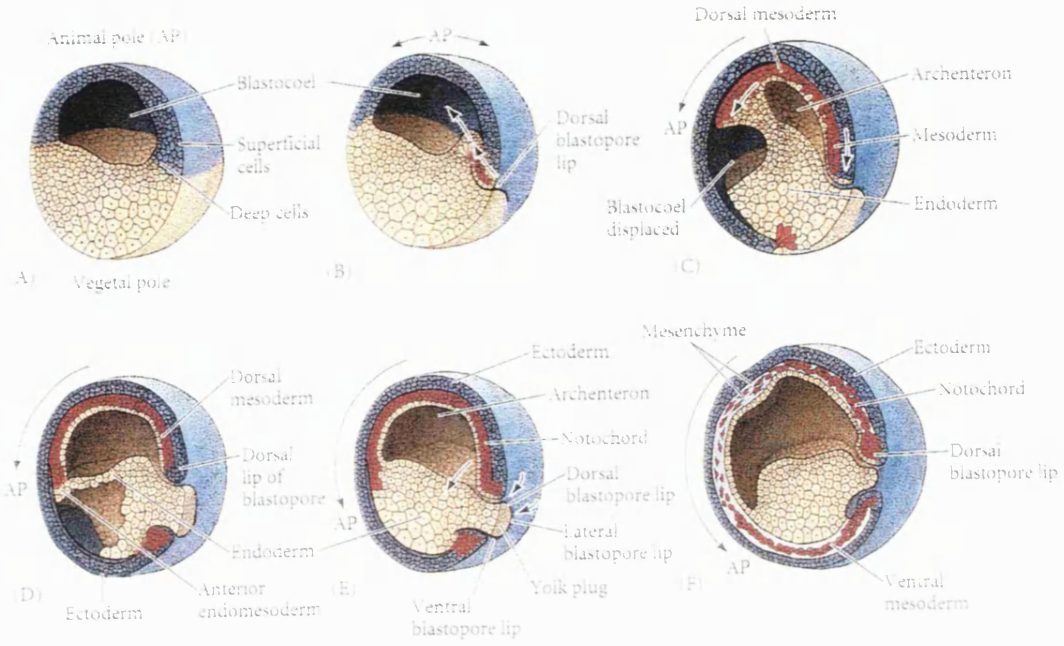


Fig. 1.1 Cell movements during gastrulation in *Xenopus*. Meridional sections cut through the middle of the embryo and positioned so that the vegetal pole is tilted to the left. The major cell movements are indicated by arrows. The ectoderm is represented in blue, endoderm in yellow and mesoderm in orange. (A, B) Early gastrulation. The bottle cells move inward to form the dorsal lip and the mesodermal precursors cells involute under the roof of the blastocoel. (C, D) Mid-gastrulation. The archenteron forms and displaces the blastocoel. Cells from the lateral and ventral lips involute into the embryo. The cells from the animal pole migrate down to cover the embryo. (E, F) End of gastrulation. The embryo becomes completely surrounded by ectoderm and the blastopore closes. The endoderm and mesoderm are now inside the embryo. From Gilbert (2000).

1.2 The Spemann's organiser and germ layer formation

An important event in the history of developmental biology was the discovery of the organiser, in the 1920's by Spemann and colleagues (Spemann, 1924; review in Hamburger, 1988). Working with newt embryos, these authors found that the transplantation of the dorsal lip of a gastrulating donor embryo into the ventral side of another embryo (recipient) results in the formation of a secondary axis, containing a neural tube, notochord and somites. Because these authors used donor and recipient embryos with different pigmentation, they could identify the origin of the induced embryonic structures. The notochord was found to originate from the graft, while the neural tube and somites originated from the recipient embryo. The inference from this result was that the graft gave an instructive signal to the tissues of the recipient embryo that mobilised them to form embryonic structures. For this reason the graft tissue became known as the "organiser". Functional homologues of the amphibian Spemann's organiser were described in other vertebrate species, including *Xenopus* (Gimlich and Cooke, 1983), zebrafish (the embryonic shield) (Shih and Fraser, 1996), chick (Hensen' s node) (Waddington, 1932) and mouse (the node) (Beddington, 1994). In fact, the existence of a group of cells with the characteristics of the Spemann's organiser is probably a feature of embryo development of all chordates (Harland and Gerhart, 1997).

Another event in early embryonic development is the formation of the three germ layers: ectoderm, mesoderm and endoderm. Nieuwkoop, in the 1960's, initiated important experiments for the interpretation of this process. This author showed that explants of amphibian embryos cultured in isolation or in combinations develop into characteristic tissues; the animal pole differentiated as epidermis and the most vegetal part of the embryo maintained its appearance and developed into a poorly differentiated endoderm. However, conjugates of animal and vegetal pole regions formed several mesodermal tissues including muscle (Nieuwkoop, 1969a). From these results Nieuwkoop concluded that the vegetal pole released signals that induced the animal pole to become mesoderm. Furthermore, Nieuwkoop and colleagues found that recombinants of dorsal vegetal cells and animal pole tissue developed into dorsal types of mesodermal tissues, including notochord and muscle, while recombinants of ventral vegetal cells and animal pole tissue developed into ventral types of mesodermal tissues such as blood and

mesenchyme (Boterenbrood, 1973). These results led to the proposal that two signals from the vegetal pole, one from the ventral side and another from the dorsal side, induce and pattern the overlaying mesodermal tissue.

This two signal model for induction of mesoderm was supported by experiments showing that embryos treated with ultra-violet (UV) radiation do not develop axial structures, such as neural tube and notochord, but develop ventral tissues of the three germ layers (ectoderm, mesoderm and endoderm) (reviewed in Heasman, 1997). These experiments showed that induction of ventral and dorsal tissues occurred by different processes, since only the induction of dorsal tissues was sensitive to UV treatment. Furthermore, it was established the existence of two periods of competence for the ventralisation of embryos by UV treatment, the first being during the maturation of the oocyte, before fertilisation of the egg, and the second shortly after sperm entry. During this second period, after fertilisation, UV prevented the microtubule mediated process of cortical rotation, in which the cortex of the egg moves in relation to the cytoplasmic core (reviewed in Gerhart et al., 1989). Embryos treated with UV during this period could be rescued to normal development of dorsal structures by tilting the embryo in respect to gravity, which permitted movements of the cytoplasm that mimicked cortical rotation. On the other hand, the existence of the pre-fertilisation period of UV sensitivity indicated that essential components for the formation of the axial structures of the embryo are present in the vegetal part of the egg before fertilisation (Holwill, 1987). During this pre-fertilisation period, UV irradiation of the oocyte did not disrupt cortical rotation of the fertilised egg and the dorsal structures were not rescued by tilting the UV treated embryos (Elinson and Pasceri, 1989). These experiments showed that two distinct but equally important steps in the process of dorsal tissue formation were being affected by UV-treatment.

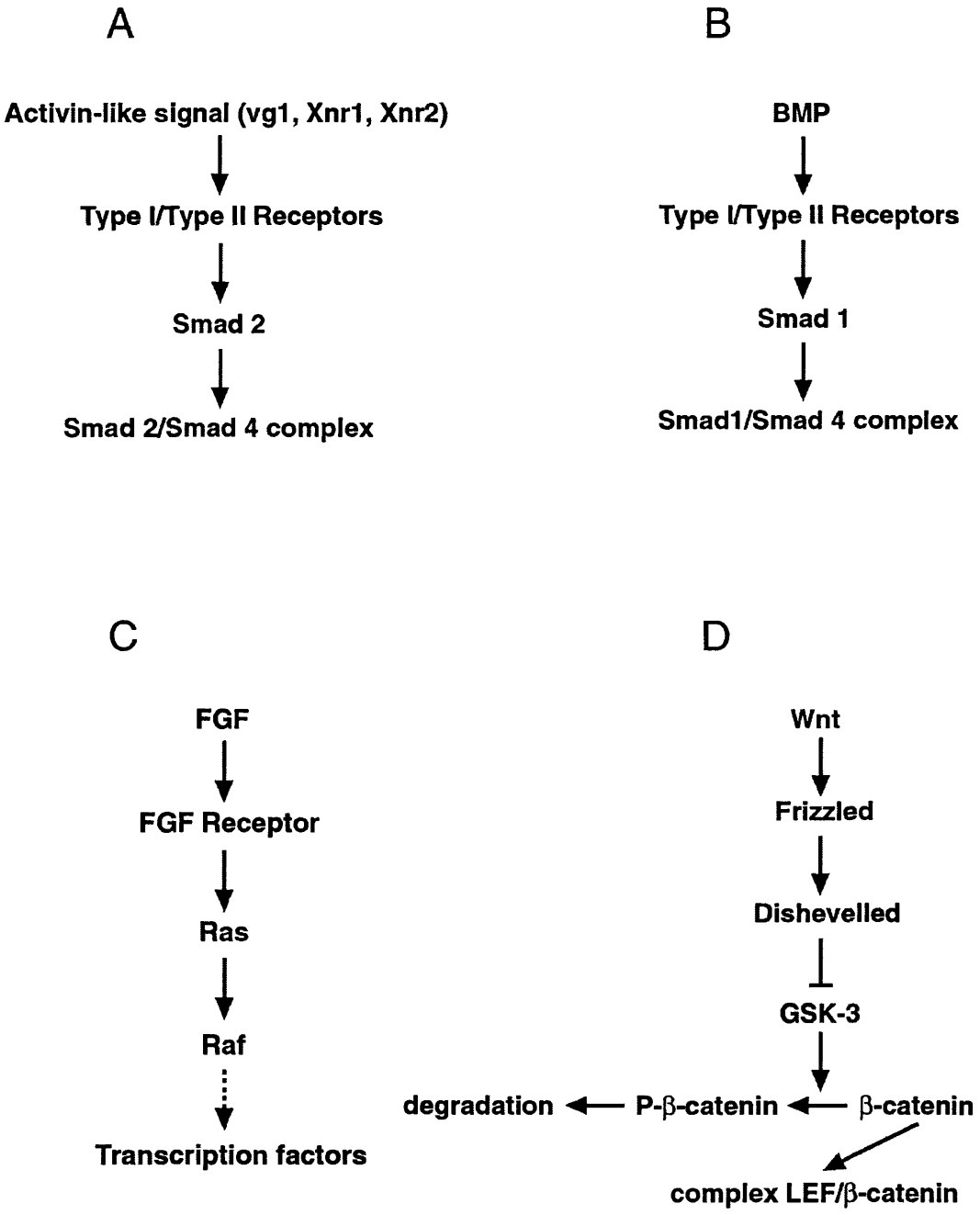
The existence of a vegetal dorsalising signal was confirmed in experiments done by Gerhart and collaborators (reviewed in Gerhart et al., 1989). These authors showed that dorsal vegetal blastomeres taken from 32 to 64-cell stage embryos, when grafted into UV treated embryos, can rescue the development of normal dorsal structures. This effect was not obtained when ventral vegetal blastomeres were grafted. When the authors labelled the dorsal vegetal cells of the graft with a lineage tracer, they found that the

progeny of these cells did not populate the dorsal structures of the rescued embryo. These results constituted further evidence of the inductive properties of the dorsal vegetal cells that were denominated by these authors as "organiser inducing region" or "Nieuwkoop center", in acknowledgement of Nieuwkoop's pioneering contribution (Gerhart et al., 1989; Harland and Gerhart, 1997).

The two signals model of mesoderm induction proposed by Nieuwkoop was modified by Slack and colleagues to include a third signal (Smith, 1989; Smith and Slack, 1983) that would act after the initial two. This third signal is produced by the newly induced dorsal mesoderm and acts over the prospective mesoderm as a dorsalising signal. Evidence for the existence of the third signal came from experiments in which ventral and dorsal mesodermal regions of early gastrula stage embryos where cultured together. This caused the ventral regions, that alone formed only blood and mesenchyme, to form more dorsal structures such as muscle (Dale, 1987b; Slack and Forman, 1980). This late dorsalising signal also explained observations made from fate mapping experiments with *Xenopus* embryos, where ventral mesodermal blastomeres contributed to the formation of muscle tissue (Dale, 1987a).

Over the last 20 years, many laboratories have developed efforts in order to identify and characterise candidate molecules for the three signals model of mesoderm induction. Substantial progress has been made and several secreted proteins and respective signalling pathways are now known to play a role in these events. These include members of the Fibroblast Growth Factor (FGF) and Transforming Growth Factor-β (TGF-β) families of secreted proteins. Intracellular components of the Wnt pathway have also been described as modifiers, with the ability to dorsalise mesoderm induced by other factors. Fig. 1.2 is a summary of the intracellular signalling pathways that are activated by each family of secreted proteins. The following sections will review literature concerning the role of each family of secreted proteins in the processes of dorsal axis induction and germ layer formation.

Fig. 1.2 Summary of signal transduction pathways that contribute to early embryonic patterning. (A) TGF-β/Activin-like signalling pathway. The ligands (Activin, Vg1, Xnr1, Xnr2) trigger the formation of a complex between the type I (ActR-IB) and the type II (ActR-II) Ser/Thr kinase receptors. The formation of this complex results in the activation of the type I receptor, which specifically activates Smad 2 or Smad 3. Activated Smad 2 forms a complex with Smad 4, which is transported into the nucleus, where it associates with other co-factors, such as Fast-1, and activates transcription of target genes. (B) BMP signalling pathway. BMPs use different type I (BMPR-IA) and type II (BMPR-II) receptors to activate Smad 1, Smad 3 or Smad 8. These Smads form a complex with Smad 4 and activate transcription of target genes in the nucleus. (C) FGF signalling pathway. FGFs induce the dimerisation and activation of the FGF Tyr kinase receptors. This activated complex binds SH2-SH3 proteins, which recruit the Ras/MAP kinase signalling pathway, leading to the transcriptional activation of target genes in the nucleus. (D) Wnt signalling pathway. Wnt ligands bind Frizzled receptors, which by a mechanism involving Dishevelled, inactivate GSK-3. The phosphorylation of β–catenin by GSK-3 promotes the proteolytic degradation of β-catenin. The Wnt induced inactivation of GSK-3 results in an accumulation of β–catenin in cytoplasm. β-catenin associates with Tcf/Lef family members and activates the transcription of target genes in the nucleus. In the absence of β-catenin, Tcf/Lef may function as repressors of transcription. Adapted from (Cadigan and Nusse, 1997; Harland and Gerhart, 1997; Massague and Chen, 2000; Nusse, 1999).


A
Activin-like signal (vg1, Xnr1, Xnr2)
Type I/Type II Receptors
Smad 2
Smad 2/Smad 4 complex
B
BMP
Type I/Type II Receptors
Smad 1
Smad1/Smad 4 complex
C
FGF
FGF Receptor
Ras
Raf
Transcription factors
D
Wnt
Frizzled
Dishevelled
GSK-3
degradation
P-β-catenin
β-catenin
complex LEF/β-catenin

1.2.1 FGFs

One of the first mesoderm inducing molecules to be isolated and characterised was bFGF (Slack et al., 1987). This was done using purified bFGF from bovine brain and ectodermal explants as an assay. Untreated ectodermal explants (animal caps) formed spheres containing only epidermal tissue. Animal caps treated with bFGF differentiated into ventral mesodermal tissues. Histological analysis of these tissues identified mesenchyme and muscle but not notochord. This led to the proposal of bFGF as being the candidate molecule for the ventral vegetal signal of the three signals model.

bFGF transcripts were found to be present in *Xenopus* oocytes and embryos (Kimelman and Kirschner, 1987). However, the fact that this molecule does not contain a signal sequence (Kimelman et al., 1988), which is required for the secretion of proteins from the cell, made bFGF an improbable candidate for the endogenous ventral vegetal mesoderm inducing signal. In support of this, neutralising antibodies for bFGF failed to inhibit the induction of ventral mesoderm, in experiments using recombinants of animal cap and ventral vegetal tissues (Slack, 1991). Other *FGFs* were cloned in *Xenopus*, such as *eFGF* (Isaacs et al., 1992), *FGF-3* (Tannahill et al., 1992) and *FGF-8* (Christen and Slack, 1997) but the spatial and temporal patterns of expression of these genes did not fit with being the ventral vegetal inducer.

Evidence in support of a more general role for FGF signalling in mesoderm formation came from experiments where this signalling pathway was blocked by the use of a dominant negative FGF receptor, XFD, which lacks the intracellular domain of the protein that is required for signalling (Amaya et al., 1991; Amaya et al., 1993). These experiments showed that FGF signalling is required not only for formation of ventral structures but also dorsal ones, such as notochord and muscle. In fact, all mesodermal tissues except head mesoderm were affected. Other experiments highlighted the role of FGFs not in the induction but in the maintenance of mesoderm (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). *eFGF* was found to be expressed during gastrula stages and to regulate the expression of *Xbra*, a gene required for mesoderm formation. This requirement of FGF signalling for the maintenance of mesodermal tissues was also supported by experiments where XFD was expressed in transgenic embryos (Kroll and Amaya, 1996). These embryos only expressed XFD after the onset of zygotic

transcription, during gastrula stages, but still failed to develop most mesodermal tissues. Furthermore, studies with an antibody that recognises the activated form of ERK, a downstream target of FGF signalling, identified spatial and temporal patterns of response to FGF signalling in the embryo. These started at late blastula stage in the dorsal region of the embryo and continue through gastrulation in the posterior part of the embryo (Christen and Slack, 1999; Curran and Grainger, 2000). Taken together, these experiments support a model where FGF signalling is required for mesoderm formation but does not seem to be the relevant signal for mesoderm induction.

1.2.2 TGF-βs

The members of the transforming growth factor-β (TGF-β) family of secreted proteins are divided in sub-families that include the TGF-βs, the Activins/Inhibins, the Nodals and the Bone Morphogenetic Proteins (BMPs). Activin was isolated from the supernatant of a *Xenopus* cell line based on its ability to induce mesoderm in animal cap explants (Smith, 1987; Smith et al., 1990). Different isoforms of the protein were detected during early stages of embryo development (Fukui et al., 1994). Experiments where Activin signalling was blocked in the embryo by overexpression of a dominant negative form of the activin type II receptor lacking the serine/threonine kinase domain in the cytoplasmic portion of the receptor, resulted in the loss of mesodermal structures (Hemmati-Brivanlou, 1992). However, this dominant negative receptor was found to block signalling by other members of TGF-β family, such as BMPs (Hemmati-Brivanlou, 1995) and Vg1 (Schulte-Merker et al., 1994). In experiments where embryos were treated with Follistatin, a more specific inhibitor of Activin, normal mesoderm formation was observed (Schulte-Merker et al., 1994), which argues against an *in vivo* role for Activin in mesoderm induction.

Vg1 is another member of the TGF-β family that has been involved in mesoderm induction. *Vg1* mRNA was detected in the vegetal part of oocytes and early embryos (Weeks and Melton, 1987) but only a small fraction of the protein was detected as the processed active form (Tannahill and Melton, 1989). Artificially processed Vg1 protein, by the use of a protein chimera where Vg1 C-terminal domain was fused to the N-terminal domain of BMP-2, induced dorsal mesoderm in animal caps and rescued axial

development in UV-treated embryos (Thomsen and Melton, 1993). This result led to the proposal that localised processing of Vg1 precursor protein in the dorsal part of the embryo would be a tightly regulated step for mesoderm formation. In support of this model, overexpression of a dominant negative Vg1 in embryos disrupted the formation of dorsal mesoderm (Joseph and Melton, 1998).

Members of the BMP family have been shown to specify the dorsal/ventral character of induced mesoderm rather than being involved in mesoderm induction. Overexpression of *BMP-4* in animal caps induced ventral mesoderm and ventralised mesoderm induced by Activin (Jones et al., 1992; Dale, 1992). This ventralisation effect was also observed in *BMP-4* injected embryos, where ventral structures formed at the expense of dorsal ones. Furthermore, BMP-4 was shown to act only during gastrula stages and not during the initial steps of mesoderm induction (Jones et al., 1996). Overexpression of *BMP-4* mRNA in embryos did not block the initial activation of dorsal genes, such as *goosecoid* and *Xnot*, but downregulated these genes and activated a ventral one, *Xhox3*, during later stages of gastrulation. Blocking BMP signalling with a truncated BMP receptor converted ventral mesoderm into dorsal, without having an effect in the total amount of mesoderm formed (Graff et al., 1994). This finding was supported by the discovery that *noggin* (Smith and Harland, 1992) and *chordin* (Sasai et al., 1994) were able to dorsalise mesoderm when overexpressed in the embryo. These genes are expressed in the Spemann's organiser and encode secreted proteins that bind BMPs and block BMP signalling (Piccolo et al., 1996; Zimmerman et al., 1996). As a consequence of these results, BMP antagonists, according to 3-signals model for mesoderm formation, have been considered as the third signal with dorsalising activity. Later in this chapter I will discuss the idea that the balance between BMPs and their antagonists may also be able to pattern the ectoderm into neural and non-neural domains.

Members of the Nodal family induce mesoderm in animal caps and rescue the development of dorsal structures in UV ventralised embryos (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000). Loss of function experiments in mouse and zebrafish showed that Nodal signalling is required for the formation of mesoderm. In mice with a retroviral insertion mutation in the *nodal* gene, formation of the primitive streak and most mesodermal derivatives was compromised (Conlon et al., 1994).

Similarly, double mutants for the zebrafish *nodal* related genes *squint* and *cyclops* failed to form head and trunk mesoderm (Feldman et al., 1998). In *Xenopus*, specific repression of Nodal signalling was obtained by the use of a dominant negative cleavage mutant of *Xnr2* (Osada and Wright, 1999) or by the carboxy-terminal fragment of Cerberus, Cer-S (Agius et al., 2000). These reagents repressed the formation of mesoderm in embryos and in recombinants of animal and vegetal cells. Despite these results, members of the Nodal family have been dismissed until recently as endogenous mesoderm inducers because their expression is only zygotic and maternal components are more likely to be involved in the early inducing events (Heasman, 1997). This view has been challenged since the identification of *VegT*, a T-box transcription factor maternally expressed in the vegetal pole and zygotically in the marginal zone of the embryo (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). Overexpression of *VegT* induced mesoderm in animal caps and secondary axis in embryos. Overexpression of a dominant negative form of VegT, consisting of a protein chimera with the transcriptional repression domain of the *Drosophila* engrailed protein, repressed the expression of mesodermal genes and blocked the development of the axial structures of the embryo (Horb and Thomsen, 1997). More importantly, VegT was shown to be required for the formation of mesoderm and endoderm, in experiments where maternal transcripts of *VegT* were depleted by injection of specific antisense oligonucleotides (Zhang et al., 1998). VegT depleted embryos developed with a strong reduction of axial structures and with an alteration of the blastula stage fate map. Endoderm did not form, mesoderm formed from the vegetal pole cells and ectoderm formed from cells of the animal pole and marginal zone of the embryo. Moreover, recombinants of vegetal and animal pole cells taken from normal and VegT depleted embryos revealed that VegT is required in the vegetal pole cells for the release of mesoderm inducing signals. Recombinants of VegT depleted vegetal cells with normal animal pole cells failed to form mesoderm, while recombinants of normal vegetal cells with VegT depleted animal pole cells formed normal mesoderm. These results indicated that essential mesoderm inducing signals have a zygotic rather that a maternal origin, since their production is dependent on transcriptional activation by VegT. In support of this idea, in VegT depleted embryos members of the *nodal* family are downregulated and VegT binding

sites were found in the *Xnr1* promoter. Finally, injection of RNA of members of the *nodal* family rescues the development of mesoderm in VegT depleted embryos (Kofron et al., 1999).

1.2.3 Wnts

The first evidence suggesting that members of the Wnt family of secreted proteins may be involved in axis formation came from experiments in which overexpression *Wnt1* and *Wnt8* in the ventral side of the embryo induced a secondary axis and rescued the development of axial structures in UV-treated embryos (McMahon, 1989; Smith and Harland, 1991; Sokol et al., 1991). This result was supported by additional experiments showing that intracellular components of the Wnt signalling pathway have identical axis inducing properties. Overexpression in the ventral side of the embryo of positive regulators of the pathway, like Dishevelled (Dsh) (Sokol et al., 1995) and β-catenin (Funayama et al., 1995), resulted in the induction of secondary axes. Overexpression studies with a negative regulator of the pathway, glycogen synthase kinase-3 (GSK-3), and a dominant negative form of this molecule with no kinase activity (dnGSK-3), confirmed the previous results (He et al., 1995; Pierce and Kimelman, 1995). More importantly, β-catenin was shown to be required for normal axis formation because depletion of the maternal protein from embryos suppressed the development of axial structures, a phenotype similar to the ventralisation caused by UV-treatment (Heasman et al., 1994).

However, none of the known Wnt proteins seems to be involved in axis formation *in vivo* because they are not expressed during the appropriated stages. The best candidates would be Xwnt8b and Xwnt11. *Xwnt8b* was shown to be maternally expressed and to have axis inducing activity when overexpressed in embryos, but its expression is stronger in animal pole than in the vegetal pole of the embryo (Cui et al., 1995). *Xwnt11* is expressed maternally in the vegetal pole and the protein was found to accumulate on the dorsal side of the embryo (Schroeder et al., 1999), but its axis inducing ability is not well established. Injection of *Xwnt-11* RNA in UV-treated embryos only partially rescued the development of axial structures (Du, 1995; Ku and Melton, 1993).

While it is possible that an as yet unidentified Wnt protein is involved in axis formation *in vivo*, experiments that used antagonists of the Wnt pathway suggest this is not the case. In one of these studies, a mutant dominant negative form of Dishevelled with a deletion of the PDZ domain (Xdd1) was able to block double axis induction by Xwnt8 when injected in the ventral side of the embryo, but failed to block induction of the primary axis when expressed dorsally (Sokol, 1996). Similar results were obtained by overexpression of extracellular antagonists of the Wnt pathway, such as, dominant negative *Xwnt8* (Hoppler et al., 1996) and *FrzA* (Xu et al., 1998), which encodes a protein similar to the extracellular domain of the Wnt receptor, Frizzled. Overexpression of these molecules in the dorsal side of the embryo failed to block primary axis induction, although they blocked the induction of secondary axes, when injected together with *Xwnt8* RNA in the ventral side of the embryo.

These results suggest that, for axis formation, stabilisation of β-catenin may occur by a mechanism that does not involve the upstream components of the pathway, Dishevelled and Wnts, but still requires the inactivation of GSK-3. In support of this, GSK-3 protein levels seem to be depleted on the dorsal side of the embryo in the same region where β-catenin enrichment was observed (Dominguez and Green, 2000). Furthermore, these authors showed that overexpression of *Xwnt8* and *Xdsh* in the ventral side of the embryo did not cause depletion of GSK-3 levels, but only affected the specific activity of the kinase. On the other hand, ventral overexpression of *GBP* (GSK-3 binding protein), an antagonist of GSK-3 that is required for development of the dorsal structures of the embryo (Yost et al., 1998), depleted GSK-3 levels without affecting the specific activity of the kinase. This indicates that the inactivation of GSK-3 that is required for axis formation may be achieved by a GBP dependent (but Wnt/Dsh independent) mechanism that targets GSK-3 to degradation. How this mechanism is regulated and how GBP action is restricted to the dorsal side of the embryo is not known.

Independently of the mechanism that leads to the inactivation of GSK-3, the stabilisation and transport of β-catenin to the nucleus, where it associates with members of Tcf/Lef family of transcription factors, causes the transcriptional activation of target genes. Among these is *Xnr3,* a member of the TGF-β family, which is induced by the Wnt/β-catenin signalling pathway through a mechanism that is dependent on the integrity

of Tcf/Lef binding sites present on its promoter (McKendry et al., 1997). Other target genes are the transcription factors *siamois* (Brannon et al., 1997) and *Xtwin* (Laurent, 1997), which are expressed in the dorsal marginal zone during blastula and early gastrula stages and have the capacity to induce a secondary axis when overexpressed in the ventral side of the embryo (Lemaire et al., 1995). For the induction of these genes, however, the Wnt/β-catenin signalling pathway may co-operate with the TGF-β pathway. Overexpression of *Xwnt8* together with *Smad 2*, which transduces Activin-type of signals, induced *siamois* more strongly than *Xwnt8* alone and overexpression of a dominant negative activin receptor in embryos caused a reduction of the expression of *siamois* (Crease et al., 1998). Additionally, β-catenin and Lef-1were shown to bind Smad 4 and to act synergistically in the induction of *Xtwin* (Nishita et al., 2000).

In addition to the role of the Wnt/β-catenin signalling pathway in dorsal axis formation, some authors have proposed a later role during gastrula stages, in the induction or maintenance of ventral mesodermal fates. In support of this, *Xwnt8* is normally expressed in the ventral and lateral regions of the embryo during gastrulation. Furthermore, ectopic expression of *Xwnt8* in the dorsal side of the embryo, by the use of a plasmid that only drives *Xwnt8* expression after the onset of zygotic transcription, caused ventralisation of the embryo (Christian and Moon, 1993). In accordance with this, inhibition of the Wnt signalling pathway on the ventral side of the embryo, by injection of the extracellular domain of XFrizzled 8 (ECD8), caused the formation of partial secondary axis, indicating that Wnt signal is required for formation of ventral cell fates (Itoh and Sokol, 1999).

1.3 Neural induction

The inductive processes that happen until late blastula stages cause the formation of the Spemann's organiser. It is though that this population of cells starts its activity at the onset of gastrulation, eventually leading to the consequences first observed by Spemann and collaborators in the 1920s. The activity of the organiser can be divided in three aspects (Harland and Gerhart, 1997):

1) Self-differentiation – cells of the organiser differentiate into a variety of tissues such as notochord and prechordal plate.

2) Morphogenesis – as a consequence of movements that first cause the internalisation of the mesoderm and endoderm, and later the convergence and extension of the embryo.

3) Induction – the cells of the organiser secrete factors that affect the three germ layers of the embryo. These factors cause, for example, dorsalisation of the mesoderm and induction of neural tissue in the ectoderm. The next sections of this introduction will focus on the process of neural induction by factors secreted from the organiser.

Classical embryological experiments analysed the problems of neural induction and patterning of the neural tissue along the anterior/posterior axis. These experiments led to the formulation of two different models (reviewed in Foley et al., 2000; Gamse and Sive, 2000; Slack and Tannahill, 1992). The first of these models, initially proposed by Spemann and collaborators (Spemann, 1931 as in Foley et al., 2000), states that the different regions along the anterior/posterior axis of the neural plate are induced by different signals arising from the corresponding regions of the underlying mesoderm. A second model, proposed by Nieuwkoop and collaborators (Nieuwkoop, 1952), argues that the induction and patterning of neural tissue occurs in two phases. During the initial "activation" phase, neuralising signals induce only an anterior type of neural tissue (forebrain). This is followed by a "transformation" phase, where subsequent signals confer a more posterior character to the neuralised tissue (midbrain, hindbrain and spinal cord). The signals of this second phase could be distributed as a gradient, with the highest concentration of signals inducing the most posterior structures.

Other studies focused on the problem of how the neural inducing signals produced by the mesoderm reach the ectodermal tissue. The traditional view, from the early experiments of the discovery of the organiser, was that neural inducing signals pass vertically from the involuting mesoderm to the overlaying ectoderm (vertical signalling). More recent experiments, that used culture of explants and recombinants of mesoderm and ectoderm, showed that some neural inducing signals may pass from the mesoderm to the ectoderm along the plane of the tissue without the requirement of mesoderm involution (planar signalling) (Dixon and Kintner, 1989; Poznanski and Keller, 1997). The culture of recombinants of mesoderm and ectoderm was also used to address the

problem of competence of the ectoderm to respond to mesodermal neural inducing signals (Sharpe and Gurdon, 1990). These authors studied the induction of specific anterior and posterior neural molecular markers in the recombinants, and showed that the competence of the ectoderm to respond to the mesodermal signals is lost by early neurula stages (stages 13-14).

Other studies led to the discovery of genes expressed in a differential manner along the AP axis. These markers allowed a more detailed characterisation of each region of the AP axis than was possible by histological analysis. Among the genes first described in *Xenopus* were *NCAM*, expressed in all neural tissue (Kintner and Melton, 1987), *En-2*, expressed in the midbrain/hindbrain barrier (Hemmati-Brivanlou et al., 1991) and *Hoxb9*, a *Hox* gene expressed in the spinal cord (Sharpe et al., 1987).

Hox genes were first described in *Drosophila* because mutations in these genes cause homeotic phenotypes, where parts of the body were replaced by others. *Hox* genes encode transcription factors that contain a common DNA binding motif, the homeobox. These genes are organised in genomic clusters and their expression patterns along the AP axis recapitulate their organisation in the genomic cluster, a property termed colinearity. Vertebrate homologues of the *Drosophila Hox* genes were found to have similar properties in terms of genomic organisation and expression patterns. Gain of function and loss of function analysis, in particular done in mice, led to homeotic transformations and confirmed the role of these genes in the establishment of AP patterning in vertebrates (Krumlauf, 1994).

Finally, some authors investigated the molecular identity of the neural inducing signals. The early efforts were unsuccessful, because existing biochemical methods were limited and the ectoderm of the most commonly used newt species had a tendency to autoneuralise, that is, to adopt neural fates in response to a variety of non specific stimuli (reviewed in Wilson and Hemmati-Brivanlou, 1997). Studies performed in the last ten years overcame these limitations and successfully identified molecules with the capacity to induce neural tissue. These studies will be reviewed next.

1.3.1 Anterior neural induction and BMP signalling repression

The traditional view of the neural induction process, derived from the classical embryological experiments of transplantation of the organiser, was that the ectoderm receives signals from the organiser that induce neural fate. In the absence of these signals, ectodermal cells adopt an epidermal fate. It was therefore surprising, when it was reported that ectodermal cells, when cultured dissociated and in the absence of any signal from the organiser, adopt neural fate (Godsave and Slack, 1989; Godsave and Slack, 1991). One of the hypotheses raised to explain this result was that ectodermal cells normally produce an inhibitor of neural induction that would be lost upon dissociation of cells. This possibility gained further support from the finding that overexpression of a dominant negative form of the Activin type II receptor caused neuralisation of intact ectodermal explants (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou, 1992). The initial interpretation of this result was that inhibition of Activin signalling was sufficient to promote neural fate and Activin signalling should promote epidermal fate. However, the dominant negative Activin receptor inhibited not only Activin but also BMP signalling and, in fact, it was not Activin but BMP-4 that induced epidermal fate in dissociated ectodermal cells that otherwise would form neural tissue (Wilson and Hemmati-Brivanlou, 1995). Similar results were obtained by overexpression of *BMP-2*, *BMP-7*, the positive intracellular regulators of the BMP pathway *Smad 1* and *Smad 5*, and a constitutively active *BMP 2/7* receptor (Suzuki et al., 1997; Suzuki et al., 1997; Wilson et al., 1997). Overexpression of negative intracellular regulators of BMP pathway, such as, *Smad 6* and *Smad 7*, induced neural tissue in intact ectodermal explants (Casellas and Brivanlou, 1998; Hata et al., 1998). Furthermore, the patterns of expression of *BMP-2*, *BMP-4* and *BMP-7* are suggestive of their role in promotion of epidermal fate and repression of neural fate. These genes are expressed throughout the ectoderm at the beginning of gastrulation and later their expression disappears from the prospective neural plate but it is maintained in the future epidermal area (Fainsod et al., 1994; Hemmati-Brivanlou, 1995).

A second line of evidence came from a variety of approaches that led to the cloning of genes with the ability to induce neural tissue, when overexpressed in ectodermal explants. These include *noggin* (Lamb et al., 1993), *follistatin* (Hemmati-

Brivanlou et al., 1994), *chordin* (Sasai et al., 1995), *cerberus* (Bouwmeester et al., 1996) and *Xnr3* (Hansen et al., 1997). All of these genes are expressed in the organiser (or in parts of the organiser) during gastrulation, which makes them candidates as organiser derived neural inducing signals.

The reconciliation of these two lines of evidence came from experiments showing that the neural inducing molecules produced by the organiser act as antagonists of BMP signalling. Noggin and Chordin bind directly to BMPs preventing the activation of the BMP receptor (Piccolo et al., 1996; Zimmerman et al., 1996). Follistatin was suggested to form an inactive trimeric complex with BMP-4 and BMP receptor (Iemura et al., 1998). Cerberus binds BMPs and also members of the Wnt and Nodal families of proteins (Piccolo et al., 1999). The mechanism of action of Xnr3 is not known but it was suggested that Xnr3 competes with BMPs for binding to the receptors (Hansen et al., 1997). The synthesis of these results led to the elaboration of the "neural default" model for the mechanism of neural induction (Wilson and Hemmati-Brivanlou, 1997). According to this model, cells of the gastrula ectoderm have an intrinsic tendency to differentiate into neural tissue. This tendency is inhibited through the action of BMP signalling that promotes epidermal differentiation. During gastrulation, BMP antagonists produced in the organiser establish a "BMP free" domain that undergoes neural differentiation and forms the neural plate.

According to the "neural default" model, the specification of neural or epidermal cell fate depends on the balance between BMPs and BMP antagonists. This balance may be regulated by several mechanisms that act at the transcriptional, translational and post-translational levels. An example of regulation at transcriptional level is the downregulation of *BMP-4* expression by XBF-2, which was reported to act as a transcriptional repressor (Mariani and Harland, 1998). Also, ectopic expression of the nuclear localised protein Geminin, caused downregulation of *BMP-4* transcripts, although in this case no DNA binding ability or transcriptional repression activity was reported (Kroll et al., 1998). Evidence supporting regulation of BMP signalling by a translational mechanism came from the cloning and characterisation of two genes related to the eukaryotic initiation factor 4A (*eIF-4A*), a component of the basal translation initiation complex. One of these genes, *eIF-4AII*, is expressed in the prospective neural plate

during gastrula stages and is able to induce neural fate when overexpressed in ectodermal explants (Morgan and Sargent, 1997). The other gene, *eIF-4AIII*, is expressed in the ventral ectoderm (prospective epidermis) and promotes epidermal fate in dissociated ectodermal cells (Weinstein et al., 1997). Finally, evidence supporting post-translation regulation of BMP signalling came from the characterisation of the metalloprotease Xolloid, which binds and inactivates Chordin, promoting BMP signalling (Piccolo et al., 1997).

The "neural default" model was based entirely on experiments with *Xenopus*. The application of this model to explain neural induction in other vertebrate systems, such as zebrafish, mouse and chick, has not been straightforward. Studies in zebrafish revealed that overexpression of the homologues of *noggin* and *follistatin* causes similar effects to the ones observed in *Xenopus*. However, while the homologue of *follistatin* is not expressed in the organiser (Bauer et al., 1998) one of the homologues of *noggin* is (Furthauer et al., 1999). The homologue of *chordin* is *dino* (or *chordino*), for which a null mutation was identified by a large-scale mutant screen in zebrafish. *Dino* mutants have a ventralised phenotype with reduction of the neural plate (Schulte-Merker et al., 1997). Other mutants, such as *swirl* and *snailhouse*, that are caused by mutations in *BMP-2* (Kishimoto et al., 1997) and *BMP-7* (Dick et al., 2000), respectively, have the opposite phenotype, with excessive development of the neural plate and dorsal mesoderm. These mutations revealed that, also in zebrafish, activation or repression of BMP signalling is required for the formation of ventral or dorsal mesodermal fates, respectively. However, because the mesoderm is affected in these mutants, it is not clear how much of the neural phenotype is caused by the direct action of the genes or by secondary action due to the affected mesoderm.

In mouse, simple targeted deletions of the BMP antagonists *noggin* (McMahon et al., 1998), *follistatin* (Matzuk et al., 1995) and *cerberus* (Belo et al., 2000) did not result in severe perturbations of neural plate formation. The double mutant for *noggin* and *chordin* presented more severe perturbations with defects in dorsal-ventral patterning of the neural tube and loss of forebrain, the most anterior part of the neural tube (Bachiller et al., 2000). These mutants, however, were still able to form a substantial part of the neural plate, which may suggest a redundancy with other, eventually yet unknown, BMP

antagonists. Targeted deletions of *BMPs* or positive regulators of BMP signalling did not give more useful information regarding the validity of the "neural default" model. Deletion of *BMP-2* (Zhang and Bradley, 1996) and *BMP-7* (Luo et al., 1995) did not cause severe effects on the neural plate. Deletion of *BMP-4* caused a variable phenotype with truncation of the posterior structures in the less severe cases and lethality prior to neurulation in the more severe cases (Winnier et al., 1995). Deletion of the *BMP type I receptor* also resulted in lethality prior to neurulation (Mishina et al., 1995).

Experiments in chick embryos, using pellets of COS cells expressing either *BMPs* or the BMP antagonist *chordin*, indicated that the "neural default" model might not be applicable in this system (Streit et al., 1998). These studies showed that both *BMP-4* and *chordin* are expressed before primitive streak formation and *BMP-4* expression is downregulated upon streak formation. Misexpression of *BMP-4* or *chordin* caused inhibition of primitive streak formation or induction of an ectopic primitive streak, respectively. However, misexpression of *chordin* was not sufficient to induce neural tissue outside the future neural plate, and misexpression of *BMP-4* did not interfere with normal neural induction. Furthermore, this study suggested that, in chick, BMP antagonists must synergise with other factors to induce neural tissue, because Chordin could stabilise the expression of early neural markers in cells that had been treated with neural inducing signals.

1.3.2 Other signalling centres in anterior neural induction: the mouse AVE, the AE and the PME

Work initially done in mouse suggested that the node, the homologue of the Spemann organiser, may not be sufficient for the induction of a complete neural axis with anterior structures, and that additional signalling centres may be required for anterior neural induction. Transplantation of the node induced partial double axes without anterior neural structures (Beddington, 1994). Furthermore, secondary axes induced by ectopic expression of *Cwnt8c* were also incomplete, lacking forebrain (Popperl et al., 1997). Additional work suggested that the extraembryonic tissues that form the anterior visceral endoderm (AVE) have an essential patterning role over the anterior neural plate. Removal of the AVE during early gastrulation stages compromised the formation of the

forebrain (Thomas and Beddington, 1996). Anterior truncations were also obtained in mutations of genes that are expressed in the AVE, such as *Hesx1* (Dattani et al., 1998), *Hex* (Martinez Barbera et al., 2000), *Lim1* (Shawlot and Behringer, 1995), *Otx2* (Acampora et al., 1995). However, of these genes, only *Otx2* (Rhinn et al., 1998) and *Lim1* (Shawlot et al., 1999) were shown to be required in the AVE, by the use of chimeras of mutant AVEs and wild-type epiblasts.

Although *Xenopus* does not have extraembryonic tissues, it has been suggested that the most anterior tissues derived from the organiser, the anterior endoderm (AE) and the prechordal plate mesendoderm (PME) are important signalling centres for the induction of anterior neural tissue (Jones et al., 1999; Kiecker and Niehrs, 2001; Niehrs, 1999). This is supported by the finding that genes expressed in the AE and PME, such as *cerberus* (Bouwmeester et al., 1996), *dickkopf-1* (Glinka et al., 1998) and *frzb-1* (Leyns et al., 1997; Wang et al., 1997), are able to induce anterior but not posterior neural structures, when misexpressed in *Xenopus* embryos.

Simultaneous inhibition of Wnt and BMP signalling on the ventral side of the embryo induces the formation of ectopic heads (Glinka et al., 1997). In accordance to this result, Cerberus was shown to inhibit BMP, Nodal and Wnt signals (Piccolo et al., 1999). Dickkopf-1 (Dkk-1) was shown to antagonise Wnt signalling and to induce ectopic heads when expressed together with BMP antagonists on the ventral side of the embryo (Glinka et al., 1998). Embryos treated with an inactivating antibody against Dkk-1 presented a posteriorised phenotype with loss of anterior neural structures such as eyes and forebrain (Kazanskaya et al., 2000). However, these embryos also presented malformations of the PME, which made it difficult to distinguish if Dkk-1 is acting directly on the neural tissue or by secondary signals produced by the PME.

The idea that Dkk-1 may act directly on the neural tissue was supported by studies with the zebrafish homologue of Dkk-1 (Hashimoto et al., 2000). These authors showed that overexpression of *dkk-1* promoted the development of anterior neural tissue in embryos that lack most of mesoderm and endoderm, due to the injection of *antivin* RNA. Another study in zebrafish provided further support to the idea that Wnt signalling repression is required in the neuroectoderm for the development of anterior structures (Kim et al., 2000). These authors found that the *headless* mutant, which presents defects

in anterior neural structures, is due to a mutation in the *tcf-3* gene. This gene is expressed in the neuroectoderm and the mutation affects the repressive but not the activating action of Tcf-3. Rescue of the mutant was obtained by injection of an amino-terminal truncated form of Tcf-3, which does not bind to β-catenin but still retains its repressive action.

1.3.3 Posteriorisation of neural tissue

Nieuwkoop's "activation/transformation" model for the formation of the CNS, states that after an initial "activation phase", where neural tissue is induced with an anterior character, a subsequent phase of signalling, "transformation phase", confers a more posterior character to the neural tissue. Further experimental support for the existence of posteriorising signals came from studies where recombinants of ectoderm with anterior or posterior mesoderm were used. In one of this studies an anterior neural marker, *XIF3*, was induced by both anterior and posterior mesoderm, but a posterior neural marker, *Hoxb9*, was only induced by posterior mesoderm (Sharpe and Gurdon, 1990). Similar results were obtained by other authors (Zoltewicz and Gerhart, 1997), that also showed, based on the differential expression of the genes *gsc*, *Xbra* and *Xnot*, that the organiser is already patterned along the AP axis during the earlier stages of gastrulation.

The zebrafish homologue of the organiser, the shield, also presents sub-regions with different inductive capacities along the AP axis. Transplantation into the ventral side of the embryo of the deep layer of cells of the shield, which express *gsc* and are localised anteriorly along the AP axis, resulted in the formation of secondary axes containing mostly anterior neural structures (heads with no trunks). On the other hand, transplantation of the superficial layer of cells, which express *flh* and are localised posteriorly along the AP axis, resulted in the formation of secondary axes containing mostly posterior neural structures (trunks without heads) (Saude et al., 2000). Taken together, these studies suggest that the more posterior regions of the organiser are a source of signals that posteriorise neural tissue. Other sources of posteriorising signals include the ventrolateral parts of the mesoderm which will develop into paraxial mesoderm (Gamse and Sive, 2000).

Recently, several secreted molecules have been identified as candidate signals for the second "transformation" phase of Nieuwkoop's model. These molecules are able to induce a more posterior character in neural tissue and include retinoic acid, members of the FGF family and members of the Wnt family. In the next sections I will review the evidence supporting the posteriorising role of each of these molecules.

1.3.3.1 Retinoic acid

Retinoic acid (RA) was one of the first molecules reported to affect the anterior/posterior (AP) pattern of the central nervous system (CNS) (Durston et al., 1989). These authors found that administration of RA to *Xenopus* embryos until early neurula stages causes anterior truncations in the CNS. These embryos developed lacking forebrain and midbrain. The interpretation of this result was that RA might act as a morphogen, transforming anterior neural tissue into a more posterior fate, without affecting the total amount of neural tissue that is formed. A more detailed analysis of the effects of low doses of RA on the CNS revealed a reduction in size of the forebrain, midbrain and the four more anterior rhombomeres of the hindbrain, which was not interpreted as a posteriorisation of the anterior neural tissue (Papalopulu, 1991). Other authors reported that not only neural tissue but also mesodermal tissues were affected by RA treatments (Ruiz i Altaba and Jessell, 1991; Sive and Cheng, 1991; Sive et al., 1990). In the last study, it was shown that RA ventralises mesoderm in animal caps previously treated with a mesoderm inducing factor, although, by itself, RA is not able to induce mesodermal markers. It was also shown that the dorsal lip mesoderm, which is able to induce secondary heads when transplanted into the ventral marginal zone of another embryo, loses this head inducing ability if treated with RA and only induces more posterior structures.

Further studies identified target genes of RA, such as *XHoxa-1* and *XHoxd-1*, two homeobox containing genes that were found to be induced by RA in animal caps and in embryos, even in the presence of the protein synthesis inhibitor, cyclohexamide, which argues in favour of a direct regulation of these genes by RA (Kolm and Sive, 1995). Another study observed that RA treatment of animal caps previously injected with *noggin* RNA causes posteriorisation of the character of the neural tissue (Papalopulu and

Kintner, 1996). In these animal caps there was downregulation of an anterior gene, *XBF-1*, and induction of a more posterior one, *Xhoxb-3*. Although it was not possible to conclude, in this case, if the effect of RA was direct or indirect, since the experiments were not done in the presence of cyclohexamide, it was shown that this posteriorisation happened in the absence of mesoderm.

Evidence supporting an endogenous role of RA in the AP patterning mechanisms came from the finding that the embryo contains RA (Durston et al., 1989) and the proteins required for retinoid signal transduction, such as the nuclear RA receptors RARs and RXRs (Blumberg et al., 1992). Furthermore, overexpression studies using constitutively active and dominant negative forms of the nuclear RA receptors showed that increasing receptor activity suppresses anterior regions and decreasing receptor activity enhances anterior regions of the embryo (Blumberg et al., 1997; Kolm et al., 1997). In the last study it was also shown that retinoid signalling is required for the induction of *XHoxd-1* during normal embryo development.

Additional studies using other vertebrate systems confirmed the role of RA in patterning the AP axis during vertebrate embryonic development. In particular, studies done in mice, revealed that RA can alter the patterns of expression of *Hoxb-1* and *Hoxb-2* in the hindbrain, causing a posterior transformation of rhombomeres (r) 2/3 into an r 4/5 identity (Marshall et al., 1992). Furthermore, the effects of RA on *Hoxb-1* expression in the hindbrain were found to be dependent of a Retinoic Acid Response Element (RARE), a regulatory region present on the *Hoxb-1* promoter that binds RAR/RXR heterodimers *in vitro*. Mutational analysis of this region in the context of the activation of a reporter gene mimicking *Hoxb-1* expression, revealed that not only is RARE essential for the response to ectopic RA, but it is also required for the expression of the reporter during normal embryo development (Marshall et al., 1994). Finally, the generation of a targeted germline mutation of this specific RARE showed that this regulatory region is required for the expression of *Hoxb-1* in the neural ectoderm (Studer et al., 1998).

Other studies done in chick and mice established a more refined map of retinoids in the developing embryo. In the chick this was achieved by using a cell line, F9, as reporter for RA activity (Maden et al., 1998) or by studying the expression patterns of *RALDH-2* and *CYP26*, two genes that encode enzymes involved in the synthesis and

degradation of RA, respectively (Swindell et al., 1999). These studies showed that during pre-somitic stages the posterior regions of the embryo contain more RA. After somite formation the gradient seems to invert, with higher levels of RA at the level of the hindbrain/spinal cord junction and lower levels of RA both anterior and posterior to this region.

The targeted deletion of *RALDH-2*, in mice, caused a severe deficiency in RA production and defects in the formation of several embryonic structures, such as somites, limb buds and heart (Niederreither et al., 1999). In the hindbrain, formation of the more posterior rhombomeres was impaired and the more anterior ones expanded posteriorly (Niederreither et al., 2000). The targeted deletion of *CYP26*, on the contrary, inhibited the degradation of RA causing phenotypes similar to the administration of exogenous RA (Abu-Abed et al., 2001; Sakai et al., 2001). In the hindbrain, this resulted in posteriorisation of anterior rhombomeres. These mutant embryos, however, also presented defects in more posterior regions, which consisted in posterior truncations and anterior transformations. The relevance of this result is that in the absence of RA degradation, the endogenous levels of RA accumulated and caused anteriorisations in the more posterior regions of the embryo. This argues against a simple model of RA action, where a gradient of RA concentrations, lower in more anterior and higher at more posterior regions, would confer progressively more posterior values along the AP axis. In addition, RA might co-operate with other factors for the establishment of AP patterning. This is supported by the fact that the early expression of markers such as *Otx-2* (anterior) and *Hoxa-1* (posterior) is not affected in *RALDH-2* or *CYP26* mutant embryos.

1.3.3.2 FGFs

Members of the FGF family of secreted proteins are also thought to be involved in the patterning of the vertebrate central nervous system as a "transforming" or posteriorising signal. Evidence supporting this idea came from gain of function experiments performed in *Xenopus*. Ectodermal explants simultaneously treated with bFGF and Noggin expressed posterior neural markers, such as *En-2* (midbrain), *Krox-20* (hindbrain) and *Hoxb9* (spinal cord) (Lamb and Harland, 1995), which were not induced by treatment with Noggin alone. These authors showed a proportional posteriorisation of

neural tissue caused by increasing concentrations of bFGF protein, suggesting that an endogenous gradient of FGF protein may specify several positional identities along the AP axis. This posteriorising effect was also observed when neural plate explants were treated with bFGF, so that forebrain tissue expressed hindbrain markers and hindbrain tissue expressed spinal cord markers (Cox and Hemmati-Brivanlou, 1995). Overexpression of *eFGF* in embryos after the mid-blastula transition, caused the suppression of anterior structures (forebrain, eyes) and the enlargement of posterior structures (proctodeum) (Isaacs et al., 1994). Furthermore, overexpression of *eFGF* during gastrula stages induced posterior neural markers such as *Hoxb9*, *Hoxc-6* and *Xcad3* (Pownall et al., 1996). In fact, *Xcad3* was shown to be an immediate target of FGF signalling and dominant negative versions of *Xcad3* blocked the posteriorising activity of FGF (Isaacs et al., 1998). This led to the proposal that Xcad3 mediates FGF activity.

Evidence from loss of function experiments, supporting the requirement of FGF signalling for posterior neural development, has been more controversial. Transgenic embryos expressing a dominant negative form of the FGF receptor 1, *XFD*, had a well patterned nervous system, despite having defects in posterior mesodermal tissues (Kroll and Amaya, 1996). However, other studies using a similar approach have found that, although posterior neural markers were present in transgenic embryos expressing *XFD*, the initial induction of these markers was delayed in comparison to normal embryos (Pownall et al., 1998). Furthermore, other authors have criticised experiments that used transgenic expression of *XFD* because this system only drives expression of XFD after the onset of zygotic transcription and sufficient XFD protein levels for an effective inhibition of FGF signalling may be present only at later stages, not affecting early signalling events. In accordance with this idea, it was shown that expression of XFD as RNA in Keller recombinants inhibited the expression of *En-2*, *Krox-20* and *Hoxb9* (Holowacz and Sokol, 1999). In whole embryos, inhibition of the posterior neural markers expression was only obtained when *XFD* RNA was targeted to dorsal and lateral regions of the embryo. Similar results were obtained by other authors, who blocked FGF signalling by using RNA injections of *XFD* and *N17Ras*, a dominant negative form of Ras (Ribisi et al., 2000). Overexpression of *XFD* and *N17Ras* in animal caps blocked the

induction of posterior neural markers by FGF, and in whole embryos, these dominant negative constructs inhibited posterior neural development.

Another controversial issue is the one regarding the role of FGF signalling in anterior neural induction. This was first raised by the observation that ectodermal explants treated with bFGF alone expressed anterior neural markers (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). Other authors suggested that this result may have been caused by dissociation of the cells of the explants during the treatments with FGFs (Xu et al., 1997). In this last study, and using a different method for treatment, bFGF did not induce anterior neural markers in ectodermal explants. Nevertheless, bFGF treatment enhanced the neuralisation of animal caps expressing a dominant negative BMP receptor. Furthermore, different results were also obtained when XFD was used to ask whether FGF signalling is required for anterior neural induction. Some authors found that injection of XFD RNA could block the induction of anterior neural markers in ectodermal explants expressing *noggin* (Launay et al., 1996). This result was not confirmed by other authors, who found that XFD overexpression could not block the induction anterior neural markers in Keller recombinants or in intact embryos (Holowacz and Sokol, 1999; Ribisi et al., 2000). Finally, it has been suggested that anterior neural induction might require FGF signalling mediated by FGF receptor 4a (XFGFR4a) instead of XFGFR1 (Hongo et al., 1999). These authors observed that the expression of *XFGFR4a* peaks between gastrula and neurula stages in the anterior part of the embryo, while the expression of *XFGFR1* is weaker during these stages and all along the AP axis. Furthermore, it was shown that embryos expressing a dominant negative form of XFGFR4a, with a deletion of the intracellular domain similar to XFD, had defects in the development of anterior neural structures.

Recent studies performed in the chick confirm the role of FGF in the induction of posterior neural markers. Administration of FGFs to the extraembryonic epiblast cells induces them to express posterior but not anterior neural markers (Storey et al., 1998). However, other authors have shown a requirement of FGF signalling during the earlier phases of neural induction. In one of these studies, although specific anterior and posterior neural markers were not analysed, FGF signalling was shown to be necessary and sufficient for the induction of *ERNI*, an early neural marker (Streit et al., 2000).

Another study in the chick showed that the onset of neural induction occurs *in utero*, before the formation of the Hensen's node, and that FGF signalling is required in this process for the downregulation of *BMP-4* and *BMP-7* in the prospective neural tissue (Wilson et al., 2000).

In mice, some members of the FGF family were deleted by homologous recombination. Mutation of *FGF3* (Mansour et al., 1993) led to late defects in ear and tail development, mutation of *FGF5* (Hebert et al., 1994) led to defects in hair growth and disruption of *FGF4* resulted in lethality before gastrulation (Feldman et al., 1995). Disruption of *FGF8* resulted in defects in the migration of mesodermal and endodermal precursors out of the primitive streak. This caused secondary defects in the neuroectoderm, where the expression of anterior markers was expanded and posterior markers failed to be expressed (Sun et al., 1999). A similar phenotype was observed in mutants for *FGFR1* (Deng et al., 1994; Yamaguchi et al., 1994). Analysis of chimeric embryos consisting of a mixture of normal and *FGFR-1* mutant cells revealed that FGFR1 was required not only in the mesodermal and endodermal cells precursors, but also, in cells of the posterior neural tube (Ciruna et al., 1997; Deng et al., 1997). A more direct role of FGF signalling in AP patterning was also suggested by experiments that used hypomorphic alleles of *FGFR1*. These mutant embryos presented posterior truncations, while mutants with a semi-dominant allele, where FGF signalling is increased, presented homeotic transformations in a posterior direction (Partanen et al., 1998). The mutants with hypomorphic alleles also presented alterations in the patterns of expression of *Hoxd-4*, *Hoxb-5* and *Hoxb9*. One of the explanations advanced for this observation was that maybe in mice, as in *Xenopus*, there is a regulatory connection between FGFs and *Hox* genes involving members of the *caudal* related family of transcription factors.

1.3.3.3 Wnts

The first evidence supporting a role of Wnts in the AP patterning of the CNS came from overexpression experiments in *Xenopus*. Simultaneous expression of *Xwnt3a* and *noggin* in ectodermal explants induced the posterior neural markers *En-2* and *Krox-20* and suppressed *Otx-2*, an anterior neural marker (McGrew et al., 1995). Furthermore,

expression of *Xwnt3a* in Keller recombinants suppressed *Otx-2* and *En-2*, inducing the more posterior markers *Krox-20* and *Hoxb9*. Similar results were obtained using overexpression of *β-catenin* or *dishevelled* (Itoh and Sokol, 1997), intracellular components of the Wnt signalling pathway. Ectopic expression of *Xwnt8* in *Xenopus* embryos during gastrula stages, by the use of a plasmid expression construct that drives *Xwnt8* expression under the control of the cytoskeletal actin promoter (pCSKA-Xwnt8), caused deletion of eyes and forebrain (Christian and Moon, 1993). Overexpression of a dominant negative version of *Xwnt8* in intact embryos and Keller recombinants repressed the expression posterior neural genes and induced anterior ones (McGrew et al., 1997). In accordance with this result, overexpression in Xenopus embryos of the Wnt antagonists *frzb-1* (Leyns et al., 1997; Wang et al., 1997) and *dickkopf-1* (Glinka et al., 1998) caused the expansion of anterior and repression of posterior structures. Furthermore, depletion of β-catenin protein in the anterior region of the embryo, by injection of "morpholino" antisense oligos in the A-tier cells of the 32-cell stage embryo, resulted in the expansion of anterior structures such as the cement gland (Heasman et al., 2000).

Wnt proteins were shown to regulate genes expressed in the posterior regions of the CNS. The induction of *Pax-3* in recombinants consisting of *Xenopus* ectodermal explants expressing *noggin* and chick posterior non-axial mesoderm was blocked by the expression of dominant negative *Xwnt8* (Bang et al., 1999). These authors also showed that overexpression of *Xwnt8* or *dnXwnt8* in *Xenopus* embryos caused expansion or repression of the normal expression of *Pax-3,* respectively. This led to the suggestion that, *in vivo*, *Pax-3* expression on the lateral/posterior part of the neural plate is dependent on *Xwnt8*, which is expressed in the underlying paraxial mesoderm.

Other authors have suggested that *En-2*, which is expressed in the midbrain-hindbrain boundary, is directly regulated by the Wnt/β-catenin pathway, via Tcf/Lef sites present in the *En-2* promoter (McGrew et al., 1999). These sites were shown to be required for the activation of a reporter gene in ectodermal explants expressing *noggin* and *Xwnt3a*, and suggested to be essential for the expression of *En-2* during embryo development. Other authors have suggested that Wnt signalling may not only be involved in the posteriorisation of the neural tube, but also in the induction of anterior neural tissue (Baker et al., 1999). In this study, expression of *mWnt8*, *mWnt3*, *Xwnt8* and *β-catenin* in

ectodermal explants resulted in the induction of the neural markers *NCAM* and *Krox-20* and suppression of *BMP-4* expression. This activity was atributed to an early maternal Wnt signalling pathway, because zygotic overexpression of *mWnt3* and *mWnt8* failed to induce neural markers in the same assay. It was also shown that in *Xenopus* embryos, overexpression of *β-catenin* leads to an expansion of the neural plate while overexpression of a dominant negative *Tcf3* blocks neural induction.

In mice, targeted deletion of *Wnt1*, *Wnt3*, *Wnt3a* and *Wnt5a* resulted in phenotypes in which development of the CNS was affected. Embryos homozygous for null alleles of *Wnt1* developed with a loss of the midbrain territory, caused by the failure in the maintenance of the expression of *En-1* (McMahon et al., 1992). It was suggested that the absence of phenotype at more posterior levels, such as the hindbrain and spinal cord, was due to the redundant action of *Wnt3a*, which is also expressed in the dorsal part of the neural tube along the AP axis. However, mice with a double deletion of *Wnt1* and *Wnt3a* still formed a normal hindbrain and spinal cord, although they present defects in the proliferation of neural crest cells progenitors (Ikeya et al., 1997). *Wnt3a* single mutants also had defects in the formation of posterior mesoderm, including somites, notochord and tailbud (Takada et al., 1994). *Wnt3* mutants failed to form a normal neural tube, but this is due to an early defect in the formation of the primitive streak (Liu et al., 1999). *Wnt5a* mutants present a reduction in the extension of the AP axis and truncation of the most posterior structures (Yamaguchi et al., 1999). This defect, however, was not caused by a direct patterning effect since the molecular markers analysed showed normal expression patterns. In this case, it was suggested that *Wnt5a* is required for the normal proliferation of the presomitic mesoderm cells that will form the somites. In zebrafish, only two mutants with defects in Wnt genes have been described so far, and none of them seem to have defects in CNS which are a direct consequence of the mutation. The *pipetail* phenotype, which has defects in tail formation, is caused by a mutation on the *Wnt5* gene (Rauch et al., 1997). The *silberblick* phenotype is due to mutations in the *Wnt11* gene, which is required for the normal convergence extension movements of mesodermal tissues (Heisenberg et al., 2000).

1.3.3.4 Transcription factors: Meis3, Xiro-1 and E2F

The previous sections reviewed the role of the secreted molecules RA, FGFs and Wnts in posteriorisation of neural tissue. In addition to these molecules, transcription factors have also been proposed to play a role in establishment of AP pattern in the developing CNS. *Xenopus Meis3*, a homologue of *Drosophila homothorax* gene, when misexpressed in embryos, caused anterior truncations leading to the loss of forebrain and anterior expansion of hindbrain and spinal cord (Salzberg et al., 1999). Overexpression of *Meis3* in ectodermal explants resulted in the induction of posterior neural markers, such as *Krox-20* and *Hoxb9*, but not anterior neural or pan-neural markers. In ectodermal explants neuralised by Noggin, overexpression of *Meis3* resulted in the suppression of anterior neural and induction of posterior neural markers.

Xiro1, a member of the Iroquois family of transcription factors, acts as a transcriptional repressor and is required for downregulation of *BMP-4* expression (Gomez-Skarmeta et al., 2001). Overexpression of *Xiro1* in ectodermal explants resulted in the induction of anterior neural markers and *Krox-20*, a gene that is not induced by repression of BMP signalling. This result suggests that Xiro1 may also act through an alternative mechanism besides downregulation of *BMP-4* expression. Endogenous *Xiro1* expression was shown to be dependent on a Wnt signal.

The transcription factor *E2F* was identified in a functional screen for genes involved in AP patterning (Suzuki and Hemmati-Brivanlou, 2000). Overexpression of *E2F* in ectodermal explants induced the posterior neural markers *Krox20*, *Hoxb4*, *Hoxb7*, *Hoxb9* and the ventral/posterior mesodermal marker *Xhox3*. The induction of these markers by E2F is direct, since it occurs in the absence of cell to cell communication and in the presence of inhibitors of protein synthesis. Furthermore, overexpression of a dominant negative form of E2F disrupted the formation of posterior structures of the embryo.

1.4 This study

The work presented in this study attempts to contribute to the understanding of the role of the Wnt/β-catenin signalling pathway in the molecular mechanisms that pattern the central nervous system during *Xenopus* embryonic development. In Chapter 3,

I describe results concerning a screen performed in order to identify factors involved in posteriorisation of neural tissue. This screen led to the isolation of truncated forms of β-catenin and a novel secreted protein, Wise. In Chapter 4, I demonstrate that activation of the Wnt/β-catenin signalling pathway in neuralised ectodermal explants induces posterior and mesodermal markers. To ask whether posteriorisation is a secondary consequence of the induction of mesoderm, I performed experiments using an inducible form of β-catenin. These experiments are described in Chapter 5 and demonstrate that activation of the Wnt/β-catenin signalling pathway during gastrula stages posteriorises neural tissue in the absence of mesoderm. I next investigated the molecular mechanism by which the Wnt/β-catenin signalling pathway posteriorises neural tissue. The results described in Chapter 6 show that induction of posterior neural markers by β-catenin occurs in a non-cell autonomous manner and requires cell-to-cell contact in the animal cap. These results suggested that additional signalling within cells is required downstream of the activation of the Wnt/β-catenin signalling pathway. Finally, I show that FGF signalling is required downstream of the Wnt/β-catenin signalling pathway for posteriorisation of neural tissue.

Chapter 2

Materials and Methods

2.1 Common molecular biology techniques

2.1.1 Preparation and storage of competent bacteria

The DH5α strain of *Escherichia coli* was rendered competent for transformation with plasmid DNA by treatment with RbCl. A single colony was placed in 5 ml of LB medium and shaken at 37°C, 200 rpm, until it reached an $OD_{550\ nm}$ of 0.3 (approximately 2 h). This culture was inoculated into 100 ml of LB and further incubated until reaching an $OD_{550\ nm}$ of 0.48 (2-3 h). The culture was cooled to 4°C and centrifuged during 5 minutes, at 7000 g. The pellet was ressuspended in 30 ml of Tf1 (0.1 M RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15% glycerol, pH 5.8) and centrifuged during 5 minutes, at 7000 g, 4°C. The pellet was ressuspended in 4 ml of ice cool Tf2 (10 mM RbCl, 75 mM $CaCl_2$, 10 mM MOPS, 15% glycerol, pH 7) and divided in 200 µl aliquots that were quickly frozen in dry ice and stored at –80°C.

2.1.2 Plasmid transformation of competent bacteria

Frozen DH5-α competent bacteria were thawed on ice. Plasmid DNA was added to 100 µl of cells. The bacterial cells were kept on ice for 20 minutes, heat shocked at 42°C for 45 seconds and cooled on ice for 2 min. After this period, 900 µl of LB was added and the mixture was incubated at 37°C for 15 minutes. Bacteria were then plated onto LB plates containing the required antibiotic (typically 100 µg/ml ampicillin) and placed in a 37°C incubator overnight.

2.1.3 Preparation of plasmid DNA

For a small scale preparation of plasmid DNA, 2 ml of a 3 ml overnight bacterial culture of transformed DH5-α bacteria in LB containing antibiotic (100 µg/ml ampicilin) was processed using the "Wizard Plus Minipreps system" (Promega) according to the instructions of the manufacturers.

For a large scale preparation of plasmid DNA, 0.5 ml of plasmid bacterial culture was placed in 200 ml of LB containing 100 µg/ml ampicillin, and shaken at 37°C overnight. The Qiagen midi or maxi-prep kit (Qiagen) was then used to isolate plasmid DNA, according to instructions of the manufacturers.

2.1.4 DNA and RNA quantification

DNA and RNA were quantified by spectrophotometry at 260 nm (optical density, OD=1 corresponds to 50 μg/ml double stranded DNA, 35 μg/ml single stranded DNA and 40 μg/ml RNA). The ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid preparation (pure preparations of DNA and RNA should have OD_{260}/OD_{280} values between 1.8 and 2.0).

2.1.5 Restriction digestions and ligation reaction

Restriction enzyme digests were performed for approximately 2 hours using commercially supplied restriction enzymes and buffers (Promega, New England Biolabs). The volume of enzyme used in the reaction was never more than 10% of the total reaction volume.

Ligations were performed in small volumes, generally 20 μl for a total DNA content of 0.5 μg. Ligations were performed overnight at 14°C using T4 DNA polymerase (Gibco BRL) and the appropriate ligation buffer (Gibco BRL).

2.1.6 Agarose gel electrophoresis

Separation and size estimation of DNA fragments were performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in 1x TAE to a final concentration of 0.8% to 2% depending on the expected size of the DNA fragment. To visualise the DNA, ethidium bromide was added to the gel to a final concentration of 0.5 μg/ml. DNA samples were mixed with 10x loading buffer and electrophoresis was performed at 5 to 10 V/cm of gel length, until the appropriate resolution was achieved. The resolved DNA was visualised using ultraviolet light at 340 nm, and the size was estimated by comparison with known size markers such as the 1 kb size markers (Gibco BRL).

2.1.7 Purification of specific DNA fragments from gels

In order to purify DNA fragments of interest, DNA was subjected to agarose gel electrophoresis and the region of the gel containing the appropriate band was excised

under ultra-violet light (365 nm). DNA was purified using the QAIquick Gel Extraction Kit, according to the instructions of the manufacturer (Qiagen).

2.1.8 *In vitro* transcription

In vitro synthesised RNA was used for injection into *Xenopus* embryos or as a digoxigenin-labeled probe for whole-mount *in situ* hybridisation. RNA was transcribed from constructs containing a promoter for SP6, T3 or T7 RNA polymerases. Plasmid DNA was first linearised with an appropriated restriction endonuclease, to produce run off transcripts of the insert. When 3'–protruding ends originated, these were converted to blunt by treatment with T4 DNA polymerase.

For RNA used in embryo injections, sense transcripts were *in vitro* synthesised using 5 µg of linearised plasmid DNA and 50 u of the adequate RNA Polymerase (Promega) in the presence of: 10 mM DTT, 1 mM of ATP, CTP, UTP, 0,1 mM GTP, 0.5 mM RNA cap analog (m7G(5')ppp(5')G), 10 µl 5xTranscription Optimized buffer (Promega) and 40 u RNasin Ribonuclease Inhibitor (Promega), in a final volume of 50 µl. After incubating at 37°C for 30 minutes, 2.5 µl of GTP (10 mM) were added and the reaction was incubated at 37°C, for more 60 minutes. To remove the DNA template after the RNA synthesis, 5 u of RQ1 DNase (Promega) were added to the reaction and this was incubated at 37°C, for 60 minutes. The synthetic RNA was purified from unincorporated nucleotides using Croma spin columns (Clontech), ethanol precipitated, ressuspendend in water and checked by measuring OD at 260 nm and by agarose gel electrophoresis.

For digoxigenin-labeled probes the procedure was similar to the described above, except that an anti-sense transcript was synthesised using a mixture of digoxigenin –labeled nucleotides. For a reaction volume of 50 µl, 5 µg of linearised plasmid DNA and 50 u of RNA Polymerase (Promega) were used in the presence of 10 µl 5xTranscription Optimized buffer (Promega), 40 u RNasin Ribonuclease Inhibitor (Promega) and 5 µl of DIG RNA labelling Mix (Boehringer). After incubating at 37°C for 2 hours, 5 u of RQ1 DNase (Promega) were added to the reaction and this was incubated at 37°C for 60 minutes. The RNA probe was purified from unincorporated nucleotides using Croma spin columns (Clontech), ethanol precipitated, ressuspendend in water and checked by measuring OD at 260 nm and by agarose gel electrophoresis.

The constructs used in this thesis as templates for *in vitro* transcription reactions are listed in Table 2.1.

Table 2.1 Constructs used as templates for *in vitro* transcription reactions

Insert	Plasmid	Linearisation site	RNA polymerase	Reference
chick somite cDNA library	pSP64T	SfiI	SP6	This thesis
noggin	pGEM5Zf(-)	NotI	SP6	(Smith and Harland, 1992)
Xβ–catenin	pCS2+	NotI	SP6	(Funayama et al., 1995)
Xwnt3a	pSP64T	EcoRI	SP6	(Wolda, 1993)
Xwnt8	pSP64T	BamHI	SP6	(Christian et al., 1991)
GR-LEFΔNβCTA	pCS2+	NotI	SP6	This thesis
XFD	pSP64T	EcoRI	SP6	(Amaya et al., 1991)
dnFGFR4a	pSP64	SalI	SP6	(Hongo et al., 1999)
dnRARα1	pCDG1	Not1	T7	(Blumberg et al., 1997)
βGal	pGEM	XhoI	SP6	(Smith and Harland, 1991)
BF1	pCS2+	XhoI	SP6	(Bourguignon et al., 1998)
En-2	pBSKS+	XbaI	T3	(Hemmati-Brivanlou et al., 1991)
Krox-20	pGEM	EcoRI	T7	(Bradley et al., 1993)
Hoxb9	pGEM	EcoRI	T7	(Sharpe et al., 1987)

2.1.9 Western blot

To make protein extracts from ectodermal explants, 10 animal caps were treated with 50 ml of extraction buffer (50 mM Tris, pH 7.4; 100 mM NaCl; 1% NP-40; 2 mM EDTA; 1% aprotinin; 2 mM PMSF), dissociated by agitation with vortex and incubated on ice, during 15 min. After centrifugation at 15000 rpm, at 4°C, during 30 minutes, 10 µl of the supernatant (equivalent to 2 animal caps) were analysed in 15% acrylamide SDS-PAGE electrophoresis gels and subsequently blotted on a polyvinylidene difluoride membrane (Sequi-Blot™ PVDF protein sequencing membrane, Bio-Rad), previously permeabilised by immersion in methanol. The electrophoretic transfer was performed in 1X CAPS Buffer/10% methanol (10X CAPS Buffer stock contains: 22.1 g of 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) in 900 ml water, titrated to pH 11 with 2 N NaOH and filled up to 1 l with water) for 30 minutes at 200 mA constant current.

The PVDF membrane was pre-blocked with 5% milk powder (Marvel) in PBS for 1 hour at room temperature with moderate shaking. Then, it was incubated with 5 to 10 μg/ml of primary antibody in 0.5% milk powder/PBS overnight at 4°C. Anti-HA mouse monoclonal antibody, 12CA5 (Boehringer), was used as primary antibodies. A series of 4 washes of 15 minutes each were performed using 0.1% Tween 20/PBS followed by a 1 to 2 hour incubation with the secondary antibody. Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was used as the secondary antibody in a 1:1000 dilution. The membrane was placed for approximately 2 minutes in Reaction Buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Detection of alkaline phosphatase activity was assayed by overlaying the membrane with 3 or 4 ml of BM Purple Substrate (Boehringer), which contains the substrates of the alkaline phosphatase reaction (Nitro Blue Tetrazolium Chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)). The membrane was kept in the dark for 10 to 40 minutes until the staining developed. The reaction was terminated with Stop Buffer (100 mM Tris, 100 mM NaCl, 10 mM EDTA) and washed in distilled water to avoid formation of salt crystals on the membrane.

2.2 Construction and sub-division into pools of the chick somite cDNA library

Somites were collected from chick embryos from stages HH 8 to HH 13 (Hamburger and Hamilton, 1951), according to the mapped activity for the ectopic induction of *Hoxb4* in pre-otic rhombomeres (Itasaki et al., 1996). RNA was extracted using the Trizol reagent (Gibco BRL) and mRNA was purified using the Dynabeads mRNA purification kit (Dynal). cDNA was synthesised using the SuperScript plasmid system (Gibco BRL). A poly d(T)/Not I primer-adapter and a XhoI adapter were used to directionally clone the cDNA population into a modified version of the pSP64T plasmid (Krieg and Melton, 1984). This originated a library with approximately 250.000 independent clones. An aliquot of the library was plated, giving origin to 100 plates with about 500 independent clones/plate. The colonies of each plate were pooled, a small aliquot was stored as a glycerol stock and the rest was used to prepare plasmid DNA,

using the Qiagen midi-prep system. These pools of plasmid DNA were used as templates in an *in vitro* transcription reaction.

The subdivision of a pool of the library was done by plating a small aliquot from the glycerol stock and re-picking single colonies into arrayed plates. These plates were used to grow bacteria for plasmid DNA preparation and were kept as master plates. Typically, for each pool with 500 clones, 1000 single colonies were picked into 10 plates (100 colonies/plate). Further subdivisions were done using the master-plates, until a single clone was reached.

2.3 *Xenopus* embryos, microinjections and ectodermal explants

Xenopus embryos were obtained according to Smith and Slack (1983). *Xenopus* females were injected with 700 units of human chorionic gonadotrophin (Sigma) the previous day. Eggs were obtained by manual extrusion and were *in vitro* fertilised by rubbing the eggs with macerated testis from a sacrificed male. Fertilised eggs were kept in 10% NAM, de-jellied using 2% cysteine hydrochloride (pH 7.9-8.1) and staged according to Nieuwkoop and Faber (1967).

Microinjection of *Xenopus* embryos was done by transferring de-jellied embryos into 75% NAM containing 4% Ficoll in Petri dishes lined with 1% agarose. Embryos were injected using a nitrogen-driven injection system (Narishige IM 300 Microinjector, Japan) with the aid of a micromanipulator (Oxford manipulator, Micro Instruments). Typically, volumes of 10 nl per injection were delivered into one or two blastomeres of two, four, eight or sixteen-cell stage embryos using a capillary glass needle. The injection volume was calculated by injecting the liquid into oil and measuring the diameter of the drop using a graticule. By treating the drop as a sphere, the injected volume was determined. For dorsal or ventral injections, the dorsal and ventral halves of the 4-cell stage embryo were distinguished due to the lighter pigmentation and smaller size of the dorsal blastomeres. For culture of injected embryos, these were transferred to 10% NAM before gastrulation, generally at stage 8 (mid-blastula). Non-specific death of embryos before stage 8 was not included in the scoring of phenotypes.

For isolation of ectodermal explants (animal caps), embryos at stage 8-9 were placed in 75% NAM. The vitelline membrane surrounding the embryo was removed

manually using sharpened watchmakers forceps. A circle of tissue from the animal-most region, comprising less than 50% of the animal hemisphere of the embryo, was cut using forceps. The tissue was placed in 75% NAM on agarose-coated dishes until sibling embryos had reached the desired stage.

Dissociation of the animal cap cells was done by culture in calcium and magnesium free medium (CMFM, Sargent et al., 1986; 88 mM NaCl, 1mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris (pH 7.6), 50 µg/ml gentamicin). Treatments with dexamethasone (DEX; Sigma) were done at the final concentration of 1 µM in 10%NAM, 75%NAM or CMFM.

2.4 RNA isolation and RT-PCR

RT-PCR was performed on ectodermal explants and embryos to analyse gene expression in these tissues. RNA was isolated from 10 to 20 ectodermal explants or 2 embryos using the Trizol reagent (Gibco BRL) in accordance to the manufacturers instructions. The samples were treated with DNase to remove genomic DNA contaminants, using 1 u RQ1 DNase (Promega), 20 u RNasin, 1 mM DTT, 5x Transcription optimized buffer, in 50 µl of final volume, at 37 °C, during 1 hour. RNA equivalent to 5 ectodermal explants was reverse transcribed as follows: RNA and 1 µl random primers at 0.1 µg/µl (Promega) were incubated at 65 °C for 4 minutes, putted on ice, and brought up to the final volume of 20 µl by the addition of dNTPs at 0.5 mM each, 1mM DTT, 20 u RNasin, 4 µl of Transcription optimised buffer and 50 u MMLV RTase (Gibco BRL). The reactions were incubated at 42 °C for 30 minutes. One-tenth of the reverse transcription reaction was used for the PCR reaction in a final volume of 25 µl. Reactions contained 100 µM of each dNTP, AmpliTaq buffer (Perkin Elmer), 1.5 mM $MgCl_2$, 0.1 µg of each primer, 1 µC ^{32}P dCTP and 1,25 u AmpliTaq DNA Polymerase (Perkin Elmer). After an initial denaturation step at 93°C, for 2,5 minutes, reactions were 25 times cycled through 30 seconds at 93°C, 1 minute at 55°C, 30 second at 72°C, and a final elongation step of 5 minutes at 72°C. The products of PCR amplification were run on 5% polyacrilamide gel electrophoresis and after dried the gel was exposed from 2 hours to overnight. The primers used in this thesis are listed in Table 2.2

Table 2.2 Sequences of the primers used in RT-PCR

Marker	Primer sequence	Reference
eF1α	5'- CAGATTGGTGCTGGATATGC 5'- ACTGCCTTGATGACTCCTAG	(Hemmati-Brivanlou and Melton, 1994)
NCAM	5'- CACAGTTCCACCAAATGC 5'- GGAATCAAGCGGTACAGA	(Hemmati-Brivanlou and Melton, 1994)
BF-1	5'- CCTCAACAAGTGCTTCGTCA 5'- TAAAGGTGAGTCCGGTGGAG	This thesis
En-2	5'- CGGAATTCATCAGGTCCGAGATC 5'- GCGGATCCTTTGAAGTGGTCGCG	(Hemmati-Brivanlou and Melton, 1994)
Krox-20	5'- CCTTTGATTCAGATGAGCGGAG 5'- CGACATGCTGCAGCTCAGGTT	This thesis
Hoxb9	5'- TACTTACGGGCTTGGCTGGA 5'- AGCGTGTAACCAGTTGGCTG	(Hemmati-Brivanlou and Melton, 1994)
Muscle actin	5'- GCTGACAGAATGCAGAAG 5'- TTGCTTGGAGGAGTGTGT	(Hemmati-Brivanlou and Melton, 1994)
Xhox3	5'- GTACCTCAACCAACGGCCTA 5'- GGACTCGGGAGAAGGGTAAC	This thesis
Xbra	5'- CACCGAGAAGGAGCTGAAGGTTAG 5'- TGCCACAAAGTCCAGCAGAACC	This thesis
α-globin	5'- TGCTGTCTCACACCATCCAGG 5'- TCTGTACTTGGAGGTGAGGACG	This thesis
Siamois	5'- GAGCCTCAGGTCAGCAAAAC 5'- GGTACTGGTGGCTGGAGAAA	This thesis
Xnr3	5'- TCCACTTGTGCAGTTCCACAG 5'- ATCTCTTCATGGTGCCTCAGG	This thesis
eFGF	5'- TATGAATGCAAAGGGGAAGC 5'- GTGGCAAGAAATGGGTCAGT	This thesis
FGF3	5'- TGGAGGGGTTTACGAACATC 5'- CCCTTTGATGGCAACAATTC	This thesis
FGF8	5'- TTAAAGGTGCGGAGACTGGT 5'- TGTCTTCGACCCTTTCCTTG	This thesis
wise	5'- GGCAGCACAACGAGTCCAGC 5'- AATCTGAAAGCAGATAGTTGG	This thesis
cβ-catenin	5'- GATATTGATGGCCAATATGCAA 5'- ATAATAGCATGGCGAGATGC	This thesis

2.5 Histology, β-galactosidase staining and whole-mount *in situ* hybridisation

For histological analyses, specimens were fixed, sectioned and stained as described (Green et al., 1990). For β-galactosidase staining tissues were fixed in MEMFA (formaldehyde at 3.7% in 1X MEM salts) for 1 hour at room temperature, washed in PBS containing 0.01% Tween-20 and stained in PBS containing 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate and

0.02% NP-40. After staining samples were refixed in MEMFA for 1 hour and processed for *in situ* hybridisation as described

For whole mount *in situ* hybridisation embryos were fixed for 2 hours at 4°C in MEMFA and were transferred to methanol for long-term storage at -20°C. Embryos were rehydrated by taking them through a methanol/water series starting with 75% methanol, 50% methanol, and then 25% methanol in PBS/Tween 0.1%. Embryos were then washed 3 times in PBS/Tween 0.1%. Proteinase K (Boehringer) at 10 μg/ml (in PBS/Tween 0.1%) was added, to increase penetration of the probe by degradation of surface proteins, and embryos were left for 10 minutes at room temperature. The embryos were washed in PBS/Tween 0.1% and refixed with freshly prepared 4% paraformaldehyde in PBS for 20 minutes. The embryos were washed 5 times with PBS/Tween 0.1%, pre-hybridised with 1 ml of Hybe solution for 2 hours at 60°C and, finally, they were hybridised overnight at 60°C in 500 μl of this solution with 2 μl of denaturated dioxigenin (DIG)-labelled probe. The Hybe solution was: 50% deionised formamide, 5X SSC pH 6, 200 μg/ml t-RNA, 100 μg/ml heparin, 1X Denhardt's (50X stock contains 1 g polyvinylpyrrolidone, 1 g Ficoll and 1 g BSA made up to 100 ml with SPH_2O), 0.1% Tween 20, 0.1% CHAPS and 5 mM EDTA.

The embryos were washed using a series of washing solutions with decreasing amounts of formamide: 50% formamide/5X SSC/0.1% CHAPS (10 minutes at 60°C), 25% formamide/2X SSC/0.1% CHAPS (10 minutes at 60°C), 2X SSC/0.1% CHAPS (30 minutes at 60°C twice), 0.2X SSC/0.1% CHAPS (30 minutes at 60°C twice). After the washes, embryos were treated with maleic acid buffer (150 mM NaCl, 0.1 M maleic acid pH 7.5) in 0.1% triton (MABT) for 5 minutes at room temperature twice. Before adding antibody, the embryos were treated with freshly prepared 2% blocking reagent (Boehringer)/10% lamb serum (heat inactivated)/MABT, for 1 hour at room temperature. Finally, embryos were incubated overnight at 4°C with gentle rotation with a 1:4000 dilution of anti-dioxigenin (DIG) Fab fragments conjugated with alkaline phosphatase (Boehringer).

On the following day, embryos were washed 4 times for 1 hour at room temperature in MABT. Then, they were treated with alkaline phosphatase buffer (0.1 M Tris.HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween 20 and 2 mM levamisol

freshly added) followed by 1 ml of BM purple alkaline phosphatase substrate (Boehringer). The staining reaction was carried out for 3 to 24 hours depending on the probe. After staining was complete, the embryos were washed in alkaline phosphatase buffer twice and fixed by a 1 hour treatment with MEMFA at 4°C with gentle rotation. Then, embryos were washed in methanol twice and bleached in 1% H_2O_2, 5% formamide, 0.5x SSC to remove the normal pigmentation of the embryos and reduce nonspecific staining.

2.6 Formulation of frequently used solutions and media

PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4
TAE	40 mM Tris.acetate, 2 mM $Na_2EDTA.2H_2O$ (pH 8.5)
TBE	89 mM Tris-Base, 89 mM Boric acid, 2 mM EDTA pH 8.0
20X SSC	3 M NaCl, 0.3 M $Na_3citrate.2H_2O$, adjust pH to 7.0 with 1 M HCl
10X gel loading buffer	50% glycerol, 10 mM Tris.HCl pH 8.0, 0.25% w/v bromophenol blue
5X sample buffer (for SDS-PAGE)	15% w/v SDS, 15% v/v β-mercaptoethanol, 50% v/v glycerol, 1.5% w/v bromophenol blue
Formamide-containing loading dye	80% v/v formamide, 1 mM EDTA, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol
10X NAM (1 litre)	110 M NaCl, 2 M KCl, 1 M $Ca(NO_3)_2$, 1 M $MgSO_4$, 0.1 M Na_2EDTA
10% NAM (500 ml)	5 ml 10X NAM, 10 ml 0.1M Na phosphate (pH 7.4), 2.5 ml 10 mg/ml gentamycin
75% NAM (500 ml)	37.5 ml 10X NAM, 10 ml 0.1M Na phosphate (pH 7.4), 5 ml 0.1M $NaHCO_3$, 2.5 ml 10 mg/ml gentamycin
2% cysteine hydrochloride pH 7.9-8.1 (200 ml)	4.4 g L-cysteine hydrochloride monohydrate, 1.33 to 1.36 g NaOH pellets, fill up to 200 ml distilled water
10 X MEM salts	1 M MOPS, 20 mM EGTA, 10 mM $MgSO_4$
MEMFA (50 ml)	5 ml 10X MEM salts, 5 ml 37% formaldehyde
LB (L-Broth)	1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl
L-agar	L-Broth supplemented with 1.5% bacto-agar

2.7 Abbreviations

BSA	Bovine serum a lbumin
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra-acetate
MOPS	3-(N-morpholino)-propanesulfonic acid
PCR	Polymerase chain reaction
SDS	Sodium lauryl sulfate
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
ATP	Adenosine 5'-triphosphate
CTP	Cytidine 5'-triphosphate
UTP	Uridine 5'-triphosphate
GTP	Guanidine 5'-triphosphate
TTP	Thymidine 5'-triphosphate

Chapter 3

A screen for a somite derived posteriorising factor

3.1 Introduction

The molecular mechanisms that establish and maintain AP patterning in the vertebrate CNS have been extensively studied in the hindbrain. The hindbrain is composed of segments, termed rhombomeres, that adopt an identity that is distinct from its neighbours. Each rhombomere forms a lineage restricted cellular compartment that contributes with neural crest cells to specific skeletal structures and with neurons to distinct cranial branchiomotor nerves. In addition, each rhombomere expresses a characteristic set of genes. *Hox* genes, in particular, are differentially expressed and are an important part of the molecular mechanisms that specify rhombomere identity. Transformations of rhombomere identity have been obtained experimentally by gain and loss of function analyses of several *Hox* genes (Krumlauf, 1994; Lumsden and Krumlauf, 1996).

Some studies used grafting techniques in the chick to explore the possibility of transforming the identity of rhombomeres by exposing them to different environments. In one of these studies, although the grafting of a particular presumptive rhombomeric territory from a caudal to a rostral position did not perturb its identity, the transposition from a rostral to a caudal position did. This resulted in the induction, in the transplant, of the *Hox* genes normally expressed in more caudal positions, in particular, *Hoxb4* (Grapin-Botton et al., 1995). It was also shown that this induction is still possible at stages following the establishment of rhombomere boundaries, suggesting that rhombomere identities are not necessarily irreversibly predetermined. Another study has confirmed these results, and showed that the anteroposterior position of the graft, but not its size, is critical for *Hox* induction (Itasaki et al., 1996). In this study, interspecies grafting experiments (mouse/chicken) showed that the induction is conserved between species. Furthermore, in experiments where posterior somitic mesoderm was grafted into an anterior location, it was found that somites have a graded ability to reprogram *Hox* expression in the rhombomeres, suggesting that plasticity is a consequence of environmental signals from paraxial mesoderm.

In a more recent study (Gould et al., 1998), it was found that the activities of two different enhancers mimic the normal *Hoxb4* expression in the hindbrain. One of these enhancers corresponds to the early phase of expression (Early Neural Enhancer - ENE)

and the other to the late phase of expression (Late Neural Enhancer - LNE). In experiments where segments of hindbrain were cultured together with somitic mesoderm, it was shown that somites are necessary to induce the activity of ENE. So, somites not only induce ectopic *Hoxb4* expression in anterior regions of the hindbrain, but are also required for the establishment of the normal pattern of *Hoxb4* expression. Ectopic induction of *Hoxb4* expression is also obtained by administration of retinoic acid (RA) and this induction is dependent on retinoic acid response elements (RARE) present in ENE. However, experiments in which membranes with various pore sizes were used between recombinants of hindbrain and somitic mesoderm showed that the signalling factor from the somitic mesoderm must be greater than 10 KDa, excluding the possibility of RA alone being the somite factor. One hypothesis is that a secreted factor from the somites induces the production of RA in the CNS, where it acts as a second messenger. Alternatively, RA produced in the somites may diffuse to the CNS, but in this case an additional factor must be required, acting either as a transporter of RA or in a non-related parallel pathway.

Other studies have described somite dependent posteriorising activities in different contexts from the induction of *Hoxb4* expression (Ensini et al., 1998; Grapin-Botton et al., 1998; Muhr et al., 1997). These studies do not identify the somite derived molecules that are involved in these signalling events. So, these activities can be due to a particular signal that is affecting AP patterning in a general way or they can correspond to different signals that occur at distinct stages of embryonic development.

In order to identify molecules involved in this somite derived posteriorising activity, a functional screen was set up to clone genes that may be involved in these events. A cDNA library was made from chick somites which induce ectopic expression of *Hoxb4* (Itasaki et al., 1996), and this library was tested in an expression cloning strategy, using *Xenopus* embryos. Ectodermal explants dissected from *Xenopus* embryos injected with *noggin* RNA express anterior neural markers. The strategy was to co-inject synthetic RNA made from pools of clones of the somite cDNA library together with *noggin* RNA and ask if any of the pools of the library can posteriorise the ectodermal explants. To determine the AP character of the ectodermal explants, RT-PCR for known AP markers, such as, *Engrailed-2* (midbrain) and *Krox-20* (hindbrain), was used. A pool

of the library with the ability to induce posterior neural markers was then sub-divided into smaller pools that were re-tested, until a single clone was reached. This type of expression cloning strategy has been successfully used in *Xenopus* for the isolation of genes involved in dorsal axis formation (Smith and Harland, 1992; Smith and Harland, 1991).

3.2 Pools of the somite cDNA library can posteriorise *Xenopus* ectodermal explants neuralised with Noggin

Experiments of interspecies grafting and *in vitro* co-culture of hindbrain segments and somites have shown that the induction of more posterior markers by a somite signal is conserved between chick and mouse. However, before starting a screen based on a *Xenopus* animal cap assay, it was necessary to show that this conservation of mechanisms is also present in *Xenopus*. To test this, an experiment was performed in which *Xenopus* ectodermal explants neuralised by injection of *noggin* RNA were co-cultured with chick somites. The result of this experiment was that in the recombinants, the ectodermal explants expressed *Engrailed-2* (*En-2*), a midbrain marker that was not expressed in ectodermal explants treated with Noggin alone (data not shown, N. Itasaky personal communication). This result demonstrated that chick somites are able to posteriorise *Xenopus* ectodermal explants neuralised with Noggin, showing conservation of mechanisms in these two species.

To construct the cDNA library, somites of chick embryos were collected from stages HH 8 to HH 13 (Hamburger and Hamilton, 1951), according to the mapped activity for the induction of *Hoxb4* expression (Itasaki et al., 1996). The library consists of approximately 250,000 independent clones from which a fraction was plated, to give origin to 100 plates containing about 500 independent clones per plate. The colonies of each plate were pooled, aliquots were stored as glycerol stocks and the rest was used to prepare plasmid DNA, which was used as template for *in vitro* transcription reactions.

The synthetic RNA of each pool was mixed with *noggin* RNA and injected into 2 cell stage *Xenopus* embryos. Ectodermal explants from these embryos were dissected at stage 8, cultured in 75%NAM and assayed by RT-PCR for the induction of posterior neural markers, when sibling embryos reached stage 22-24. Fig. 3.1 is an example of an

RT-PCR experiment, where pools that were positive in a first round of screening were re-tested. Ectodermal explants injected with *noggin* and synthetic RNA from pools 14, 22, 33, 35, 70, 88, 91, 94, 98 and 100 expressed *En-2*, which was not expressed in ectodermal explants injected with *noggin* RNA alone or uninjected ectodermal explants. The marker *NCAM*, which is ubiquitously expressed in neural tissue, confirms that the ectodermal explants were neuralised and *eF1α* was used to show that similar amounts of RNA were loaded in each lane. The induction of *muscle actin*, a mesodermal marker, in ectodermal explants injected with RNA from pools 88 and 100 may reveal that in these cases, the induction of the posterior neural markers was not direct, but secondary to mesoderm induction. In these cases, clones present in the pools should be responsible for the induction of mesoderm in the ectodermal explants. Table 3.1 summarises the results obtained from the screening of 100 pools of the cDNA library, only referring the pools that induced at least one posterior neural marker in the assay.

Table 3.1 Identification of pools that induced posterior neural markers in ectodermal explants. The posterior neural markers *En-2*, *Krox-20* and the mesodermal marker *muscle actin* were analysed by RT-PCR of ectodermal explants expressing synthetic RNA from 100 pools of the library. Only the pools that were positive for the induction of at least one posterior neural marker are listed. The markers were induced when (+) and not induced when (-).

Pool	*En-2*	*Krox-20*	*M.Actin*	Pool	*En-2*	*Krox-20*	*M.Actin*
5	+	-	-	70	+	-	-
11	+	-	-	76	+	-	-
14	+	-	-	81	+	+	+
22	+	-	-	88	+	+	+
33	+	+	-	89	+	+	+
35	+	+	-	91	+	-	-
46	+	-	-	94	+	-	-
47	+	-	-	96	+	-	-
51	+	-	-	98	+	-	-
63	+	-	-	100	+	+	+

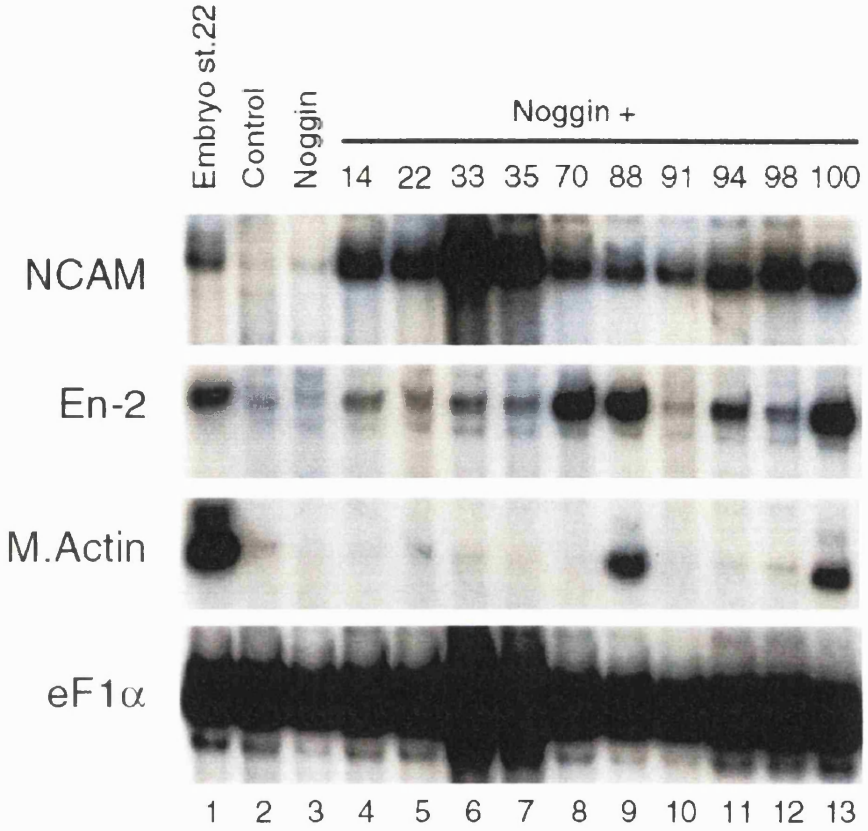


Fig. 3.1 – Pools of the cDNA library induced *En-2* in *noggin* injected ectodermal explants. RT-PCR of ectodermal explants of embryos injected with 500 pg/embryo of *noggin* RNA (lane 3) or 500 pg/embryo of *noggin* RNA and 6 ng/embryo of RNA from each pool (lanes 4 to 13). Sibling embryos were used as a positive control (lane 1). Uninjected ectodermal explants (lane 2) do not express *NCAM*, *En-2* or *M.Actin*. Ectodermal explants injected with *noggin* RNA express *NCAM* but not *En-2* and *M.Actin*. *eF1α* was used as a loading control. All samples were analysed when sibling embryos reached stage 22.

3.3 Sub-division of pools positive for the induction of posterior neural markers

The subdivision of pools positive for the induction of posterior neural markers was done by plating a small aliquot of the glycerol stock of each pool and picking individual colonies from these plates into grided plates. For each pool of 500 clones, 1000 colonies were picked into 10 plates (100 colonies/plate) that were kept as master-plates and used, by re-picking and growing bacteria in liquid media (LB), to make plasmid DNA preparations. The plasmid DNAs of each plate containing 100 clones were pooled and used for *in vitro* transcription reactions. These RNAs were used together with *noggin* RNA for injection into embryos, from which ectodermal explants were dissected, cultured and assayed by RT-PCR for the induction of the posterior neural markers. A positive pool at this level was further subdivided, by re-picking colonies from the respective master-plate of 100 clones and repeating the process, using pools containing 10 clones each. In the final step, the 10 colonies of a positive pool were assayed individually.

The subdivision of pools 46 and 47 was done previously in the lab (data not shown, N.Itasaki personal communication) and led to the identification of two single clones responsible for the activity of these pools. From pool 46 was isolated an amino-terminally truncated form of *β-catenin*, lacking the first 239 bp of the coding region. Based on the published sequence of chick *β-catenin* (Lu et al., 1997), this clone presumably encodes a truncated protein lacking the first 87 amino acids. This domain contains phosphorylation sites for GSK-3, which are required for the degradation of β-catenin (Yost, 1996). This clone is therefore likely to encode a stable, and presumably more active form of β-catenin. From pool 47 a second clone was isolated, encoding a novel secreted protein with no matches in GeneBank. Based on its characterisation and functional relationship to the Wnt signalling pathway it was denominated Wise (Wnt inhibitor/activator in surface ectoderm) (Itasaki, submitted).

Before subdividing the remaining positive pools, they were analysed for the presence of *β-catenin* and *wise*, to avoid the re-isolation of these two clones. PCR was performed using specific primers for *β-catenin* and *wise* and, as template, plasmid DNA of each of the positive pools listed in Table 3.1. *β-catenin* was detected in pools 5, 33, 35,

63, 81, 88, 89, 96 and 100. *wise* was detected in pools 11, 22, 91 and 96. Table 3.2 summarises the results obtained. Based on these findings, it was decided not to subdivide pools that contained *β-catenin* or *wise*, although the possibility that an additional clone with posteriorising activity might be present in these pools cannot be excluded. Pools 14, 51, 70, 94 and 98, which were judged not to contain *β-catenin* or *wise,* were subdivided as they may contain other clones with posteriorising activities.

Table 3.2 Identification of pools of the library containing *β-catenin* or *wise*. PCR was performed using specific primers for *β-catenin* and *wise*, and plasmid DNA of each positive pool as a template (pools of 500 clones/pool listed in Table 3.1). The clones are present in a particular pool when (+) and absent when (-).

Pool	*β-catenin*	*wise*	Pool	*β-catenin*	*wise*
5	+	-	70	-	-
11	-	+	76	-	-
14	-	-	81	+	-
22	-	+	88	+	-
33	+	-	89	+	-
35	+	-	91	-	+
46	+	-	94	-	-
47	-	+	96	+	+
51	-	-	98	-	-
63	+	-	100	+	-

The subdivision of pool 70 is represented in Fig. 3.2. For the sake of simplicity, only the RT-PCR results for the posterior neural markers *En-2* and *Krox-20* are shown. As mentioned above, 10 pools containing 100 clones each were generated from pool 70 and 6 ng/embryo of RNA from each pool were co-injected with 500 pg/embryo of *noggin* RNA. Several of these pools induced *En-2* in ectodermal explants (Fig. 3.2A). Pool 70.1 was subdivided into smaller pools containing 10 clones each. At this stage, 600 pg/embryo of RNA from each pool was co-injected with 500 pg/embryo of *noggin* RNA. From the assay of these pools resulted that only pool 70.1.2 induced *En-2* (Fig. 3.2B, lane

5). Only 4 of the 10 single clones from pool 70.1.2 contained insert. Single clone 70.1.2.9 induced *Krox-20* (Fig. 3.2C, lane 6). Sequencing the insert of 70.1.2.9 indicated this was another truncated form of *β-catenin*. This clone lacks the first 263 bp of the coding region and presumably encodes a protein that starts with the methionine in position 98. This *β-catenin* clone was not detected by PCR analysis of the pool 70, because one of the primers used spans nucleotides 240 to 262 of the coding region, which are not present in 70.1.2.9.

The subdivision of pool 51 is shown in Fig. 3.3. From the analysis of pools 51.1 to 51.10 (pools with 100 clones/each), resulted that pools 51.1 and 51.9 induced *En-2* without inducing *muscle actin* (Fig. 3.3A, lanes 4, 10). Pool 51.1 was subdivided into 10 pools containing 10 clones each (Fig. 3.3B, lanes 4-13), but none were able to induce *En-2*. Pool 51.1 was assayed (Fig. 3.3B, lane 14) together with the sub-pools and did not induce En-2. A positive control for the induction of posterior markers, β-catenin, induced *En-2* and *muscle actin* (Fig. 3.3B, lane 15). Pool 51 is an example where subdivision was unsuccessful, because the activity was lost during the process. Similar results were obtained in the sub-division of pools 14, 94 and 98.

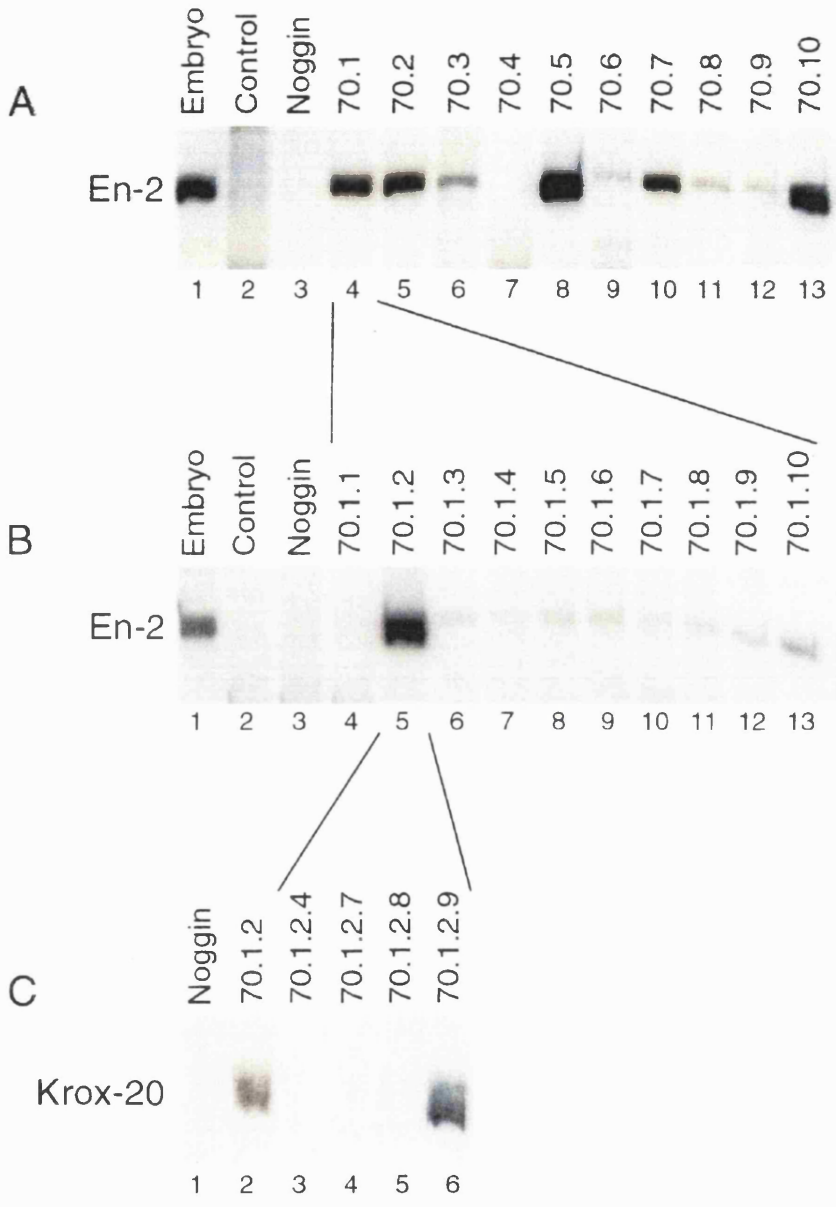
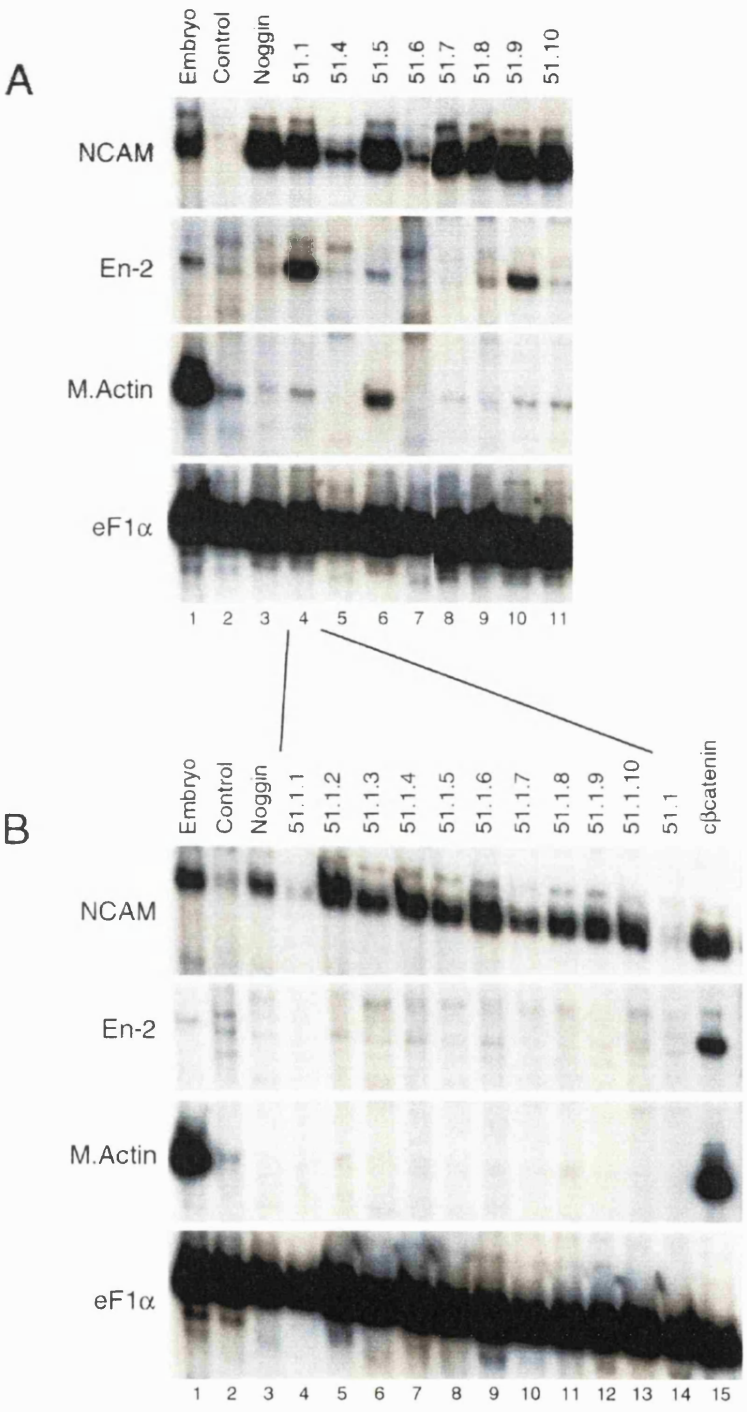


Fig 3.2 Subdivision of pool 70. RT-PCR results obtained for the posterior neural markers *En-2* and *Krox-20* during the subdivision of pool 70. (A) Analysis of the pools of 100 clones revealed that pool 70.1 (lane 4) is able to induce *En-2*. (B) Analysis of pools 70.1.1 to 70.1.10 (pools with 10 clone/each). Pool 70.1.2 induces *En-2* in ectodermal explants (lane 5). (C) Analysis of the single clones derived from 70.1.2. Single clone 70.1.2.9 induces Krox-20 in ectodermal explants (lane 6). No posterior neural markers are induced in *noggin* injected or in uninjected ectodermal explants. All tissues were analysed when sibling embryos reached stage 22.

Fig. 3.3 Subdivision of pool 51. RT-PCR results obtained during the subdivision of pool 51. All tissues were analysed when sibling embryos reached stage 22. (A) Analysis of pools containing 100 clones, revealed that pools 51.1 (lane 4) and 51.9 (lane 10) induced *En-2* expression in ectodermal explants. (B) Pools containing 10 clones each, from subdivision of pool 51.1, did not induce any posterior neural marker. Embryo (lane 1) is a positive control and uninjected ectodermal explants were used as a negative control (lane 2). Ectodermal explants injected with *noggin* RNA express *NCAM* but not *En-2* or *muscle actin*. *eF1α* was used as a loading control.


A
Embryo
Control
Noggin
51.1
51.4
51.5
51.6
51.7
51.8
51.9
51.10
NCAM
En-2
M.Actin
eF1α
1 2 3 4 5 6 7 8 9 10 11
B
Embryo
Control
Noggin
51.1.1
51.1.2
51.1.3
51.1.4
51.1.5
51.1.6
51.1.7
51.1.8
51.1.9
51.1.10
51.1
cβcatenin
NCAM
En-2
M.Actin
eF1α
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

3.4 Discussion

In this chapter I have presented results concerning a screen that was performed in order to identify factors able to posteriorise neural tissue. This was done by injection of RNA from pools of a chick cDNA somite library together with *noggin* RNA into *Xenopus* embryos. Ectodermal explants were isolated from these embryos, cultured and assayed for the expression of neural markers by RT-PCR. Positive pools for the induction of posterior neural markers were then sub-divided and re-tested, in order to identify single clones responsible for the posteriorising activity. The outcome of this process was the isolation of two clones, *β-catenin* and *wise.*

3.4.1 Isolation of *β-catenin* and *wise*

In this screen, one hundred pools, each containing approximately 500 clones (covering 50,000 independent clones of the library), were assayed. From these pools, 20 were able to induce posterior neural markers (Table 3.1). The subdivision of the first two pools (pool 46 and 47) led to the identification of two clones, *β-catenin* and *wise.* The inductive properties of β-catenin and its role in AP patterning of neural tissue will be considered later in this thesis. The functional characterisation of Wise revealed that this secreted protein behaves as an agonist and antagonist of Wnt signalling in a context dependent manner (Itasaki, submitted).

The remaining pools that induced posterior neural markers were analysed for the presence of these two clones. *β-catenin* was found in 10 pools and *wise* was found in 5 pools. These pools were not subdivided to avoid re-isolation of *β-catenin* or *wise,* although additional clones with posteriorising activity may be present in these pools. The 4 remaining pools were subdivided but this did not lead to the successful isolation of any other clone, because the activity was lost during the process. The reasons for this are unclear. One possibility is that the posteriorising activity of these pools depended on the conjugated action of more than one clone. Upon subdivision of the pools these clones would be separated and the activity lost. In this case, combinations of the sub-pools may restore the activity, but this was never attempted because it would be a very time consuming and complex procedure. Another possibility is that during the isolation of the animal caps for the assay of these pools, marginal zone cells were accidentally

incorporated in the explants. Such cells would have formed dorsal mesoderm and induced posterior neural markers as a secondary effect. This may explain why some pools induced posterior neural markers in an inconsistent manner. Nevertheless, induction of posterior neural or mesodermal markers in animal caps from uninjected and *noggin* injected embryos was never observed.

The ability of members of the Wnt pathway (McGrew et al., 1995) and FGFs (Lamb and Harland, 1995) to induce posterior neural markers in neuralised ectodermal explants was reported previously. The results of the screen are consistent with these reports, since β-catenin is an intracellular component of the Wnt signalling pathway. However, no clones related to the FGF family were isolated from the screen, which may indicate that no FGFs are present in the library. On the other hand, *β-catenin* is highly represented in the library since it was found in 11 pools. Furthermore, amino-truncated clones of β-catenin were active in the assay. In fact, these clones may behave as a constitutively active form, since the amino terminal domain contains phosphorylation sites for GSK-3, which are required for the degradation of β-catenin (Yost, 1996).

3.4.2 What is the identity of the somite derived posteriorising factor?

The main goal of this screen was to identify candidate secreted molecules for the somite derived posteriorising factor (Itasaki et al., 1996). The screen resulted in the isolation of *wise* and *β-catenin*, raising the question of whether the proteins encoded by these genes can be responsible for the posteriorising activity of somites. The evidence collected so far, however, does not support this idea and will be discussed further.

β-catenin is an intracellular and not a secreted protein. This does not exclude that, downstream of β-catenin, signals secreted by the somites can posteriorise the neural tube. Alternatively, since *β-catenin* is ubiquitously expressed, it may act within the neural tube. In this case, a Wnt ligand expressed in the somites would activate the Wnt signalling pathway in the neural tube. However, overexpression of *β-catenin* or *Wnts* in the chick neural tube by "in ovo" electroporation was not able to ectopically induce the expression of *Hoxb4* as the grafting of somites was (N.Itasaki, personal communication). *wise* encodes a secreted protein, but it is not expressed in the somites. Moreover, ectopic

expression of *wise* in the neural tube also failed to induce *Hoxb4* expression (N.Itasaki, personal communication).

Despite the arguments discussed above, the hypothesis that a clone present in the somite cDNA library is responsible for the posteriorising activity of the somites cannot be excluded. Firstly, only one fifth of the library was analysed (50,000 out of 250,000 clones). Secondly, it may be necessary to use higher concentrations of RNA than the ones used, to detect putative inducers in the animal cap assay. In the screen of pools with 500 clones, 6 ng of RNA were injected in each embryo, which represents 12 pg of RNA/clone (assuming that 500 different clones are equally represented in each pool). Thirdly, the assay using *Xenopus* animal caps may be inadequate for the identification of a chick somite derived posteriorising factor, if the mechanism that operates in chick is not conserved in *Xenopus*. However, chick somites are able to posteriorise ectodermal explants neuralised by Noggin. Furthermore, the mouse enhancer of *Hoxb4* responsive to the somite signal, ENE (Gould et al., 1998), is also able to direct expression of a reporter gene in transgenic experiments in *Xenopus* (S.Mercurio, personal communication).

Chapter 4

Overexpression of components of the Wnt/β-catenin signalling pathway in ectodermal explants

4.1 Introduction

The previous chapter describes a functional screen designed to identify molecules able to posteriorise neural tissue. This screen led to the isolation of an amino terminal truncated form of β-catenin and a novel secreted protein, Wise. β-catenin is an intracellular component of the Wnt signalling pathway. The functional characterisation of Wise revealed that the activity of this protein depends on the Wnt pathway; the posteriorising activity of Wise is blocked by dominant negative forms of components of the Wnt pathway and Wise physically binds the Wnt Frizzled receptor (Itasaki, submitted). These results show that activation of the Wnt/β-catenin signalling pathway posteriorises neural tissue. Similar results were previously obtained by other authors (McGrew et al., 1997; McGrew et al., 1995). In these reports β-catenin and Xwnt3a posteriorised ectodermal explants injected with *noggin* RNA, inducing the expression of the posterior neural markers *En-2* and *Krox-20* and suppressing *Otx-2*, an anterior neural marker. Furthermore, these authors showed that activation of the Wnt/β-catenin signalling pathway posteriorises neural tissue in the absence of dorsal mesoderm, suggesting that this is a direct effect in the neural tissue. In contrast with this result, in the previous chapter I have shown that Noggin and β-catenin can induce posterior neural and dorsal mesodermal markers in ectodermal explants (Fig. 3.3).

In this chapter I further characterise the activity of the Wnt/β-catenin signalling pathway in ectodermal explants, using overexpression of *Xwnt3a*, *Xwnt8* and truncated or full-length forms of *β-catenin* with and without *noggin*.

4.2 Posterior neural and dorsal mesodermal markers are induced in ectodermal explants injected with *noggin* and truncated *β-catenin* RNA

In order to characterise the activity of the Wnt/β-catenin signalling pathway in neuralised animal caps, I injected a range of RNA concentrations of the truncated form of *β-catenin* together with *noggin* RNA into 2 cell stage *Xenopus* embryos. Animal caps were isolated from the injected embryos at stage 8, cultured in 75% NAM and assayed for gene expression by RT-PCR when sibling embryos reached stage 20 (Fig. 4.1). The results show that a low concentration of *β-catenin* RNA (20 pg/embryo; Fig. 4.1, lane 5) induces the expression of the posterior neural markers *En-2*, *Krox-20* and *Hoxb9*, but not the dorsal mesodermal marker *muscle actin*. At higher concentrations of *β-catenin* RNA (100 pg/embryo; Fig.4.1, lane 6 and 500 pg/embryo; Fig. 4.1, lane 7), in addition to the posteriorisation of neural tissue (induction of *En-2*, *Krox-20* and *Hoxb9* and suppression of the anterior neural marker *BF-1*), induction of *muscle actin* is observed, demonstrating the formation of dorsal mesoderm in the animal caps.

4.3 Posterior neural and dorsal mesodermal markers are induced in ectodermal explants injected with *noggin* and other components of the Wnt/β-catenin signalling pathway

To determine if the induction of dorsal mesoderm in animal caps shown in Fig. 4.1 is a specific property of this truncated form of β-catenin or a general property resulting from the activation of the Wnt/β-catenin signalling pathway, I tested other members of the pathway in the same assay. *Xwnt8*, *Xwnt3a, Xβ-catenin* (full length) and *wise* RNAs were injected together with *noggin* RNA into 2 cell stage *Xenopus* embryos. Animal caps were isolated from the injected embryos at stage 8, cultured in 75% NAM and assayed for gene expression by RT-PCR when sibling embryos reached stage 22 (Fig. 4.2A and B). Truncated β-catenin, Xβ-catenin, Xwnt8 and Xwnt3a induced *En-2* and *muscle actin* in animal caps neuralised with Noggin. On the other hand, Wise induced *En-2* but not *muscle actin*. Therefore, the ability to induce both posterior neural and dorsal mesodermal markers appears to be a property common to Xwnt8, Xwnt3a and β-catenin but not to Wise.

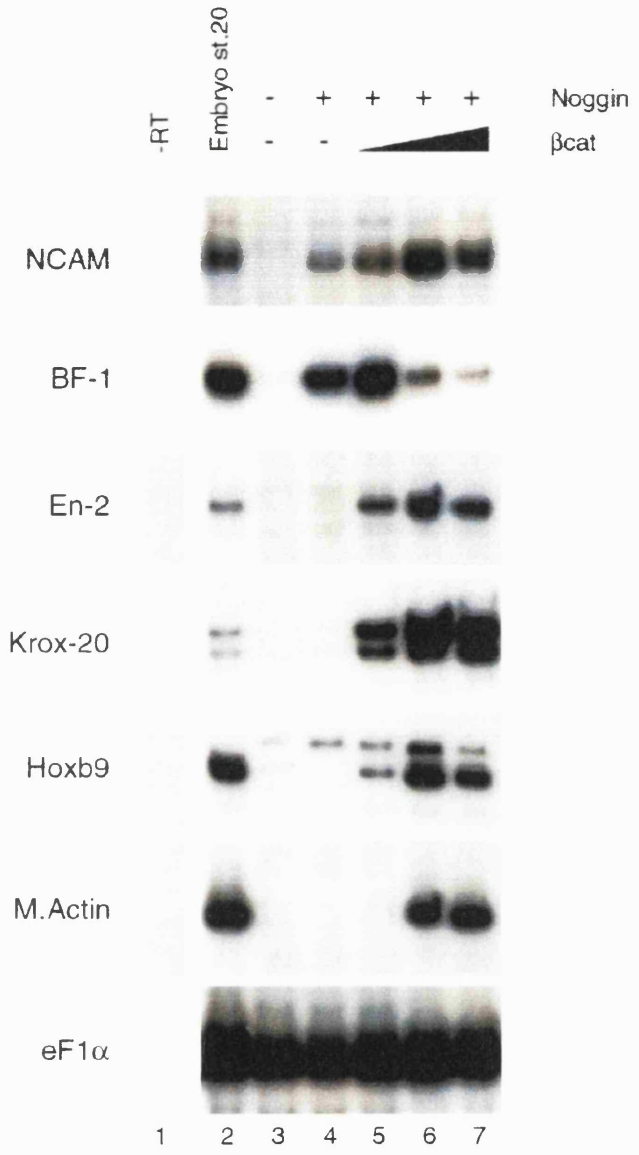
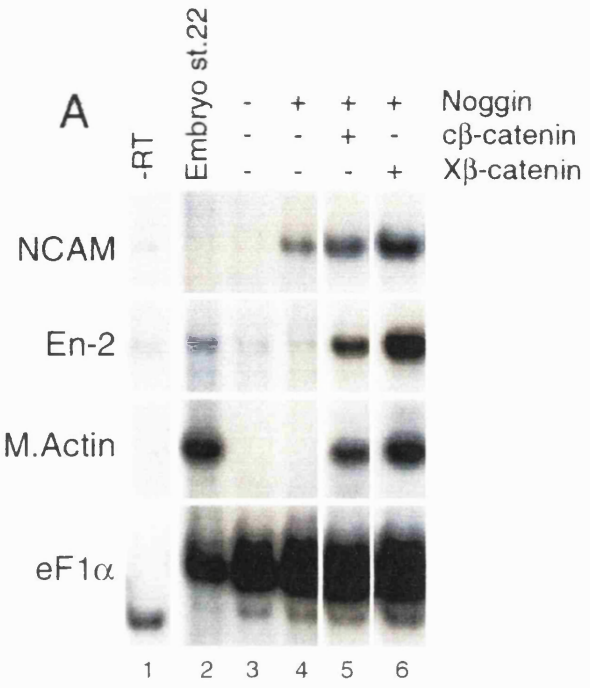
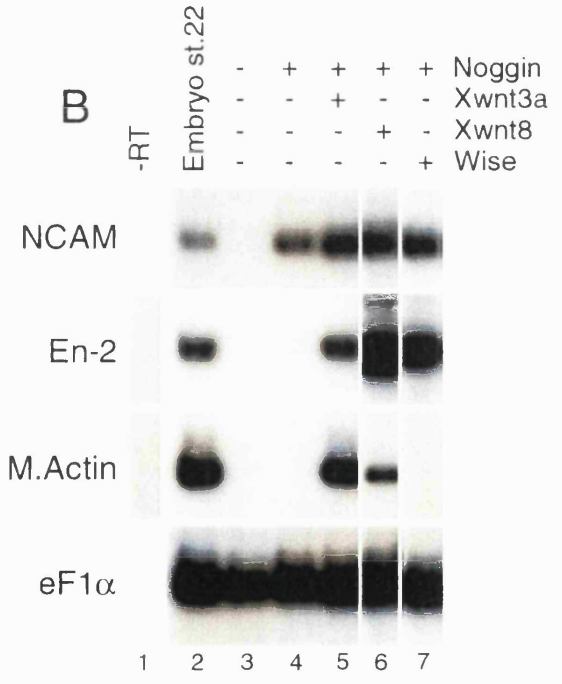


Fig. 4.1 An amino terminal truncated *β-catenin* induces posterior neural and mesodermal markers in animal caps neuralised with Noggin. RT-PCR of animal caps injected with 500 pg/embryo of *noggin* RNA and 20 pg/embryo (lane 5), 100 pg/embryo (lane 6), 500 pg/embryo (lane 7) of a truncated *β-catenin*. –RT (lane 1) is a negative control where no reverse transcriptase was added to embryo RNA during RT reaction. Embryo RNA was used as positive control (lane 2). All samples were frozen for analysis at stage 20. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers and animal caps injected only with *noggin* RNA (lane 4) expressed *NCAM* and *BF-1*, but not posterior neural or mesodermal markers. Animal caps injected with *noggin* and truncated *β-catenin* express posterior neural and mesodermal markers. *eF1α* was used as loading control.

Fig. 4.2 Truncated β-catenin, Xβ-catenin, Xwnt8 and Xwnt3a induce *En-2* and *muscle actin* in animal caps neuralised with Noggin. (A) Animal caps injected with 500 pg/embryo of *noggin* RNA and 100 pg/embryo of truncated *β-catenin* RNA (lane 5) or 500 pg/embryo *Xβ-catenin* RNA (lane 6) express *En-2* and *muscle actin.* (B) Animal caps injected with 500 pg/embryo of *noggin* RNA and 50 pg/embryo of *Xwnt3a* RNA (lane 5) or 50 pg/embryo of *Xwnt8* RNA (lane 6) express *En-2* and *muscle actin.* Animal caps injected with 500 pg/embryo of *noggin* RNA and 600 pg/embryo *wise* RNA (lane 7) express *En-2* but not *muscle actin.* –RT (lane 1) is a negative control where no reverse transcriptase was added to embryo RNA during RT reaction. Embryo RNA was used as positive control (lane 2). Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers and animal caps injected only with *noggin* RNA (lane 4) expressed *NCAM*, but not *En-2* or *muscle actin.* All samples were frozen for analysis at stage 22. *eF1α* was used as loading control.


A
-RT
Embryo st.22
- + + + Noggin
- - + - cβ-catenin
- - - + Xβ-catenin
NCAM
En-2
M.Actin
eF1α
1 2 3 4 5 6


B
-RT
Embryo st.22
- + + + + Noggin
- - + - - Xwnt3a
- - - + - Xwnt8
- - - - + Wise
NCAM
En-2
M.Actin
eF1α
1 2 3 4 5 6 7

4.4 Ventral mesodermal markers are induced in ectodermal explants injected with components of the Wnt/β-catenin signalling pathway

Other authors have reported that activation of the Wnt/β-catenin signalling pathway in animal caps causes formation of mesoderm. *Xwnt8* was reported to induce dorsal mesoderm in animal caps isolated at the late blastula/early gastrula stages (Sokol, 1993). These animal caps expressed *muscle actin* and underwent severe morphogenetic movements (elongation), which are a sign of dorsal mesoderm formation (Symes and Smith, 1987). Other reports showed that animal caps injected with RNA or DNA of members of the Wnt family of proteins differentiate into ventral mesodermal tissues, including mesothelium and mesenchyme, but not dorsal structures such as muscle (Chakrabarti et al., 1992; Christian and Moon, 1993). In these cases the animal caps did not elongate but formed vesicles that are characteristic of ventral mesoderm formation.

Therefore, it is important to characterise the type of mesoderm that is induced by the activation of the Wnt/β-catenin signalling pathway in the presence or absence of Noggin. For this purpose, *Xenopus* embryos were injected with *Xwnt8* RNA alone or together with *noggin* RNA. Animal caps were isolated at stage 8 and assayed for the induction of dorsal and ventral mesodermal markers at stage 14 (Fig. 4.3B) and stage 24 (Fig. 4.3A). Animal caps from embryos injected with *noggin* and *Xwnt8* express *muscle actin* and *Xbra* but not the ventral markers *α-globin* and *Xhox3* (Fig. 4.3A and B; lane 5). In contrast, animal caps from embryos injected with *Xwnt8* express the ventral markers *α-globin* and *Xhox3,* but not *muscle actin* and *Xbra* (Fig. 4.3A and B; lane 6). In this case induction of *Hoxb9* and, to a lower extent, *NCAM* is also observed (Fig. 4.3A, lane 6). In support of the molecular data, by comparison to controls (Fig. 4.4, panels a and b), animal caps from embryos injected with *noggin* and *Xwnt8* underwent elongation movements typical of dorsal mesoderm formation (Fig. 4.4, panel d). Animal caps from embryos injected with *Xwnt8* did not elongate but instead formed vesicles characteristic of ventral mesoderm induction (Fig. 4.4, panels f and h).

These results show that Xwnt8 itself has the ability to induce ventral mesoderm in animal caps, and that co-injection of *noggin* RNA dorsalises the character of the mesoderm induced by *Xwnt8*. Similar results were obtained with truncated *β-catenin*.

Animal caps from embryos injected with *noggin* and *β-catenin* express *muscle actin* but not *α-globin* (Fig. 4.5, lane 5). In contrast, animal caps from embryos injected with *β-catenin* express *α-globin,* but not *muscle actin* (Fig. 4.5, lane 6).

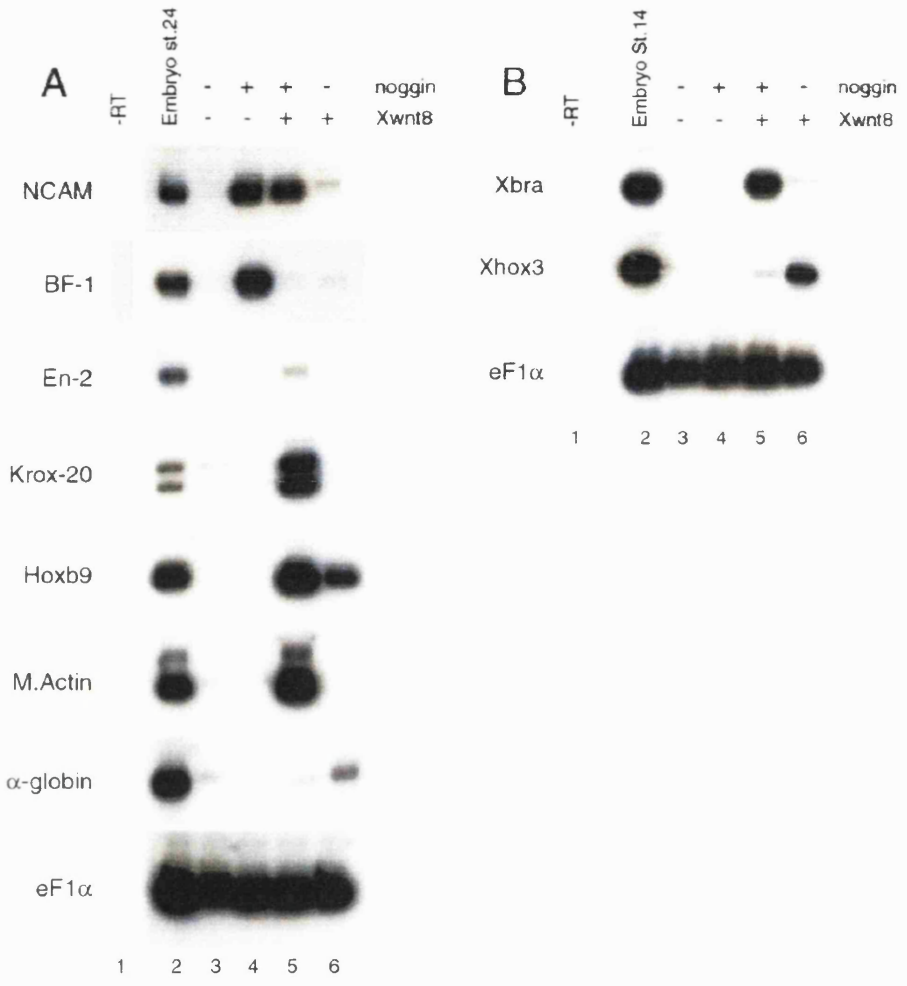


Fig. 4.3 *Xwnt8* induces posterior neural and dorsal mesodermal markers in animal caps when co-injected with *noggin* and ventral mesodermal markers when injected alone. (A) RT-PCR of animal caps assayed when sibling embryos reached stage 24. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while explants from embryos injected with noggin RNA (500 pg/embryo; lane 4) expressed *NCAM* and *BF-1* but not posterior neural or mesodermal markers. Posterior neural markers and the dorsal mesoderm marker *muscle actin* were induced in explants co-injected with noggin and *Xwnt8* (50 pg/embryo; lane 5). *Xwnt8* RNA alone (lane 6) induced the ventral mesoderm marker *α-globin* and *Hoxb9*. (B) RT-PCR of animal caps assayed at stage 14 to examine mesodermal markers. *Xwnt8* induced *Xhox3* and co-injection of *noggin* resulted in the expression of *Xbra*. Embryo RNA was used as a positive control. –RT is embryo RNA processed without reverse transcriptase and served as a negative control. *eF1α* was used as loading control.

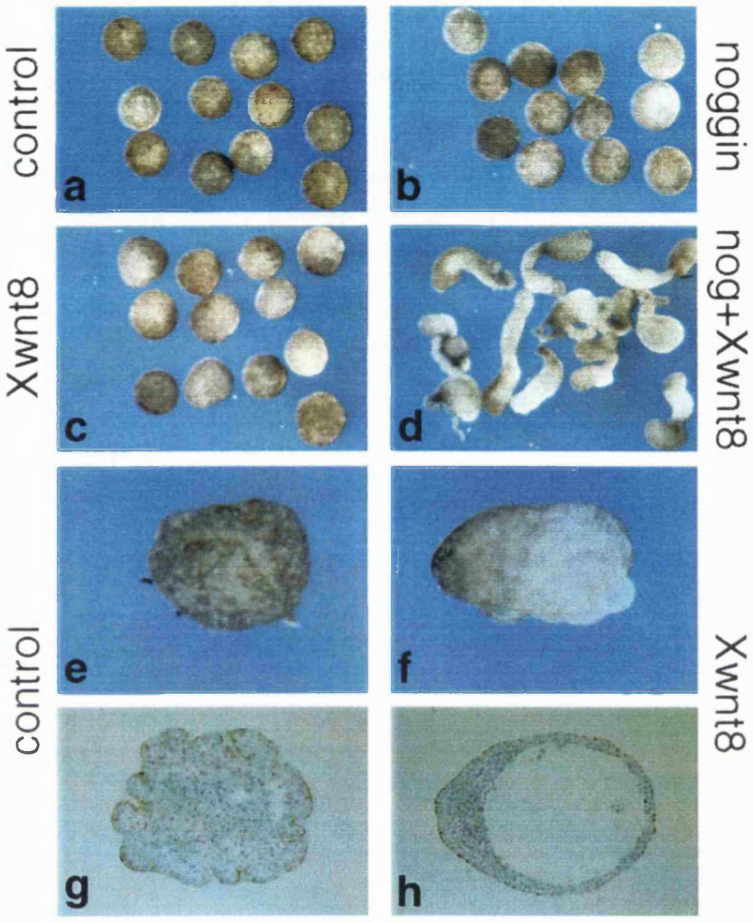


Fig. 4.4 Morphology of animal caps from embryos injected with *Xwnt8* and *noggin* RNA or *Xwnt8* RNA alone. Animal caps were isolated at stage 8 and fixed either at stage 18 (a-d) or stage 38 (e-f). Explants from uninjected (a), *noggin*-injected (b) and *Xwnt8*-injected (c) embryos did not elongate. Animal caps co-injected with *noggin* and *Xwnt8* elongated (d). Animal caps from uninjected embryos (e and g) did not form vesicles, while those injected with *Xwnt8* RNA (f and h) formed vesicles characteristic of ventral mesoderm.

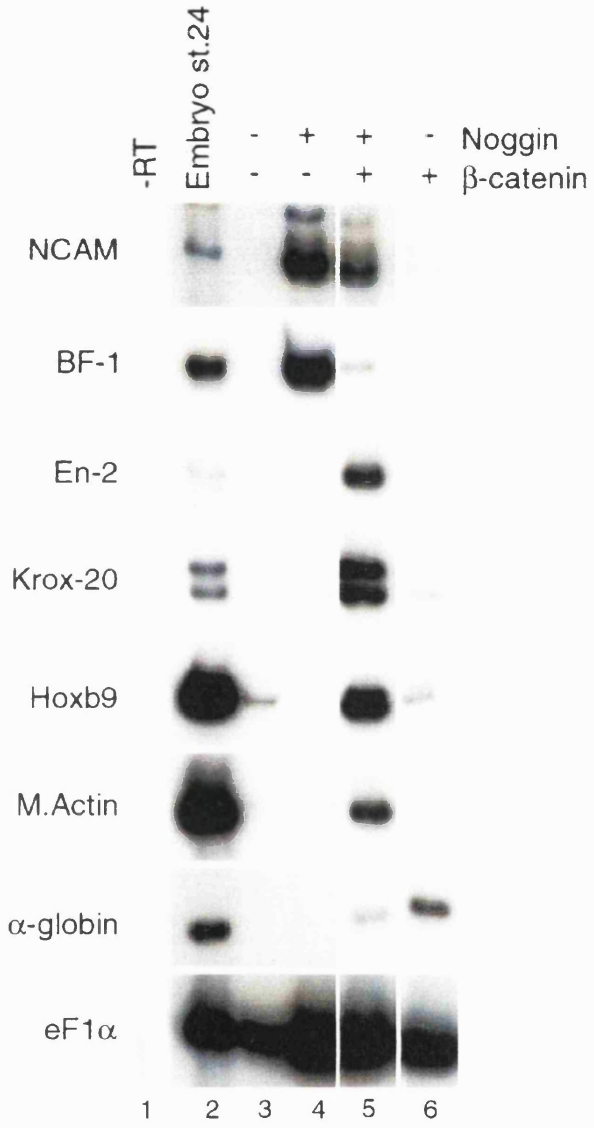


Fig. 4.5 Truncated *β-catenin* induces posterior neural and dorsal mesodermal markers in animal caps when co-injected with *noggin* and ventral mesodermal markers when injected alone. RT-PCR of animal caps assayed when sibling embryos reached stage 24. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while explants from embryos injected with *noggin* RNA (500 pg/embryo; lane 4) expressed *NCAM* and *BF-1* but not posterior neural or mesodermal markers. Posterior neural markers and the dorsal mesoderm marker *muscle actin* were induced in explants co-injected with *noggin* and truncated *β-catenin* (500 pg/embryo; lane 5). Truncated *β-catenin* RNA alone (lane 6) induced the ventral mesoderm marker *α-globin* but not *muscle actin*.

4. 5 Discussion

In this chapter *Xwnt8*, *Xwnt3a*, *Xβ-catenin* and truncated *β-catenin* functioned similarly when co-injected with *noggin* in animal caps. Posterior markers such as *En-2, Krox-20*, and *Hoxb9* were induced together with the dorsal mesoderm marker *muscle actin*. Animal caps injected with truncated *β-catenin* or *Xwnt8* RNA expressed the ventral mesodermal markers *Xhox3* and *α-globin* but not *muscle actin*.

Previously, Xwnt3a and Xβ-catenin have been shown to induce posterior neural markers in the absence of mesoderm in neuralised animal caps (McGrew et al., 1997; McGrew et al., 1995). The reasons causing the differences between these results and those reported here are not clear, but could be due to the amount of RNA injected. We also observe induction of posterior neural markers in the absence of mesoderm with low concentrations of truncated *β-catenin* RNA (Fig. 4.1, lane 5).

Other reports have shown that activation of the Wnt signalling pathway in animal caps causes the formation of mesoderm. Overexpression of *Drosophila wingless* by injection of RNA (Chakrabarti et al., 1992) or *Xwnt8* (Christian and Moon, 1993) using a plasmid which drives expression after the mid-blastula transition led to the induction of ventral mesoderm in animal caps. On the other hand, overexpression of *Xenopus dishevelled* (Itoh and Sokol, 1997) or *Xwnt8* (Sokol, 1993) caused the formation of dorsal mesoderm in animal caps. In this last study, the formation of dorsal mesoderm was shown to occur only when the explants were isolated at late blastula/early gastrula stages.

In our assays, Xwnt8 and truncated β-catenin induced ventral mesoderm in animal caps and co-injection of *noggin* led to the dorsalisation of this mesoderm. These results are in accordance with the reported capacity of Noggin to dorsalise whole embryos and ventral marginal zone explants (Lamb et al., 1993; Smith and Harland, 1992). Xwnt8 and truncated β-catenin also induced the neural markers *NCAM* and *Hoxb9*. This may reflect induction of neural tissue by members of the Wnt pathway, as previously reported (Baker et al., 1999).

Chapter 5

Posteriorising activity of an inducible form of β-catenin, GR-LEFΔNβCTA, in ectodermal explants and intact embryos

5.1 Introduction

In the previous chapter I have shown that activation of the Wnt/β-catenin signalling pathway in animal caps neuralised with Noggin leads to the induction of posterior neural and mesodermal markers. This result raises the question of whether the posteriorisation of neural tissue is a direct consequence of the activation of the Wnt/β-catenin signalling pathway or is secondary to the induction of mesoderm in the animal caps. To address this point, I asked whether it is possible to separate these two processes.

For this purpose an inducible form of β-catenin was used. A fusion protein consisting of the LEF-1 DNA binding domain and the C-terminal trans-activation domain of β-catenin (LEFΔNβCTA) (Vleminckx et al., 1999) was shown to mimic the activation of the Wnt/β-catenin signalling pathway in the context of double axis formation and induction of the direct target gene *siamois* (Brannon et al., 1997). Inducibility was achieved by fusing in frame LEFΔNβCTA to the ligand binding domain of the human glucocorticoid receptor (GR) creating GR-LEFΔNβCTA. Constructs based on GR-fusions encode proteins that remain inactive in the cytoplasm until addition of the ligand, dexamethasone (DEX), and have been shown to be a useful system to conditionally activate transcription factors during *Xenopus* embryonic development (Kolm and Sive, 1995; Tada et al., 1997).

It was previously shown that the competence of animal caps to respond to mesoderm inducing factors such as activin or FGF is lost during gastrula stages (Green et al., 1990). If Wnt/β-catenin mediated induction of posterior neural markers is independent of the induction of mesoderm, activation of GR-LEFΔNβCTA during gastrula stages should induce posterior neural markers in the absence of mesoderm.

In this chapter I used GR-LEFΔNβCTA in order to activate the Wnt/β-catenin signalling pathway in ectodermal explants and intact *Xenopus* embryos at specific stages of embryonic development.

5.2 GR-LEFΔNβCTA can posteriorise neural tissue in the absence of mesoderm

In preliminary experiments, GR-LEFΔNβCTA and GR-β-catenin (a fusion of *GR* to full-lenght *Xβ-catenin*) were compared in their abilities to induce double axes when activated in the ventral side of the embryo and to induce the direct target genes *siamois* and *Xnr3* (McKendry et al., 1997) in animal cap explants (data not shown). Both proteins were able to induce double axes and the expression of *siamois* and *Xnr3*, but GR-β-catenin was active even in the absence of DEX. For this reason GR-LEFΔNβCTA was used in the remaining experiments.

To investigate whether the induction of posterior neural markers by the Wnt/β-catenin signalling pathway is independent of the induction of mesoderm, *noggin* and *GR-LEFΔNβCTA* RNAs were injected into the animal pole of 2 cell stage *Xenopus* embryos. Animal caps were isolated at stage 8, cultured in presence or absence of DEX until late neurula stages and assayed for gene expression of neural and mesodermal markers by RT-PCR. When DEX was added at stage 9, suppression of the anterior marker *BF-1* and induction of *En-2, Krox-20, Hoxb9* and *muscle actin* was observed (Fig. 5.1, lane 6). When DEX was added at stage 12, *En-2* and *Krox-20* were induced but not *Hoxb9* or *muscle actin* and *BF-1* was only partially suppressed (Fig. 5.1, lane 7). When DEX was added at stage 15, none of the posterior neural markers were induced and *BF-1* levels were similar to the *noggin* injected animal caps (Fig. 5.1, lane 8).

Mesodermal markers were also analysed at stage 14. Addition of DEX at stage 9 caused the induction of *muscle actin*, *Xbra* and *Xhox3* (Fig. 5.2A, lane 6). None of these markers were induced when DEX was added at stage 12 (Fig. 5.2A, lane 7). These results show that activation of GR-LEFΔNβCTA at stage 9 leads both to posteriorisation of neural tissue and mesoderm induction, but activation at stage 12 leads to the induction of *En-2* and *Krox-20* in the absence of mesoderm. *Hoxb9* was induced when DEX was added at stage 9 but not at stage 12, which may indicate that the induction of this marker is dependent on the induction of mesoderm. These results also show that the competence of the animal caps to respond to the activation of GR-LEFΔNβCTA is finished by mid-neurula stages. Hence, Wnt/β-catenin signalling can posteriorise neural tissue in the absence of mesoderm in a stage specific manner.

To rule out the possibility that the distinct activities of GR-LEFΔNβCTA at different stages were a consequence of degradation of the protein over time, the protein levels were analysed by western blot (Fig. 5.2B). The result shows that the levels of GR-LEFΔNβCTA remain stable until at least stage 16. This is consistent with other reports indicating that GR fusion proteins are remarkably stable (Kolm and Sive, 1995; Tada et al., 1997).

5.3 Timing of induction of the posterior neural markers by GR-LEFΔNβCTA

In neuralised animal caps, the activation of GR-LEFΔNβCTA at stage 9 induced *En-2*, *Krox-20* and *Hoxb9*, but at stage 12 only *En-2* and *Krox-20* were induced (Fig. 5.1). This difference in the induction of the posterior neural markers could be caused by the difference in the length of time that the animal caps were treated with DEX. To address this point, embryos were co-injected *GR-LEFΔNβCTA* and *noggin* RNA and animal caps were isolated at stage 8. DEX was added at stage 9 or stage 12 and animal caps were collected for assay at stage 13, 15, 18 and 20. The results show that regardless of whether DEX is added at stage 9 or stage 12, the posterior neural markers are only first detected at stage 15 (Fig. 5.3, lanes 7 and 11), correlating with the time at which they are normally expressed during embryonic development (Hemmati-Brivanlou et al., 1991; Bradley et al., 1993; Sharpe et al., 1987). The expression of *Hoxb9* was detected when DEX was added at stage 9 (Fig. 5.3, lanes 6-9) but not at stage 12 (Fig. 5.3, lanes 10-13), independently of the period of time that the animal caps were cultured in the presence of DEX. Hence, the activity of GR-LEFΔNβCTA depends on the stage at which DEX is added but not on the length of the treatment.

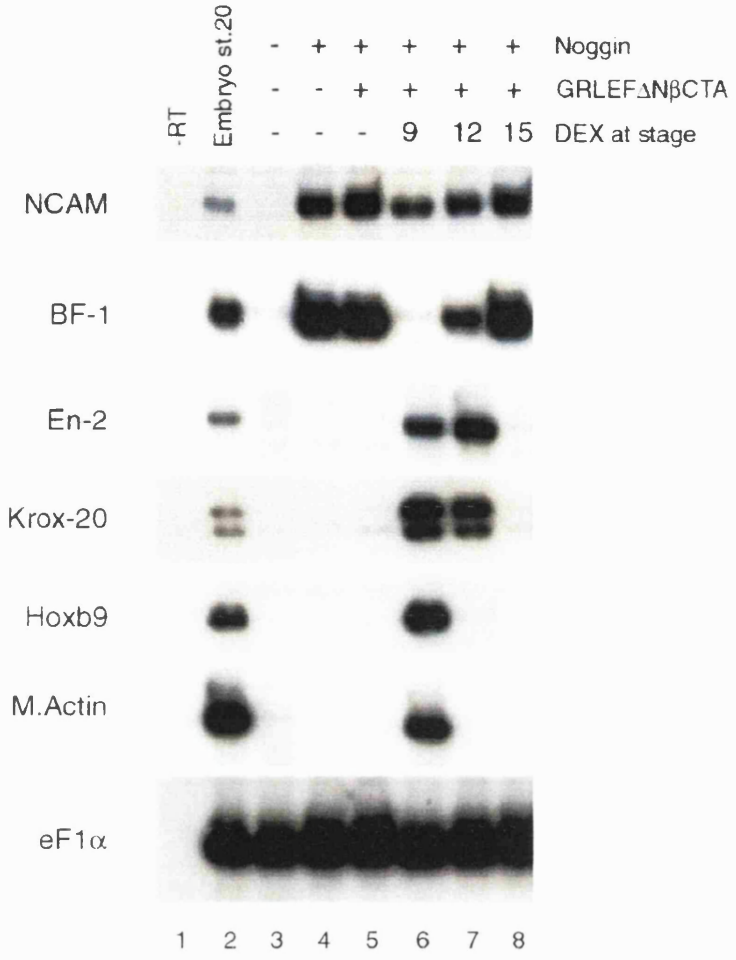


Fig. 5.1 An inducible form of β-catenin, GR-LEFΔNβCTA, upregulates both posterior neural and mesodermal markers when activated at stage 9, but only posterior neural markers when activated at stage 12. RT-PCR of animal caps analysed at stage 20. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while explants from embryos injected with *noggin* RNA (500 pg/embryo; lane 4) expressed *NCAM* and *BF-1* but not posterior neural or mesodermal markers. Posterior neural and mesodermal markers were detected in tissues from embryos co-injected with *noggin* RNA and *GR-LEFΔNβCTA* RNA (50 pg/embryo; lanes 5-8) after the addition of DEX at stage 9 (lane 6). Posteriorisation occurred in the absence of mesoderm induction when DEX was applied at stage 12 (lane 7). When DEX was added at stage 15 (lane 8) neither posteriorisation nor mesoderm induction occurred. In the absence of DEX, *NCAM* and *BF-1* are expressed but not posterior neural or mesodermal markers (lane 5). All samples were assayed at stage 20. – RT is a negative control (lane 1), embryo is a positive control (lane 2) and *eF1α* served as loading control.

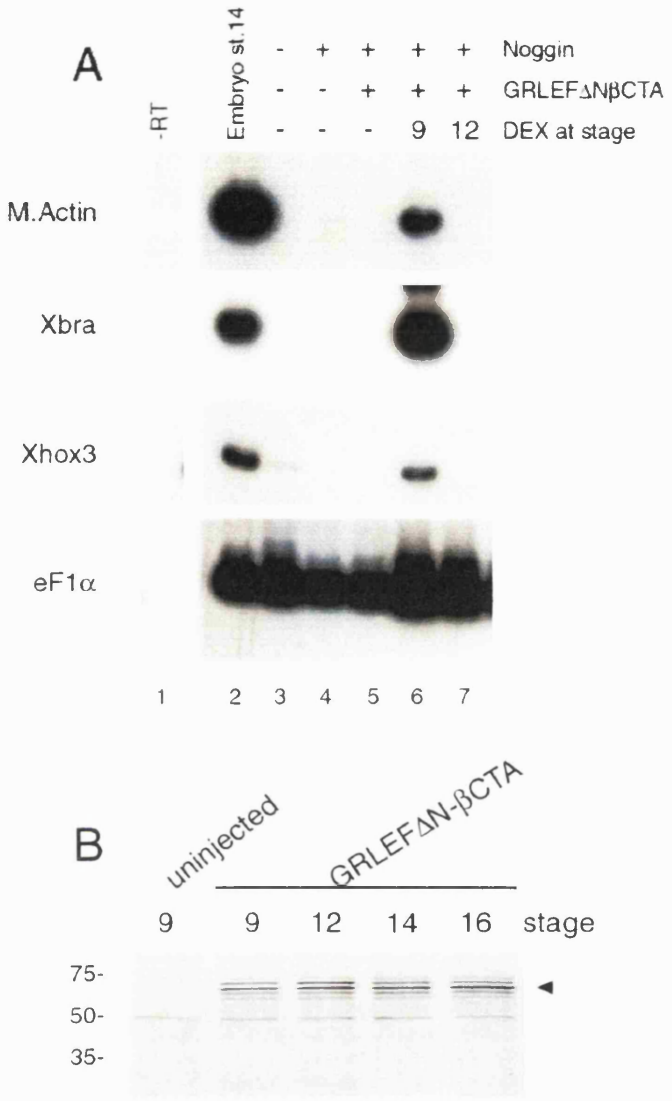


Fig. 5.2 GR-LEFΔNβCTA induces mesodermal markers when activated at stage 9 but not at stage 12. (A) RT-PCR of animal caps assayed for mesodermal markers at stage 14. Mesodermal markers were detected in animal caps co-injected with *noggin* and *GR-LEFΔNβCTA* RNA when DEX was added at stage 9 (lane 6) but not at stage 12 (lane 7). In the absence of DEX, the expression of mesodermal markers was not observed (lane 5). –RT is a negative control (lane 1), embryo is a positive control (lane 2) and *eF1α* served as loading control. (B) GR-LEFΔNβCTA protein levels are similar between blastula and neurula stages. Embryos were injected with 50 pg/embryo of *GR-LEFΔNβCTA* RNA, ectodermal explants were isolated at stage 8 and collected at the indicated stages. Two cap equivalents of protein were analysed by western blotting using the mouse anti-HA monoclonal antibody 12CA5 after SDS-PAGE. The band corresponding to GR-LEFΔNβCTA is indicated by an arrowhead and the positions of molecular mass markers (KDa) are indicated on the left of the panel.

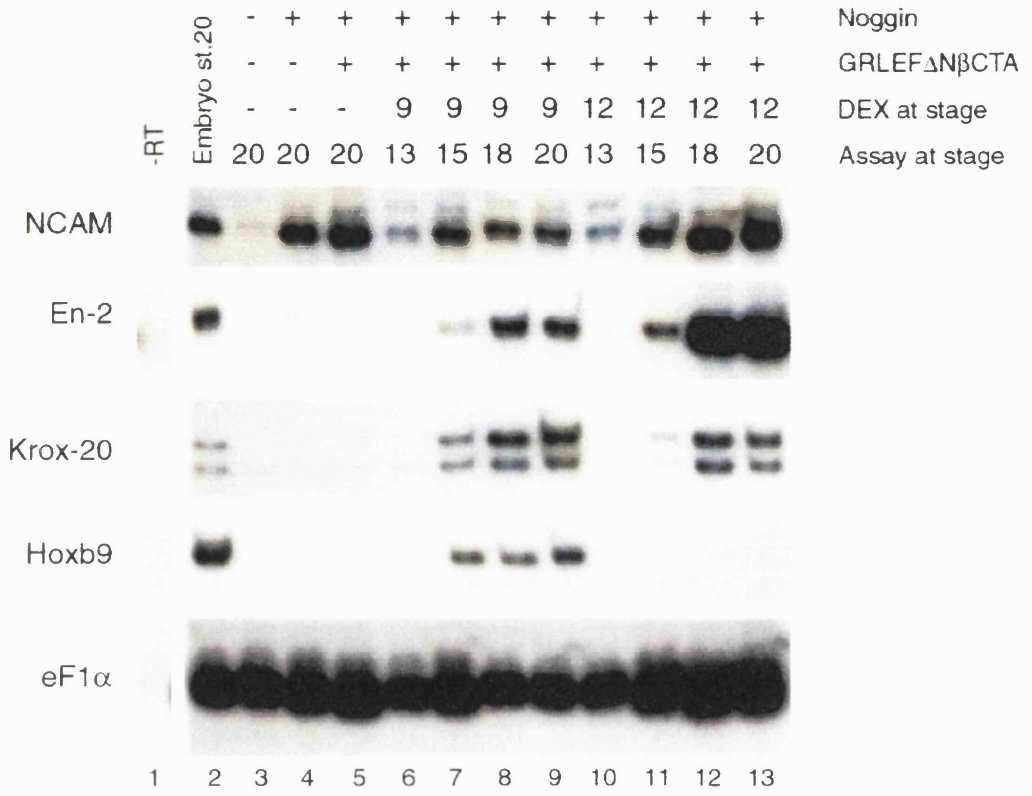
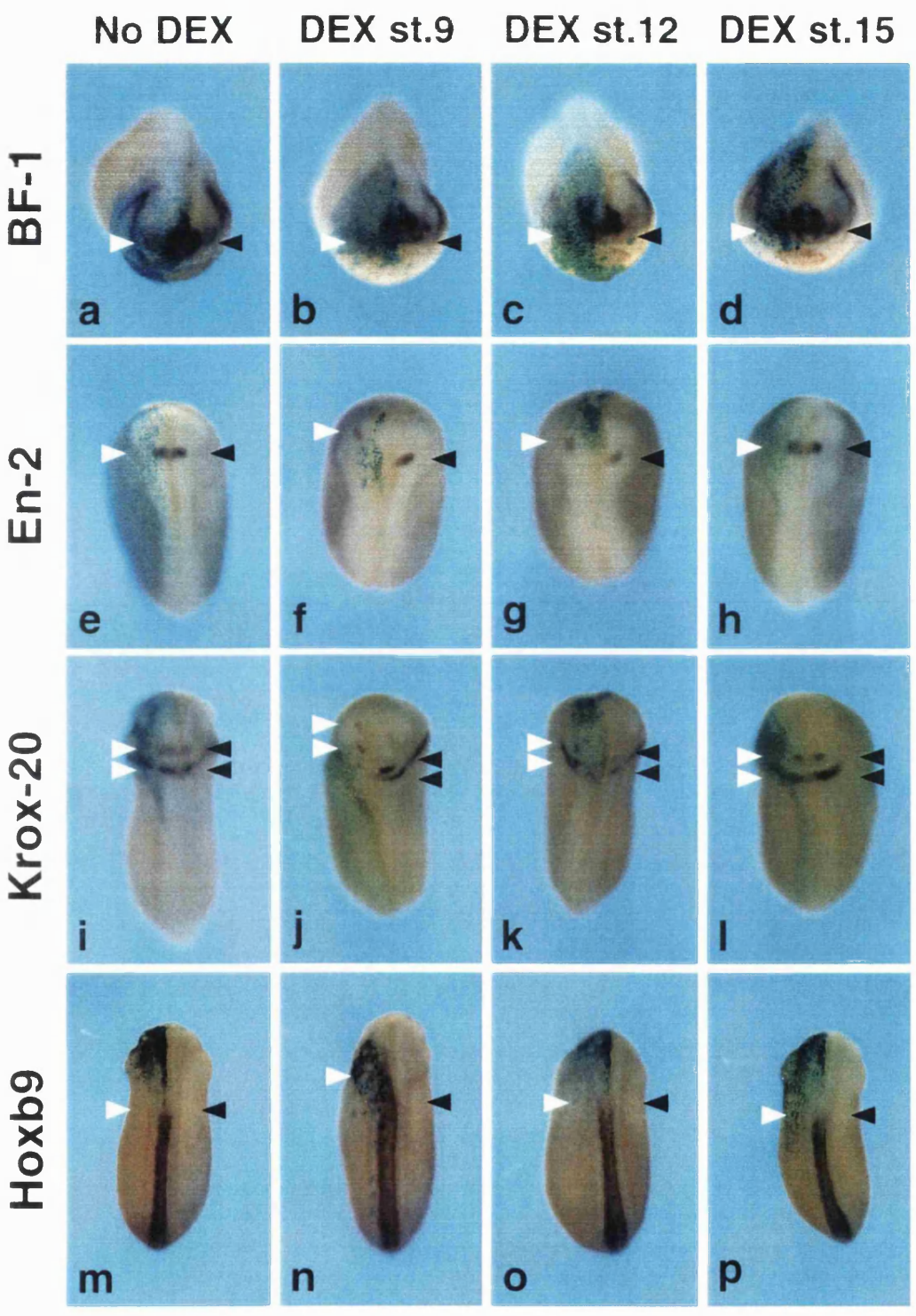


Fig. 5.3 Timing of induction of the posterior neural markers by GR-LEFΔNβCTA. RT-PCR analysis of animal caps from embryos injected with 500 pg/embryo *noggin* RNA (lane 4) or 500 pg/embryo *noggin* RNA and 50 pg/embryo *GR-LEFΔNβCTA* RNA (lanes 5-13). When DEX was not added, *NCAM* is expressed but not posterior neural markers (lane 5). Addition of DEX at stage 9 (lanes 6-9) or stage 12 (lanes 10-13) resulted in the induction of posterior neural markers. Animal caps were collected at several stages for assay: stage 13 (lanes 6, 10), stage 15 (lanes 7, 11), stage 18 (lanes 8, 12) and stage 20 (lanes 9, 13). Posterior neural markers are first observed at stage 15 independently of the stage when DEX was added to the culture media. Animal caps from uninjected embryos (lane 3) did not express any neural marker. –RT is a negative control (lane 1) and embryo (lane 2) a positive control. *eF1α* was used as loading control.

5.4 Activation of GR-LEFΔNβCTA in intact *Xenopus* embryos

I next investigated the consequences of activating GR-LEFΔNβCTA in intact *Xenopus* embryos. *GR-LEFΔNβCTA* and *β-gal* RNA was injected into one animal/dorsal blastomere of 8 cells stage embryos. These embryos were cultured in the presence or absence of DEX and the expression of neural markers was assayed by whole-mount *in situ* hybridisation. When DEX was added at stage 9, suppression of *BF-1* expression (Fig. 5.4, b) and anterior shifts in the pattern of expression of *En-2*, *Krox-20* and *Hoxb9* (Fig. 5.4, f, j and n respectively) were observed. Addition of DEX at stage 12 resulted in similar effects on the expression of *BF-1*, *En-2* and *Krox-20* (Fig. 5.4, c, g and k) but *Hoxb9* was unaffected (Fig. 5.4, o). Addition of DEX at stage 15 did not affect the expression of any of the neural markers (Fig. 5.4, d, h, l and p). In the absence of DEX the markers were identical on the non injected and injected (light blue β-gal staining) sides of the embryos (Fig. 5.4, a, e, i and m). Overall, these findings show that activation of GR-LEFΔNβCTA in whole embryos results in neural markers being expressed in more anterior locations, suggesting that anterior tissues acquire a more posterior character. Furthermore, the competence for the posteriorising action of GR-LEFΔNβCTA in whole embryos is similar to the animal cap assay. In both assays no effects were observed after stage 15.

Fig. 5.4. Activation of GR-LEFΔNβCTA in intact *Xenopus* embryos results in posteriorisation of the neural tube. One animal/dorsal blastomere of 8 cell stage embryos was injected with 50 pg of *GR-LEFΔNβCTA* RNA and 200 pg of *β-gal* RNA. Embryos were fixed at stage 18 to 22, processed for β-gal staining to reveal the injected side (light blue staining) and for whole mount *in situ* hybridisation with probes specific for *BF-1* (a-d), *En-2* (e-h), *Krox-20* (i-l) and *Hoxb9* (m-p) (purple staining). With no addition of DEX, the markers show an identical pattern of expression on the injected and uninjected sides (a, 24/25; e, 20/20; i, 20/20; m, 24/24). When DEX was added at stage 9, suppression of *BF-1* expression (b, 25/30) and anterior shifts in the expression of *En-2* (f, 26/30), *Krox-20* (j, 25/28) and *Hoxb9* (n, 20/32) resulted. Addition of DEX at stage 12 affected *BF-1* (c, 20/32), *En-2* (g, 22/28) and *Krox-20* (k, 17/25) but not *Hoxb9* (o, 3/29). Addition of DEX at stage 15 did not affect any of the markers (d, 3/28; h, 2/27; l, 2/28; p, 1/25). The position of the markers is indicated with a white arrowhead on the injected side and a black arrowhead on the control side. The numbers in brackets represent (number of affected embryos/total number of injected embryos). Panels a-d are frontal views (dorsal is up) all other panels are dorsal views (anterior is up).


No DEX
DEX st.9
DEX st.12
DEX st.15
BF-1
En-2
Krox-20
Hoxb9
a
b
c
d
e
f
g
h
i
j
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5.5 Discussion

In this chapter, I have used an inducible form of β-catenin, GR-LEFΔNβCTA, in order to activate the Wnt/β-catenin signalling pathway in a stage specific manner in *Xenopus* ectodermal explants and intact embryos.

The results of activation of GR-LEFΔNβCTA in animal caps neuralised with Noggin were stage dependent. Activation of GR-LEFΔNβCTA at stage 9 resulted in the induction of posterior neural and dorsal mesodermal markers. This is consistent with the results obtained using the truncated form of β-catenin, Xβ-catenin, Xwnt3a and Xwnt8 (Chapter 4). Activation of GR-LEFΔNβCTA at stage 12 resulted in the induction of *En-2* and *Krox-20* but not *Hoxb9* or mesodermal markers. This suggests that the induction of *En-2* and *Krox-20* does not depend on the induction of mesoderm. On the other hand, the induction of *Hoxb9* may depend on mesoderm induction. By stage 15 the ability of GR-LEFΔNβCTA to induce posterior neural markers is lost, showing that the competence of the animal cap to respond to the activation of GR-LEFΔNβCTA is finished by mid-neurula stages.

Activation of GR-LEFΔNβCTA in intact *Xenopus* embryos resulted in posteriorisation of the neural tube in a stage dependent manner. Activation of GR-LEFΔNβCTA at stage 9 suppressed the expression of anterior neural marker *BF-1*, and caused anterior shifts in the expression of *En-2, Krox-20* and *Hoxb9*. At stage 12, activation of GR-LEFΔNβCTA affected *BF-1*, *En-2* and *Krox-20*, but not *Hoxb9*. At stage 15, activation of GR-LEFΔNβCTA did not affect any of the neural markers assayed. These results are in accordance with previous reports, in which the deletion of anterior structures of the embryo was caused by expression of *Xwnt8* DNA (Christian and Moon, 1993; Fredieu et al., 1997), or by ubiquitous expression of *Xwnt8* in transgenic embryos under an inducible heat-shock promoter (Wheeler et al., 2000).

Furthermore, there is good correlation between the results obtained by activating GR-LEFΔNβCTA in animal caps and those in intact embryos. In both assays activation of GR-LEFΔNβCTA at a specific stage affected the same neural markers. Also in both assays no posteriorising effects were observed after stage 15.

Chapter 6

Mechanism of induction of the posterior neural markers by the Wnt/β-catenin signalling pathway

6.1 Introduction

In the previous chapter I have shown that activation of the Wnt/β-catenin signalling pathway induces posterior neural markers and suppresses anterior ones in a stage dependent manner, both in neuralised animal caps and in whole embryos. However, the molecular mechanism by which the Wnt/β-catenin signalling pathway causes these effects is not clear. It has been suggested that *En-2* is directly regulated by the Wnt/β-catenin signalling pathway, in a mechanism that is dependent on Tcf sites present in the *En-2* promoter (McGrew et al., 1999). On the other hand, the results obtained in Chapter 5 for the induction of *Hoxb9* (Fig. 5.1) indicate that this may occur via an indirect mechanism since the induction of *Hoxb9* seems to depend on the formation of mesoderm. Furthermore, it is not clear if the induction of *Krox-20* and the suppression of *BF-1* occur via a direct or indirect mechanism.

In this chapter I ask whether the induction of *En-2* and *Krox-20* by GR-LEFΔNβCTA occurs in a cell or non-cell autonomous manner. Since GR-LEFΔNβCTA acts as a transcriptional activator, a cell autonomous induction of these posterior neural markers should occur if they are directly regulated by β-catenin. On the other hand, a non-cell autonomous induction of *En-2* or *Krox-20* by GR-LEFΔNβCTA suggests that this happens by an indirect mechanism and further signalling between cells must be required. Furthermore, I ask whether dissociation of the animal cap cells, by treatment with calcium and magnesium free medium, can block the induction of posterior neural markers by GR-LEFΔNβCTA.

Finally, I ask if other signalling molecules involved in posteriorisation of neural tissue, such as RA and FGFs, are required downstream of the activation of the Wnt/β-catenin signalling pathway for the induction of posterior neural markers. For this purpose, I assayed if dominant negative RARα1 and two dominant negative FGF receptors, XFD and dnFGFR4a, are able to block the induction of posterior neural markers by GR-LEFΔNβCTA.

6.2 GR-LEFΔNβCTA induces the expression of *En-2* and *Krox-20* in a non-cell autonomous manner

In order to determine if the induction of *En-2* and *Krox-20* by the Wnt/β-catenin signalling pathway occurs in a cell or non-cell autonomous manner, I injected *noggin*, *GR-LEFΔNβCTA* and *β-gal* RNA as a lineage tracer into one animal blastomere of the 16 cell-stage embryos. Animal caps were isolated at stage 8, cultured in the presence or absence of DEX, fixed at stage 20 and processed for β-gal staining and *in situ* hybridisation with *En-2* or *Krox-20* probes. In the absence of DEX, no induction of *En-2* or *Krox-20* occurs (Fig. 6.1 a, b). Addition of DEX at stage 9 (Fig. 6.1, c, d) or stage 12 (Fig. 6.1, e, f) resulted in the induction of *En-2* and *Krox-20*. In all cases, it was possible to observe expression of these genes outside of the β-gal stained area. This result indicates that the induction of *En-2* and *Krox-20* by GR-LEFΔNβCTA can occur by a non-cell autonomous mechanism.

6.3 Posteriorisation of neural tissue by GR-LEFΔNβCTA requires cell-to-cell contact

The results described above indicate that downstream of the activation of the Wnt/β-catenin pathway, signalling between cells is required for the induction of the posterior neural markers. Therefore, I tested if cell communication within the animal cap is necessary for the induction of posterior neural markers. Embryos were injected with *noggin* and *GR-LEFΔNβCTA* RNA and animal caps isolated at stage 8. The animal caps were cultured in the presence or absence of DEX, in calcium and magnesium free medium (CMFM), which causes dissociation of cell-to-cell contact. As a positive control, intact animal caps expressed posterior neural markers (Fig. 6.2A, lane 6) when DEX was added to the culture medium. Dissociation of the animal cap cells completely blocked the induction of *En-2* and *muscle actin* and prevented the suppression of *BF-1* (Fig. 6.2A, lanes 7, 8). *Krox-20* expression was detected at low levels but this was not DEX dependent. The induction of *Hoxb9* was strongly inhibited (compare Fig. 6.2A lanes 5, 6 with 7, 8). On the other hand, induction of the known direct targets of the Wnt/β-catenin signalling pathway *siamois* and *Xnr3*, was not affected by the dissociation treatment, since these genes show similar levels of activation in intact and dissociated animal caps

(Fig. 6.2B, lanes 5, 6 and 7, 8). Altogether, these results demonstrate that the induction of the posterior neural markers by GR-LEFΔNβCTA occurs in a non-cell autonomous manner and requires cell-to-cell contact dependent signals.

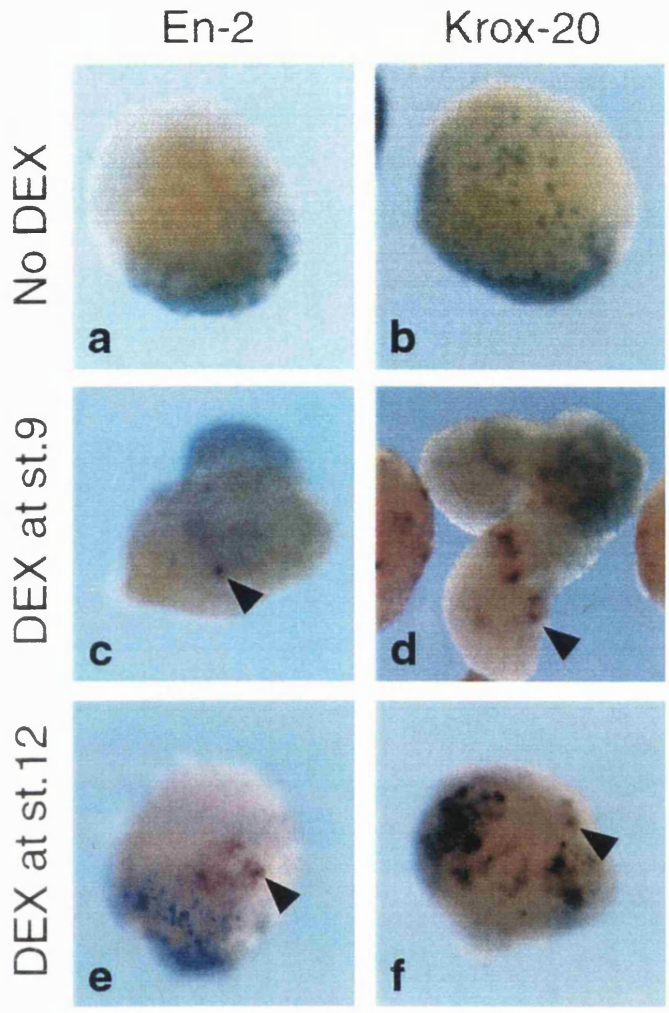
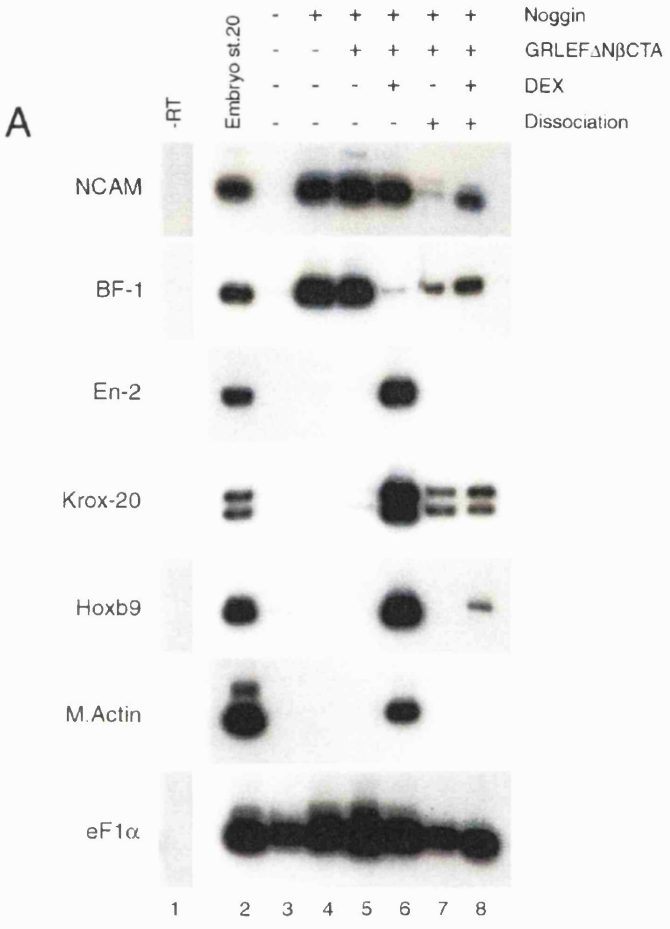


Fig. 6.1 Induction of *En-2* and *Krox-20* by GR-LEFΔNβCTA occurs in a non-cell autonomous manner. One animal blastomere of 16 cell stage embryos was injected with 500 pg *noggin* RNA, 50 pg *GR-LEFΔNβCTA* RNA and 200 pg of *β-gal* RNA as a lineage tracer. Animal caps were dissected at stage 8, cultured in the absence or presence of DEX, fixed at stage 20, stained for β-gal (light blue staining) and processed for whole mount *in situ* hybridisation with probes specific for *En-2* (a, c and e) and *Krox-20* (b, d and f) (purple staining). When DEX was not added to the culture media, induction of *En-2* (a, 0/25) or *Krox-20* (b, 0/30) was not observed. Addition of DEX at stage 9 and stage 12 resulted in the induction of *En-2* (c, 22/26 and e, 18/24), and *Krox-20* (d, 26/32 and f, 22/28) in a non-cell autonomous manner. Arrowheads indicate patches where the markers were induced outside the cells expressing the lineage tracer. The numbers in brackets above represent (number of affected embryos/total number of injected embryos).

Fig. 6.2 The induction of posterior neural markers by GR-LEFΔNβCTA requires cell-to-cell contact. (A) RT-PCR analysis showing that intact explants from embryos co-injected with *noggin* (500 pg/embryo) and *GR-LEFΔNβCTA* (50 pg/embryo) RNA expressed posterior neural and mesodermal markers when DEX was added at stage 9 (lane 6). Dissociation of the explants blocked the induction of the posterior neural and mesodermal markers (lane 8). As a negative control, DEX was not added to the culture medium (lanes 5, 7). (B) Dissociation of animal cap cells did not affect the induction of direct targets of Tcf/β-catenin. RT-PCR detected the induction of *siamois* and *Xnr3* in both intact (lane 6) and dissociated (lane 8) explants. As a negative control, DEX was not added to the culture medium (lane 5, 7). Animal caps were dissected at stage 8 and cultured intact (in 75%NAM; lanes 3-6) or dissociated in calcium and magnesium free medium (CMFM; lanes 7,8) until stage 11 (B) or stage 20 (A). Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while *noggin* injected explants expressed only anterior neural markers. *eF1α* served as loading control, –RT as a negative control and embryo RNA as a positive control.


A
-RT
Embryo st.20
- + + + + +
- - + + + +
- - - + - +
- - - - + +
Noggin
GRLEFΔNβCTA
DEX
Dissociation
NCAM
BF-1
En-2
Krox-20
Hoxb9
M.Actin
eF1α
1 2 3 4 5 6 7 8
B
-RT
Embryo st.11
- + + + + +
- - + + + +
- - - + - +
- - - - + +
Noggin
GRLEFΔNβCTA
DEX
Dissociation
Xnr-3
Siamois
eF1α
1 2 3 4 5 6 7 8

6.4 Dominant negative RARα1 partially blocks the posteriorising effect of GR-LEFΔNβCTA

I next sought to identify the signals that are required downstream of the activation of GR-LEFΔNβCTA for the induction of the posterior neural markers. Possible candidates include retinoic acid (RA) and members of the FGF family of secreted proteins, which have been shown to posteriorise *Xenopus* embryos and animal caps neuralised with Noggin (see Chapter 1).

To determine whether RA is required for the induction of posterior neural markers by Wnt/β-catenin signalling, I injected embryos with *noggin*, *GR-LEFΔNβCTA* and *dnRARα1* RNA. Animal caps were isolated at stage 8 and cultured in the presence or absence of DEX. In animal caps injected with *noggin* and *GR-LEFΔNβCTA*, addition of DEX at stage 9 resulted in the induction of posterior neural markers and *muscle actin* (Fig. 6.3, lane 6). Co-injection of 100 pg/embryo of *dnRARα1* RNA (Fig. 6.3, lane 7) did not alter the expression of the markers tested. Co-injection of 1000 pg/embryo *dnRARα1* RNA (Fig. 6.3, lane 8) resulted in the complete suppression of *En-2* and partial suppression of *Krox-20*, but did not affect any other marker. In explants cultured in DEX from stage 12, the presence of dnRARα1 did not significantly affect the expression of any neural marker (Fig.6.3, lanes 9, 10 and 11). These results indicate that the requirement for signalling by RA after activation of GR-LEFΔNβCTA is restricted to the induction of *En-2* and *Krox-20*.

6.5 FGF signalling is required for the posteriorising effect of GR-LEFΔNβCTA

To test if FGF signalling is required for the posteriorising effect of GR-LEFΔNβCTA, I used two dominant negative FGF receptors, dnFGFR1 (XFD) and dnFGFR4a. *Xenopus* embryos were injected with *noggin*, *GR-LEFΔNβCTA* and *XFD* RNA, ectodermal explants were isolated at stage 8 and cultured in the presence or absence of DEX until stage 22. In animal caps from embryos injected with *noggin* and *GR-LEFΔNβCTA*, addition of DEX at stage 9 induced the expression of posterior neural markers and *muscle actin* (Fig. 6.4A, lane 6). Co-injection of a low dose of *XFD* (200

pg/embryo) caused the suppression of *muscle actin* and an anteriorisation of the explants. The expression of *Hoxb9* was suppressed and the expression of *BF-1* was rescued (Fig. 6.4A, lane 7). Co-injection of a high dose of *XFD* (1000 pg/embryo) completly suppressed the expression of all neural markers, including *NCAM* (Fig. 6.4A, lane 8). Addition of DEX at stage 12 to *noggin* and *GR-LEFΔNβCTA* injected animal caps caused the induction of *En-2* and *Krox-20* (Fig. 6.4A, lane 9). Co-injection of *XFD* suppressed the induction of these markers (Fig. 6.4A, lanes 10, 11). In summary, inhibition of FGF signalling suppressed the posteriorising effect of GR-LEFΔNβCTA but also suppressed the induction of neural tissue at high *XFD* RNA concentrations.

I next used dnFGFR4a to determine if inhibition of FGF signalling by this dominant negative receptor could also block the induction of posterior neural markers by GR-LEFΔNβCTA. *Xenopus* embryos were injected with *noggin*, *GR-LEFΔNβCTA* and *dnFGFR4a* RNA, ectodermal explants were isolated at stage 8 and cultured in the presence or absence of DEX until stage 22. In animal caps from embryos injected with *noggin* and *GR-LEFΔNβCTA*, addition of DEX at stage 9 led to the induction of the posterior neural markers and *muscle actin* (Fig. 6.4B, lane 6). Co-injection of 200 pg/embryo of *dnFGFR4a* RNA suppressed the expression of *muscle actin* and *Hoxb9* and rescued the expression of *BF-1* and *En-2* (Fig. 6.4B, lane 7). Co-injection of 1000 pg/embryo *dnFGFR4a* RNA almost suppressed all posterior neural markers, but did not affect the expression of *BF-1* and *NCAM* (Fig. 6.4B, lane 8). Addition of DEX at stage 12 to *noggin* and *GR-LEFΔNβCTA* injected animal caps induced the expression of *En-2* and *Krox-20* (Fig. 6.4B, lane 9). Co-injection of *dnFGFR4a* RNA suppressed the induction of these markers but did not affect the expression of *BF-1* or *NCAM* (Fig. 6.4B, lanes 10, 11). In summary, inhibition of FGF signalling by dnFGFR4a suppressed the posteriorising effect of GR-LEFΔNβCTA but did not affect the induction of neural tissue by noggin.

XFD and *dnFGFR4a* were introduced in the embryo as RNA, which is translated soon after injection. It was therefore possible that the suppression of the posterior neural markers was due to an early effect caused by these dominant negative forms on the competence of the animal cap to respond to the activation of GR-LEFΔNβCTA. To address this question, I used the specific inhibitor of the tyrosine kinase activity of the

FGF receptor, SU5402 (Calbiochem) (Mohammadi et al., 1997), which can be applied simultaneously with DEX. I injected embryos with *noggin* and *GR-LEFΔNβCTA* RNA, isolated animal caps at stage 8 and cultured them in the presence of DEX and SU5402. Increasing amounts of SU5402 completely suppressed the induction of posterior neural markers (Fig. 6.4C, lanes 7 and 8), providing further evidence for the requirement of FGF signalling in the induction of posterior neural markers by GR-LEFΔNβCTA.

Finally, I investigated if *FGFs* themselves are induced in animal caps following activation of GR-LEFΔNβCTA. Animal caps isolated from embryos injected with *noggin* and *GR-LEFΔNβCTA* RNA were analysed by RT-PCR. Activation of GR-LEFΔNβCTA at stage 9 or stage 12 induced *FGF3* and *FGF8* (Fig. 6.4D, lanes 6 and 7), but *eFGF* was induced only when DEX was added at stage 9 and not at stage 12. This result shows that *FGFs* are induced as a consequence of the activation of Wnt/β-catenin pathway.

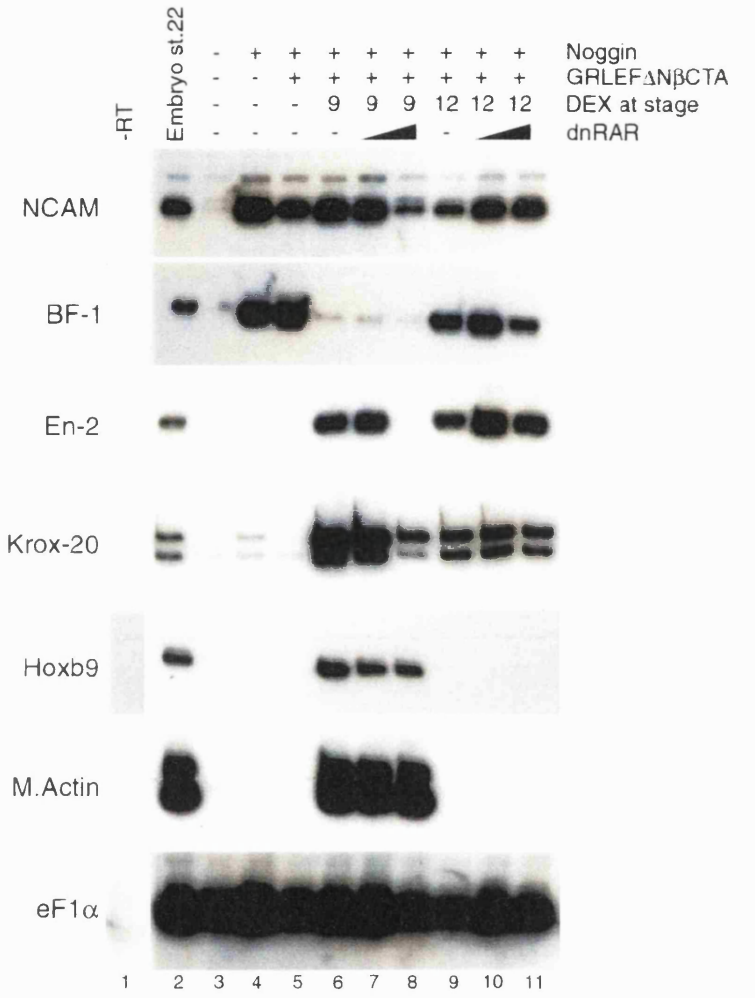
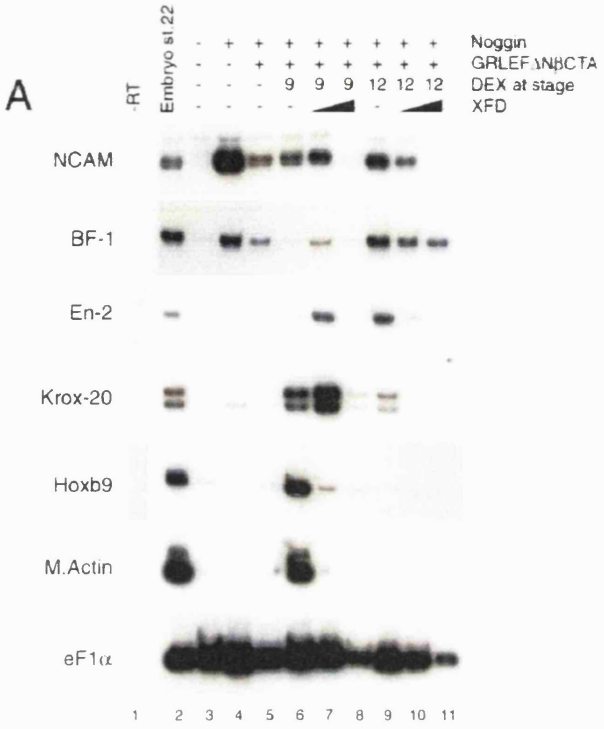


Fig. 6.3 The dominant negative RARα1 partially blocks the induction of posterior neural markers by GR-LEFΔNβCTA. RT-PCR detected the induction of posterior and mesodermal markers in explants co-injected with *noggin* (500 pg/embryo) and *GR-LEFΔNβCTA* (50 pg/embryo) when DEX was added at stage 9 (lane 6). Co-injection of 100 pg/embryo *dnRARα1* RNA did not affect any of the markers (lane 7). Co-injection of 1000 pg/embryo *dnRARα1* RNA suppressed *En-2* and *Krox-20* but did not affect other markers (lane 8). When DEX was added at stage 12, dnRARα1 did not affect any neural marker (lanes 10, 11). – RT is a negative control and embryo is a positive control. *eF1α* served as loading control.

Fig. 6.4 FGF signalling is required for induction of posterior neural and mesodermal markers by GR-LEFΔNβCTA. (A) XFD suppressed the induction of the posterior neural and mesodermal markers by GR-LEFΔNβCTA. RT-PCR detected the induction of posterior and mesodermal markers in explants co-injected with *noggin* (500 pg/embryo) and *GR-LEFΔNβCTA* (50 pg/embryo) RNA, when DEX was added at stage 9 (lane 6). Co-injection of 200 pg/embryo *XFD* RNA suppressed *muscle actin* and *Hoxb9* expression and rescued *BF-1* and *En-2* expression (lane 7). Co-injection of 1000 pg/embryo *XFD* RNA suppressed all neural and mesodermal markers (lane 8). When DEX was added at stage 12, XFD blocked the induction of all posterior neural markers (lanes 10, 11). (B) dnFGFR4a suppressed the induction of the posterior neural and mesodermal markers by GR-LEFΔNβCTA. RT-PCR detected the induction of posterior and mesodermal markers in explants co-injected with *noggin* (500 pg/embryo) and *GR-LEFΔNβCTA* (50 pg/embryo) RNA when DEX was added at stage 9 (lane 6). Co-injection of 200 pg/embryo *dnFGFR4a* RNA suppressed *muscle actin* and *Hoxb9* expression and rescued *BF-1* and *En-2* expression (lane 7). Co-injection of 1000 pg/embryo *dnFGFR4a* RNA completely suppressed *muscle actin*, *Hoxb9*, and *En-2*, partially suppressed *Krox-20*, but did not affect *BF-1* and *NCAM* expression (lane 8). When DEX was added at stage 12, dnFGFR4a blocked the induction of all posterior neural markers (lanes 10, 11). (C) The FGF signalling inhibitor SU5402 blocked the induction of posterior neural markers when added simultaneously with DEX at stage 9. Both 10 μM (lane 7) and 50 μM (lane 8) of SU5402 suppressed the induction of posterior neural and mesodermal markers. (D) FGFs are induced by GR-LEFΔNβCTA. RT-PCR of animal caps analysed when sibling embryos reached stage 15 detected the expression of *eFGF*, *FGF3* and *FGF8* when DEX was added at stage 9 (lane 6). Lower levels of *FGF3* and *FGF8* expression were detected when DEX was added at stage 12 (lane 7). –RT is a negative control and embryo is a positive control. *eF1α* served as loading control.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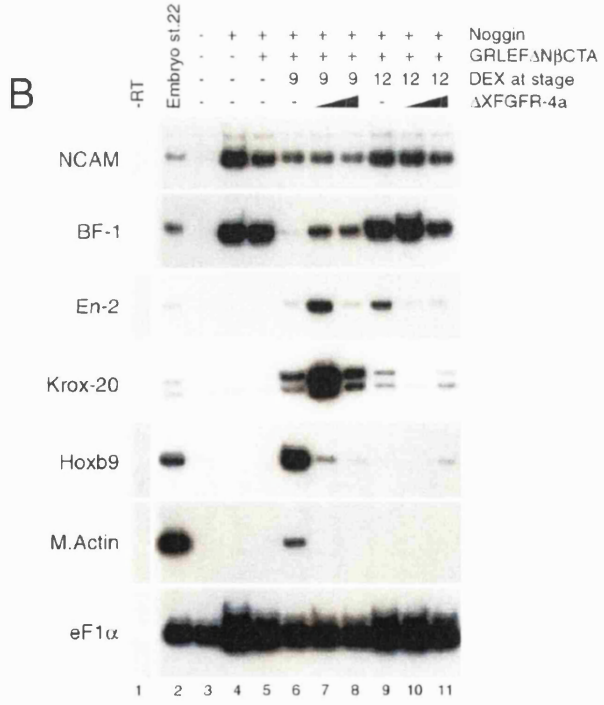
En-2
Krox-20
Hoxb9
M.Actin
eF1α
1 2 3 4 5 6 7 8 9 10 11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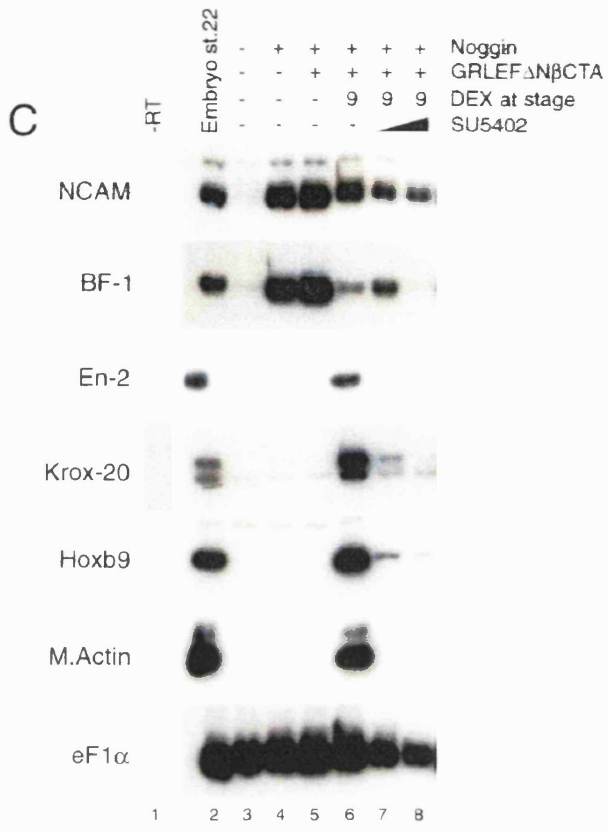
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En-2
Krox-20
Hoxb9
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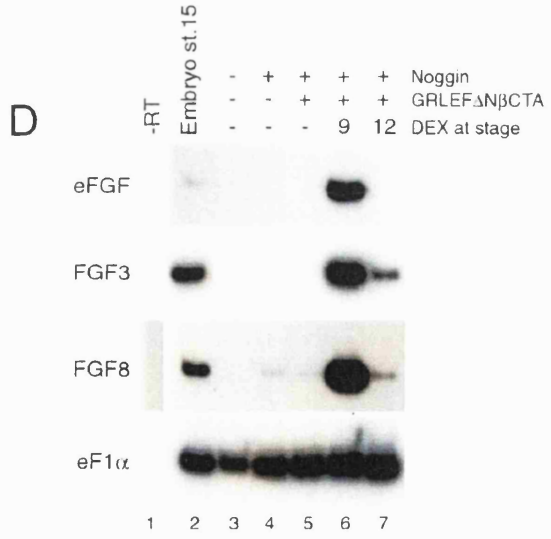
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Embryo st.15
- + + + + Noggin
- - + + + GRLEFΔNβCTA
- - - 9 12 DEX at stage
eFGF
FGF3
FGF8
eF1α
1 2 3 4 5 6 7

6.6 Discussion

6.6.1 Indirect mechanism of posteriorisation of neural tissue by the Wnt/β-catenin signalling pathway

In this chapter I have investigated the mechanism of induction of posterior neural markers by the Wnt/β-catenin signalling pathway and the results obtained suggest that this occurs via an indirect mechanism. The induction of *En-2* and *Krox-20* expression by GR-LEFΔNβCTA in animal caps neuralised with Noggin occurred in a non-cell autonomous manner, as the expression of these markers was observed in cells not containing the β-gal lineage tracer. This result is consistent with the effects of GR-LEFΔNβCTA on intact embryos (Fig. 5.4), where in some cases it is possible to observe an anterior displacement of the markers, beyond the β-gal stained area, also suggesting a non-cell autonomous mechanism. Dissociation of animal cap cells, by culture in calcium and magnesium free medium, blocked the induction of *En-2*, *Krox-20* and *Hoxb9* and the suppression of *BF-1*. This dissociation treatment did not block the induction of the known direct targets of the Wnt/β-catenin pathway, *siamois* and *Xnr3*.

It has been suggested that *En-2* is directly regulated by the Wnt/β-catenin pathway, via Lef/Tcf sites present in the *En-2* promoter (McGrew et al., 1999). These sites are required for the activation of a reporter by Noggin and Xwnt3a in animal caps, and were suggested to be essential for the Wnt1-dependent expression of *En-2* during embryo development. However, in mice, Wnt1 is required for the maintenance of *En-2* expression but not for its initiation (McMahon et al., 1992; Danielian and McMahon, 1996). Although other Wnt family members may play a direct role in the initiation of *En-2* expression, this has not been shown. The indirect mechanism for activation of the posterior neural markers suggested by the results described here indicates that activation of *En-2* expression by Wnt/β-catenin signalling may occur independently of these Lef/Tcf sites.

Recombination experiments in which dorsal mesoderm was co-cultured with ectoderm (Fredieu et al., 1997) provide more evidence in support of an indirect mechanism for induction of posterior neural markers by the Wnt/β-catenin signalling pathway. In these experiments, expression of *β-catenin* (or treatment with lithium) in the

dorsal mesoderm reduced the capacity of the recombinants to form anterior structures such as eyes and cement gland. These authors suggested that the Wnt/β-catenin pathway might trigger the production of a "dominant posteriorising morphogen" in the dorsal mesoderm, which would then act on the ectodermal/neural tissue. The results presented in this thesis suggest that the Wnt/β-catenin signalling pathway may also induce posteriorising signals in the absence of mesoderm.

6.6.2 FGF signalling is required for posteriorisation of neural tissue by the Wnt/β–catenin signalling pathway

The finding that posteriorisation of neural tissue by the Wnt/β-catenin signalling pathway may occur by an indirect mechanism, raised the question of whether other known posteriorising molecules, such as retinoic acid and FGFs are required for the induction of posterior neural markers by the Wnt/β-catenin pathway. I have shown that dnRARα1 is able to block the induction of *En-2* and *Krox-20* by GR-LEFΔNβCTA when DEX is added at stage 9 but not at stage 12. Since activation of GR-LEFΔNβCTA at stage 9, but not at stage 12, leads to mesoderm formation, this result may indicate that RA is part of the posteriorising signals that are produced in the mesoderm and act in the neural tissue. Alternatively, dnRARα1 may affect the character of the mesoderm, restricting the capacity to produce posteriorising signals. Blocking RA signalling when GR-LEFΔNβCTA is activated at stage 12 does not affect the induction of any posterior neural marker, which indicates that the posteriorising activity of GR-LEFΔNβCTA at stage 12 does not depend on RA signalling.

I have shown that FGF signalling is required in a more general manner for the induction of posterior neural markers by the Wnt/β-catenin signalling pathway. The dominant negative FGF receptor 1, XFD, blocked the induction of posterior neural and mesodermal markers in animal caps injected with *noggin* and *GR-LEFΔNβCTA*. In a previous report (McGrew et al., 1997), XFD was shown to block the suppression of the anterior gene *Otx-2*, but not the induction of *En-2* and *Krox-20*, when co-injected with *noggin* and *Xwnt3a*. In the experiments described here, low doses (200 pg/embryo) of XFD gave a similar result when GR-LEFΔNβCTA was activated at stage 9. Higher doses

(1000 pg/embryo) blocked completely the induction of all neural markers, in accordance with results previously obtained by other authors (Launay et al., 1996). When DEX was added at stage 12, 200 pg/embryo of XFD suppressed the expression of *En-2* and *Krox-20*. This shows that XFD can inhibit the posteriorising effect of GR-LEFΔNβCTA, both when this construct was activated at stage 9 or stage 12.

Similar results were obtained with dnFGFR4a, except that in this case, suppression of *BF-1* and *NCAM* was not observed. Dominant negative FGFR4a was shown to block the induction of anterior neural markers in recombinants of ectoderm and dorsal mesoderm and in ectodermal dissociated cells (Hongo et al., 1999). These authors suggested that FGF signalling mediated by FGFR4a is required for anterior neural induction. The results described here do not confirm these findings since dnFGFR4a did not block the induction of *BF-1* and *NCAM* in animal caps injected with *noggin* and *GR-LEFΔNβCTA*. On the other hand, both XFD and dnFGFR4a blocked the induction of *En-2*, *Krox-20*, *Hoxb9* and *muscle actin*.

The ability of SU5402 to suppress the induction of the posterior neural markers when added simultaneously with DEX, suggests that FGF signalling is required after the activation of GR-LEFΔNβCTA and not before. Finally, I have shown that the expression of *FGF3* and *FGF8* is induced in animal caps as a consequence of the activation of GR-LEFΔNβCTA. During the gastrula stages of embryonic development *FGF3* (Tannahill et al., 1992) and *FGF8* (Christen and Slack, 1997) are expressed in the posterior ectoderm and mesoderm, which is consistent with an eventual role of these proteins as posteriorising factors. Altogether, these results suggest that FGF signalling is required for the posteriorising action of the Wnt/β-catenin signalling pathway.

Chapter 7

Final Discussion

7.1 Posteriorisation of neural tissue by the Wnt/β-catenin signalling pathway

In this thesis I have analysed the mechanism by which the Wnt/β-catenin signalling pathway patterns the central nervous system of developing *Xenopus* embryos. The starting point of this project was a functional screen designed to identify factors involved in posteriorisation of neural tissue, from which clones were isolated that encoded truncated forms of β-catenin. The role of β-catenin and Wnt proteins as inducers of posterior neural markers in neuralised animal caps had been reported previously (McGrew et al., 1997; McGrew et al., 1995). In contrast with these reports, I have shown that β-catenin, Xwnt8 and Xwnt3a induce not only posterior neural markers but also mesodermal markers in neuralised animal caps. This result raised the question of whether the posteriorisation of neural tissue is a direct consequence of the activation of the Wnt/β-catenin signalling pathway or secondary to the induction of mesoderm in the animal caps. To address this question, I used an inducible form of β-catenin (GR-LEFΔNβCTA), which allows the activation of the Wnt/β-catenin signalling pathway at specific stages of embryonic development. The activation of GR-LEFΔNβCTA during blastula stages induced posterior neural and mesodermal markers in neuralised animal caps, while activation of GR-LEFΔNβCTA during gastrula stages induced the expression of *En-2* and *Krox-20* in the absence of mesoderm. This result shows that the induction of posterior neural markers by the Wnt/β-catenin signalling pathway in neuralised animal caps does not depend on the induction of mesoderm.

7.2 Indirect mechanism of posteriorisation of neural tissue by the Wnt/β-catenin signalling pathway

I showed that *En-2* and *Krox-20* are induced in a non-cell autonomous manner by GR-LEFΔNβCTA in neuralised animal caps. Moreover, dissociation of the animal cap cells by culture in calcium and magnesium free medium, blocked the induction of posterior neural markers by GR-LEFΔNβCTA, suggesting that signalling between cells is required downstream of the activation of GR-LEFΔNβCTA. Blocking FGF signalling by the use of dnFGFR1, dnFGFR4a or the compound SU5402, resulted in the suppression of the posteriorisation caused by GR-LEFΔNβCTA, suggesting that FGF signalling is

required downstream of the Wnt/β-catenin signalling pathway for the posteriorisation of neural tissue. Previously, it was shown that dnWnt8 suppresses the posteriorisation of neural tissue caused by bFGF (McGrew et al., 1997). Taken together, these results raise the possibility of the existence of a regulatory loop between the Wnt/β-catenin and FGF signalling pathways, required for the posteriorisation of neural tissue.

Another example where the Wnt/β-catenin signalling pathway was shown to cross-talk with FGF signalling is during the initiation of limb development in the chick (Kawakami et al., 2001). This study shows that *Wnt2b* and *Wnt8c* control the expression of *FGF-10* in the presumptive forelimb and hindlimb regions, respectively. Moreover, the induction of *FGF8* in the limb ectoderm by *FGF10* is mediated by *Wnt3a.*

In intact *Xenopus* embryos, the posteriorising action of FGFs and BMPs but not of RA is rescued by Dkk1, a Wnt inhibitor (Kawakami et al., 2001). The authors concluded from this result that the posteriorising activity of FGFs and BMPs is mediated by a Wnt signal. However, it is not possible to determine if these posteriorising activities act directly in the neural tissue since the experiments were performed in intact embryos. In fact, ectopic expression of *dkk1* affects mesodermal and endodermal tissues in addition to neural tissue. Embryos injected with *dkk1* RNA have an expanded prechordal plate (Kawakami et al., 2001) and ectopic expression of *dkk1* in the ventral marginal zone is sufficient to induce cardiogenesis (Schneider and Mercola, 2001). Moreover, the mechanism of action of Dkk1 is unknown and is possible that Dkk1 promotes anterior fates by a mechanism that is independent of its Wnt antagonistic action.

7.3 Which Wnt protein acts as a posteriorising factor during embryonic development?

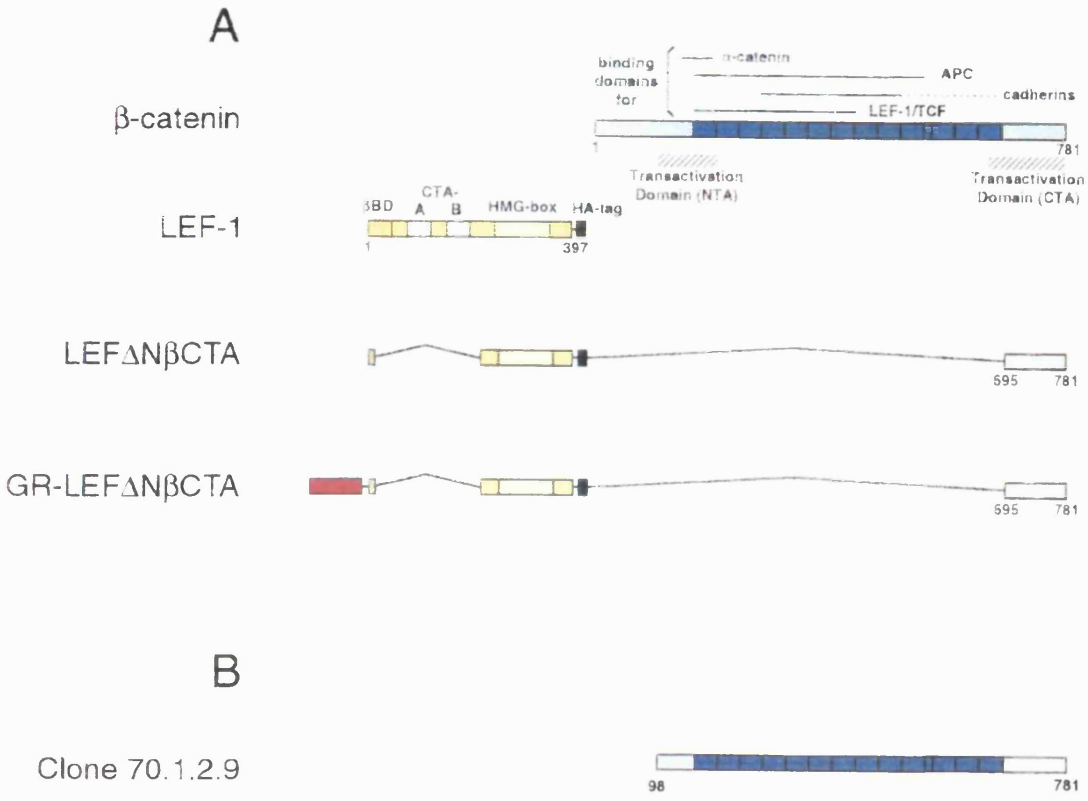
Xwnt3a and Xwnt1 have been proposed as neural posteriorising factors on the basis of gain-of function experiments in *Xenopus* embryos and explants (McGrew et al., 1997; McGrew et al., 1995). However, it was shown in this thesis that Xwnt8, Xwnt3a and β-catenin have similar posteriorising activities in overexpression assays in animal caps. This result indicates that all Wnt proteins able to activate the Wnt/β-catenin signalling pathway may have a similar posteriorising activity in this assay. In order to determine which Wnt acts as the endogenous posteriorising signal during embryonic

development is necessary to perform loss-of-function analyses for each Wnt gene. In mice targeted deletion of *Wnt1* has revealed that this gene is required for the development of the midbrain but not more posterior neural structures (McMahon et al., 1992). The absence of defects at more posterior levels of the neural tube was attributed to the redundant action of *Wnt3a*. However, mice with a double deletion of *Wnt1* and *Wnt3a* formed a normal posterior neural tube, although they presented defects in the proliferation of neural crest cells progenitors (Ikeya et al., 1997). While it is possible that an as yet unknown Wnt protein co-operates with Wnt1 and Wnt3a for the induction of posterior neural tube, it was recently shown that mice with a conditional deletion of *β-catenin* in the region of *Wnt1* expression do not show additional defects in the posterior neural tube to the ones observed in the *Wnt1*, *Wnt3a* double deletion (Brault et al., 2001). This result suggests that no additional Wnt protein in the neural tube co-operates with Wnt1 and Wnt3 in the patterning of posterior neural tube.

In *Xenopus*, *Xwnt1* is only expressed at tailbud stages and *Xwnt3a* starts to be expressed at gastrula stages (Bang et al., 1999; McGrew et al., 1997; Wolda, 1993). In Chapter 5 it was shown that the competence to posteriorise neuralised animal caps and intact embryos by activation of the Wnt/β-catenin signalling pathway is finished by stage 15. This result suggests that *Xwnt1* cannot be the endogenous posteriorising signal since it is only expressed after the period of competence for posteriorisation is finished. On the other hand, *Xwnt3a* expression is compatible with a role as posteriorising signal.

Wnt8 is maybe the best candidate for the endogenous posteriorising signal. In *Xenopus*, *Xwnt8* is expressed in the ventral/lateral mesoderm during gastrulation and was proposed to induce or maintain the expression of *Pax3* in the posterior/lateral domains of the presumptive neural plate (Bang et al., 1999). The ventral/lateral mesoderm is fated to become the posterior paraxial mesoderm (Dale, 1987a), which has been described as a source of posteriorising signals in *Xenopus* (Bang et al., 1999; Bang et al., 1997), zebrafish (Woo and Fraser, 1997) and chick (Itasaki et al., 1996; Muhr et al., 1997). *Xhox3*, a gene that has been involved in the promotion of posterior neural fates is expressed in the posterior paraxial mesoderm (Ruiz i Altaba and Melton, 1989) and *E2F* promotes posterior neural and ventral/lateral mesodermal fates (Suzuki and Hemmati-Brivanlou, 2000). In mice, *Wnt8* expression is first detected in the posterior region of the

epiblast of early primitive streak stage embryos. During gastrulation, *Wnt8* is expressed in newly formed mesoderm and in the posterior neuroectoderm (Bouillet et al., 1996). The expression of Wnt8 in mice is consistent with a role in the promotion of posterior neural fates. Unfortunately, the results of *Wnt8* loss-of-function are not known. All together these results indicate that Wnt8 is a good candidate for the endogenous posteriorising signal.



(A) Schematic representation of LEF-1, β-catenin, LEFΔNβCTA and GR-LEFΔNβCTA. The numbers represent the amino acid positions. In LEF-1, the β-catenin binding domain (βBD), two context dependent transcriptional activation domains (CTA-A and CTA-B) and DNA binding domain (HMG-box) are indicated. In β-catenin are represented the binding sites for LEF-1/TCF, cadherins, α-catenin, APC and the N-terminal and C-terminal transactivation domains. In GR-LEFΔNβCTA, the ligand binding domain of the human glucocorticoid receptor (red box) was fused in frame to LEFΔNβCTA with a pentaglycine bridge.

(B) Schematic representation of clone 70.1.2.9, a truncated β-catenin lacking the first 263 bp of the coding region. This clone presumably encodes a protein that starts with the methionine in position 98. Adapted from Vleminckx et al (1999).

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