Specification of the Lens and Olfactory Placodes and Dorsoventral Patterning of the Telencephalon

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Cover image: A glimpse of development.
Pictures showing: 1) A chick embryo transfected with a Noggin vector and a control GFP vector using the electroporation method. 2) The olfactory placode visualized with fluorescent antibodies detecting HuC/D (green) and Keratin (red). Cell nuclei are stained with Dapi (blue). 3) A stage 4 olfactory/lens placode explant expressing *Raldh3* mRNA.
Till Mamma, Åsa och Gunilla

Amor vincit omnia
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ABSTRACT

The vertebrate nervous system is a highly complex and intriguing structure with diverse functions. To understand the functional nervous system, we first have to be aware of how it is assembled during development. In this thesis the mechanism of early diversification and regionalization necessary for subsequent formation of part of the nervous system, namely the telencephalon and the placodes, will be addressed. We have identified signalling molecules involved in the dorsoventral patterning of the telencephalon and we propose a mechanism for the induction and differential specification of the olfactory and lens placodes.

The telencephalon is regionalized along the dorsoventral axis during development. The cells situated dorsally will give rise to the cerebral cortex while the ventral and intermediate cells are mainly progenitors for the basal ganglia. The cerebral cortex is associated with higher cognitive functions whereas the basal ganglia control movements. We provide evidence that dorsal and intermediate telencephalic cells are re-specified from cells with an intrinsic ventral character. Dorsal telencephalic cells are specified at stage 10 in chick, while the intermediate cells are specified a few hours later, at stage 14. The expression of Wnt and Fibroblast growth factors (Fgf$s$) coincides with the time point when the dorsal cells are specified, and we provide evidence that Wnt and FGF signals act in a sequential way to specify dorsal telencephalic cells. The retinoic acid (RA) synthesising enzyme Raldh3 is expressed in proximity to the telencephalon, and our result suggests that RA is both required and sufficient to induce intermediate telencephalic cell types. Additionally, Fgf8 is expressed in the anterior neural ridge and the ventral telencephalic cells require FGF signals that oppose RA to maintain their character.

The olfactory and lens placodes contribute to the special sense organs associated with olfaction and vision, respectively. Olfactory and lens placodes are specified at gastrula stage in chick, and become spatially separated at the neural fold stage. We provide evidence that Bone morphogenetic protein (BMP) signalling is required for the induction of a pool of placodal progenitor cells. Furthermore, time of exposure to BMP signals plays a key role in the differential specification of the olfactory and lens placodes, where continued exposure to BMP signals promotes lens character at the expense of olfactory placodal cells.
PAPERS IN THIS THESIS

This thesis is based on the following papers referred to in the text by their roman numerals (I-III).


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANR</td>
<td>Anterior neural ridge</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic protein</td>
</tr>
<tr>
<td>BMPR</td>
<td>Bone Morphogenetic protein receptor</td>
</tr>
<tr>
<td>βgal</td>
<td>Beta galactosidase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRABP</td>
<td>Cellular retinoic acid binding protein</td>
</tr>
<tr>
<td>D</td>
<td>Dorsal</td>
</tr>
<tr>
<td>Dlx</td>
<td>Vertebrate <em>Distalless</em> homologues</td>
</tr>
<tr>
<td>DV</td>
<td>Dorsoventral</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>FB</td>
<td>Forebrain</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FoxG1 (BF-1)</td>
<td>Forkhead-box G1 (Brain Factor 1)</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth/differentiation factor</td>
</tr>
<tr>
<td>HH</td>
<td>Hedgehog</td>
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<tr>
<td>I</td>
<td>Intermediate</td>
</tr>
<tr>
<td>LGE</td>
<td>Lateral ganglionic eminence</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>mFrz</td>
<td>Mouse frizzled receptor protein</td>
</tr>
<tr>
<td>MGE</td>
<td>Medial ganglionic eminence</td>
</tr>
<tr>
<td>OLP</td>
<td>Olfactory and lens placode</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RALDH</td>
<td>Retinaldehyde dehydrogenase</td>
</tr>
<tr>
<td>RARE</td>
<td>Retinoic acid responsive element</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>Smad</td>
<td>Small mothers against decapentaplegic</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>V</td>
<td>Ventral</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless/Int</td>
</tr>
<tr>
<td>Xbra</td>
<td>Xenopus Brachyury</td>
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INTRODUCTION

Every day, every second we are influenced by our environment – voices, paintings, perfumes, food and faces. These perceptions form the foundation of our knowledge about the world. Information received from peripheral receptors that sense the environment is analyzed by the brain and translated into components that give rise to perceptions, some of which are stored in memory. For example, chemicals floating in the air can enter the nose and bind to olfactory receptor neurons, a signal is then transferred to the brain and may be recognized as the wonderful smell of a flower.

Our ability to take in, sort out, and associate information from the world around us in an organized manner is what creates the entire image of our lives. To understand how the functional nervous system works, we first have to be aware of how it is assembled during development. This thesis will address the mechanisms of early diversification and regionalisation that are necessary for subsequent formation of the adult nervous system.

Organisation of the Nervous System

The adult central nervous system (CNS) can be divided into six anatomical regions: the spinal cord, the medulla, the pons and cerebellum, the midbrain, the diencephalon and the cerebral hemispheres. The cerebral hemispheres are by far the largest region of the brain. They, in turn, consist of the cerebral cortex, the underlying white matter, the basal ganglia, the hippocampus and the amygdala.

The cerebral cortex is the highly convoluted surface of the cerebral hemispheres. It is the centre of higher cognitive and perceptual functions, which include processing sensory information and integrating cortical output that is important for control of movements.

The basal ganglia are important in control of movement, but they also contribute to cognition. Postmortem examinations in patients with Parkinson’s and Huntington’s disease have revealed pathological changes in
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the basal ganglia. The major components of the basal ganglia are the striatum and the pallidum.

The peripheral nervous system is both responsible for register and transfer of sensory input to the CNS and for execution of the motor commands generated in the brain and spinal cord. The sensory system can be divided into specialised systems (olfaction, vision, touch etc.) and each of these modalities is mediated by a separate system.

The olfactory epithelium consists of 1) olfactory receptor cells, 2) sustentacular or supporting cells and 3) basal cells (stem cells). The olfactory epithelium contains a few million olfactory sensory neurons that project their axons to glomeruli in the olfactory bulb. The olfactory epithelium is unique in the way that it retains stem cell capacity and generates differentiated neurons throughout life.

The visual system is the most complex of all the sensory systems; the optic nerve contains about one million fibres. The crystalline lens is located just behind the iris, and its purpose is to focus light onto the retina. The lens consists of an elastic capsule, a layer of epithelium and lens fibres.

The nervous system is organized along two major axes that are established early in development- the rostral-caudal (head to tail) axis and the dorso-ventral (back to belly). Due to the cephalic flexure that occurs in higher vertebrates, the “top of the skull to jaw” direction will be denoted dorso-ventral.

Development of the Nervous System

A widespread view of cell differentiation is that cells start from a primitive state of differentiation and progressively become more diversified. Cells share less features as development proceeds and finally, the cells are committed to a particular fate, meaning that they are unable to change to another developmental fate. One of the three germ layers, the ectoderm, will generate cells of the central and the peripheral nervous system as well as the epidermis. The first morphological sign of a subdivision of the ectoderm is
the delimitation of the neural plate. The neural plate, containing prospective CNS cells, is first visualised at the gastrula stage where it forms around the node (Fig 1a). The prospective epidermal ectoderm is situated in the more lateral areas of the ectodermal layer (Fig 1c), and cells that later will give rise to the peripheral nervous system are situated at the border of the neural plate and epidermal ectoderm (Fig 1b).

![Fig 1: Schematic Pictures of Stage 4 Chick Embryos. a) The neural plate, b) the prospective peripheral nervous system region and c) the epidermal ectoderm.](image)

Shortly after the neural plate has formed, its edges thicken and move upward to form the neural folds. The neural folds will subsequently move toward the midline of the embryo and eventually fuse to form the neural tube. The caudal part of the neural tube will give rise to the spinal cord, while the rostral part will form three primary vesicles that later on will give rise to the forebrain, midbrain and hindbrain. As development continues, five secondary vesicles will form where the most anterior of the primary vesicles, the forebrain vesicle, will be subdivided into the telencephalic and diencephalic vesicles\textsuperscript{10}.

Fate maps established in a variety of vertebrate species have shown that already at the gastrula stage there is a subdivision where the most anterior region of the neural plate will give rise to forebrain structures while
progressively more posterior regions give rise to the midbrain, hindbrain, and spinal cord\textsuperscript{11,12}.

**Signalling Molecules**

Classical embryological experiments have shown the importance of tissues being able to signal to each other\textsuperscript{10,13-15}. Today there are many known signalling centres where the cells secrete proteins called “inducing factors”\textsuperscript{16}. There are surprisingly few families of signalling molecules that have been found involved in directing cells into specific fates during early development: the transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP), fibroblast growth factor (FGF), the Wingless/Int (Wnt) and the Hedgehog families (HH) (**Table 1**). The small lipophilic molecule retinoic acid (RA) has also been shown to regulate gene expression during development of tissues and organs. RA is produced from retinol in two steps; first it is converted to retinal by the alcohol dehydrogenase (Adh) enzymes and then to RA by the spatially restricted, rate limiting retinaldehyde dehydrogenases (Raldh) enzymes\textsuperscript{17}.

<table>
<thead>
<tr>
<th><strong>Produced by</strong></th>
<th><strong>Receptors</strong></th>
<th><strong>Intracellular effectors</strong></th>
<th><strong>Antagonists</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wnt</strong></td>
<td>Frizzled1-10</td>
<td>β-catenin</td>
<td>Sfrp, Dickkopf</td>
</tr>
<tr>
<td><strong>FGF</strong></td>
<td>FGFR1-4</td>
<td>MAPK, Ras</td>
<td>Sprouty, Pyst1</td>
</tr>
<tr>
<td><strong>HH</strong></td>
<td>Patched, Smoothened</td>
<td>Gli1-3</td>
<td>Gli3</td>
</tr>
<tr>
<td><strong>TGFβ/BMP</strong></td>
<td>BMPRI, BMPRII</td>
<td>SMAD1-5, 8</td>
<td>Chordin, Noggin, Follistatin, Dan SMAD6-7</td>
</tr>
<tr>
<td><strong>Retinoids</strong></td>
<td>RAR, RXR</td>
<td>CRABP</td>
<td>CYP26</td>
</tr>
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</table>

**Table 1: Inducing Factors and Selected Proteins Involved in the Respective Pathway.**
The observation that a small number of signalling pathways regulate a large variety of developmental processes leads to the conclusion that the cellular response to the same signals varies and will depend on the status of the recipient cell. This type of recycling scenario is exemplified in the following section (and Fig2) by the different roles of Wnt signalling in the development of the central nervous system.

At the blastula stage, Wnt signalling inhibits the specification of neural cells by blocking the ability of epiblast cells to respond to FGF signals\textsuperscript{18}. About 20h later, at the gastrula stage, Wnt signalling in combination with FGF inhibits the generation of telencephalic cells and specifies cells of the caudal neural tube\textsuperscript{19,20}. After an additional 10h, at the neurula stage, the telencephalic cells that are exposed to Wnt signals will maintain telencephalic character, and exposure to Wnt signals at this stage promotes the acquisition of a dorsal telencephalic fate (paper I).

Thus, the same signalling pathway can be involved in several steps of the development of a single tissue. However, the intriguing question of how signalling inputs are interpreted to generate cell-type specific patterns of gene expression and response remains.

\textbf{Fig2: Recycling Signalling Molecules.} Cells that are protected from Wnt signalling at the blastula and gastrula stage become telencephalic. However, when these cells are exposed to Wnt at the neurula stage, they respond by acquiring a dorsal telencephalic character.
Combinatorial Signalling of Inducing Signals

In the example above, Wnt and FGF interact in all the three events, but in totally different ways. At blastula stage, Wnt signals oppose FGF activity, while at gastrula stage the same signals converge to caudalize neural cells and finally at the neurula stage, Wnt and FGF acts sequentially to specify dorsal telencephalic cells. Hence, depending on the “maturity” of the cell it will respond differently to the same combination of signals.

Two different mechanisms of interaction between pathways are exemplified in the process of neural induction in the blastula chick embryo. FGF signals act by repressing Bmp mRNA expression and thereby promoting neural fate, while Wnt signals promote epidermal induction by blocking the ability of lateral epiblast cells to respond to the FGF signal. Thus, the interaction between FGF and BMP occurs at a transcriptional level while the FGF-Wnt interaction possibly occurs at a protein level. Smad proteins have been suggested to be a key node for pathway integration. Smads are known mediators of BMP receptor signalling, and they become active by phosphorylation of the carboxy-terminal region. Recently, Pera et al. suggested that MAPK-mediated phosphorylation of the linker region of the Smad protein inhibits the transduction of the BMP signal, possibly by nuclear exclusion of Smad. Accordingly, Smad proteins provide a link between the FGF and the BMP pathways. Thus, signalling protein can interact in different combinations at different levels, giving a few proteins great possibilities to specify a large number of cell-types.

Spatial and Temporal Gradients

More than a century ago, evidence began to accumulate that cells receive “positional information” that instructs them to develop in specific ways depending on their location within a tissue. During embryonic development, signalling molecules act instructively to pattern fields of cells and to govern cell fate decisions. In some cases, these molecules form a concentration gradient as they spread from a localized source into the surrounding tissue, resulting in different concentrations of the signal, the “morphogen”, specifying different cell fates. To be defined as a morphogen, the signal has to be able to generate at least two different cell types at
different concentrations\textsuperscript{28,29}. A classical example is the patterning of the ventral neural tube, where Shh acts as a graded signal inducing five different neuronal subtypes in the spinal cord at different concentration thresholds\textsuperscript{30}.

Sometimes it is difficult to separate a spatial gradient from a temporal gradient. In a study by Gurdon \textit{et al.} low concentration or short duration of Activin both induce Xbra at short range, whereas with increasing time or higher concentration, Xbra was expressed in cells located at a longer distance from the source of the signal\textsuperscript{31}. In another system, time of exposure and morphogen concentration is not equivalent. In development of the digits, it has been shown that digit 3-5 are composed of cells that have been exposed to the same concentrations of Shh, but for differing amounts of time during development. Thus, this suggests that a temporal gradient, and not a spatial gradient, is responsible for specifying distinct digits in the posterior half of the limb\textsuperscript{29,32,33}. In paper III, we suggest that time of exposure is a key feature in the differential specification of olfactory and lens placodes.

\textit{In vivo and In vitro Methods}

Genetic models, primary in mouse and zebrafish, have been of great importance for the understanding of developmental biology. Unfortunately, some animals with mutations in one of the signalling pathways show severe defects early in development making it very difficult to draw any conclusions about the importance of these signals in later developmental events. Redundancy among signals from the same protein family is also a factor that complicates the analysis of mutants. Developing the \textit{in vitro} explant assay gave us the opportunity to isolate pieces of a single germ layer at a specific developmental time point and culture it under controlled conditions. This makes it possible to exclude indirect effects and circumvent early requirements for the signal.
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*Figure 2: The Explant Method.*
a) Ectodermal explants are isolated from other germ layers and cultured under serum free conditions in collagen drops alone or together with inducing or inhibiting factors. b) The explants are fixed, frozen and sectioned. c) Consecutive sections are stained by immunohistochemistry or in situ hybridization and then analyzed.

**Chick as a Model Organism**
The chick is widely used as a model system in developmental studies. It has been demonstrated to have great similarities in expression of genes and the future functional subdivisions with mammals\(^\text{34,35}\), and has technical advantages due to its accessible development. Chick is a well established model used for studying lens and CNS development, while the development of the olfactory epithelium is not as well characterised in this system. Although the morphology of the adult nasal region differs widely between vertebrates, the functional characteristics are comparable. Olfaction is mediated by a large number of olfactory receptors in a wide spectrum of vertebrates, from the lamprey to humans\(^\text{36}\). The early development of the olfactory placode also seems to share great similarities between different species, both morphologically and concerning signalling molecules and transcription factors expressed in the area\(^\text{37}\) (*Fig 3*).
Figure 3: Features of the Olfactory Placode are Conserved between Chick and Mouse.
Upper panel, molecular markers expressed in the chick E3.5 nasal pit, and lower panel, markers expressed in the mouse E10.

Development of the Central Nervous System
The vertebrate neural tube is regionally patterned along both the anterior-posterior and dorsal-ventral axes. The generation of distinct classes of neurons at defined positions is a fundamental step in the development of the vertebrate central nervous system. The early neural plate is already subdivided into a complex grid of molecularly distinct progenitor regions. A unique combination of transcription factors will be expressed over time in each progenitor domain and it is these transcriptional codes that specify distinct regions of the CNS, but secreted signalling molecules determine the combination of transcription factors that will be expressed in a specific region depending on temporal and spatial restrictions of the inducing factors and associated proteins\textsuperscript{38,39}. 

The Spinal Cord
The vertebrate spinal cord is the most caudal part of the CNS, responsible for local reflexes, the relay of sensory information to higher brain centres
and organized movements. It is composed of a variety of cell types including different motor neurons, local interneurons and projection neurons\(^\text{40}\). Experimentally, it presents a well-characterized region of the CNS - the physiology and anatomy of the neurons are well defined and functions have been assigned to many of the identified neurons. Two main signalling pathways, Shh and BMP, have been suggested to pattern the spinal cord into ventral and dorsal domains\(^\text{25,30,41-45}\).

**Ventral Patterning of the Spinal Cord**

Shh signalling emanating from the notochord and floorplate is critical in the development of the spinal cord since embryos lacking *Shh* function fail to generate motor neurons and most ventral interneurons\(^\text{46}\). Together with the piece of evidence that Shh is sufficient to induce ventral interneurons in neural plate explants, this suggests that Shh signalling specify ventral neuronal fates\(^\text{41}\). Shh has been shown to exert its function by inducing class I homeodomain (HD) proteins and blocking class II proteins, thereby creating a variety of progenitor cells in the caudal neural tube. In 2003 Novitch *et al.* showed that Shh is only one of three classes of signalling factors that control the temporal and spatial pattern of class I and II HD protein expression in ventral progenitor cells, with retinoids and FGF signals having crucial accessory roles\(^\text{47}\).

**Dorsal Patterning of the Spinal Cord**

The identity of dorsal neurons depends initially on BMP-mediated signals derived from the epidermal ectoderm that induce dorsal midline cells of the roof plate. Roof plate cells provide a secondary source of TGF\(\beta\)-related signals that are required for the generation of distinct classes of dorsal interneurons\(^\text{44}\). GDF7 and other BMP family members expressed by the roof plate have non-redundant functions *in vivo*, exemplified by the fact that the GDF7-null mutant lacks only the D1A neurons, while the D1B and other dorsal interneurons are unaffected\(^\text{43}\).

Several Wnt proteins are expressed in or adjacent to the dorsal spinal cord, and it has been suggested that Wnt has both a mitogenic and a patterning
function in the spinal cord. Dickinson et al. put forward that ectopic Wnt-1 expression leads to consistent changes in the relative proportions of dorsal and ventral regions of the spinal cord, but that Wnt-1 does not appear to act as a primary patterning signal\textsuperscript{48}. However, Chesnutt et al. looked at more fine tuned markers and found that Isl1 and LH2 neurons were largely absent from the dorsal margin in \textit{Wnt1–/–;Wnt3a–/–} embryos. This observation indicates that the activity of Wnt proteins is required for proper generation of the interneuron subclasses D1 and D2 in the spinal cord\textsuperscript{49}.

**The Telencephalon**

The telencephalon is the most complex region of the adult vertebrate brain, yet it has a relatively simple structure in the embryo. The telencephalon derives from the most anterior part of the developing CNS. It consists of two main subdivisions — the dorsal telencephalon (the pallium) and the ventral telencephalon (the subpallium). The dorsally located pallium will give rise to the cerebral cortex, which is a region of the brain associated with higher cognitive functions, while the subpallium will mainly give rise to the basal ganglia. The subpallium can be further subdivided into two domains in mammals, the more ventrally located medial ganglionic eminence (MGE) and the lateral ganglionic eminence (LGE). MGE progenitors give rise to the basal ganglia called the Pallidum. The main derivative of the LGE progenitors is the Striatum, the input nucleus that together with the other basal ganglia is responsible for the control of movement\textsuperscript{7,50,51}.

**Induction of the Telencephalon**

In 1952 Niewkoop proposed a two-step model for induction of different regions of the nervous system; 1) activation: neural fate is induced and the cells have a forebrain like character, 2) transformation: the neuroectoderm becomes caudalized. This model has been supported in many more recent studies\textsuperscript{52-55}, and a couple of molecules have been suggested to be important in specifying caudal cell types, among them a combination of Wnt and FGF\textsuperscript{19,56}. Mutants in negative regulators of the Wnt signalling pathways in mouse (\textit{dickkopfl}) and zebrafish (\textit{masterblind} and \textit{headless}) all show
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reduced or total lack of the forebrain\textsuperscript{57-59}, showing the importance of inhibiting Wnt signalling for generation of the telencephalon. \textit{Fgf8} expression can be detected in the anterior neural ridge (ANR), the rostral-most portion of the neural plate, close to the forebrain progenitors. Explant studies have shown that ANR transplantation and \textit{Fgf8} beads can induce FoxG1, suggesting that FGF8 plays an inductive role in forebrain development\textsuperscript{60}. However, both the mouse and zebrafish \textit{Fgf8}-mutants with reduced FGF8 function generate a telencephalon, although it is hypoplastic and displays morphological defects in the midline region\textsuperscript{61-63}.

**Dorsoventral Patterning of the Telencephalon**

Members from all the previously mentioned families of signalling molecules are expressed in, or in the vicinity of, the developing forebrain\textsuperscript{64-67}. Genetic evidence based on loss-of-function and gain-of-function studies indicates that they are involved in regional specification of the telencephalon. The regionalization of the telencephalon is accompanied by expression of region-specific codes of transcription factors, which in turn regulate neuronal development\textsuperscript{68}. Mechanisms of patterning similar to those in the spinal cord have started to emerge also in the telencephalon, although less is known about this region.

**Ventral Telencephalon**

Shh signalling plays a key role in ventral patterning throughout rostro-caudal levels of the nervous system. Shh is believed to act through the inhibition of Gli3 repressor activity. Even though the \textit{Shh-/-} mutant lacks the ventral telencephalon\textsuperscript{46}, the double knockout mice \textit{Shh-/-Gli3-/-} show an almost normal telencephalic phenotype\textsuperscript{69}.

Prospective telencephalic cells isolated from chick embryos at gastrula stage acquire a ventral character after culture, which can be blocked by inhibiting Shh\textsuperscript{1}. This indicates that all telencephalic cells are initially specified as ventral and thereafter a subpopulation acquires a more dorsal character. However, since the \textit{Shh-/-} mouse still has a dorsal telencephalic structure there is no absolute requirement for the cells to first be specified as ventral.
Moreover, since the double knockouts Shh-/-Gli3-/- generate a telencephalon with both dorsal and ventral structures\textsuperscript{69}, Shh seems to have an important regulatory role, but is perhaps dispensable for DV patterning in this region.

**Dorsal Telencephalon**

The major part of the telencephalon that is positioned dorsally and laterally, the part that further on will be referred to as the dorsal telencephalon (Fig 4a), will give rise to the cerebral cortex. The cells generated from the more medial part (Fig 4b) will differentiate into the non-neural, cerebrospinal fluid secreting structure: the choroid plexus\textsuperscript{6}. Several transcription factors have been reported to have an important role in the maintenance and patterning of the dorsal telencephalon, namely Gli3, Pax6, Lhx2, Ngn1/2 and Emx2\textsuperscript{70-75}. Less is known, however, about the signalling molecules that induce the dorsal telencephalon.

![Figure 4: Schematic Picture of a Chick E3.5 Telencephalon.](image)

\textit{a)} The progenitor cells positioned dorsal-laterally will give rise to the cerebral cortex and \textit{b)} the dorsal-medial part will give rise to the choroid plexus.

Analogous to the spinal cord, members of the BMP family (Bmp2, 4, 5, 6 and 7) are expressed in proximity to the prospective dorsal telencephalon but in contrast, it has been difficult to show any specific role for BMP signalling in the specification of cerebral cortex progenitor cells. Analysis has been difficult since mice mutants (e.g BMP4-/- and BMPR1a-/-) show severe early phenotypes and die at gastrulation, or do not show any apparent CNS deficiency (BMPR1b-/-). To circumvent this problem Hébert et al. constructed a conditional knockout mouse, where the Bmpr1a gene was disrupted throughout the telencephalon from its earliest stages of development\textsuperscript{6}. These mutants show normal dorsoventral patterning of the
telencephalon, except from a morphologically defective midline structure and a great reduction of the early choroid plexus marker transthyretin (Trt). A BMP4 conditional knockout was created using the same system, however, these mice develop without any apparent telencephalic phenotype. Thus, BMP signalling seems to be dispensable for specification of the dorsal telencephalon, except perhaps the midline structures.

Another strong candidate for patterning the telencephalon is the Wnt family of genes. Expression of Wnt genes can be detected in the medial margin of the telencephalon in a variety of species. For example, Wnt2b, 3a, 5a and 7a, are strongly expressed in mouse E12.5 cortical hem. The telencephalic deficiencies in the Wnt3a and Lef1 mutants, which lack the hippocampus, indicates that Wnt signalling plays an important role in the patterning of the telencephalon.

Intermediate Telencephalon

The region between the ventral and dorsal part of the telencephalon is characterised in mammals by a ventricular bulge that is called the lateral ganglionic eminence (LGE). In mammals the LGE is a clear morphological, visible structure that gives rise to the striatum, the interneurons of the olfactory bulb and the amygdala. Even though the embryological LGE structure is not as prominent in chick, the pattern of transcription factors expressed and its prescribed functions seems to be largely conserved among vertebrates.

Experiments in mouse by Toresson et al. have shown that the homeobox gene Meis2 is expressed in differentiated neurons in the striatal region as well as in the precursor cells in early stages, and that glial cells in this area are a local source of retinoids. This study also demonstrated that local retinoid signalling within the LGE regulates striatal neuron differentiation. However, the question of retinoid involvement in the specification of the early prestriatal/intermediate cell types remains to be answered.
**Cell Proliferation and Differentiation**

In combination with specification of cell fates, regulation of cell proliferation is essential for the proper development of the telencephalon and its derivatives\(^{85}\). Some of the signalling molecules and transcription factors involved in the patterning of the telencephalon are also involved in regulating the size of this structure\(^{85}\). Knockout mice that lack the transcription factors Pax6 or Foxg1 (BF-1) show abnormal cell cycle duration in dorsal precursor cells\(^{86,87}\). Estivill-Torrus *et al.* provide evidence that Pax6 is required in cortical progenitors to control cell-cycle duration and the onset of expression of neural-specific markers. Other transcription factors that may be involved in regulation of cell proliferation and differentiation are Lhx2 and Emx2. Mice in which either of these genes is mutated have reduced telencephalic (e.g., hippocampal) regions\(^{71,88}\). FGFs and BMPs have been suggested to promote proliferation of the telencephalic vesicles early in development\(^{89}\), and BMP signals trigger neuronal differentiation of neocortical precursors within the ventricular zone at later stages\(^{90}\).

**Development of the Peripheral Nervous System**

The sensory nervous system in the vertebrate head arises from two different cell populations: neural crest and placodal cells, while in the trunk it originates from the neural crest only. All cranial sensory ganglia receive contributions from both cell populations, whereas crucial components of the special sense organs – the eye, ear, nasal epithelium and lateral line – are largely derived from sensory placodes\(^{91}\). Placode and neural crest cell populations have many features in common; i) they arise from the border of the neural plate and epidermis, ii) both have the ability to give rise to different cell types, including glia, neurons and secretory cells; and iii) development of both cell populations involves changes in cell shape. Although these two cell populations both arise from areas near the border of the neural plate and develop similarly in several respects, where neural crest has attracted much attention and been intensively studied, placodal development has long been neglected.
INTRODUCTION

Placodal Primordium

The ectodermal, horseshoe-shaped region lateral to the neural plate has been denoted placodal primordium or preplacodal region. Fate maps established in a variety of species have shown that all placodes originate from this region\textsuperscript{92,93}. The placodal region is at the head-fold and neurula stage characterised by expression of genes such as members from the \textit{Six}, \textit{Dlx} and \textit{Eya} families, although the adjacent epidermal ectoderm also expresses some of these factors\textsuperscript{94,95} (and paper III). It is still an unresolved question whether particular placodes are specified individually or whether all placodes originate from a common placodal primordium. The transcription factors that are expressed in the placodal field continue to be expressed in most of the placodes and subsequently their sensory organ. Mutations in these genes have been made in a variety of species to evaluate their importance in placodal development. The drosophila \textit{Eya1-/-} mutants do not develop any eyes, but there have been no reports on eye deficiency in the corresponding mouse mutants. However, there is one study suggesting that mutations in the \textit{Eya1} gene in humans can cause congenital cataract\textsuperscript{96}. No apparent phenotype was detected in the \textit{Six4}-deficient mice\textsuperscript{97}. \textit{Six1}-deficient mice generate an olfactory placode but the olfactory cavity is severely disorganized in E18.5 \textit{Six1-/-} embryos\textsuperscript{98}. Both single and double knockouts of the \textit{Six1} and \textit{Eya1} genes show that these genes are not required for the induction of the otic placode, but there is a striking degree of disorganisation at the inner ear later on in these mutants\textsuperscript{98-101}. \textit{Dlx5-/-} mutants generate an olfactory placode but have a hypoplastic olfactory epithelium that fails to form normal axonal connections with the olfactory bulb\textsuperscript{102}.

Taken together, “preplacodal genes” do not seem to be important for the initial specification of the placodes, but rather for size regulation, cell shape changes and neurogenesis at later stages\textsuperscript{103}. However, the question of a common placodal primordium, from which all placodes derive, remains unanswered.
INTRODUCTION

Signalling Molecules Involved in the Induction of the Placodes

Xhip protein has been described to inhibit Hedgehog signalling, but is also suggested to interfere with the Wnt and FGF pathways. Loss of Xhip function results in a suppression of olfactory and lens placode formation. A tight regulation of these pathways is important for placode induction, but signalling through them might not be required.

BMP2/4 homologues appear to have a very ancient role in distinguishing neural from non-neural ectoderm. In Drosophila, amphioxus and vertebrates, BMP2/4 homologues are expressed in non-neural ectoderm and function in distinguishing neural from non-neural ectoderm. Several studies in amphibians and fish, suggest that a BMP gradient specifies different ectodermal fates, where intermediate levels of BMP signals induce placodal cells. Other studies, in chick and Xenopus, argue that FGF signalling is important for induction of the placodes, but that inhibition of BMP is also required. In paper III, we suggest that BMP induces placode character, but that FGF signalling is also necessary, mainly to prevent the cells from acquiring an epidermal fate. The expression pattern of BMP2 and BMP4 in the same horseshoe-shaped region from where the placodes arise, indicates that BMP signalling is involved in the generation of the placodes.

There is also data from genetic studies in zebrafish and mouse that support the importance of BMP signalling in placodal development. No otic or olfactory placode was in zebrafish Bmp2 (swirl) mutant embryos. In advanced Bmp4tm1 homozygous mouse mutant embryos, neither lens placode nor expression of Sox2 is induced in the prospective lens ectoderm, although the nasal placode is formed. Similarities in expression pattern and homology between BMP2 and BMP4 can result in a milder phenotype than might be expected in these mutants, due to functional redundancy. In the mouse BMP2 mutant the proamniotic canal fails to close and heart development proceeds abnormally, which precludes the analysis of the role of BMP2 in ectodermal development. Thus, BMP seems to be important for the generation of the placodes, although the mechanism behind is difficult to evaluate in genetic studies, due to redundancy and the fact that...
the same genes are essential for generation of other structures and tissues (mesoderm for example) very early in development.

**Development of the Lens**

The lens develops in close relationship with the retinal primordium. A classic experiment by Spemann and Lewis was performed in the beginning of the last century, suggesting that signals from the presumptive retinal region are required and sufficient for the lens to form in frogs\textsuperscript{13,14}. However, studies reported shortly after this, and also more recent studies suggest, that the lens is able to form in the absence of the optic cup, and that ectopically formed lenses in transplantation studies derive from contaminating donor cells\textsuperscript{115,116}. Lens development is today believed to involve several steps, where the signals from the optic vesicle act late in this process, maybe to “fully activate” the genetic program involved in lens development, while the induction of lens character occurs earlier, before the formation of the optic vesicle\textsuperscript{117}. There are several conserved transcription factors, for example Pax6, Sox-1, -2, -3, Six3 and Maf, that are important for lens formation. Three large Maf family proteins, L-Maf, c-Maf, and MafB, are important for lens development. L-Maf is expressed first and induces $\delta$-crystallin most efficiently\textsuperscript{118}. L-Maf expression is initiated in the thickened lens placode and binds and transactivates expression of crystallin proteins\textsuperscript{117}. The crystalline proteins are a group of structural proteins that confer transparency and refractivity to the lens, $\alpha$- and $\beta$- crystallins are conserved throughout the vertebrates while $\delta$-crystallins are only found in reptiles and birds\textsuperscript{117}.

FGF signalling has been implicated in lens development, since all four FGFR genes are expressed in the developing lens and at least 13 of the FGF genes are expressed in the eye\textsuperscript{119}. By generating mouse mutants, today, no individual FGF ligand or receptor have proven to be essential for lens development, although, given the large numbers of ligands and receptors expressed in this area, there might be functional redundancy among these genes\textsuperscript{119}. However, *in vitro* studies in chick and rat suggest that FGF signals are not involved in induction of the lens placode, but might be responsible for the polarity of the lens and stimulate proliferation of epithelial cells\textsuperscript{119}.
Since both BMP4 and BMP7 are expressed in the presumptive lens ectoderm and both the BMP4-/- and BMP7-/- knockout mice fail to form a lens placode, BMP signalling is a strong candidate for inducing the lens113,120.

Development of the Olfactory Epithelium

The olfactory placode develops in close proximity to the forebrain, and expression of Emx2, Pax6, Meis2, Otx2, FoxG1 and Sox2 is common to both regions103. However, at present there are no convincing data that the forebrain has any inductive effect on the olfactory placode, although it has been suggested that the interaction of olfactory sensory neurons with the presumptive forebrain is important for the development of the olfactory bulb121,122. In lower vertebrates, mesoderm has been shown to have an inductive capacity, while this does not seem to be the case in higher vertebrates91. Although BMPs, Wnts, Retinoids and FGFs are all mentioned in discussions concerning the induction of the olfactory placode, today there is no strong candidate.

Differential Specification of the Olfactory and Lens Placode

The lens and olfactory placode precursors are initially intermingled and show a similar pattern of expression of transcription factors during development93,103,123. Oct-1, Sox2, and Pax6 are examples of transcription factors that are co-expressed in and important for both the lens and nasal placode induction124. At neurula stage the placodal cells have segregated and are located at two different positions in the embryo. Dlx and Pax6 are initially co-expressed in the olfactory–lens placode region, but after segregation Dlx5 is lost from the lens and Pax6 expression is transiently down-regulated in nasal precursors92. Down-regulation of these transcription factors seems to be required for the placodal cells to acquire their respective characters, but occurs to late too explain the segregation.

Bailey et al. suggests that the entire preplacodal region is initially specified as lens tissue125. They also suggest that FGF signalling imparts olfactory character in a subpopulation of these cells, although, Bailey et al. never
show that FGF signals are able to induce switching from one fate to the other. The study only shows that FGF induces olfactory character in prospective olfactory explants, not ectopically in lens explants, and conversely, that blocking FGF in prospective olfactory explants only inhibits the olfactory markers but does not ectopically induce lens markers. So, there is still an open question whether FGF signals control placode segregation or if the specification of the lens and olfactory placodes is regulated by any of the other previously discussed signalling pathways.
AIMS

The aim of this thesis was to understand when and how cells in the anterior region of the early embryo that will give rise to structures of the central and peripheral nervous system acquire their initial regional identity.

Specific aims were:

- To determine when telencephalic progenitor cells acquire intermediate and dorsal character.
- To determine which signalling molecules are involved in the specification of the intermediate and dorsal telencephalon, and the origin of these signals.
- To determine when the olfactory and lens placodes are induced and what signalling mechanisms are involved in this initial induction.
- To determine the molecular mechanism of the differential specification of the olfactory and lens placodes.
RESULTS

**Specification of Dorsal Telencephalic Character by Sequential Wnt and FGF Signalling (paper I)**

The telencephalon is regionalized along the dorsoventral axis into different progenitor domains that will give rise to neurons in specific parts of the adult brain. To define the positional character of these cells we analyzed region-specific transcription factors. In a stage 15 chick embryo we could detect Pax6\(^+\) cells and a few Ngn2\(^+\) cells in the dorsal region, while in a stage 22 embryo we detected cells that co-expressed Pax6, Ngn2 and also Emx1. Thus, at stage 15 the dorsal telencephalic cells have started to acquire a dorsal character, and at stage 22 the cells have reached a definitive dorsal Pax6\(^+\)/Ngn2\(^+\)/Emx1\(^+\) state.

![Diagram of telencephalon with expression patterns](image)

*Figure 5: Expression of Transcription Factors in an E3.5 Chick Embryo.*

To analyze when the cells acquire their dorsal character, from which tissue the inducing signals derive and the molecular nature of these signals, we mainly used an *in vitro* culturing assay (*Fig 2*). As an *in vivo* complement to this *in vitro* method, we have also used the New Culture method.
Early prospective telencephalic cells are specified as ventral cells at stage 4-6, but at stage 8-10 two different telencephalic populations have been specified. Fate maps have shown that cells that are fated to generate the dorsal telencephalon are located in a posterior position of the telencephalic region (Fig 6) while the ventral telencephalic progenitor cells arise from a more anterior position in stage 8 and 10 chick embryos.

**Figure 6: Telencephalic Progenitor Cells are Located at Different Positions Along the Rostrocaudal Axis of the Anterior Prosencephalon.** Light grey- ventral, dark grey- dorsal and grey – intermediate telencephalon.

**Telencephalic Cells Acquire their Dorsal Character Gradually**

Explants isolated from the prospective dorsal telencephalic region of stage 8 embryos generated Pax6+ and Ngn2+ cells, but no Emx1+ cells. Dorsal explants from a stage 10 embryo generated in addition some Emx1+ cells after culture, and in dorsal explants from a stage 15 embryo most cells expressed Pax6, Ngn2 and Emx1. Thus, at stage 8 the dorsal cells have acquired an early dorsal character, and at stage 15 the cells are specified to express a profile of transcription factors characteristic of a definitive dorsal telencephalic identity.

**Signals from the Epidermal Ectoderm Induce Dorsal Telencephalic Cells**

The signals that induce dorsal character must be present in the region between stage 8 and 10, and an apparent candidate tissue is the adjacent
neural fold. Non-neural ectoderm induces dorsal cell fates in the spinal cord, so to test if non-neural ectoderm could induce dorsal telencephalic cell fate we cultured dorsal stage 8 explants together with the flanking neural fold region. These explants generated cells expressing Pax6, Ngn2 and Emx1—definitive dorsal telencephalic character. To further test this we cultured chick dorsal neural fold together with quail ventral telencephalic explants. In these co-cultured explants the expression of the ventral marker Nkx2.1 was blocked and the cells started to express Pax6, Ngn2 and Emx1. Conversely, quail dorsal neural fold together with chick ventral explants, also generated cells expressing dorsal markers (data not shown). Together these results suggest that dorsal telencephalic character is generated by a re-specification of cells of intrinsic ventral character by signals from the non-neural ectoderm.

**Wnt Signals Induce Early Dorsal Character**

The indication that the signal that induces dorsal telencephalic character originates from the non-neural ectoderm raises the question about the molecular nature of this signal(s). Wnt1 and Wnt4 are expressed in the neural folds at stage 8 and at stage 10 Wnt8b is expressed in the neural ectoderm giving rise to the dorsal telencephalon\(^{126}\).

To test if Wnt signalling is required for the generation of dorsal telencephalic cells, we exposed both explants and whole embryos to the Wnt inhibitor, soluble mouse frizzled receptor 8 protein (mFrz8). Stage 8 dorsal telencephalic neural fold explants cultured with the Wnt inhibitor did not generate Pax6\(^+\), Ngn2\(^+\) and Emx1\(^+\) cells, instead the cells started to express the ventral marker Nkx2.1. In whole embryos cultured with the Wnt inhibitor, the expression of Pax6 and Emx1 was lost and in some embryos the expression of Nkx2.1 was expanded dorsally.

To test whether Wnt signalling through the canonical pathway is sufficient to induce dorsal cells, we exposed stage 8 ventral explants to soluble Wnt3A. Exposure to Wnt3A generated dorsal telencephalic cells in a concentration dependent manner. In the presence of 30ng/ml of Wnt3A, the expression of Nkx2.1 was blocked and most of the cells expressed the dorsal
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cell markers Pax6, Ngn2 and Emx1. This was confirmed in vivo by implanting beads soaked in control conditioned media or conditioned media containing Wnt3A close to the prospective ventral forebrain in stage 9 embryos. The embryos were then allowed to develop to stage 20-22. While the control embryos showed normal morphology and dorsoventral patterning of the telencephalon, the embryos exposed to Wnt signals no longer expressed Nkx2.1. Some embryos (2/5) also ectopically expressed Pax6 in the ventral cells, and Emx1 expression was shifted more ventrally.

Together this suggests that Wnt signals are both required and sufficient for the generation of dorsal telencephalic progenitor cells that later will give rise to the cerebral cortex.

Sequential Wnt and FGF Induce Definitive Dorsal Character

Stage 8 ventral explants exposed to Wnt signals generate cells expressing Pax6, Ngn2 and Emx1. In contrast when stage 8 dorsal explants are exposed to Wnt, the number of Pax6+ cells is increased but no Emx1+ cells are generated. This implicates that prospective ventral but not dorsal cells at this stage are exposed to a signal that is able to promote the generation of Emx1+ cells.

The expression pattern of Fgf8 makes it a promising candidate. At stage 8, Fgf8 is expressed in the anterior neural ridge close to the prospective ventral forebrain, while at stage 10 the expression has extended to the non-neural ectoderm close to the prospective dorsal telencephalic cells. To test whether Emx1+ cells require FGF signalling, we exposed the stage 8 dorsal neural fold explants, which normally express Pax6, Ngn2 and Emx1, to the FGF signalling inhibitor SU5402. The generation of Emx1+ cells was blocked in stage 8 dorsal neural fold explants exposed to SU5402, but Pax6+ and Ngn2+ cells were still generated. In whole embryos grafted with SU5204 beads in the prospective dorsal telencephalon, the number of Emx1+ cells was severely reduced, while the number of Pax6+ and Nkx2.1+ cells was normal. Thus, FGF seems to be required for the generation of definitive dorsal Emx1+ cells.
RESULTS

To test if FGF alone is sufficient to induce definitive dorsal character, we exposed stage 8 dorsal explants to FGF8, but this did not induce Emx1+ cells. However, when we added FGF8 in combination with Wnt3A to the dorsal explants, the cells started to express Emx1 in addition to Pax6 and Ngn2. Together these results suggest that dorsal telencephalic cells are generated by sequential Wnt and FGF signalling.

Retinoic Acid Signalling Specifies Intermediate Character in the Developing Telencephalon (paper II)

The findings that SHH is required for the ventral telencephalon and sequential Wnt and FGF for the generation of the dorsal region give rise to the question of how the intermediate region is specified. At stage 14, but not earlier, we were able to isolate a region expressing exclusively the intermediate markers Meis2 and Pax6 (Fig 5). Thus, the domain of telencephalic cells of intermediate character is established later than the ventral and dorsal cell-types.

Retinoic Acid is Required for the Induction of Intermediate Cells

RA has been suggested to be involved in patterning of neuronal progenitor cells in diverse areas. In the spinal cord, RA is required for the specification of the intermediate region. Meis2 was originally identified as a retinoid-inducible gene in P19 carcinoma cells127, and in addition the retinoic acid synthetic enzyme gene Raldh3 is expressed in the olfactory placode located adjacent to the developing forebrain128. Thus, telencephalic cells seem to be exposed to RA at the time point when they become specified as intermediate telencephalic cells.

To examine whether the specification of intermediate cells require RA signalling, we isolated stage 14 intermediate explants and blocked RA signalling using the RAR antagonist BMS-1895453 (BMS453). Under these conditions the generation of Meis2+ cells was blocked, Pax6+ cells reduced and few Nkx2.1+ cells were generated.
To confirm this, beads soaked in BMS453 were placed unilaterally adjacent to the neuroepithelium in the prospective intermediate region in intact embryos. The embryos were cultured to stage 20 and analyzed for the expression of *Meis2*. *Meis2* mRNA is widely expressed in the intermediate region at this stage compared to the Meis2 protein that is only expressed in a few cells. After culture to stage 20, the expression of *Meis2* was normal on the control side while in the cells that had been exposed to the RA antagonist, *Meis2* was severely reduced or absent. Thus, RA signalling is required for the specification of cells of intermediate character.

**RA Induces Cells of Intermediate Character**

At stage 8, the expression of *Raldh3* has not been initiated, and thus, the telencephalic cells have perhaps not been exposed to RA signals. When we exposed dorsal and ventral telencephalic explants to RA, cells expressing Meis2, Pax6 and Isl1 were generated. In stage 8 dorsal telencephalic explant exposed to RA, one region of cells expressing Meis2 and Pax6 and a separate region of cells expressing Meis2 and Isl1 was generated, similar to the expression pattern in the dorsal and ventral parts of the intermediate domain *in vivo*. The induction of intermediate cells was less efficient in stage 8 ventral explant exposed to RA, although both Meis2⁺ and Pax6⁺ cells were generated.

Next we implanted beads soaked in RA adjacent to the dorsal neuroepithelium of stage 10 embryos. In control embryos Pax6, Nkx2.1 and Emx1 were expressed and only a few intermediate cells expressed Meis2 protein. In contrast, in all embryos grafted with RA beads, Meis2 was expressed throughout the dorsal and intermediate telencephalon and Emx1 was blocked. Collectively, this suggests that retinoic acid signalling induces intermediate character in telencephalic cells.

**FGF Signals Maintain Cells of Ventral Character by Blocking RA**

Cells in the ventral telencephalon maintain their ventral character despite apparent exposure to RA, as suggested by the pattern of expression of
RESULTS

*Raldh3*. In our previous experiments, inducing cells of intermediate character was much more efficient in dorsal compared to ventral explants; this indicates that ventral cells are or have been exposed to a factor that makes them less competent to respond to RA. A mechanism where RA and FGF oppose each other has been suggested in the spinal cord\(^4^7\), and *Fgf8* is expressed in the prospective ventral telencephalon\(^5\). To test if this mechanism is also active in the anterior part of the neural tube, we exposed stage 10 ventral explants to soluble FGFR4 to block FGF signalling. Blocking FGF in ventral explants induced Pax6\(^+\) and Meis2\(^+\) cells and the expression of Nkx2.1 was blocked. Adding a combination of RA and FGF to stage 8 dorsal explants did not lead to any induction of Meis2 and Isl1 in contrast to the effect of addition of RA alone.

Next we used intact embryos and implanted beads soaked in soluble FGFR4 adjacent to the prospective ventral telencephalon. In grafted embryos, cells did not express Nkx2.1, and in 4/10 embryos Meis2 was ectopically expressed in the ventral region.

Collectively, these results provide evidence that FGF signalling maintains cells of ventral character and suppresses intermediate character by inhibiting the cell’s response to RA signalling.
RESULTS

Figure 7: Model of the Initial Dorsoventral Patterning of the Telencephalon. At gastrula stages, most or all prospective telencephalic cells become specified as ventral Nkx2.1+ cells in response to node-derived Shh signals. Wnt signals derived from adjacent dorsal ectoderm induce early dorsal Pax6+ cells. RA promotes the generation of intermediate Meis2+ and Meis2+/Pax6+ cells. FGF signals derived from the anterior neural ridge maintains ventral character by opposing RA signaling in ventral cells, and FGF signals derived from the dorsal midline region induce definitive dorsal Pax6+/Emx1+ cells. The specification of the most dorsal midline cells of the telencephalon appears to require BMP signalling, not indicated in this model.

Time of Exposure to BMP Signals Plays a Key Role in the Specification of the Olfactory and Lens Placodes (paper III)

The olfactory epithelium and lens are ectodermal structures of the vertebrate head, which develop in close proximity to the telencephalon and optic regions. To find out when and how these placodes are induced and differentially specified, we used the explant assay (Fig 2). Fate maps in chick have shown that placodal progenitors are intermingled at the neural plate and epidermal border at the late gastrula stage (stage 4), but that the olfactory and lens progenitors are spatially separated at the neural fold stage (stage 8). We isolated explants from stage 3, 4 and 6 embryos and analyzed them at a time-point corresponding to a stage 17 (E2.5) embryo.
RESULTS

when the olfactory epithelium and lens are distinct morphological structures in vivo and express different molecular markers (Fig 8). Explant from the anterior border of the olfactory and lens placodal region were denoted OLP.

Explant from the border region of a stage 3 embryo did not generate any placodal markers after culture, however stage 3 border explants cultured in the presence of the Wnt inhibitor mFrz8CRD generated both olfactory and lens placodal markers in distinct domains (Suppl. Fig 1). In contrast, stage 4 and 6 OLP explants generated Raldh3+, HuC/D+ and Keratin+ cells in a distinct domain and δ-crystallin+ and Keratin+ cells in a separate, non-overlapping domain of the explant. Separate stage 8 lens explants (LP) generated δ-crystallin+, L-Maf+, and Keratin+ cells characteristic of the lens placode, whereas stage 8 olfactory explants (OP) generated Raldh3+, HuC/D+, Dlx+ and Keratin+ cells characteristic of the olfactory placode. Thus, consistent with fate maps, the olfactory and lens placodal cells are specified at stage 4, but spatially separated at stage 8.

BMP Activity is Important for Induction of Olfactory and Lens Placodes

BMPs represent one class of secreted signals that play important roles during patterning of embryonic ectoderm. At gastrula stages, we can detect
both Bmp2 and Bmp4 \textsuperscript{111} and also pSmad-1 - an indicator of ongoing BMP signalling\textsuperscript{129}, at the anterior border region. To test if BMP signalling is required for the initial induction of placodal cells, we blocked BMP signals in stage 4 OLP explants. Under these conditions both the olfactory and lens placodal markers were blocked and the cells acquired a neural forebrain character. To find out if BMP signals are sufficient to induce placodal cells, we isolated stage 4 forebrain (FB) explants and cultured those in the presence of BMP4, these cells acquired a placodal character, expressing Raldh3, HuC/D and Keratin in a distinct domain and δ-crystallin and Keratin in a separate, non-overlapping domain of the explant. Different concentrations (10-50ng/ml) of BMP4 did not generate any significant difference in the numbers of cells expressing olfactory compared to lens markers. Although, when forebrain cells were exposed to higher concentrations than 50ng/ml only Keratin\textsuperscript{+} cells were generated, characteristic of epidermal ectoderm, in agreement with previous results\textsuperscript{26,130}. Thus, BMP signals are required and sufficient to induce olfactory and lens placodal cells, and different concentrations do not affect the differential specification.

**BMP Signals Induce a Pool of Placodal Progenitor Cells**

Cells that will differentiate into placodal cells express transcription factors from the Six and Dlx families\textsuperscript{94,103,131,132}. In a stage 8 chick embryo, prospective placodal cells express Six1 and Dlx5\textsuperscript{94,95}. To test if isolated neural cells exposed to BMP undergo this developmental step we cultured explants from stage 4 to the equivalent of a stage 8 embryo. In agreement with the placodal \textit{in vivo} situation, FB explant cultured in the presence of BMP4 generated Six1\textsuperscript{+} and Dlx5\textsuperscript{+} cells (Suppl. Fig 2). Moreover, in OLP explants, Noggin blocked the expression of these genes (Suppl. Fig 2). Thus, BMP signals are required and sufficient to induce a pool of progenitor cells that later differentiate into olfactory and lens placodal cells.
Time of Exposure to BMP Signals Mediates the Differential Specification of Olfactory and Lens Placodal Cells

*Bmp4* and pSmad1/5/8 are preferentially expressed in the prospective lens ectoderm, compared to the prospective olfactory placodal region. Hence, if concentrations of BMP signals do not have an effect on the differential specification, could it be the time of exposure to BMP signals that is important? We cultured stage 4 OLP explants for 12-15h alone and then either added Noggin to block BMP signalling or BMP4 protein to induce signalling, and continued the culture to the equivalent of an E2.5 chick embryo. Under these conditions Noggin blocked the generation of δ-crystallin+ cells and most of the cells expressed Raldh3, HuC/D and Keratin. Addition of BMP blocked olfactory placodal cells and most cells acquired a lens character. As a complement, we did the analogous experiment and cultured stage 4 FB explants in the presence of BMP4 (35ng/ml) for 12-15h, then added Noggin or additional BMP4 and continued the culture. Inhibition of BMP signalling after 12-15h generated cells that expressed olfactory placodal markers, while addition of BMP4 generated cells of lens placodal character.

To further provide evidence that it is the time of exposure and not the concentration of BMP that plays a key role in the differential specification of the olfactory and lens placodes, we cultured FB explants in the presence of high BMP (100ng/ml) (Suppl. Fig 3). To avoid the generation of epidermal cells, we also added mFrz8CRD to block Wnt signalling. Under these conditions the generation of epidermal cells was blocked and δ-crystallin+ lens cells, but no Raldh3+ or HuC/D+ olfactory placodal cells were generated. To exclude the possibility that the generation of olfactory placodal cells requires Wnt activity, stage 4 OLP explants were cultured in the presence of mFrz8CRD, but under these conditions cells characteristic of both the olfactory placode and lens placode were still generated.

To examine short-term exposure to high levels of BMP we exposed stage 4 FB explants to BMP4 (100ng/ml) and mFrz8CRD for 15h, then added Noggin to block further BMP activity for the additional 27-29h of culture (Suppl. Fig 3). Under these conditions, Raldh3+, Keratin+ and HuC/D+ olfactory placodal cells, but no lens cells were generated. Thus, short-term
exposure to high levels of BMP signals induces olfactory placodal but no lens cells, whereas prolonged exposure to high levels of BMP signals generates lens at the expense of olfactory placodal cells.

Thus, taken together these results show that a temporal gradient of BMP signals mediates the differential specification of olfactory and lens placodal cells, and that continued exposure to BMP signals promotes the generation of lens cells while inhibiting olfactory placodal cells.

BMP Signals Induce Lens Cells at the Expense of Olfactory Placodal Cells at the Neural Fold Stage
To further examine the differential specification we isolated olfactory and lens placodal explants from the neural fold stage, when the cells have migrated away and are spatially separated from each other. In these experiments we, in addition, used an antibody detecting proteins from the Dlx family and an in situ probe detecting L-Maf. The homeodomain box genes Dlx3 and Dlx5 are expressed in the olfactory epithelium in chick and L-Maf is a transcription factor expressed in the lens. To test if BMP activity beyond stage 8 affects the differential specification of placodal cells, we isolated stage 8 OP and LP explants and cultured them in the presence of Noggin or BMP4 to the equivalent of an E2.5 embryo. In LP explants, Noggin blocked the generation of L-Maf+ and δ-crystallin+ cells, and most cells expressed Raldh3, HuC/D, Dlx and Keratin, characteristic of the olfactory placode. In OP explants the cells still expressed olfactory placodal markers when cultured with Noggin, but in the presence of BMP4 the expression of olfactory markers was blocked and the cells expressed lens placodal markers. Together these results suggest that by stage 8 the olfactory placode is independent of BMP, while the lens placode requires continued exposure to BMP signals.

Lens Cells Require Continued Exposure to BMP Signals Beyond Stage 8 in Vivo
Next we used an in vivo assay: electroporation and culture in ovo (Fig 9), to test the differential requirement for BMP signals in whole embryos. We
RESULTS

used stage 8 chick embryos and transfected vectors into the anterior neural fold region on one side of the embryo using electroporation. We transferred a control vector, a Noggin vector\(^2\), or a constitutively active ALK6 (BMPR1b) vector\(^3\). Co-injection of a Green fluorescent protein vector (pCaggs-GFP)\(^{136}\) was used to monitor the transfection efficiency and the embryos were allowed to develop to approximately E2.5.

Control embryos (n=10) showed a normal morphology and normal expression of molecular markers. In contrast, all Noggin transfected embryos (n = 11) lacked a lens on the electroporated side and no δ-crystallin expression could be detected, while the olfactory placode appeared to develop normally (Suppl. Fig 4). In embryos electroporated with the ALK6 vector (n=4) no Raldh3 expression could be detected and the expression of Dlx was reduced (Suppl. Fig 5). However, the lens placode appeared normal in ALK6 electroporated embryos. Thus, these results confirm that continued exposure to BMP signals is required for development of the lens, while the olfactory placodal character is inhibited by BMP signals at the neural fold stage.

**Figure 9: The Electroporation Method.**

DNA encoding a) Noggin or b) caALK6 was cloned into the Beta-actin based vector pMiwIII\(^{23}\). c) DNA (0.5-1μg/μl) was transferred into the prospective placodal region by the use of a Electro Square Porator ECM 830.
Figure 10: Model of the Specification of Lens and Olfactory Placodal Cells. At the gastrula stage, cells exposed to an intermediate level of BMP are specified as placodal progenitor cells. Cells that are exposed to high levels of BMP are specified as epidermal ectoderm. The placodal cells that continue to be exposed to BMP are specified as lens cells while olfactory placodal character is inhibited by continued BMP signalling.
DISCUSSION

In summary, we provide evidence that dorsal and intermediate telencephalic cells are re-specified from cells with an intrinsic ventral character. Dorsal telencephalic cells are specified at stage 10 in chick, while the intermediate cells are specified a few hours later, at stage 14. Expression of Wnt and FGF coincides with the time point when the dorsal cells are specified, and we provide evidence that Wnt and FGF signals act in a sequential way to specify dorsal telencephalic cells. The retinoic acid producing enzyme Raldh3 is expressed in proximity to the telencephalon, and our results provide evidence that retinoic acid is both required and sufficient to induce intermediate telencephalic cell types. Furthermore, Fgf8 is expressed in the anterior neural ridge and our results provide evidence that the ventral telencephalic cells require FGF signalling primarily to oppose retinoic acid to maintain their ventral character.

Olfactory and lens placodes are specified at gastrula stage in chick, and become spatially separated at the neural fold stage. We provide evidence that bone morphogenetic protein (BMP) signalling is required for the induction of a pool of placodal progenitor cells. Furthermore, time of exposure to BMP signals plays a key role in the differential specification of the olfactory and lens placodes, whereby continued exposure to BMP promotes lens character at the expense of olfactory placodal cells.

The Development of the Telencephalon Compared to the Spinal Cord

The spinal cord is formed from cells initially specified as forebrain cells and then re-specified to become spinal cord cells. The cells also subdivide into ventral and dorsal cell-types, a process that is well studied in the spinal cord. In the telencephalon, the general idea has been that the dorsoventral patterning of the telencephalon is induced by equivalent signals to those patterning the spinal cord. While the ventralizing role of Shh is conserved throughout the neural tube, in the telencephalon, BMP signalling is only important for the specification of the dorsal-most cells that will generate the non-neural structure the choroid plexus. However, Wnt signalling is also...
suggested to induce dorsal cell types in the neural tube, analogous with our results in the telencephalon. Wnt signalling also has a proliferative effect, and it is difficult to separate the mitogenic from the patterning effect. However, Zechner et al. showed that Wnt signalling through $\beta$-catenin induces the transcription factor Olig3, which is crucial for the induction of a subpopulation of spinal cord neurons (dl2-3). The proliferative effect was sustained, but the dl2-3 neurons were lost in $\beta$-catenin gain-of-function Olig3-/- cells, indicating that Wnt actually has a patterning role in the spinal cord that is separate from its mitogenic role. In addition, there are epistatic interactions between Wnt and BMP, which makes it difficult to analyze the effects of these signalling pathways. However, we provide evidence that Wnt is sufficient to induce dorsal telencephalic cells in the absence of BMP signals (Suppl. Fig 6). The specification of the most dorsal, non-neural midline cells of the telencephalic vesicle appears, however, to require BMP signalling. The role of BMP as an inducer of non-neural cells is in agreement with results at the blastula and gastrula stage, where BMP signals block neural fate (paper III).

Our result that Wnt induces dorsal telencephalic character has been confirmed in conditional $\beta$-catenin mutant mice, where expression of dorsal markers Emx1, Emx2 and Ngn2 are down-regulated and ventral/intermediate markers Gsh2, Mash1 and Dlx2 are ectopically up-regulated in the pallium. Furthermore, when ES cells that first were allowed to differentiate into telencephalic (BF1+) cells were then exposed to Wnt signals, the number of cells that expressed Pax6 increased significantly. These results support the idea that Wnt signalling suppress ventral character and promote the initial dorsal telencephalic specification.

Regarding the specification of the intermediate cells, there are also great similarities between the spinal cord and telencephalon. Novitch et al. showed that retinoid signalling promotes the transition of progenitors located in the intermediate spinal cord into Olig2+ motor neurons. The mechanism whereby FGF inhibits RA signalling in ventral progenitors also seems to be rather conserved along the rostrocaudal axis. The intermediate spinal cord marker Pax6 requires RA signalling and is inhibited by FGF signalling. However, in the spinal cord there is a clear involvement of Shh in generation of the intermediate progenitor cells, but in the
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telencephalon the expression of the subpallium specific markers Gsh2 and RBP-1 is maintained in the Shh-/- mutant69,142.

Thus, dorsoventral patterning of the telencephalon shares many features with patterning of the spinal cord, but there are also differences. Shh induces ventral cell types in both structures and retinoids are involved in generating intermediate cells in both telencephalon and spinal cord. However, in the spinal cord, BMP signals are believed to be the main inducer of dorsal cells, while in the telencephalon Wnt signalling plays the key role and BMP signalling is only required for non-neural midline cells.

Dorsal Telencephalic Cells are Re-specified from Cells of Ventral Character

Cells isolated from the prospective forebrain region of a stage 4-6 chick embryo express ventral telencephalic markers after culture while no expression of dorsal markers can be detected1. At stage 8 it is possible to isolate cells that after culture express markers characteristic of the dorsal telencephalon. This indicates that a subset of the cells that once were specified to become ventral are later re-specified as dorsal cells. However both the conditional Fgfr1-/-Fgfr2-/- mutant143 and the Shh-/- mutant46 that both lack ventral telencephalic progenitors still generate the dorsal telencephalon, suggesting that there is not an absolute requirement for the dorsal cells to first become specified as ventral.

Sequential Wnt and FGF Signalling Induces Dorsal Telencephalic Cells

Wnt signalling alone is not sufficient to induce Emx1; this induction seems to require an additional signal. At stage 10 Fgf8 is expressed in the non-neural cells that abut the prospective dorsal telencephalon, and our results show that FGF signalling is required, together with Wnt, to generate Emx1+ cells. Wnt and FGF signals have also been shown to act in concert in the early caudalization of the neural tube, where a gradient of Wnt signals, in the presence of FGF, specify caudal cell-types19,137.
In conditional knockout mice that lack the Fgfr1 receptor after E13.5, the volume of the hippocampal pyramidal layer was decreased, while the hippocampal field was normally patterned and cell differentiation was normal\textsuperscript{144}. This suggests that FGF signalling has an important additional role in proliferation at later stages. A recent article shows that in the Sip-/- mice, that lack hippocampal formation, the Wnt antagonist Sfrp1 is up-regulated and Wnt/JNK signalling is down-regulated\textsuperscript{145}. Thus, there is also a requirement for both FGF and Wnt later in forebrain development, at least for the generation of a functional hippocampus.

The Specification of Intermediate Telencephalic Cells

To test if any of the signalling pathways that previously have been implicated in forebrain development are important for intermediate cells, we blocked the SHH, Wnt, BMP and FGF pathways in stage 14 intermediate explants. None of these conditions inhibited the expression of Meis2, and blocking Wnt signalling only inhibited generation of Pax6\textsuperscript{+} cells. Meis2 is induced by RA in P19 carcinoma cell-lines\textsuperscript{146,147}, showing that this intermediate marker has the ability to respond to RA signalling. We were able to induce Meis2 by adding RA to prospective telencephalic cells, and in addition we induced two other markers, Pax6 and Isl1, confirming that it was cells of an intermediate telencephalic character that were generated in response to RA treatment.

Intermediate telencephalic cells are specified at stage 12-14, later than ventral and dorsal cells. This indicates that intermediate cells are re-specified from either ventral or dorsal telencephalic cells. Even though it is possible to generate intermediate cells from both prospective ventral and dorsal cells \textit{in vitro} by adding RA, generation of ectopic intermediate cells is more efficient in dorsal explants. This and the fact that intermediate Pax6\textsuperscript{+} cells seem to depend on early Wnt signalling, indicates that intermediate cells are re-specified from dorsal telencephalic cells.

RA Specifies the Caudal Hindbrain and Rostral Spinal Cord

Nordström \textit{et al.} have shown that early Wnt and FGF signalling act at the gastrula stage and provide a positional context for the later actions of RA
and FGF signals in specifying the rostrocaudal identity of hindbrain and spinal cord cells\textsuperscript{137}. \textit{Raldh2} starts to be expressed at the gastrula stage\textsuperscript{128} and shortly thereafter RA signalling has been shown to specify caudal hindbrain cells and, in concert with FGF, rostral spinal cord cells\textsuperscript{137}. The same mechanism can be observed in the dorsoventral patterning of the telencephalon in chick, where Wnt, FGF and Shh first establish a dorsal and ventral region of the telencephalic vesicle. After this first dorsoventral context is generated, \textit{Raldh3} starts to be expressed close to the telencephalon and RA acts in a subsequent step to induce intermediate cells (paper II).

\textbf{Is RA Required to Induce Meis2 in Mouse?}

It is difficult to study the requirement for RA in the generation of Meis2\textsuperscript{+} intermediate cells in mouse, since the \textit{Raldh2}\textsuperscript{−/−} and \textit{Raldh2}\textsuperscript{−/−}:\textit{Raldh3}\textsuperscript{−/−} mutant mice die at E8.75 and Meis2 is not expressed until E9.5. To circumvent this problem, Molotkova \textit{et al}. performed a maternal dietary RA supplementation of the \textit{Raldh2}\textsuperscript{−/−}:\textit{Raldh3}\textsuperscript{−/−} embryos from E6.75 to E8.5\textsuperscript{148}. They state that this RA treatment does not stimulate RA activity in the forebrain, because there is no detectable \textit{RARE-lacZ} activity at E8.5 in the \textit{Raldh2}\textsuperscript{−/−} RA-rescued forebrain. However, earlier studies\textsuperscript{149} have shown that 12h after the last RA dose (corresponding to E8.75) \textit{RARE-lacZ} activity was detected throughout almost the whole embryo in both \textit{Raldh2}\textsuperscript{−/−} and wild-type embryos. In the RA-rescued \textit{Raldh2}\textsuperscript{−/−}:\textit{Raldh3}\textsuperscript{−/−} embryos, Meis2 expression was still observed in the intermediate region of the telencephalon. The authors draw the conclusion that RA is not required to initiate Meis2 expression in the forebrain in mouse. However, we think that the Meis2\textsuperscript{+} cells could be induced by the maternally administered RA. If there is still exogenous RA activity at E8.75, this may be enough to induce intermediate telencephalic character.

\textbf{RA in Further Differentiation of Intermediate Telencephalic Cells}

Later in development, both \textit{Raldh1} and \textit{Raldh3} are present and RA signalling is active in the mammalian homologue of the intermediate part of the telencephalon, the lateral ganglionic eminence (LGE)\textsuperscript{68,150}. In addition,
several members from the family of retinoic acid receptors (RAR) are expressed in the telencephalon\textsuperscript{68,84}. RAR\textbeta{} and RXR\gamma{} and the cellular retinol binding protein (CRBP1) are selectively expressed in the LGE\textsuperscript{68,84}. In a study by Liao \textit{et al}., explants from different telencephalic regions at mouse E15 embryos were co-cultured with retinoid reporter cells in the presence or absence of retinol\textsuperscript{150}. In the absence of retinol, few $\beta$gal-positive cells are present, regardless of the origin of the explants. However, in LGE explants cultured in the presence of retinol, a large number of cells were $\beta$gal-positive, whereas explants from the medial ganglionic eminence (MGE) or cortex were still negative in the presence of retinol\textsuperscript{150}. This indicates that the LGE cells, but not cells from the cortex or MGE, have the ability to respond to retinoids. Toresson \textit{et al}.

The Role of FGF Signalling in Dorsoventral Patterning of the Telencephalon

As previously discussed, FGF signalling is required to generate definitive dorsal telencephalic cells. In addition in paper II, we show a role for FGF signals in the ventral telencephalic cells. Retinoic acid is known to be a diffusible molecule and both the expression pattern of the Raldh enzymes and the \textit{RARE-lacZ} reporter mice indicate that the ventral telencephalon is exposed to retinoic acid; however, these cells maintain their ventral character. In our assay, we were able to induce intermediate telencephalic cells by adding RA to both ventral and dorsal explants, although much more efficiently in dorsal cells. This might be due to the fact that ventral telencephalic cells are exposed to \textit{Fgf8} from the anterior neural ridge (ANR) at the gastrula stage and onwards. In many other systems FGF and RA signals have been shown to have opposite effects\textsuperscript{47,137,152,153}. In agreement with this, we have shown that in the presence of FGF8, RA is not able to
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induce intermediate telencephalic cells. In limb development, RA signalling is required to maintain Meis activity, and RA synthesis and signalling is inhibited by FGF activity\textsuperscript{152}. FGF is suggested to oppose RA in two different ways in the limbs; first by down-regulating Raldh2, and second by interfering with the intracellular RA signalling pathway\textsuperscript{152}. The mechanism by which FGF opposes RA signalling in the telencephalon is not clear. However, the effects of addition or inhibition of FGF signals from explants where the Raldh enzymes are not present show that there is another mechanism involved in the telencephalon other than down-regulation of the Raldh enzymes.

The requirement of FGF signalling for ventral telencephalic cells was confirmed by a conditional knockout, where \textit{Fgfr1} and \textit{Fgfr2} were deleted in telencephalic cells. This mutant lacked ventral precursor cells and the additional deletion of \textit{Gli3} could not rescue the phenotype indicating that FGF signalling can act independently of Shh signalling to generate ventral telencephalic cells\textsuperscript{143}.

**The Existence of a Preplacodal Primordium**

Is there a common preplacodal primordium from which all the specified placodal cells arise or are the placodal cells only considered to have a common origin because they derive from a restricted region? There is conflicting evidence whether or not the cells are already specified as a particular placode when they are situated in the border region of the gastrula embryo. The so called preplacodal genes (\textit{Six1}, \textit{Eya2}, \textit{Dlx5}) are expressed in large parts of the non-neural ectoderm, also including the epidermal ectoderm\textsuperscript{94,95}. A complete loss of placodal derivatives has not been observed in any of the mouse mutants for these genes. Thus, these genes are not exclusive and do not seem to be a prerequisite for the placodes. We were unable to identify a stage when the cells are specified as preplacodal but not specified to express differentiated lens and olfactory placodal markers. Explants isolated and cultured from stage 3 did not generate any preplacodal markers, but at stage 4 the cells from the anterior border region are specified as placodal, expressing both \textit{Six1} and \textit{Dlx5} (Suppl. Fig. 2) and later more differentiated markers (paper III). In addition, exposing prospective forebrain explant to BMP4 generate cells that first express \textit{Six1} and \textit{Dlx5}
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(Suppl. Fig. 2), and later more differentiated placodal markers. Taken together, these results suggest that differentiation from a \(\text{Six}1^+/\text{Dlx}5^+\) cell to a lens or olfactory placodal cell is a subsequent process that does not need signalling from any other tissue.

The early ectoderm seems to be a very plastic tissue, which can produce a variety of cell-types given the correct series of inductive signals. What is described as a placodal primordium seems to be more of a reflection of competence of early ectoderm to generate placodal structures.

The Role of BMP in the Induction of Rostral Placodal Progenitors

Previous results have suggested that blockade of BMP signalling is required for the generation of placodal progenitor cells\(^{94,107,109,110}\). In these studies, however, placodal cells were generated from tissues that are probably exposed to high BMP activity\(^{26,105,130}\), raising the possibility that under these conditions a reduction rather than a complete inhibition of BMP signalling promoted the generation of placodal progenitor cells. We suggest that an intermediate concentration induces placodal cells, but high levels of BMP induce epidermal cells (paper III). Depending on the origin of the manipulated tissue and the intrinsic levels of BMP within it, it can appear that either Noggin or BMP4 can act ectopically as the inducer of placodal cells; however, \textit{in vivo} it seems that it is an intermediate level of BMP signals that specify placodal fate. As both \textit{Bmp2} and \textit{Bmp4} are expressed throughout the preplacodal region in the gastrula embryo, and also p-Smad1, it seems unlikely that a complete blockade of BMP signalling would be necessary for the induction of the placodes.

Are all Placodal Cells First Specified as Lens?

Lens tissue develops ectopically in zebrafish \textit{Syu (Shh)} and \textit{Yot (Gli2)} mutants, probably originating from the adenohypophysis anlage\(^{154}\). This suggests that Shh signalling promotes adenohypophysis specification at the expense of lens cells. However, loss of \textit{Xhip (described as an Shh antagonist)} function resulted in a suppression of both olfactory and lens placode formation\(^{104}\), which could indicate that Shh suppress both these
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placodes, but as there are indications that Xhip also interfere with the FGF and Wnt pathways, it is difficult to draw any conclusions.

Bailey et al. showed explants from the placodal region of a stage 6 embryo expressing \textit{L-Maf} in the whole explant\textsuperscript{125}. They suggest that all placodal cells are first specified as lens and then re-specified into other placodal fates. However, only one olfactory placodal marker; Foxg1 (BF1), was analysed and found negative in the stage 6 explants, which were cultured for a longer time than the explants where \textit{L-Maf} was detected and no other olfactory markers were analysed. The apparent lack of olfactory placodal markers could also be explained by the absence of sectioned explants in this study. Sectioning the explant and not only looking at the surface could possibly reveal that part of the explant from the anterior border region was actually specified as olfactory placodal cells also in this study.

The studies described above do not show convincing evidence that sensory placodes have a common lens property before they diverge, and we have not been able to isolate explants expressing only the olfactory or lens markers either at stage 4 or 6; the explants always contained cells of both characters.

**Spatial and Temporal Gradients**

The fact that cells can respond to different levels of signalling molecules is now well established\textsuperscript{19,26,28,30,39,137}. However, the mechanism whereby graded information is transferred from the receptor to the nucleus is not fully understood. The theory that there are different receptors or that the signalling cascades divide to give different responses does not seem to be a general explanation. However, a graded activity of BMP and Shh can be mimicked by a corresponding change in the intracellular effectors Smad or Gli3, respectively\textsuperscript{29}. This suggests that the difference in concentration is retained throughout the signalling cascade. For Activin, Shh and DPP a two-to threefold change in concentration has been shown to be sufficient to switch cells between alternative fates\textsuperscript{29}. This seems to coincide also with levels of BMP signals in the ectoderm, since our results show that 35ng/ml BMP4 induces placode while 100ng/ml induces epidermal cells: a \( \sim \) threefold increase.
Recently, temporal gradients have been suggested to be an additional patterning mechanism. Digits 2-5 in mouse are Shh dependent while digit 1 forms in the absence of Shh signalling. Cholesterol is essential for long range Shh signalling. In mouse models that lack cholesterol, digits 1, 4 and 5 are still formed, suggesting that there is another Shh dependent mechanism where diffusion is not necessary, acting to differentiate digit 4 from 5\textsuperscript{155}. Showing that all the cells of digit 4 and 5 are a subset of ZPA cells that expresses Shh, and that cells that form digit 5 express Shh for a longer time than cells that form digit 4, Harfe \textit{et al.} propose a combination of spatial and temporal gradients that specify the digits. Digit 1 is Shh independent, 2 and 3 form at different concentrations whereas 4 and 5 are separated by the time of exposure to Shh\textsuperscript{33}. It is less clear how cells respond to temporal gradients; however, a long period of exposure can be important both for allowing the persistence of the expressed gene and the inhibition of negative regulators\textsuperscript{29}.

Although a traditional spatial gradient of BMP signals seems to explain the subdivision of ectodermal cells into neural crest or epidermis\textsuperscript{112,156} as well as placode or epidermis, the choice between olfactory and lens placode could not be accomplished with different BMP concentrations. In contrast, changing the time of exposure to BMP changed the fate of these cells; longer duration of exposure to BMP in combination with higher levels promotes lens cells at the expense of olfactory placodal cells.

As well as induction of a particular cell fate by the presence of a signal, it seems likely that the absence of a signal can induce different cell fates. When the cells no longer receive input through this signalling pathway, repression is relived and the cells can transcribe new mRNA or translate new proteins permitting acquisition of a different fate. The opposite, the need for constant input through a signalling pathway for transcription of mRNA can not be accomplished by increasing the concentration of the signalling molecule at a certain time point; it is the continuous exposure that is important.

In anterior placodal development, the initial BMP concentration determines whether the cells will become epidermal or placodal but the further time of exposure to BMP signals decides if the placodal cells will acquire a lens or
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olfactory fate. When the olfactory placodal cells are excluded from the BMP signal, either by migrating away from the source or by being exposed to inhibitors, they will respond by repressing genes essential for lens specification and activating genes responsible for olfactory specification.

Differential Specification of the Olfactory and Lens Placodes
The olfactory and lens placodal cells are intermingled at the neural plate border at gastrula stage, but subdivided into two separated domains at the neurula stage. Bailey et al. suggest that FGF signalling is responsible for the differential specification of the olfactory and lens placodes. This statement is based on experiments where prospective olfactory explants (POE) were exposed to FGF and started to generate olfactory placodal markers, in combination with the result that expression of olfactory markers decreased in POE when exposed to an FGF inhibitor. However, lens cells never generated olfactory markers when exposed to FGF in this study. This suggests that FGF is involved in proliferation or maintenance, rather than specification of the olfactory placode. In our explants, isolated from the same stage and approximately the same areas, we can switch between the different placodal fates by adding or inhibiting BMP signals, indicating that BMP plays a key role in the differential specification. We have also done the FGF experiments in our assay, and in agreement with the result of Bailey et al., we provide evidence that stage 8 lens explants exposed to FGF signals still generated δ-crystallin+ and L-Maf+ cells, and blocking FGF signalling in olfactory explant did not induce lens cells. Thus, we provide evidence of a key role for BMP in the differential specification of the placodes, but the role for FGF in this developmental process needs to be further evaluated.

Significance of Investigating the Development of the Nervous System
An interesting characteristic of the olfactory epithelium is that it contains cells giving rise to olfactory neurons throughout life. Gaining more information about the olfactory placode and the neurons that continuously differentiate from the olfactory epithelium, could help the general understanding of how neurons are generated from stem cells.
Defects in sensory placode development are associated with a number of human syndromes, for example blindness due to defects in lens development or Kallmann syndrome due to defective olfactory development⁹. One of the loci associated with Kallmann syndrome contains a gene encoding FGFR1, showing that signalling molecules and their receptors are important for development of the sensory organs in humans¹⁵⁸. Age-related cataract is the leading cause of blindness in the world, particularly in developing countries¹⁵⁹. Although cataract surgery is a very effective treatment, the fact that it has become the most frequent surgical procedure in people aged 65 years or older in the Western world makes it a considerable financial burden to the health care system¹⁶⁰. Getting more insight into how the lens is generated could be a first step in understanding and perhaps halting, the formation of a cataractous lens.

Another less obvious sector of application is cancer treatment. Most of the signalling molecules we have analysed also have mitogenic functions. Over-activation of the Shh pathway, for example, has been associated with basal cell carcinoma, medulloblastoma and pancreatic cancer¹⁶¹,¹⁶². Hence, while the knowledge of which signalling molecule induces a specific neuron is not immediately clinically relevant, the more profound understanding of signalling molecules in general has potential for improving clinical applications in the future.
CONCLUSIONS

- Dorsal telencephalic character is specified by sequential Wnt and FGF signalling.

- Retinoic acid is required and sufficient to induce cells of intermediate telencephalic character.

- Ventral telencephalic cells require FGF signalling to maintain their character, and FGF signals act in part by opposing RA.

- BMP signals are required and sufficient to induce olfactory/lens placodes in gastrula stage embryos.

- Time of exposure to BMP signals is important for the differential specification of olfactory and lens placodal character.

In conclusion, the data presented in this thesis show that signalling molecules can act in different ways to induce and pattern embryonic ectodermal tissues. We provide evidence that signalling molecules can act in synergy as well as oppose each other and also that temporal gradients can be important for specification of cells in the nervous system.
Tidigt under embryoutvecklingen bildas olika populationer av celler som sedan utvecklas och ger upphov till olika strukturer i den vuxna individen. Denna avhandling handlar om hur delar av det centrala och perifera nervsystemet regionaliseras, vilka signalmolekyler som styr detta, vilka vävnader som signalerna kommer från och vid vilken tidpunkt detta sker.

Hjärnan genereras från fem vesiklar i neuralröret. Vesiklarna ger upphov till olika delar av hjärnan; telencephalon, diencephalon, mesencephalon, metencephalon och myelencephalon medan den mest caudala delen av neuralröret kommer att bilda ryggmärgen. Den dorsala delen av telencephalon som ger upphov till hjärnbarken är den mest avancerade delen av den mänskliga hjärnan. De intermediära och ventrala delarna ger upphov till basal ganglierna som är viktiga främst för viljestyrd rörelser. Tidigare forskning har visat att signalmolekylen Sonic hedgehog specificerar den ventrala delen av telencephalon. För att ta reda på vad som specificerar den intermediära och dorsala delen använde vi oss av kycklingembryon och en in vitro odlings metod; ”Explant metoden” samt olika metoder där man manipulerar och sedan odlar intakta embryon. Våra resultat tyder på att signalmolekylerna Wnt och fibroblast growth factor (FGF) är viktiga för att specificera dorsala telencephalon och detta sker vid stadie 8-10, medan specificeringen av den intermediära delen sker senare, vid stadie 14. Specificeringen av intermediära celler kräver signallering av den lipofila molekylen retinoic acid (RA). Vi föreslår även att pågående FGF signallering krävs för att de ventrala cellerna ska behålla sin karaktär och att detta delvis sker genom att FGF inhiberar RA.

En viktig del av det perifera nervsystemet är de sensoriska organen, som hjälper oss att kunna se, höra och lukta. Linsplakoden och luktplakoden är förstadium till linsen i ögat respektive luktepitelet. Dessa strukturer bildas från ett område mellan neuralplattan och det blivande epidermet. Våra resultat tyder på att redan vid gastrulastadiet är dessa plakoder speciferade och vid neurulastadiet är de dessutom spatialt uppdelade. Vidare så visar vi att båda plakoderna induceras av bone morphogenetic protein (BMP) och att
tiden som cellerna exponeras för denna signal är viktig för uppdelningen mellan dem; kort exponeringstid leder till att luktplakodceller bildas medan lång exponeringstid ger linsceller.

Sammanfattningsvis tyder denna avhandling på att signalmolekyler kan fungera tillsammans på olika sätt, de kan ha en synergistisk effekt men även arbeta antagonistiskt i specificeringen av ektodermal strukturer. Vi har även framlagt bevis på att exponeringstiden för en signalmolekyl kan vara avgörande för vilken typ av cell som bildas inom det rostrala nervsystemet.
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REFERENCES


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Supplementary Figures

Suppl. Fig 1:

Suppl. Fig 2:
Supplementary Figure 1: Blocking Wnt Signalling in Stage 3 Anterior Border Cells Generates Lens and Olfactory Placodal cells.

(A) Stage 3 OLP explants (n=30) cultured for 50h alone generated a distinct region of Keratin+ cells and a few HuC/D+ cells and a separate region of cells expressed L5+ cells. No cells expressed δ-crystallin or Raldh3+. 

(B) Stage 3 OLP explants (n=25) cultured for 50h in the presence of mFrz8CRD generated a distinct region of Raldh3+, HuC/D+ and Keratin+ cells and a separate region of cells expressed δ-crystallin and Keratin, but L5+ cells was blocked.

Supplementary Figure 2: BMP Signals are Required and Sufficient to Induce Six1+ and Dlx5+ Progenitor Cells.

The line in the schematic figure indicates the level of the transverse sections shown in the corresponding panel.

(A) Six1 and Dlx5 are expressed in the anterior border region and prospective epidermal ectoderm, but are excluded from the neural plate. Six1 is also expressed in the underlying mesoderm.

(B) Stage 4 OLP explants (n=20) cultured for 15h generated Six1+ and Dlx5+ cells.

(C) In stage 4 OLP explants (n=20) cultured in the presence of Noggin for 15h, the generation of Six1+ was blocked and Dlx5+ cells was greatly reduced.

(D) In stage 4 FB explants (n=20) cultured for 15h no cells expressed Six1 and no or a few cells expressed Dlx5.

(E) In stage 4 FB explants (n=20) cultured in the presence of BMP4 (50ng/ml) for 15h cells expressed Six1 and Dlx5.
Supplemantary Figures

**Suppl. Fig 3:**

Stage 4

<table>
<thead>
<tr>
<th>BMP4 (100ng/ml) st 4 FB</th>
<th>Raldh3</th>
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**Suppl. Fig 4:**

<table>
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<th>Keratin</th>
<th>β-crystallin</th>
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<table>
<thead>
<tr>
<th>pCAGG + Noggin - GFP</th>
<th>olfactory placode</th>
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</table>
Supplementary Figure 3: Time of Exposure to BMP Signals Controls the Specification of the Olfactory and Lens Placodes.

(A-C) The explants were cultured for 42-44h in total.

(A) Stage 4 FB explants (n=25) cultured in the presence of BMP4 (100ng/ml) generated Keratin+ cells, but no placodal or neural cells were detected.

(B) In stage 4 FB explants (n=20) cultured in the presence of BMP4 (100ng/ml) and mFrz8CRD, the cells expressed δ-crystallin+ and Keratin+ cells.

(C) In stage 4 FB explants (n=20) cultured in the presence of BMP4 (100ng/ml) and mFrz8CRD for 15h and then exposed to Noggin, the cells expressed Raldh3+, HuC/D+ and Keratin+ cells. Scale bar, 100 μm.

Supplementary Figure 4: BMP Signals are Required for the Generation of the Lens in Whole Embryos.

(A-C) E2.5 chick embryos derived from stage 8 embryos transfected with the control vector pMiwIII or pMiwIII-Noggin in the anterior neural fold region using an electroporation technique, followed by culturing in ovo. A Green fluorescent protein vector (pCaggs-GFP) was co-injected to monitor the transfection efficiency.

(A) Embryos (n=10) transfected with control vector pMiwIII and pCaggs-GFP showed a normal morphology of the lens and expression of GFP, Keratin and δ-crystallin was detected in the lens.

(B) In embryos (n=11) transfected with pMiwIII-Noggin and pCaggs-GFP, GFP was detected in the ectoderm next to the eye region. The embryos lacked a morphological lens and no cells expressed δ-crystallin, changes in invagination of the prospective retina were observed, but otherwise the embryos showed normal morphology.

(C) Embryos (n=11) transfected with pMiwIII-Noggin and pCaggs-GFP, showed a normal morphology of the olfactory placode and expression of GFP, Raldh3, HuC/D and Keratin was detected in the olfactory placode.
SUPPLEMENTARY FIGURES

Suppl. Fig 5:

Suppl. Fig 6:
Supplementary Figure 5: Ectopic BMP Receptor Signalling Inhibit the Generation of Olfactory Placodal Cells At the Neural Fold Stage.

(A-B) E2.5 chick embryos derived from stage 8 embryos transfected with the control vector pMiwIII or pMiwIII-Alk6 in the anterior neural fold region using an electroporation technique, followed by culturing in ovo. A Green fluorescent protein vector (pCaggs-GFP) was co-injected to monitor the transfection efficiency.

(A) Embryos (n=10) transfected with control vector pMiwIII and pCaggs-GFP showed a normal morphology of the olfactory placode and expression of GFP, Keratin, Dlx and Raldh3 was detected in the olfactory placode.

(B) In embryos (n=4) transfected with pMiwIII-Alk6 and pCaggs-GFP, GFP and Keratin was detected in the prospective olfactory placodal ectoderm, while no Raldh3+ were generated in this region, and the number of Dlx+ cells were greatly reduced.

Supplementary Figure 6: Wnt Signals are Sufficient to Induce Dorsal Telencephalic Cells in the Absence of BMP Signals.

(A) Stage 8 Ventral (V) telencephalic explant (n=20) cultured alone generated Nkx2.1+ cells, but no Meis2+ or Pax6+ cells were generated.

(B) Stage 8 V explants (n=15) cultured in the presence of Wnt signals generated Pax6+ cells, but no Meis2+ or Nkx2.1+ cells were detected.

(C) Stage 8 V explants (n=10) cultured in the presence of Wnt signals and the BMP inhibitor Noggin generated Pax6+ cells, but no Meis2+ or Nkx2.1+ cells were detected.