Glypicans and FGFs in CNS Development and Function

Glypicans en FGFs tijdens CZS Ontwikkeling en Functie

(met een samenvatting in het Nederlands)

Proefschrift

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Cover: Distribution of *emx-2*, *en-2*, *krox-20* and *hox-b9* transcripts in early tail bud *Xenopus* embryos during central nervous system patterning.

Ai miei genitori

Avere la consapevolezza
di cio' che si sa;
conoscere d'ignorare
quello che non si sa:
ecco il vero sapere.

Confucio

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Introduction

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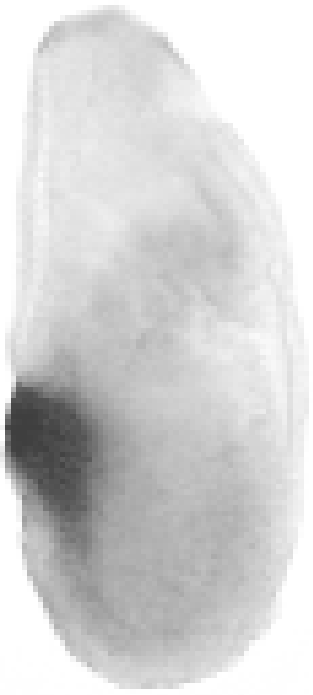


The nervous system consists of hundreds of differentiated cell types organized in intricate spatial networks. It consists of two main compartments: the central nervous system (CNS), which is composed of the brain and the spinal cord, and the peripheral nervous system, which is composed of ganglia and peripheral nerves that lie outside the brain and the spinal cord. During embryonic development, neurons acquire their cell identities by the action of regulatory signals, which ultimately regulate the transcription of specific genes (reviewed by Rubenstein and Beachy, 1998). My research aimed at understanding the cell-to-cell signaling mechanisms that pattern the embryonic forebrain and spinal cord. In order to provide the conceptual context of the studies presented, this introduction highlights the principles of early neural development. Figure 1 illustrates the anatomical subdivisions of the developing vertebrate CNS. The prosencephalon or forebrain arises from the most anterior region of the embryonic neural tube. It will give rise to the telencephalon and diencephalon as development proceeds. The spinal cord will develop from the more posterior embryonic neural tube.

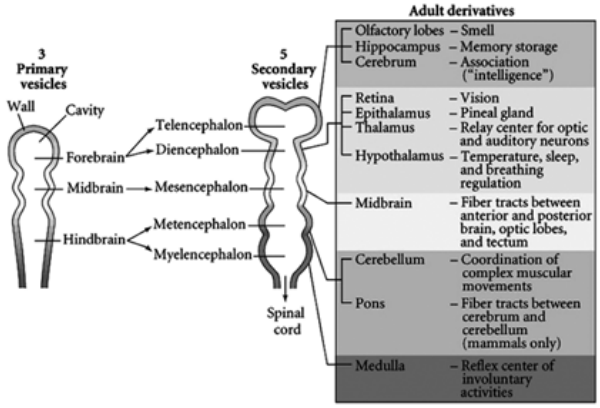


Figure 1. *Schematic representation of early human brain development. Reproduced from Gilbert, (Gilbert, 2000). The three primary brain vesicles are subdivided in five vesicles as development continues. At the right is a list of the adult derivatives formed by the walls and cavities of the brain.*

1 Neural Induction: the Role of the Organizer

The earliest step in the generation of the nervous system is the determination of ectodermal fate or neural induction. Neural tissue is derived from the embryonic ectoderm, which gives rise also to the epidermis. The term neural induction (or neuralization) denotes the phase where embryonic ectodermal cells become specified as neural stem or precursor cells. Studies performed mainly using *Xenopus* embryos as animal model have lead to the proposal that neural induction occurs by means of a "default" mechanism (reviewed by Weinstein and Hemmati-Brivanlou, 1999). In particular, ectodermal cells will adopt a neuronal fate in the absence of signaling by bone morphogenetic proteins (BMPs). The first insights into this mechanism came from transplantation experiments in amphibian embryos performed by Spemann and Mangold in the 1920s. These studies led to the discovery that a region of the gastrula-stage embryo, the dorsal lip of the blastopore (also known as "Spemann's organizer") has the ability to induce neighboring cells to acquire neural fates (Spemann and Mangold, 1924; Spemann and Mangold, 2001). It is now established that the Spemann's organizer produces diffusible BMP antagonists that act as neural inducers. Among these neural

inducers there are *Noggin*, *Chordin*, *Follistatin*, *Nodal-related-3* (*Xnr-3*) and *Cerberus* (reviewed by Harland and Gerhart, 1997; reviewed by Weinstein and Hemmati-Brivanlou, 1999). According to the default model, these molecules antagonize BMP signaling and enable the surrounding cells to execute their default tendency to give rise to neural tissue (reviewed by Hemmati-Brivanlou and Melton, 1997). In conclusion, neural induction in amphibians is initiated during gastrulation, in the time period of Spemann's organizer action and depends on BMP antagonists secreted by cells of the organizer. In *Drosophila* and amphibians, BMP antagonists obviously play a major role in neural induction, while studies in chicken and in mouse embryos show that neural induction requires coordinate signaling by fibroblast growth factors (FGFs) and Wnt family members (reviewed by Wilson and Edlund, 2001). In particular, FGFs inhibit *Bmp* genes expression at blastula stage and Wnt signals can in turn attenuate the action of FGFs. The action of WNTs and FGFs occurs before formation of the organizer, suggesting that neural induction starts prior to gastrulation in the amniotes (reviewed by Munoz-Sanjuan and Brivanlou, 2002; reviewed by Stern, 2002). The molecular network regulating neural induction is illustrated in Figure 2. Beside its neural induction potential, Spemann's organizer fulfils other functions and abilities. It forms dorsal mesoderm (prechordal plate, chordamesoderm, etc.), it dorsalizes the surrounding mesoderm into lateral mesoderm and is able to initiate the gastrulation movements and therefore establish the antero-posterior embryonic axis (Harland and Gerhart, 1997).

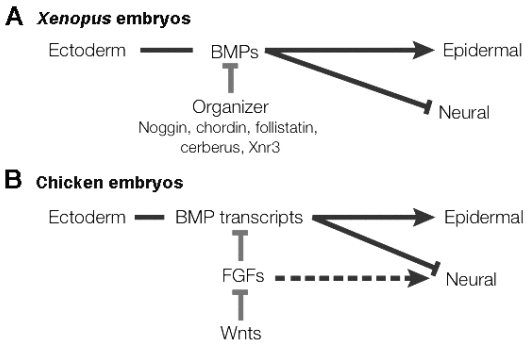


Figure 2. *Neural induction. Modified from Munoz-Sanjuan, 2002. Models proposed in Xenopus (A) and chicken (B) embryos. Overall, the abrogation of BMP signals is central to the acquisition of neural fates across species. (A) In amphibians, neural induction is thought to occur mostly through the clearance of BMP ligands by BMP antagonists secreted from the organizer. (B) In chicken embryos, BMP inhibition takes place initially through the transcriptional downregulation of BMP messages from the prospective neural territory. FGF and Wnt signals have been shown to mediate this transcriptional regulation. FGFs have been proposed to promote neural fates (broken line) independently of BMP inhibition, although the exact mechanism of action and its requirement for neural induction is unclear.*

The Hensen's node ("the node") in chicken and in mouse embryos is homologous of Spemann's organizer (reviewed by Weinstein and Hemmati-Brivanlou, 1999). It also gives rise to similar embryonic tissues as the prechordal mesoderm, the notochord and gut endoderm and will induce a secondary axis upon transplantation. However,

unlike Spemann's organizer, transplantation of the chicken and mouse node does not result in duplication of the most anterior CNS structures. Moreover, mouse embryos deficient for the transcription factor HNF3β lack the node but still express markers of anterior neural structures (Ang and Rossant, 1994). Therefore, the anterior CNS is established before formation of the node or primitive streak in mouse and chicken embryos (Beddington, 1994; Storey et al., 1992). More recent studies have shown that forebrain development is under the control of signaling by the Anterior Visceral Endoderm (AVE; Thomas and Beddington, 1996).

1.1 Morphogenetic Signaling by the AVE

The anterior visceral endoderm or AVE is a cell layer of the extra-embryonic endoderm that overlies the epiblast. Cell lineage studies have shown that the AVE is derived from endodermal cells at the tip of the pregastrulating mouse embryo, which express the transcription factor *Hex* (Thomas et al., 1998). During pre-streak stages, these distal visceral endoderm cells move to the prospective anterior side of the embryo, thus generating the AVE (reviewed by Beddington and Robertson, 1999). Cell movements and molecular signals involved in AVE function are schematized in Figure 3. The importance of the AVE for forebrain development comes from the observation that its removal from the egg cylinder (common name use to indicate mouse embryos at early post implantation stage) at the early streak stage leads to a failure of anterior neural structures to develop. Furthermore, a number of transcription factors necessary that its removal from the egg cylinder (common name use to indicate mouse embryos at early post implantation stage) at the early streak stage leads to a failure of anterior neural structures to develop. Furthermore, a number of transcription factors necessary- that its removal from the egg cylinder (common name use to indicate mouse embryos at early post implantation stage) at the early streak stage leads to a failure of anterior neural structures to develop. Furthermore, a number of transcription factors necessary- for head formation such as *Otx-2*, *Lim-1*, *Goosecoid*, *Cerberus-1*, *Hesx* and *Hex* are expressed by the AVE at least 12 hours before the streak becomes apparent (reviewed by Beddington and Robertson, 1999) and loss-of-function mutations in these genes disrupt forebrain development (reviewed by Stern, 2002). However, transplantation of the AVE adjacent to non-neural ectoderm does not generate an ectopic forebrain in the mouse; this is only achieved when the AVE is combined with both the anterior epiblast and the posterior epiblast fragment that contains the tip of the early primitive streak (early gastrula organizer; Tam, 1990). Therefore, formation of anterior neural structures requires the synergistic interaction of anterior germ layer tissue and early gastrula organizer. Transplantation experiments performed using chicken hypoblast (equivalent to the mouse AVE; Foley et al., 2000) together with the molecular analysis of several OTX-2 mutants (Kimura et al., 2000) lead to the proposal that signals from the AVE "protect" prospective forebrain cells from posteriorizing signals (reviewed by Stern, 2001). Thus, specification of anterior neural cells in mouse appears to be initiated before the onset of gastrulation and before the AVE is positioned adjacent to the prospective forebrain. The AVE, in turn, will stabilize the neural forebrain fate by antagonizing the subsequent action of posteriorizing signals (reviewed by Stern, 2001).

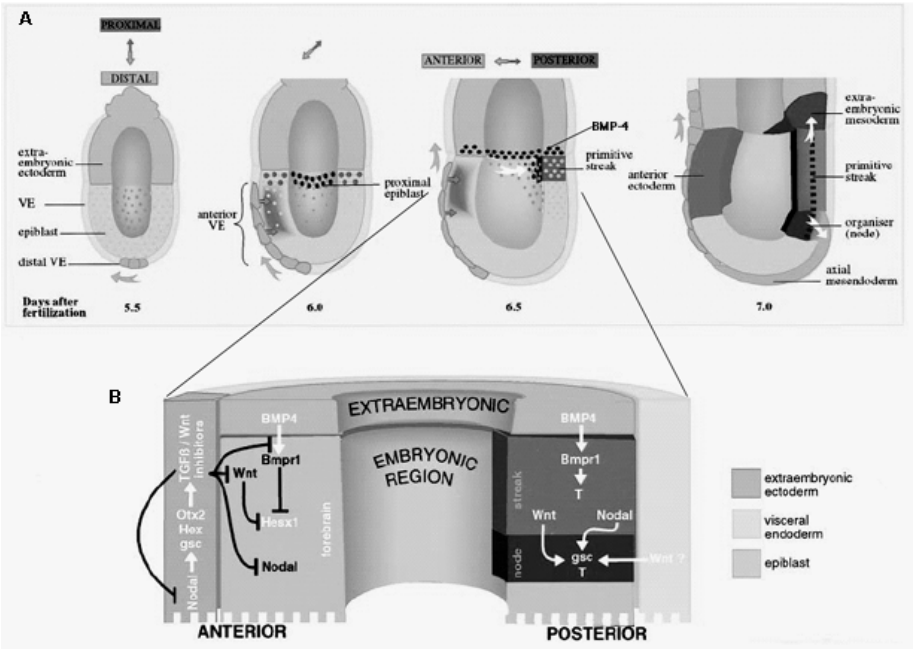


Figure 3. *Cell Movements and Molecular Signals Controlling Axis Formation. Reproduced from Beddington, 1999. (A) Changes in cell movement and gene expression that mark the conversion of the proximal–distal axis into the antero-posterior axis of the embryo. At 5.5 days of embryonic development (E 5.5), distal visceral endodermal cells, marked by Hex expression, give rise only to anterior progeny, which populate the AVE and eventually move into the extra-embryonic region (gray arrows). The AVE induces anterior character in the underlying epiblast. Genes expressed in the future primitive streak such as Cripto are ubiquitously expressed in the epiblast of an E 5.5 embryo but restricted to the proximal rim of epiblast at E 6.0, where other markers of the primitive streak, such as Brachyury (T) start to be expressed. Bmp-4 is transiently expressed in the adjacent extra-embryonic ectoderm. Caudal cell movement in the proximal epiblast (white arrow) results in the primitive streak forming opposite to the AVE at E 6.5. By E 7.0 the extra-embryonic mesoderm, is produced from the posterior aspect of the streak while anteriorly the organizer (the "node") forms. (B) Possible scheme of the molecular interactions regulating antero-posterior axis development at E 6.5. One role of the AVE would be to provide the requisite TGF-b and WNT inhibitors while the streak is being induced in order to allow expression of anterior markers such as Hesx1.*

2 Neural Patterning

The future nervous system manifests itself first shortly after gastrulation through a thickening of the ectoderm on the dorsal side, which demarcates the future neural plate. The neural plate undergoes morphogenetic movements that first create a neural groove by raising the lateral edges of the plate and ultimately the neural tube forms by dorsal closure of the lateral edges of the neural plate. The neural tube then differentiates to create patterns of distinct cell types along its three main axes. The result of these patterning events gives rise to a mature nervous system consisting of four major compartments along the antero-posterior embryonic axis: forebrain, midbrain, hindbrain and

spinal cord. Further patterning along the dorso-ventral axis of the neural tube leads to the development of structures like the telencephalic pallium and the thalamus in the dorsal and subpallium and hypothalamus in the ventral neural tube (reviewed by Altmann and Brivanlou, 2001). After formation of the neural plate, signaling centers, or secondary organizers are established along the antero-posterior axis. These signaling centers are essential for induction and patterning of the different CNS territories. I will briefly describe those required for forebrain and dorso-ventral spinal cord patterning.

2.1 The Anterior Neural Ridge

The anterior neural ridge (ANR) is morphologically defined as a cell layer lying at the junction of the rostral neural plate margin and the non-neural ectoderm. It is required for induction and patterning of the telencephalon (Figure 4A, B). For example, removal of the ANR results in loss of expression of the telencephalic marker *Brain factor-1* (*Bf-1*). In contrast, transplantation of an additional ANR can induce ectopic *Bf-1* expression in both mouse and zebrafish embryonic neural plate explants (Houart et al., 1998; Shimamura and Rubenstein, 1997). Both *Fgf-8* and *Fgf-3* are expressed by ANR cells and there is evidence that FGFs are key mediators of ANR functions. FGF-8 soaked beads restore *Bf-1* expression and rescue widespread cell death in anterior neural plate explants denuded of the ANR (Shimamura and Rubenstein, 1997). Accordingly, *Bf-1* expression is downregulated in the presence of FGF signal inhibitors (Ye et al., 1998). Interestingly, telencephalic formation proceeds in the absence of FGF-8 in both zebrafish and mouse embryos (Shanmugalingam et al., 2000; Wilson and Rubenstein, 2000). However, these mutant embryos lack anterior and ventral telencephalic structures (Wilson and Rubenstein, 2000). Therefore, FGF-8 has a more general role in telencephalic patterning and differentiation rather than acting only as an inducing factor.

2.2 The Prechordal Plate

The prechordal plate is the other signaling center regulating generation of the discrete subdivisions within the anterior neural plate. It is a mesendodermal derivative of the node and forms the anterior end of the axial mesendoderm that underlies the most rostral midline of the neural plate (Figure 4A, B). The prechordal plate confers dorso-ventral pattern to the overlying tissue via secreted molecules such as Sonic Hedgehog (SHH), BMP-7, Noggin and WNT antagonists as *Dickkopf-1* (*Dkk-1*) and *Frzb-1* (reviewed by Kiecker and Niehrs, 2001). Surgical ablation of the prechordal plate in *Xenopus*, mouse, chicken and zebrafish embryos causes dorso-ventral patterning defects in the forebrain (Camus et al., 2000; Li et al., 1997; Pera and Kessel, 1997; Saude et al., 2000). In particular, these embryos show cyclopia similar to mouse and zebrafish embryos deficient for SHH function (Chiang et al., 1996; Karlstrom et al., 1999). These and other results show that SHH specifies the ventral midline of the anterior CNS in conjunction with BMP-7 and in parallel with Nodal signals secreted by the axial mesendoderm (reviewed by Kiecker and Niehrs, 2001). Gain- and loss-of-function experiments in chicken and mouse embryos revealed an additional important role of the prechordal plate in patterning the neural plate along its antero-posterior axis through Wnt and BMP antagonists (Anderson et al., 2002; Hashimoto et al., 2000; reviewed by Kiecker and Niehrs, 2001).

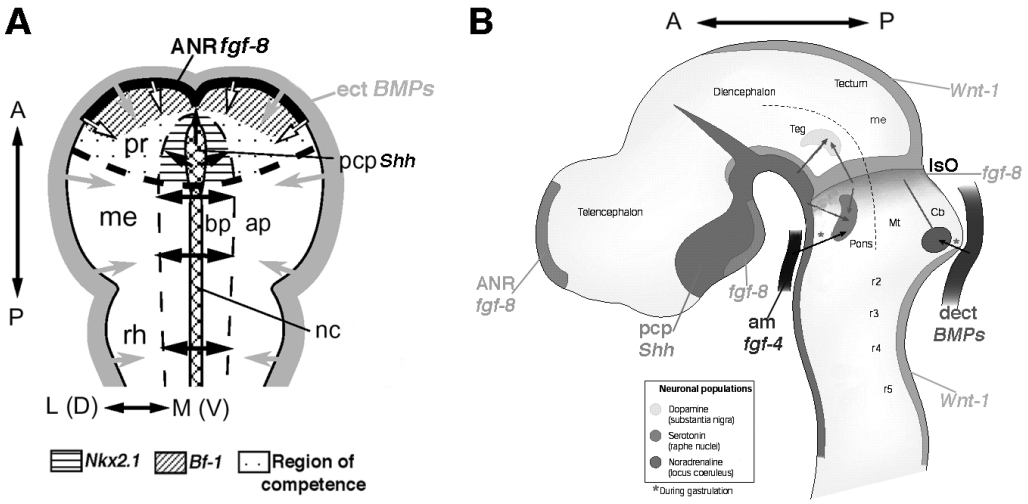


Figure 4. *Signaling centers regulating CNS patterning. Adapted from Shimamura, 1997 and Wurst, 2001. (A) Model of patterning mechanism that regionalize the anterior neural plate. Antero-posterior patterning mechanism generates transverse subdivisions: prosencephalon (pr) and mesencephalon (me) (indicate by thick broken line) that have distinct responses to medial and local signals. Medial or ventral patterning signals (SHH; black arrow), which induce the primordial of the basal plate (bp) or ventral forebrain and expression of ventral forebrain marker such as Nkx2.1, arise from the axial mesendoderm (prechordal plate, pcp). Lateral or dorsal patterning signals (BMPs; gray arrow), which arise from the non-neural ectoderm (ec) flanking the neural plate, induce expression of Msx1 and pattern the primordial of the alar plate (ap) or dorsal forebrain. Local signals, arising from the ANR (indicate by thick black line) (FGF-8; white arrows) induce expression of Bf-1, which regulates development of specific forebrain structures (telencephalon and optic vesicles). (B) Sagittal view of a mouse neural tube at E 11; anterior is to the left. Inductive influences derived from different growth factors, secreted by signaling centers and non-neural tissues during gastrulation, control antero-posterior and dorso-ventral patterning. They also control neuronal identities. Additional abbreviations: A, anterior; am, anterior mesoderm; Cb, cerebellum; D, dorsal; dect, dorsal ectoderm; L, lateral; M, medial; Mt, metencephalon; P, posterior; r, rhombomeres; Teg, tegmentum; Tel, telencephalon; V, ventral.*

2.3 Dorso-Ventral Patterning of the Telencephalon

The developing forebrain is first subdivided into telencephalon and diencephalons along its antero-posterior axis. These brain compartments are in turn subdivided into distinct dorso-ventral domains such as the dorsal cerebral cortex and the ventral subpallium, which constitutes most of the basal ganglia (Wilson and Rubenstein, 2000). As discussed previously, the ANR and the prechordal plate have important roles in establishment of the telencephalic territory and patterning of its ventral structures (Foley et al., 2000). However, less is known about the signals that promote dorsal telencephalic fate. Embryological and genetic studies have shown that dorsal telencephalic development is controlled largely by BMP, Wnt and FGF family members (Gunhaga et al., 2000). For example, several BMP ligands are expressed by dorsal midline cells and

by epidermal ectoderm surrounding the prospective telencephalon (Faure et al., 2002). In addition, exposure of the developing telencephalon to BMPs perturbs the generation of ventral telencephalic cells (Furuta et al., 1997; Golden et al., 1999). After neural tube closure, cells of the developing dorsal telencephalon express Wnt genes and genetic studies have shown that WNT signaling is required to maintain *Emx-2* expression (Hollyday et al., 1995; Theil et al., 2002). After neural tube closure, *Fgf-8* expression extends posteriorly in the dorsal midline to overlap the domains of *Fgf-15*, *Fgf-17* and *Fgf-18* (reviewed by Ford-Perriss et al., 2001; Shimamura and Rubenstein, 1997). Reduced *Fgf-8* expression in mouse and zebrafish mutants impairs growth in the telencephalon and results in miss-specification of dorsal midline cells (Shanmugalingam et al., 2000). Storm et al. (2003) reported that ectopic FGF-8 signaling in telencephalic mouse explants induces a structure that resembles a rostral midline. These findings have lead to the hypothesis that FGF-8 is required for the generation of dorsal midline cells within the telencephalon. Furthermore, it has been shown that FGF-8 acts as a cell survival factor during forebrain development (Storm et al., 2003). Recent studies by Gunhaga et al. (2003) begin to clarify the roles for WNT and FGF signaling in the early specification of dorsal telencephalic cells. In particular, they establish that sequential WNT and FGF signaling specifies cells of dorsal telencephalic character in chicken embryos (Gunhaga et al., 2003).

2.4 Dorso-Ventral Patterning of the Developing Spinal Cord

The initial dorso-ventral patterning of the spinal cord is controlled by the opposing action of signals produced by the dorsal epidermis and the ventral notochord, a mesodermal node derivative (reviewed by Altmann and Brivanlou, 2001). The notochord secretes SHH that in turn induces the overlying ventral neural tube cells to become floor plate (Roelink et al., 1994). Floor plate cells also secrete SHH, which forms a concentration gradient highest in the most ventral portion of the neural tube. This gradient of SHH signaling is responsible for cell fate specification of the different neuronal subtypes in the ventral-intermediate region of the developing spinal cord (reviewed by Altmann and Brivanlou, 2001). In particular, progenitors adjacent to the floor plate (receiving high SHH concentrations) become the ventral neurons, while the next group of progenitors, exposed to slightly less SHH signaling become fated to motoneurons (Roelink et al., 1995). More dorsal cells (receiving progressively less SHH) become interneurons. The identity of the most dorsal progenitors is instead controlled by the interaction of BMP-4, BMP-7, Dorsalin and Activin produced by the epidermis (Liem et al., 1997; Liem et al., 1995). Similar to the notochord and the floor plate, the dorsal epidermis establishes a signaling center by inducing *Bmp-4* expression in the roof plate cells of the neural tube. *Bmp-4* expression, in turn, induces a cascade of Transforming Growth Factor-β (TGF-β) superfamily proteins that act in a concentration dependent manner to specify neuronal identities in the dorsal spinal cord (reviewed by Altmann and Brivanlou, 2001). As result of this patterning process sensory neurons will develop in the most dorsal region of the spinal cord, while motoneurons will arise in its ventral part and interneurons differentiate in the medial region. The generation of these distinct neuronal subtypes is the basis for establishment of neuronal diversity in the developing spinal cord. Spinal cord neurons undergo further diversification as developmental proceeds. Interestingly, *Fgf-2* and *Fgf receptor-1* (*Fgfr-1*) are expressed in the intermedio-lateral neurons during dif-

ferentiation of the preganglionic neurons of the autonomic nervous system (Stapf et al., 1997). In addition, FGF-2 deficient mice are hypotensive and suffer from an autonomic dysfunction that impairs blood pressure regulation (Dono et al., 1998). Therefore, I have analyzed the potential role of FGF-2 during development of the neuronal regulatory circuits involved in blood pressure regulation in chapter 4.

3 Heparan Sulphate Proteoglycans and Signaling

Ligand-receptor interactions are regulated at different levels by means of signal agonists and antagonists (reviewed by Freeman and Gurdon, 2002). Genetic analysis of cell-to-cell signaling during *Drosophila* development brought to the attention another type of molecules, which modulates ligand-receptor interaction at the cell surface. Heparan Sulphate Proteoglycans (HSPGs) are abundant cell-surface glycoproteins, which act as co-receptors in signaling processes (reviewed by Bernfield et al., 1999). Structurally they consist of a core protein to which heparan sulphate glycosaminoglycan chains (GAG) are attached. Due to their sulphate groups, the GAG chains are negatively charged, which confers them the specificity of their interaction (reviewed by Nakato and Kimata, 2002).

3.1 Glypicans

Glypicans together with the Syndecans form one of the largest families of HSPGs. Glypicans are unique feature among HSPGs as they are linked to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (Veugelers et al., 1999). To date six glypican family members (GPC-1 to GPC-6) have been identified in mammals (Figure 5), two in *Drosophila* (*dally* and *dally-like*), one in zebrafish (*knypek*), and one in *C.elegans* (reviewed by De Cat and David, 2001). The core protein is similar among different Glypicans (Figure 5). They all encode an N-terminal secretory signal peptide and a hydrophobic domain required for the carboxy-terminal addition of the GPI anchor (Figure 6). Another characteristic shared by all Glypicans is the location of the sites for attachment of the heparan sulphate side-chains. Heparan sulphate side-chains are in general attached to the most carboxy-terminal domain, placing in this way the side-chains close to the cell membrane (reviewed by Song and Filmus, 2002). Furthermore, the positions of 14 cysteine residues, which form intramolecular disulfide bonds, are conserved suggesting that the three-dimensional structure of Glypicans is very similar. On the base of amino acid homologies, mammalian Glypicans can be subdivided in two distinct groups. One group comprises GPC-1, GPC-2, GPC-4, GPC-6 and they displays 35-63% sequence similarity, while the second group includes GPC-3 and GPC-5, which share 54% similarity (Veugelers et al., 1999).

3.1.1 Expression of Mammalian Glypicans during CNS Development

So far most studies to analyze Glypican expression have been focused on later developmental stages (from embryonic day 13 onwards). One of the family members *Gpc-2*, or *Cerebroglycan*, is predominantly expressed in the developing CNS. Interestingly, *Gpc-2* is expressed by neuronal progenitors as they enter in the final cell division and is downregulated again during migration and differentiation of post-mitotic neurons (Stipp et al., 1994). In contrast, *Gpc-1* is expressed both by proliferating progenitors and post-mitotic neurons (Carey and Stahl, 1990). *Gpc-3* is the only glypican

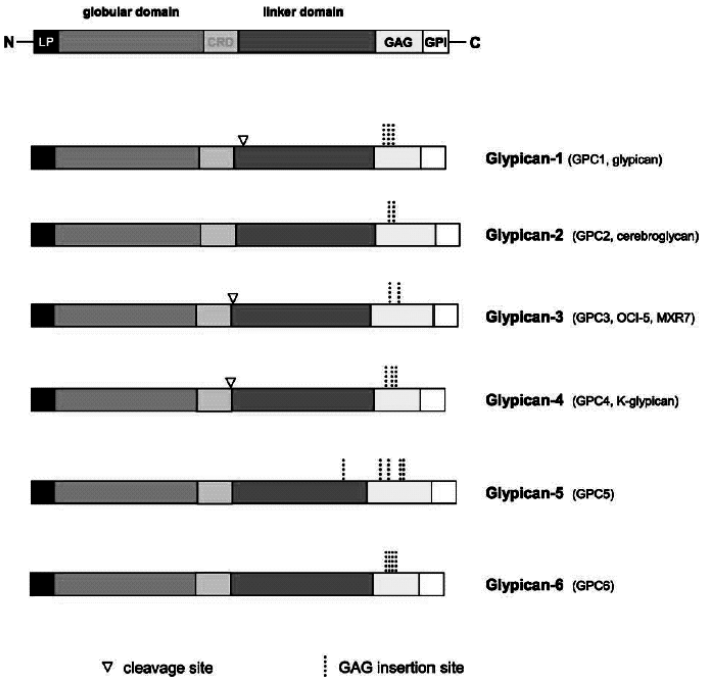


Figure 5. *Schematic representation of mammalian Glypicans. Reproduced from Song, 2002. The mature glypican core protein, approximately 500 amino acid in length and 60-70 kDa, contains two structural domains: the linker and an N-terminal globular domain. The globular domain carries 11 of the 14 conserved cysteine residues and a putative cysteine-rich domain (CRD), which is commonly found in Wnt binding molecules such as Wnt receptors, Frizzled, and Frizzled-Related Proteins. In the C-terminus part is placed the GAG domain for the attachment of the HS chains. In addition at the C-terminal part, the proteins contain the GPI anchor.*

gene not expressed during CNS development, but in mesenchymal cell types (Pellegrini et al., 1998). *Gpc-5* is expressed by dorsal root ganglia and during differentiation of the CNS (Saunders et al., 1997). *Gpc-6* is expressed by the neuroectodermal cells surrounding the lateral ventricles of the developing telencephalon (Veugelers et al., 1999). In the developing CNS, *Gpc-4* is highly expressed by the telencephalon and during neuronal differentiation its expression is downregulated (Hagihara et al., 2000). *Gpc-4* transcripts are also expressed in the tubular epithelium of the kidney, adrenal glands, smooth muscles of the endothelium and intestine and in the mandibular and maxillary regions (Watanabe et al., 1995). The major topic of chapter 2 was to determine the distribution of *Gpc-4* during early stages of CNS development.

3.1.2 Functions of Glypicans during Embryonic Development

First insights into the function of Glypicans have come from genetic and molecular studies performed in *Drosophila* embryos. The *Drosophila* glypican gene, *division abnormally delayed* (*dally*), was identified in a genetic screen aimed to identify mutations affecting cell division patterns in the eye and larval brain (Nakato et al., 1995). Further analysis of several mutant alleles showed that DALLY is also required for proper morphogenesis of wing, antenna and genitalia imaginal disks. Furthermore, difficulties in isolating complete loss-of-function *dally* alleles suggest that it is a haplo-lethal locus, i.e. 50% reduction in its expression is already lethal (Lin and Perrimon, 1999). Consistent with the proposal that HSPGs modulate intercellular communication, it has been shown that patterning defects in eye, antenna and wing of *dally* mutants are caused by a reduction of *decapentaplegic* (*dpp*; homolog of vertebrate BMPs) and *wing-*

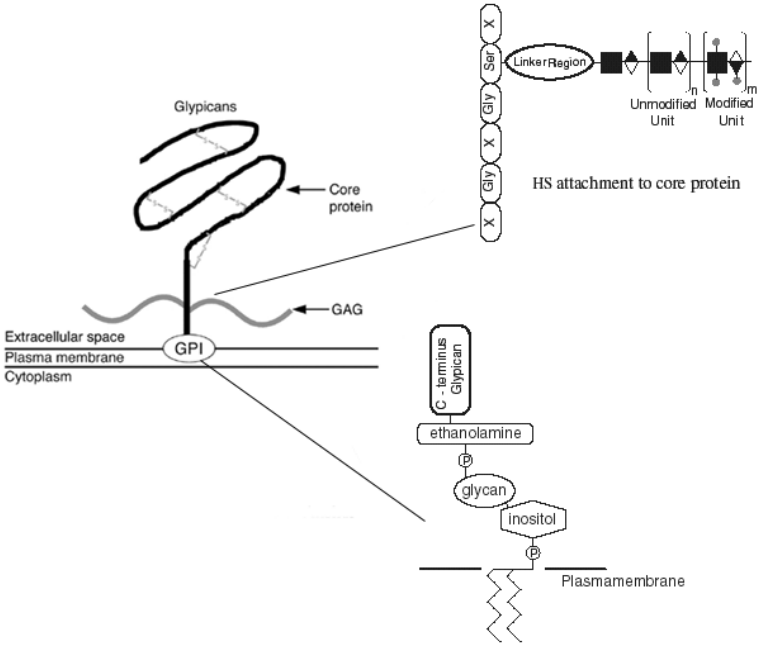


Figure 6. *Schematic representation of the Glypican structure. Reproduced from De Cat, 2001. The Glypican core protein is located in extra-cellular compartments. Numerous disulfide bridges organize the core protein as a high compact globular domain. HS chains are covalently bound to serine residues that form part of Ser-Gly-X-Gly motifs in the polypeptide chain. A Xylose-galactose-galactose-glucoronic acid sugar sequence links the HS chain to the core protein. HS is a linear polymer formed by the alternating addition of N-acetyl glucosamine (filled square) and glucoronic acid residues to the linker region (unmodified unit). As the polysaccharide assembles, it undergoes a series of modifications that include sulphatations and epimerizations (modified unit). The protein is covalently linked to the plasmamembrane at its carboxy-terminus, via a GPI anchor. The glycan component of the GPI anchor consists of a trimannose-glucosamine core.*

less (*wg*; homolog of vertebrate WNTs) signaling (Fujise et al., 2003; Tsuda et al., 1999). Accordingly, ectopic expression of *dally* can enhance the patterning activity of DPP. More recently, a second *Drosophila* glypican gene, *dally-like* (*dly*), has been isolated. *Dly* colocalises with *Wg* and is required for Wg signaling (Baeg et al., 2001). Indeed, RNA interference inhibition of *dly* phenocopies the epidermal patterning abnormality of *wg* mutant embryos. Also during vertebrate embryogenesis Glypicans modulate WNT signaling. For example, genetic studies in zebrafish show that *knypek* acts during vertebrate gastrulation to positively modulate non-canonical WNT signaling during establishment of cell polarization, which underlies convergent extension movements during gastrulation (Topczewski et al., 2001). In addition, studies performed in *Xenopus* embryos have confirmed the role of GPC-4 as a positive regulator of non-canonical WNT signaling during gastrulation (Ohkawara et al., 2003).

Our understanding of the developmental function of Glypicans in mammals comes predominantly from studies analyzing GPC-3. The *Gpc-3* gene is mutated in patients affected by a X-linked disorder characterized by pre- and post- natal overgrowth

known as the Simpson Golabi Behmel Syndrome (SGBS; Pilia et al., 1996). These mutations are microdeletions in different exons encoding the gene that result in a non-functional GPC-3 protein. Human patients affected by SGBS show pleiotropic phenotypes including facial defects, macroglossia, polydactyly, supernumerary nipples, kidney and congenital heart defects, rib and vertebral abnormalities, umbilical and inguinal hernias and an increased risk of Wilm's tumors (Hughes-Benzie et al., 1996). The phenotype varies from mild forms in carrier females to infantile lethality in some affected males. The involvement of Gpc-3 in SGBS was confirmed by the analysis of GPC-3 deficient mice, which display developmental overgrowth and kidney defects. Consistent with the overgrowth phenotype, it has been proposed that GPC-3 acts as negative regulator of cell proliferation and can induce cell apoptosis (Cano-Gauci et al., 1999). In particular, GPC-3 plays a key role in regulating BMP-2, BMP-7 and FGF-7 signaling during kidney development (Grisaru et al., 2001). In summary, Glypicans seem to act in a cell-type specific manner to modulate distinct signaling pathways during embryonic development (see below).

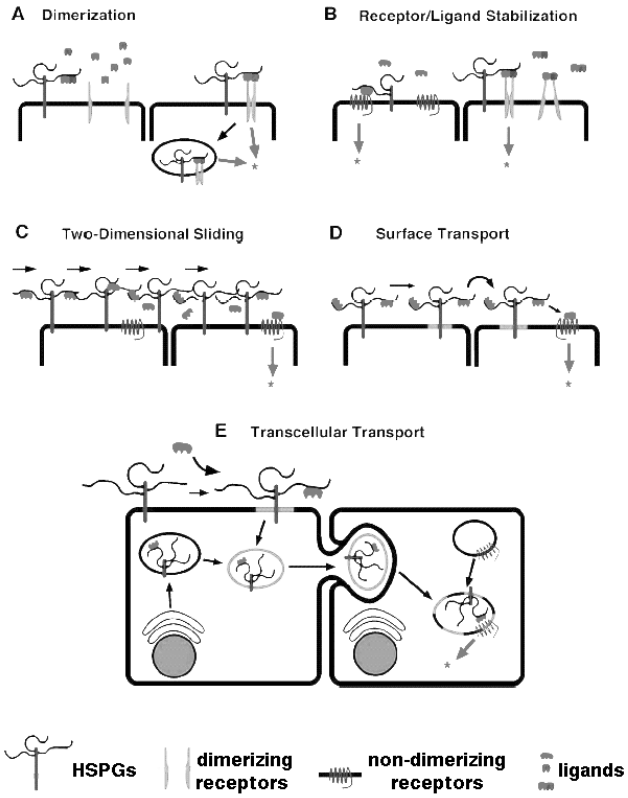
3.1.3 How do HSPGs such as Glypicans Regulate Cell-to-Cell Signaling?

A variety of biochemical, crystallographic and cell culture studies implicate HSPGs in modulation of FGF signaling (reviewed by Bernfield et al., 1999). FGF receptors are capable of binding FGFs in the absence of HSPGs in cell culture, but FGF signaling is potentiated in the presence of HSPGs. It has been proposed that HSPGs function to promote FGF ligand dimerization and/or stabilize the receptor-ligand complexes. HS GAGs chains may stabilize or induce the formation of FGF dimers or a ternary complex composed of ligand plus high and low affinity receptors (Figure 7A, B; Hagihara et al., 2000; reviewed by Ornitz and Itoh, 2001). For example, FGF signal transduction is altered when the synthesis of HS GAG side-chains is compromised. A loss-of-function mutation in enzymes critically involved in HS chains biosynthesis such as *sugarless* (*sgl*) and *sulfateless* (*sfl*) in *Drosophila* embryos results in mesoderm migration defects identical to the ones seen in *heartless* mutant embryos (*htl*; Michelson et al., 1998a; Michelson et al., 1998b; Perrimon and Bernfield, 2000). Interestingly, *htl* encodes one of two FGF receptors in *Drosophila* (Michelson et al., 1998b). Indeed, overexpression of the FGF ligand *branchless* (*bnl*) in *Drosophila* embryos partially rescues both *sgl* and *sfl* phenotypes (Lin et al., 1999).

With respect to Wg and Hedgehog (HH) signaling it appears that HSPGs are primarily involved in the transport/diffusion of the signal away from producing cells through either surface or trans cellular transport (Figure 7D, E). Evidence suggests that HSPGs regulate endocytosis, which has also been shown to regulate the range of Wg signaling (reviewed by Nybakken and Perrimon, 2002). Recent studies establish that DALLY and Wg colocalise in membrane vesicles called argosomes in the *Drosophila* imaginal disks epithelium. Argosomes derive from basolateral membranes and are capable to traffic through adjacent cells. It has been proposed that argosomes act as transport vehicles to spread signaling molecules. Therefore, Glypicans may participate in transport of Wg through argosomes (S. Eaton, personal communication; Greco et al., 2001). Two other models have been proposed with regards to the function of HSPGs in Wg signaling. Genetic experiments performed with *dally* mutant led to the proposal that Dally acts as a co-receptor for the Wg-transducing receptor encoded by the seven-transmembrane protein Frizzled-2 (Lin and Perrimon, 1999). Therefore, HSPGs may be

required for stabilizing Wg-receptor complexes. Alternatively, HSPGs may restrict the extra-cellular diffusion of the ligand by tethering. This model is supported by the observation that ectopic expression of *dly* in the developing wing imaginal disks increases the level of extra-cellular Wg protein but at the same time inhibits Wg signaling in the developing wing margin (Figure 7C; Baeg et al., 2001).

On the base of these studies five different models, none mutually exclusive have been proposed and are represented in Figure 7 (reviewed by Nybakken and Perrimon, 2002).



4 The Role of Fibroblast Growth Factors during CNS Development

Fibroblast Growth Factors (FGFs) are potent modulators of cell proliferation, migration, differentiation and survival. To date, 23 different members (Fgf-1 to Fgf-23) have been identified in vertebrates on the basis of a conserved 120 amino acid core region. This conserved domain confers a common tertiary structure and the ability to bind heparin (reviewed by Ford-Perriss et al., 2001). Most FGFs are secreted and signal to target cells by binding and activating cell-surface tyrosine kinase FGF receptors (FGFR; reviewed by Ornitz and Itoh, 2001). Four FGFR genes (Fgfr-1 to Fgfr-4) encode

Figure 7. *Models of HSPG regulation of developmental signaling. Reproduced from Nybakken, 2002. In all models, the grey arrows pointing to an asterisk indicate activation of the signaling pathway. (A) HSPG control of ligand dimerization. Many signaling ligands must dimerize or oligomerize to initiate effective signaling. HSPGs may be required for dimerization or oligomerization of ligands and/or presentation of these oligomers to their appropriate signaling receptors. As represented in the model, this could occur on the surface or in intracellular vesicles. (B) Receptor–ligand stabilization. HSPGs may not be required for ligand dimerization, but rather to stabilize the ligand/receptor signaling complex and promote maximal signaling from the receptors. (C) Two-dimensional sliding. In this model, HSPGs regulate the distribution of ligands by binding the ligands and allowing them to "slide" along the HS chain. This limits the ligand distribution to the plane formed by cell surfaces. (D) Surface transport of ligands by HSPGs. HSPGs might be required for active transport of signaling ligands away for the producing cell. This transport could occur in two different ways. The ligand-bound HSPGs could actually move between cells, perhaps via lipid rafts which can move between cells (represented by the pale gray cell membrane region), or by one HSPG passing its bound ligand to a HSPG on adjacent cell. Once localized to the adjacent cell, the signaling molecule can bind its receptor and activate signaling. (E) Transcellular transport. This model is similar to the surface transport model, but in this model, the ligand-bound HSPG is placed into a vesicle and this vesicle is then expelled from the ligand-producing cell and taken up by an adjacent cell. The HSPG-ligand-containing vesicle can then fuse with a vesicle containing the ligand's receptor and signaling is activated. The pale gray molecules represent individual, dimerizing receptors such as tyrosine kinase receptors. The multiple pass transmembrane proteins represent non-dimerizing receptors. Ligands are depicted in various shades of gray.*

multiple receptor isoforms with unique ligand-binding properties, which are generated by alternative splicing of the carboxy-terminal regions. Initiation of FGFR signal transduction starts by binding of the FGF ligand to the extra-cellular domain of the receptor, the dimerization of the receptor itself and subsequent phosphorylation of tyrosine residues. The regulation of FGF-FGFR interactions is complex and modulated by HSPGs (see before). Several FGF ligands and receptors are expressed during CNS development (reviewed by Dono, 2003). In particular, *Fgf-1*, *Fgf-2* and *Fgf-15* are expressed by both the developing and adult CNS, while *Fgf-8* and *Fgf-17* are expressed during the early phase of neurogenesis. Expression studies performed in chicken, mouse and *Xenopus* embryos have shown that *Fgfr-1* and *Fgfr-3* transcripts are present in the ventricular zone of the neural tube during neurogenesis (Golub et al., 2000; Wilke et al., 1997). During postnatal CNS development, *Fgfr-1* is expressed by neurons such as motoneurons of the spinal cord while *Fgfr-3* becomes confined to glia cells (Peters et al., 1993). *Fgfr-2* is expressed in the developing forebrain, midbrain and hindbrain, while *Fgfr-4* is expressed in the ventricular zone of the developing spinal cord and in dorsal root ganglia (Marcelle et al., 1994).

Gain- and loss-of-function studies have shown that FGF signaling fulfils multiple roles during CNS development. FGF signaling functions first during neural induction and subsequently in most events resulting in specification and terminal differentiation of different CNS regions during embryogenesis (reviewed by Dono, 2003).

4.1 FGFs and Antero-Posterior Patterning

Studies performed in chicken and *Xenopus* embryos have shown that FGFs

promote posterior neural fate during early antero-posterior patterning of the neural plate (reviewed by Dono, 2003). Implantation of FGF-soaked beads in chicken embryos leads to the formation of ectopic posterior neural structures (Alvarez et al., 1998; Storey et al., 1998). Signaling through the FGFR promotes development of the posterior nervous system by maintaining a proliferative stem zone of progenitors that contribute to the posterior neural tube during posterior regression of Hensen's node (Mathis et al., 2001). In *Xenopus* embryos, transcription factors such as *Brain factor-2* (*Xbf-2*) and *Xmeis3* induce posterior neural tissue through activation of the FGF dependent RAS-MAP kinase pathway (Ribisi et al., 2000). In addition to its function in the ANR, FGF-8 is a key mediator of the isthmic organizer (IsO) functions (reviewed by Wurst and Bally-Cuif, 2001). IsO is a signaling center lying at the junction of mid- and hindbrain that regulates proper development of the mesencephalic and metencephalic derivatives. IsO cells express *Fgf-8* and application of FGF-8 beads to ectopic locations induces ectopic expression of genes normally present at the mes/metencephalic junction (Martinez et al., 1999). In agreement with gain-of-function studies, FGF-8 loss-of-function mutations in zebrafish and mice cause loss of the IsO (Meyers et al., 1998). After establishment of the mid/hindbrain junction, other FGF family members such as *Fgf-17* and *Fgf-18* are expressed by the IsO and adjacent territories (Xu et al., 2000). FGF-17 deficient mouse embryos display a loss of proliferative precursors in the midline of the primordial cerebellum (vermis), which functions in motor coordination and maintenance of body equilibrium. This phenotype is enhanced by the loss of FGF-8, which demonstrates that both molecules cooperate in regulating the size of the precursor cell pool giving rise to the cerebellar vermis (Xu et al., 2000). Interestingly, FGF-8 produced by the ANR and the IsO, in combination with FGF-4 and SHH, can induce dopaminergic and serotonergic neurons in neural plate explants. These results led to the proposal that these signaling molecules create a grid of positional information in the neural tube (Figure 4B; Ye et al., 1998).

4.2 FGFs and Cerebral Cortex Development

FGFs are part of the instructive signaling molecules required for correct growth and patterning of the neocortex (reviewed by Dono, 2003). They are produced locally and act on progenitor cells in neuroepithelial cell layer lining the lumen of the telencephalic vesicles (ventricular zone). Throughout neurogenesis ventricular zone cells express several Fgf ligands and Fgf receptors (review by Ford-Perriss et al., 2001). In particular, FGF-2 proteins are abundant during early developmental stages and nearly absent at the end of neurogenesis (Raballo et al., 2000). Cell culture experiments have shown that FGF-2 stimulates proliferation and survival of ventricular zone progenitors (Murphy et al., 1990). FGF-2 not only influences the proliferation and survival of these cells, but also their potential differentiation. In particular, FGF-2 alone or in combination with neurotrophins promotes differentiation of neocortical precursor cells (Murphy et al., 1990). Furthermore, multipotent mouse cortical stem cells generate neurons or glia in response to different concentrations of FGF-2 ligand (Qian et al., 1997). FGF-2 deficient mice display defects in the neuronal density and cytoarchitecture of the developing neocortex due to defects in neuronal migration (Dono et al., 1998). Taken together, these studies indicate that FGF-2 regulates neuronal precursors during the early stages of neocortex development. However, other FGF family members are also expressed during neocortex development (reviewed by Dono, 2003). For example, *Fgf-7* is expressed

by the ventricular zone and *Fgf-18* transiently by the prechordal plate (Hu et al., 1998; Mason et al., 1994). In addition, FGF-8 and FGF-17 are produced by anterior medial cells of neocortical primordium, a signaling center important for antero-posterior patterning of the developing neocortex (Crossley and Martin, 1995; Xu et al., 1999).

4.3 FGFs and Spinal Cord Development

Most of the findings regarding FGF function during spinal cord development come from experiments performed in chicken embryos. During development and adulthood, survival and function of spinal motoneurons are dependent on trophic factors such as Ciliary Neurotrophic Factor (CNTF), Leukaemia Inhibitory Factor (LIF), neurotrophins, FGFs, and Insulin-like Growth Factors (IGFs) (Eisen, 1999). These neurons express different Fgf ligands (for example *Fgf-1* and *Fgf-9*) and Fgfrs. *In vivo* and *in vitro* experiments have shown that FGF-2, FGF-5 and FGF-9 promote cell survival (reviewed by Ford-Perriss et al., 2001). Furthermore, experimental evidences suggest that FGF-2 and FGF-9 act in a dose-dependent manner to regulate terminal differentiation of cholinergic spinal cord neurons (Grothe et al., 1991; Kanda et al., 1999). However, as FGF-2 deficient mice do not display defects in motoneuron functions (Dono et al., 1998), it remains to be established whether FGF-2 plays an essential role during motoneuron development. In contrast, FGF-2 deficient mice display an impaired baroreceptor reflex function, which points to defects in the autonomic compartment of the spinal cord (Figure 8; Dono et al., 1998).

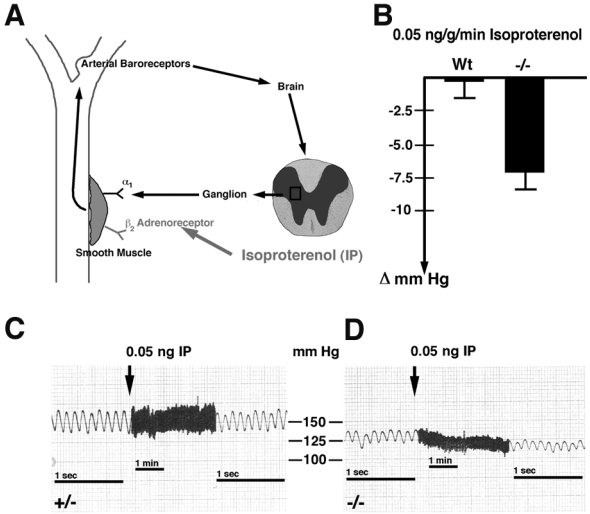


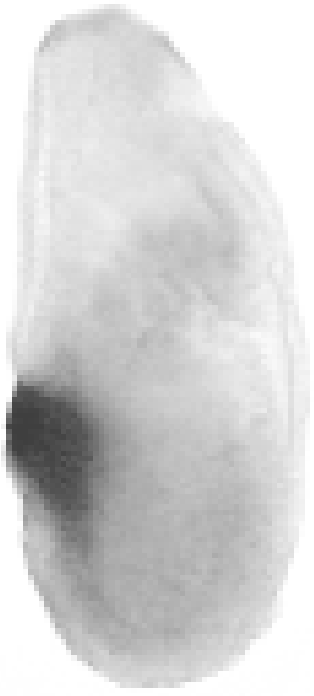
Figure 8. *Defective reflex control of blood pressure in FGF-2 deficient mice. Modified from Dono, 1998. Adult FGF-2 mutant mice fail to compensate a hypotensive challenge induced by intravenous infusion of the b_1/b_2-adrenoreceptor agonist isoproterenol. (A) Schematic representation of baroreceptor reflex (neural control of blood pressure). (B-D) Effects of isoproterenol infusions on resting blood pressure (measured as arterial blood pressure) in awake control and FGF-2-deficient mice. (B) Average responses to isoproterenol infusion in wild-type (Wt) and FGF-2 deficient mice (-/-). Note that resting blood pressure was significantly reduced by isoproterenol in FGF-2 deficient but not in control mice. (C) Typical recording in a heterozygous mouse. (D) Typical recording in an FGF-2 deficient mouse. The arrows indicate the time when the isoproterenol infusion was started.*

5 Aim of this thesis

The major aim of this thesis was to elucidate the functions of the glypican family member Glypican-4 (GPC-4) and its interaction with FGF signaling during vertebrate CNS development. I first analyzed the spatial and temporal distribution of *Gpc-4* during early mouse embryogenesis (chapter 2). In light of its expression during CNS development, I then analyzed its *in vivo* function during early forebrain patterning using *Xenopus* embryos as model. *Xenopus* embryos allow a rapid assessment of gene function through gain- and loss- of function studies (chapter 3). I show that GPC-4 binds and is able to positively modulate the function of FGF-2 during dorsal forebrain development (chapter 3). Mice deficient for FGF-2 function are affected by an autonomic dysfunction in addition to defects in neocortex formation. I have therefore, analyzed the role of FGF-2 signaling in development and function of the autonomic nervous system in the spinal cord (chapter 4).

Glypican-4 expression pattern

chapter 2



Expression of the *Glypican-4* gene during early mouse embryogenesis

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In preparation for GEP

Abstract

Glypicans are a family of heparan sulfate proteoglycans that are essential modulators of cell signaling during development. Little is known about their *in vivo* functions during central nervous system (CNS) morphogenesis. We show here that the *Glypican-4* (*Gpc-4*) gene is the only murine glypican gene highly expressed by the cells of the anterior visceral endoderm (AVE) and the anterior neural ridge (ANR), two signaling centers essential for induction and patterning of the forebrain. Furthermore, *Gpc-4* transcripts are also present in the telencephalic progenitor cells underlying the ANR, which suggests possible roles for GPC-4 in the regulation of cell signaling in both the producing and responding cells. As development proceeds, *Gpc-4* expression persits in progenitor cells and early post-mitotic neurons of the cerebral cortex. These results indicate that GPC-4 may be part of the signaling network regulating cortical layer development.

1. Results and discussion

Glypican-4 (*Gpc-4*) belongs to a family of heparan sulfate proteoglycans (HSPGs) that are linked to the cell surface through a covalent glycosyl-phosphatidylinositol (GPI) linkage. To date, six glypican genes have been identified in mammals (*Glypican-1* to *Glypican-6*). All glypican family members are secreted and GPI anchored and in addition to their conserved amino acid sequence contain two to four consensus sites for the attachment of glycosaminoglycan (GAG) chains (reviewed by Song and Filmus, 2002). Most likely, these cell surface molecules modulate the activity ranges of various morphogens and growth factors such as Fibroblast growth factors (FGFs), Bone morphogenetic proteins (BMPs), Wnts, Hedgehog (HHs) and Insuline-like growth factors (IGFs). (Perrimon and Bernfield, 2000). Several Glypicans are expressed during development (reviewed by De Cat and David, 2001), in particular, during central nervous system (CNS) morphogenesis. For example, neural precursor cells generally express *Gypican-1* (*Gpc-1*; Litwack et al., 1998) and *Glypican-4* (*Gpc-4*; this study and Watanabe et al., 1995) in the ventricular zone of the cerebral wall. Neural precursors of the striatal primordium express *Glypican-5* (*Gpc-5*; Saunders et al., 1997). In addition, *Glypican-2* (*Gpc-2*; Stipp et al., 1994), *Gpc-1* and *Gpc-5* transcripts are also found in early post mitotic neurons during differentiation. Recently, we have shown that *Xenopus Gpc-4* is highly expressed during gastrulation and neurulation and in particular by the anterior neural plate cells during forebrain patterning. In agreement with its temporal and spatial distribution, we have established that GPC-4 is required for positive modulation of FGF signaling during dorso-ventral forebrain patterning in *Xenopus* embryos (Galli, 2003 and chapter 3).

In this report, we characterize the spatial and temporal distribution of *Gpc-4* transcripts during early mouse embryogenesis. In addition, we compare its expression pattern to that of other glypican family members.

As shown in Fig. 1A, *Gpc-4* is not expressed by blastocyst stage mouse embryos while specific transcripts are amplified in mouse embryos at embryonic day 9.5 (E 9.5; Fig. 1A). These results indicate that *Gpc-4* expression may be initiated during or shortly after implantation. Therefore, we analyzed *Gpc-4* expression in post implantation mouse embryos by whole mount *in situ* hybridization. Before the onset of gastrulation (E 5.6) *Gpc-4* transcripts become progressively restricted to the region where the anterior visceral endoderm (AVE; arrow in Fig. 1B) will develop. The AVE is the extra embry-

onic tissue that overlies the epiblast destined to form the anterior CNS (forebrain). It has been shown that the AVE together with the node and its derivatives is one of the signaling centers essential for the correct specification of the anterior neural ectoderm (reviewed by Beddington and Robertson, 1999). In addition, embryological and genetic studies show that the AVE is necessary to initiate anterior neural plate patterning (Martinez-Barbera and Beddington, 2001). Analysis of *Gpc-4* transcripts distribution on transverse sections of early streak embryos (E 6.6) confirmed that AVE cells express *Gpc-4* while it is not expressed by the epiblast (insert in Fig. 2). *Otx-2* is one of the transcription factors expressed by the AVE and required for positioning the AVE at the anterior side of the gastrulating embryo (Perea-Gomez et al., 2001). In OTX-2 deficient embryos *Gpc-4* is expressed in distal visceral endoderm in contrast to anterior expression in wild-type littermate embryos (Fig. 2). Similar to *Gpc-4*, AVE markers such as *Hex* (Thomas et al., 1998) and *Cer1* (Belo et al., 1997) remain distally in OTX-2 deficient embryos (Perea-Gomez et al., 2001). These results establish Gpc-4 as a "bonafide" AVE marker.

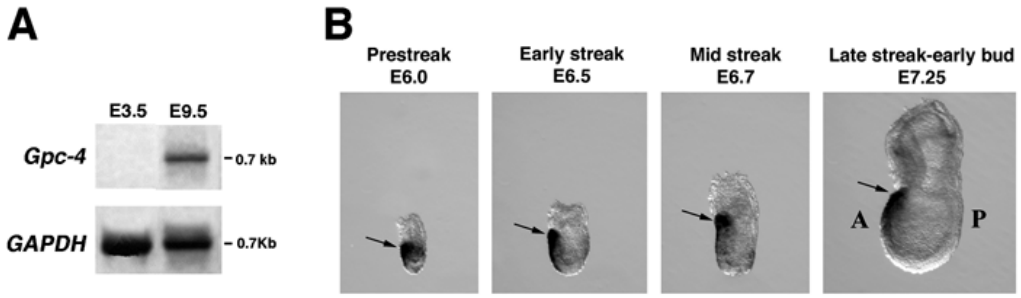


Figure 1. *Early expression of Gpc-4 during embryogenesis. (A) RT-PCR using Gpc-4 specific oligonucleotides. Gpc-4 is expressed in E 9.5 embryos (E 9.5) but not in blastocysts (E 3.5). GAPDH was used to normalize samples. (B) Whole mount in situ hybridization show the spatial and temporal distribution of Gpc-4 transcripts at early post-implantation stages. Arrow point to the high expression of Gpc-4 in the AVE. Anterior (A) is on the left, posterior (P) is on the right.*

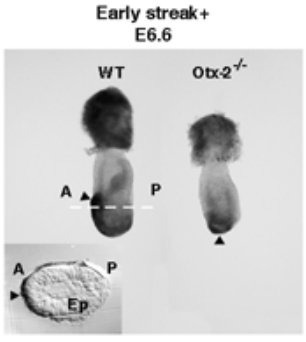


Figure 2. *Gpc-4 is an AVE marker. Expression of Gpc-4 detected by whole mount in situ hybridization at early streak-plus stage (E6.6) in wild type (on the left side) and in Otx-2$^{-/-}$ embryo (on the right side). The broken line indicates the level of the section represented in the inset (bottom left side). The inset shows Gpc-4 expression only in the extra-embryonic (AVE, arrowhead) and not in the epiblast (ep). Note that in the Otx-2$^{-/-}$ embryo the AVE did not move anterior and Gpc-4 is express distally (compare arrowhead in wt and Otx-2$^{-/-}$). Anterior (A) is on the left and posterior is on the right (P).*

To determine the distribution of other glypican family members in early post-implantation mouse embryos available ESTs for Gpc-1, Gpc-3 and Gpc-6 were used for

whole mount *in situ* hybridization. These studies show that none of these glypican family members is expressed during early gastrulation stages (data not shown).

Expression of *Gpc-4* persists in early somites stage mouse embryos (Fig. 3). At E 8.0 *Gpc-4* transcripts are restricted to two distinct regions of the developing embryo (Fig. 3A): anteriorly they are confined to the head region while posteriorly they are restricted to the gastrulating tail bud (inset in Fig. 3A). Interestingly, the anterior distribution of *Gpc-4* transcripts overlaps with the expression of *Fgf-8* (Fig. 3B) in the anterior neural ridge (ANR) and *Bf-1* in the anterior neuroectoderm (Fig. 3C). The ANR is the rostral-most junction between neural and non-neural neuroectoderm, and forms a key-signaling center regulating forebrain patterning (reviewed by Rubenstein et al., 1998). Cells of the ANR produce FGF-8 that in turn induces the expression of *Bf-1* in the underlying neural plate cells (Shimamura and Rubenstein, 1997). The distribution of *Gpc-4* transcripts at this developmental stage suggests that GPC-4 may modulate signaling in both signaling and responding cells. In contrast to *Gpc-4*, *Gpc-1* (Fig. 3D) is expressed at low levels throughout the entire antero-posterior (A-P) axis of the E 8.0 mouse while *Gpc-3* transcripts were only found in the presomitic mesoderm (Fig. 3E). Interestingly, *Gpc-6* is also expressed by anterior neuroectoderm cells but in a domain more posterior than *Gpc-4* (compare Fig. 3F to 3A). This *Gpc-6* expression overlaps with the presumptive midbrain territory. In contrast, *Gpc-2* is not expressed during embryonic day 8.0 (data not shown).

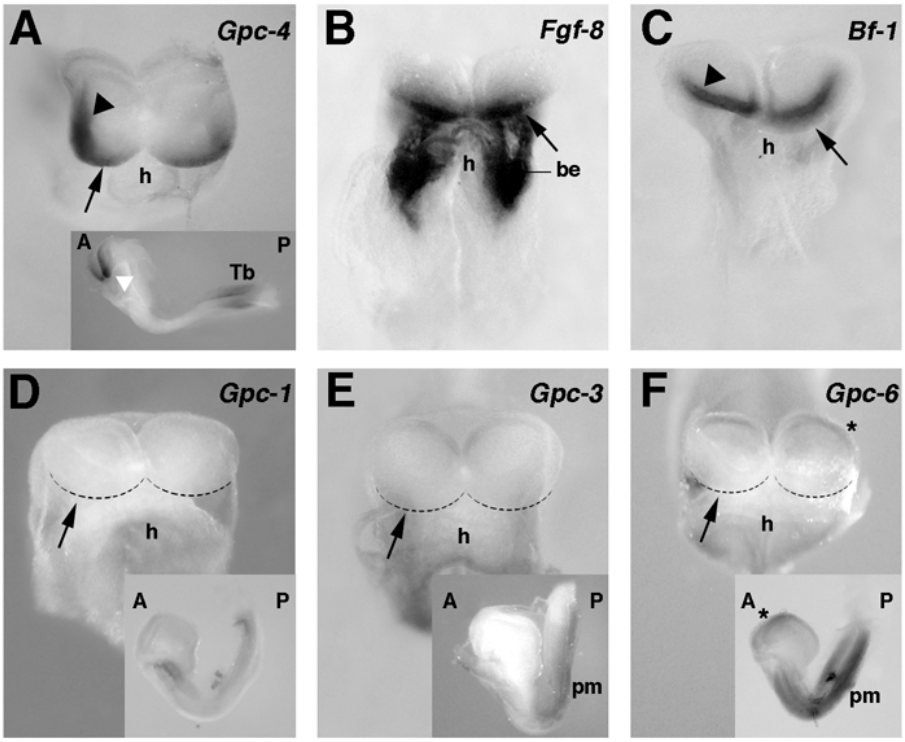


Figure 3. *Expression of Gpc-4 in the anterior neural fold (A-C). (A) Distribution of Gpc-4 transcripts at 4 to 6 somites stage. Gpc-4 is expressed in the anterior neural folds (white arrowhead in the inset). This expression domain encompasses the one of Fgf-8 in the ANR (compare panel A to B, arrow) and also the expression domain of Bf-1 in the responding ANR cells (compare panel A to C, black arrowhead). Note that Gpc-4 is also express in the tail bud (Tb) (inset panel A). (B) Expression of Fgf-8 in the ANR (arrow) and in the branchial ectoderm (be). (C) Expression of Bf-1 in the developing telencephalon and not in the ANR (arrow). (D-F) Other glypican family members are not express in the anterior neural fold (compare arrow in A to D, F, E). (D) Gpc-1 is expressed at low levels throughout the embryo. (E) Gpc-3 is highly expressed in the presomitic mesoderm but not in the developing forebrain. (F) Gpc-6 transcripts in the presomitic mesoderm (pm) and in the midbrain (asterisk). The broken lines in D, E, and F mark the anterior neural fold. A to F: frontal view of E 8.0 mouse embryos. Inserts in A, D, E, and F show lateral view of e 8.0 mouse embryos. A: anterior is to the left, P: posterior is to the right. Other abbreviation: h, heart.*

Expression of *Gpc-4* in neural tissue persists during neurogenesis. Shortly after neural tube closure (E 9.0) *Gpc-4* transcripts are abundant in the developing forebrain in both dorsal and ventral regions (Fig. 4A, B). Other domains of *Gpc-4* expression include the first and second branchial arches, somites and the posterior tail bud. In contrast, *Gpc-1* is expressed widely within the developing CNS at E 9.0 (Fig. 4C). In agreement with earlier studies by Pellegrini et al. (1998), *Gpc-3* is expressed by somites, first branchial arches and in the posterior diencephalic wall of the developing brain (Fig. 4D). Finally, *Gpc-6* transcripts are present in mesenchymal tissues and only at low levels in the neuroepithelium of the developing CNS (Fig. 4E). At stage E 10.25 *Gpc-4* remains abundantly expressed by the developing forebrain and in somites and a novel site of expression appears in the midbrain (Fig. 4F). In contrast, expression of *Gpc-1*, *Gpc-3* and *Gpc-6* remains unchanged at this developmental stage (Fig. 4H, I and J).

In situ hybridization analysis of serial coronal sections of E 12.5 mouse brains show that high level of *Gpc-4* transcripts are present in the neuroepithelium lining the telencephalic lateral ventricles (ventricular zone; Fig. 5A). Within the ventricular zone (VZ), the neural progenitors of the cerebral cortex proliferate and cell fates are determined (Parnavelas, 2000). *Gpc-4* expression in cortical progenitors persists during subsequent development, as also previously reported by Hagihara et al. (2000) (Fig. 5A). Interestingly, cells of the developing cortical plate specifically express *Gpc-4* during E 14.5 to E 16.5 (Fig. 5B and data not shown).

These studies identify GPC-4 as prime mouse glypican gene expressed by two signaling centers important for induction and patterning of the developing forebrain, the AVE and the ANR. The AVE is source of Wnt, BMP and Nodal signal antagonists (Borges et al., 2002; Glinka et al., 1998; Zakin et al., 2000), while the ANR produces FGFs. Therefore GPC-4 could function during these early determinate patterning events to modulate signaling from both AVE and ANR, similar to what we have recently shown for its function during dorsal forebrain patterning in *Xenopus* embryos. Later during development, *Gpc-4* is predominantly expressed in neural precursor cells of the developing cerebral cortex, where it may also modulate response to morphogenetic signals. The expression pattern of *Gpc-4* indicates that this signaling modulator functions to regulate major signaling pathways in a dynamic and developmental stage specific manner.

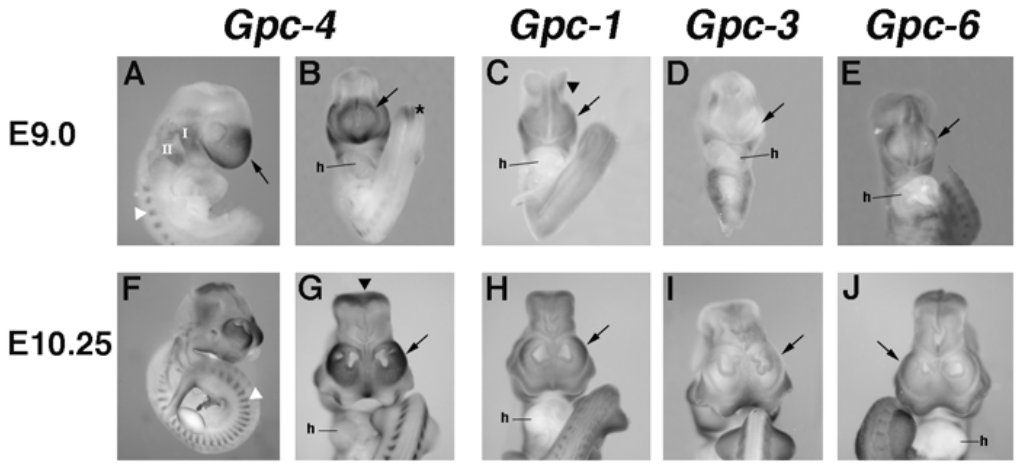


Figure 4. *Gpc-4 is highly expressed in the developing forebrain. (A, B) Gpc-4 is expressed in E 9.0 mouse embryo in the forebrain (arrow), in the first (I) and second branchial arches (II), in the somites (white arrowhead) and in the tail bud (asterisk). (C) Gpc-1 distribution at E9.0. Note that Gpc-1 is expressed by the neuroepithelium of the entire CNS. Arrow indicates Gpc-1 transcripts at the level of the forebrain. The black arrowhead shows the expression in the midbrain. (D) Gpc-3 is not expressed in CNS but only in mesenchymal derivatives. (E) Gpc-6 is expressed at low levels by the neuroepithelium. (F, G) Gpc-4 expression in the developing forebrain (arrow), in the midbrain (black arrowhead) and somites (white arrowhead) of E 10.25 mouse embryo. (H) Gpc-1 expression at E 10.25 mouse embryo in the developing brain (arrow). (I) Gpc-3 expression at E 10.25 mouse embryo in the developing brain (arrow). (J) Gpc-6 expression at E 10.25 mouse embryo in the developing brain (arrow). Note that Gpc-4 is the only glypican gene predominantly expressed in the developing telencephalon (compare arrow in C, D, E to the ones in A and B for E 9.0 mouse embryos and compare arrow in H, I, J and the ones in F and G for E 10.25 mouse embryos). A, F are side view with anterior to the right; B-E are frontal view, G-J are frontal view of the head region. Other abbreviation: h, heart.*

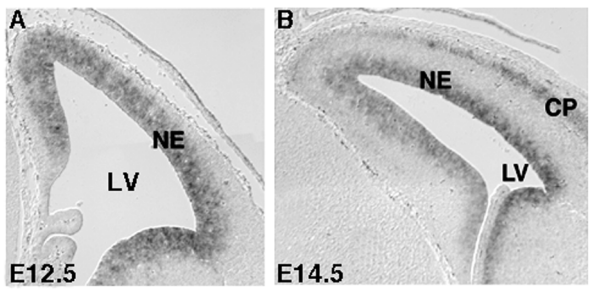


Figure 5. *Neural precursor cells of the developing cerebral cortex express Gpc-4. In situ hybridization performed on coronal sections at the level of the telencephalic vesicles of E 12.5 (A) and E 14.5 (B) mouse embryonic brains. Gpc-4 transcripts are present in the ventricular zone of the lateral ventricle at both stages. Note that at E 14.5 Gpc-4 is also expressed by cells of the cortical plate. Abbreviations: LV, lateral ventricle; NE, neuroepithelium; CP, cortical plate.*

2. Experimental procedures

2.1. Embryos and cell culture

Pre and post-implantation embryos were obtained from F1 crosses between C57Bl6 females and CBA males. E8.0 to E10.0 mouse embryos were generated from F1 CD1 crosses. Otx-2 heterozygous mutant mice (Acampora et al., 1995) on C57BL6/CBA current background (from K. Lawson) were intercrossed to obtain homozygous mutant embryos. For RT-PCR analysis: blastocysts were collected from the uterus as described in Hogan et al. (Hogan et al., 1986).

2.2. RNA isolation, cDNA synthesis and PCR

Total RNA was extracted from blastocysts and total embryos according to the Atlas^{TM} Pure Total RNA Labeling System User Manual (Clontech). RNA extraction from blastocysts: 30 µg tRNA was added as a carrier and 3 µg of RNA were used to synthesize cDNA. For ES cells and E9.5 mouse embryos, 12 µg of total RNA sample were reverse transcribed with oligo(dT) and Superscript II (Gibco-BRL). 1/10 of the resulting cDNA was used for PCR amplification using Gpc-4 gene specific primers designed in exon 1 (5'-CTGCTTTCCATCGGGTCTCATTCTG-3') and exon 4 (5'-AGGTCCTGGCTGCAACA AATGCTC-3'). Samples were normalized by PCR using GAPDH primers as it has been shown to be a validating housekeeping gene (Fig. 1A; Murphy and Polak, 2002).

2.3. Identification of Glypicans cDNA and antisense digoxygenin-labeled RNA

Glypican-1, 2, 3, 6 clones were identified by a BLAST search of the GenBank EST database using the corresponding cDNA sequence available from the NCBI database (Glypican-1, Gpc-1: accession number AF185613; Glypican-2, Gpc-2: accession number NM_172412; Glypican-3, Gpc-3: accession number BC036126 and Glypican-6, Gpc-6: accession number AF105268). The corresponding EST clones with highest homology were obtained from the RZPD Consortium (www.rzpd.de/cgi-bin/db). Gpc-1: RZPD clone ID IMAGE480151; Gpc-2: RZPD clone ID IMAGp952N2135; Gpc-3: RZPD clone ID IMAGp998O1010961; Gpc-6: RZPD clone ID IMAGp 998I1610907. These ESTs clones were confirmed by restriction enzyme digestion. Antisense digoxygenin-labeled RNA were synthesized according to the manufacturer's instruction (Boheringer) from the following templates: Gpc-1 and Gpc-2 were cloned in pT7 T3D-PacI, lynearized with EcoRI and transcribe with T3 RNA polymerase; Gpc-3 and Gpc-6 both cloned in pCMVSport6 were cut with PstI and SalI, respectively and transcribed with T7 RNA polymerase. These antisense probes were tested performing whole mount *in situ* hybridization at later developmental stages to ensure for each gene the published expression pattern .The Glypican-4 (Gpc-4) cDNA was previously cloned by RT-PCR in our laboratory and the antisense digoxygenin-labeled RNA containing the 3' coding region of the gene was obtained using BamHI and transcribe with T3 RNA polymerase.

2.4. RNA in situ hybridization

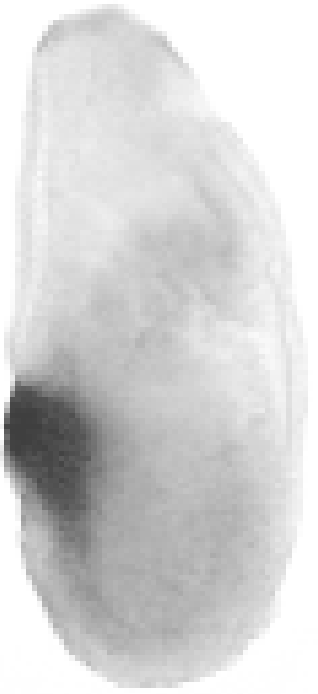
Whole mount RNA *in situ* hybridization using digoxygenin-labeled RNA probes was performed as previously described (Haramis et al., 1995). For young embryos (E5.6 to E7.25 mouse embryos) minor modification were used: embryos were preblock in 10% sheep serum in TBST for 2 hours, α-digoxygenin (α-Dig) was used 1:2000. After

α-Dig incubation embryos were washed 5 times 60 minutes and overnight in TBST-2mM Levamisole at RT. RNA *in situ* hybridization on sections: coronal sections of 7µm were processed and hybridized using digoxygenin-labeled riboprobes as described (Dono et al., 1998).
For sectioning of embryos after whole mount *in situ* hybridization, embryos were first dehydrated through a graded series of ethanol and embedded in cold glycol methacrylate (Technovit 8100) overnight at 4^{o}C. Transversal sections were cut at 10µm.

Acknowledgements
We are grateful to Jeroen Korving for technical expertise. This study was supported by the faculty of biology, University of Utrecht.

Glypican-4 and forebrain patterning

chapter 3



Glypican-4 modulates FGF signalling and regulates dorso-ventral forebrain patterning in *Xenopus* embryos

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SUMMARY

Heparan sulphate proteoglycans such as Glypicans are essential modulators of intercellular communication during embryogenesis. In *Xenopus laevis* embryos, the temporal and spatial distribution of *Glypican-4* (*Gpc-4*) transcripts during gastrulation and neurulation suggests functions in early development of the central nervous system. We have functionally analysed the role of *Xenopus Gpc-4* by antisense morpholino oligos and show that *Gpc-4* is part of the signalling networks patterning the forebrain. Depletion of GPC-4 proteins results in a pleiotropic phenotype affecting both primary axis formation and early patterning of the anterior central nervous system. Molecular analysis shows that posterior axis elongation during gastrulation is affected in GPC-4 depleted embryos, while head- and neural induction are apparently normal. During neurulation, loss of GPC-4 disrupts expression of dorsal forebrain genes like *Emx-2*, whilst genes marking the ventral forebrain and posterior central nervous system remain expressed. This loss of GPC-4 activity also causes apoptosis of forebrain progenitors during neural tube closure. Biochemical studies establish that GPC-4 binds FGF-2 and modulates FGF signal transduction. Inhibition of FGF signal transduction by adding the chemical SU5402 to embryos from neural plate stages onwards phenocopies the loss of gene expression and apoptosis in the forebrain. We propose that GPC-4 regulates dorso-ventral forebrain patterning by positive modulation of FGF signalling.

INTRODUCTION

The vertebrate forebrain consists of anatomically and functionally distinct domains patterned along their antero-posterior and dorso-ventral axis (reviewed by Rubenstein et al., 1998). For example, the telencephalic subpallium and hypothalamus are ventral forebrain structures, while the telencephalic pallium and epithalamus are located dorsally. As these different forebrain structures arise from the anterior neural plate (Rubenstein et al., 1998), the identification of the mechanisms that control anterior neural plate regionalization is central to understanding morphogenesis of the forebrain. Fate mapping studies and molecular analysis of embryos from different vertebrate species have established that the anterior neural plate is regionalized through restricted activation of key transcription factors (Rubenstein et al., 1998). For example, cells of the medial anterior neural plate activate the Nkx-2.1 homeobox gene and expression persists later in the presumptive ventral telencephalon and hypothalamus (Hollemann and Pieler, 2000; Wilson and Rubenstein, 2000). Genetic analysis has shown that *Nkx-2.1* is essential for ventral forebrain identity (Wilson and Rubenstein, 2000). In contrast, the presumptive dorsal forebrain territory predominantly expresses *Emx-1* and *Emx-2* (Pannese et al., 1998; Simeone et al., 1992). Dorsal forebrain patterning is disrupted in *Emx* deficient mouse embryos (Bishop et al., 2003; Yoshida et al., 1997) and mutations in the human *Emx-2* gene are linked to schizencephaly, a congenital brain malformation characterized by clefts in the human cerebral cortex (Brunelli et al., 1996).

Different types of signalling molecules, their antagonists and receptors regulate regionalization of the anterior neural plate (Rubenstein et al., 1998; Wilson and Rubenstein, 2000). For example, antagonism of WNT signalling is necessary for correct subdivision

of the anterior neural plate into telencephalon, diencephalon and eye territories (Houart et al., 2002; Wilson and Rubenstein, 2000). BMP-7 and SHH signalling by the prechordal mesoderm induces *Nkx-2.1* and directs neural plate cells towards hypothalamic fate (Wilson and Rubenstein, 2000). Other BMP family members are produced by the non-neural ectoderm adjacent to the anterior neural plate and regulate expression of anterior neural markers and dorsal forebrain development in a dose-dependent manner (Hartley et al., 2001; Wilson and Rubenstein, 2000). Accordingly, inactivation of the BMP antagonists *Chordin* and *Noggin* in mouse embryos causes defects in forebrain patterning (Wilson and Rubenstein, 2000). The role of FGFs during forebrain morphogenesis appears widespread as several *Fgfs* like *Fgf-8*, *Fgf-2* and *Fgf-9* are expressed by the anterior neural plate and forebrain primordia (reviewed by Dono, 2003). For example, embryological and genetic studies have shown that FGF-8, produced by the anterior neural ridge participates in inducing the telencephalon and differentiation of anterior midline cells (Eagleson and Dempewolf, 2002; Rubenstein et al., 1998). Moreover, FGF-8 acts in a dose-dependent manner to control cell survival in the developing forebrain in the mouse (Storm et al., 2003). In zebrafish embryos, FGFs also regulate dorso-ventral forebrain patterning as evidenced by genetic analysis (Shanmugalingam et al., 2000) and transient inhibition of FGF signal transduction by the chemical inhibitor SU5402 (Shinya et al., 2001). These latter studies showed that FGF-8 and FGF-3 cooperate to promote *Nkx-2.1* expression and morphogenesis of the ventral telencephalon. In addition, FGF-8 and FGF-2 can induce dorsal forebrain genes such as *Emx*-1 in neuralized *Xenopus* animal cap explants (Lupo et al., 2002).

Cell-cell signalling interactions are modulated by cell surface proteins including glypicans. Glypicans like other heparan sulphate proteoglycans (HSPG) bind FGFs, WNTs and BMPs through their heparan sulphate glycosaminoglycan (HS-GAG) side-chains (Hagihara et al., 2000; Nybakken and Perrimon, 2002). It has been proposed that glypicans regulate cell signalling by either promoting or stabilizing the interactions of ligands with their cognate high affinity receptors (Nybakken and Perrimon, 2002). For example, vertebrate Glypican-1 binds FGFs, thereby favouring assembly of the ligand-receptor complex (Steinfeld et al., 1996). Alternatively, glypicans such as *Drosophila Dally-like* may shape ligand gradients by restricting their diffusion within the extra-cellular matrix (Baeg et al., 2001). *Dally*, another *Drosophila* glypican regulates imaginal disc patterning and morphogenesis by positive and differential modulation of *wingless* (*wg*) and *decapentaplegic* (*dpp*) signalling (Nybakken and Perrimon, 2002). Genetic analysis of the zebrafish *knypek* shows that this glypican functions to potentiate non-canonical WNT signalling. By modulating WNT-11 activity, *knypek* regulates the convergent-extension movements during zebrafish gastrulation (Topczewski et al., 2001). In mice, Glypican-3 is required for cellular response to BMP and FGF signalling during organogenesis (Grisaru et al., 2001). Furthermore, several glypican family members are expressed in the developing central nervous system (CNS; reviewed by Song and Filmus, 2002). One of them, Glypican-4 (*Gpc-4*) is predominantly expressed in the presumptive forebrain territory during head-fold stages in mouse embryos (Galli et al., unpublished). Subsequently, its expression persists in neuronal progenitors of the developing forebrain (Hagihara et al., 2000).

In the present study, we functionally analyse the *Xenopus Gpc-4* gene by translational

inhibition through specific antisense morpholino oligos and by gain-of-function experiments. Depletion and increase of GPC-4 activity in developing embryos results in gastrulation and axis elongation defects similar to the phenotype of *knypek* deficient zebrafish embryos. Furthermore, we identify GPC-4 as a key regulator of dorso-ventral forebrain patterning. In particular, loss of GPC-4 activity results in down-regulation of dorsal forebrain identity genes from early neural plate stages onwards and massive cell death in the anterior CNS during neural tube closure. We show that GPC-4 binds FGF-2 and inhibition of FGF signalling by SU5402 (Mohammadi et al., 1997) results in dorsal forebrain phenotypes similar to those of GPC-4 depleted embryos. We conclude that establishment and patterning of the dorsal forebrain territory requires modulation of FGF signalling by GPC-4.

MATERIALS AND METHODS

Identification of the *Xenopus* Gpc-4 gene and generation of antisense morpholino oligos

A *Xenopus laevis Gpc-4* cDNA clone was identified by a BLAST search of the GenBank EST database using the mouse *Gpc-4* sequence (Watanabe et al., 1995). The corresponding clone (RZPD clone ID:IMAGE998F078241Q2; see: www.rzpd.de/cgi-bin/db) was obtained from the RZPD Consortium. The entire EST (2569 bases) was sequenced to show that it contains the complete ORF and part of the 5' and 3'UTRs. The *Xenopus Gpc-4* DNA sequence is 100% identical to the sequence available from the NCBI database (Accession number ABO82534). The 5´UTR of an additional *Xenopus Gpc-4* allele was isolated by 5´RACE PCR (GeneRacer kit, Invitrogen). Based on the sequence of the two alleles, a 25-nucleotide antisense morpholino oligo against the 5'UTR of *Gpc-4* (Gpc-4Mo) was designed to inhibit translation from both alleles (Gene Tools, USA). The Gpc-4Mo is complementary to a sequence 70 bases upstream of the ATG start codon (5´-TGCAAAGTGCTGAGAATCCCCTAGT-3'). An antisense morpholino oligo against the human β-globin gene (CoMo) was used as standard control and injected at the same concentration as the Gpc-4Mo. Injection of 60 to 80 ng of Gpc-4Mo per embryo gave rise to the phenotypes described in this study. Injection of a second independent morpholino complementary to the *Gpc-4* RNA sequence surrounding the ATG start codon resulted in similar phenotypes (data not shown; see also Ohkawara et al. 2003). In vitro translation of capped *Xenopus Gpc-4* mRNA: transcripts were synthesized using SP6 RNA polymerase (Ambion). 50 ng of the capped mRNA (Ambion) was translated by using rabbit reticulocyte lysate (Promega) and [^{35}S] methionine in the presence of increasing amounts of Gpc-4Mo (0.1, 0.4, 1.6 and 4 μg) or equal amounts of CoMo.

Embryo manipulations

Xenopus laevis eggs were fertilized and cultured following standard protocols (Sive et al., 2000). For the functional analysis of GPC-4, two cells stage embryos were injected with 30-40 ng of antisense morpholino oligo per blastomere at the animal pole. To test the efficiency of the Gpc-4Mo in vivo, 600 pg of capped *Gpc-4GFP* mRNA was injected in two-cell stage embryos. Subsequently, a total of 100 ng of Gpc-4Mo or CoMo was

injected in either one or both blastomeres. Overexpression studies: mouse *Gpc-4* capped mRNA (*mGpc-4*) was injected into the animal pole of one cell stage embryo at different concentration ranging from 0.25 ng to 2.5 ng; "control" embryos were uninjected. To rescue the molecular and morphological defects of Gpc-4Mo injected embryos, a total of 60 ng of Gpc-4Mo (or CoMo) was injected into both blastomeres of two-cell stage embryos. After completion of the second division, a total of 800 pg of the mouse *Gpc-4* capped mRNA was injected into the two dorsal blastomeres. Inhibition of FGF signalling by SU5402 (Calbiochem) treatment of embryos: embryos were cultured in normal medium (MBS; Sive et al., 2000) until the onset of neurulation (stage 13). From stage 13 onwards embryos were cultured in MBS supplemented with SU5402 (0.1 mg/ml final concentration; dissolved in DMSO) or DMSO (same final concentration) until harvesting them between stages 15 and 21-22 for analysis.

Whole mount in situ hybridisation and detection of apoptotic cells

Whole mount in situ hybridisation was performed as previously described (Sive et al., 2000) and pigment granules were bleached as described (Song and Slack, 1994). Apoptotic cells were detected by using the in situ cell death detection kits (sections: fluorescein; whole mount: POD, Roche) according to the manufacturer instructions with only minor modifications.

Proteins binding assays and immunoblot analysis

For binding assays, NIH3T3 cells were transfected with 10 μg of the mouse Gpc-4-C-myc plasmid. Cells were lysed 36 hours after transfection in PBS containing 0.5% NP40. After sonication, GST-FGF-2 binding assays were performed as described (Fumagalli et al., 1994). Proteins were separated by 8% SDS-PAGE and c-myc epitope tagged GPC-4 detected by anti-myc antibodies. For analysis of ERK and SMAD1 phosphorylation levels embryos were lysed and proteins separated on a 15% gel. Proteins were immunoblotted using anti-pSMAD1 (Persson et al., 1998), anti-pERK (Cell Signalling) and anti-α–TUBULIN antibodies (Sigma).

RESULTS

Distribution of *Gpc-4* transcripts in *Xenopus* embryos

We identified the *Xenopus Gpc-4* gene by searching an expressed sequence tag (EST) database with the mouse *Gpc-4* cDNA (see Materials and Methods). The predicted *Xenopus* GPC-4 protein core is encoded by 556 amino acids and is orthologous to mouse *Gpc-4* (71.4% identity, 81% similarity; (Watanabe et al., 1995) and most likely to zebrafish *knypek* (57.4% identity, 71% similarity; (Topczewski et al., 2001).

Xenopus Gpc-4 is a maternally expressed gene as transcripts are detected in the animal hemisphere from two-cell up to blastula stages (Fig. 1A and data not shown). At the onset of gastrulation, expression expands to the marginal zone (Fig. 1B). During progression of gastrulation (Fig. 1C,D), *Gpc-4* transcripts become progressively localized to the dorsal side of the embryo. In particular, high levels of *Gpc-4* transcripts are detected in the area of Spemann's organizer during gastrulation (Fig. 1C,D). At this stage, the *Gpc-4* transcript domain encompasses the ones of *Noggin* (compare Fig. 1D,E; Smith and Harland, 1992) and *Chordin* (data not shown), which indicates that *Gpc-4* is expressed by the prechordal endomesoderm and chordamesoderm (see also Ohkawara et al. 2003). In addition, the *Gpc-4* expression domain also encompasses the one of *Sox-2* (Mizuseki et al., 1998), an early marker for neural fates (compare Fig. 1D,F). This latter result shows that presumptive neuroectodermal cells express *Gpc-4* during neural cell fate specification.

During neurulation, *Gpc-4* expression is high in presomitic mesoderm and the developing CNS (Fig. 1G,I,J,L). In the posterior neural plate, *Gpc-4* expressing neuroectodermal cells form two longitudinal stripes spanning the presumptive spinal cord (white arrow in Fig. 1G). In the anterior neural plate, *Gpc-4* expressing cells form a single arch, which crosses the midline (black arrow in Fig. 1G) and borders the *Fgf-8* expressing anterior neural ridge (data not shown). This anterior *Gpc-4* expression domain overlaps that of *Bf-1* (compare Fig. 1G,H), which is the earliest known marker for telencephalic cell fates (Bourguignon et al., 1998). *Gpc-4* transcripts are present in both the epithelial and sensory layers of the neuroectoderm (Fig. 1I), while expression in the underlying prechordal plate fades away (white arrowhead in Fig. 1I).

By mid-neurulation (Fig. 1J), the anterior *Gpc-4* expression resolves into two distinct domains. The posterior one overlaps with *Emx-2* (compare Fig. 1J,K), one of the earliest genes expressed in presumptive dorsal forebrain territories (Pannese et al., 1998). In the developing dorsal forebrain, *Gpc-4* transcripts persist up to early neural tube stages (Fig.1L and data not shown). From tail bud stages onwards, other predominant sites of *Gpc-4* expression include the developing branchial arches, somites and pronephric ducts (data not shown).

GPC-4 is required for gastrulation and nervous system patterning in *Xenopus* embryos

An antisense morpholino oligonucleotide directed against the 5' leader of the *Xenopus Gpc-4* mRNA was used to block GPC-4 protein translation. Initially, we assessed the efficiency of two candidate oligos (see Materials and Methods). One of these, namely

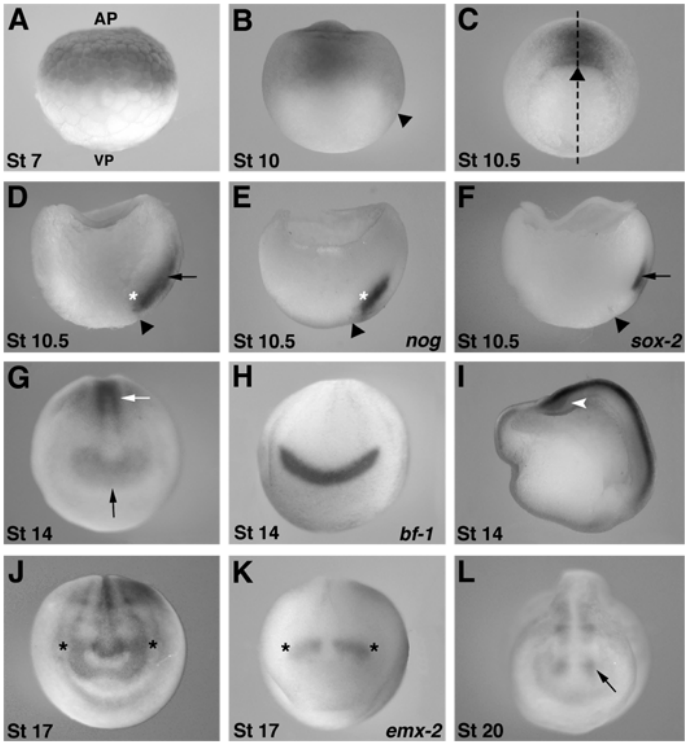


***Figure 1**. Gpc-4 expression during early development of Xenopus embryos. Arrowheads in (B) to (F) point to the dorsal blastopore lip. (A) Blastula (stage 7) showing localization of Gpc-4 transcripts in the animal hemisphere. AP: animal pole VP: vegetal pole. (B) Expression of Gpc-4 at the onset of gastrulation (stage 10). (C) Dorso-vegetal view of an early gastrula stage embryo (stage 10.5). The broken line indicates the plane of the hemi-sections shown in panels (D) to (F) and (I). (D-F) Hemi-sections of embryos cut along the dorso-ventral axis (stage 10.5). (D) Gpc-4 transcripts in prechordal endomesoderm and chordamesoderm (asterisk) and in the neuroectodermal cell layer (arrow). (E) Distribution of Noggin transcripts in the prechordal endomesoderm and chordamesoderm (asterisk). (F) Sox-2 in the neuroectodermal cell layer (arrow). (G) Frontal view of an early neural plate embryo (stage 14). Note Gpc-4 transcripts in the anterior neural plate (black arrow) and presumptive spinal cord (white arrow). (H) Frontal view of a stage 14 embryo showing Bf-1 expression in the anterior forebrain. (I) Expression of Gpc-4 in a hemi-sectioned embryo (stage 14). Anterior is to the left. White arrowhead points to decreasing expression in the prechordal plate. (J) Frontal view of a mid-neurula (stage 17). Asterisks point to Gpc-4 transcripts in the presumptive dorsal forebrain. (K) Emx-2 expression in the presumptive dorsal forebrain (stage 17, asterisks). (L) Expression of Gpc-4 following closure of the anterior neural tube (stage 20). Arrow points to transcripts in the forebrain.*

Gpc-4Mo, blocks translation of *Gpc-4* mRNA very efficiently both in vitro (Fig. 2A, upper panel) and in vivo (Fig. 2C,D). Therefore, Gpc-4Mo and an unrelated control antisense morpholino oligo (CoMo, Fig. 2A lower panel and Fig. 2B) were used for all studies shown.

Injection of Gpc-4Mo into both blastomeres of two-cell stage embryos severely alters embryogenesis (Fig. 2F,H,J; 86%, n=193), whilst CoMo injected embryos develop nor-

mally (Fig. 2E,G,I; 91%, n=107). Gpc-4Mo injected embryos develop normally up to gastrulation (data not shown) and gross-morphological defects appear from gastrulation onwards (Fig. 2F,H,J). Initially, a delay in blastopore closure becomes apparent as a large open blastopore remains at a stage by which gastrulation is almost complete in control embryos (compare Fig. 2E,F). At the end of neurulation, the anterior neural tube remains open in GPC-4 depleted embryos and the brain vesicles are less pronounced (compare Fig. 2G,H). By the tail bud stage, GPC-4 depleted embryos are shorter with a kinked axis and their dorsal fin and head structures are reduced (compare Fig. 2I,J). Both eye fields are present but significantly reduced in size (indicated by circles in Fig. 2I,J), while the cement gland appears normal (compare Fig. 2I,J). GPC-4 depleted embryos fail to reach the swimming tadpole stage (data not shown). These phenotypes are less severe than those recently described by Ohkawara et al. (2003). However, injections of higher amounts of Gpc-4Mo result in embryos with spina bifida (data not shown) as described by Ohkawara et al. (2003).

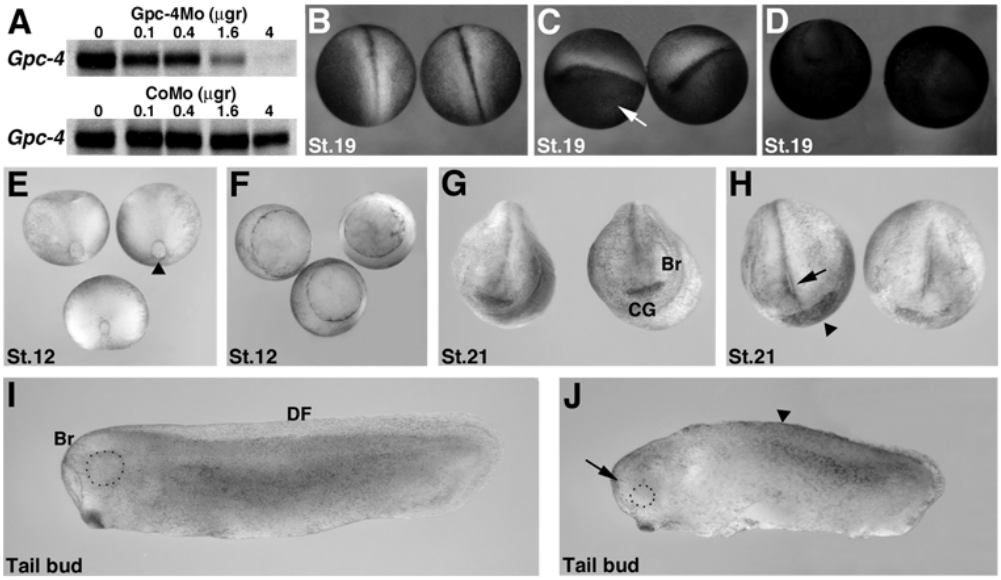


***Figure 2**. GPC-4 is required for early embryonic development. (A) The Gpc-4Mo inhibits translation of Gpc-4 mRNA in vitro. Capped mRNA was in vitro translated in the presence of increasing amounts (indicated in mgr) of Gpc-4Mo (top panel) or CoMo (bottom panel). (B-D) Gpc-4Mo specifically inhibits translation of Gpc-4 transcripts in vivo. (B) Embryos injected with chimeric Gpc-4GFP transcripts and the CoMo. (C,D) Embryos injected with Gpc-4GFP mRNA and Gpc-4Mo in one blastomere (arrow in C) or both blastomeres (D). Injection of Gpc-4Mo inhibits Gpc-4GFP mRNA translation as evidenced by lack of GFP activity. (E-J) Two-cell embryos were injected with CoMo (E,G,I) or Gpc-4Mo (F,H,J) and analysed at different developmental stages. (E,F) GPC-4 functions during gastrulation. Dorso-vegetal view of stage 12 embryos. (E) Blastopore has closed in embryos injected with CoMo (stage 12). (F) Blastopore remains open in embryos injected with Gpc-4Mo (stage 12). (G,H) GPC-4 is required for anterior CNS development. Frontal view of stage 21 embryos. Embryos injected with Gpc-4Mo (H) retain an open anterior neural tube (arrow) but develop a cement gland (arrowhead). (I,J) Side view of tail bud stage embryos. In contrast to control embryos (I), embryos injected with Gpc-4Mo (J) are shorter, lack the dorsal fin and have small heads. arrowhead in (J) points to the missing dorsal fin, arrow to microcrphaly. The developing eyes are encircled. CG: cement gland; Br: brain; DF: dorsal fin.*

To investigate the molecular and cellular defects underlying the gross-morphological alterations of GPC-4 depleted embryos (Fig. 2), we analysed the expression of genes regulating gastrulation and neurulation. The expression of *Goosecoid* (*Gsc*; Cho et al., 1991) appears initially normal indicating that GPC-4 does not affect establishment of Spemann's organizer (compare Fig. 3A,B; n=3/3). During gastrulation, *Gsc* expressing cells ingress and move toward the anterior of the embryo. Because of this anterior expansion, the *Gsc* expression domain narrows and elongates in control embryos (Fig. 3C), while it remains broad in Gpc-4Mo injected embryos (Fig. 3D; n=8/8). Changes in the spatial distribution of mesodermal and neuroectodermal genes become more apparent towards the end of gastrulation. For example, *Xenopus brachyury* (*Xbra*; Smith et al., 1991) is detected in the developing mesoderm around the blastopore and in the presumptive notochord in control embryos (Fig. 3E). In Gpc-4Mo injected embryos, the length of the presumptive notochord is very much reduced (arrow in Fig. 3F; n=9/10) and *Xbra* expression remains predominantly around the enlarged blastopore. Accordingly, analysis of *Noggin* expression in the prospective notochord (Smith and Harland, 1992) shows that the posterior extension of its expression domain is shorter and remains wider in comparison to control embryos (compare Fig. 3G,H; n=13/17). In contrast, the anterior *Noggin* (asterisks in Fig. 3G,H; n=13/17) and *Dkk-1* expression domains (asterisks in Fig. 3I,J; n=9/10; (Glinka et al., 1998)), which mark the anterior endoderm and prechordal endomesoderm, seem normal. Neural induction is also not affected, as expression levels of the pan-neural marker *Sox-2* (Mizuseki et al., 1998) are normal (compare Fig. 3K,L). However, the posterior neuroectoderm lacks the characteristic neural plate morphology (asterisk in Fig. 3L; n=9/10) apparent in control embryos (asterisk, Fig. 3K) in agreement with altered *Xbra* and *Noggin* expression in the notochord (compare Fig. 3F,H). Finally, analysis of *Et* expression (Li et al., 1997) in GPC-4 depleted embryos shows that two retinal and eye primordia develop (compare Fig. 3M,N; n=9/10). These findings are in agreement with normal *Shh* expression in the prechordal plate and ventral midline (arrows, Fig. 3O,P; n=10/10; Ekker et al., 1995). Taken together, these results show that inhibition of GPC-4 function during gastrulation affects antero-posterior axis elongation, while the head organizer, specification of the anterior neuroectoderm and ventral midline formation seem normal.

To better clarify the in vivo function of GPC-4 we performed gain-of-function studies. Injection of increasing amounts of mouse *Gpc-4* capped mRNA (*mGpc-4;* ranging from 0.5 ng to1.6 ng) into one-cell embryos caused trunk and tail truncations and a severe dorsal flexure phenotype (Fig.4). Injection of less than 0.5 ng of mRNA per embryo has no phenotypic effects while injection of more than 1.6 ng causes embryonic lethality (data not shown). Injection of 0.5 ng leads to a mild phenotype such as apparent trunk and tail shortening (Fig. 4B). The impaired axial elongation and dorsal flexure becomes more severe by increases the amount of *mGpc-4* injected transcripts (Fig. 4C,D). The most severe phenotype (Fig. 4D) results in very significant trunk shortening, little or no tail development and spina bifida in most embryos. In contrast head structures seem less abnormal. Morphological analysis of gastrulating embryos shows that overexpression of GPC-4 causes an incomplete blastopore closure (data not shown). To investigate the molecular defects underlying the body axis phenotypes caused by the overexpression of GPC-4 we analysed the expression of molecular markers for gastrulation and neurulation processes. During gastrulation the distribution of *Chordin* and *Noggin*

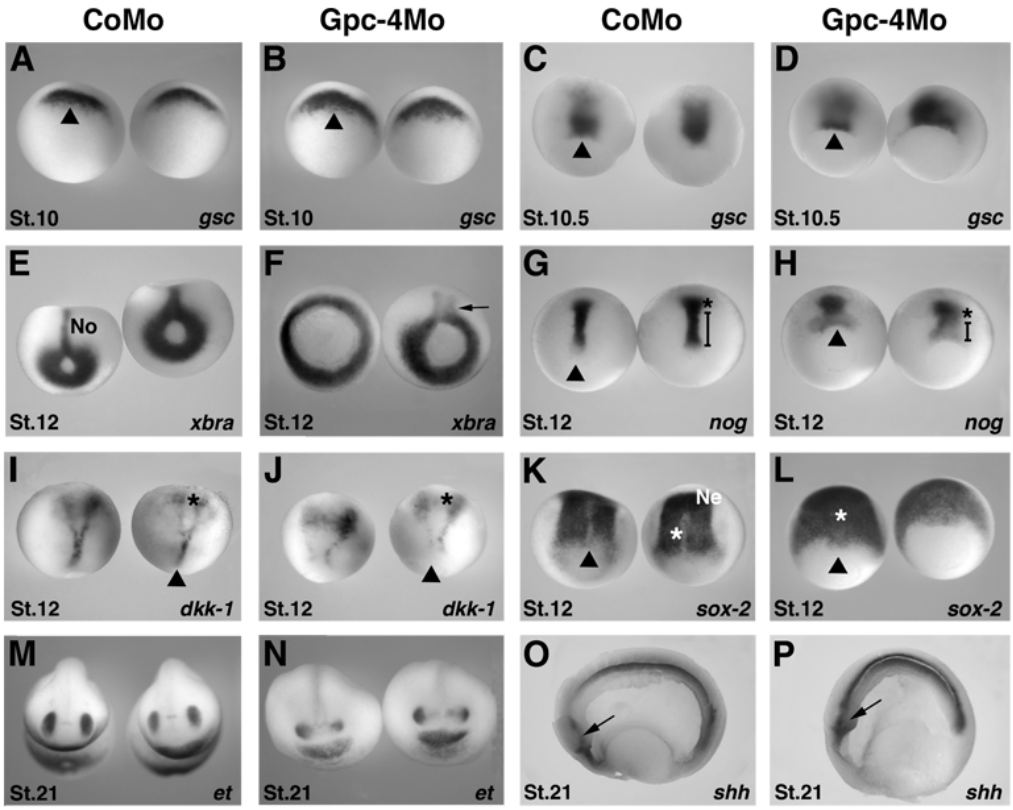


***Figure 3**. Changes in gene expression become apparent during gastrulation of GPC-4 depleted embryos. Dorso-vegetal view of embryos injected with CoMo in panels (A,C,E,G,I) and Gpc-4Mo in panels (B,D,F,H,J). Arrowheads in panels (A to D, G to L) indicate the blastopore lip. (A-L) Anterior is to the top. (A-D) Gsc expression during gastrulation (stage 10 to 10.5). (E,F) Xbra expression during late gastrulation (stage 12). No: Expression in the developing notochord in control embryos (E). Arrow in (F) indicates reduced length of notochord expression in GPC-4 depleted embryos. (G,H) Noggin transcripts at stage 12. Asterisk indicates anterior most expression. Bar indicates length of expression domain in the presumptive notochord. Noggin transcripts are normal in the anterior mesendoderm (asterisk), but the length of the presumptive notochord is reduced in GPC-4 depleted embryos (compare G to H). (I,J) Dkk-1 expression at stage 12. Asterisk indicate that the expression of dkk-1 in the anterior endoderm is not affected in GPC-4 depleted embryos (compare I to J). (K,L) Sox-2 expression in neuroectoderm (Ne) at stage 12. Asterisk in (K): Sox-2 is not expressed in the posterior midline of control embryos. Asterisk in (L): Sox-2 expression is not excluded from posterior midline in Gpc-4Mo injected embryos. (M,N) Frontal view of Et expression (stage 21) to show that two retina fields form in CoMo (M) and Gpc-4Mo injected embryos (N). (O,P) Expression of shh in hemi-sectioned embryos at stage 21. Anterior is to the right. Note that shh transcripts are normal in the prechordal plate (arrow).*

(data not shown) is not altered in *Xenopus* overexpressing GPC-4 embryos. Only towards the end of gastrulation changes in the spatial distribution of mesodermal genes become apparent. The expression of *Xbra* around the blastopore is normal while its expression in the forming notochord is altered. In particular, in *mGpc-4* injected embryos

the length of the notochordal expression domain is shorter and remains wider (bar, Fig.4F) with respect to control embryos (bar, Fig. 4E). Head and anterior neural plate induction is also not affected, as *Otx-2* expression remains normal (Fig. 4G,H). In addition, ectopic *mGpc-4* expression seems not to alter early patterning of the CNS as shown by expression of several markers of early morphogenesis, such as *Otx-2* (Fig. 4I,J) and *Sox-2* (Fig. 4K,L), *Krox-20* and *En-2,* (data not shown) remain normal. These results show that overexpression of GPC-4 during early development alters mainly the establishment and elongation of the antero-posterior axis, while induction and specification of mesoderm and neuroectoderm and specification of the anterior neuroectoderm seem to occur normally.

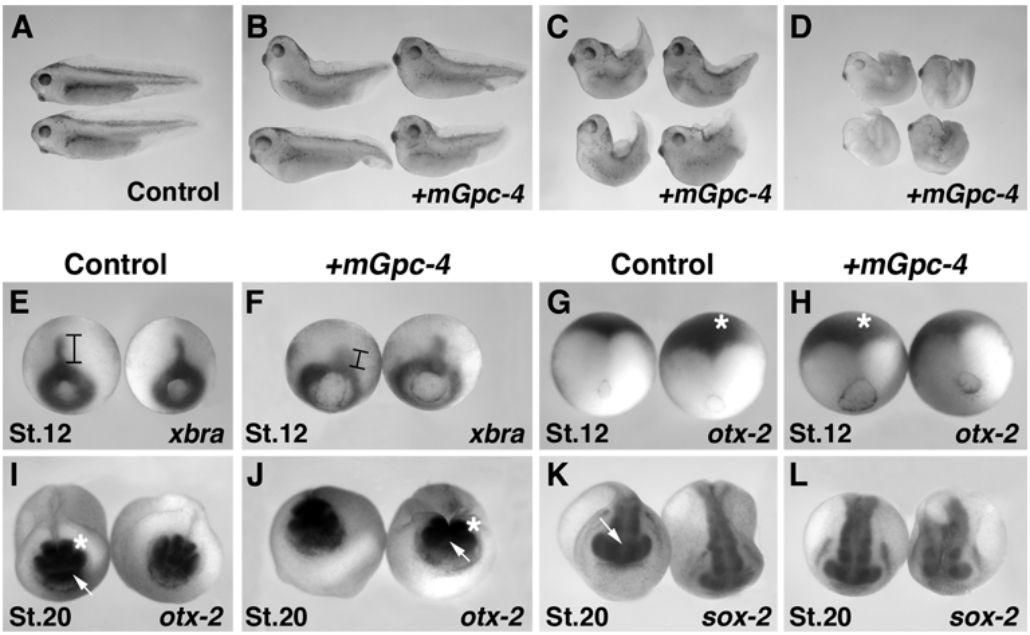


Figure 4. *Over-expression of mGpc-4 truncates the antero-posterior embryonic axis. (A-D) Side view. Gross morphological phenotypes of embryos injected with increasing amounts of mGpc-4 capped mRNA: (B) 0.5 ng, (C) 0.8 ng and (D) 1.6 ng; (A) control: uninjected embryos. (E-L) Molecular analysis of embryos injected with 1.6 ng of mGpc-4 transcripts in comparison to control embryos. (E-H) Dorso-vegetal view. (E, F) Xbra transcripts distribution. Note that the altered expression of Xbra in the presumptive notochord territory (compare E to F). Bar indicates the length of Xbra expression domain in the presumptive notochord. (G, H) Otx-2 expression. Asterisk in (G, H) point to the expression in the neuroectoderm. (I, L) Frontal view. (I, J) Otx-2 transcripts at stage 21. Arrow: forebrain expression; Asterisk: midbrain expression. (K, L) Expression of the neural marker Sox-2 during stage 21. Note that over-expression of mGpc-4 does not significantly alter Sox-2 distribution (arrow points to forebrain territory).*

Gpc-4 (Fig. 1) and other family members are expressed in the developing neural tube (Song and Filmus, 2002), but their functions during CNS morphogenesis remain to be identified. To get insight into the roles of Glypicans in this process, we further investigated the brain defects observed in GPC-4 depleted *Xenopus* embryos. Analysis of the

Sox-2 distribution after neural tube closure (Fig. 5A) reveals the phenotypic alterations of the neural tube morphology (Fig. 5E; n=10/11). Histological sections of the embryonic CNS demonstrate that patterning of the forebrain and midbrain are predominantly affected (compare Fig. 5B,F and Fig. 5C,G). In particular, the size of the forebrain is reduced, the mesencephalon and eye vesicles are less pronounced and neural tube closure has not occurred correctly (white arrowheads Fig. 5F,G). These results show that anterior CNS structures are severely affected in GPC-4 depleted *Xenopus* embryos, while the spinal cord appears rather normal (compare Fig. 5D,H).

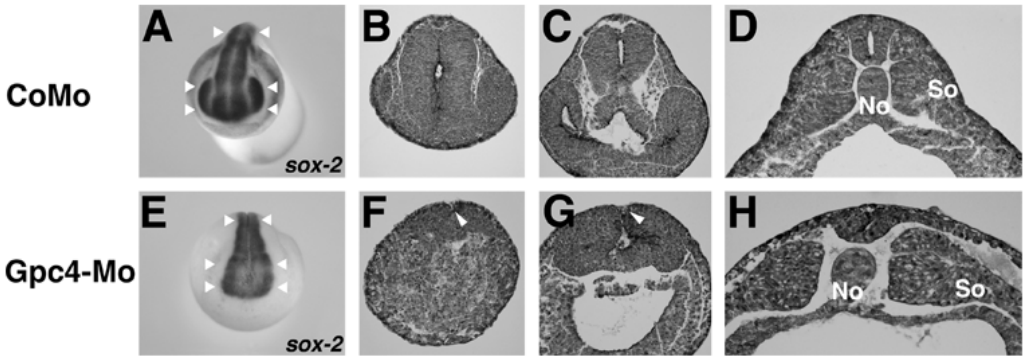


Figure 5. *Forebrain defects in GPC-4 depleted embryos. (A-D) Embryos injected with CoMo. (E-H) Embryos injected with Gpc-4Mo. (A-E) Frontal view of Sox-2 distribution at stage 21. White arrowheads indicate the level of the transverse sections shown in panels (B-D) and (F-H). (B-D, F-H) Histological sections are at the level of the forebrain in panels (B) and (F), midbrain in panels (C) and (G) and spinal cord in panels (D) and (H). Arrowhead in panel (F) and (G) points to defects in dorsal neural tube closure. No: notochord; So: somites.*

GPC-4 regulates expression of transcription factors required for dorsal forebrain development

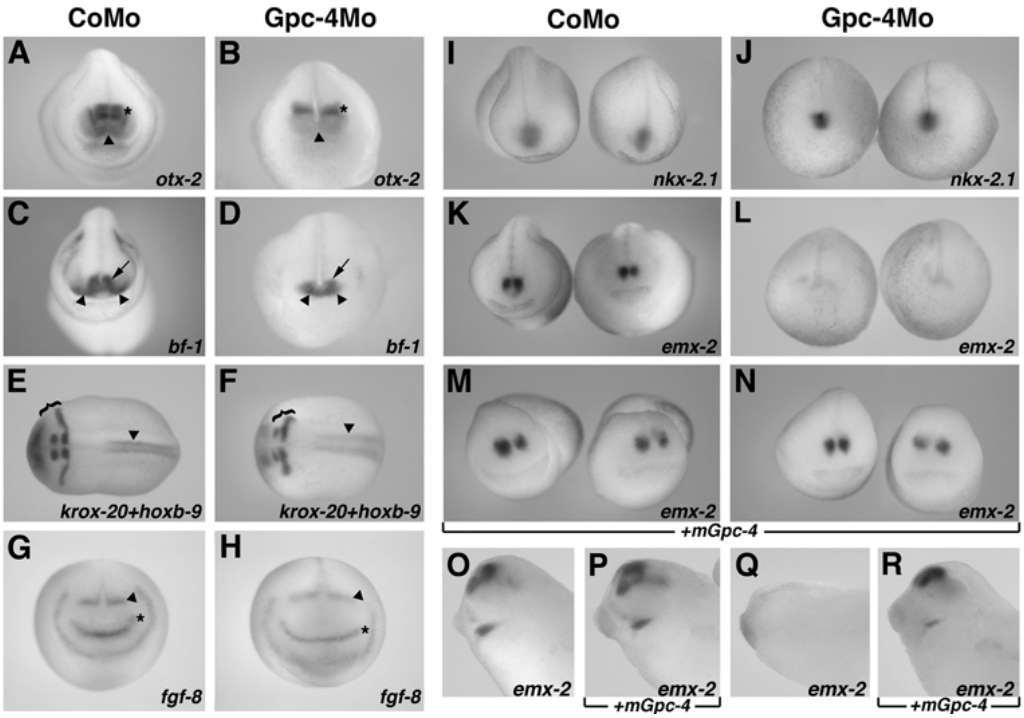
The *Otx-2* gene is expressed by the fore- and midbrain during CNS patterning (Pannese et al., 1995). Depletion of GPC-4 eliminates most of the *Otx-2* expression in the forebrain (arrowheads in Fig. 6A,B; n=13/16) while its midbrain expression domain is less affected (asterisks in Fig. 6A,B). Similarly, *Bf-1* expression is reduced in the developing telencephalon (arrows in Fig. 6C,D; n=6/8). In contrast, *Hoxb-9* in the spinal cord (arrowheads in Fig. 6E,F; n=10/10; Cho et al., 1988), *Krox-20* in the hindbrain (brackets in Fig. 6E,F; n=16/16; Bradley et al., 1993), and *Fgf-8* in the isthmus and anterior neural ridge appear normal (asterisk in Fig. 6G,H; n=9/9; Eagleson and Dempewolf, 2002).

Following neural induction, the vertebrate forebrain is also regionalized along its dorsoventral axis. One hallmark of these early patterning events is the expression of *Emx-2* in the dorsal and *Nkx-2.*1 in the ventral forebrain territories (Hollemann and Pieler, 2000). In GPC-4 depleted embryos, *Emx-2* expression is drastically reduced or absent following neural tube closure (compare Fig. 6K,L; n=31/37), while *Nkx-2.1* remains

expressed (compare Fig. 6I,J; n=12/12). Similar to *Emx-2*, the expression of other dorsal forebrain genes such as *Emx-1* and *Eomesodermin* is also down regulated (data not shown).

Rescue of forebrain patterning defects by co-injection of *mouse Gpc-4* (*mGpc-4*) mRNA

The following rescue experiment was performed to assess that the molecular and morphological defects in forebrain patterning are specifically caused by interference of Gpc-4Mo with GPC-4 functions. *Xenopus* embryos were co-injected with the Gpc-4Mo and the *mGpc-4* mRNA, which lacks the Gpc-4Mo target sequence (data not shown). Such co-injection rescues *Emx-2* expression in 69% of all embryos (Fig. 6N and Table 1). Furthermore, forehead morphology and *Emx-2* distribution in the dorsal forebrain of rescued tail bud embryos (Fig. 6R) are similar to control embryos (Fig. 6O). In contrast, *mGpc-4* mRNA does not significantly alter *Emx-2* expression and dorsal forebrain patterning upon co-injection with CoMo (Fig. 6M,P). Taken together, these results demonstrate that GPC-4 function is required to regulate expression of dorsal forebrain identity genes.



GPC-4 is required for establishment of the *Emx-2* expression domain and survival of forebrain cells

As *Emx-2* is one of the earliest known genes expressed in the presumptive dorsal forebrain territory, we determined whether GPC-4 is required to establish *Emx-2* expression

***Figure 6**. GPC-4 regulates expression of dorsal forebrain markers. Molecular analysis of neural markers in CoMo and Gpc-4Mo injected embryos. (A-D) and (G-N) frontal views; (E,F) dorsal views, (O-R) side view; anterior is to the left. (A,B) Otx-2 expression (stage 21); arrowhead: forebrain expression; asterisk: midbrain expression. (C,D) Bf-1 expression (stage 21); arrows: expression in the developing telencephalon; arrowhead: olfactory placodes. (E,F) Expression of the posterior neural markers Krox-20 (bracket) and Hoxb-9 (arrowhead) in stage 21 embryos. (G,H) Fgf-8 expression (stage 17); asterisk: anterior neural ridge, arrowhead: isthmus. (I,J) Nkx-2.1 expression in the ventral forebrain (stage 21); note that Nkx-2.1 expression remains in GPC-4 depleted embryos (J). (K,L) Emx-2 expression in the dorsal forebrain of developing embryos (stage 21); note that Emx-2 expression is drastically reduced in GPC-4 depleted embryos (L). (M) Emx-2 expression in embryos co-injected with CoMo and mGpc-4 mRNA; note that over-expression of mGpc-4 does not affect Emx-2. (N) Rescue of Emx-2 expression in embryos co-injected with Gpc-4Mo and mGpc-4 mRNA. (O) Emx-2 expression in a tail bud embryo injected with CoMo. (P) Emx-2 expression in a tail bud embryo co-injected with CoMo and mGpc-4 mRNA. (Q) Loss-of Emx-2 expression in a tail bud embryo injected with Gpc-4Mo. (R) Rescue of Emx-2 expression and forehead morphology in a tail bud embryo co-injected with Gpc-4Mo and mGpc-4 mRNA.*

Table 1. *mGpc-4* mRNA rescues *Emx-2* expression in Gpc-4Mo injected embryos

		Emx-2 expression		
Injection	N. of embryos	Normal	Reduced	Absent
CoMo	14	100%		
Gpc-4Mo	15	20%	20%	60%
Gpc-4Mo+*mGpc-4*	39	69%	18%	13%
CoMo+*mGpc-4*	17	95%	5%	

mGpc-4: *mouse Gpc-4* mRNA
This table summarizes the results of analysing *Emx-2* expression in two independent experiments.

or only to maintain its expression during neural tube closure (Fig. 7K-N and Table 1). Analysis of *Xenopus* embryos prior to neural tube closure (from stage 14 to 17) shows that GPC-4 is required for *Emx-2* expression in the dorsal forebrain from early neural plate stages onwards (compare Fig. 7A,B; absent: n=17/28, low: n=11/28 and data not shown). The reduced forebrain vesicles (Fig. 2J, 5F, 6Q) of Gpc-4Mo injected embryos prompted us to analyse possible effects of GPC-4 depletion on cell survival. No differences in the level of apoptotic cells are detected when comparing control (Fig. 7C) and GPC-4 depleted embryos prior to anterior neural tube closure (stage 17; Fig. 7D; n=3/4). In contrast, massive apoptosis is observed in the CNS of GPC-4 depleted embryos during closure of the anterior neural tube (stage 20; compare Fig. 7E,F, n=6/8). In particu-

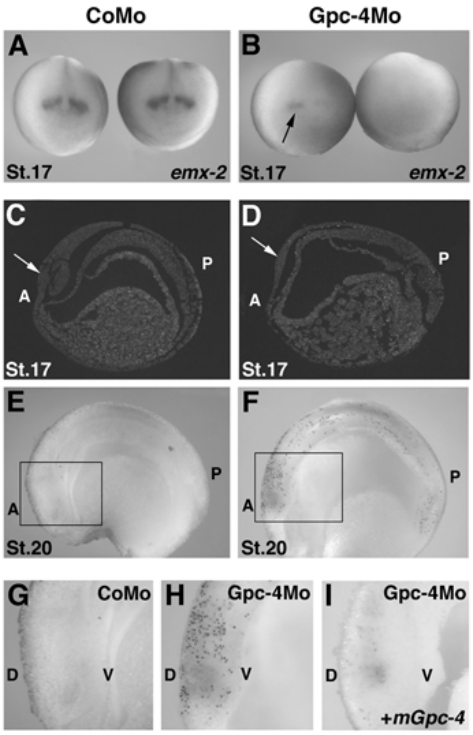


Figure 7. *GPC-4 is required for establishment of Emx-2 expression and survival of anterior CNS cells. (A,B) Emx-2 transcript distribution during neurulation (stage 17) in CoMo (A) and Gpc-4Mo injected embryos (B). Frontal view; Emx-2 expression in GPC-4 depleted embryos is either very low (arrow) or absent. (C-H) TUNEL assays to detect apoptotic cells in neurulating embryos. (C,E,G) CoMo injected embryos. (D,F,H) Gpc-4Mo injected embryos. Anterior is to the left. (C,D) Fluorescence analysis of cell death on sagital sections of a stage 17 embryo. Fluorescence was used, as it is more sensitive for detection of low numbers of apoptotic cells. Arrows in (C) and (D) point to the presumptive forebrain. (E-I) Detection of apoptotic cells by whole mount analysis of hemi-sectioned embryos (stage 20). Massive cell death is apparent in the brain of Gpc-4Mo injected embryos (F) in contrast to CoMo injected embryos (E). Boxed areas in panels (E) and (F) indicate the enlargements shown in panels (G) and (H). (I) Cell death is rescued in embryos co-injected with Gpc-4Mo and mGpc-4 mRNA. A: anterior; P: posterior; D: dorsal; V: ventral.*

lar, cell death is abundant in the anterior brain encompassing the dorsal forebrain (compare Fig. 7G,H). This cell death is rescued in Gpc-4Mo embryos co-injected with *mGpc-4* mRNA (Fig. 7I). These results show that down regulation of *Emx-2* (Fig. 7B) long precedes the onset of apoptosis (Fig. 7H) and that GPC-4 functions are required for survival of neural progenitors in the developing forebrain (Fig. 7I).

Evidence for a role of GPC-4 in modulating FGF signalling during dorsal forebrain development

Members of the BMP and FGF signalling families have been implicated in regulation of vertebrate forebrain morphogenesis (Wilson and Rubenstein, 2000). In particular, the FGF-2 protein is distributed in a pattern similar to *Gpc-4* transcripts during early *Xenopus* forebrain development (compare Fig. 1G,I,J to Song and Slack, 1994), raising the possibility of a direct interaction. Biochemical analysis reveals that a glutathione S-transferase (GST)-FGF-2 fusion protein retains the fully heparan-sulphated GPC-4 protein of about 200 kDa (arrow Fig. 8A), but not the unmodified 60 kDa protein (asterisk Fig. 8A). Furthermore, the two proteins can be co-immunoprecipitated from chicken embryonic fibroblast protein extracts (data not shown) indicating that GPC-4 complexes with FGF-2 in vivo. These biochemical studies suggest that GPC-4, like other glypicans (Grisaru et al., 2001), modulates FGF signalling. ERK protein kinases are targets of FGF signalling in neurulating *Xenopus* embryos, (Christen and Slack, 1999). Therefore, their phosphorylation levels serve as an intra-cellular indicator of FGF signal transduc-

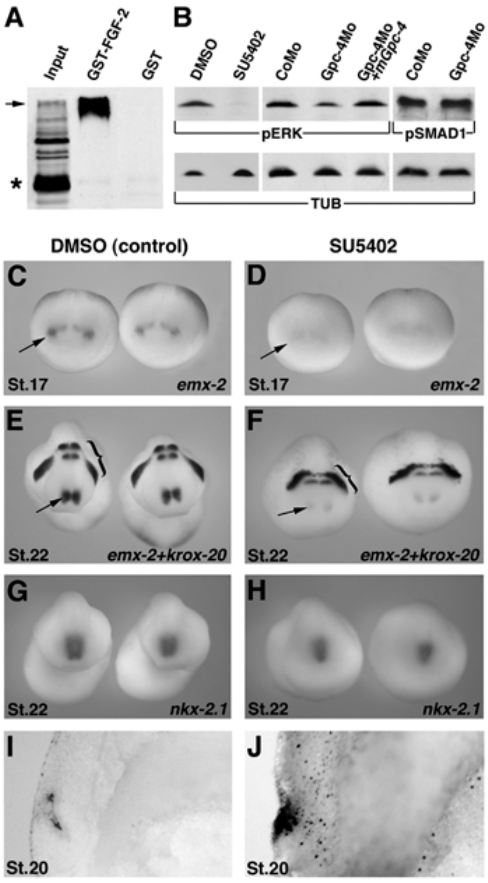


Figure 8. *GPC-4 modulates FGF signalling during neurulation. (A) Immunoblot analysis of the GPC-4/ FGF-2 complexes as detected by anti-myc antibodies. Lane "Input": NIH3T3 cells transfected with the c-myc epitope tagged mouse Gpc-4 cDNA. The HS-GAG modified mouse GPC-4 proteins have an apparent MW of around 200 kDa (arrow), while the unmodified proteins run at 60 kDa (asterisk). Lane "GST-FGF-2": only the modified 200 kDa GPC-4 protein (arrow) binds to FGF-2. Lane "GST": mouse GPC-4 does not bind to GST (control). (B) Immunoblot analysis of phosphorylated ERK (pERK) and SMAD1 (pSMAD1) proteins in Xenopus embryos (stage 15). Levels of phosphorylated proteins were determined in embryos cultured in the presence of the FGF inhibitor SU5402 (0.1 mg/ml, lane "SU5402"); with DMSO as a control (lane "DMSO"); injected with CoMo (lane "CoMo"), with Gpc-4Mo (lane "Gpc-4Mo") and with Gpc-4Mo + mGpc-4 mRNA (lane "Gpc-4Mo + mGpc-4"). TUB: a-TUBULIN levels in the extracts are determined to normalize samples. (C-H) Molecular analysis of embryos cultured with DMSO (panels C,E,G) and with SU5402 (0.1 mg/ml; panels D,F,H). Arrows in panels (C) to (F) indicate Emx-2 expression. (C) Emx-2 expression in control embryos cultured with DMSO (stage 17). (D) Down-regulation of Emx-2 in embryos cultured with SU5402 (stage 17). (E) Emx-2 and Krox-20 (bracket) expression in control embryos cultured with DMSO (stage 22). (F) Down regulation of Emx-2, but not Krox-20 (bracket) in embryos cultured with SU5402 (stage 22). (G) Nkx-2.1 expression in embryos cultured with DMSO (stage 22). (H) Nkx-2.1 expression in embryos cultured with SU5402 (stage 22). (I) Lack of cell death in the forebrain region of an embryo cultured with DMSO (stage 20). (J) Apoptotic cells detected in the forebrain region of an embryo cultured with SU5402 (stage 20).*

tion (Fig. 8B). Biochemical analysis of *Xenopus* embryos shows that ERK phosphorylation levels are reduced about 2-3 fold when injected with Gpc-4Mo at the two-cell stage (pERK, Fig. 8B, compare lane "CoMo" to "Gpc-4Mo" and data not shown). This down regulation of ERK phosphorylation in Gpc-4Mo injected embryos is rescued following co-injection of *mGpc-4* mRNA (Fig. 8B lane "Gpc-4Mo + *mGpc-4*"). In contrast, phosphorylation of the SMAD1 protein, indicative of BMP signal transduction, is not altered (pSMAD1, Fig. 8B; Persson et al., 1998). These studies show that GPC-4 interacts with FGF ligands and although it is not essential for FGF signalling, it is required to enhance FGF signal transduction during neurulation of *Xenopus* embryos. The potential roles of FGFs during forebrain patterning were further investigated by blocking FGF signal transduction using SU5402 (Mohammadi et al., 1997). To avoid perturbing gastrulation, SU5402 was added to *Xenopus* embryos from early neural plate stages onwards (stage 13, see Materials and Methods). Analysis of SU5402 treated embryos shows that *Emx-2* expression is down regulated from stage 17 (compare Fig. 8C,D; n=5/5) onwards (compare Fig. 8E,F; n=11/11). In contrast, expression of *Nkx-2.1* (Fig. 8G,H; n=5/5) and *Krox-20* (bracket in Fig. 8E,F; n=7/7) are only slightly affected. Similar to Gpc-4Mo injected embryos, inhibition of FGF signalling by SU5402 results in death of forebrain cells at the onset of anterior neural tube closure (Fig. 8J). In summary, inhibition of either GPC-4 function or FGF signal transduction affects *Emx-2* expression in similarly (compare Fig. 7B to 8D and Fig. 6L to 8F). These findings indicate that GPC-4 regulates *Emx-2* expression and thereby dorsal forebrain development by positive modulation of FGF signalling.

DISCUSSION

We have functionally analysed the *Glypican-4* gene in developing *Xenopus* embryos using Gpc-4Mo antisense morpholino oligos and ectopic expression of mouse *Gpc-4*. Gpc-4Mo specifically blocks GPC-4 protein translation as evidenced by biochemical analysis and phenotypic rescue by *Gpc-4* transcripts. Injection of Gpc-4Mo and *mGpc-4* into *Xenopus* embryos causes defects in axis elongation during gastrulation. In particular, ectopic expression of *mGpc-4* leads to embryos, which are phenotypically indistinguishable from the ones recently described by Ohkawara et al. (2003). Our molecular analysis shows that tissue specification and differentiation occur normally in embryos with impaired GPC-4 function. In contrast, the spatial distribution of mesodermal and neuroectodermal markers is affected in GPC-4 depleted embryos. These phenotypes are typical of alterations in the function of genes involved in convergent-extension movements during gastrulation. Interestingly, ectopic expression of *mGpc-4* also results in a dorsal flexure phenotype while Gpc-4Mo injected embryos display a very mild dorsal flexure. A possible explanation for this discrepancy is that the signalling mechanism that regulates morphogenetic movements during gastrulation (reviewed by Wallingford et al., 2002) is less sensitive to inhibition of GPC-4 activity then over-expression. Alternatively, the loss of GPC-4 activity may affect primarily morphogenetic movements of the posterior neural plate (Wallingford and Harland, 2002). Similar differences have been previously described in embryos with impaired *Strabismus* function, a gene implicated in regulation of convergent extension movements in *Xenopus* (Darken et al.,

2002). The short body axis of *Xenopus* embryos with altered GPC-4 activity is reminiscent of the phenotype of *knypek* deficient zebrafish embryos (Topczewski et al., 2001). The Glypican encoded by *knypek* is highly homologous to the *Gpc-4* and *Gpc-6* genes and regulates cell polarity during convergent extension movements. Similar to *knypek, Gpc-4* is expressed in tissues undergoing extensive movements during gastrulation (reviewed by Wallingford et al., 2002). These tissues include the involuting mesoderm and the posterior neuroectoderm. Moreover, embryos with alterated Gpc-4 activity show defects in neural tube closure, which is often associated with defects in gastrulation movements (Wallingford and Harland, 2002). Taken together, these findings are consistent with previous studies of *knypek* mutant zebrafish embryos. This genetic analysis established that *knype*k promotes non-canonical WNT signalling (WNT-11), which is required for convergent-extension movements during zebrafish gastrulation. Indeed, Ohkawara et al. (2003) showed that GPC-4 also regulates convergent extension movements by modulation of the non-canonical WNT pathway during *Xenopus* gastrulation. Therefore, the present study focuses on analysing key GPC-4 functions during early forebrain patterning and provides evidence that GPC-4 is required to enhance FGF signalling.

GPC-4 is required for forebrain patterning in *Xenopus* embryos

It is unlikely that GPC-4 acts during head and anterior neural plate induction as the cement gland, ventral forebrain, two eye primordia and olfactory placodes form. The latter two structures derive from the most anterior neural plate (Rubenstein et al., 1998), which indicates that the most anterior brain structures are present in GPC-4 depleted *Xenopus* embryos. In agreement, *Otx-2*, the earliest anterior neural plate marker (Rubenstein et al., 1998), is expressed during gastrulation and only down-regulated during neurulation. In contrast to abrogation of GPC-4, inhibition of *Dkk-1* and *Igf*, which regulate head- and anterior neural plate induction, results in severe microcephaly and complete loss of the cement gland and eyes (Glinka et al., 1998; Pera et al., 2001). Moreover, abrogation of *Tlc* and *Axin*, two inhibitors of WNT signalling, disrupts anteroposterior regionalization of the forebrain, causing loss of both ventral and dorsal forebrain and eye fields (Houart et al., 2002; Wilson and Rubenstein, 2000). These phenotypes are much more severe and their appearance significantly precedes the ones observed in GPC-4 depleted *Xenopus* embryos.

Subsequently, inductive signals emanating from the prechordal plate (e.g. SHH) and anterior neural ridge (e.g. FGF-8) act on anterior neural plate cells to establish regional differences such as specification of dorsal and ventral forebrain identities (Rubenstein et al., 1998). *Gpc-4* is expressed by the prechordal endomesoderm during gastrulation and the anterior neural plate at the time when these signalling centers are active. However, the *Shh* and *Fgf-8* expression domains are established correctly in Gpc-4Mo injected *Xenopus* embryos. Inactivation of *Shh* and *Fgf-8* causes ventral forebrain defects (Rubenstein et al., 1998) in contrast to interfering with GPC-4 activity (this study). Therefore, the dorsal forebrain defects observed in Gpc-4Mo injected embryos arise most likely by altering the reception of signals targeted to dorsal neuroectodermal cells prior to closure of the anterior neural tube (see below).

In *Xenopus* and mouse embryos, cells of the presumptive forebrain begin to express

Gpc-4 during neurulation (this study and chapter 2) and in the embryonic mouse brain expression persists in telencephalic neural precursors (Hagihara et al., 2000). Mutations in human *Gpc-3* and *Gpc-4* genes, which are next to one another on the X-chromosome, have been linked to the *Simpson-Golabi-Behmel* syndrome (SGBS). The SGBS syndrome is characterized by general pre- and postnatal overgrowth (reviewed by DeBaun et al., 2001). A fraction of SGBS patients also show mental retardation, seizures and a high risk for neuroblastoma (DeBaun et al., 2001). In the present study, we show that abrogation of GPC-4 activity in *Xenopus* embryos disrupts forebrain patterning, cell survival and causes microcephaly. Therefore, our findings raise the possibility that some of the CNS abnormalities affecting SGBS patients may arise as a consequence of disrupting *Gpc-4* gene function during neurulation. In GPC-4 depleted *Xenopus* embryos, the expression of dorsal forebrain identity genes like *Emx-2* and *Emx-1* is disrupted already during neurulation. Previous genetic analysis of *Emx* genes in mice has established that they regulate regionalization and expansion of the dorsal forebrain compartment and subsequent cerebral cortex morphogenesis (Mallamaci et al., 2000; Yoshida et al., 1997). In particular, *Emx-1* and *Emx-2* compound mutant embryos have greatly reduced telencephalic vesicles already prior to initiation of cerebral cortex development (Bishop et al., 2003). Therefore, the dorsal forebrain defects observed in GPC-4 depleted *Xenopus* embryos could be a consequence of mainly disrupting expression of the *Emx* genes during neurulation.

GPC-4 modulates FGF signalling in the developing dorsal forebrain

Patterning of the vertebrate CNS depends to a large extent on extra-cellular regulation of signals (Rubenstein et al., 1998; Wilson and Rubenstein, 2000). Glypicans regulate signalling by modulating formation of receptor-ligand complexes (Nybakken and Perrimon, 2002). In agreement, abrogation of GPC-4 function in neurulating *Xenopus* embryos reduces phosphorylation of ERK protein kinases, which are specific targets of FGF signalling (Christen and Slack, 1999). This result shows that GPC-4 participates in enhancing FGF signal transduction during embryogenesis. In agreement, genetic studies in *Drosophila* show that formation of an active FGF receptor-ligand complex depends on the presence of HSPGs (Lin et al., 1999). Inhibition of FGF signalling by SU5402 in *Xenopus* embryos phenocopies aspects of depleting GPC-4 function such as loss-of *Emx-2* expression and increased apoptosis of forebrain progenitors. Several FGF ligands and their cognate receptors are expressed during patterning of the vertebrate CNS (Dono, 2003). Genetic and functional analysis established that two of these ligands, FGF-8 and FGF-3 function during formation of mid-hindbrain and rhombomere boundaries, respectively, in vertebrate embryos. Moreover, both FGF ligands participate in patterning of the anterior telencephalic midline and the anterior and post-optic commissure (Shinya et al., 2001; Wilson and Rubenstein, 2000). The present study establishes that FGF signalling also regulates dorsal forebrain development, but the involved FGF ligand(s) remain to be identified. Candidates are FGF-9 (Song and Slack, 1996) and in particular FGF-2, as this FGF ligand is present throughout the brain during *Xenopus* neurulation (Song and Slack, 1994) and binds GPC-4 (this study). FGF-2 deficient mice display defects in dorsal telencephalon patterning, albeit only much later during cerebral cortex layer formation (Dono, 2003). Therefore, further functional and genetic analysis is necessary to identify and study the FGF ligands interacting with GPC-4 in embryos.

Comparative analysis of GPC-4 depleted and SU5402 treated *Xenopus* embryos suggests that modulation of BMP and/or WNT signalling does not significantly contribute to *Emx-2* regulation in the dorsal forebrain. In contrast, the similarities in the axis defects between GPC-4 depleted *Xenopus* (Ohkawara et al., 2003) and *knypek* deficient zebrafish embryos points to possible effects on non-canonical WNT signalling during gastrulation (see before). Therefore, Glypicans may control the activity of different ligands in a stage- and/or tissue-specific manner as shown for *Drosophila dally*, which regulates *wg* during embryonic and *dpp* signalling during post-embryonic development (Nybakken and Perrimon, 2002). Modifications of proteins by HS-GAG side chains are not uniform and changes in the distribution of sulphate groups affect ligand-binding properties. Enzymes involved in HSPG biosynthesis modify the HS-GAG side chains of Glypicans and regulate their ability to bind signal peptides during *Drosophila* embryogenesis (Giraldez et al., 2002). It will be important to determine if and to what extent alterations of HS-GAG side-chains of GPC-4 may confer it with the ability to bind WNT during gastrulation and FGF ligands during neurulation (this study). Such alterations may explain cell-type and developmental-stage specific modulation of ligand-receptor interactions by glypicans during vertebrate embryogenesis.

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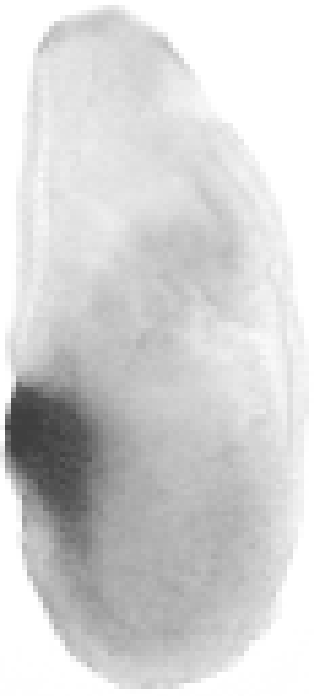
FGF2 in Blood Pressure Control

chapter 4



FGF2 signaling is required for the development of neuronal circuits regulating blood pressure

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Abstract

Fibroblast growth factor 2 (FGF2) signaling is involved in angiogenesis, vascular contractility, and cardiac hypertrophy. Mice lacking a functional *FGF2* gene ($FGF2^{-/-}$) are hypotensive, but the primary physiological role of FGF2 in cardiovascular homeostasis remained unknown. Using a chicken *FGF2* (*cFGF2*) transgene under control of the *Wnt-1* promotor, we selectively re-expressed FGF2 in the developing nervous system of $FGF2^{-/-}$ (transgenic FGF2 mutant) embryos. Expression of the *cFGF2* transgene in the developing nervous system including its autonomic region was limited to the period between embryonic day 9.5 and 14.5. Significantly, no *FGF2* re-expression was detected in developing heart and blood vessels. Pharmacological analysis revealed a normalization of blood pressure response to isoproterenol-induced vasodilation in adult transgenic FGF2 mutant mice. In addition, the hypotensive phenotype was rescued in one line (of two) transgenic FGF2 mutant adult mice having expressed higher levels of cFGF2 proteins during nervous system development. These genetic studies indicate that FGF2 signaling is essential for complete development of the neural circuitry required for central regulation of blood pressure, while it appears dispensable for blood pressure control in the healthy adult.

Introduction

Fibroblast growth factor 2 (FGF2) is a signaling molecule with important functions for growth and differentiation, particularly in the central nervous system (CNS). From neurulation onwards, *FGF2* is expressed at high levels in the CNS,{Dono & Zeller 1994 ID: 168} and mice lacking a functional *FGF2* gene show distinct defects in the developing cerebral cortex and adult spinal cord.{Dono, Texido, et al. 1998 ID: 916}{Ortega, Ittmann, et al. 1998 ID: 942}{Vaccarino, Schwartz, et al. 1999 ID: 1000}

Several observations indicate that FGF2 is also involved in cardiovascular homeostasis. In the adult, *FGF2* is expressed in smooth muscle and endothelial cells.{Lindner & Reidy 1993 ID: 833} Infusion of FGF2 causes vasodilation and hypotension in rats, dogs, and man{Cuevas, Carceller, et al. 1991 ID: 842}{Lazarous, Scheinowitz, et al. 1995 ID: 826}{Lazarous, Unger, et al. 2000 ID: 935}{Unger, Goncalves, et al. 2000 ID: 923} via opening of ATP-sensitive K^+ channels and enhanced release of nitric oxide.{Cuevas, Carceller, et al. 1991 ID: 842} Endogenous release of FGF2 from vascular smooth muscle cells is triggered by an increase in mechanical strain above normal levels,{Cheng, Briggs, et al. 1997 ID: 830} possibly causing transient elevations in cell membrane permeability.{Kaye, Pimental, et al. 1996 ID: 825} Together these studies suggest, that FGF2 functions in an anti-hypertensive vasodilator cascade.

Surprisingly, targeted inactivation of the *FGF2* gene ($FGF2^{-/-}$) in mice leads to chronically decreased blood pressure levels in adults.{Dono, Texido, et al. 1998 ID: 916},{Zhou, Sutliff, et al. 1998 ID: 925} $FGF2^{-/-}$ mice display a markedly attenuated myocardial growth response to mechanical overload,{Schultz, Witt, et al. 1999 ID: 940}

raising the possibility that an impaired left ventricular function may contribute to the hypotension. Furthermore, the spontaneous contractile activity of isolated portal veins from FGF2$^{-/-}$ mice is reduced.{Zhou, Sutliff, et al. 1998 ID: 925} This may cause alterations in blood volume distribution and cardiac filling pressure. In addition, if this defect affects directly the contractile apparatus, it may also affect arterial resistance vessels. Finally, the neural control of blood pressure is impaired in FGF2$^{-/-}$ mice.{Dono, Texido, et al. 1998 ID: 916} However, the relative importance and possible contributions of these various deficits to the observed hypotensive phenotype was unclear. Accordingly, the primary physiological role of FGF2 in blood pressure homeostasis has remained obscure.

During CNS development, *FGF2* is expressed by progenitor cells of neuronal circuits involved in the central regulation of blood pressure, for example in the myelencephalon{Dono, Texido, et al. 1998 ID: 916} and the intermedio-lateral neurons in the spinal cord.{Stapf, Lück, et al. 1997 ID: 859} Furthermore, *FGF2* is also expressed by migrating trunk neural crest cells,{Savage, Hart, et al. 1993 ID: 195} which give rise to peripheral cardiovascular nerves. This strongly suggests an essential role of FGF2 during the normal development of cardiovascular reflex control. If the hypotensive phenotype of FGF2$^{-/-}$ mice were indeed primarily caused by a congenital malformation of neuronal circuits regulating blood pressure,{Dono, Texido, et al. 1998 ID: 916} then restricted re-expression of *FGF2* during nervous system development should rescue the defects in cardiovascular homeostasis in adult mice. To directly test this hypothesis, we used a chicken *FGF2* (*cFGF2*) transgene under the control of the *Wnt-1* promoter,{Zuniga Mejia Borja, Murphy, et al. 1996 ID: 729}{Echelard, Vassileva, et al. 1994 ID: 147} to target *FGF2* re-expression specifically to the developing nervous system in FGF2 mutant embryos.

Materials and Methods

Transgenic mice

The generation of transgenic mouse lines expressing the three chicken *FGF2* (*cFGF2*) protein isoforms{Dono & Zeller 1994 ID: 168} in the dorsal aspect of the neural tube has been described previously.{Zuniga Mejia Borja, Murphy, et al. 1996 ID: 729} Two transgenic lines expressing the transgene appropriately in the embryos (Tg^A and Tg^C; see ref. {Zuniga Mejia Borja, Murphy, et al. 1996 ID: 729}) were used for all experiments in the present study. The transgenic mice were crossed to FGF2$^{-/-}$ mice{Dono, Texido, et al. 1998 ID: 916} to generate adult mice and embryos of all genotypes. All control and mutant mice were littermates of a mixed C57BL/6J x 129/Sv genetic background. Mice and embryos carrying the *FGF2* loss-of-function allele and/or the *cFGF2* transgene were genotyped by PCR analysis of tail biopsies or embryonic yolk sac DNA using specific PCR primers.

In situ hybridization and antibody staining

Expression of the *cFGF2* transgene was determined using digoxigenin-labeled riboprobe complementary to coding exon 2/3 of the chicken *FGF2* gene.{Zuniga Mejia

Borja, Murphy, et al. 1996 ID: 729} This probe does not detect endogenous mouse *FGF2* transcripts. Embryos and embryonic CNS were dissected into PBS, fixed in 4% paraformaldehyde and processed for whole-mount *in situ* hybridization. Alternatively, embryos were embedded in paraffin and 10 µm transverse sections were processed for RNA *in situ* hybridization.{Dono, Texido, et al. 1998 ID: 916} Parallel sections were processed for immunohistochemistry. Ectopic cFGF2 proteins were detected using affinity purified FGF2 antibodies.{Dono & Zeller 1994 ID: 168} Endogenous mouse FGF2 proteins were not detected under the conditions used.

Immunoblot analysis
Embryonic brains and spinal cords were dissected in PBS and immediately frozen in liquid nitrogen. Normalized protein extracts were prepared and analyzed as previously described.{Dono & Zeller 1994 ID: 168} Samples were further normalized for protein content using monoclonal ß-tubulin antibodies (Sigma 1:2000).

Cardiovascular studies
All cardiovascular studies were performed in awake, unrestrained female mice (body weight 21-42 grams; age 5–10 months) in accordance with national guidelines for the care and use of research animals. Animals were anesthetized with ketamine (100 µg per g body weight i.p.) and xylazine-HCL (4 µg per gram body weight i.p.). Chronic catheters were implanted in the left femoral artery and vein under aseptic conditions as described previously.{Just, Faulhaber, et al. 2000 ID: 933}{Plüger, Faulhaber, et al. 2000 ID: 939} Blood pressure was measured in the abdominal aorta via the femoral artery catheter (transducer PRC-21K, amplifier MIO-0501, FMI) and continuously recorded at 500 Hz (80586, DAS-0216, Keithley-Metrabyte; LabTech Notebook pro 10.2.1, Labtech). Drugs were infused via the femoral vein catheter by using a calibrated pump (Precidor 5003, Infors AG). Baseline values of arterial blood pressure and heart rate were determined for 1 hour in each mouse in its own cage on day 2 or day 3 (i.e. 48-72 hours) after surgery. To test the cardiovascular response to an acute hypotensive stimulus, on day 1 (i.e. >24 hours) after surgery the $ß_1/ß_2$-adrenoceptor agonist isoproterenol (Sigma) was infused after a control period of 5 minutes at increasing doses (0.05, 0.1, 0.2 and 0.4 ng/min per gram body weight). Isoproterenol does not cross the blood-brain barrier and therefore has no direct effects on the central nervous system.{Goodman, Gilman} Before each dose of isoproterenol, a baseline recording of 5 minutes was performed. Isoproterenol was infused at a constant rate for 3 minutes. Blood pressure and heart rate responses were averaged over each infusion period. Between each dose of isoproterenol, mice were allowed to recover until blood pressure and heart rate returned to their baseline levels over the entire experimental period.

Statistical analysis
Values are expressed as mean ± SEM. Statistical comparisons were made by one-way ANOVA (1-hour baseline recordings) or two-way ANOVA (infusion studies) followed by the Bonferroni's Multiple Comparison test. An error level of $P<0.05$ was considered as significant.

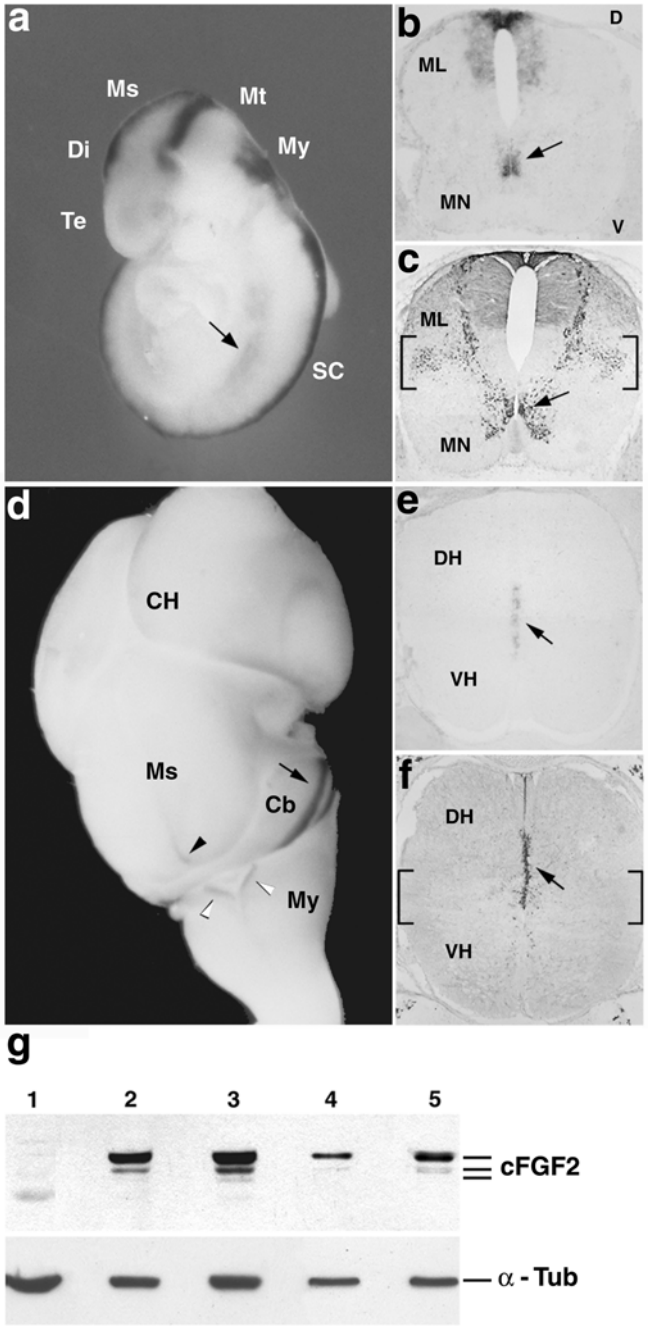
Results

Temporal and spatial expression of the cFGF2 transgene

Chicken *FGF2* (*cFGF2*) is functionally conserved to its mouse counterpart and its ectopic expression does not alter morphology of the wild-type CNS.{Zuniga Mejia Borja, Murphy, et al. 1996 ID: 729} In the dorsal spinal cord and developing autonomic nervous system, the transgene distribution overlaps with that of endogenous *FGF2* (Figure 1; compare to ref. {Dono, Texido, et al. 1998 ID: 916}){Stapf, Lück, et al. 1997 ID: 859}. Therefore, two independent *cFGF2* transgenic lines (called Tg^A and Tg^C; see ref. {Zuniga Mejia Borja, Murphy, et al. 1996 ID: 729}) were intercrossed to $FGF2^{-/-}$ mice{Dono, Texido, et al. 1998 ID: 916} to generate compound transgenic FGF2 mutant embryos and adult mice ($FGF2^{-/-}$; Tg^A and $FGF2^{-/-}$; Tg^C) for developmental and physiological analysis.

The temporal and spatial distribution of the *cFGF2* transgene was analyzed in transgenic FGF2 mutant ($FGF2^{-/-}$; Tg) and wild-type littermate embryos ($FGF2^{+/+}$; Tg and/or $FGF2^{-/+}$; Tg) using *cFGF2* specific probes and antibodies (Figure 1). *cFGF2* transcript and protein distributions were identical for both transgenic lines (Tg^A, Tg^C) in wild-type and FGF2 mutant embryos (Figure 1 and data not shown). The *cFGF2* transgene was expressed in the developing CNS from early neural fold stages onwards (data not shown; see also ref. {Echelard, Vassileva, et al. 1994 ID: 147}). By embryonic day 9.5, the *cFGF2* transgene was abundantly expressed in the CNS and at lower levels in developing sympathetic ganglia (arrow, Figure 1A). In the CNS, the *cFGF2* transgene was expressed mainly by the dorsal diencephalon, midbrain, myelencephalon and spinal cord, while no expression was detected in the telencephalon (Figure 1A). Most relevant to the purpose of this study, the *cFGF2* transgene was not expressed by the developing heart and blood vessels (embryonic day 9.5 to 16.5; see e.g. Figure 1A). From embryonic day 9.5 to 14.5, the *cFGF2* transgene expression pattern remained the same in the CNS, while it was lost from differentiating sympathetic ganglia (data not shown).

During embryonic day 11, immature neurons of the developing autonomic nervous system separate from the ventral pool of motor neurons, migrate to the intermediate spinal cord layers,{Yip, Yip, et al. 2000 ID: 924} where they undergo terminal differentiation.{Phelps, Barber, et al. 1991 ID: 920} The intermedio-lateral neurons of the thoracic spinal cord are a major component of the developing autonomic nervous system.{Phelps, Barber, et al. 1991 ID: 920} Therefore, the cellular distribution of the *cFGF2* transgene was determined in transverse sections of thoracic spinal cords at embryonic day 12.5 (Figure 1B, 1C). Highest levels of *cFGF2* transcripts were detected in the dorsal aspect of the neural tube (Figure 1B) and a ventral region bordering motor neurons (arrow, Fig 1B). Using more sensitive immunohistochemistry, cFGF2 proteins were detected in broader areas than transcripts in both dorsal and ventral spinal cord regions (compare Figure 1B to 1C). In particular, cFGF2 proteins were detected in the intermediate part of the spinal cord during differentiation of autonomic neurons (bracketed region, Figure 1C). This pattern is specific for cFGF2 proteins as no signal was detected in non-transgenic littermates (data not shown).



During later developmental stages, expression of the *cFGF2* transgene is progressively down-regulated. By embryonic day 16.5, expression in the brain was limited to the

Figure 1. *Expression of the cFGF2 transgene is restricted to the developing nervous system.* ***A****, cFGF2 transgene distribution in FGF2 mutant mouse embryos carrying transgene C (FGF2$^{-/-}$; Tg^{C}) during embryonic day 9.5. Note dorsal cFGF2 expression in the diencephalon (Di), mesencephalon (Ms), myelencephalon (My) and spinal cord (SC). The transgene is also expressed at the mesencephalic/metencephalic junction and in neural crest derivatives located in the trunk (arrow). No expression is detected in the metencephalon (Mt) and telencephalon (Te).* ***B****, Distribution of the cFGF2 transcripts in the spinal cord during embryonic day 12.5. Transverse section (10 µm) showing the transcript distribution in a FGF2$^{+/-}$; Tg^{A} spinal cord. The section is located at the level of the forelimb. Highest expression is detected in the dorsal aspect of the spinal cord.{Echelard, Vassileva, et al. 1994 ID: 147} Note the second expression domain in ventral part of the spinal cord (arrow). ML: mantle layer, MN: motor neurons.* ***C****, Distribution of the cFGF2 proteins as detected by immunohistochemistry in a section adjacent to the one shown in Figure 1B. Note that the cFGF2 proteins are more widespread than cFGF2 transcripts. Ectopic cFGF2 proteins are also detected in the intermedio-lateral region of the spinal cord (indicated by brackets) and ventral spinal cord regions (arrow).* ***D****, Brain of an FGF2$^{-/-}$; Tg^{C} mouse embryo (embryonic day 16.5). Note the down-regulation in mesencephalon (Ms; arrowhead) and low expression in the roof of the ependymal diverticulum of the fourth ventricle (open arrowhead) and up-regulation in the developing cerebellum (Cb; arrow). CH: cerebral hemisphere; My: myelencephalon.* ***E****, Transverse section (10 µm) to show cFGF2 transcript distributions in an FGF2$^{+/-}$; Tg^{C} spinal cord during embryonic day 16.5. The section is at the level of the forelimb. Note the restricted expression in the ependymal layer (arrow).* ***F****, Distribution of cFGF2 proteins in a section adjacent to the one shown in Figure 1E. cFGF2 proteins are now restricted to the ependymal layer (arrow) and no longer expressed in the intermedio-lateral region (indicated by brackets). DH: dorsal grey horn; VH: ventral grey horn.* ***G, Upper panel:*** *immunoblot analysis of total proteins prepared from brain and spinal cords of mouse embryos (embryonic day 12.5) using FGF2 antibodies. All three cFGF2 protein isoforms are translated in transgenic mouse embryos.* ***Lane 1:*** *non-transgenic control embryos (10 µl of total extract loaded);* ***lane 2 and 4****: FGF2$^{-/-}$; Tg^{A} (5 µl and 2.5 µl loaded, respectively);* ***lane 3 and 5:*** *FGF2$^{-/-}$; Tg^{C} (5 µl and 2.5 µl loaded, respectively). Note that transgenic embryos of line C (lanes 3,5) express at least 2-fold higher levels of cFGF2 protein than line A (lanes 2, 4).* ***G, Lower panel:*** *immunoblot analysis of the same protein extracts using ß-tubulin antibodies.*

developing cerebellum (Figure 1D). In the spinal cord, both *cFGF2* transcripts and proteins were restricted to the ependymal layer (Figure 1E, 1F). Interestingly, expression was no longer detected in regions containing differentiating autonomic neurons (indicated by brackets, Figure 1F). This significant decrease of *cFGF2* transgene expression was confirmed by semi-quantitative RT-PCR (data not shown). Finally, immunoblot analysis of normalized protein extracts prepared from dissected CNS (embryonic day 12.5) showed that all three cFGF2 protein isoforms{Dono & Zeller 1994 ID: 168} are expressed (Figure 1G). It is important to note that cFGF2 protein levels were at least 2-fold higher in embryos carrying Tg^{C} (Figure 1G, lanes 3 and 5) than Tg^{A} (Figure 1G, lanes 2 and 4). In summary, these studies establish that expression of the *cFGF2* transgene in the developing autonomic nervous system within the CNS is limited from embryonic day 9.5 to about 14.5. The transgene was never expressed outside the developing nervous system (Figure 1; see also ref. {Zuniga Mejia Borja, Murphy, et al. 1996 ID: 729}, {Echelard, Vassileva, et al. 1994 ID: 147}).

Rescue of blood pressure regulation by the cFGF2 transgene

The results shown in Figure 1 indicate that the *cFGF2* transgenic lines are suited to determine whether re-expression of *FGF2* in the developing nervous system of $FGF2^{-/-}$ embryos rescues the defects in blood pressure regulation. Adult $FGF2^{-/-}$ mice fail to compensate a hypotensive challenge induced by intravenous infusion of the $ß_1/ß_2$-adrenoceptor agonist isoproterenol.{Dono, Texido, et al. 1998 ID: 916} Therefore, the effects of increasing doses of isoproterenol on mean arterial blood pressure and heart rate were comparatively analyzed in conscious, adult mice of all genotypes (Figure 2 and Table 1). Over the entire concentration range, $FGF2^{-/-}$ mice displayed significantly lower blood pressures than their wild-type littermates (Figure 2A). In contrast, FGF2 mutant mice carrying transgene C ($FGF2^{-/-}$; Tg^C) or A ($FGF2^{-/-}$; Tg^A), respectively, responded normally to increasing doses of isoproterenol (Figure 2B, 2C). This can best be seen by comparing the dose-response curves of transgenic FGF2 mutant mice to those of their wild-type ($FGF2^{+/+}$) and transgenic wild-type ($FGF2^{+/+}$; Tg^C and $FGF2^{+/+}$; Tg^A; see Figure 2B, 2C) litter mates. Heart rates were not different between FGF2 mutant mice ($FGF2^{-/-}$, $FGF2^{-/-}$; Tg^A, and $FGF2^{-/-}$; Tg^C) and their corresponding wild-type controls ($FGF2^{+/+}$, $FGF2^{+/+}$; Tg^A and $FGF2^{+/+}$; Tg^C ; see Table 1). These results indicate that cardiovascular reflex control of blood pressure is normalized

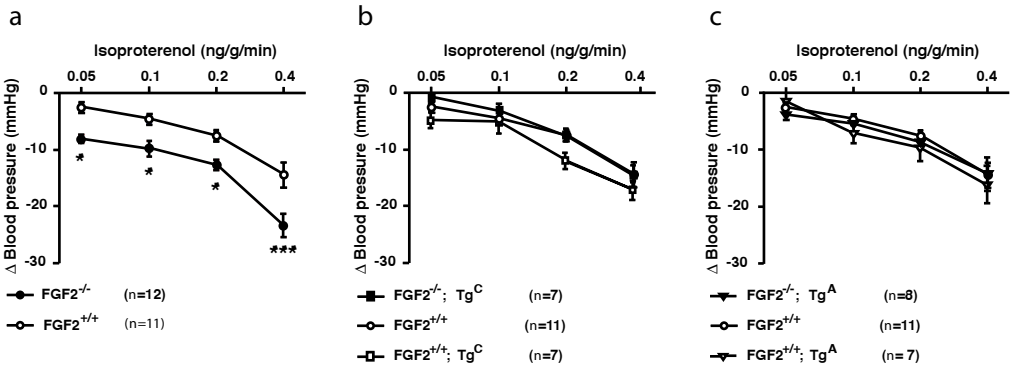


Figure 2. *Rescue of the autonomic dysfunction in transgenic, FGF2 mutant mice.* ***A****, Effects of increasing doses of isoproterenol on the mean arterial blood pressure in wild-type and* $FGF2^{-/-}$ *mice. Isoproterenol infusion at 0.05 ng/min per gram body weight already induced a larger decrease in mean arterial blood pressure in* $FGF2^{-/-}$ *than in wild-type mice. Note that increasing doses of isoproterenol caused a progressive decrease of mean arterial blood pressure in wild-type mice. However,* $FGF2^{-/-}$ *mice always showed a significantly greater reduction in blood pressure than wild-type controls (P<0.001).* ***B****, Effects of increasing doses of isoproterenol on mean arterial blood pressure in FGF2 mutant mice carrying transgene C (*$FGF2^{-/-}$*;* Tg^C*) and wild-type controls (*$FGF2^{+/+}$ *and* $FGF2^{+/+}$*;* Tg^C*). No significant differences in response to isoproterenol infusion were detected between the three genotypes.* ***C****, Effects of increasing doses of isoproterenol on mean arterial blood pressure in FGF2 mutant mice carrying transgene A (*$FGF2^{-/-}$*;* Tg^A*) and wild-type controls (*$FGF2^{+/+}$ *and* $FGF2^{+/+}$*;* Tg^A*). No significant differences in response to isoproterenol infusion were detected between the three genotypes. Data for wild-type mice (*$FGF2^{+/+}$*) are identical in all three panels. All data are mean ± SEM. n: number of mice. *P<0.05, ***P<0.001 vs. wild-type by two-way ANOVA over all genotypes followed by Bonferroni's Multiple Comparison test.*

TABLE 1. Baseline heart rates and heart rate response to isoproterenol

Genotype		Baseline	Response to Isoproterenol				
	n		n	0.05	0.1	0.2	0.4
$FGF2^{+/+}$	12	643 ± 21	11	66 ± 24	76 ± 23	90 ± 23	85 ± 19
$FGF2^{-/-}$	13	607 ± 38	12	79 ± 9	76 ± 14	99 ± 20	152 ± 21
$FGF2^{+/+}$; Tg^{a}	7	665 ± 16	7	66 ± 26	74 ± 25	92 ± 17	70 ± 11
$FGF2^{-/-}$; Tg^{a}	8	630 ± 14	8	73 ± 17	74 ± 12	75 ± 12	100 ± 21
$FGF2^{+/+}$; Tg^{c}	7	633 ± 16	7	70 ± 31	120 ± 25	107 ± 16	150 ± 27
$FGF2^{-/-}$; Tg^{c}	7	647 ± 24	7	93 ± 27	166 ± 20	148 ± 23	155 ± 36

Data were obtained 1-3 days following surgery in conscious, unrestrained mice and represent mean ± SEM. Baseline refers to mean heart rates obtained in one-hour recordings. Responses to isoproterenol denote changes in heart rate relative to 5 minutes control recordings obtained immediately before staring the infusion of isoproterenol. n: number of adult mice analyzed.

in adult FGF2 mutant mice, which as embryos expressed the *cFGF2* transgene specifically in their developing nervous system (Figure 1).

Potential rescue of the hypotensive phenotype in FGF2 mutant mice (Figure 3A; see ref. {Dono, Texido, et al. 1998 ID: 916}){Zhou, Sutliff, et al. 1998 ID: 925} carrying either transgene was assessed by determining the mean arterial blood pressure in conscious, unrestrained adult mice over prolonged recording periods. Indeed, FGF2 mutant mice carrying transgene C ($FGF2^{-/-}$; Tg^{C}) displayed normal blood pressure levels (Figure 3B), whereas FGF2 mutant mice carrying transgene A ($FGF2^{-/-}$; Tg^{A}) remained hypotensive (Figure 3C; compare to Figure 3A). Interestingly, baseline heart rates did not differ between the genotypes (Table 1). The differential rescue of blood pressure may result from a lower level of cFGF2 protein expression in transgenic embryos of line A (Figure 1G), which is probably due to differences in transgene copy numbers and/or integration site. These results indicate that higher levels of FGF2 proteins are required to rescue the hypotensive phenotype in addition to cardiovascular reflex control.

Discussion

Although physiological, pharmacological, and genetic studies have demonstrated that FGF2 full-fills important functions in cardiovascular homeostasis, its primary role in blood pressure regulation has remained elusive. We now show that FGF2 signaling in the developing nervous system, but not in vascular smooth muscle cells nor cardiac

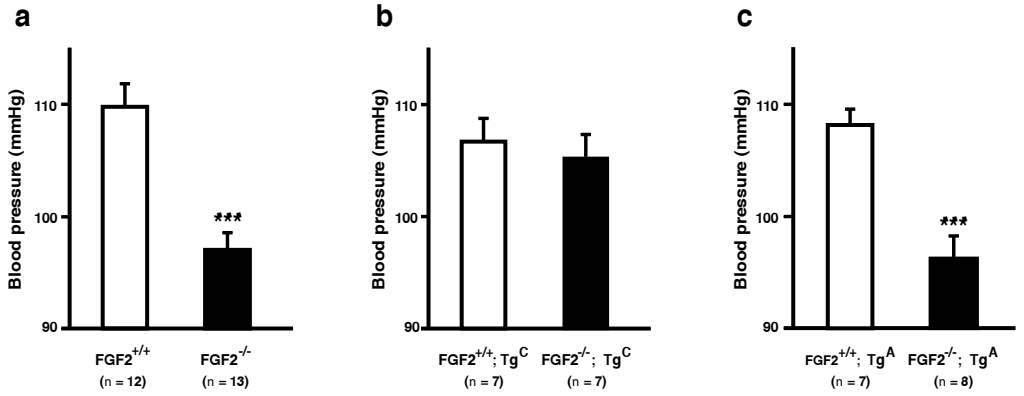


Figure 3. *Analysis of resting mean arterial blood pressure in transgenic, FGF2 mutant mice.* ***A****, Resting mean arterial blood pressure in wild-type and FGF2$^{-/-}$ mice. FGF2$^{-/-}$ mice always displayed lower blood pressures than their wild-type litter mates (97±2 vs. 110±2 mmHg; P<0.001).* ***B****, Resting mean arterial blood pressure in FGF2 mutant mice carrying transgene C (FGF2$^{-/-}$; Tg^{C}) and wild-type transgenic controls (FGF2$^{+/+}$; Tg^{C}). Blood pressure levels were nearly identical in both groups (105±2 vs. 107±2 mmHg; P=non-significant).* ***C****, Resting mean arterial blood pressure in FGF2 mutant mice carrying transgene A (FGF2$^{-/-}$; Tg^{A}) and wild-type transgenic controls (FGF2$^{+/+}$; Tg^{A}). Despite the fact that response to isoproterenol induced vasodilation was normal in FGF2 mutant mice carrying transgene A (Figure 2C), they displayed a significantly lower blood pressure than their wild-type transgenic litter mates (96±2 vs. 108±2 mmHg; P<0.001). All data are mean ± SEM. n: number of mice. ***P<0.001 vs. corresponding wild-type control by one-way ANOVA over all genotypes followed by Bonferroni's Multiple Comparison test.*

myocytes is required to establish normal blood pressure control mechanisms.

In particular, these findings indicate that FGF2 signaling is essential for normal development of the neuronal regulatory circuits involved in central regulation of blood pressure. An essential role during embryonic development is in agreement with the observed high expression of FGF2 during development of the autonomic nervous system in embryos.{Savage, Hart, et al. 1993 ID: 195}{Stapf, Lück, et al. 1997 ID: 859}{Dono, Texido, et al. 1998 ID: 916} However, the specific target cells of FGF2 signaling in these determinative processes remain to be identified. Interestingly, neither baseline heart rates nor heart rate responses to isoproterenol were affected in FGF2 mutant mice and their wild-type littermates. This argues against the cardiovascular phenotype of FGF2$^{-/-}$ mice being caused by a major defect in regulation of heart rates. Since the slopes of the blood pressure response to isoproterenol are similar in all genotypes, an impairment of the afferent limbs of the baroreceptor reflex seems also unlikely. Rather, the physiological results would be compatible with a dysfunction of pre-ganglionic intermedio-lateral neurons in the spinal cord. These neurons relay the central sympathetic outflow to the target organs. Their degeneration reduces baseline sympathetic tone and excitatory sympathetic reflexes and is associated with impaired baroreceptor reflex

function and hypotension.{Bannister & Mathias 1992 ID: 927} However, the differential rescue of cardiovascular response to isoproterenol and baseline blood pressure in FGF2 mutant mice carrying transgene A ($FGF2^{-/-}$; Tg^{A}) and transgene C ($FGF2^{-/-}$; Tg^{A}) suggests that spinal and supraspinal mechanisms are potentially differentially affected in $FGF2^{-/-}$ mice.

Since FGF2 mutant mice carrying the transgene do not express *FGF2* outside the nervous system, it is unlikely that FGF2 signaling in the peripheral cardiovascular system participates in maintaining blood pressure above hypotensive levels in the healthy adult. However, this does not exclude that FGF2 may serve important functions under patho-physiological conditions. For example, cardiomyocytes release FGF2 upon elevated mechanical load,{Clarke, Caldwell, et al. 1995 ID: 828} and FGF2 mutant mice display severely reduced hypertrophic myocardial growth in response to pressure overload.{Schultz, Witt, et al. 1999 ID: 940} These studies suggest an important role of FGF2 signaling during cardiac hypertrophy. Furthermore, we showed previously {Dono, Texido, et al. 1998 ID: 916} that $FGF2^{-/-}$ adult mice respond to continuous infusion of angiotensin II (over 6 days) with an exaggerated increase in blood pressure. This observation supports the notion that FGF2 released from smooth muscle cells and/or endothelial cells upon elevated mechanical stress may exert substantial vasodilator effects under patho-physiological conditions.

Interestingly, only FGF2 mutant mice expressing higher levels of the *cFGF2* transgene ($FGF2^{-/-}$; Tg^{C}) displayed normal baseline blood pressure levels. These data indicate that the hypotensive phenotype of $FGF2^{-/-}$ mice is not *per se* linked to the dysfunction of cardiovascular reflex control. This observation corroborates the proposal that short- and long-term blood pressure regulation depends on different neural reflex mechanisms.{Brooks & Osborn 1995 ID: 928}{DiBona 2000 ID: 930} Further comparative physiological analysis of both transgenic FGF2 mutant mouse strains may help to further dissect these pathways. More importantly, the present study demonstrates that neural control mechanisms are an essential component of long-term blood pressure regulation. The identification of the precise nature of this FGF2-dependent pathway will greatly enhance our understanding of the interplay between neural, vascular and renal mechanisms in controlling blood pressure.

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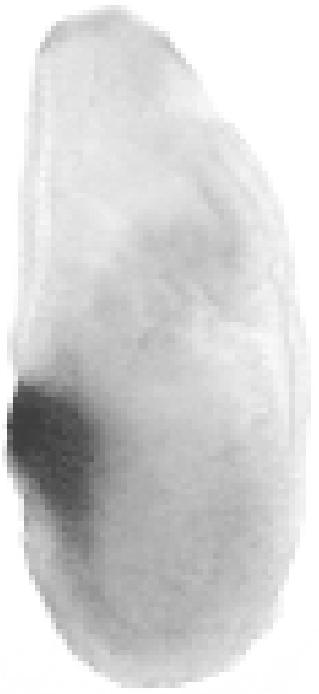
Summarizing Discussion

chapter 5



Cell-to-cell signaling is a fundamental process during early vertebrate CNS development. Communication between cells implicates interaction between a ligand (a signaling molecule) and its receptor. For example, in the developing embryo secreted signaling molecules such as Hedgehog, BMPs, WNTs and FGFs bind to their cognate high affinity receptors on responsive cells. The activation of these pathways leads to an intracellular signal transduction that culminates in a precise cellular response. To ensure that cell-to-cell communication evolves correctly these ligand-receptor interactions are tightly regulated so that cells receive a particular type of signal at the correct time and in the correct location. The regulation of ligand-receptor interaction occurs at different levels and involves types of regulatory molecules such as signal agonists and antagonists produced by organizing centers and signaling modulators. The main topic of the research presented in this thesis was to investigate the role of FGF signaling and the signaling modulator Glypican-4 (GPC-4) during vertebrate CNS development with special emphasis on investigating their functions during forebrain patterning.

Glypicans and Patterning of the Brain

As described in chapter 3, I establish that GPC-4 is required for dorso-ventral patterning of the forebrain during *Xenopus* embryonic development. In particular, Gpc-4 is required for activation and maintenance of dorsal forebrain identity genes like *Emx-2* and for survival of forebrain progenitor cells during neural tube closure. Furthermore biochemical and embryological analysis establishes that GPC-4 regulates dorso-ventral forebrain patterning via positive modulation of FGF signaling. It is known that HSPGs modulate different types of growth factor signals (Perrimon and Bernfield, 2000). Previous studies have indeed shown that patterning of the dorsal forebrain also requires the coordinate action of BMP and WNT signaling expressed by the dorsal midline and dorsal ectoderm, respectively (reviewed by Wilson and Rubenstein, 2000). In addition, evidence by others implicated FGF signaling in the specification of dorsal midline cells in the telencephalon (Shanmugalingam et al., 2000). My studies now establish that modulation of FGF signaling by GPC-4 is required for the establishment of dorsal forebrain character. This conclusion is supported by recent studies of Gunhaga et al. (2003), which establish that Wnt and FGF signaling interact to induce dorsal telencephalic character in chicken embryos.

While the morphology of dorsal (adult pallial) and ventral (subpallial) telencephalic structures varies enormously between different vertebrate species during early development, the activation of transcription factors in specific domains within the forming telencephalon is very similar among all vertebrates (Fernandez et al., 1998; Puelles et al., 2000). Moreover, evolutionary conserved signaling pathways control regionalization of the telencephalon from zebrafish through to mammals (reviewed by Wilson and Rubenstein, 2000). Therefore, it is to be expected that GPC-4 also participate in dorsal forebrain patterning in mammals. Indeed, the spatial and temporal distribution of *Gpc-4* and *Fgf* receptors is highly conserved during CNS development of *Xenopus* and mouse embryos (my studies and Golub et al., 2000).

The dorsal telencephalon gives rise to several regions of the adult brain including the cerebral cortex, the most complex CNS structure in mammals. I establish that *Gpc-4* is the only of several glypican genes highly expressed in the ventricular zone of

the developing telencephalon in the mouse. In the ventricular zone, the neural progenitors of the cerebral cortex are born, proliferate and their cell fates are determined. Loss of GPC-4 function during *Xenopus* CNS development leads to downregulation of the dorsal telencephalic identity gene *Emx-2*. In humans, mutations in *EMX-2* gene are associated with schizoencephaly, a genetic malformation characterized by uni- or bi- lateral cleft extending from the pial surface of the cortex all the way to the ventricular surface (reviewed by Walsh, 1999). Furthermore, in mouse embryos EMX-2 is required for normal development of the cerebral cortex (Mallamaci et al., 2000). *Emx-2* is expressed in a pattern overlapping with *Gpc-4* in the cortical progenitors of the dorsal telencephalic neuroepithelium (Simeone et al., 1992). Interestingly, the expression of *Gpc-4* in neural stem cells overlaps with the distribution of *Fgf*, *Wnt* and *Bmp* receptors (reviewed by Dono, 2003; Kim et al., 2001; Zhang et al., 1998). During early corticogenesis FGF-2 ligand is abundantly expressed in the ventricular zone and is required to promote proliferation and migration of ventricular zone neuroepithelial cells *in vivo* (Dono et al., 1998; Raballo et al., 2000). Taken together, these studies lead to the hypothesis that GPC-4 may be involved in regulating proliferation and/or fates of *Emx-2* expressing cells and/or future migratory paths of neural stem cells during corticogenesis by regulating their cellular response to FGFs and/or other growth factor signals. This proposal can be tested by generating GPC-4 deficient mice through gene targeting (see below).

In the genome GPC-4 is located on the X chromosome adjacent to GPC-3. Mutations in human GPC-3 and GPC-4 genes are associated with the Simpson Golabi Behemel Syndrome (SGBS), a pleiotropic syndrome characterized by general overgrowth. A fraction of SGBS patients also display mental retardation, seizures and a high risk for neuroblastomas (DeBaun et al., 2001). In addition to the SGBS, the Gustavson syndrome maps to the genomic region of *Gpc-4*. Patients affected by this syndrome display mental retardation associated with microcephaly, optic atrophy, sever impaired vision or blindness, hearing defects, spasticity, epileptic seizures and death during infancy. My studies establish that disruption of GPC-4 function in *Xenopus* embryos results in microcephaly. Our findings suggest a potential involvement of GPC-4 in the development of these genetic malformation affecting brain development and function. Therefore, the generation of GPC-4 deficient mice may provide a genetic model to study this type of neurological disorders.

Glypicans and Early Patterning Events

During early neurulation of mouse embryos, *Gpc-4* is expressed in tissues such as the AVE, the ANR and the prechordal plate. These signaling centers have been implicated in early steps of anterior brain specification. In particular, the AVE is important for maintenance of the forebrain territory. The AVE seems to not express signaling molecules but seems to be a source of antagonists like Cerberus and Dkk-1 (Borges et al., 2002; Glinka et al., 1998; Zakin et al., 2000). These antagonists limit the action of Wnt, Nodal and BMP signals to the posterior side of the embryo, where the node and the primitive streak are forming (reviewed by Beddington and Robertson, 1999). Biochemical and cell culture studies have shown that HSPGs bind the BMP antagonist Noggin at the cell surface (Paine-Saunders et al., 2002). Therefore, it is possible that GPC-4 participates in modulation of cell-to-cell signaling at the AVE through modulating

ligand-antagonist interactions rather than only ligand-receptor interactions. The expression of *Xenopus Gpc-4* in the anterior endoderm and the prechordal endomesoderm partially overlaps with the distribution of *Cerberus* and *Dkk-1* (our unpublished observations). The anterior endoderm and the prechordal endomesoderm of *Xenopus* embryos seem homologous to the mouse AVE (reviewed by de Souza and Niehrs, 2000). Therefore, the expression of mouse *Gpc-4* in these signaling centers is conserved during the evolution.

How does Gpc-4 Modulate Cell-to-Cell Signaling?

As presented in the introduction different models have been proposed regarding the way HSPGs regulate cell-to-cell signaling. From the studies presented here using *Xenopus* embryos, it seems that GPC-4 acts as a positive modulator to enhance FGF signaling during forebrain patterning. Therefore, GPC-4 may participate in FGF ligand dimerization and/or stabilization of ligand-receptor complexes. However, GPC-4 seems not absolutely essential for FGF signaling as low levels of phosphorylated ERK proteins are still detected in GPC-4 depleted embryos when compared to embryos with completely blocked FGF signaling. In contrast to ERKs, survival of forebrain cells and *Emx-2* expression are more affected by lack of GPC-4 than by complete blocking FGF signaling. These findings suggest that in addition to FGFs, GPC-4 may also modulate other signals during forebrain patterning. Indeed, during *Xenopus* gastrulation GPC-4 controls convergent extension morphogenetic cell movements (chapter 3 and Ohkawara et al., 2003; Topczewski et al., 2001) by modulating the non-canonical WNT signaling pathway in zebrafish and *Xenopus* embryos (Ohkawara et al., 2003; Topczewski et al., 2001). It is possible that GPC-4 regulates these different signaling pathways in a stage and tissue specific manner as previously shown for the *Drosophila* glypican *dally*. This cell and developmental stage specific signal modulation may depend on changes of the HS GAG side-chains (Perrimon and Bernfield, 2000).

Gpc-4 Deficient Mouse and Future Prospective

The studies that I performed in *Xenopus* start to reveal the *in vivo* functions of GPC-4 during embryonic development. However, in light of the possible involvement of GPC-4 in X-linked human genetic disorders, such as the SGBS and Gustavson syndrome (see before) the generation of a loss-of-function Gpc-4 mutation in the mouse is important and may provide a genetic model to study these diseases. I have attempted to generate GPC-4 deficient mice using gene targeting in mouse embryonic stem cells (ES; Figure 1). However these studies have not been successful, possibly due to the fact that the *Gpc-4* gene is located on the X chromosome (Veugelers et al., 1998) and the ES cell lines used were all male. Therefore, a conditional gene targeting approach should be used to circumvent the possibility of a phenotype in ES cells and early lethality of chimeric mice due to GPC-4 deficiency. Moreover, Cre recombinase mediated tissue-specific gene inactivation will allow to study GPC-4 functions specifically during cerebral cortex formation. Furthermore, *Gpc-4* is also expressed during kidney and limb development (Watanabe et al., 1995). Interestingly, patients affected by SGBS display also kidney and limb malformations. Therefore, the development of these structures

may be altered in GPC-4 deficient mouse embryos.

Last but not least, changes in glypicans expression have been associated with tumor progression (reviewed by Filmus and Selleck, 2001). This is not surprising considering the ability of glypicans to regulate the activity of growth and survival factors. Manipulation of cell-to-cell signaling and cell growth promoting factors are interesting targets for designing therapies against cancer. Therefore, it is important to better define the functions of glypican genes and HSPGs in modulation of signaling processes by a combination of genetics, biochemistry and cell biological studies.

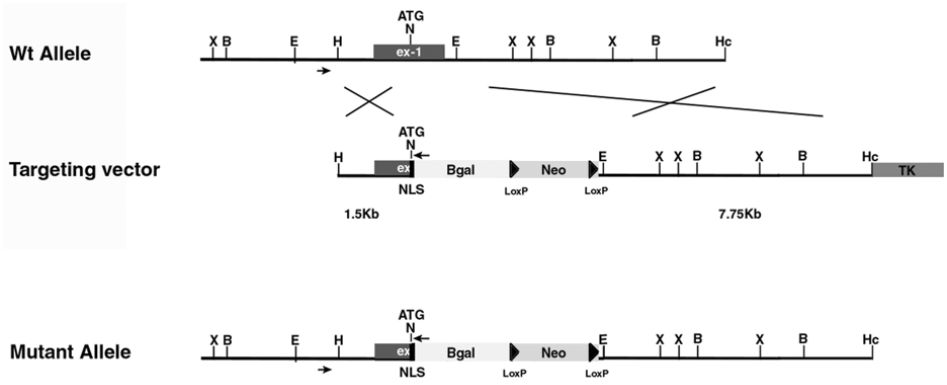


Figure 1. *Schematic representation of the GPC-4 gene targeting strategy used for my studies. Top: structure of part of the Gpc-4 locus containing the first exon and the starting ATG codon. This ATG codon is fused in frame with the β-galactosidase gene in the targeting vector (middle). The β-galactosidase reporter gene will allow following the cells that normally express Gpc-4. The targeting vector also contains a neomycin cassette, flanked by two loxP sites, and a timidyne kinase cassette for positive and negative selection of the transfected ES cells. Homologous recombination should result in the Gpc-4 mutant allele shown at the bottom.*

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English Summary

The research presented in this thesis aims at understanding the intercellular communication regulating vertebrate forebrain and spinal cord development. Cell-to-cell signaling involves the interaction between secreted signaling molecules (ligands) and their receptors. Ligand-receptor interaction is a tightly regulated process and is modulated at the cell surface by the action of glycoproteins such as Glypicans. Glypicans consist of a core protein to which heparan sulphate glycosaminoglycan chains (GAG) are attached. Due to their sulphate groups, the GAG chains are negatively charged. This feature confers them specificity of ligand binding. To date, six glypican genes have been identified in mammals (*Glypican-1* to *Glypican-6*) however little is known about their function during CNS development.

Chapter 2 described the spatial and temporal distribution of vertebrate *Glypican-4* (*Gpc-4*) and other glypican family members during early mouse embryogenesis. *Gpc-4* is the prime mouse glypican gene expressed by the cells of the anterior visceral endoderm (AVE) and the anterior neural ridge (ANR), two signaling centers essential for induction and patterning of the forebrain. As development proceeds, *Gpc-4* expression persists in progenitor cells and early post-mitotic neurons of the cerebral cortex. Taken together these results indicate that GPC-4 may function during early forebrain patterning events to modulate morphogenetic signaling by the AVE and ANR. At later developmental stages, Gpc-4 may also be part of the signaling network regulating cerebral cortex development.

In chapter 3 I show that the expression of *Gpc-4* in the AVE and in the ANR is conserved during evolution. Indeed, the *Xenopus Gpc-4* is expressed by the ANR cells and by the anterior endoderm and the prechordal endomesoderm, which are considered functionally equivalent to the mouse AVE. In light of *Gpc-4* expression during CNS development, I have performed gain- and loss- of function studies in *Xenopus* embryos to analyze GPC-4 function during forebrain development. During neurulation, loss of GPC-4 activity disrupts the expression of dorsal forebrain genes like *Emx-2*. In contrast, ventral forebrain genes remain expressed. In addition to this, loss of GPC-4 causes apoptosis of forebrain neural progenitors during neural tube closure. Biochemical studies establish that GPC-4 binds Fibroblast Growth Factor 2 (FGF-2) and enhances FGF signal transduction. Therefore, we propose that GPC-4 regulates dorso-ventral forebrain patterning by positive modulation of FGF signaling.

FGF signaling plays important roles in modulating cell proliferation, migration, differentiation and survival during CNS morphogenesis. For example, FGF-2 deficient mice show defects in neocortex formation and are affected by an autonomic dysfunction leading to a hypotensive phenotype. In chapter 4, I have analyzed the role of FGF-2 signaling in development and function of the autonomic nervous system. In particular, FGF-2 was selectively re-expressed in the developing CNS of FGF-2 deficient embryos using a chicken *FGF-2* (*cFGF-2*) transgene under the control of the *Wnt-1* promotor. Pharmacological analysis revealed a normalization of the neural control of blood pressure in transgenic FGF-2 mutant adult mice having expressed cFGF-2 proteins during CNS development. In addition, high levels of cFGF-2 also rescued the hypotensive phenotype. These studies indicate that FGF-2 signaling is essential for complete development of the neural circuitry required for central regulation of blood pressure.

In the final chapter, I discuss the role of GPC-4 in brain patterning also in light

of its potential involvement in two human syndromes. Glypicans are known to modulate different growth factor signaling in a cell type specific manner. Developmental-stage specific modulation of ligand-receptor interactions by GPC-4 during CNS development is also discussed in details.

Samenvatting in het Nederlands

Het onderzoek dat in dit proefschrift wordt beschreven, heeft als doel te begrijpen hoe bij vertebraten intercellulaire communicatie de ontwikkeling van de voorbrein en het ruggenmerg reguleert. Bij deze cel-cel communicatie speelt de interactie tussen afgescheiden signalerende moleculen (liganden) en hun receptoren een cruciale rol. De ligand-receptor interactie is een strak gereguleerd proces en wordt aan de celoppervlakte gemoduleerd door de actie van glycoproteïnen zoals Glypicans. Glypicans bestaan uit een kernproteïne waaraan ketens van heparaansulfaat-glycosaminoglycan verbonden zijn. Deze ketens zijn door de sulfaatgroepen negatief geladen. Deze eigenschap stelt hen er toe in staat om specifiek bepaalde liganden te binden. Tot op heden zijn zes glypican genen geïdentificeerd in zoogdieren (*Glypican-1* tot *Glypican-6*), over hun functie tijdens CZS ontwikkeling is echter nog weinig bekend.

Hoofdstuk 2 beschrijft de mRNA distributie van *Glypican-4* (*Gpc-4*) en andere glypican familieleden tijdens vroege muis embryogenese. *Gpc-4* is het eerste muis 'glypican' gen dat tot expressie komt in de cellen van het 'anterior visceral endoderm' (AVE) en de 'anterior neural ridge' (ANR), twee signalerende centra die essentieel zijn voor inductie en formatie van de voorbrein. Later tijdens de ontwikkeling komt *Gpc-4* blijvend tot expressie in cellen die het toekomstige hersenschors zullen gaan vormen en in de vroege post-mitotic neuronen van de hersenschors. Deze resultaten suggereren dat GPC-4 tijdens de vroege patroonvorming van de voorhersenen de morfogene signalen van de AVE en ANR zou kunnen moduleren. Tijdens latere ontwikkelingsstadia, zou Gpc-4 ook deel uit kunnen maken van het signalerende netwerk dat hersenschorsontwikkeling regelt.

In hoofdstuk 3 toon ik aan dat de expressie van *Gpc-4* in AVE en in ANR tijdens evolutie geconserveerd is gebleven. *Xenopus Gpc-4* komt namelijk tot expressie in de cellen van de ANR, anterior endoderm en prechordal endomesoderm, die functioneel als gelijkwaardig aan de AVE in de muis kunnen worden beschouwd. Om de functie van van *Gpc-4* tijdens voorbrein ontwikkeling te kunnen bestuderen hebben we in *Xenopus* embryo's zowel de expressie niveau van GPC-4 verhoogd als sterk gereduceerd. Een verlies van GPC-4 activiteit tijdens neurulatie verstoort de expressie van genen, zoals *Emx-2*, in de dorsale voorhersenen. Expressie patronen van genen die specifiek tot expressie komen in de ventrale voorhersenen veranderen niet. Verder veroorzaakt het verlies van GPC-4 activiteit apoptosis bij de neurale progenitors van de voorbrein tijdens de neurale buis sluiting. Daarnaast stellen biochemische studies vast dat GPC-4 aan de Fibroblast Growth Factor 2 (FGF-2) bind en hierdoor FGF signalering versterkt wordt. Deze experimenten suggereren dat GPC-4 de dorsale-ventrale patroonvorming van de voorhersenen regelt door positieve modulatie van FGF signalering.

FGF signalering speelt een belangrijke rol bij het moduleren van celproliferatie, migratie, differentiatie en overleving tijdens CZS morfogenese. Muizen waarbij bijvoorbeeld FGF-2 ontbreekt, vertonen defecten tijdens de ontwikkeling van de neocortex en hebben last van een autonome dysfunctie waardoor een te lage bloeddruk ontstaat. In hoofdstuk 4, heb ik de rol van FGF-2 signalering bestudeerd tijdens de ontwikkeling en functie van het autonome zenuwstelsel. In FGF-2 deficiënte embryo's is met behulp van een transgene lijn dat het kippen *FGF-2* gen (*cFGF-2*) onder controle heeft van de *Wnt-1* promotor, FGF-2 expressie specifiek teruggebracht in het zich ontwikkelende CZS. Farmacologische analyse liet een normalisatie zien van de neurale controle van bloeddruk in transgene FGF-2 mutant volwassen muizen, die cFGF-2 proteïnen tot expressie

hadden gebracht tijdens CZS ontwikkeling. Bovendien werd door de expressie van cFGF-2 ook de bloeddruk genormaliseerd. Deze studies wijzen erop dat FGF-2 signalering essentieel is voor de volledige ontwikkeling van het neurale circuit dat nodig is voor de centrale regeling van het bloeddruk.

In het laatste hoofdstuk bespreek ik de rol van GPC-4 tijdens de patroonvorming van hersenen in relatie tot zijn potentiële betrokkenheid bij twee humane syndromen. Het is bekend dat Glypicans de signalering van verschillende groeifactoren kunnen moduleren die celtype afhankelijk kan zijn. Ontwikkelingsstadium specifieke modulatie van ligand-receptor interactie door GPC-4 tijdens CZS ontwikkeling wordt ook in detail besproken.

RIASSUNTO

La ricerca scientifica presentata in questa tesi ha lo scopo di capire la comunicazione intercellulare che regola lo sviluppo della parte anteriore del cervello embrionale ("forebrain") e della spina dorsale nei vertebrati. La comunicazione cellulare coinvolge l'interazione fra le molecole-segnale (ligandi), che sono secrete, ed i loro rispettivi recettori. L'interazione tra ligando e recettore è un processo strettamente regolato durante lo sviluppo embrionale ed e' modulato, a livello della superficie cellulare, da glicoproteine quali i Glipicani. Questi ultimi sono proteine costituite da un nucleo principale a cui catene di zuccheri ("heparan sulphate glycosaminoglycan") sono attaccate. Queste catene di zuccheri sono cariche negativamente per la presenza di gruppi solfurici. Questa caratteristica attribuisce ai Glipicani specificità nell'interazione con diversi tipi di ligandi. Ad oggi, sei geni della famiglia dei Glipicani sono stati identificati nei mammiferi (da "Glypican-1" a "Glypican-6"), tuttavia poco si sa circa la loro funzione durante lo sviluppo del sistema nervoso.

Il capitolo 2 di questa tesi descrive la distribuzione spazio-temporale del *Glypicano-4* (*Gpc-4*) e di altri membri della famiglia dei Glipicani durante le prime fasi dello sviluppo embrionale nel topo. Nel topo, *Gpc-4* è il principale gene della famiglia ad essere espresso dalle cellule dell' "anterior visceral endoderm" (AVE) e da quelle dell' "anterior neural ridge" (ANR), due centri di segnalazione essenziali per induzione e successivo sviluppo del forebrain. Durante le successive fasi dello sviluppo embrionale Gpc-4 e' anche espresso dalle cellule staminali neuronali e dai primi neuroni post-mitotici che formano la corteccia cerebrale. Insieme questi risultati indicano che GPC-4 può funzionare durante i primi eventi fondamentali per lo sviluppo embrionale del forebrain modulando i segnali morfogenetici prodotti dall' AVE e dall' ANR. Nelle fasi successive dello sviluppo embrionale, Gpc-4 può anche fare parte della rete di segnalazione che regola lo sviluppo della corteccia cerebrale.

Nel capitolo 3 viene indicato che l'espressione di *Gpc-4* nell' AVE e nell' ANR e' conservata durante l'evoluzione. Infatti, anche il Gpc-4 dello *Xenopus* è espresso dalle cellule dell' ANR e anche dall'endoderma anteriore e dall'endomesoderma precordale, due tessuti che sono considerati dal punto di vista funzionale equivalenti all'AVE del topo. Alla luce dell'espressione di *Gpc-4* durante lo sviluppo del sistema nervoso centrale, ho effettuato esperimenti di "gain- e loss- of function" usando embrioni di *Xenopus* cosi' da poter analizzare la funzione di GPC-4 durante lo sviluppo del forebrain. Durante il processo di neurulazione, la perdita di attività del GPC-4 interrompe l'espressione di geni che marcano il territorio dorsale del forebrain come ad esempio *Emx-2*. In contrasto, geni che marcano il territorio ventrale del forebrain rimangono ancora espressi. In aggiunta, la perdita di GPC-4 causa anche la morte cellulare delle cellule neuronali staminali del forebrain durante la chiusura del tubo neurale. Studi biochimici dimostrano che GPC-4 lega la molecola-segnale: Fibroblast Growth Factor 2 (FGF-2) ed e' in grado di aumentare la traduzione del segnale FGF. Sulla base di questi risultati, viene proposto che la glicoproteina GPC-4 regoli lo sviluppo dorso-ventrale del forebrain modulando positivamente le molecole-segnale della famiglia degli FGF.

Molecole-segnale come gli FGF hanno ruoli importanti durante lo sviluppo in quanto regolano la proliferazione, la migrazione, il differenziaziamento e la sopravvivenza cellulare durante lo sviluppo del sistema nervoso centrale. Per esempio, topi mancanti del gene FGF-2 mostrano difetti nella formazione della corteccia cerebrale e soffrono di una disfunzione del sistema nervoso autonomo che conduce ad un fenotipo

ipoteso. Nel capitolo 4, viene analizzato il ruolo del segnale FGF-2 durante lo sviluppo e il funzionamento del sistema nervoso autonomo. In particolare, il gene FGF-2 è stato ri-introdotto selettivamente nel sistema nervoso centrale di embrioni di topo mancanti del gene FGF-2. Il gene e' stato ri-introdotto utilizzando un *FGF-2* trans-gene dil pollo (*cFGF-2*) sotto il controllo del promotore di *Wnt-1*. Analisi farmacologiche hanno rivelato una normalizzazione del controllo neuronale della pressione sanguigna in topi adulti che ri-esprimono l'FGF-2 durante lo sviluppo del sistema nervoso grazie alla presenza del transgene. Inoltre, alti livelli della proteina FGF-2 recuperano il fenotipo ipoteso. Questi studi indicano che la molecola-segnale FGF-2 è essenziale per il completo sviluppo dei circuiti neuronali richiesti per la regolazione centrale della pressione sanguigna.

Nel capitolo finale, viene discusso il ruolo di GPC-4 nello sviluppo del cervello anche alla luce della sua potenziale implicazione in due sindromi umane. Inoltre i Glipicani sono conosciuti come glicoproteine in grado di modulare in maniera specifica, a seconda del tipo di cellula, diverse molecole-segnale essenziali durante lo sviluppo embrionale. Inoltre la modulazione dell'interazione ligando-recettore, specifica per un determinato stadio dello sviluppo embrionale da parte di GPC-4 durante lo sviluppo del sistema nervoso centrale, è discussa in dettaglio.

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Milano, The Acknowledgement Day

CURRICULUM VITAE

Antonella Galli was born on the 9th of July 1971, in Milan, Italy. She studied Biology in the University of Milan: “Universita’ degli Studi”. She graduated in 1998, doing her experimental thesis in the laboratory of Developmental Biology, DIBIT-San Raffaele Hospital, Milan, Italy under the supervision of Prof. Dr. E. Boncinelli. In the same laboratory she also performed her postgraduate internship. Since the 1st of November 1999 she works in the Department of Developmental Biology, in the University of Utrecht investigating the role of Glypican-4 and Fibroblast Growth Factors in intercellular communication during central nervous system development. The research described in this thesis was carried out under the supervision of Dr. Rosanna Dono. From January 2004 she will start to wok as a postdoctoral fellow in the Developmental genetics group of Prof. R. Zeller at the University of Basel Medical School.

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