Development 129, 917-933 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV1755

spiel-ohne-grenzen/pou2 mediates regional competence to respond to Fgf8 during zebrafish early neural development

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Accepted 27 November 2001

SUMMARY

Neural patterning of the vertebrate brain starts within the ectoderm during gastrulation and requires the activity of organizer cell populations in the neurectoderm. One such organizer is located at the prospective midbrain-hindbrain boundary (MHB) and controls development of the midbrain and the anterior hindbrain via the secreted signaling molecule Fgf8. However, little is known about how the ability of neural precursors to respond to Fgf8 is regulated. We have studied the function of the zebrafish *spiel-ohne-grenzen* (*spg*) gene in early neural development. Genetic mapping and molecular characterization presented in the accompanying paper revealed that *spg* mutations disrupt the *pou2* gene, which encodes a POU domain transcription factor that is specifically expressed in the MHB primordium, and is orthologous to mammalian Oct3/Oct4. We show that embryos homozygous for *spg/pou2* have severe defects in development of the midbrain and hindbrain primordium. Key molecules that function in the formation of the MHB, such as *pax2.1*, *spry4*, *wnt1*, *her5*, *eng2* and *eng3*, and in hindbrain development, such as *krox20*, *gbx2*, *fkd3* and *pou2*, are all abnormal in *spg* mutant embryos. By contrast, regional definition of the future MHB in the neuroectoderm by complementary expression of *otx2* and *gbx1*, before the establishment of the complex regulatory cascade at the MHB, is normal in *spg* embryos. Moreover, the Fgf8 and Wnt1 signaling pathways are activated normally at the MHB but become dependent on *spg* towards the end of gastrulation. Therefore, *spg* plays a crucial role both in establishing and in maintaining development of the MHB primordium. Transplantation chimeras show that normal *spg* function is required within the neuroectoderm but not the endomesoderm. Importantly, gain-of-function experiments by mRNA injection of *fgf8* and *pou2* or Fgf8 bead implantations, as well as analysis of *spg-ace* double mutants show that *spg* embryos are insensitive to Fgf8, although Fgf receptor expression and activity of the downstream MAP kinase signaling pathway appear intact. We suggest that *spg*/*pou2* is a transcription factor that mediates regional competence to respond to Fgf8 signaling.

Key words: Competence, Fgf8, *pou2*, Oct3/4, Pou5f1, MHB, Isthmus, *pax2*, *spiel-ohne-grenzen*, Hindbrain, Zebrafish

INTRODUCTION

The organization of the vertebrate brain and its differentiation into functionally and anatomically distinct areas is based on early patterning and regional specification of the neural plate during embryonic development. Both vertical signals that emanate from the mesendoderm and planar signals travelling within the plain of the neuroectoderm itself are thought to be involved in neural plate patterning (Ruiz i Altaba, 1994; Gurdon et al., 1995; Kelly and Melton, 1995; Lumsden and Krumlauf, 1996; Wilson et al., 2002). Embryonic development of the midbrain and the anterior hindbrain in particular depend on an ectodermal population of cells located at the midbrain-hindbrain junction [the mid-hindbrain boundary (MHB) or isthmic organizer]. The organizer potential was initially demonstrated by transplantation experiments in chicken embryos, where isthmic tissue grafts induced midbrain and cerebellum ectopically. In its normal location, the MHB organizer was then proposed to regulate polarized morphological differentiation of the adjacent tectum and elaboration of the cerebellar anlage (Martinez and Alvarado-Mallart, 1990; Marin and Puelles, 1994; Garda et al., 2001; Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Evidence for this concept has come from functional studies of the molecules involved. Several transcription factors of the Pax, Otx, Gbx and Lim class play pivotal roles during development and function of the MHB organizer. In zebrafish, null mutations for the *pax2.1* gene (*pax2a* – Zebrafish Information Network) (*no isthmus* or *noi*) or inactivation of the *eng2* and *eng3* genes causes absence of the midbrain, MHB and cerebellum (Brand et al., 1996; Lun and Brand, 1998; Pfeffer et al., 1998; Scholpp and Brand, 2001), similar to the cognate mouse phenotypes (Millen et al., 1994; Wurst et al., 1994; Favor et al., 1996; Urbanek et al., 1997; Schwarz et al., 1997). The secreted signaling molecules Wnt1 (McMahon et al., 1992) and Fgf8 are thought to mediate organizer function. Fgf8 in particular is expressed in the MHB organizer and when

added ectopically can mimic the organizing activity (Crossley et al., 1996). Functional studies of the *acerebellar*/*fgf8* (*ace*) mutant in zebrafish and targeted disruption in mice highlight the crucial function of Fgf8 in this process (Brand et al., 1996; Reifers et al., 1998; Meyers et al., 1998; Picker et al., 1999). Like other Fgfs, Fgf8 is thought to signal through the MAP kinase pathway (Basilico and Moscatelli, 1992), resulting in activation of the specific target genes *gbx2*, *spry2*, *spry4*, *erm* and *pea3* after exposure to Fgf8 (Liu et al., 1999; Hidalgo-Sanchez et al., 1999; Martinez et al., 1999; Chambers et al., 2000; Fürthauer et al., 2001; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001; Lun et al., 2002). Furthermore, Fgf8 is required during formation of the heart field, for limb development, neural induction, telencephalon patterning, left-right asymmetry, gastrulation and ear development, among others (Brand et al., 1996; Shimamura and Rubenstein, 1997; Meyers and Martin, 1999; Sun et al., 1999; Reifers et al., 2000b; Shanmugalingam et al., 2000; Moon and Capecchi, 2000; Streit et al., 2000). Thus, Fgf8 elicits very different responses in different embryonic target cells, raising the important question of how the differential competence of the responding cells arises.

We have analyzed the function of the zebrafish spiel-ohne-grenzen (spg) gene during neural development. We find that spg allows the early neuroectodermal cells around the MHB to respond to Fgf8. spg alleles have been isolated in several mutagenesis screens as mutations affecting MHB morphology (Schier et al., 1996a; Burgess et al., 2002). In the accompanying paper (Burgess et al., 2002), we report that spg mutants affect the gene encoding the transcription factor Pou2, an ortholog of the mammalian Oct3/Oct4/Pou5f1 gene. We now show that spg serves a key function during development of the MHB and the hindbrain. Such a function has not been described for the mammalian gene; therefore, we have identified a novel component of the MHB genetic hierarchy. We show that spg/pou2 functions specifically in patterning the neuroectoderm where it is necessary to establish and maintain the MHB organizer and the hindbrain primordium; whereas, it appears dispensable for the earliest stage of subdividing the neuroectoderm. Importantly, RNA injections and Fgf8-bead implantations demonstrate that the early neural primordium of spg mutants is insensitive to the effects of Fgf8. In particular, both Pou2 and Fgf8 are necessary for the initiation of gbx2 expression at the MHB primordium. We, thus, suggest that spg/pou2 is required to make neuroectodermal cells competent to respond to Fgf8, as assayed by their ability to activate the correct target genes.

MATERIALS AND METHODS

Fish maintenance

Zebrafish were maintained under standard conditions (Westerfield, 1994; Brand and Granato, 2001). Embryos were staged as described elsewhere (Kimmel et al., 1995) or by hours post fertilization (hpf) at 28°C.

Fish lines

Alleles of spg^{m216}, spg^{e713} and spg^{hi349} are described elsewhere (Schier et al., 1996b; Burgess et al., 2002), the latter-most allele probably being a null allele [see Burgess et al. (Burgess et al., 2002) for a description of the molecular nature of these alleles]. The ace^{ti282}/*fgf8* allele has been described previously (Brand et al., 1996; Reifers et al., 1998). Heterozygous double carriers for spg^{m216} and ace^{ti282} were identified by random intercrosses.

Staining of living embryos

Confocal microscopy of Bodipy-Ceramide (Molecular Probes) was as described previously (Picker et al., 1999). Acridine Orange (2 μg/ml, Molecular Probes) was added into the medium surrounding dechorionated embryos for 4 hours during gastrulation, at the 5 somite stage and at the 12 somite stage.

Analysis of gene expression

Standard methods for whole-mount RNA in situ hybridization (ISH) were used, with laboratory modifications as described elsewhere (Reifers et al., 1998). Probes for the following genes were used: *krox20* (egr2 – Zebrafish Information Network) (Oxtoby and Jowett, 1993); *pax2.1* (Krauss et al., 1991a); *pax6* (Macdonald et al., 1994); *eng2* (*eng2a* – Zebrafish Information Network) (Ekker et al., 1992; Fjose et al., 1988); *shh* (Krauss et al., 1993); *ephA4* (*efna4* – Zebrafish Information Network) (Xu et al., 1994); *wnt1* (Kelly et al., 1993); *wnt4* (Ungar et al., 1995); *otx2* (Mori et al., 1994); *pax7* (Seo et al., 1998a); *fgf8* (Reifers et al., 1998); *spry4* (Fürthauer et al., 2001); *six3* (Seo et al., 1998b); *gbx1* and *gbx2* (Lun et al., 2002); *fkd3* (*foxb1.2* – Zebrafish Information Network) (Odenthal and Nusslein-Volhard, 1998); *valentino* (Moens et al., 1996); *wnt8b* (Kelly et al., 1995); *emx1* (Morita et al., 1995); and *Oct3*/*Oct4* (Schoeler et al., 1989).

Immuno- and histochemistry

Antibody staining against acetylated tubulin was carried out as previously described (Macdonald et al., 1997). Embryos for histological sections were embedded in epoxide resin, sectioned with a microtome (1 μm sections) and stained with Methylene Blue-Toluidine Blue, as described elsewhere (Kuwada et al., 1990). Brain morphology and staining (embryos were mounted in 70% glycerol after in situ hybridization) were documented on a Zeiss axiophot.

RNA injections

cDNA of murine *Oct3/4*, *fgf8*, *pou2* and nuclear *lacZ*, subcloned into pCS2+ (Rupp et al., 1994), were linearized and transcribed using the SP6 message mMachine kit (Ambion). The amount of mRNA injected was estimated from the concentration and volume of a sphere of RNA solution (0.25M KCl, 0.2% Phenol Red) injected into oil at the same pressure settings. RNA solution was backloaded into borosilicate capillaries prepared on a Sutter puller and injected into the cytoplasm of one cell of 2-cell stage embryos (about 100 pg *fgf8* mRNA or 200 pg of *pou2* mRNA per embryo). The injected mRNA has a strong tendency to stay in the progeny of the injected blastomere, as monitored by the unilateral distribution of co-injected *lacZ* mRNA, detected by staining with anti-β-gal antibody (Promega, 1:500) after in situ hybridization.

Bead implantation

Bead implantation was carried out as previously described (Reifers et al., 2000b). Beads coated with Fgf8b or phosphate-buffered saline (PBS) control beads were implanted at indicated regions of wild-type and *spg* mutant embryos at the 13 somite stage, embryos were fixed at 26 hpf.

Transplantation

Zygotes of wild-type embryos were labeled by injection of 10% HRP-coupled tetramethylrhodaminedextran (M_r=10,000, Molecular Probes D-1817) in 0.25M KCl and raised together with unlabeled host embryos from a heterozygous cross of *spg* carriers. Heterotopic transplantations of wild-type donor cells into host embryos were made between sphere and shield stage using a trimmed borosilicate capillary. Host embryos were fixed at the tailbud stage. After in situ hybridization, transplanted cells were

stained combining the Vectastain ABC system (VectorLabs) and the DAB system (Sigma).

Inhibitor treatment

For inhibition of the Fgf pathway, wild-type and *spg* embryos were treated with the chemical inhibitor SU5402, which blocks activity of all Fgf receptors (Calbiochem) (Mohammadi et al., 1997). The inhibitor was applied at 20 μM into embryo medium at 28°C in the dark and embryos were incubated from end of gastrulation until fixation at the eight-somite stage.

RESULTS

spiel-ohne-grenzen (*spg*) is required for midbrain-hindbrain boundary and hindbrain development

At 24 hpf, the MHB of wild-type embryos is marked by a prominent inward fold of the neuroepithelium, which develops into the isthmic constriction of the brain. The formation of this fold is disturbed in living homozygous *spg* embryos (Fig. 1) (Schier et al., 1996a). Results are based on analysis of the spg^{e713} allele and the likely null allele spg^{hi349}; they give an identical phenotype of slightly variable expressivity, with the exception of the *pou2* staining [see Burgess et al. (Burgess et al., 2002), for a discussion of molecular defects of the alleles]. Optical sections of live embryos stained with Bodipy-Ceramide and histological sections show that in *spg* mutants, both the prominent inward fold at the MHB and the cerebellar primordium which abuts the MHB are missing, and that the tectum opticum is variably reduced in size (Fig. 1C-F,I,J). After 26 hours of development, a small aggregate of cells is visible at the MHB of *spg* mutants (Fig. 1D,F) that is absent in *acerebellar* (*ace*) mutants (Reifers et al., 1998). In addition, *spg* mutants have smaller otic vesicles with often only one otolith, and a curved, slightly shortened tail with misshaped somites (not shown). From day 4 onwards, 30-50% of mutant larvae show a slightly reduced frequency of heartbeat and develop edema, although both the atrium and ventricle are initially present, unlike in *acerebellar* embryos (Reifers et al., 2000b). *spg* mutants feed far less efficiently than wild-type embryos and die after 14 to 19 days for unknown reasons.

Acridine Orange specifically interacts with DNA of non-condensed, fragmented chromatin and can be used in zebrafish to detect cells undergoing cell death (Brand et al., 1996). We detected dying cells in the prospective MHB and tectum of *spg* mutants from the 14-somite stage onwards until the pharyngula period, most prominently during late somitogenesis (Fig. 1G,H). Cell death is particularly apparent within the hindbrain around the 22 somite stage, in two transverse stripes (Fig. 1H, insert) that probably correspond to r3 and r5 (see Fig. 5). Weaker incorporation of Acridine Orange occurs in the optic stalk, tail-tip and the dorsal midline of the tail and trunk regions (Fig. 1H and not shown). Because dying cells are detectable from mid-somitogenesis stages onwards, cell death probably results from earlier defects. In addition, the cell death probably contributes to the development of the MHB phenotype of *spg* mutants at 24 hpf. Anti-acetylated-tubulin staining demonstrates that the axonal scaffold of *spg* embryos is specifically disrupted not only in midbrain, but also hindbrain development: longitudinal and transverse axon bundles, normally located at rhombomeric boundaries, are not tightly fasciculated and show imprecise scaffolding, and the distance between forebrain and hindbrain commissures is reduced in *spg* embryos, probably owing to tissue elimination by cell death (Burgess et al., 2002) (Fig. 1H). Indeed, *spg* embryos lack a recognizable trochlear nerve within the MHB (not shown).

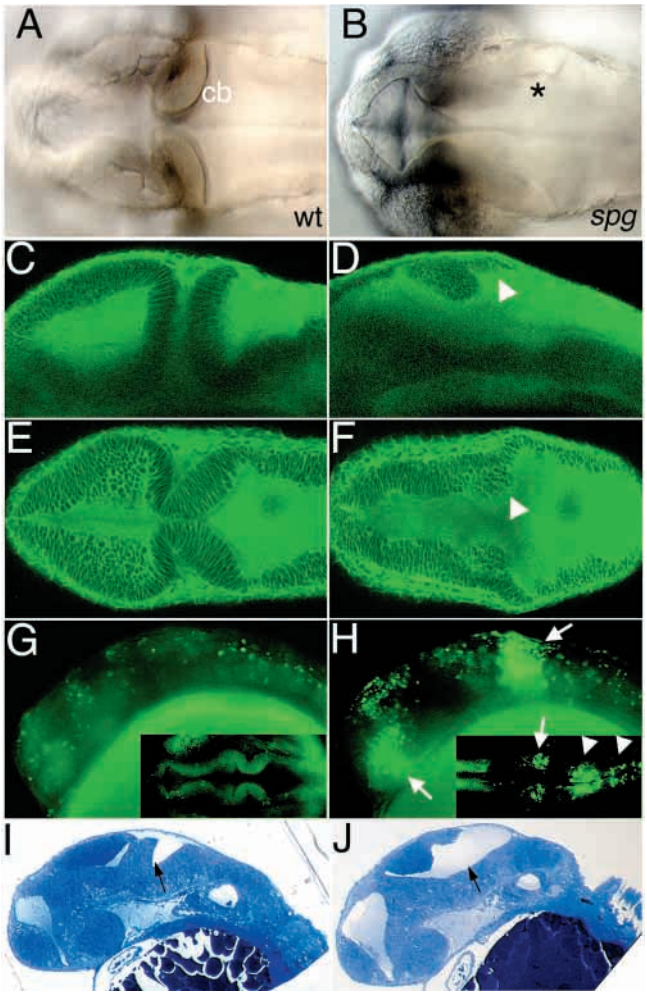


Fig. 1. Brain phenotype of *spg*-embryos at pharyngula stages. (A,C,E,G,I) Wild-type and (B,D,F,H,J) homozygous *spg* mutant embryos. (A,B,E,F and small pictures in G,H) dorsal views; (C,D,G,H,I,J) lateral views. In the wild-type embryo (I), the MHB is marked by an arrow; The asterisk in B and the arrow in J indicate lack of the MHB in mutant embryos. (D,F) An arrowhead indicates a likely rudimentary tissue of the posterior cell row and the cerebellum after 28 hpf. (A,B) Phenotype of living embryos. (C-F) Optical sections of living embryos stained with fluorescent Bodipy-Ceramide. (G,H) Fluorescent staining with Acridine Orange indicates cell death at the prospective MHB and the optic stalk in *spg* embryos (H) at the 17-somite stage, indicated by arrows. Cell death is also detected in two transverse bands within the rhombencephalon at the 22-somite stage (arrowheads, insert in H; the arrow points to the MHB). (I,J) Sagittal histological sections.

Establishment and maintenance of the MHB is affected in *spg* mutants

The above observations, previous data (Schier et al., 1996a) and the expression pattern of *pou2* (the gene affected in *spg* mutants) (Burgess et al., 2002) all suggest that early neural

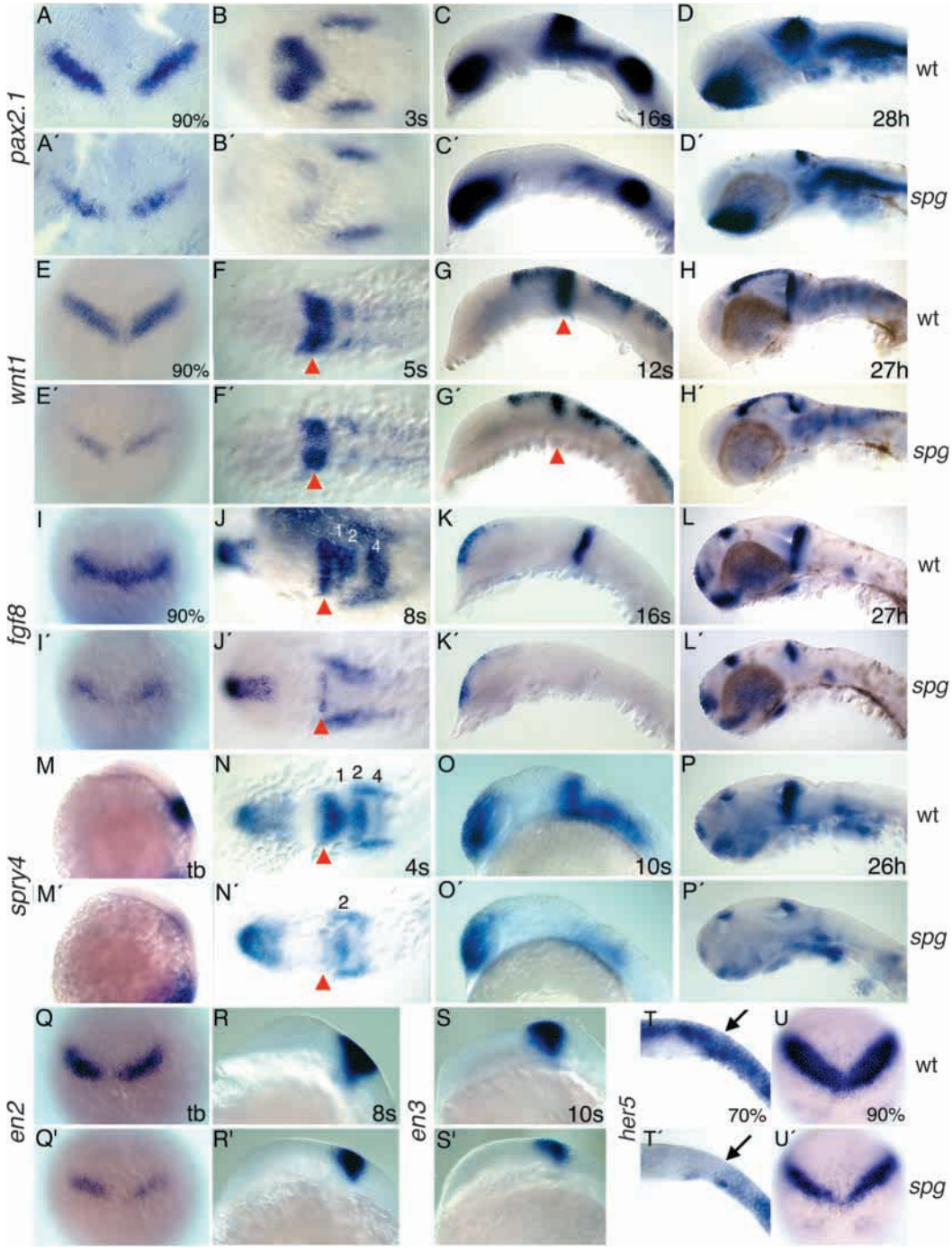


Fig. 2. The primordia of the MHB and the hindbrain affected in *spg* embryos. (A-B′,E-F′, I-J′,N-N′,Q,Q′,U-U′) Dorsal views; embryos in the remaining pictures are shown from lateral view. Gene expression, stages and genotypes are noted. Red arrowheads indicate expression of genes at the MHB throughout. (A-D′) In *spg* embryos, *pax2.1* shows reduced expression at the MHB from its onset onwards (A′,B′), is lost during midsomitogenesis (C′) and re-expressed as a dorsal patch after 24 hpf. Expression of *pax2.1* within the otic placode (B-D′) is not affected in mutant embryos. (E-H′) *wnt1* is normally expressed at its onset at 80% of epiboly (not shown), but becomes subsequently downregulated in mutant (E′) at the time when *pax2.1* is initiated. During somitogenesis (F-G′), the expression of *wnt1* at the MHB (arrowhead) and within rhombomeres is downregulated in *spg* embryos. At pharyngula stages, *wnt1* expression is continued within a dorsal patch at the MHB. (H,H′) The midsagittal expression in the diencephalon seems unaffected, but MHB expression is reduced to a dorsal patch in mutant embryos. (I-I′) *fgf8* expression, like that of *wnt1*, is not affected in *spg* embryos at its onset of expression at the MHB (not shown), but soon becomes downregulated at around 90% of epiboly. (J) *fgf8* expression caudally continues in r1, r2 and r4 in wild-type embryos. (J′) In mutant embryos, *fgf8* expression is strongly reduced within r1 and abolished within r2 and r4. During somitogenesis, *fgf8* expression is completely lost from the MHB but, like *pax2.1* and *wnt1*, recovers at a dorsal patch at the MHB. (M-P′) *spry4* is not properly initiated in *spg* embryos. At the four-somite stage, *spry4* is strongly reduced at the MHB and in r1, r2 and r4 (N′). MHB expression of *spry4* during somitogenesis and pharyngula stages follows the same mode as *fgf8* and *pax2.1*. (Q,Q′) *en2* is normally initiated at the MHB at the end of gastrulation. In *spg* mutant embryos, *en2* is downregulated from its initiation of expression. (R) *en2* is expressed in the prospective tectum in a graded fashion during somitogenesis but is strongly reduced in *spg* embryos (R′). (S) *en3* is encompassed within the *en2* domain at the tectum in wild-type embryos. (S′) In *spg* embryos, *en3* is downregulated in a similar fashion as *en2*. (T,T′) half sides of transverse sections through the *her5* positive domain at the spatial level of the future MHB; arrows point to the neuroectoderm. (T) *her5* is normally initiated within the neuroectoderm around 70% of epiboly, overlying mesendodermal expression. (T′) *her5* is not properly initiated in *spg* embryos. (U,U′) *her5* expression at the MHB is reduced in mutant embryos at the end of gastrulation.

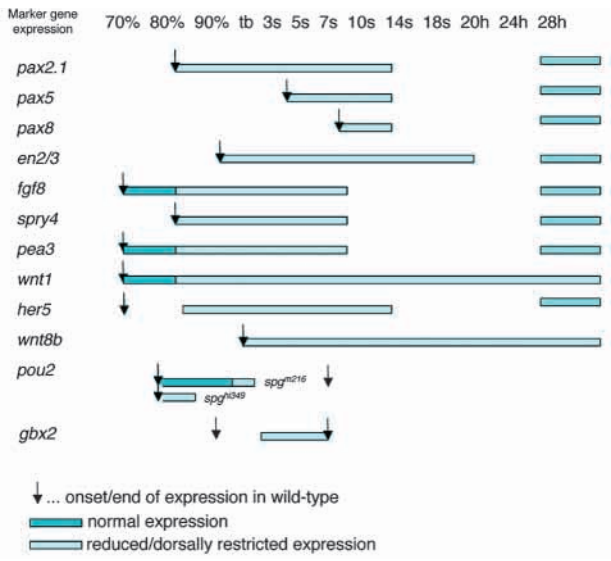


Fig. 3. Marker gene expresison at the MHB in *spg*.

development is abnormal in *spg* mutants. Using in situ hybridization with antisense RNA, we therefore followed in detail the expression of MHB and hindbrain marker genes. Four representative stages are shown to illustrate the results (Fig. 2, summarized in Fig. 3). Expression of *pax2.1* is downregulated already at the onset of expression at 80% of epiboly (Fig. 2A′), as reported previously (Schier et al., 1996a; Burgess et al., 2002), as is *spry4* (Fig. 2M′). *pax2.1* expression remains strongly reduced during early somitogenesis stages (Fig. 2B′), is completely eliminated during midsomitogenesis stages (Fig. 2C′) and is re-expressed in a dorsal patch at the prospective MHB after 24 hpf (Fig. 2D′). Similarly, expression of *eng2*, *eng3* and *her5* is affected both during initiation and maintenance (Fig. 2Q′-U′). The kinetics of *fgf8* expression at the MHB in *spg* mutants is overall similar to that of *pax2.1* and the other markers (Fig. 2I′-L′; red arrowheads). By contrast, however, expression of *fgf8* and *wnt1* is initiated normally at 70-80% of epiboly, and only become decreased at 80-90% of epiboly (Fig. 2E′,I′). During early somitogenesis stages, *fgf8* and *spry4* are expressed in rhombomeres 1, 2 and 4 (Reifers et al., 1998; Fürthauer et al., 2001). In these rhombomeric domains, *fgf8* and *spry4* expression is likewise strongly reduced in *spg* mutants (Fig. 2J′,N′). Conversely, initiation of *pou2* expression is not affected during gastrulation stages in *noi/pax2.1* and *ace/fgf8* mutants, or in wild-type embryos in which all Fgf signaling is blocked pharmacologically (data not shown). Beginning at the one- to two-somite stage, *pou2* expression is gradually lost in *ace* mutants or in inhibited embryos, as described previously for many other markers, but *pou2* expression remains normal in *noi* mutants at least until the six-somite stage (data not shown) (Reifers et al., 1998). Thus, *spg/pou2* is required to initiate expression of *pax2.1*, *eng2*, *eng3* and *her5*, and is required to maintain, but not initiate, expression of *wnt1* and *fgf8*.

Although the tectum expresses *otx2* (see Fig. 5Q), tectum development is abnormal in *spg* mutants. Expression of the engrailed genes *eng2* and *eng3* is reduced in *spg* mutants throughout embryonic development (Fig. 2R′,S′), consistent

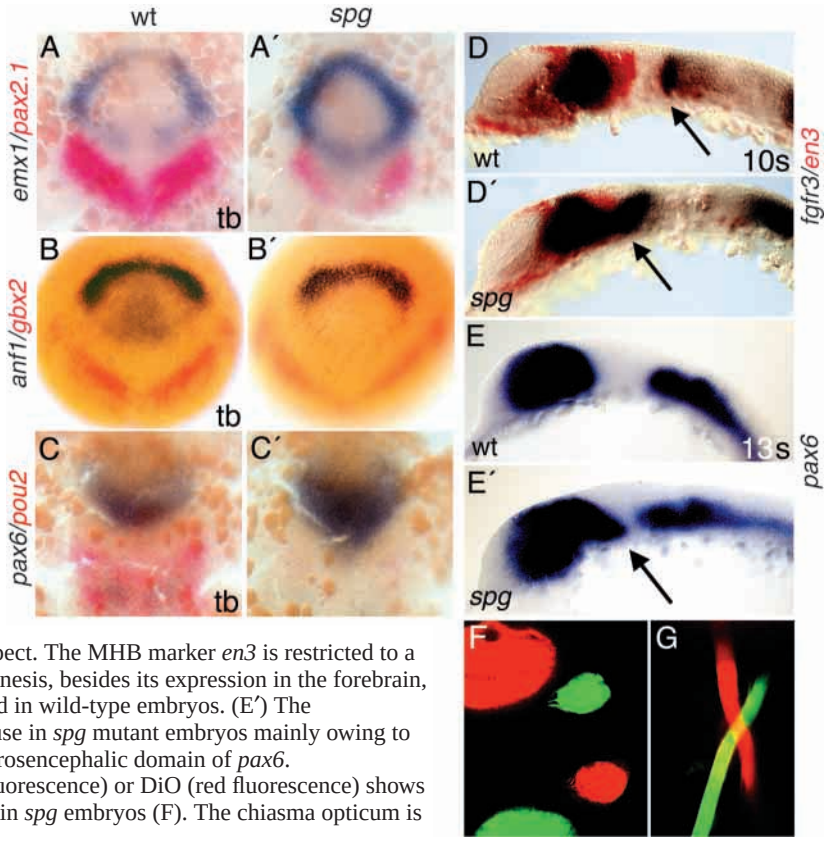


Fig. 4. Prosencephalic markers expand posteriorly in *spg* embryos. (A) *emx1* is expressed in telencephalic precursors from end of gastrulation onwards in wild-type embryos. The posterior transverse expression domain marks the di-mesencephalic boundary. *pax2.1* expression at the anterior MHB is shown in red. (A′) In *spg* embryos, defined by the impaired expression of *pax2.1* at the MHB, *emx1* expression is generally elevated but reduced in its spatial lateral extent. The posterior border of *emx1* expands caudally. (B) *anf1*, like *emx1*, is expressed at the anterior neural border with a patch of expression centering around the midline of the neuroectoderm. *gbx2* expression at the posterior MHB is shown in red. (B′) *anf1* is lost within the midline expression domain in *spg* embryos, defined by impaired *gbx2* expression (see Fig. 3 for *gbx2* expression). (C) *pax6* is initiated within the forebrain at the end of gastrulation. *pou2* expression at the MHB is seen in red. (C′) *spg* embryos, identified by loss of *pou2* expression, show a posterior expansion of *pax6* expression into the territory of the prospective MHB. (D,D′) Double in situ hybridization with *fgfr3* (blue) and *en3* (red) at the 10-somite stage show the hindbrain domain of *fgfr3* (arrow in D,D′) is fused with the diencephalic domain of *fgfr3*, particularly at its ventral aspect. The MHB marker *en3* is restricted to a dorsal patch in mutant embryos (D′). (E) During somitogenesis, besides its expression in the forebrain, *pax6* is also expressed within the hindbrain and spinal cord in wild-type embryos. (E′) The prosencephalic and the rhombencephalic domain nearly fuse in *spg* mutant embryos mainly owing to strong posterior expansion of the posterior border of the prosencephalic domain of *pax6*. (F,G) Anterograde filling of whole eyes with DiI (green fluorescence) or DiO (red fluorescence) shows a proper contralateral retinotectal mapping of RGC axons in *spg* embryos (F). The chiasma opticum is properly formed in *spg* mutant embryos (G).

Fig. 5. The hindbrain primordium is affected in *spg* embryos. Embryos are photographed from the dorsal side, with the exception of D,D′ (transverse sections at the level indicated by an arrow in C,C′) and E, E′ (lateral views). Dorsal is upwards in D,D′. Anterior is towards the top in A-C′,F,F′,R,R′,S,S′; anterior is to the left in the remaining pictures. Embryos are at the tailbud stage unless indicated differently. (A) *gbx1* expression is strictly posteriorly adjacent to the MHB domain of *pax2.1* in wild-type embryos during gastrulation. (A′) In *spg* embryos, *pax2.1* and *gbx1* are expressed in the same mutually exclusive fashion as seen in wild-type embryos at the end of gastrulation. However, in mutant embryos *pax2.1* expression is reduced at the MHB. (B) In wild-type embryos, *otx2* expression partially overlaps *pax2.1* expression at the MHB at the end of gastrulation. (B′) *spg* embryos show a proper spatial relationship of *otx1* and *pax2.1* at the prospective MHB at the end of gastrulation. (C,D) In wild-type embryos, *gbx2* becomes activated at around 90% of epiboly within the neural ectoderm, shortly after onset in the underlying mesendoderm. (C′,D′) In mutant embryos, the mesendodermal domain of *gbx2* is initiated normally (red arrowhead in D′) but the neurectodermal domain of *gbx2* is not initiated. Two longitudinal stripes in the non-neural ectoderm are unaffected (black arrow in D′). (E,E′) In contrast to *spg* mutant embryos, *gbx2* is lost in both the mesendodermal and the neuroectodermal germ layer in *ace* mutant embryos. (F) The hindbrain domain of *fkd3* is lost in mutant embryos (F′). (G) In wild-type embryos, *krox20* stains r3 and r5, and *six3* is expressed within the prosencephalon, including the prospective eye field. (G′) In mutant embryos, *six3* seems not affected but *krox20* is strongly reduced. (H) *ephA4* is expressed in wild-type embryos within the prosencephalon and the rhombencephalon, in particular within rhombomeres 1, 3 and 5. (H′) Rhombomeric expression of *ephA4* is strongly affected and the prosencephalic domain shows massive posterior expansion. (I) *wnt8b* is normally expressed within the diencephalon, at the MHB and within rhombomeres 1, 3 and 5. (I′) In *spg* embryos, MHB expression of *wnt8b* is strongly reduced (arrowhead) and rhombomeric expression is strongly downregulated; in particular, r1 cannot be discriminated from and possibly fuses with the MHB domain. (J) Double in situ staining for *hoxb1a*, expressed in r4, and *hoxb4a*, expressed within the spinal cord with an anterior limit at the border between r6 and 7 in wild-type embryos. The bracket indicates the gap between r4 and 7. (J′) In mutant embryos, the gap between r4 and r7, indicated by the bracket, is strongly reduced. (K,K′,L) *pou2* expression becomes refined during early somitogenesis within distinct bilateral clusters, according to r2 and r4, and to a patch of expression at the posterior border of the MHB. (L′) In early somitogenesis, embryos of the allele *spg*^{e713} show strongly reduced rhombomeric expression of *pou2*, whereas in embryos carrying the insertional allele *spg*^{hi349}, *pou2* expression is totally abolished (L″). (M) *val* is normally expressed within r5 and 6. (M′) In mutant embryos, *val* expression is nearly abolished in r5 but the expression in r6 is not affected. *val* is also expressed within precursor cells of the neural crest (indicated by arrows in M,M′), which is not affected in mutant embryos. (N) *fkd3* is expressed at inter-rhombomeric borders at late somitogenesis stages in wild-type embryos. (N′) In *spg* embryos, inter-rhombomeric expression is strongly reduced. (O) *zath1* is normally expressed at the prospective cerebellum and along the dorsal rim of the fourth ventricle. This expression is also maintained during later pharyngula stages (P). (O′) *zath1* expression is lost from the cerebellar anlage in *spg* embryos (arrow) but expression recovers partially at later stages (arrow in P′). (Q) In wild-type embryos, expression of *otx2* at pharyngula stages covers the midbrain and the MHB, in particular the concise stripe of the posterior cell row (arrow) marking the transition between the tectum and the cerebellar anlage. (Q′) In *spg* embryos, expression of *otx2* partially recovers within this particular posterior cell row (arrow) at late pharyngula stages. The spatial extent of the midbrain territory of *otx2* is apparently smaller than in wild-type embryos. (R) Among the proneural genes, *ngn1* is expressed in precursors of primary neurons in wild-type embryos at the beginning of somitogenesis. (R′) *ngn1* is strongly abrogated in mutant embryos. (S) *sox17* is normally expressed within the endodermal precursors in a punctate pattern during gastrulation (inset: transversal section at 70% epiboly, showing *pou2* expression restricted to the neuroectodermal layer). (S′) *sox17* expression is strongly affected in mutant embryos. (T) *myod* is expressed within the paraxial mesoderm and muscle precursors within somites during somitogenesis. (T′) *myod* expression is strongly reduced in the somitic mesoderm of *spg* embryos but the paraxial domain seems unaffected.

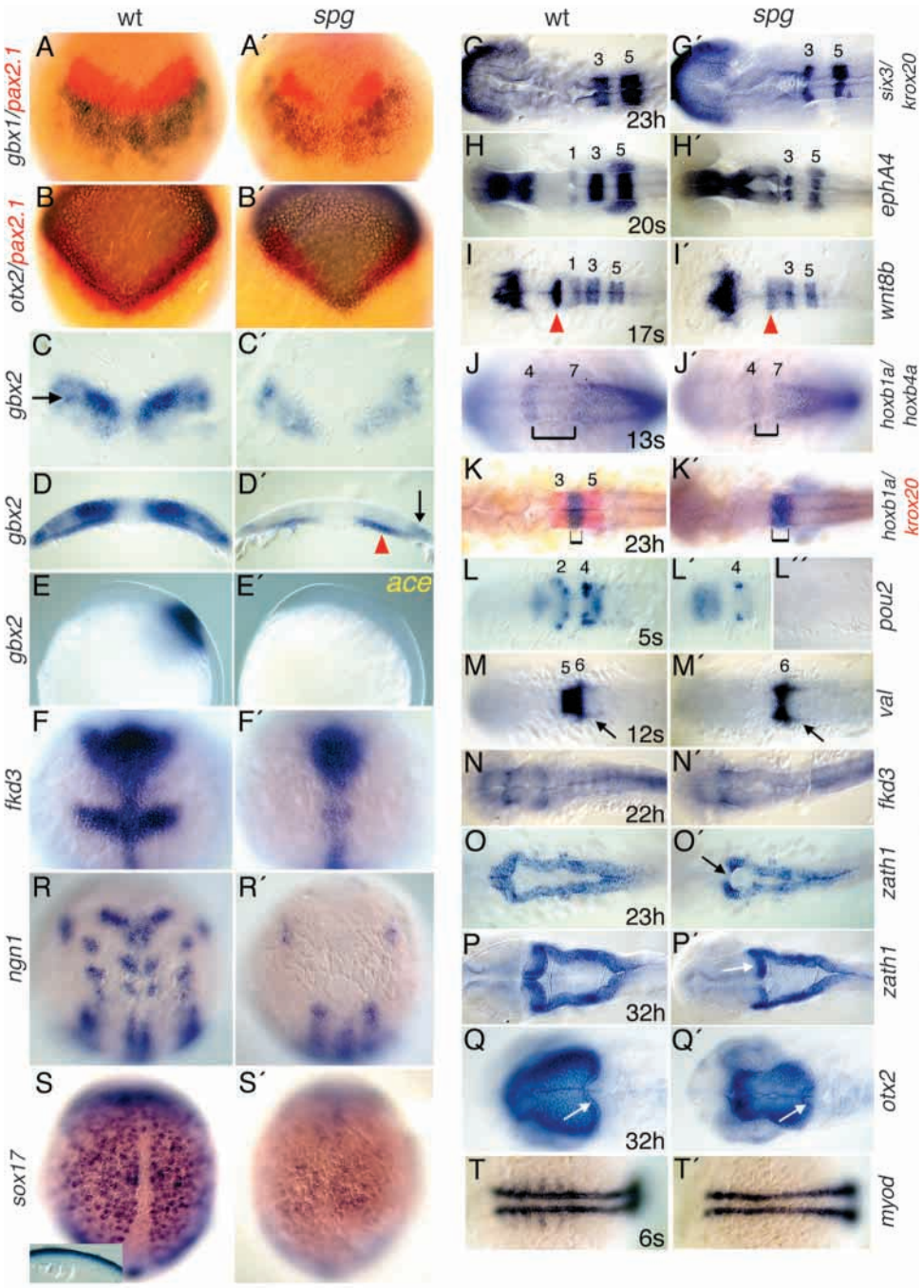
with their role as target genes of *pax2.1* (Lun and Brand, 1998; Scholpp and Brand, 2001). Moreover, the tectum-specific ephrins ephrinA5a and ephrinA5b, the genes for which are probably the target of engrailed proteins, are strongly restricted to a dorsal patch but never completely abolished in *spg* mutants (not shown). *her5* encodes a bHLH transcription factor expressed at 70% of epiboly in the MHB primordium and the underlying mesendoderm (Fig. 2T) (Müller et al., 1996; Lun and Brand, 1998). In *spg* mutants, *her5* expression is initially normal in the mesendodermal layer (Fig. 2T′), but is not initiated within the overlying neuroectoderm (arrow in Fig. 2T′,U′). At 90%, *her5* expression is downregulated in the mesendoderm of wild-type and mutant embryos. The expression of additional MHB markers in *spg* mutants is similar to those above and is summarized in Fig. 3. MHB markers are typically more strongly affected in the ventral MHB of *spg* mutants during early somitogenesis, before the expression is eventually lost completely (see Fig. 8O; and not shown). This is not due to defective midline tissue, as *shh* and *twhh*, which encode two secreted Hedgehog-family members expressed throughout the ventral CNS midline, are expressed normally in *spg* mutants. Tailbud expression of these genes was slightly reduced (not shown), possibly explaining the slightly twisted tail of the mutants.

Positioning of the MHB is normal in *spg* embryos

Studies in several vertebrates suggest that *Otx2* and *Gbx2*, which are expressed in mutually exclusive territories of the hindbrain and fore/midbrain, respectively, are involved in positioning the organizer at the MHB (Wassarman et al., 1997; Broccoli et al., 1999; Millet et al., 1999; Rhinn and Brand, 2001). In zebrafish, *gbx1* is the functional equivalent of the murine *Gbx2* gene (Lun et al., 2002). In wild-type embryos, *gbx1* is expressed in the hindbrain primordium, in a domain complementary to the expression of *otx2*, which partially includes the *pax2.1* activation domain (Lun et al., 2002) (see Fig. 5A,B). In *spg* mutants, recognizable by their reduced expression of *pax2.1*, the spatial relationship between *gbx1*, *otx2* and *pax2.1* expression appears normal (see Fig. 5A′,B′), indicating that the initial subdivision of the neurectoderm into an *otx2*-and a *gbx1*-positive domain occurs normally in *spg* mutants.

Caudal expansion of prosencephalic gene expression

Morphological, histological and immunohistochemical inspection at pharyngula stages showed that forebrain architecture was largely normal in *spg* mutants (Fig. 1). *ace*/*fgf8* mutants show abnormal retinotectal projection and a defective



optic chiasm (Picker et al., 1999; Shanmugalingam et al., 2000), which prompted us to study forebrain marker expression and to examine specifically the visual system in *spg* mutants using anterograde fills with DiI. In all of seven examined *spg* mutants, we observed normal contralateral retinotectal mapping of retinal ganglion cell axons (Fig. 4F) and a properly elaborated decussation of the optic nerve (Fig. 4G). In our marker analysis, we find evidence for abnormal development of the forebrain neural plate, especially of the diencephalic primordium. *emx1* is expressed in the telencephalic primordium at the end of gastrulation, lining the anteriormost border of the developing brain, and in a bilateral transverse stripe of expression in the posterior diencephalon, which does not fuse at the midline (Morita et al., 1995). These bilateral stripes are parallel to, yet separate from, the *pax2.1* stripes at the MHB (Fig. 4A). In *spg* mutant embryos, *emx1* expression appears to be generally upregulated in the telencephalic primordium, and the bilateral transverse stripes in the diencephalon almost fuse with the strongly reduced MHB domain of *pax2.1* (Fig. 4A′; *pax2.1* expression is shown in red). *anf1* is expressed in a similar way to *emx1* but with a triangular domain in the diencephalon (Fig. 4B) (Shanmugalingam et al., 2000; Kazanskaya et al., 1997) that is not seen in *spg* embryos (Fig. 4B′). Furthermore, expression of *pax6* at the di-mesencephalic boundary (Macdonald et al., 1994) is upregulated and strongly expanded caudally from its onset of expression (Fig. 4C′).

During somitogenesis stages, *fgfr3* and *pax6* are expressed in wild-type embryos in the diencephalon and in r1, i.e. in territories abutting the midbrain and MHB (Fig. 4D,E) (Krauss et al., 1991b; Sleptsova-Friedrich et al., 2001). In *spg* embryos, these expression domains almost fuse (Schier et al., 1996a). As strong cell death is not yet detectable at this stage, this fusion may be due to a transformation of the intervening mis-specified midbrain and MHB tissue, rather than a simple elimination. In double in situ hybridization analysis with *fgfr3* and *eng3* (red staining in Fig. 4D,D′), *eng3* is still expressed in a mesencephalic remnant posteriorly adjacent to the diencephalic territory of *fgfr3* but is reduced to a faint dorsal patch as expected for *spg* embryos (Fig. 4D′). This suggests that during early somitogenesis, the remnant expression of tectal or MHB markers is still able to specify some rudimentary tissue between the forebrain and the hindbrain, which prevents forebrain gene expression from invading into this distinct dorsal tissue. Later in development, when gene expression is generally absent at the MHB in *spg* embryos, prosencephalic markers are not only posteriorly expanded on the ventral but also on the dorsal side, exemplified by the expanded dorsal thalamic domain of *ephA4* (Fig. 5H). In contrast to telencephalic and diencephalic gene expression domains, midbrain expression domains (i.e. those of engrailed genes or of *otx2*) are never caudally expanded in *spg* embryos (Fig. 5Q). Unlike diencephalic marker expression, hindbrain marker expression is not markedly expanded towards the anterior. For example, the anterior limit of the hindbrain expression domains of *pax6* or *gbx1* (Fig. 4E′ and not shown)

appear normal in *spg* embryos, and even in double in situ hybridization analysis with *krox20* and *pax6*, we found no relative expansion of the anterior border of *pax6* expression in the hindbrain of *spg* mutants (not shown). Similarly, rostral borders of the diencephalic expression domains of *dlx2*, *otx1*, *wnt8b*, *emx1*, *pax6*, *ephA4*, *six3* and *anf1* were not altered during early somitogenesis stages in *spg* mutants (not shown).

Early failure of hindbrain gene expression in *spg* mutants

During the analysis of MHB-specific genes such as *fgf8* and *spry4*, we found that these genes are also affected in their hindbrain expression (Fig. 2J′,N′). *pou2* is itself expressed in the hindbrain primordium at the end of gastrulation (Burgess et al., 2002; Hauptmann and Gerster, 1995) prompting us to examine establishment of gene expression in the hindbrain primordium of *spg* embryos. We find that gene expression in the hindbrain fails from late gastrula stages onwards, as is most clearly evident from the analysis of *gbx2* expression. *gbx2* expression is initiated first in the mesendoderm around 80% of epiboly and subsequently around 90% within the overlying hindbrain neuroectoderm (Lun et al., 2002) (Fig. 5C-E). Studies of *acerebellar* (*ace*) mutants have shown that both tissue layers absolutely require *fgf8* to express *gbx2* (Fig. 5E′) (Lun et al., 2002). By contrast, in *spg* mutants, neuroectodermal expression of *gbx2* is not initiated at all (Fig. 5D′), whereas the underlying mesendodermal expression of *gbx2* occurs normally (Fig. 5C′,D′; arrowhead in D′). In addition to *gbx2*, ectodermal expression of the forkhead domain transcription factor *fkd3* (Odenthal and Nüsslein-Volhard, 1998) is absent from the hindbrain primordium of *spg* embryos (Fig. 5F′). Therefore, although positioning of the MHB in the neuroectoderm appears normal, global gene expression in the hindbrain primordium, a known site of *pou2* expression (Burgess et al., 2002), is already severely disrupted before the end of gastrulation in *spg* mutants.

During the early segmentation period, *pou2* expression becomes confined to distinct cell populations in r2 and r4 (Fig. 5L) (Hauptmann and Gerster, 1995). Genes that mark the segmental organization of the hindbrain, such as *krox20*, *ephA4*, *wnt8b*, *hoxb1a* and *hoxb4a* are all strongly affected in their expression (Fig. 5G-K′), probably owing to a mixture of a global and a rhombomere specific requirement for *pou2* in the hindbrain. Next to the *pou2*-expressing rhombomeres r2 and r4, *krox20*, *ephA4* and *wnt8b* are normally expressed within r3 and r5. In *spg* mutants, the size of r3 and r5 appears reduced, whereas that of the intermittent r4 appears normal or enlarged (Fig. 4G′-I′). Thus, in addition to the early gene expression defects of the hindbrain primordium, rhombomeres also show specific defects during segmentation stages that differ depending on the rhombomere considered. This may reflect a later, rhombomere-restricted function of *pou2*. *six3* expression in the prospective telencephalon and eye field is not altered in *spg*, whereas *ephA4* expression in the otic placode is reduced, and diencephalic expression is posteriorly expanded into the midbrain and MHB (Fig. 5H′), as described above for other diencephalic markers, except for *wnt8b*, which is not altered (Fig. 5I′). Expression of the Hox genes *hoxb1a* in rhombomere 4 (r4) and *hoxb4a* from r7 into the spinal cord (Fig. 5J,J′) is mildly affected in *spg* embryos at midsomitogenesis. The *hoxb1a* domain appears more diffuse compared with the wild type and is even wider towards the end of somitogenesis (Fig. 5K′), but more strikingly, the gap between the *hoxb1a* and *hoxb4a* domains in the r4-r7 territory is significantly reduced in *spg* embryos, as indicated by brackets in Fig. 5J′. At late somitogenesis stages, *hoxb1a* is more strongly affected in *spg* mutants. Concomitant with the reduced odd-numbered rhombomeres, r4 apparently enlarges at the expense of r3 and 5, as judged from *hoxb1a*/*krox20* double staining (Fig. 5K,K′). The expression of *pou2* itself provides one of the clearest example for a function of *spg/pou2* in specific hindbrain rhombomeres. In embryos that carry the spg^{e713} point allele, the discrete patches of *pou2* expression in r2 and r4 are strongly reduced or absent in *spg* mutants (Fig. 5L′). Embryos carrying the apparent null allele spg^{hi349} show complete absence of expression in all *pou2* domains (Fig. 5L″). Expression of the bZIP transcription factor *valentino/Kreisler* (Manzanares et al., 1999; Cordes and Barsh, 1994) in r5 and r6 is abrogated in r5 but unaffected in r6, including the neural crest streaming from r6 (Fig. 5M′). Consistent with the disorganization of hindbrain commissures (Burgess et al., 2002), expression of *fkd3* (Fig. 5N) (Odenthal and Nusslein-Volhard, 1998) at rhombomere boundaries is nearly abolished in *spg* embryos (Fig. 5N′).

Neurogenesis, as labeled by *zath1* expression, is reduced in *spg* mutants in the ventricular zone of the hindbrain ventricle at 24 and 32 hpf (Fig. 5O′,P′). *zath1* expression in the mutants occurs also in a position corresponding to the cerebellar anlage/posterior MHB in the wild-type (arrow). Expression in this tissue might either reflect an expanded rhombic lip, or a partial reformation of the cerebellum at later stages in the mutants. This tissue does not express the fore/midbrain marker *otx2* (Fig. 5Q), and we therefore tentatively suggest that this is the result of a partial re-formation of cerebellar tissue after 30 hpf in the *spg* mutants, explaining some of the observed variation in morphological strength. Earlier stages of neurogenesis, as labeled by the proneural bHLH transcription factor *ngn1* are also affected. *ngn1* is expressed in trigeminal precursors and in proneural cell clusters in the brain primordium already at the end of gastrulation, and this expression fails to be initiated in *spg* mutants (Fig. 5R,R′). Expression in three rostrocaudal rows of cells within the presumptive spinal cord containing the precursors of motoneurons, interneurons and sensory neurons is, however, initiated normally in *spg* mutants, although the rows are compressed into a narrower space.

Mesendoderm development in *spg* embryos

In addition to the brain phenotype, *spg* mutants have a curved and malformed tail with misshapen somites (Burgess et al., 2002), suggesting the existence of non-neural defects. We find that *myod* expression is strongly reduced in somitic precursors (Fig. 5T), but unaffected in adaxial cells. Somitic expression of other markers like *snail1* (Hammerschmidt and Nüsslein-Volhard, 1993), *eng2* (Devoto et al., 1996) and *fgf4.1* (Grandel et al., 2000) are also reduced in *spg* embryos during somitogenesis (not shown). However, unlike in *acerebellar* mutant embryos (Reifers et al., 1998), somitic alterations are not morphologically distinguishable before the beginning of pharyngula stages. Because induction of muscle pioneers is dependent on signals from the notochord (Halpern et al., 1993), we analyzed markers expressed in the midline mesoderm. The

expression of the pan-mesodermal gene *ntl* (Schulte-Merker et al., 1994), the early mesendodermal marker *wnt8* (Kelly et al., 1995) and the early axial mesoderm marker *flh*/*znot* (Talbot et al., 1995) are not altered at gastrulation stages (not shown). Similar to *her5* (Fig. 2T), the expression of the anterior prechordal plate marker *gsc* (Schulte-Merker et al., 1994) is normal at 70% of epiboly and tailbud stage, but shows reduced midline expression at the four-somite stage in the mutant. The intermediate mesodermal expression of *pax2.1* is never affected in *spg* mutants. By contrast, expression of the endoderm specific marker *sox17* (Alexander and Stainier, 1999) is strongly reduced at the tailbud stage (Fig. 5S′) but not at its onset around 50% of epiboly, although *pou2* expression is restricted to the ectoderm during gastrulation (inset). The reduction in *sox17* expression may be due to the general expression of *pou2* at pre-gastrula stages. Expression of *nkx2.5* in the heart primordium (Chen et al., 1996; Reifers et al., 2000b) is only slightly reduced at the eight-somite stage (not shown).

Table 1. Summary of *fgf8, pou2* and mouse *Oct3/Oct4* overexpression studies and transplantation experiments

Injected mRNA	Amount injected	Genotype of treated embryo	Embryonic response*	Number injected	Rescue of expression
fgf8	25 pg	*spg*$^{-/-}$	100%	56 (total)	0
pou2	250 pg	*ace*$^{-/-}$	100%	40 (total)	0
lacZ	500 pg		0%	32 (total)	0
Mouse *Oct3/Oct4*	300 pg	*spg*$^{-/-}$		25 (total)	20

Total numbers indicate mutant embryos only.

*****Embryos misexpressing *fgf8* mRNA are typically dorsalized from mid-gastrulation onwards. Embryos misexpressing *pou2* mRNA show altered cell movements during gastrulation and lateral expansion of MHB expression domains.

Of 72 mutant embryos with transplanted cells in the MHB region, 64 show 'rescue' of *gbx2* expression at tb; of 23 mutant embryos with transplanted cells in the mesendoderm, none shows 'rescue' of *gbx2* expression at tb; and of 19 mutant embryos with transplanted cells in the MHB region, all show 'rescue' of *pax2.1* expression at 20 s.

Specific requirement for *spg/pou2* in the neuroectoderm

The abnormal development of both the endomesodermal and ectodermal layers led us to ask in which germlayer normal *spg*/*pou2* activity is required to allow normal hindbrain development (Fig. 6). We transplanted wild-type cells before onset of gastrulation into the prospective ectoderm or mesoderm of *spg* mutants. After developing until the tailbud stage, chimeric embryos were examined for expression of *gbx2* or *pax2.1*. Previous data have shown that both mesendodermal and neuroectodermal expression of *gbx2* expression requires Fgf8 (Fig. 5E′, Fig. 6G) (Lun et al., 2002). By contrast, only the neuroectodermal *gbx2* expression requires *spg*, and expression in the underlying mesendoderm is intact (Fig. 5C′,D′). When the transplanted wild-type cells were located in the neuroectoderm, they expressed *gbx2* in *spg* embryos, whereas a location in the mesoderm was not sufficient to restore neuroectodermal expression (Fig. 6A,B,E) (Table 1); Cross-sections confirmed that the expressing cells were confined to the ectoderm (Fig. 6B, bracket). Chimeric *spg* embryos with neuroectodermal clones fixed during mid-somitogenesis also showed rescue of *pax2.1* at the MHB (Fig. 6C,D). We conclude that the neuroectoderm of *spg* mutants is permissive for proper *gbx2* and *pax2.1* expression of wild-type cells, whereas wild-type cells located in the mesodermal layer do not support ectodermal expression of these markers (Fig. 6E). Together with the fact that *pou2* is expressed only in the neuroectodermal germlayer of the gastrula, these results strongly suggest that *spg/pou2* specifically functions in the neuroectoderm during gastrulation, independently of its ubiquitous expression during pre-gastrula stages (Fig. 6G).

Mouse *Oct3/Oct4* can functionally replace zebrafish *pou2*

While determining the molecular nature of *spg* alleles, we found that *pou2* is the likely to be the zebrafish ortholog of the mouse *Oct3/Oct4* gene (Burgess et al., 2002), which is widely known for its involvement in differentiation of the inner cell mass and of germ cells, but for which a role in brain development had not been reported. We therefore examined the expression of *Oct3/Oct4* in mouse embryos, and found that *Oct3/Oct4* was expressed at E8-8.5 throughout the neural plate, though the expression is apparently not restricted to the midbrain-hindbrain domain (Fig. 6F) (Schoeler et al., 1989), as in zebrafish [see Fig. 4 by Burgess et al. (Burgess et al., 2002) for comparison]. We reasoned that *Oct3/Oct4* as an ortholog of *pou2* might be able to restore the phenotype of *spg* mutant embryos if injected, and found that this was indeed the case. Injection of synthetic mRNA for *Oct3/Oct4* into one cell at the two-cell stage rescued the expression of *pax2.1*, which is normally severely reduced in *spg/pou2* mutants at this stage (Fig. 6H), in the same manner as does injection of *pou2* mRNA (Burgess et al., 2002). These results suggest that Oct3/Oct4 may function in activation of Pax2 also in normal mouse development.

Combinatorial roles for *pou2* and *fgf8* in the hindbrain

The phenotypic similarities between *ace/fgf8* and *spg/pou2* mutants raised the possibility that these genes might act in the same pathway, or in synergistic pathways. *fgf8* transcription is initiated normally in *spg* mutants at 70% epiboly, but becomes downregulated by the end of gastrulation and is completely lost during somitogenesis (Fig. 2I-K′). We therefore injected *fgf8* mRNA unilaterally into wild-type and *spg* two-cell stage embryos to determine if *fgf8* was capable of rescuing any aspect of the *spg* phenotype. We used *gbx2* and *spry4*, known early downstream targets of Fgf8, as markers to assay the effects of *fgf8* mis-expression at the end of gastrulation in *spg* embryos. In wild-type embryos, *fgf8* mis-expression caused a strong dorsalization of the whole embryo (Fürthauer et al., 1997; Reifers et al., 1998), which is visible as a pronounced dorsoventral expansion of *spry4* and *gbx2* in the injected half (Fig. 7A,C, arrow). As in the wild type, *fgf8*-mRNA injection into *spg* mutants results in strong lateral expansion of the endogenous mesendodermal domain of *gbx2* (Fig. 7B), confirming that *fgf8* can also exert its dorsalizing activity in *spg* embryos; the residual, weak expression of *spry4* may be similarly expanded (Fig. 7D). Moreover, in the neuroectoderm of wild-type embryos, *fgf8* mRNA injection also caused upregulation of the endogenous expression domains of both *gbx2* and *spry4* (Fig. 7A,C). Unexpectedly, and in contrast to

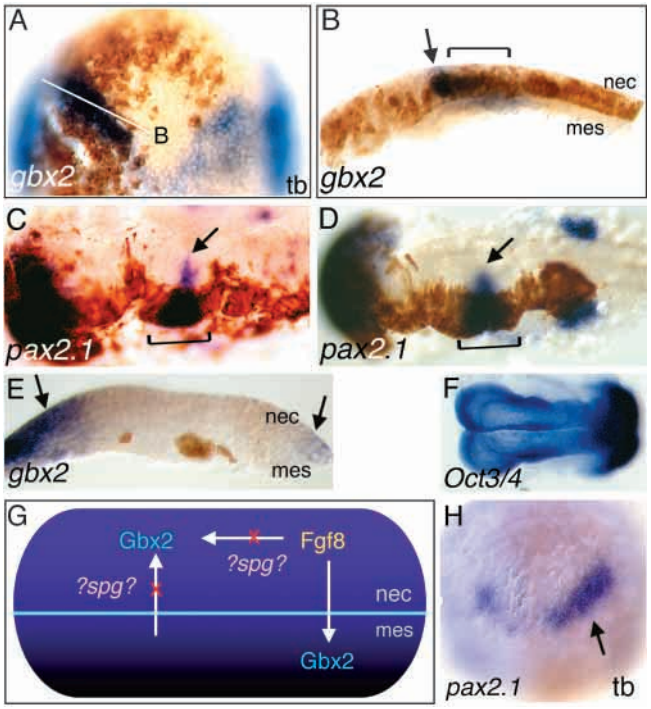


Fig. 6. *spg/pou2* requirement in the neuroectoderm. (A) Transplanted wild-type cells (brown) in *spg* embryos express *gbx2* cell autonomously. All blue cells carry the brown transplantation marker. The right half of the embryo serves as a control: it is devoid of wild-type cells. As is normally seen in *spg* mutants (compare with Fig. 5D), *gbx2* expression is found only in the mesendoderm, but not the overlying neuroectoderm. Dorsal view of a *spg* chimera, anterior is upwards. The white line indicates the plane of the transversal section in B along the *gbx2* domain. (B) Cross-section of the embryo in A showing that the transplanted wild-type cells expressing *gbx2* (bracket; arrow indicates the unaffected non-neural ectoderm domain, see also Fig. 5D′) are located in the neuroectoderm. Other cells that are only brown lie outside the normal domain of *gbx2* expression. (C,D) Transplanted wild-type cells (brown) in *spg* embryos also express *pax2.1* normally at the MHB. Arrows point to the residual *pax2.1* expression at the MHB which is retained in *spg* embryos until late stages of somitogenesis. (E) Clones of wild-type cells within the mesoderm cannot restore *gbx2* expression in *spg* mutant embryos at the tailbud stage. The plane of section is similar to B. Arrows point to the unaffected non-neural ectoderm domain. (F) Mouse *Oct3/Oct4/Pou5f1* is globally expressed within the neural plate at day 8.0 p.c. (dorsal view, anterior to the left). (G) *spg*/*pou2* might be required to activate Fgf8-dependent *gbx2* expression either for a planar or vertical signal. The transplantation experiments presented here show a requirement in the neuroectoderm. (H) Mouse *Oct3/Oct4* mRNA and *lacZ* mRNA were co-injected into one cell of a two-cell stage zebrafish embryo. *pax2.1* expression can be restored in *spg* mutant embryos by mouse *Oct3/Oct4* mRNA (arrow, *lacZ* expression is indicated by the brown color) (Burgess et al., 2002). nec, neuroectoderm; mes, mesendoderm; tb, tailbud stage.

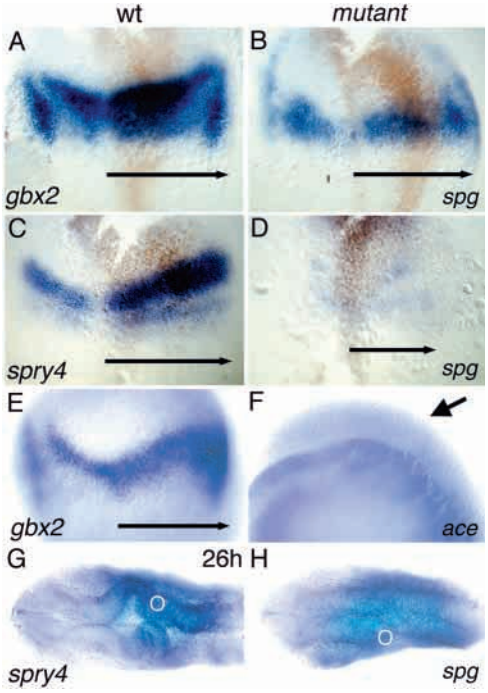


Fig. 7. Relationship between *pou2* and *fgf8*. All embryos are depicted dorsally with the exception of the embryo in F, which is depicted laterally. (A-D) Gain of *fgf8* function by unilateral misexpression of *fgf8* mRNA into one cell of two-cell stage embryos. To determine the effect caused by *fgf8* overexpression, *gbx2* (A,B) and *spry4* (C,D), both markers for the prospective hindbrain, were used. The activity of misexpressed *fgf8* can be judged from dorsalization of the embryos indicated by lateral expansion of endogenous *gbx2* and *spry4* expression, indicated by arrows (A-E) at the injected side of the embryo. Deposition of co-injected *lacZ* mRNA is visualized by staining for anti-β-gal antibody (brown), reflecting the location of injected *fgf8* mRNA (not visualized). Distribution of injected mRNA is restricted to one half of the embryo, allowing for comparison with the contralateral side as a control. In *spg* embryos, neither expression of *gbx2* (B) nor *spry4* (D) could be rescued or upregulated, respectively, by *fgf8* overexpression. (E,F) In a reversed experiment, *pou2* mis-expression into *ace* embryos (carried out in the same unilateral fashion described for *fgf8* injection above), *pou2* overexpression and *fgf8* itself can provoke dorsalization of the injected half of the embryo (obviously seen in the wild-type embryo in E, but not in the *ace* embryo in F, owing to complete loss of the readout marker *gbx2*) but cannot rescue expression of *gbx2* in *ace* mutant embryos. (H) A bead soaked with Fgf8 protein can not rescue the morphology of the isthmic constriction at the MHB but can evoke ectopic *spry4* expression in wild-type and *spg* embryos (G,H; white circles indicate the implanted bead).

the mesendodermal expression domain, the neuroectodermal expression of *gbx2* and *spry4* could not be initiated (*gbx2*) or restored to the wild-type level (*spry4*) in *spg* embryos injected with *fgf8* mRNA (Fig. 7B,D). The *gbx2* expression seen in Fig. 7B is the mesendodermal domain that is unaffected in *spg* mutants (see also Fig. 5C′,D′). Equivalent results were obtained with *fgf8* injection when wild-type and *spg* mutant embryos were fixed at early and mid-somitogenesis stages (not shown). Thus, the hindbrain and MHB primordium of *spg/pou2* mutants appear to be insensitive to Fgf8 signaling. In mice, *Fgf8* and *Gbx2* are thought to act in a feedback loop (Garda et al., 2001); the loop could simply be interrupted between *fgf8* and *gbx2* by the absence of *pou2*, if *pou2* acts within this loop upstream of *gbx2*. We therefore tested whether injection of *pou2* mRNA into *ace* embryos could restore *gbx2* expression, and found that this was not the case (Fig. 7E,F). These findings show that both Fgf8 and Pou2 are required for *gbx2* and *spry4* expression in the ectoderm. In addition to *gbx2*

and *spry4*, we also found transcriptional activation of *fkd3* within the hindbrain primordium to be dependent on both *pou2* and *fgf8* (not shown).

To further test the idea that *spg* embryos might be regionally insensitive to Fgf8, we implanted beads soaked with Fgf8 protein into the prospective MHB territory of *spg* mutants. For technical reasons, these implantations were made at the 13-somite stage. In *ace* mutants, this treatment rescues the formation of the MHB constriction, and leads to re-expression of the target gene *spry4* (F. Reifers and M. B., unpublished) (Fürthauer et al., 2001). In wild-type embryos examined at 26 hpf, the MHB constriction is clearly visible. The localized source of Fgf8 protein provided by the bead was not able to restore the MHB constriction in *spg* embryos. However, after in situ staining, ectopic expression of *spry4* was readily observed both in wild-type and in mutant embryos (Fig. 7G,H; compare with Fig. 2P,P′). This finding corroborates the results of the *fgf8* mRNA injections and furthermore indicates that the MAP kinase pathway through which Fgf8 exerts its effect on *spry4* induction is functional at least at later stages of MHB development in *spg* embryos. We found normal expression of the known Fgf receptors 1, 3 and 4 at tailbud stage in *spg* mutants (not shown), suggesting that the pathway is also intact around the normal time of *gbx2* and *spry4* onset. In summary, analysis of the loss- and gain-of-function experiments in *spg* and *ace* embryos suggests that Fgf8 and Pou2 do not act in a simple linear pathway, but genetically act in parallel in a stage- and tissue-specific manner, in order to initiate and maintain the developing MHB (Fig. 8Q).

To examine how specific *pou2* function might be for Fgf8, we studied the phenotype of *spg*/*ace* double homozygous mutants. At 90% epiboly, *spg*/*ace* double mutants embryos show no *gbx2* expression, in the same way as *ace* single mutants (not shown; see also Fig. 5E′). At later stages, however, the double mutants are easily distinguishable, because their MHB and their ear and tail phenotypes are stronger than that of either single mutant. The prospective tectal region is strongly reduced in size, and the otic placodes are extremely small and never develop into otic vesicles (Fig. 8J). In situ analysis shows that in the double mutants, *pax2.1* expression is already almost completely abolished at the MHB during early somitogenesis stages, whereas it is still recognizably expressed in either single mutant embryo (Fig. 8E,H,K). This finding suggests that at later stages, *pou2* might also function independently of Fgf8, possibly in conjunction with other Fgfs. Given the often redundant nature of Fgf signaling, a stronger phenotype might arise from a *pou2* requirement for mediating the effects of Fgfs other than Fgf8 that are also expressed at the MHB (Reifers et al., 2000a). We therefore compared the double mutant phenotype to the phenotype of embryos where all Fgf signaling is blocked, owing to pharmacological inhibition with SU5402 in a *spg/pou2* mutant background (Fig. 8M-P). At the eight-somite stage *pax2.1* expression is reduced at the MHB in inhibitor-treated wild-type embryos, resembling *pax2.1* expression in *ace* mutants at the same age (Reifers et al., 1998), although the expression domain is more reduced than in *ace* mutants (Fig. 8N). Inhibitor treatment of *spg* mutant embryos, which normally show a dorsally restricted *pax2.1* MHB domain at the eight-somite stage, leads to complete abrogation of *pax2.1* expression at the MHB (Fig. 8O,P), almost mimicking the *spg*/*ace* double mutant phenotype (Fig. 8K). These findings suggest that both additional Fgfs and non-Fgf-dependent pathways contribute to the enhanced phenotype of *spg/ace* double mutant embryos.

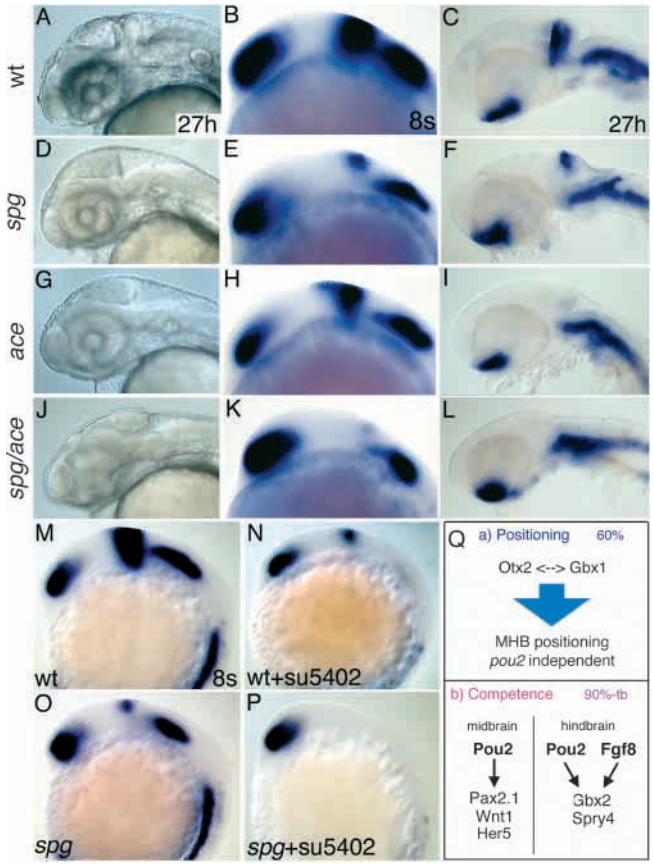


Fig. 8. The double mutant *spg-ace* shows a more severe brain phenotype (J, living embryo) than each mutant alone (D, G, living embryos). (B,C,E,F,H,I,K,L,M-P) *pax2.1* expression. At midsomitogenesis, the MHB expression of *pax2.1* is severely reduced and completely missing at pharyngula stages in the double mutant embryos (K,L). (M-P) Phenocopy of the double mutant phenotype produced by blocking Fgf receptors using the inhibitor SU5402. (N,P) *spg* embryos treated with the inhibitor (+ SU5402, P) reveal strong similarity to *spg/ace* double mutant embryos, which is reflected by *pax2.1* staining (compare P with K; expression of *pax2.1* within the otic placode is also strongly reduced by inhibition (S. Léger and M. B., unpublished). (Q) During the first steps of regionalization of the MHB and the hindbrain, positioning of the MHB is independent of Pou2 (a). During the establishment phase of the MHB organizer, Pou2 is upstream of several cognate MHB markers (b). In the hindbrain primordium, *spg/pou2* and *ace/fgf8* serve a combinatorial role in initiation of *gbx2* and *spry4*.

DISCUSSION

We have analyzed the function of *spg/pou2* during zebrafish brain development, and have found that *spg/pou2* is essential for proper development of the mid-hindbrain boundary and hindbrain territories. Our present analysis of early marker genes shows that *spg*/*pou2* functions during the initial establishment of these brain regions, and may also function

during their maintenance, in particular in hindbrain rhombomeres 2 and 4. In addition, *spg/pou2* functions also during development of the forebrain, in particular the diencephalon, and in differentiation of the paraxial mesoderm and endoderm. Most importantly, the results of our cell transplantations, mRNA injections and bead implantation experiments show that *spg* embryos are regionally insensitive to Fgf8 in the early hindbrain neuroectoderm. We therefore suggest that *spg/pou2* encodes the first example of a tissue-specific competence factor for Fgf8 signaling. In the accompanying paper, we show that *spg* mutations affect the *pou2* gene, an ortholog of mammalian Oct3/Oct4 (Burgess et al., 2002).

spg functions during establishment of the MHB- and hindbrain neuroectoderm

Key molecules that control MHB development, such as Fgf8, Pax2.1 and Wnt1, are already expressed during the earliest, establishment, phase of MHB organizer development (Reifers et al., 1998; Lun and Brand, 1998). Investigation of *no isthmus (noi)*/*pax2.1* and *acerebellar (ace)*/*fgf8* mutant embryos has revealed that *pax2.1*, *fgf8* and *wnt*1 define three separate and independent signaling pathways during this initial phase of MHB development at around 80% of epiboly (reviewed by Rhinn and Brand, 2001). During early somitogenesis, these genes become mutually dependent, demarcating the transition from the establishment to the maintenance phase of MHB development. Fgf8 serves a key function both in the hindbrain primordium and during maintenance of the MHB organizer (Reifers et al., 1998; Fürthauer et al., 2001; Raible and Brand, 2001), and the phenotype of *spg/pou2* mutants suggests that the function of *spg*/*pou2* is closely related, but not identical to that of *ace*/*fgf8*.

Given that *pou2* is also expressed maternally and in the pregastrula zygote (Takeda et al., 1994; Hauptmann and Gerster, 1995), it was important to determine whether this pregastrula expression phase influences the later neuroectodermal function of *spg/pou2*. The loss of endodermal *sox17* expression that we have observed in *spg* mutants may reflect a *spg/pou2* function at the pregastrula-stage, because after the onset of gastrulation, *pou2* is no longer expressed in the endomesoderm. This phenotype needs further examination. Mild Oct4 overexpression in mouse ES cells triggers endoderm and mesoderm differentiation, also suggesting a possible role of this gene in endoderm development (Niwa et al., 2000). Interestingly, POU-type transcription factors often heterodimerize with HMG-domain proteins (Kamachi et al., 2000). Pou2 might therefore be a binding partner for the zebrafish HMG domain protein Casanova, a crucial regulator of endoderm development and *sox17* expression (Dickmeis et al., 2001; Kikuchi et al., 2001). With respect to neuroectodermal development, the results of the transplantation experiments, together with the expression pattern of *pou2*, argue that the effect on the neuroectoderm is a specific function of *spg/pou2*, and not a secondary consequence of altered endodermal development. For MHB development, a crucial event is the positioning of the organizer in the gastrula neuroectoderm. In mice and chick, positioning is reflected in formation of a molecular interface between the *Otx2* and *Gbx2* genes (Hidalgo-Sanchez et al., 1999; Millet et al., 1999; Broccoli et al., 1999). In zebrafish, this situation is very similar, but not identical, as the function of *gbx2* appears to have switched to *gbx1* (Lun et al., 2002; Rhinn and Brand, 2001). In this respect, our observation that the *otx2/gbx1* interface is formed normally in *spg* mutants is important, as is the finding that expression of *fgf8* and *wnt1* is initiated in the correct spatial domain in *spg* mutants. Together, this shows that the neuroectoderm is not generally defective in *spg* mutants.

Shortly after the initial formation of the *otx2/gbx1* interface (around 70% of epiboly), the gene expression program in *spg* mutants becomes specifically abnormal in the MHB and the hindbrain primordium, coincident with the time and place of restricted *pou2* expression in the neuroectoderm. By contrast, anterior neural plate markers such as *six3* or *otx2* are not or only mildly affected in the mutants, consistent with the notion that *spg*/*pou2* acts specifically within the MHB and hindbrain primordium. The strong reduction in *pax2.1* staining and *wnt1* staining illustrates the function in midbrain development (Fig. 8Q). In fact, given its expression profile and requirement in *pax2.1* activation, *spg/pou2* encodes the first candidate regulator of *pax2.1* expression; this regulation may well be direct, as a functional *pax2.1* promoter fragment contains putative POU protein binding sites (Picker et al., 2002) (A. Picker and M. B., unpublished). The requirement for early hindbrain development is most clearly seen by the effects on the markers *gbx2*, *fkd3* and *spry4*, all of which become activated at this stage in the hindbrain primordium. Expression of these marker genes has been clearly linked to Fgf signaling (Chambers and Mason, 2000; Liu et al., 1999; Fürthauer et al., 2001; Darlington, 1999) (K. Lun and M. B., unpublished), further strengthening the case for a relation between *spg/pou2* function and Fgf8 signaling. Given that expression of these genes and *fgf8*, *wnt1* and *her5* becomes abnormal from 80% of epiboly onwards, this marks the time when *pou2* first exerts a crucial function in the MHB and hindbrain neuroectoderm. These genes could require *spg/pou2* directly or indirectly for their expression. Many of the gene expression defects we observed at later stages in *spg* mutants are also likely to be due ultimately to this early failure to express *gbx2*, *spry4*, *pax2.1* and *fkd3* (e.g. the reduced *eng2* and *eng3* expression is probably due to loss of *pax2.1* expression, since *pax2.1* is absolutely required for *eng* gene expression) (Lun and Brand, 1998). In summary, our results show that *spg* is required for proper development of the MHB organizer and the hindbrain primordium, positively regulating expression of *pax2.1*,, *krox20*, *gbx2*, *fgf8*, *spry4* and *fkd3* at the end of gastrulation.

Competence to respond to Fgf8 in the early hindbrain requires *spg/pou2*

Fgf8 is expressed in several domains in or around the early neuroectoderm, and the same molecule functions differently in different tissues. The different potential to respond must therefore be encoded by the developmental state or history of the target tissue, referred to as competence to respond in a specific way to an inductive signal, in this case Fgf8. The factors that mediate competence to respond to Fgf8 are so far unknown; our analysis suggests that Pou2 is one such factor. Our transplantation assays revealed that *spg/pou2* is cell autonomously required in the neuroectoderm, in accordance with its expression pattern and the function of *pou2* as a transcriptional regulator. While addressing the mechanism by which *pou2* exerts its effects in the earlier neuroectoderm

through *fgf8* mRNA injection and bead implantation assays, we found that *spg* mutants were regionally insensitive to the effects of *fgf8* expression. Providing *fgf8* mRNA or Fgf8 protein to *spg* embryos was not sufficient to restore expression of the target genes *gbx2* and *spry4* to *spg* mutants, although other effects that characteristically result from Fgf8 treatment, e.g. dorsalization, were still evident. Conversely, providing *pou2* mRNA back to *ace/fgf8* mutants, which normally lack *gbx2* expression, failed to restore *gbx2* and *fkd3* expression, although *pou2* injections clearly rescued the *spg* mutant phenotype (Burgess et al., 2002). Together with the results of the *spg/ace* double mutant phenotype, these data suggest that *pou2* and *fgf8* do not act in a simple linear pathway that leads to *gbx2, fkd3* and *spry4* activation, but rather are both required to synergistically activate these genes (Fig. 8Q). Mechanistically, Pou2 might, for example, require an activating signal that is under the control of Fgf8, such as phosphorylation, for its activity (this signal would be absent in *ace* mutants) or both a target of the MAP kinase pathway and Pou2 could act together in a transcriptional complex controlling *gbx2* expression. Further evidence to support the theory that *pou2* is not simply downstream of *fgf8* comes from the analysis of embryos where Fgf signaling has been pharmacologically inhibited, and from studying *pou2* expression in *ace* mutants. In both conditions, *pou2* expression is initially normal, and becomes only reduced from somitogenesis stages onwards, when maintenance mechanisms start to operate (G. R., unpublished). In summary, initial processes involving the spatiotemporal set up of the MHB primordium and the hindbrain during mid-gastrulation stages are independent of *pou2*, whereas the MHB- and hindbrain primordium is made competent to respond to the effects of Fgf8 by expressing *pou2* from the establishment phase at the end of gastrulation onwards. In particular, we propose that *pou2* and *fgf8* are jointly required to initiate expression of *gbx2, spry4* and *fkd3* in the hindbrain primordium.

Our work raises several new questions with respect to the issue of competence. If the spatially restricted expression of *pou2* serves to make neuroectoderm competent, how in turn is the expression of *pou2* set up? The answer to this question will be of particular interest, because the interface between *otx2* and *gbx1* that may position the MHB is forming normally in *spg* mutants. A further question that we have not yet addressed is whether *pou2* mediates competence for other Fgfs as well, or indeed for other classes of signaling molecules. The similarities between the *acerebellar/fgf8* mutant phenotype and the *spg* mutant phenotype argues for a relatively high degree of specificity to mediate the effects of Fgf8. By contrast, the differences to the *ace* mutant phenotype for example in forebrain development or heart development and the *pou2* expression pattern argue that *pou2* is not a ubiquitous competence factor for Fgf8. Furthermore, our analysis of the *spg/ace* double mutants, and the comparison between the mutants and Fgf-inhibitor treated embryos, suggests that *pou2* also serves roles that are not linked to Fgf(8) signaling. By what molecular mechanism *spg/pou2* mediates competence remains undetermined. The Fgf8 bead implantation experiments show that in other tissues or at later stages, *spg* mutants are able to respond to Fgf8, as evidenced by *spry4* expression or dorsalisation, suggesting that the MAP kinase signaling pathway that is thought to mediate the effects of Fgf8 is not generally disrupted in the mutants. An obvious possibility is that Spg/Pou2, as a transcription factor, might control expression of some component of the MAP kinase cascade in a tissue-specific manner. However, at least with respect to the Fgf receptors 1, 3 and 4 we have not detected any abnormal expression in the early hindbrain primordium of *spg* mutants (G. R., unpublished). POU type homeodomain transcription factors assemble into transcription factor complexes that include, for example, ETS type transcription factors that serve to integrate the activity of several signaling pathways, including Fgf signaling (Fitzsimmons et al., 1996; Raible and Brand, 2001). Oct4 specifically forms a complex with Ets2, and thus silences transcription of the tau interferon promoter in trophectoderm (Ezashi et al., 2001). An appealing mechanism of action is therefore that Spg/Pou2 might be necessary to form a stable transcription factor complex that serves as a target for Fgf signaling in downstream gene activation or repression.

spg/pou2 functions during maintenance of the MHB and hindbrain primordium

The lack of *pou2* also has consequences for later stages of MHB development. The requirement of *spg* during the maintenance phase of MHB development can be subdivided into two aspects. During early somitogenesis stages, *spg* seems required for MHB development along the entire dorsoventral axis at the prospective MHB, as MHB marker expression is lost from this region in mutant embryos, increasing from ventral to dorsal. This difference may reflect a graded requirement for Fgf signaling along the dorsoventral axis (Reifers et al., 1998; Köster et al., 1997; Carl and Wittbrodt, 1999). In contrast to *ace* mutants, however, which exhibit a gradual narrowing of MHB markers from dorsal to ventral, MHB gene expression in *spg* mutants is completely lost from the ventral part, but always remains detectable in a dorsal patch in the dorsal neural tube. Alternatively, this phenotype may be a later consequence of the early failure of MHB- and hindbrain gene expression domains to fuse at the midline that is already evident by the end of gastrulation. Midline marker gene expression, e.g. of *shh*, is not altered, raising the possibility that perception of midline signals might be affected in *spg*/*pou2* embryos. During pharyngula stages, we observed that MHB markers recover in their expression in the dorsal-most neural tube. Morphologically, this coincides with a partial dorsal re-formation of the isthmic fold, as reflected by expression of *otx2* and *zath1*. This recovery is observed in both weak and strong *spg* alleles, suggesting that alternative and *pou2*-independent regulatory mechanism(s) might exist that allow for later induction of dorsal parts of the MHB.

The second aspect of *spg/pou2* function during the maintenance period is related to the specific subdomains in the hindbrain that express *pou2*. From the beginning of somitogenesis until the seven-somite stage, *pou2* is expressed specifically in rhombomeres 2 and 4 of the hindbrain (Takeda et al., 1994; Hauptmann and Gerster, 1995; Burgess et al., 2002). In particular, gene expression of *krox20*, *ephA4*, *wnt8* or *val* in odd numbered rhombomeres 1, 3 and 5 strongly requires *pou2*, suggesting that *pou2* may act on these rhombomeres in a non-autonomous fashion via a diffusible signal. Notably, *fgf8* expression is strongly reduced in r2 and r4, making Fgf8 an excellent candidate for the signal controlled by *pou2*. The

situation is likely to be more complex, as development of the even-numbered rhombomeres itself becomes abnormal, and signaling molecules like Wnt8 in odd-numbered rhombomeres are also affected. In contrast to odd-numbered rhombomeres, which are reduced in size, r2 and 4 are not, yet they show strong downregulation of *pou2* expression. Moreover, at late somitogenesis stages, r4 spatially expands at the expense of r3 and 5, as indicated by *hoxb1a* staining. Furthermore, we found indications that *pou2* is necessary to maintain rhombomeric integrity. This is reflected by the loss of *fkd3*, a marker for inter-rhombomeric boundaries, and by the strongly reduced distance between r4 and 7, which further illustrates the reduced size of r5. A key question that remains to be addressed is of course to what extend the defects in rhombencephalon development during early somitogenesis stages are due to the specific requirement of *spg*/*pou2* within rhombomere 2 and 4, as might be suspected on the basis of its expression, or due to the failure to express early markers of the hindbrain primordium at the end of gastrulation, as we describe here. Similarly, it will be interesting to determine whether *pou2* functions directly in proneural gene activation of *ngn1* clusters in the rhombencephalon.

One consequence of the failure to specify the MHB and hindbrain primordia properly in *spg/pou2* mutants is that these tissues are not or only partially formed in a pharyngula stage embryo. The actual loss of tissue is probably the result of two very different basic mechanisms. The localized cell death we observe in the forming midbrain and hindbrain during late somitogenesis stages may well be a direct consequence of the earlier mis-specification of these tissues. In addition, however, we also observe that neighboring territories to the most strongly affected areas appear expanded in size. This is particularly noticeable for the posterior forebrain (Fig. 4D′,E′) and more weakly also for the anterior hindbrain, and within the hindbrain for the rhombomeres bordering on the most strongly deleted rhombomeres r2 and r4. The processes maintaining the major brain subdivisions are poorly characterized, but seem to require integrity of neighboring brain regions. Posterior forebrain expansion is, for example, also seen in the murine *Pax2/Pax5* double mutants (Schwarz et al., 1997) and in the zebrafish *noi/pax2.1* mutants (S. Scholpp and M. B., unpublished), which exhibit loss of the midbrain, the MHB and the cerebellum, coupled to a posterior expansion of the rostral *pax6* domain and partial fusion with the *pax6* hindbrain domain. This may result from the lack of *eng2* and *eng3* gene expression in the mutants, as misexpression of engrailed-type genes can suppress forebrain development during chick and Medaka development (Araki and Nakamura, 1999; Ristoratore et al., 1999). The lack of MHB expression during mid-late somitogenesis in *spg* therefore probably contributes to the observed fusion of gene expression domains of forebrain and hindbrain markers as a secondary consequence. Interestingly, however, expansion of forebrain markers is already evident during the establishment phase of the MHB primordium, raising the possibility that *pou2* on its own has an active role in suppression of forebrain markers. Better fate maps and proliferation assays will be needed to address this issue further.

Control of totipotency versus differentiation switch

In the accompanying paper (Burgess et al., 2002), we have presented evidence that *spg/pou2* is likely to be the zebrafish ortholog of the mammalian *Oct3/Oct4/Pou5f1* gene, which is suggested to control totipotency of stem cells, i.e. the inner cell mass or ES cells derived from it, and germline determination (Pesce and Scholer, 2000). We have not yet addressed a possible function in germline development in *spg* mutants. A brain-specific function of *Oct3/Oct4* is not known for the mouse gene; the conventional deletion of this gene causes developmental arrest of mouse embryogenesis around implantation, which has so far precluded studying a possible later role in neural development. Although it is conceivable that the brain specific function was lost in the mammalian lineage, or was secondarily acquired in the teleost lineage, our results argue that this is less likely to be the case. We find that injection of mouse *Oct3/Oct4* mRNA into *spg* mutant zebrafish embryos rescues *pax2.1* expression (Fig. 6H, arrow; Table 1), and that in mice, *Oct3/Oct4* is strongly expressed throughout the neural plate until day 8.0-8.5 p.c. (Fig. 6F). However, gene expression is not confined to the midbrain-hindbrain area in mice, as it is seen for the zebrafish ortholog *pou2*. Either *Oct3/Oct4* functions in a different way in the mouse neuroectoderm, or not at all, or the mechanism is slightly different. For example, a pairing partner of Oct3/Oct4, e.g. a Sox or Ets protein, could provide the spatial specificity in the mouse neural plate, which would alleviate the need to restrict expression to the midbrain-hindbrain domain in the mammalian lineage. Regardless of the exact evolutionary origin, the phenotype of *spg* mutants appears more specific than would be expected for a gene that controls totipotency in all embryonic cells (Pesce and Scholer, 2000). In zebrafish, *spg/pou2* is clearly shut down in much of the neuroectodermal primordium during early somitogenesis stages, and appears to function as a transcriptional regulator for specific target genes in the cells in which it is expressed. Many, but not all cells either begin or have already undergone a significant differentiation at the time when they still express *pou2*. Therefore, if *spg/pou2* were to perform a similar function in controlling totipotency in zebrafish as in mice, this function would very likely be restricted to a specific, early step of differentiation. Instead of controlling totipotency, *spg/pou2/Oct3/Oct4* might serve more generally as a switch that controls the ability to respond to signals like Fgf8, and probably other signals of the Fgf subfamily, that act repeatedly during several developmental decisions. It is interesting to note that Fgfs are also important signals in the initial cell divisions of the mouse embryo (Chai et al., 1998). Accordingly, the decision to follow the embryonic fate, and eventually the germline fate, would be specific to the type of binary decisions controlled by this gene.

We thank Noriyuki Morita and Christiane Klisa who isolated the new *spg* alleles during our ENU mutagenesis screen, Alexander Picker and Frank Reifers for help with the eye labeling experiment and the bead implantations, Tobias Langenberg for help with the confocal microscopy, Andrea Hellwig for histological sectioning, and Florian Raible, Shawn Burgess, Carl-Philipp Heisenberg and Muriel Rhinn for critical reading of the manuscript. Independently, the *spg* gene has also been studied and identified in the laboratory of W. Driever, University of Freiburg. This work was supported by grants from the DFG, the European Union and the Max Planck Society.

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