

Endogenous Cerberus activity is required for anterior head specification in *Xenopus*

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Summary

We analyzed the endogenous requirement for Cerberus in *Xenopus* head development. ‘Knockdown’ of Cerberus function by antisense morpholino oligonucleotides did not impair head formation in the embryo. In contrast, targeted increase of BMP, Nodal and Wnt signaling in the anterior dorsal-endoderm (ADE) resulted in synergistic loss of anterior head structures, without affecting more posterior axial ones. Remarkably, those head phenotypes were aggravated by simultaneous depletion of Cerberus. These experiments demonstrated for the first time that endogenous Cerberus protein can inhibit BMP, Nodal and

Wnt factors in vivo. Conjugates of dorsal ectoderm (DE) and ADE explants in which Cerberus function was ‘knocked down’ revealed the requirement of Cerberus in the ADE for the proper induction of anterior neural markers and repression of more posterior ones. This data supports the view that Cerberus function is required in the leading edge of the ADE for correct induction and patterning of the neuroectoderm.

Key words: Cerberus, Head induction, Morpholino, Targeted activation, *Xenopus laevis*

Introduction

In amphibians, the formation of the anterior-posterior (AP) axis is dependent on Spemann’s organizer activity (Spemann and Mangold, 1924). Classic transplantation experiments demonstrated that the inductive properties of the organizer change in the course of development. The early organizer can induce a complete secondary body axis including head, whereas the late organizer can only induce trunk-tail structures (Spemann, 1931). This led to the concept of two organizing centers: the head and the trunk-tail organizers. Recently, molecules that are expressed in the Spemann’s organizer have been identified in *Xenopus* (reviewed by De Robertis et al., 1997). When ectopically expressed in the ventral side of *Xenopus* embryos, some of these factors, like *gooseoid*, *noggin* or *chordin*, can induce secondary body axis (Cho et al., 1991; Smith and Harland, 1992; Sasai et al., 1994). In contrast to these axis-inducing factors, secreted proteins such as Cerberus and Dickkopf-1 are only able to induce head-like structures (Bouwmeester et al., 1996; Glinka et al., 1997). In *Xenopus*, *cerberus* is expressed in the non-involuting anterior dorsal endoderm (ADE), but not in the involuting mesoderm. The presence of the strong head-inducing factor Cerberus in the ADE raised the possibility that this region could be the head organizing center in *Xenopus* (Bouwmeester et al., 1996; Bouwmeester and Leyns, 1997).

Biochemical analysis in *Xenopus* showed that Cerberus can bind to Xnr1, BMP4 and Xwnt8 and thereby blocks their

function (Piccolo et al., 1999). These inhibitory properties of Cerberus are considered essential for the head inducing activity of this secreted factor.

A gene homologous to *cerberus* has been isolated in the mouse (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998). The expression of mouse *cerberus-like* (*cer-l*) and other markers such as *Hesx1*, *Lim1* and *Otx2* in the anterior visceral endoderm (AVE), led to the hypothesis that this region is the topological mouse equivalent of the ADE in *Xenopus* (Acampora et al., 1995; Thomas and Beddington, 1996; Belo et al., 1997; Bouwmeester and Leyns, 1997). Therefore, the AVE was proposed to be the head organizer in the mouse. This view is supported by the finding that in chimeric mutant mouse embryos composed of AVE that lacks either *Otx2*, *Lim1* or *Hnf3β*, and wild-type epiblast, the head is not properly induced (Rhinn et al., 1998; Shawlot et al., 1999; Dufort et al., 1998). Surprisingly, in generated *cer-l* knockout (KO) mouse lines no phenotypic head and axis defects were observed, arguing against a role of *cer-l* in early embryogenesis (Belo et al., 2000; Shawlot et al., 2000; Stanley et al., 2000).

In *Xenopus*, the endogenous function of Cerberus in the ADE remains unclear because of the lack of loss-of-function data. In order to characterize the function of Cerberus in head formation, a novel combination of strategies was employed. Endogenous Cerberus was ‘knocked down’ using an antisense morpholino oligonucleotide that specifically blocked the translation of the *cerberus* mRNA (CerMO). In addition, the relative levels of the signaling molecules BMP4, Xnr1 and

Xwnt8, which are antagonized by Cerberus, were raised in the ADE. This was achieved by driving their expression under the control of a mouse *cer-1* promoter fragment that is specifically activated in the ADE and closely resembles the spatiotemporal expression pattern of endogenous *cerberus*. Dorsal-vegetal injection of the CerMo does not cause visible head defects in the *Xenopus* embryo. In contrast, targeted increase of BMP, Nodal or Wnt activity in the ADE resulted specifically in the loss of head, but not trunk-tail structures. These factors synergistically inhibited head structures when simultaneously expressed in the ADE. Remarkably, these phenotypes caused by BMP, Nodal or Wnt were strongly enhanced when, in addition, Cerberus function in the ADE was blocked by the CerMo. The endogenous function of Cerberus in head formation, revealed in this sensitized system, could also be demonstrated in an explant recombination assay. ADE can induce forebrain markers when conjugated with dorsal ectoderm (DE) but not when Cerberus function was knocked down by the morpholino oligo. Furthermore, concomitantly, the ADE represses the expression of more caudal neural markers through the activity of Cerberus.

We demonstrate that endogenous Cerberus can inhibit BMP, Nodal and Wnt in vivo, and that this activity is required in the ADE for proper head induction/patterning in *Xenopus*.

Materials and methods

Plasmid constructs and morpholino oligonucleotide

An *EcoRI* genomic fragment containing the first exon of *cer-1* and 4 kb of non coding upstream region was isolated from a mouse genomic library generated in λ Fix II (Stratagene) and subcloned in pBluescriptIIKS+ (Stratagene). The 4.0 kb upstream region was subcloned in pBSIIKS+ and a *NcoI* site was introduced at the ATG translation start site by PCR-based mutagenesis, generating McerP. The plasmid McerP-*lacZ* was constructed by inserting a *NcoI*-*Bam*HI fragment, containing a β -galactosidase CDS with a nuclear localization signal and the SV40 poly(A) signal, at the *cer-1* ATG site of McerP.

To obtain the misexpression constructs the CDS from *Xnr1*, *XBMP4* and *Xwnt8* cDNAs were amplified by PCR with primers that introduced a *NcoI* site at the ATG translation start.

Primers used:

Xnr1-F (5'-TTTACTAGTCCATGGCATTCTGACAGCAGTCC-3') and *Xnr1*-R (5'-TTTGTGCGACTTAACTGCACCCACATTCCTC-3'); *XBMP4*-F (5'-TTTACTAGTCCATGGGAATTCTGTGTAACCGAATGCTG-3') and *XBMP4*-R (5'-TTTGTGCGACTCAACGGCACCCACACCTTCC-3'); *Xwnt8*-F (5'-TTTACTAGTCCATGGGACAAAACACCACTTTGTTCATCC-3') and *Xwnt8*-R (5'-TTTGTGCGACTCATCTCCGGTGGCCTCTG-3').

Each of these amplified CDSs was digested with *NcoI* and inserted at the ATG of McerP. A 263 bp *XhoI*-*Apal* fragment containing the SV40 poly(A) signal from pCS2+ was inserted downstream of each stop codon, generating McerP-*Xnr1*, McerP-BMP4 and McerP-*Xwnt8*. The plasmids CMV-*Xnr1*, CMV-BMP4 and CMV-*Xwnt8* were constructed by cloning the respective CDS PCR fragments at the *EcoRI* (filled in)-*XhoI* sites of pCS2+.

The *cerberus* morpholino oligonucleotide, obtained from Gene Tools LLC, was designed to target the 5' UTR region between bases -35 and -11 upstream of the AUG (5'-CTAGACCCTGCAGTGTCTGAGCG-3'). To express the C-terminal HA-tagged Cerberus protein in *Xenopus* embryos, a 1.4 kb *EcoRI*-*XhoI* fragment from pCDNA-*Xcer*-HA (containing bases from -50 in the 5' UTR) was subcloned in pCS2+. The *Xcer*-HA rescue construct was generated by subcloning a 1.36 kb *EcoRV*-*XhoI* fragment from

pCDNA-*Xcer*-HA, which only includes 11 bases upstream of the ATG, into the *EcoRI* site of pCS2+.

mRNA synthesis and microinjection

Capped sense mRNAs were synthesized using the Ambion mMessage mMachine kit. *Xenopus* eggs were obtained as described by Medina et al. (Medina et al., 2000) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). In vitro fertilization and microinjection of *X. laevis* embryos were performed as described previously (Bouwmeester et al., 1996).

Conjugate assays

Dorsal ectoderm and anterior dorsal endoderm were dissected from stage 10.5 embryos in 1 \times MBS-H. Conjugates were made by recombining the DE with the ADE and were grown in 0.5 \times MBS-H until siblings reached late tailbud stage 30/31. The conjugates were assayed by RT-PCR for expression of the neural markers *eomes*, *Xemx1*, *XBF1*, *En2* and *Krox20*.

In situ hybridization and β -galactosidase staining

Whole-mount and hemi-section in situ hybridization and antisense probe preparation was carried out as described by Belo et al. (Belo et al., 1997). The plasmids containing *XBF1*, *Xotx2* and *Xshh* fragments were linearized using *XbaI*, *EcoRI* and *KpnI* respectively, and transcribed using T3 RNA polymerase. Plasmids containing *lacZ*, *Xcer*, *Xhex*, *XKrox20* and *Xnot2* were cut with *SallI*, *EcoRI*, *NofI*, *EcoRI* and *EcoRI*, respectively, and transcribed using T7 RNA polymerase. Stained embryos (stage 21 and above) were bleached by illumination in 1% H₂O₂, 4% formamide and 0.5 \times SSC pH 7.0. For β -galactosidase staining, embryos were fixed in MEMFA (room temperature for 1 hour), rinsed in PBS and stained by using X-gal (Steinbeisser et al., 1989).

RT-PCR

Total RNA was prepared from embryos or conjugates with Trizol reagent (Gibco-BRL) and treated with RNase-free DNase I (Promega). First strand cDNA primed by random hexamers was synthesized with AMV reverse transcriptase (Roche) and PCR was performed using standard conditions and the following sets of primers: *Engrailed2*-F (5'-ATGAGCAGAATAACAGGGAAGTGGA-3') and *Engrailed2*-R (5'-CCTCGGGGACATTGACTCGGTGTG-3'), 28 cycles; *eomes*-F (5'-GCCTACGAAACAGACTACTCT-3') and *eomes*-R (5'-TAATGGAGGGAGGGGTTTCTAC-3'), 28 cycles; *Krox20*-F (5'-AACCGCCCCAGTAAGACC-3') and *Krox20*-R (5'-GTGTCAGCCTGTCTCTGTAG-3'), 24 cycles; *Nkx2.1*-F (5'-CTGACATATTGAGTCCCCTGGAGG-3') and *Nkx2.1*-R (5'-CCAGGTTTCCCAATTGCCATTGC-3'), 30 cycles; *ODC*-F (5'-CAGCTAGCTGTGGTGTGG-3') and *ODC*-R (5'-CAACATGGAAACTCACACC-3'), 21 cycles; *Xag*-F (5'-CTGACTGTCCGATCAGAC-3') and *Xag*-R (5'-GAGTTGCTTCTCTGGCAT-3'), 23 cycles; *XBF1*-F (5'-AAAGTGGACGGCAAAGACGGTG-3') and *XBF1*-R (5'-CCAATGAACACATCGTCGCTGC-3'), 26 cycles; *Xemx1*-F (5'-GCAGAAGCCTTTGTTCAGTGG-3') and *Xemx1*-R (5'-CTCCAGTTTCTGCCTCTTG-3'), 31 cycles.

In vitro translation and western blot analysis

For in vitro transcription/translation the TNT®* Coupled Reticulocyte Lysate System (Promega) was used according to the manufacturer's instructions. Protein extraction of embryos was carried out as described previously (Munchberg et al., 1999). Proteins were heated in sample buffer and separated by denaturing SDS-PAGE using a 13.5% polyacrylamide gel (Laemmli, 1970). Subsequently, proteins were transferred to a nitrocellulose membrane (Townbin et al., 1979), detected with monoclonal rabbit anti- α -HA antibody (Santa Cruz) for *Xcer*-HA or monoclonal mouse anti-c-myc (Oncogene) for Δ N Moesin-myc and developed using a chemiluminescent substrate (Pierce).

Results

Antisense morpholino oligonucleotide inhibits Cerberus activity

Antisense morpholino oligonucleotides are able to inhibit the translation of mRNAs in embryos (Heasman, 2002). To block the endogenous function of *Xenopus cerberus* in the ADE, we designed a morpholino oligonucleotide complementary to the 5' UTR region between bases -35 and -11 of the *cerberus* mRNA (CerMo; Fig. 1A).

The sequence of the morpholino oligo was compared with all the available *Xcerberus* EST sequences present in the general publicly accessible Databases. In all the found entries for *Xcerberus*, the 5' UTR sequence complementary to the

oligo was present. This strongly indicates that in the frog embryo, no additional *cerberus* messages with a different 5' UTR exist. We tested the ability and specificity of this CerMo to inhibit translation of *cerberus* mRNA in a cell free transcription/translation system and in *Xenopus* embryos. Western blot analysis for the HA-tagged Cerberus protein, demonstrated that only mRNAs containing the 5' UTR sequences complementary to CerMo (5' UTR cer-HA) were efficiently blocked in both systems (Fig. 1B,C). Standard control morpholino (CoMo) did not inhibit translation of *cerberus* mRNA. Neither CoMo nor CerMo interfered nonspecifically with the translation of an unrelated control mRNA ΔN Moesin-myc (Fig. 1B,C).

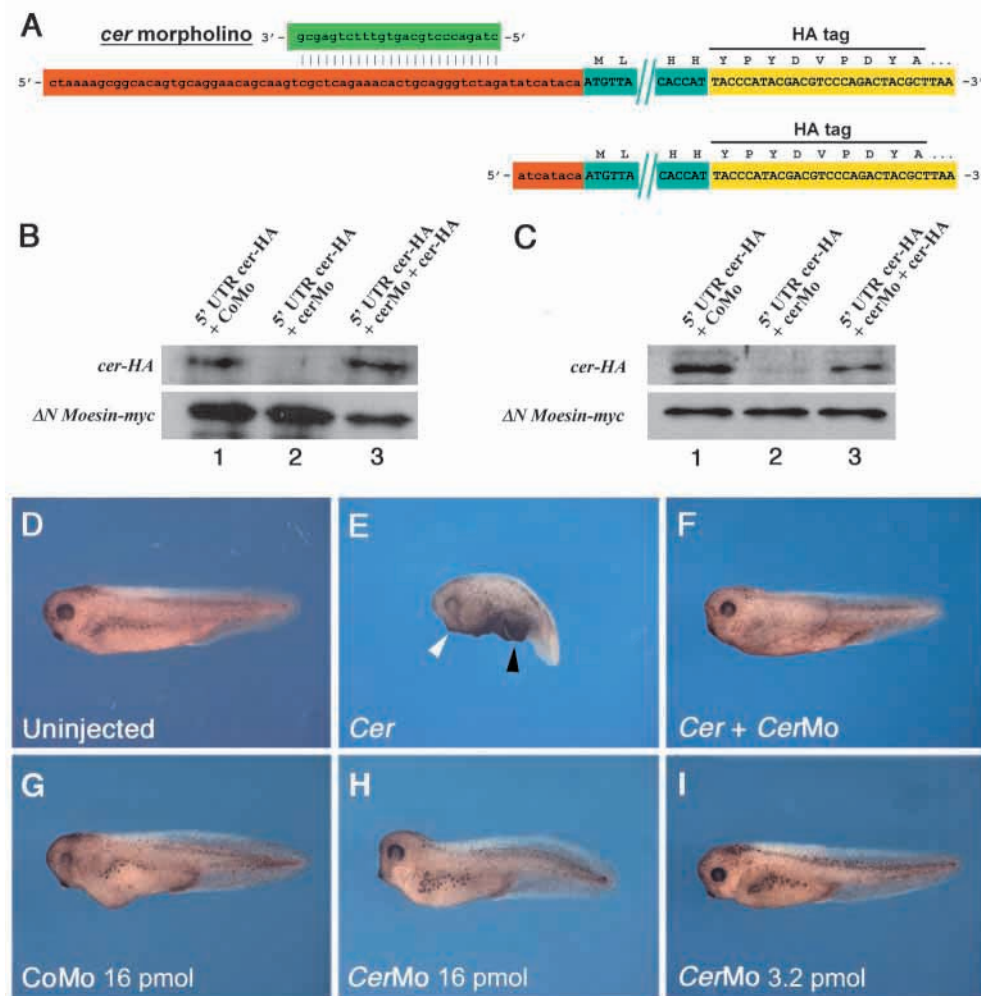


Fig. 1. Cer morpholino inhibits translation of *cerberus* mRNA. (A) Scheme of the HA-tagged Cerberus expression construct (5' UTR cer-HA; top), the HA-tagged Cer rescue construct (cer-HA; bottom) and the morpholino sequence targeting the *cerberus* 5' UTR sequences. (B,C) Western blot analysis of HA-tagged Cerberus and myc-tagged ΔN -moesin proteins. (B) In vitro transcription/translation of Cerberus protein in reticulocytes from 220 ng of plasmid was blocked by 20 pmol of the CerMo (lane 2) but not by control morpholino (CoMo) (lane 1). Transcription/translation from an equal amount of rescue plasmid was not blocked by the CerMo (lane 3). (C) Four-cell stage embryos injected in the animal pole with a total of 120 μ g of 5' UTR cer-HA construct were grown till stage 10.5 and one embryo equivalent protein extracts were used per lane in western blots. Translation of 5' UTR cer-HA was blocked by coinjection with 1.6 pmol of CerMo (lane 2), but not with 2.0 pmol CoMo (lane 1). Coinjection of 80 pmol of the rescue construct was able to overcome the CerMo effect (lane 3). (D-F) Ectopic head-like structures induced by the injection of 700 μ g of 5' UTR cer-HA capped mRNA in the ventral side of four-cell stage embryos (E) are suppressed by coinjection of 3.2 pmol of CerMo (F). White arrowhead indicates the cement gland of the primary axis while the black arrowhead points to the ectopic cement gland. (G-I) No significant anterior defects are visible in embryos microinjected in the two dorsal-vegetal blastomeres at four-to-eight cell stage either with a total of 16 pmol of CoMo (G) or with 16 pmol (H) and 3.2 pmol (I) of CerMo.

Morpholino knockdown of Cerberus in the ADE does not prevent head formation

Synthetic *cerberus* mRNA can induce head-like structures when microinjected in the ventral side of *Xenopus* embryo (Fig. 1E) (Bouwmeester et al., 1996). This induction was not observed when the 5' UTR *cerberus* mRNA and the CerMo were coinjected (Fig. 1F), demonstrating that the morpholino can efficiently inhibit Cerberus activity in the embryo. In order to assess the phenotypic effect of knocking down endogenous Cerberus, four- to eight-cell stage embryos were injected with CerMo in the two dorsal-vegetal blastomeres, the clonal descendants of which include the ADE cells (Bauer et al., 1994). Despite the ability of the CerMo to block Cerberus activity, we did not observe any abnormal phenotypes in embryos injected with 3.2 pmol of CerMo (Fig. 1I). Mild axis defects were observed when the maximal possible dose (16 pmol) of either CoMo or CerMo was injected (Fig. 1G,H). Using this morpholino-mediated knockdown strategy, we conclude that reducing Cerberus activity in the ADE was not sufficient to impair head formation in the *Xenopus* embryo.

Gain of BMP, Nodal or Wnt function in the ADE perturbs head formation

Cerberus protein can bind to and antagonize BMP4, Xnr1 and Xwnt8 molecules (Piccolo et al., 1999). We reasoned that an alternative way to modulate Cerberus activity in the ADE would be by locally raising the levels of BMP, Nodal and Wnt proteins. This changes the balance between the agonists (BMP, Nodal and Wnt) and the antagonist Cerberus. Such a strategy requires that these factors be expressed strictly in the ADE, as their presence in the dorsal ecto-mesoderm strongly interferes with axis formation. Unfortunately, according to the available fate maps, the dorsal-vegetal blastomeres of the eight-cell stage embryo will give rise not only to the ADE, but also to ectomesodermal cells (Bauer et al., 1994). This compromises the usefulness of the injection of RNA or constitutive expression constructs in these blastomeres. Therefore, the precise targeting of gene expression to the ADE, can only be achieved through the use of a promoter, specific for that region.

A 4.0 kb mouse *cerberus-like* promoter fragment isolated from a genomic library, was found to be specifically activated in the AVE of transgenic mouse lines (M. F., unpublished data). This promoter fragment was fused to a NLS-*lacZ* reporter gene (generating McerP-*lacZ*; Fig. 2A) and microinjected into *Xenopus* embryos. Surprisingly the mouse *cer-l* promoter was only activated in the dorsal side of gastrula embryos and β -galactosidase (β -gal) activity could only be detected in the ADE (Fig. 2B,C). In contrast, CMV-driven *lacZ* expression could be detected in both dorsal and ventral tissues (Fig. 2D,E). The temporal and spatial specificity of this promoter was confirmed by in situ hybridization (Fig. 2F,F',G,G') and by RT-PCR (not shown). *Xenopus* embryos were injected dorsally at the four- to eight-cell stage with the McerP-*lacZ* construct and sagittally sectioned through the dorsal lip, at stage 10⁺ and 11. The left halves of these embryos were hybridized with an antisense *lacZ* probe (Fig. 2F,G). The corresponding right halves were hybridized with a probe against *Xcer* (Fig. 2F',G'). The region of *lacZ* expression precisely matched the endogenous *cerberus* expression domain, detected in the corresponding half embryos. This finding enabled us to use the mouse *cer-l* promoter as a tool to precisely target expression

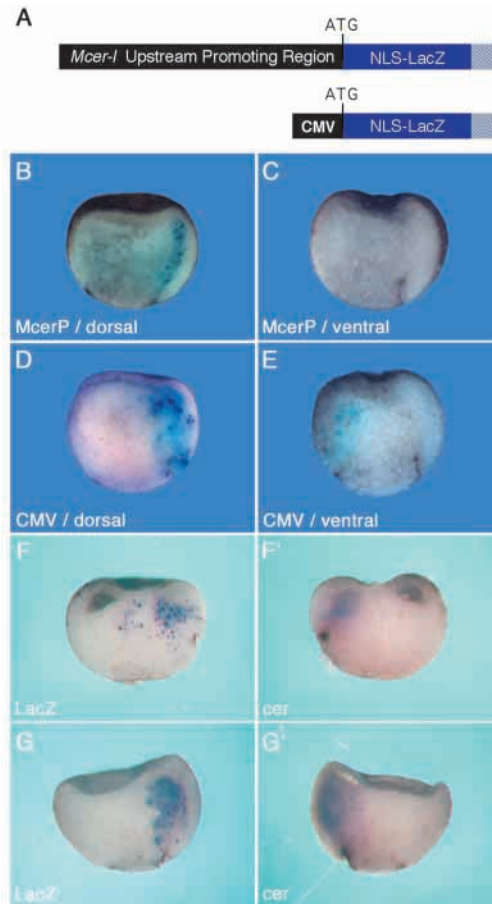


Fig. 2. *lacZ* expression driven by the Mcer promoter mimics endogenous *cerberus* expression domain in the early frog embryo. (A) Schematic of the McerP-*lacZ* and CMV-*lacZ* constructs. (B-E) β -gal staining of embryos injected at the four- to eight-cell stage either in the two dorsal-vegetal (B,D) or ventral-vegetal (C,E) blastomeres with McerP-*lacZ* or CMV-*lacZ* constructs. Embryos were injected at the four- to eight-cell stage in both dorsal-vegetal blastomeres with McerP-*lacZ*, grown to stage 10⁺ (F-F') or 11 (G-G'), sagittally sectioned and each half was hybridized with a *lacZ* (F,G) or a *Xcer* (F',G') probe.

of BMP, Nodal and Wnt proteins to the ADE of *Xenopus* embryos. To that end, we fused the *cer-l* promoter to *BMP4*, *Xnr1* or *Xwnt8* cDNAs generating McerP-BMP4, McerP-Xnr1 and McerP-Xwnt8, respectively. These constructs were injected in the two dorsal-vegetal blastomeres of eight-cell stage embryos. When 80 pg of either McerP-BMP4, McerP-Xnr1 or McerP-Xwnt8 were injected, head development was markedly affected in stage 35 embryos, whereas the trunk-tail structures appeared normal (Fig. 3D,G,J). In contrast, the injection of 80 pg of CMV-driven *BMP4*, *Xnr1* or *Xwnt8* expression constructs resulted in severe axial defects (Fig. 3A-C), leading to either a complete ventralization (CMV.BMP4; Fig. 3B) or dorsalization (CMV.Xnr1; Fig. 3B) of the embryo. When McerP-*lacZ* was coinjected to monitor targeting efficiency, β -gal activity was only detected in the anterior gut/liver/heart region of the *Xenopus* embryos (Fig. 3E,H,K). Due to its stability, β -gal protein can act as a lineage tracer for

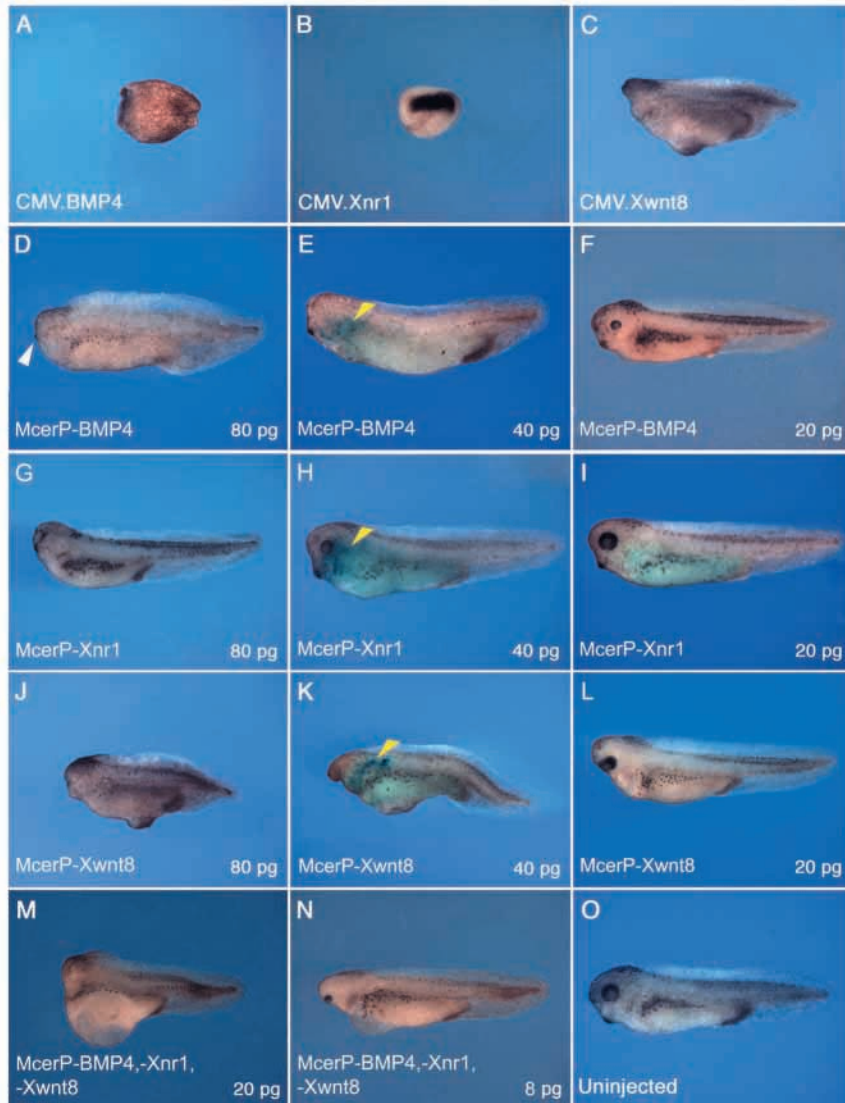


Fig. 3. Head defects induced by McerP-BMP4, Xnr1 and Xwnt8 microinjection in the frog embryo. Embryos were injected at the four-to-eight-cell stage in the two dorsal-vegetal blastomeres. (A-C) Injection of 80 pg of CMV.BMP4 (A; 100%, $n=22$), CMV.Xnr1 (B; 94%, $n=17$) and CMV.Xwnt8 (C; 20%, $n=25$) led to very severe phenotypes. (D-F) Injection of 20 pg (F; 60%, $n=20$), 40 pg (E; 62%, $n=34$) and 80 pg (D; 50%, $n=24$) of McerP-BMP4 showed a concentration dependent increase in head truncation. (G-I) A progressive head reduction and loss of eye were observed when 20 pg (I; 100%, $n=26$), 40 pg (H; 24%, $n=21$) and 80 pg (G; 59%, $n=34$) of McerP-Xnr1. (J-L) Increasing the amount of McerP-Xwnt8 from 20 pg (L; 60%, $n=25$) to 40 pg (K; 62%, $n=28$) to 80 pg (J; 74%, $n=19$) resulted in loss of cement gland and cyclopia and ultimately in complete truncation of the head. (M,N) Synergistic effect of McerP-BMP4, Xnr1 and Xwnt8 is shown by the coinjection of 8 pg (N; 65%, $n=23$) and 20 pg (M; 63%, $n=46$) of each construct which resulted in more severe defects than the ones observed in embryos injected with equal amounts of the individual constructs (F,I and L). 20 pg of McerP-*lacZ* was coinjected to access the correct targeting of the promoters to the ADE (yellow arrowheads in E, H and K).

the cells where it was originally expressed. Its detection in the aforementioned tissues, which had already been shown to originate from the ADE (Bouwmeester et al., 1996), provides further evidence that the activation of *Mcer-1* promoter in the ADE recapitulates the expression pattern of *cerberus*.

The severity of the induced head defects was dependent on the amount of McerP-Xnr1, -BMP4 or -Xwnt8 constructs injected (20, 40 and 80 pg/embryo). However, not all anterior structures were equally affected by the different constructs. Increasing doses of McerP-Xnr1 caused the graded reduction of brain, eye and cement gland structures (Fig. 3I,H,G). McerP-BMP4 also caused the graded loss of brain and eye structures (Fig. 3F,E,D), but residual cement gland tissue was still visible at high dosage of BMP4 (Fig. 3D). In contrast, the cement gland was the first structure to be lost in McerP-Xwnt8-injected embryos (Fig. 3L). A severe reduction of the head and a cyclopic eye were also observed in this case. A further increase in Xwnt8 dose completely eliminated the head (Fig. 3K,J). In summary, we observed that graded but distinct defects are obtained by the targeted increase of each of the signaling molecules in the ADE.

BMP, Nodal and Wnt activities synergistically suppress head formation

Independently raising the levels of Xnr1, BMP4 or Xwnt8 in the ADE led to defects in head formation. We therefore tested whether those three factors could synergistically inhibit head structures. Simultaneous microinjection of the three *Mcer-1* promoter expression constructs, at a concentration of 8 pg each per embryo, resulted in loss of cement gland, reduction of the brain and a small cyclopic eye (Fig. 3N). In embryos injected with a combination of 20 pg of each construct, the rostral head, including eyes, was completely lost (Fig. 3M). These experiments clearly demonstrated that BMP, Nodal and Wnt activity in the ADE synergize to inhibit head formation.

Next we tested whether the local increase of BMP, Nodal and Wnt activity in the ADE can affect the patterning of this tissue. Such patterning defects could be responsible for the head phenotypes observed in tadpoles. To address this issue, embryos were injected dorsally with a mixture of McerP-BMP4, McerP-Xnr1 and McerP-Xwnt8 (20 pg of each per embryo) and grown until stage 10⁺ or 12. These embryos, and uninjected siblings, were then hemi-sectioned and analyzed by

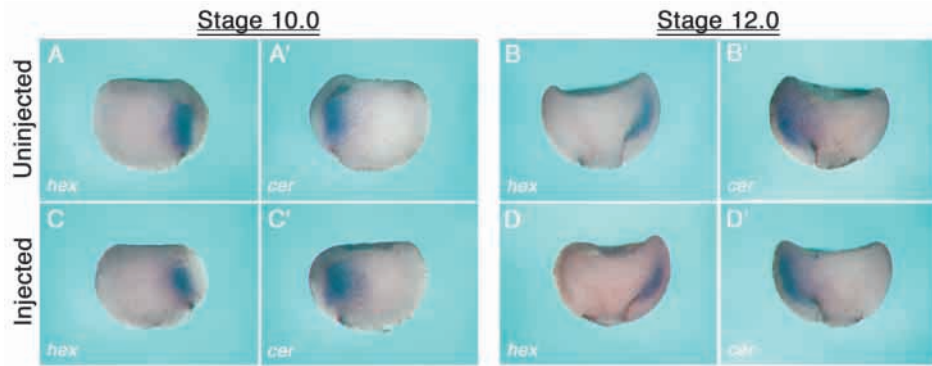


Fig. 4. Misexpression of BMP4, Xnr1 and Xwnt8 does not interfere with anterior endomesoderm patterning. (A-A' and C-C') Stage 10 and (B-B' and D-D') 12 embryos halves from uninjected (A-B') or injected twice dorsally at the four-to-eight cell stage embryos, with a mixture of 20 pg each of McerP-BMP4, McerP-Xnr1 and McerP-Xwnt8 (C-D'), were sagittally sectioned and each half was hybridized with a *Xhex* or a *Xcer* probe. The expression of these endomesodermal markers was unchanged in the injected embryos (C,C',D,D') when compared to the uninjected embryos (A,A',B,B').

in situ hybridization for typical ADE markers (Fig. 4). At stage 10⁺, the expression domains of *cerberus* and *Xhex* (Fig. 4A,A') were unaltered in injected embryos (Fig. 4C,C'). Also, no visible changes in *cerberus* and *Xhex* expression were observed in stage 12 embryos (Fig. 4B,B',D,D'). These results demonstrated that the ADE patterning is not perturbed by elevated levels of BMP, Nodal and Wnt signaling.

Increased BMP, Nodal and Wnt signaling in the ADE inhibits the formation of anterior neural tissue

In order to trace back the molecular events leading to the observed head phenotypes, we analyzed the expression of neural marker genes by RT-PCR, at different stages of development. This method allowed us to determine when, in embryogenesis, the perturbation of head development was initiated. Furthermore, we were able to establish at which level the AP axis of the neural tissue was affected. For this purpose, embryos were injected dorsally with a mixture of McerP-BMP4, McerP-Xnr1 and McerP-Xwnt8 (20 pg each/embryo). At stages 12, 13 and 15, RNA was extracted from pools of 5 randomly picked injected embryos or uninjected siblings. RT-PCR analysis showed that expression of the anterior neural markers *XBF1*, *Xemx1* and *Nkx2.1* (Bourguignon et al., 1998; Pannese et al.,

1998; Hollemann and Pieler, 2000; Small et al., 2000) was reduced in the injected embryos (Fig. 5A). Expression of *XBF1*, a pan telencephalic marker, was clearly reduced by stage 13. The same expression profile was observed for the ventral forebrain marker *Nkx2.1*. *Xemx1*, a marker for the dorsal telencephalon, was already downregulated by stage 12. In contrast, the expression of more posterior neural markers was not affected. Both *Engrailed-2* (Hemmati-Briuanlou et al., 1991), a mid-hindbrain boundary marker, and *Krox20* (Bradley et al., 1993), a marker for rhombomeres 3 and 5, were not reduced in the injected embryos (Fig. 5A). The cement gland marker *Xag* (Aberger et al., 1998) was also downregulated. From this analysis we conclude that increased levels of BMP, Nodal and Wnt in the ADE, repress the expression of anterior neural markers down to the mid-hindbrain level, as early as stage 12.

We further extended the molecular characterization of the induced head phenotype by performing an in situ hybridization analysis for neural markers in stage 22/24 embryos. The anterior neural expression of *XBF1*, *Xotx2* and *Xnot2* (Bourguignon et al., 1998; Blitz and Cho, 1995; Gont et al., 1993) was absent in the injected embryos (Fig. 5B-G). Expression of *Xnot2* in the chordoneural hinge (Fig. 5G) and of *XBF1* in the olfactory placodes (Fig. 5C) was not affected.

Similarly, the expression domain of *Krox20* was unchanged in injected embryos, despite the obvious loss of structures rostral to rhombomere 3 (Fig. 5H,I). *Xshh* (Stolow and Shi, 1995; Ekker et al., 1995), a gene expressed in the ventral

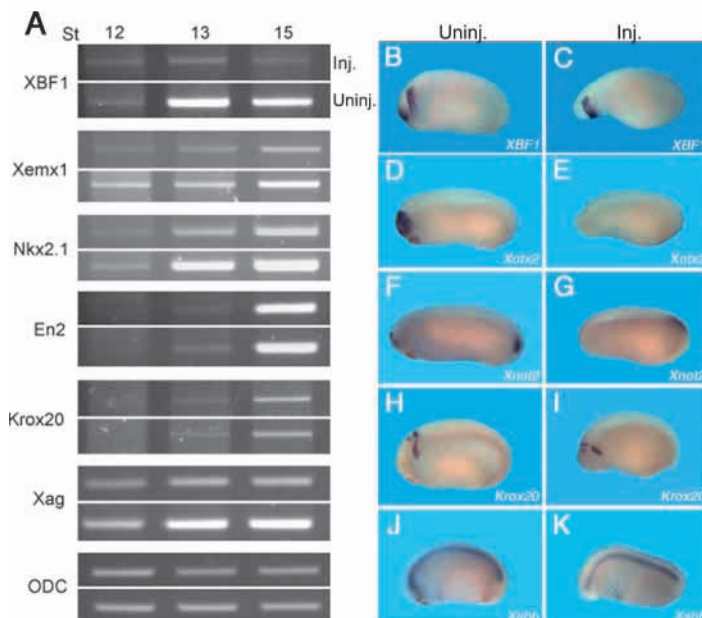


Fig. 5. Molecular markers analysis after microinjection of McerP-BMP4, -Xnr1 and -Xwnt8 in frog embryos. (A) RT-PCR analysis at stages 12, 13 and 15 show that the cement gland and anterior neural markers *Xag*, *XBF1*, *Xemx1* and *Nkx2.1* are downregulated in embryos coinjected with McerP-BMP4, McerP-Xnr1 and McerP-Xwnt8 (20 pg each), when compared with uninjected controls, while the levels of more posterior neural markers, like *En2* and *Krox20*, are not changed. *ODC* was used as a loading control. RNA extracts used for the RT-PCRs were made from pools of 5 randomly picked embryos. (B-K) In situ hybridization analysis for different molecular markers at stages 22/24. The injection of McerP-BMP4, McerP-Xnr1 and McerP-Xwnt8 (20 pg each) leads to the suppression of the anterior domains of expression of *XBF1* (B,C), *Xotx2* (D,E) and *Xnot2* (F,G). Expression of the hindbrain marker, *Krox20* (H,I), was not significantly changed in the injected embryos as well as in the controls. (J,K) *Xshh* expression in injected embryos does not extend as anteriorly as it does with the uninjected sibling embryos.

neural tube and notochord along the entire AP axis (Fig. 5J), was not detected in the rostral end of the injected embryo (Fig. 5K), while its expression in the remaining embryonic regions was identical to the uninjected controls. This in situ hybridization analysis confirmed and extended the previous RT-PCR data, demonstrating that elevated levels of BMP, Nodal and Wnt signaling in the ADE specifically inhibit the formation of forebrain and midbrain structures.

Cerberus morpholino oligonucleotide specifically enhances the head defects induced by BMP, Nodal and Wnt

Since CerMo by itself had no visible effect on head formation (Fig. 1H,I), we tested whether a possible function of Cerberus could be revealed in a sensitized experimental system. We simultaneously raised the levels of the agonists BMP, Nodal and Wnt up to a threshold level, sufficient to titrate their antagonists but without producing a severe phenotype. Hence, we analyzed whether this phenotype could be aggravated by simultaneously reducing Cerberus activity in the ADE. Dorsal injection of low doses (8 pg each) of a mixture of McerP-BMP4, McerP-Xnr1 and McerP-Xwnt8 caused a partial loss of the head (Fig. 6B and Fig. 3N). Remarkably, coinjection of CerMo strongly increased the head phenotype (Fig. 6C). The phenotype caused by CerMo was specific, since it could be rescued by injection of a full-length Cerberus expression

construct that cannot be blocked by the morpholino oligonucleotide (Fig. 6D).

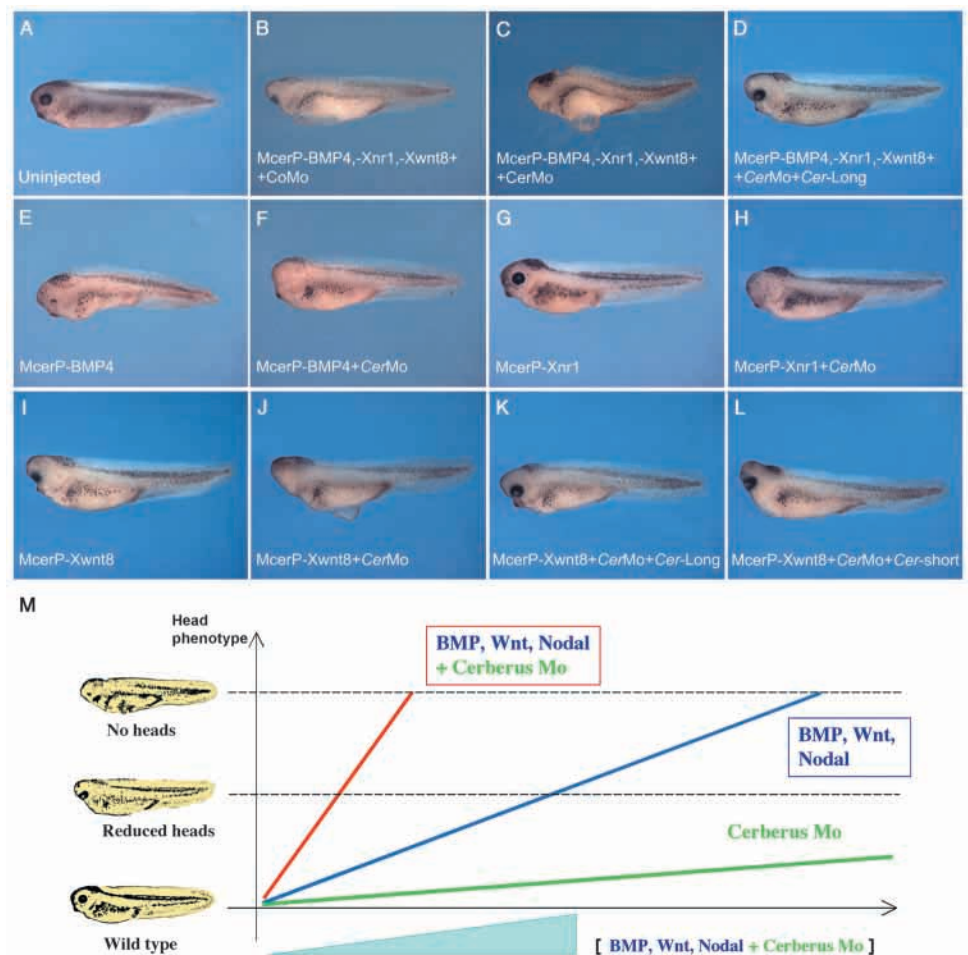
We next determined whether CerMo could also enhance the phenotypes caused by single injection of either McerP-BMP4, McerP-Xnr1 or McerP-Xwnt8. Microinjection of 20 pg of McerP-BMP4, McerP-Xnr1 or McerP-Xwnt8 led to the already described head defects (Fig. 6E,G,I). In all cases, coinjection of CerMo strongly enhanced these phenotypes (Fig. 6F,H,J). We had also shown that the cement gland was very sensitive to elevated Xwnt8 levels (Fig. 3L). Eyes and cement gland were absent in McerP-Xwnt8/CerMo-injected embryos, and both structures could be rescued by co-expression of full-length Cerberus protein (Fig. 6K). However, Cerberus-Short, which only binds to Nodal, could partially rescue the eye phenotype, but not the cement gland (Fig. 6L). This last result also suggests an interplay between Wnt and Nodal signaling, which would explain why the Nodal inhibitor Cer-S could partially rescue the eye phenotype induced by Wnt misexpression in the ADE.

In conclusion, these results clearly demonstrate that endogenous Cerberus protein can inhibit BMP4, Xnr1 and Xwnt8 activities in vivo.

Cerberus function in the ADE is required for the activation of forebrain markers

The ADE when combined with stage 10.5 DE explants induces

Fig. 6. Knockdown of endogenous Cerberus enhances the head phenotypes induced by microinjection of McerP-BMP4, -Xnr1 and -Xwnt8. (A-D) The head defects observed by the coinjection of McerP-BMP4, -Xnr1 and -Xwnt8 (8 pg each) together with the CoMo (B; 64%, $n=62$) can be aggravated when endogenous *cerberus* is knock-down by 1.6 pmol of CerMo (C; 65%, $n=46$). The specificity of this sensitization was verified by the coinjection of Cer-Long plasmid, which could rescue the phenotype (D; 58%, $n=42$). (E-J) The mild phenotypes obtained by individually injecting McerP-BMP4 (E; 30%, $n=44$), McerP-Xnr1 (G; 40%, $n=40$) or McerP-Xwnt8 (I; 66%, $n=47$) were also aggravated by the coinjection of 1.6 pmol CerMo (F; 25%, $n=41$. H; 60%, $n=39$. J; 58%, $n=44$ respectively). (K-L) CerMo dependent aggravation of the McerP-Xwnt8 phenotype could be completely rescued by Cer-Long construct (K; 66%, $n=48$), but only partially rescued if Cer-Short plasmid (L; 55%, $n=44$) is used instead. These results were observed in two independent experiments. (M) Graphical representation of the range of phenotypes observed by increasing amounts of BMP, Wnt and Nodal misexpressed in the ADE, showing the requirement of lower amounts of these signals to generate the same phenotypes when endogenous Cerberus is depleted.



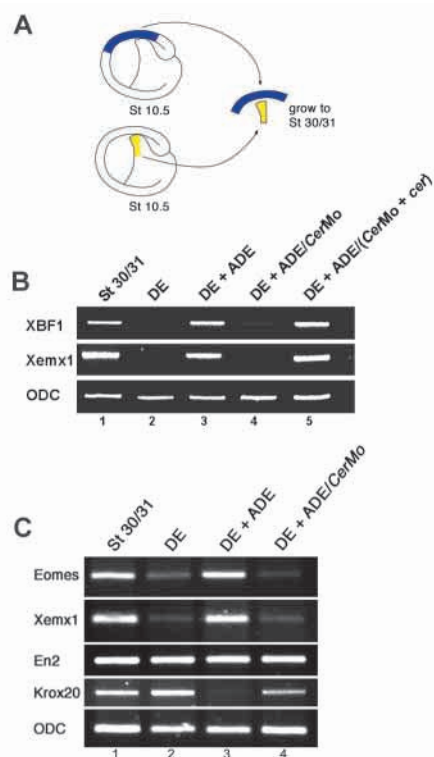


Fig. 7. Endogenous Cerberus activity is required for correct expression of neural markers in a tissue recombination induction assay. (A) ADE (yellow) explanted from either stage 10.5 uninjected embryos or from ones injected dorsol-vegetally with 3.2 pmol of CerMo, were conjugated with isochronic dorsal ectoderms (blue). Conjugates were grown until sibling embryos reached stage 30/31. (B) RT-PCR analysis of telencephalic markers in DE/ADE conjugates at stage 30/31. DE explants show expression of *XBF1* and *Xemx1*. When DE is conjugated with control ADE, dorsal telencephalic markers are up-regulated (lane 3). CerMo injected ADEs are no longer able to up-regulate neither *XBF1* nor *Xemx1* in the DE/ADE conjugates (lane 4). Cer-HA DNA construct, which lacks the 5'UTR sequences complementary to CerMo, can rescue the induction of both telencephalic markers in the DE/ADE CerMo conjugates. (C) RT-PCR analysis of neural markers from the DE/ADE conjugates at stage 30/31. DE show expression of *Xemx1* and *eomes* but, when conjugated with control ADE, marked up-regulation of these dorsal telencephalic markers (lane 3). ADE CerMo conjugated with dorsal ectoderm suppresses expression of *Xemx1* and *eomes* (lane 4). Expression levels of the mid-hindbrain marker *En2* are unchanged both in unconjugated DE (lane 2) as well as in DE/ADE (lane 3) or DE/ADE CerMo conjugates (lane 4). *Krox20* is downregulated in the DE/ADE conjugates (lane 3) but its expression levels are partially rescued in DE conjugates with CerMo injected ADE (lane 4).

dorsal telencephalic markers (Lupo et al., 2002). We have shown that in the embryo, modulating Cerberus activity in the ADE by raising BMP4, Nodal and Xwnt8 levels represses the expression of forebrain markers, including *XBF1* and *Xemx1* (Fig. 5A). To test whether the Cerberus function in the ADE was required for the activation of dorsal telencephalic markers in the neuroectoderm, we used a modified explant recombination system (Fig. 7A). RT-PCR analysis of ADE/DE conjugates revealed that uninjected ADE induced expression of both a pan telencephalic marker, *XBF1*, and a dorsal

telencephalic marker, *Xemx1* (Fig. 7B). In contrast, ADE explants in which Cerberus function had been knocked down with CerMo failed to induce both telencephalic markers (Fig. 7B). The CerMo effect was specific because expression of *XBF1* and *Xemx1* could be rescued by coinjection of a *cerberus* DNA construct that can not be blocked by the CerMo.

Cerberus activity in the ADE also modulates the expression of posterior neural markers. DE explants express *Krox20*, a hindbrain marker, and *En2*, which demarcates the mid-hindbrain boundary (Fig. 7C, lane 2). In contrast to the more anterior neural markers, expression of *Krox20* was inhibited in the DE/ADE but not in the DE/ADE CerMo conjugates (Fig. 7C). These experiments demonstrate that Cerberus activity in the ADE is required for the induction of forebrain markers and for the simultaneous repression of more posterior ones, such as *Krox20*.

Discussion

Targeted increase of BMP, Nodal and Wnt activities in the ADE affects head formation

The currently accepted model of head formation in the vertebrate embryo, postulates the existence of a head organizing center. The anterior dorsal endoderm in the amphibian, as well as the anterior visceral endoderm in the mouse embryo have been implicated as head organizers (reviewed by Beddington and Robertson, 1999). Simultaneous inhibition of BMP, Wnt and Nodal signaling in the ventral mesoderm of *Xenopus* embryos results in the formation of ectopic head-like structures (Bouwmeester et al., 1996; Piccolo et al., 1999; Glinka et al., 1997; Glinka et al., 1998). The dorsal mesendoderm in *Xenopus* expresses secreted antagonists for BMP, Wnt and Nodal proteins such as Cerberus, Dickkopf, Frzb and Crescent (Bouwmeester et al., 1996; Glinka et al., 1998; Leyns et al., 1997; Pera and de Robertis, 2000). According to this model, Cerberus would play an important role in the head organizer center. The presence of Cerberus, which can inhibit XWnt8, Xnr1 and XBMP4, in the ADE would generate a trunk-signaling free zone in the anterior region of the embryo, therefore restricting the trunk territory to the opposite side of the embryo, the posterior part.

To test in vivo the requirement for this Cerberus-mediated triple inhibition in the ADE, one cannot rely on dorsal microinjection of RNA or CMV-driven DNA constructs coding for either BMP4, Xnr1 or Xwnt8 proteins. When those constructs are microinjected in the dorsal blastomeres, their activation in the ecto-mesodermal layers leads to strong axial defects, ranging from strong ventralization (in the case of *BMP4* injection; Fig. 3A) to strong dorsalization (*Xnr1* injection; Fig. 3B). Using a mouse *cer-1* promoter fragment we were able to drive the expression of these signaling molecules in the ADE of *Xenopus* embryos. Since the activation of this promoter closely resembles the spatial and temporal expression of the *Xcer* gene, one could use it to very precisely target the expression of a given molecule to the ADE. Targeted expression of increasing doses of BMP4 led to head defects with progressive severity (Fig. 3D-F). Remarkably, neither the AP axis nor the cement gland were affected. Even at higher doses, of 80 pg, cement gland tissue was present, although the head was severely reduced. This phenotype was very different from the one observed after injection of CMV.BMP4. When

the ventralizing and anti-neural activities of BMP4 (Fainsod et al., 1994; Wilson and Hemmati-Brivanlou et al., 1995) are spatially restricted to the ADE only head defects were observed, while the axial structures remained undisturbed. A similar phenotype had already been reported for the misexpression of BMP4 in the anterior neural plate, driven by a *Pax-6* promoter fragment in transgenic frog embryos (Hartley et al., 2001). After gastrulation, the expression of *BMP4* in the *Pax-6* domain downregulated most anterior neural markers, leading to the suppression of anterior brain and eye formation. This revealed that the interplay between BMP signaling and localized inhibitors was necessary for the correct patterning of the anterior neural structures.

Injection of increasing amounts of McerP-Xnr1 resulted in gradual loss of the eye and reduction of anterior brain structures (Fig. 3G). This was surprising because the Nodal proteins are TGF- β factors with strong dorsalizing activity. Ectopic Nodal signaling in the entire mesoderm results in expanded Spemann's organizer tissue and excess of dorsoanterior structures (Jones et al., 1995; Smith et al., 1995; Joseph and Melton, 1997). Similar results were obtained when the Nodal inhibitor *Lefty1* was knocked down in *Xenopus* by antisense morpholino oligos (Branford and Yost, 2002). This led to the increase of Nodal signaling in the marginal zone causing an upregulation of Nodal responsive organizer genes. In contrast, expression of such genes in the ADE was unchanged, indicating that the level of Nodal signaling was not elevated there.

The *cer-1* promoter construct drove the expression of Xnr1 in the ADE but not in the organizer, thereby eliminating the dorsalizing effect on the mesoderm and, instead, revealing the anti head activity of this protein. This is in agreement with experiments in zebrafish, where overexpression of Nodal protein converted forebrain into more posterior neural or mesodermal tissue. Elevating the level of the Nodal inhibitor Antivin caused the loss of posterior ectoderm but did not influence forebrain and eye structures (Thisse et al., 2000).

Microinjection of McerP-Xwnt8 resulted in severe head defects ranging from cyclopia (at 20 pg; Fig. 3L), to a severe truncation of the head when a higher dosage of this construct was used. Interestingly, in this case, and in contrast to the McerP-BMP4 injected embryos, the cement gland was the first structure to disappear (compare Fig. 3D,L). Kiecker and Niehrs (Kiecker and Niehrs, 2001), have shown that a gradient of Wnt/ β -catenin signaling was involved in the anteroposterior neural patterning of *Xenopus* embryos. Wnt activity in the posterior neural plate is required for the differentiation of posterior neural cells. Our own results strongly indicate, however, that a targeted increase of Wnt activity in the ADE also prevented formation of anterior neural structures, but did not affect more posterior neural tissue. These observations are supported by genetic data from the zebrafish model. Increased Wnt signaling in the anterior head due to a mutation in the *axin* gene, a negative regulator of the Wnt signaling pathway, resulted in the loss of forebrain structures (Heisenberg et al., 2001).

When the activity of the signaling molecules BMP4, Xnr1 and Xwnt8 was simultaneously upregulated in the ADE driven by the McerP constructs, a strong synergistic defect in head structures could be observed (Fig. 3M,N). Interestingly, the targeted activation of these molecules in the ADE did not affect

the normal patterning of locally expressed genes such as *Xhex* and even *cerberus* itself (Fig. 4). Although it has been shown that *Xnr1* was able to induce *cerberus* expression, that seems to occur in a specific time frame. In particular, *cerberus* was only induced by the injection of *Xnr1* mRNA, but not by a *Xnr1* DNA construct, which is only expressed after midblastula transition (Piccolo et al., 1999). Interestingly, we observed that the adjacent anterior neuroectoderm was severely affected upon targeted expression of BMP4, Xnr1 and Xwnt8 proteins in the ADE (Fig. 5). The anterior neural markers *XBF1*, *Xemx1* and *Nkx2.1* and the cement gland marker *Xag* showed a marked decrease in the injected embryos, as shown by RT-PCR and in situ hybridization. However, more posterior markers such as *En2*, expressed in the mid and hindbrain, and *Krox20*, expressed in rhombomeres 3 and 5, were not affected by the gain of function of BMP4, Xnr1 and Xwnt8 in the ADE. Nevertheless, these embryos display a severe truncation of the head region rostral to these structures. In conclusion, these results strongly indicate that the combined increase of BMP, Wnt and Nodal activities in the ADE severely compromised the head formation program, suggesting the necessity for a tight locally controlled inhibition of those activities.

Correct balance of agonists versus antagonists in the ADE was essential for head formation

In some cases, the requirement for a given gene during embryonic development can only be demonstrated by the use of sensitized or compound system approaches. The mouse *cerberus-like* gene has been inactivated in ES cells (Belo et al., 2000; Shawlot et al., 2000; Stanley et al., 2000), failing to produce a mutant phenotype during mouse embryogenesis. Mutant mouse embryos lacking both Nodal inhibitors *Cer-1* and *Lefty1* (*cer-1^{-/-};Lefty1^{-/-}*) displayed striking early embryonic phenotypes not observed in the single mutants (Perea-Gomez et al., 2002). Furthermore, in this sensitized compound mutant background, removal of a single copy of *Nodal* can partially rescue the *cer-1^{-/-};Lefty1^{-/-}* mutant phenotypes. Therefore, the requirement for the redundant activities of *cerberus-like* and *Lefty1* at the level of nodal inhibition could only be assessed using this genetic system. In the *cer-1^{-/-};Lefty1^{-/-}* mice, *nodal* signaling is enhanced in the entire embryo. This has profound consequences on the formation of the primitive streak. Similar results were obtained in chicken embryos where nodal activity was enhanced in the epiblast, and simultaneously the hypoblast expressing the *cerberus* homologue *caronte* was removed (Bertocchini and Stern, 2002). In our *cerberus-like* promoter based assay, nodal activity is only enhanced in the ADE and therefore the formation of the trunk is not affected. Both the mouse, chick and frog experiments demonstrate that Cerberus function in vivo can only be revealed in sensitized assay systems.

As in the mouse, suppression of *Xenopus* Cerberus does not impair head formation (Fig. 1H,I). Similar results were obtained when the ADE region was extirpated from DMZ explants (Schneider and Mercola, 1999) and such explants still developed partial head-like structures. In order to reveal a putative role of Cerberus in head formation we established a novel sensitized assay system in the *Xenopus* embryo.

We tested the biological relevance of the Cerberus inhibitory activity in the ADE by simultaneously knocking down Cerberus activity and elevating the levels of the agonists

BMP4, Xnr1 and Xwnt8. When mild doses of these 3 proteins were targeted to the ADE the resulting weak head phenotype was strongly enhanced when Cerberus was knocked down by coinjection of the CerMo (compare Fig. 6B and C). This indicated that the agonists (BMP, Wnt and Nodal) must reach a critical threshold level in order to inhibit head formation. This threshold level could be lowered through the suppression of the antagonist Cerberus by CerMo, resulting in an aggravation of the phenotype (Fig. 6M). When the relative balance of agonists versus antagonists was restored by coinjection of a full-length *cerberus* construct that was not targeted by CerMo, the head phenotype was rescued almost completely. This novel approach clearly demonstrated that Cerberus is a functional inhibitor of BMP4, Xnr1 and Xwnt8 activities in vivo (Fig. 6E-L) and that this biological activity in the ADE is required for the correct specification of the head.

Endogenous Cerberus activity and anterior neural patterning

Cerberus was able to induce anterior neural markers including the dorsal telencephalic markers *eomes* and *Xemx1* in animal cap explants (Bouwmeester et al., 1996; Lupo et al., 2002). Similar results were obtained when the activities of the BMP inhibitor Chordin and the Cerberus truncated protein cer- Δ C1 were combined (Fetka et al., 2000; Lupo et al., 2002). This N-terminal fragment of Cerberus can inhibit Wnt activity (Fetka et al., 2000) and retains a residual Nodal inhibiting activity (Lupo et al., 2002). In contrast, the coinjection of *chd* and *cer-S* mRNA was unable to induce the same set of markers, pointing to the simultaneous requirement of the anti-BMP and anti-Wnt activities of Cerberus in this process. Induction of *XBF1*, *Xemx1* and *eomes* expression in dorsal ectoderm explants (DE) was also observed when they were conjugated with ADE, a tissue where endogenous *cerberus* was expressed (Lupo et al., 2002).

Knocking down Cerberus function in the ADE with a morpholino oligo, resulted in a loss of *XBF1*, *Xemx1* and *eomes* induction in ADE/DE conjugates (Fig. 7B,C). Furthermore, uninjected ADE repressed the expression of the more posterior neural marker *Krox20* in the explanted DE, but this marker was activated in conjugates of DE/ADE injected with CerMo (Fig. 7C, lane 4). In embryos injected with CerMo however, *eomes* and *Xemx1* expression in the brain was not significantly changed (data not shown). This indicates that in the embryonic context other molecules may compensate for the reduced Cerberus activity. This could also explain the reported formation of head in the DMZ explants lacking Cerberus-expressing ADE tissue (Schneider and Mercola, 1999). The completeness of these head structures, however, was not demonstrated because markers identifying only forebrain were not analyzed. Cerberus is the only known factor expressed in the leading edge of the ADE with anti-BMP, -Nodal and -Wnt activity. Thus, the anterior neural patterning activity of Cerberus in ADE/DE conjugates could be revealed through CerMo-mediated loss-of-function, since no other factors could compensate for it in this system. When the formation of the AP axis in *Xenopus* embryos is perturbed by interfering with gastrulation movements very often neural patterning defects were observed. It is tempting to speculate that these defects are the result of the incorrect positioning of the ADE and that spatially altered Cerberus activity causes aberrant neural patterning.

In conclusion, in the ADE/DE explant system (Fig. 7) a dual novel role for the ADE is described: not only does ADE induce the expression of anterior neural markers but it also represses the expression of more caudal ones through the activity of Cerberus. This clearly demonstrates that the endogenous Cerberus activity in the leading edge of the anterior dorsal endoderm is required for the correct induction and patterning of the brain.

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