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CORRECTION

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Supplementary figures S3, S4 and S5 were incorrect and have been replaced.

The authors apologise to readers for this mistake.

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RESEARCH ARTICLE

An essential role for LPA signalling in telencephalon development

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ABSTRACT

Lysophosphatidic acid (LPA) has wide-ranging effects on many different cell types, acting through G-protein-coupled receptors such as LPAR6. We show that *Xenopus lpar6* is expressed from late blastulae and is enriched in the mesoderm and dorsal ectoderm of early gastrulae. Expression in gastrulae is an early response to FGF signalling. Transcripts for *lpar6* are enriched in the neural plate of *Xenopus* neurulae and loss of function caused forebrain defects, with reduced expression of telencephalic markers (*foxg1*, *emx1* and *nkx2-1*). Midbrain (*en2*) and hindbrain (*egr2*) markers were unaffected. *Foxg1* expression requires LPAR6 within ectoderm and not mesoderm. Head defects caused by LPAR6 loss of function were enhanced by co-inhibiting FGF signalling, with defects extending into the hindbrain (*en2* and *egr2* expression reduced). This is more severe than expected from simple summation of individual defects, suggesting that LPAR6 and FGF have overlapping or partially redundant functions in the anterior neural plate. We observed similar defects in forebrain development in loss-of-function experiments for ENPP2, an enzyme involved in the synthesis of extracellular LPA. Our study demonstrates a role for LPA in early forebrain development.

KEY WORDS: LPAR6, FGF, ENPP2, Forebrain, Telencephalon, *Xenopus*

INTRODUCTION

Lysophosphatidic acid (LPA) is a small ubiquitous phospholipid that acts as an extracellular signal and is believed to be involved in numerous physiological and pathological processes. It evokes a wide range of cellular responses from different cell types, including effects on cell proliferation, migration, adhesion, shape changes and death (Noguchi et al., 2009; Skoura and Hla, 2009; Choi et al., 2010). These diverse cellular functions are mediated by at least six members of the large superfamily of G-protein-coupled receptors (GPCR): LPA receptors 1 to 3 (LPAR1-3) belong to the Endothelial differentiation gene (EDG) subgroup, which also includes receptors for the bioactive lipid Sphingosine-1-phosphate, whereas LPA receptors 4 to 6 (LPAR4-6) belong to the Purinergic receptor (P2Y) subgroup, which includes receptors for extracellular Adenosine triphosphate (ATP) and Uridine triphosphate (UTP) (Choi et al., 2010). All six receptors couple to multiple G-protein subtypes that regulate multiple intracellular signalling pathways, including Cyclic adenosine monophosphate (cAMP), Ca^{2+}, Mitogen-activated protein (MAP) kinase, and Rho GTPases.

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LPA receptors are widely expressed in vertebrate embryos, with distinct but overlapping expression patterns (Ohuchi et al., 2008; Massé et al., 2010a). Several LPA receptors are expressed in the developing nervous system (Ohuchi et al., 2008) but loss-of-function studies have provided few clues as to their role in neural development. Only minor phenotypic changes are observed, which presumably reflects redundant functions among LPA receptors (Contos et al., 2000; Contos et al., 2002; Ye et al., 2005; Lee et al., 2008). Abnormalities have been described in the cerebral cortex of *Lpar1*$^{-/-}$ mutant mice, including a reduction in neuronal progenitors (Estivill-Torrús et al., 2008) – a phenotype consistent with *Lpar1* expression in the ventricular zone of the developing cortex (Hecht et al., 1996). *Lpar5* is strongly expressed in a subset of neurons in dorsal root ganglia and loss-of-function studies have indicated a role in neuropathic pain (Lin et al., 2012). Severe neural defects have also been described in embryos lacking Ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2; also known as Autotaxin and Lysophospholipase D), a secreted enzyme involved in the synthesis of extracellular LPA (Tokumura et al., 2002; Umezu-Goto et al., 2002). *Enpp2*$^{-/-}$ mutant mice fail to complete cranial neural tube closure and exhibit defects in the forebrain and at the midbrain-hindbrain boundary (MHB) (Fotopoulou et al., 2010; Koike et al., 2010; Koike et al., 2011).

LPAR6 is the most recently characterised member of the family of LPA receptors (Chun et al., 2010). Identified as an orphan GPCR (Kaplan et al., 1993), it was subsequently named P2Y5 because of homology to nucleotide receptors (Webb et al., 1996). However, it failed to elicit detectable responses to extracellular nucleotides (Li et al., 1997). More recently, LPAR6 was shown to be a receptor for LPA, activating $G\alpha_i$ and $G\alpha_{12/13}$ G proteins, inhibiting Adenylyl cyclase, phosphorylating ERK1/2, and activating Rho GTPase (Pasternack et al., 2008; Lee et al., 2009; Pasternack et al., 2009; Yanagida et al., 2009). Little is known about the role of LPAR6 in cellular physiology except that it is required for human hair growth (Pasternack et al., 2008; Shimomura et al., 2008; Pasternack et al., 2009; Shimomura et al., 2009a). *LPAR6* is expressed in the inner root sheath of hair follicles (Pasternack et al., 2008; Shimomura et al., 2008) and loss-of-function mutations in this receptor are found in families with autosomal hair defects (Pasternack et al., 2008; Pasternack et al., 2009; Shimomura et al., 2008). Similar hair defects have been observed in families carrying loss-of-function mutations in the *Lipase H* (*LIPH*) gene, which is also expressed in the inner root sheath of hair follicles and encodes a secreted enzyme involved in the synthesis of extracellular LPA (Kazantseva et al., 2006; Shimomura et al., 2009b). However, both *LPAR6* and *LIPH* are widely expressed in human tissues, indicating that they have multiple roles.

In this study, we show that *lpar6* is expressed in *Xenopus* embryos, from late blastulae through to tadpoles, and that loss of function disrupts neural development. Embryos injected with antisense morpholinos (AMO) to *lpar6* had greatly reduced expression of telencephalic markers (*foxg1*, *emx1* and *nkx2-1*) and

Development (2014) doi:10.1242/dev.104901

reduced expression of eyefield markers (*rax* and *pax6*). Midbrain (*en2*) and hindbrain (*egr2*) markers were not affected, demonstrating that defects were restricted to the developing forebrain. *Foxg1* expression requires LPAR6 within the ectoderm and not the mesoderm. Remarkably, defects caused by injecting *lpar6*-AMO were enhanced by also inhibiting FGF signalling, with midbrain (*en2*) and hindbrain (*egr2*) markers being greatly reduced in these embryos. This suggests that LPAR6 and FGF signalling interact in anterior neural development. Finally, we show that an AMO targeting *Xenopus* ENPP2 causes similar defects to those of *lpar6*-AMO. Our study indicates that LPA signalling is required to specifying cell fates in the anterior nervous system, a role that may involve cooperation with FGF signalling.

RESULTS

Lpar6 is expressed during embryonic development

Scanning expressed sequence tag (EST) databases, we identified *Xenopus* cDNAs encoding a protein of 345 amino acids, sharing 78% identity with human LPAR6 and only 15-51% identity with human LPAR1-5 (supplementary material Fig. S1). The corresponding gene is nested within the largest intron of the *Xenopus rb1* gene and transcribed in the opposite direction (supplementary material Fig. S2), an identical arrangement to that of human *LPAR6* and *RB1* (Herzog et al., 1996). Sequence conservation and genomic synteny demonstrate that we have identified *Xenopus lpar6*.

To determine whether *lpar6* is expressed during embryonic development we performed reverse transcription polymerase chain reaction (RT-PCR) on staged *Xenopus* embryos (Fig. 1A). Transcripts were detected from early gastrula (stage 10) through to tadpoles (stage 40), with reduced expression in late gastrulae (stage 13). Microarrays (Branney et al., 2009) showed that *lpar6* was transcribed from late blastulae (stage 9), on a similar timescale to *fgf8* but preceded the FGF target genes *bra* and *cdx4* (Fig. 1B). RT-PCR found that *lpar6* expression was greatest in the marginal zone of early gastrulae, with a low level of expression in the animal hemisphere and no expression in the vegetal hemisphere (Fig. 1C). Whole-mount *in situ* hybridisation confirmed that *lpar6* is expressed in the marginal zone of early gastrulae, with strongest expression above the dorsal blastopore lip (Fig. 2A-C). A bisected embryo shows that expression is localised to the involuting mesoderm (Fig. 2B). We also observed expression in the dorsal-animal hemisphere, the prospective neural plate, of early gastrulae (Fig. 2D). In neurulae, *lpar6* is expressed in the neural plate, with strongest expression in anterior regions (Fig. 2E-H). At tailbud stages, *lpar6* expression is strongest in the head, branchial arches, notochord and myotome (Fig. 2I,J).

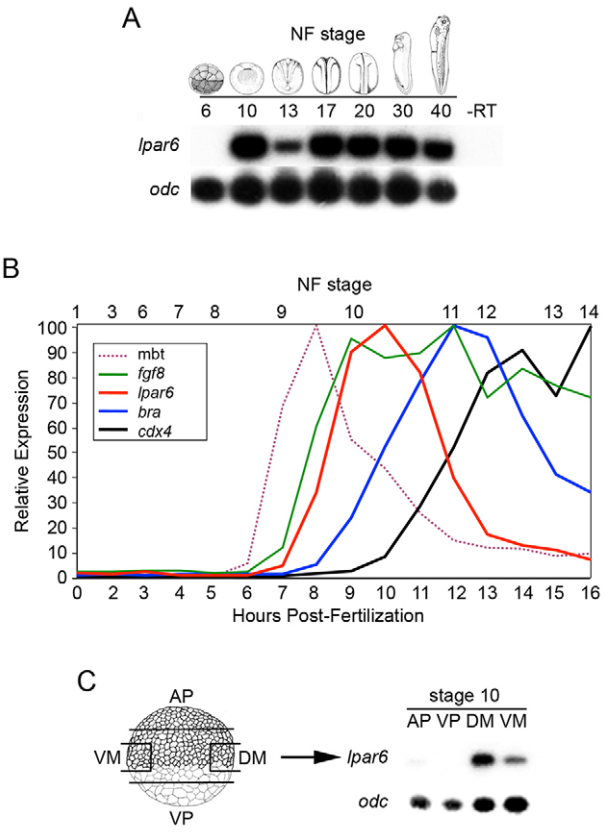

Fig. 1. Temporal expression of *lpar6*. (A) RT-PCR analysis for *lpar6* and *ornithine decarboxylase* (*odc*) in staged *Xenopus* embryos, showing *lpar6* expression from stage 10 (early gastrula) to stage 40 (tadpole). Note the drop in expression at stage 13 (late gastrula). Minus reverse transcriptase control (–RT) was performed at stage 40. (B) Microarray analysis for expression of *lpar6*, *fgf8*, *bra*, *cdx4* and a marker for the mid-blastula transition (MBT). *Xenopus* embryos were collected 0-16 hours post-fertilisation (23°C). (C) RT-PCR analysis for *lpar6* and *odc* in dissected stage 10 embryos. AP, animal pole; DM, dorsal marginal zone; VM, ventral marginal zone; VP, vegetal pole.

Lpar6 is a direct target of FGF signalling

Lpar6 was previously shown to be positively regulated by FGF signalling (Branney et al., 2009), with expression levels 60-80% lower in gastrulae expressing dominant-negative FGF receptors (Fig. 3A). To confirm this, we isolated animal caps from blastulae (stage 8) and incubated them in media containing FGF4. RT-PCR showed that FGF4-induced expression of *lpar6*, consistent with the microarray data (Fig. 3B). *Lpar6* expression was also induced by Activin (Fig. 3B), a mesoderm-inducing factor belonging to the TGF-β family (Smith et al., 1990). To determine whether *lpar6* induction is an immediate-early response to FGF signalling, we incubated animal caps in media containing both FGF4 and cycloheximide (a protein synthesis inhibitor). However, cycloheximide alone induced strong expression of *lpar6* in animal caps (data not shown), replicating the effect previously described for chick *lpar6* in cultured spleen cells (Kaplan et al., 1993). As an alternative, we sought to determine how quickly induction of *lpar6* transcription occurs in response to FGF signalling. Animal caps were incubated in media containing FGF4, then removed at different time points and analysed by RT-PCR for *lpar6* transcripts. We detected weak expression of *lpar6* after 30 minutes of FGF4 exposure, with stronger expression after 60 minutes (Fig. 3C). Our results show that transcription of *lpar6* is an early response to FGF signalling.

Inhibition of LPAR6 disrupts head development

To determine the role of LPAR6 during development we adopted a loss-of-function approach, using antisense morpholino oligonucleotides (AMO1 and AMO2) that inhibit translation of *Xenopus lpar6* (Fig. 4A,B). *Xenopus* embryos were injected at the two-cell stage with 20 ng per blastomere of either AMO1 or AMO2 and the embryos were examined for developmental defects. Neither AMO had any effect on mesoderm formation in early gastrulae (supplementary material Fig. S3). The first defects were observed at early tailbud stages, with stage 28 embryos displaying a reduced anteroposterior axis length (3.4 mm compared with control length of 4.0 mm, *t*-test $P<0.005$) and head defects (Fig. 4C-E). Whole-

Development (2014) doi:10.1242/dev.104901

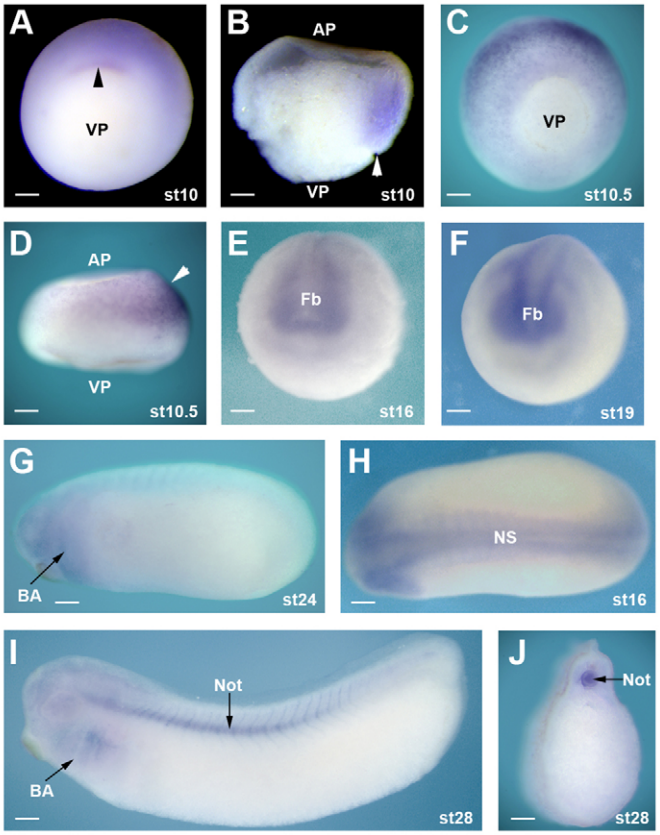

Fig. 2. Spatial expression of *lpar6*. Whole-mount *in situ* hybridisation with antisense probe for *laprr6*. (A) Stage 10, vegetal view, with dorsal blastopore lip (arrowhead). (B) Stage 10, bisected, with dorsal blastopore lip (arrowhead). (C) Stage 10.5, vegetal view. (D) Stage 10.5, lateral view, with dorsal-animal expression (arrowhead). (E) Stage 16, anterior view. (F) Stage 19, anterior view. (G) Stage 24, lateral view with head to the left. (H) Stage 16, dorsal view with head to the left. (I) Stage 28, lateral view with head to the left. (J) Stage 28, trunk section. Scale bars: 200 µm. AP, animal pole; BA, branchial arch; Fb, forebrain; Not, notochord; NS, nervous system; VP, vegetal pole.

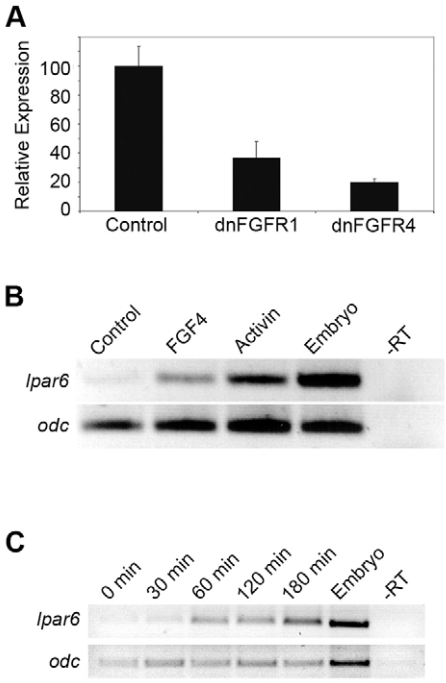

Fig. 3. FGF regulates expression of *lpar6*. (A) Microarray analysis for *lpar6* transcripts in early gastrulae expressing dnFGFR1 or dnFGFR4. (B) RT-PCR analysis for *lpar6* and *odc* in animal caps incubated for 5 hours (18°C) with either FGF4 or Activin. Sibling embryos were used for embryo and –RT controls. (C) RT-PCR analysis for *lpar6* and *odc* in animal caps incubated with FGF4 for up to 180 minutes (18°C). Sibling embryos were used for whole embryo and –RT controls.

mount *in situ* hybridisation showed that both AMO reduced telencephalic expression of *foxg1* (Fig. 4F-H). The frequency and severity of head defects were always greater with AMO2, which was used in all subsequent experiments. To demonstrate specificity, we attempted to rescue the head defect by co-injecting 40 ng of AMO2 with human *LPAR6* mRNA. Translation of this mRNA is not inhibited by AMO2 (Fig. 4B). Embryos co-injected with 200 pg of *LPAR6* mRNA failed to rescue the head defect and embryos injected with 600-800 pg usually died as gastrulae. In two experiments, embryos injected with 400 pg of *LPAR6* mRNA survived gastrulation and formed tailbud embryos with a normal head (Fig. 4I-K). We also injected *Xenopus tropicalis* embryos with a species-specific AMO for *lpar6* and obtained tadpoles with a smaller head and reduced *foxg1* expression (supplementary material Fig. S4). Our results suggest that forebrain defects are caused by specific inhibition of LPAR6 function.

Inhibition of LPAR6 disrupts neural development

Evidence from the use of the AMO injections suggested that LPAR6 has a role in anterior neural development. We therefore injected two-cell embryos with AMO2 and used whole-mount *in situ* hybridisation to study neural-specific gene expression. Initially, we injected a single blastomere with 20 ng of AMO2 and observed an increase in the width of *sox2* (Fig. 5A,B) and *cdx4* (Fig. 5I,J) expression on the injected side of neurulae, coupled with a reduction in epidermal keratin (*k81a1*) expression (Fig. 5C,D). We also observed loss in expression of *snai2*, a neural crest marker (Fig. 5E,F), whereas *myod1*, a mesodermal marker, was unaffected (Fig. 5G,H). Next, we injected 20 ng of AMO2 into each blastomere at the two-cell stage and analysed gene expression in the anterior neural plate. Expression of the telencephalon marker *foxg1* was greatly reduced in AMO2-injected neurulae (Fig. 5K,L), whereas the eye field markers *rax* and *pax6* were reduced to a lesser extent (Fig. 5Q-T). The MHB marker *en2* (Fig. 5O,P) and the hindbrain marker *egr2* (Fig. 5M,N) were expressed at normal levels, although their expression domains appear to have shifted towards the anterior neural plate border. Telencephalon development is regulated by signals from adjacent cells, including FGF8 from the anterior neural ridge (ANR) (Wilson and Houart, 2004; Hoch et al., 2009). We therefore analysed *fgf8* expression in AMO2-injected neurulae and observed that it was reduced, both in the ANR and more posteriorly at the MHB (Fig. 5U,V). There was also a reduction in diphospho-ERK (dpERK) staining in the anterior neural plate (Fig. 5W,X), demonstrating that FGF signalling was reduced in AMO2-injected embryos. Loss-of-function experiments demonstrate that LPAR6 is required for forebrain development in *Xenopus* embryos, perhaps by regulating FGF signalling.

To investigate forebrain development in more detail, we allowed AMO2-injected embryos to develop until tailbud stages (stage 26) and analysed them for telencephalon-specific gene expression (Fig. 6). In addition to *foxg1*, we also analysed expression of *nkx2-1*, a marker for the ventral telencephalon, and *emx1*, a marker for the

Development (2014) doi:10.1242/dev.104901

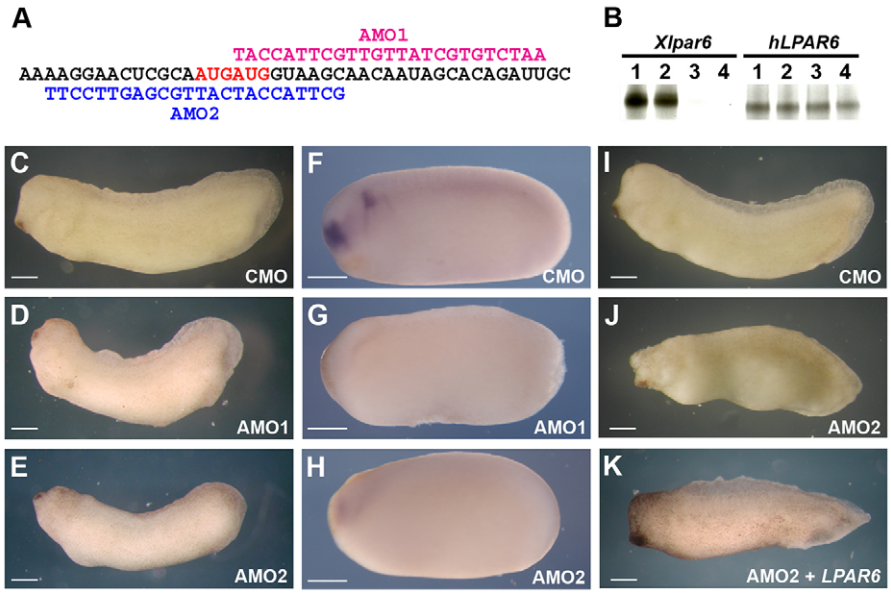

Fig. 4. LPAR6 is required for forebrain development. (A) Sequence (black letters, 5′-3′) of *Xenopus lpar6* mRNA (translational start site in red) aligned with sequence for both AMO1 (pink letters 3′-5′) and AMO2 (blue letters 3′-5′). (B) *In vitro* translation of *Xenopus lpar6* and human *LPAR6* in the presence of morpholinos. Lane 1, no MO. Lane 2, control MO. Lane 3, AMO1. Lane 4, AMO2. (C-E) Stage 28, lateral view (head to left), injected with 40 ng of morpholino. (C) Normal embryo injected with control MO (100%, *n*=90). (D) AMO1-injected embryo with head defect (60%, *n*=95). (E) AMO2-injected embryo with head defect (93%, *n*=75). Anteroposterior axis length of control-MO-injected embryos was 4.0 mm (s.d.=0.21, *n*=44) and that of AMO-injected embryos 3.4 mm (s.d.=0.17, *n*=44). Defects in D and E are statistically significant (Fisher's exact test, $P<0.001$). (F-H) Whole-mount *in situ* hybridisation, with antisense *foxg1* probe. Stage 24, lateral view (head to left), injected with 40 ng of morpholino. (F) Normal embryo injected with control MO (100%, *n*=30). (G) AMO1-injected embryo with reduced *foxg1* expression (60%, *n*=30). (H) AMO2-injected embryo with reduced *foxg1* expression (87%, *n*=30). Defects in G and H are statistically significant (Fisher's exact test, $P<0.001$). (I-K) Stage 28, lateral view (head to left), injected with 40 ng of AMO2 and 400 pg of human *LPAR6* mRNA. (I) Normal embryo injected with control MO (100%, *n*=30). (J) AMO2-injected embryo with head defect (80%, *n*=35). (K) AMO2 plus *hLPAR6* mRNA-injected embryo with normal head (66%, *n*=35). Rescue of head development in K is statistically significant (Fisher's exact test, $P<0.001$). Scale bars: 400 µm.

dorsal telencephalon (Small et al., 2000; Bachy et al., 2002). Transcripts for *foxg1* (Fig. 6A,B) and *emx1* (Fig. 6E,F) were not detected in AMO2-injected embryos, and only faint signals were detected for *nkx2-1* (Fig. 6C,D). Our results suggest that LPAR6 is required for the development of both dorsal and ventral regions of the *Xenopus* telencephalon.

LPAR6 is required in the ectoderm for telencephalon development

As *lpar6* is expressed in both mesoderm and ectoderm, we wished to determine its germ layer requirement for telencephalon development and initially targeted AMO injections to pairs of blastomeres (5 ng per blastomere) at the eight-cell stage (Fig. 7A). Fate maps (Dale and Slack, 1987; Moody, 1987) have shown that the nervous system is predominantly formed by dorsal-animal blastomeres, whereas the mesoderm that underlies the forebrain is formed by dorsal-vegetal blastomeres (Fig. 7A). AMO2 disrupted tadpole morphology in all four injection sets, but forebrain defects were confined to dorsal-animal injections (Fig. 7B-F). This suggests that LPAR6 is required in the ectoderm for forebrain development. Next, we exploited the ability of dorsal mesoderm to induce *foxg1* expression in animal cap ectoderm (Lupo et al., 2002). Embryos were injected at the four-cell stage, with 10 ng per blastomere of AMO2, and both the dorsal marginal zone (DMZ) and animal cap isolated from early gastrulae (stage 10). They were grafted together (Fig. 7G) and incubated until sibling embryos were late neurulae (stage 18). RT-PCR showed that *foxg1* expression was reduced when an AMO2-injected animal cap was grafted with a control DMZ, but not when a control animal cap was combined with an AMO2-injected DMZ (Fig. 7H). Expression of the general neural plate marker *sox2* was similar in all grafts. Our results demonstrate that LPAR6 is required in ectoderm, and not mesoderm, for telencephalon development. A requirement for LPAR6 in the ectoderm was also demonstrated in animal caps expressing Noggin, a Bone morphogenetic protein (BMP) inhibitor that induces *foxg1* expression (Papalopulu and Kintner, 1996). AMO2 reduced Noggin-induced expression of *foxg1* without affecting *sox2* expression (supplementary material Fig. S5). As mesoderm is not induced by Noggin, the inhibitory effect of AMO2 must reside within the ectoderm.

LPAR6 and FGFs act together in head development

While looking at the control of *lpar6* expression by FGF signalling, we observed enhanced head defects when embryos were co-injected with 40 ng of AMO2 and dominant-negative FGFR1 (*dnfgfr1*) mRNA (Fig. 8). Injection of AMO2 alone gave the expected forebrain defects (Fig. 8B), whereas *dnfgfr1* mRNA (plus control MO) disrupted posterior development but had no discernible effect on head development (Fig. 8C). Remarkably, embryos co-injected with AMO2 and *dnfgfr1* mRNA appeared to lack all head structures (Fig. 8D). This is a more severe phenotype than expected from the individual defects, indicating that LPA and FGF signalling might interact during the development of the anterior nervous system. We repeated this experiment using SU5402, a chemical inhibitor of FGF receptors (Mohammadi et al., 1997). Embryos injected with 40 ng of AMO2 were incubated in 10 µM SU5402, from late blastulae through early tailbud stages. This concentration of SU5402 alone gave an almost identical phenotype to injecting *dnfgfr1* mRNA. We also noted that it did not affect expression of *lpar6* (data not shown), which we attribute to the late application of the reagent. Embryos were analysed by RT-PCR for expression of the neural plate markers

Development (2014) doi:10.1242/dev.104901

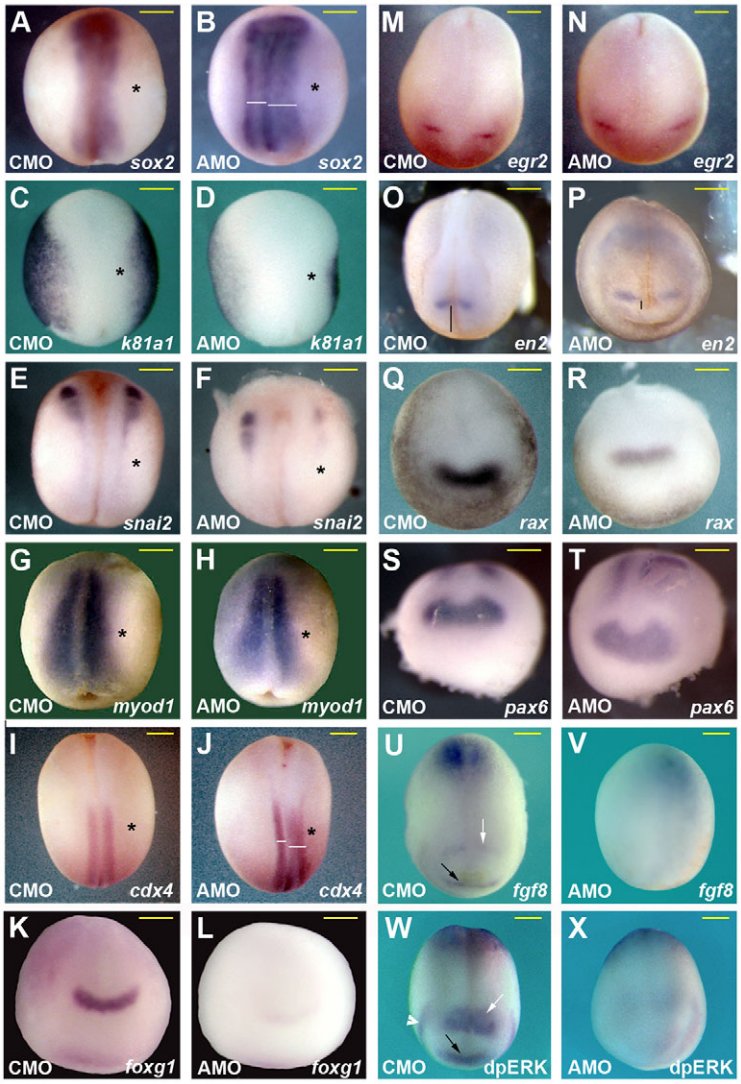

Fig. 5. LPAR6 is required for neural development. Whole-mount *in situ* hybridisation analysis of MO injected neurulae. (A-J) Dorsal views of neurulae injected with 20 ng of morpholino into a single blastomere at the two-cell stage. Head at the top and injected side (asterisk) on the right. (A,B) Neural plate marker *sox2*, with increased width on the AMO2-injected side (70%, *n*=40). (C,D) Epidermal marker *k81a1*, with decreased expression on the AMO2-injected side (75%, *n*=40). (E,F) Neural crest marker *snai2*, with reduced expression on the AMO2-injected side (69%, *n*=35). (G,H) Skeletal muscle marker *myod1*, with no defect (100%, *n*=38). (I,J) Posterior neural plate marker *cdx4*, with increased width on the AMO2-injected side (80%, *n*=40). (K-X) Anterodorsal views of neurulae injected with 40 ng of morpholino. (K,L) Telencephalon marker *foxg1*, with reduced expression in the AMO2-injected embryo (79%, *n*=38). (M,N) Hindbrain marker *egr2*, with normal expression in the AMO2-injected embryo (100%, *n*=35). (O,P) MHB marker *en2*, with normal expression in the AMO2-injected embryo (100%, *n*=35). Expression usually moved anteriorly (74%, *n*=35). (Q,R) Eyefield marker *rax*, with reduced expression in the AMO2-injected embryo (70%, *n*=40). (S,T) Eyefield marker *pax6*, with reduced expression in the AMO2-injected embryo (60%, *n*=30). (U,V) Anterior neural plate marker *fgf8*, with reduced expression in AMO2-injected embryos at the ANR (black arrow) and MHB (white arrow) (100%, *n*=23). (W,X) Whole-mount immunostaining for dpERK, with reduced ERK activity in AMO2-injected embryos at the ANR (black arrow), MHB (white arrow) and branchial arches (white arrowhead) (92%, *n*=36). All defects are statistically significant (Fisher's exact test, $P<0.001$). Scale bars: 200 µm.

foxg1, *otx2*, *rax*, *en2*, *egr2* and *sox2*, and the muscle marker *myod1* (Fig. 8E). SU5402 alone had no effect on the expression of any of the neural plate markers tested but greatly reduced expression of *myod1*, a gene known to be regulated by FGF signalling (Standley et al., 2001; Fisher et al., 2002). AMO2 alone only reduced expression of *foxg1* and *otx2*, the most anteriorly expressed genes tested. By contrast, AMO2-injected embryos treated with SU5402 displayed reduced expression of all five anterior neural plate markers (*foxg1*, *otx2*, *rax*, *en2* and *egr2*), but not the general neural plate marker *sox2*. Our results indicate that signalling pathways activated by LPAR6 and FGF interact in a redundant fashion to pattern the anterior nervous system.

Inhibition of ENPP2 disrupts forebrain development

To further test the role of LPA in forebrain development we turned to loss-of-function experiments for ENPP2, a secreted phospholipase

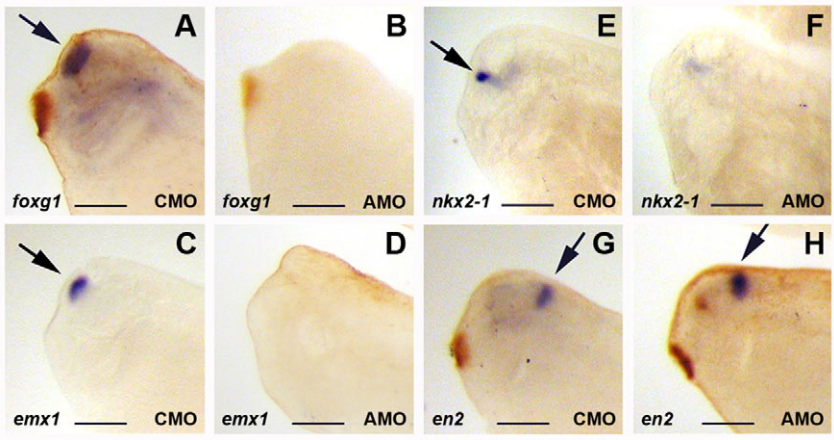

Fig. 6. LPAR6 is required for telencephalic development. Whole-mount *in situ* hybridisation of tailbud embryos injected with 40 ng of morpholinos; lateral views of the head. (A,B) Telencephalon marker *foxg1*, with loss of expression in the AMO2-injected embryo (71%, *n*=35). (C,D) Dorsal telencephalon marker *emx1*, with loss of expression in the AMO2-injected embryo (75%, *n*=32). (E,F) Ventral telencephalon marker *nkx2-1*, with reduced expression in the AMO2-injected embryo (67%, *n*=33). (G,H) MHB marker *en2*, with normal expression in the AMO2-injected embryo (100%, *n*=32). All defects are statistically significant (Fisher's exact test, $P<0.001$). Scale bars: 200 µm.

Development (2014) doi:10.1242/dev.104901

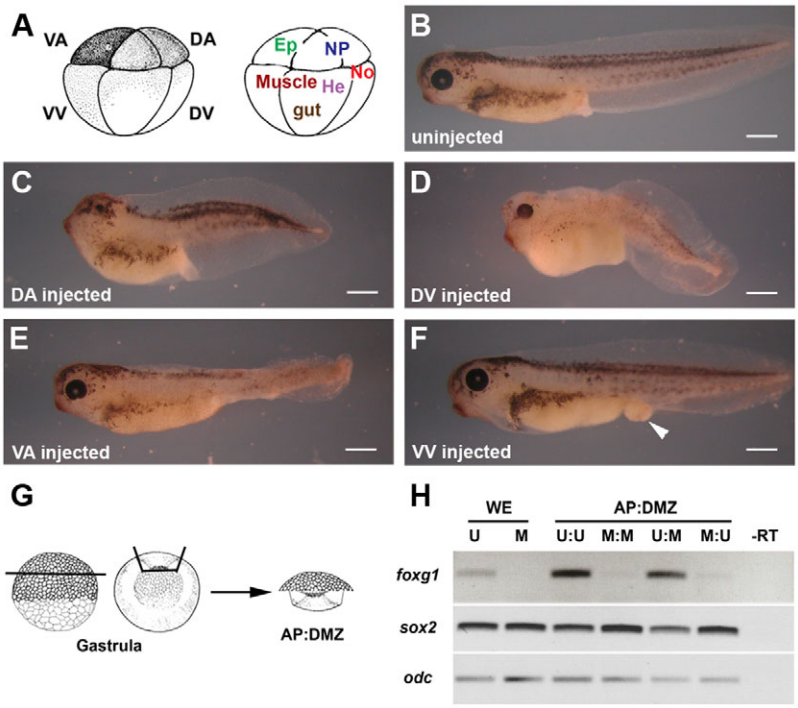

Fig. 7. LPAR6 is required in the ectoderm. (A) Schematic diagrams of an eight-cell *Xenopus* embryo, indicating injected blastomere pairs and their normal fate. (B-F) Stage 40 embryos, lateral view (head to left), injected with 5 ng per blastomere of AMO2. (B) Uninjected normal embryo (*n*=50). (C) DA injected embryo with head defect (86%, *n*=69). (D) DV injected embryo with dorsal defect but a normal head (61%, *n*=54). (E) VA injected embryo with tail defect but a normal head (76%, *n*=66). (F) VV injected embryo with defect in the posterior endoderm (arrowhead) but a normal head (60%, *n*=60). All defects are statistically significant (Fisher's exact test, *P*<0.001). (G) Schematic diagram of animal pole (AP) and dorsal marginal zone grafts. (H) RT-PCR analysis for *foxg1*, *sox2* and *odc* expression in AP:DMZ grafts. Grafts were made between uninjected (U) and AMO2-injected (M) fragments. Scale bars: 500 µm. –RT, minus reverse transcriptase control, uninjected stage 19 embryos; AP, animal pole; DA, dorsal-animal; DV, dorsal-vegetal; Ep, epidermis; He, heart; M:M, AMO2-injected AP and AMO2-injected DMZ; M:U, AMO2-injected AP and uninjected DMZ; No, notochord; NP, neural plate; U:M, uninjected AP and AMO2-injected DMZ; U:U, uninjected AP and uninjected DMZ; VA, ventral-animal; VV, ventral-vegetal; WE, stage 19 embryo.

that synthesises extracellular LPA (Tokumura et al., 2002; Umezu-Goto et al., 2002). *Xenopus enpp2* is expressed throughout development and transcripts are enriched in the nervous system of neurulae (Massé et al., 2010b). An AMO targeting the translational start site of both *enpp2a* and *enpp2b* was designed (Fig. 9A) and injected into *Xenopus* embryos with *enpp2a* mRNA. Western blot analysis showed that this AMO efficiently inhibited translation of co-injected mRNA (Fig. 9B). Next, we injected 10 ng of *enpp2* AMO into each blastomere at the two-cell stage and the first defects were detected at the end of neurulation, when AMO-injected embryos were found to be shorter than controls (Fig. 9C,D). The average anteroposterior axis length of AMO-injected embryos was only 72% of that of control embryos (2.9 mm compared to 4.0 mm, *t*-test, *P*<0.005). It was also clear that the anterior neural plate had failed to close in most AMO-injected embryos (74%, *n*=105), consistent with loss-of-function studies in mice (van Meeteren et al., 2006; Fotopoulou et al., 2010; Koike et al., 2011). To determine whether ENPP2 is required for forebrain development, we used whole-mount *in situ* hybridisation to analyse expression of anterior neural plate markers. Expression of the telencephalic markers *foxg1* (Fig. 9E,F), *nkx2-1* (Fig. 9G,H) and *emx1* (Fig. 9I,J) were greatly reduced in AMO-injected neurulae, whereas expression of the eyefield marker *rax* (Fig. 9K,L) was unaffected. Expression of the midbrain-hindbrain marker *en2* was normal in most embryos but reduced in a small number (Fig. 9M,N). We also observed a reduction in expression of *fgf8* in the ANR and MHB (Fig. 9O,P),

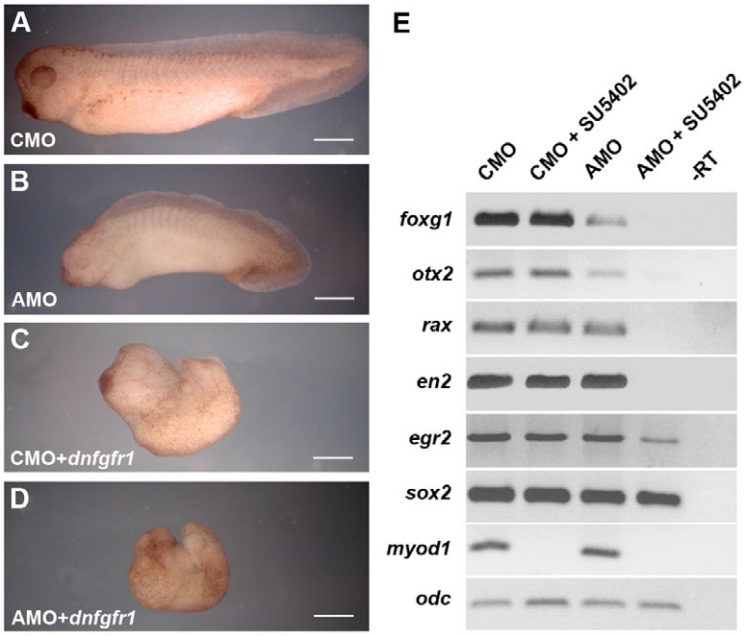

Fig. 8. LPAR6 and FGF co-regulate neural development. (A-D) stage 32, lateral view (head to left), injected with 40 ng per blastomere of MO plus or minus 1 ng of dominant-negative FGFR1 (*dnfgfr1*) mRNA. (A) Normal embryo injected with control MO (100%, *n*=27). (B) AMO2-injected embryo with head defect (81%, *n*=37). (C) Control MO plus *dnfgfr1*-injected embryo with posterior defect but normal head (92%, *n*=25). (D) AMO2 plus *dnfgfr1*-injected embryo with both head and posterior defects (100%, *n*=28). Note that the head defect is more severe than with AMO alone. All defects are statistically significant (Fisher's exact test, *P*<0.001). (E) RT-PCR analysis of MO-injected embryos incubated with or without 10 µM SU5402, from stage 9 to stage 16. Embryos were analysed for expression of the *foxg1* and *otx2* (forebrain), *rax* (eyefield), *en2* (MHB), *egr2* (hindbrain), *sox2* (neural plate), *myod1* (skeletal muscle) and *odc* (control). Scale bars: 500 µm. –RT, control-morpholino-injected embryos.

Development (2014) doi:10.1242/dev.104901

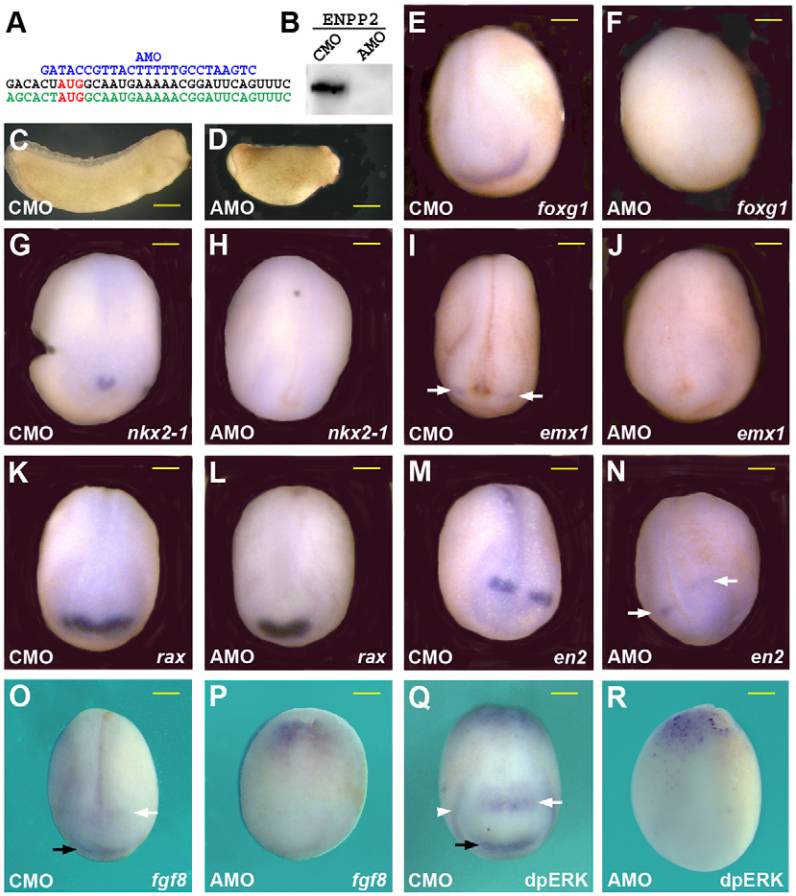

Fig. 9. ENPP2 is required for forebrain development. (A) Sequence of *Xenopus enpp2a* (black lettering, 5′-3′) and *enpp2b* (green lettering, 5-3′), translational start site in red, aligned with sequence for AMO (blue letters, 3′-5′). (B) Western blot analysis of *Xenopus* embryos injected with 1 µg of *enpp2a.myc* mRNA and 40 ng of morpholino. (C,D) Stage 28, lateral views (head to right) injected with 20 ng of either control MO or *enpp2*-AMO. Anteroposterior axis length of control-morpholino-injected embryos was 4.0 mm (s.d.=0.13, *n*=45) and that of AMO-injected embryos 2.9 mm (s.d.=0.22, *n*=45). (E-P) Whole-mount *in situ* hybridisation analysis of neurulae injected with 20 ng of either control (CMO) or *enpp2*-AMO. All embryos are viewed from anterodorsal perspective. (E,F) Telencephalon marker *foxg1*, with reduced expression in AMO-injected embryo (57%, *n*=82) (G,H) Ventral telencephalon marker *nkx2-1*, with reduced expression in AMO-injected embryo (59%, *n*=70). (I,J) Dorsal telencephalon marker *emx1*, with reduced expression in AMO-injected embryo (72%, *n*=53). (K,L) Eyefield marker *rax*, with normal expression in AMO-injected embryo (100%, *n*=77). (M,N) MHB marker *en2*, with reduced expression in AMO-injected embryo (16%, *n*=73 – 6% in controls, *n*=50). (O,P) Anterior neural plate marker *fgf8*, with reduced expression in AMO-injected embryo in both ANR (black arrow) and MHB (white arrow) (71%, *n*=21). (Q,R) Whole-mount immunolocalisation for dpERK. Note reduced ERK activity in AMO-injected embryos in the ANR (black arrow), the MHB (white arrow) and the branchial arches (white arrowhead) (96%, *n*=26). All defects are statistically significant (Fisher's exact test, $P<0.001$) except for *en2* (M,N). Scale bars: 600 µm in C,D; 200 µm in E-P.

as well as a reduction in dpERK staining in the anterior neural plate (Fig. 9Q,R). Our results demonstrate a role for ENPP2 in anterior neural plate development. These defects are similar to those caused by LPAR6 loss of function (above), confirming a role for LPA signalling in forebrain development.

DISCUSSION

Xenopus LPAR6

In this study we describe *Xenopus* LPAR6, a GPCR for the bioactive lipid LPA (Choi et al., 2010; Chun et al., 2010). We have shown that it is transcribed from late blastulae and enriched in the mesoderm of early gastrulae, with transcription regulated by FGF signalling. The distribution of *lapr6* transcripts in early gastrulae is very similar to that of *fgf4* and *fgf8* (Isaacs et al., 1995; Christen and Slack, 1997; Lea et al., 2009), and to FGF-dependent Extracellular-signal-related kinase (ERK) activity (Christen and Slack, 1999). Transcription is rapidly induced (30 minutes to 1 hour) by FGF4 in blastula stage animal caps and inhibited in early gastrulae by dominant-negative FGF receptors. We also find that *lpar6* is one of the first FGF responsive genes to be transcribed in late blastulae, suggesting that it is transcribed as an immediate-early response to FGF signalling. Inhibition of FGF signalling causes specific defects in posterior mesoderm formation (Amaya et al., 1991; Isaacs et al., 1998) and similar defects have been observed following inhibition of a number of FGF target genes, including *bra* and *cdx4* (Conlon et al., 1996; Isaacs et al., 1998). Surprisingly, we were unable to detect any effect of inhibiting LPAR6 function on mesoderm formation. Either LPAR6 is not an effector of FGF signalling in these processes, or different LPA receptor subtypes can compensate for defective LPAR6 signalling. Transcripts for *lpar1*, *lpar2*, *lpar4* and *lpar5* have been detected in *Xenopus* embryos (Lloyd et al., 2005; Massé et al., 2010a). As different LPA receptor subtypes have been shown to activate similar intracellular signalling pathways within a single cell (Dubin et al., 2010), it is possible that one or more of these *Xenopus* receptors could compensate for defective LPAR6 function at this stage.

LPA is required for anterior neural development

Xenopus lpar6 is expressed in the neural ectoderm of both early gastrulae and neurulae, and it is in the ectoderm of neurulae that we observed the first defects in loss-of-function experiments. We found that the width of the neural plate was increased throughout its length, as demonstrated by *sox2* and *cdx4* expression. This may be caused by defects in convergent-extension, coordinated cell intercalation that both narrows and extends the neural plate (Elul and Keller, 2000). Further studies will be required to confirm this, but we observed a reduced anteroposterior axis length in *lpar6*-AMO-injected embryos, expected of embryos with defective convergent-extension. However, we note that the neural tube of *lpar6*-AMO-injected embryos is fully closed, whereas neural tube defects are common in embryos with disrupted convergent-extension movements (Wallingford and Harland, 2002). A reduction in the size of the forebrain was also observed in *lpar6*-AMO-injected embryos, as demonstrated by reduced expression of the telencephalic marker *foxg1*. Both dorsal (*emx1* expression) and ventral (*nkx2-1* expression) regions of the telencephalon were affected. We also observed reduced *fgf8* expression and ERK activity (in the ANR and MHB), as well as reduced *rax* and *pax6* expression (in the eyefield). More posterior regions of the brain (*en2* and egr2 expression) were not affected. Reduced *foxg1* expression was dependent upon

Development (2014) doi:10.1242/dev.104901

inhibiting LPAR6 within the ectoderm, indicating that the role of LPAR6 is intrinsic to the developing forebrain. Our results suggest that LPA signalling through LPAR6 is required in the anterior neural ectoderm for telencephalic development.

We observed similar defects in embryos injected with an AMO for ENPP2, a secreted enzyme that synthesises extracellular LPA (Tokumura et al., 2002; Umezu-Goto et al., 2002). *Enpp2* is expressed throughout *Xenopus* development and enriched in the neural plate of neurulae, then the anterior nervous system and neural crest following the completion of neurulation (Massé et al., 2010b). The most obvious defect that we observed following AMO injection was truncation of the anteroposterior axis, which was often accompanied by defects in anterior neural tube closure. Both defects are consistent with a role for ENPP2 in regulating convergent-extension in the neural plate. Further studies will be required to confirm this. We also observed reduced expression of the telencephalic markers *foxg1*, *emx1* and *nkx2-1* in neurulae, indicating that both dorsal and ventral regions of the telencephalon were affected. Both *fgf8* expression and ERK activity was reduced in the anterior neural plate of AMO-injected embryos. In contrast to *lpar6*-AMO-injected embryos, we did not observe reduced expression of the eyefield marker *rax*. Our results are consistent with studies on mice homozygous for mutations in *Enpp2*. E8.5 homozygous mutant embryos display defects in the anterior nervous system, both neural tube defects and reduced expression of anterior neural markers (van Meeteren et al., 2006; Fotopoulou et al., 2010). Reduced expression was described for *Otx2*, *Six3*, *Tcf4* and *Fgf8*, indicating defects in forebrain development (Koike et al., 2011). In contrast to our results in *Xenopus*, expression of the ventral telencephalic marker *Nkx2-1* was not reduced in *Enpp2*$^{-/-}$ mice (Koike et al., 2011). However, we note that reduced expression of *nkx2-1* (but not *foxg1* or *emx1*) in *enpp2*-AMO-injected *Xenopus* embryos was only transient, with expression restored by early tailbud stages (supplementary material Fig. S6). The similarities between the results of loss-of-function studies in *Xenopus* and the mouse suggest a key role for LPA in regulating telencephalon development. Moreover, they suggest that *Xenopus* is an ideal organism for studying these defects. *Xenopus* embryos are accessible at all stages of development and can survive with the vascular defects that kill *Enpp2*$^{-/-}$ mouse embryos by embryonic day (E) 9.5-10.5 (van Meeteren et al., 2006; Fotopoulou et al., 2010).

Functional cooperation between LPAR6 and FGF signalling

The telencephalon is the most anterior region of the vertebrate forebrain and will eventually form the cerebrum, including cerebral hemispheres, olfactory system and basal ganglia. Its development is regulated by numerous signals from organising centres in adjacent regions of the embryo, including the ANR (Hébert and Fishell, 2008; Hoch et al., 2009). This region is a source for a number of FGF signals, including FGF8, and FGF will induce ectopic *foxg1* expression in the anterior neural plate (Shimamura and Rubenstein, 1997; Eagleson and Dempewolf, 2002). Furthermore, *fgf8* mutant zebrafish and mouse embryos display telencephalic defects (Meyers et al., 1998; Shanmugalingam et al., 2000; Walshe and Mason, 2003). In addition, progressively more severe telencephalic defects have been described in mouse embryos with single, double and triple mutations for *Fgfr1*, *Fgfr2* and *Fgfr3* (Paek et al., 2009). These results highlight the importance of FGF signalling for telencephalon development. We note that AMO for both *lpar6* and *enpp2* reduce *fgf8* expression in the ANR of *Xenopus* neurulae and dpERK in the anterior neural plate, demonstrating reduced FGF signalling in the presumptive telencephalon. This suggests an explanation for our results in which LPA signalling is required for *fgf8* expression in the ANR, with reduced expression of *fgf8* being responsible for defects in telencephalic development. However, *fgf8*-AMO injection experiments in *Xenopus* have failed to detect a role for FGF8 in forebrain development (Fletcher et al., 2006). This might reflect redundant functions among different FGFs, as both *fgf3* and *fgf8* are required for telencephalon development in zebrafish (Walshe and Mason, 2003).

A link between LPAR6 and FGF signalling in anterior neural development was also demonstrated in experiments in which we inhibited both pathways. dnFGFR1 disrupts the development of the trunk and tail of *Xenopus* embryos but has very little effect on the head (Amaya et al., 1991; Isaacs et al., 1998). Yet, when combined with inhibition of LPAR6 we observed a dramatic reduction in head development, far greater than the forebrain defects observed by inhibiting LPAR6 alone. We observed the same effect when FGF signalling was inhibited by SU5402, a small molecule inhibitor of FGF receptors (Mohammadi et al., 1997). Phenotypic enhancement, as observed here, is usually an indicator of genetic interaction and probably reflects a degree of functional redundancy. We suggest that LPAR6 and FGF signalling are required throughout the developing brain but only the telencephalon is sensitive to reductions in signalling by LPAR6 alone. Only by inhibiting both pathways is a broader role in brain development revealed. Further studies are required to determine the level at which these signalling pathways interact, but ERK1/2 is a potential candidate. ERK1/2 is a key component of the canonical FGF signalling pathway (Dorey and Amaya, 2010; Pownall and Isaacs, 2010) and also a target of LPAR6 signalling (Lee et al., 2009). Although activation of ERK1/2 in early *Xenopus* embryos is predominantly FGF dependent (LaBonne and Whitman, 1997; Christen and Slack, 1999) a degree of LPAR6 dependency cannot be excluded. How LPA signalling regulates ERK activity in *Xenopus* embryos needs to be explored but it might involve the G protein $G\alpha_i$, as LPA-induced ERK activation in hBRIE 308i cells was blocked by the $G\alpha_i$ inhibitor pertussis toxin (Lee et al., 2009). An alternative mechanism is suggested by a study on hair follicle development in mice, which showed that LPAR6 acts through $G\alpha_{13}$ to stimulate TNFα converting enzyme (TACE)-mediated ectodomain shedding of TGFα (Inoue et al., 2011). TGFα stimulates ERK activity via the Epidermal growth factor (EGF) receptor. It is of interest that AMO for the EGF-like receptor ERBB4 generates posterior defects similar to those caused by dnFGFR1, defects rescued by increasing ERK activity (Nie and Chang, 2007).

Conclusions

Our results show that LPA signalling, acting through the LPAR6 receptor, is required in the initial specification and/or maintenance of the telencephalon, the most anterior region of the vertebrate brain. This is only the second LPAR receptor, after LPAR1 (Estivill-Torrús et al., 2008), that has been shown to have a role in early neural development, even though multiple receptor subtypes are expressed in the developing nervous system (Ohuchi et al., 2008; Massé et al., 2010a). The cellular and molecular basis of this role will require further studies, as will identifying the source of the LPA signals. Previous studies have shown that LPAR6 is required for hair growth in humans, but no evidence for a role in brain function was obtained. Either LPAR6 has evolved different roles in amphibians and mammals, or functional redundancy among the different LPA receptor subtypes masks the role of LPAR6 in mammalian forebrain development.

Development (2014) doi:10.1242/dev.104901

MATERIALS AND METHODS

Embryo methods

Xenopus laevis embryos were injected at either the two, four or eight-cell stage and incubated at 14-18ºC. Animal caps were isolated at stage 8 and cultured in 50% Normal Amphibian Medium containing either 10 u/ml of *Xenopus* FGF4 (Isaacs et al., 1992) or 10 u/ml human Activin A (Sigma). Stage 10.5 DMZ and animal cap explants were grafted together and cultured until stage 18. Embryos were incubated in 10 µM SU5402 (Calbiochem) from stage 9 to stage 16. Whole-mount *in situ* hybridisation was performed using digoxigenin-labelled antisense probes (Sive et al., 2000). Immunostaining was performed using anti-diphospho-ERK (Sigma) (Christen and Slack, 1999). Animal procedures were performed under license, as required by the Animals (Scientific Procedures) Act 1986 (UK).

Sequences and AMO

EST for *Xenopus laevis lpar6* (IMAGE:3420557; Accession number BG552112) and *enpp2* (IMAGE:5570505; Accession number BQ736035) were purchased from Geneservice (Cambridge, UK) and sequenced. The Accession number for *lpar6* is HF558446, and *enpp2* is identical to NM_001087057. *Lpar6* was inserted into pCS2^{+} and *enpp2* into pCS2^{+}myc. AMO for *lpar6* (AMO1, 5′-TACCATTGCTTGTTATCGTGTCTAA-3′; AMO2, 5′-TTCCTTGAGCGTTACTACCATTGC-3′), *ennp2* (5′-CTGAATCCGTTTTTCATTGCCATAG-3′), and standard control (5′-CTGAATCCGTTTTTCATTGCCATAG-3′) were purchased from Gene Tools. *Lpar6* AMO were tested by adding 1 µg to an SP6 TNT coupled reticulocyte lysate cell free translation system (Promega), loaded with 1 µg of *lpar6* cDNA and ^{35}S-methionine (GE Healthcare). Products were separated on NuPAGE 4-12% bis-Tris gels (Invitrogen) and exposed to autoradiographic film (GE Healthcare). *Enpp2* AMO was tested by co-injecting *Xenopus* embryos with 40 ng of AMO and 1 µg of *enpp2.myc* mRNA. Proteins were isolated at stage 9 and ENPP2 detected by western blot using 9E10 monoclonal antibody (Santa Cruz Biotechnology) (Geach and Dale, 2008). Capped mRNA for human LPAR6 (Janssens et al., 1997), ENPP2.myc, dnFGFR1 (Amaya et al., 1991) and dnFGFR4 (Hongo et al., 1999) were transcribed using the SP6 mMessage mMachine kit (Ambion).

RT-PCR

Total RNA was purified using RNeasy columns (Qiagen) and cDNA synthesised using ImProm II reverse transcriptase and random primers (Promega). PCR was performed with Taq polymerase (New England Biolabs), using 0.5 µl of cDNA in a final volume of 25 µl for 26-28 cycles. Primers (5′-3′) used were: *egr2* (CCGGCCCATCCTCAGACCCAGAAA and CGCC-ACGCCGCTGTTGCCGAGTTC), *en2* (ATGAGCAGAATAACAGGGA-AGTGGA and CCTCGGGGACATTGACTCGGTGGTG), *foxg1* (AAAG-TGGACGGCAAAGACGGTG and CCAATGAACACATCGTCGCTGC), *lpar6* (AGCATCTATCACCAGCAGCAGG and TGCCGCAACCTTACTG-AGACAG), *myod1* (AGGTCCAACTGCTCCGACGGCATGAA and AGGAGAGAATCCAGTTGATGGAAACA), *odc* (CAGCTAGCTGTGGT-GTGG and CAACATGGAAACTCACACC), *otx2* (GGATGGATTTGT-TGCACCAGTC and CACTCTCCGAGCTCACTTCTC), *rax* (AGACTG-GTGGCTATGGAG and ATACCTGCACCCTGACTT), *sox2* (GAGG-ATGGACACTTATGCCCAC and GGACATGCTGTAGGTAGGCGA).

Microarray assays

Microarray data were derived from published data sets (Branney et al., 2009). Affymetrix Cel files are available at EMBL ArrayExpress, accession numbers E-MEXP-2058 and E-MEXP-2059.

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Competing interests

The authors declare no competing financial interests.

Author contributions

The project was designed by T.J.G., C.D., H.V.I. and L.D. Experiments were performed in the laboratories of L.D. and H.V.I. All authors contributed to the figures presented and interpreted the results. L.D., H.V.I. and T.J.G. wrote the manuscript, with remaining authors commenting on the manuscript.

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Supplementary material

Supplementary material available online at
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