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Glypicans are differentially expressed during patterning and neurogenesis of early mouse brain

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Abstract

Glypicans are essential modulators of cell signalling during embryogenesis. Little is known about their functions in brain development. We show here that mouse *glypicans* (*gpc-1* to *gpc-6*) are differentially expressed in embryonic brains during key morphogenetic events. In gastrulating embryos, *gpc-4* is the only *glypican* expressed in anterior visceral endoderm. During neural tube closure, *gpc-4* transcripts are restricted to the anterior neural ridge and telencephalon. At this stage, *gpc-1* expression shifts from trunk and head mesenchyme to neural tube. *Gpc-3 mRNA* appears across the ventral neural tube, then in the lamina terminalis and hypothalamus. *Gpc-2* and *gpc-6* transcripts are in all brain compartments. *Gpc-5* is found in ventral brains as neurogenesis starts. Onset of neurogenesis also coincides with differential expression of *glypican* genes either in neural progenitors or in differentiating neurons. The novel expression sites of *glypicans* shown here contribute to the identification of signalling molecules involved in brain patterning.

Keywords: Glypicans; HSPGs; Cell signalling; Signalling modulators; Morphogens; Brain development; Anterior visceral endoderm; Neural progenitors

Glypicans are heparan sulphate proteoglycans (HSPGs) that are linked to the cell surface through a glycosyl-phosphatidylinositol (GPI) anchor (reviewed in [1,2]). To date, six *glypican* genes have been identified in mammals (*gpc-1* to *gpc-6*). In addition to the GPI modification, mammalian glypicans share a significantly conserved amino acid sequence and contain two to four consensus sites for the attachment of heparan sulphate glycosaminoglycan (HS-GAG) chains [2]. Biochemical and genetic studies have shown that Glypicans play important roles in modulating cell–cell signalling interactions. For example, they regulate the action of extracellular signals such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), Wnts, Hedgehog (HHs) and HGF [1,3–5]. It has been proposed that Glypicans function by promoting and/or stabilizing the interaction of these molecules with their cognate high affinity receptors [2] or by shaping their activity gradients [6]. Consistent with this, mutations in *Drosophila*, zebrafish and mouse *glypican* genes generate phenotypes reminiscent of loss-of-function mutations in Wnts, BMPs and HH [4,7,8].

Glypicans are predominantly expressed during morphogenesis of different embryonic structures such as the mammalian brain [2]. For example, neural precursors in the ventricular zone of the telencephalic vesicles express the *gpc-1* [9], *gpc-4* (this study, [3,10] and *gpc-6* genes [11]. Neural precursors of the striatum primordium express *gpc-5* [12]. *Gpc-1* and *gpc-5* transcripts are also found in post-mitotic neurons during differentiation and in adult brains. Post-mitotic neurons also express *gpc-2*, but the timing of its expression correlates with that of neuronal migration and axonal growth [13,14]. These studies show the expression of most

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glypican genes during brain neurogenesis (i.e., beyond embryonic day 11). Ybot-Gonzalez et al. have shown that *gpc-4* exhibits a distinct and dynamic expression pattern in the anterior forebrain neuroepithelium and adjacent tissues at embryonic days (E) 7–10.5, and they suggested its potential involvement in one or more signalling pathways regulating forebrain development [15]. However, it remains to be established whether most *glypicans* are expressed during earlier brain patterning events, which are known to be controlled by their potential interactors [16].

In *Xenopus laevis gpc-4* is highly expressed during neurulation by anterior neural plate cells of the presumptive forebrain [17]. In agreement with this, Gpc-4 acts as positive modulator of FGF signalling during *Xenopus* dorso-ventral forebrain patterning [17]. Here we investigate a possible glypican-signalling requirement during mouse gastrulation and early brain morphogenesis by characterizing and comparing spatial and temporal expression patterns of known *glypican* genes.

Materials and methods

Embryo collection. E6.5 and E7.25 embryos were from (C57BL6×CBA) F1 matings kept under inverted day–night cycles. E8.0 to E10.0 embryos were generated from CD1 mice. Otx-2 heterozygous mutants on C57BL6/CBA background were intercrossed to obtain homozygous. Embryonic days were counted considering midday post-coitum as E0.5. Research was performed according to institutional review body and ethics committee guidelines.

Identification of glypicans cDNA and antisense digoxygenin-labelled RNA. Gpc-1, *gpc-3* and *gpc-6* clones were identified by BLAST search of GenBank EST database using cDNA sequences of the NCBI database (*gpc-1*: Accession No. AF185613; *gpc-3*: Accession No. BC036126; *gpc-6*: Accession No. AF105268). Corresponding EST clones were obtained from RZPD Consortium (www.rzpd.de/cgi-bin/db). *Gpc-1*: RZPD clone ID-IMAGE480151; *gpc-3*: RZPD clone ID-IMAGp998O1010961; *gpc-6*: RZPD clone ID-IMAGp 998I1610907. ESTs clones were confirmed by restriction enzyme digestion and sequence analysis. Digoxygenin RNA probes were synthesized according to the manufacturer's instruction (Roche). Probes were tested by whole mount *in situ* hybridisation on embryos at later developmental stages, whose expression pattern is published. *Gpc-4* cDNA was cloned by RT-PCR of mouse embryonic kidney. Gpc-4 F(5′CTGCTTTCCATCGGGTCTCATTCTG3′) and R(5′GTCAGCTTTCTCGTTGGCACTCTTCC3′). The *gpc-2* and *gpc-5* cDNAs were cloned by RT-PCR of postnatal mouse brains and spinal cords, respectively. The following primers were used: Gpc-2 F(5′GAGCACCAGCACCCCGAGAAGAG3′) and R(5′GTAGCCCCCTTCCACTCCTCCTCA3′); Gpc-5 F(5′CATCAACACCACGGACCACATACAC3′) and R(5′CTCCACAGCCATCTTCGTCATCACAG3′). PCR products were cloned in pGEM-T-EasyVector (Promega).

RNA in situ hybridisation. Whole mount and paraffin sections RNA *in situ* hybridisation was performed as previously described [18]. For sectioning, early streak stage, embryos were embedded in cold glycol methacrylate (Technovit 8100) or in gelatine for cryosections. E6.7 embryos were cut transversally at 10 μm. Vibratome sections of E8.5 embryos were at 100 μm. Paraffin sections of E9.5 and E10.5 embryos were at 8 μm.

Results

We first analysed *glypican* gene expression in gastrulating mouse embryos (E6.5–E7.25) by whole mount *in situ* hybridisation and found that only *gpc-4* was clearly expressed at this developmental stage (Fig. 1A and data not shown). In particular, *gpc-4 mRNA* becomes restricted to the region of the anterior visceral endoderm (AVE) as gastrulation proceeds (arrows in Fig. 1A). Analysis of the distribution of *gpc-4* transcripts in transverse sections of early streak stage embryos confirmed that AVE cells express *gpc-4*, while the epiblast does not (left inset in Fig. 1B). The AVE is the extra embryonic tissue that overlies the epiblast that will form the anterior CNS and surface ectoderm. It is essential for specifying the anterior neural ectoderm and initiating anterior neural plate patterning [19]. The AVE is generated by anterior displacement of distal VE cells to the prospective anterior side of gastrulating embryos [19] and *otx-2* is one transcription factor required for this process [20]. In agreement with this, AVE markers such as *hex* and *cer1* remain distal in *otx-2* deficient embryos [20]. Fig. 1B shows that *gpc-4* is also mislocalised to distal visceral endoderm in *otx-2* deficient embryos (see also right inset). Thus, *gpc-4* can be considered as a marker of the AVE at these developmental time points.

We next examined the expression of *glypicans* in mouse embryos at neural plate to two-somite stage (E7.5) and found that the genes are selectively turned on. Expression of *gpc-4* is restricted to the developing anterior embryonic structures and posteriorly from the node (Fig. 2A). *Gpc-3*

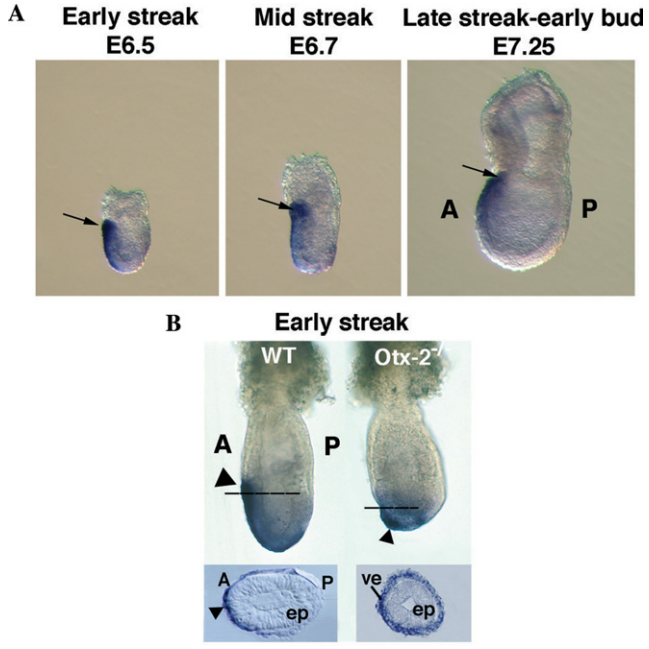

Fig. 1. *Gpc-4* is expressed in the anterior visceral endoderm (AVE). (A) Spatial and temporal distribution of *gpc-4* transcripts during early gastrulation. Arrows point to *gpc-4* in the AVE. Anterior (A) is left, posterior (P) is right. (B) Expression of *gpc-4* at advanced early streak wild type and Otx-$2^{-/-}$ embryos. The broken line indicates the level of sections in the insets of similar embryos. The left inset shows *gpc-4* expression strongest anteriorly in the AVE (arrowhead) and not in the epiblast (ep). In Otx-$2^{-/-}$ embryos the AVE has not moved anteriorly and *gpc-4* is expressed distally (compare arrowhead in WT and Otx-$2^{-/-}$). The right inset shows *gpc-4* expression in the distal visceral endoderm.

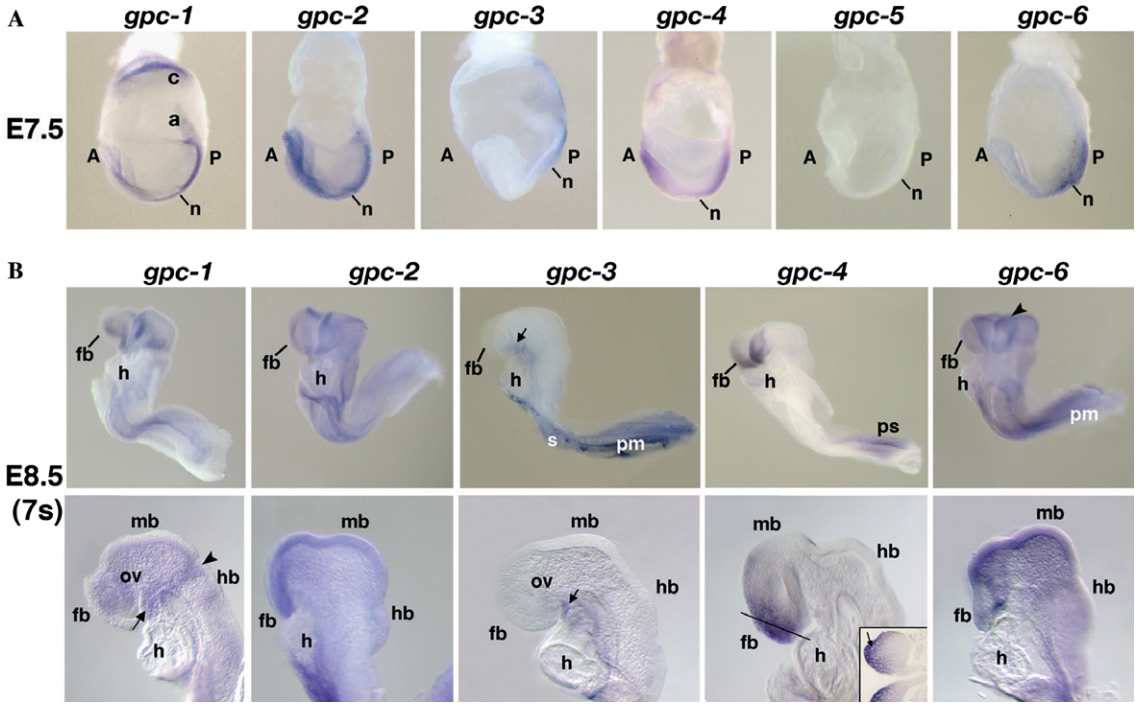

Fig. 2. Expression of *glypicans* at neurula and early somite stages. (A) Expression of *gpc-1* to *gpc-6* at E7.5 (neural plate to two-somite stage), lateral view. *Glypicans* are differentially turned on in both embryonic and extraembryonic tissues. Extraembryonically *gpc-1* is in the chorion (c) and allantois (a). (B) Expression of *gpc-1* to *gpc-6* at seven somite stage (E8.5) as revealed by whole mount in situ hybridisation (top panels) or by vibratome sagittal sections at the level of the head region (bottom panels). *Gpc-2* and *gpc-6* are expressed along the entire antero-posterior CNS axis. *Gpc*-1 is not expressed in embryonic brains, except for the optic vesicle neuroectoderm (ov). Note expression in the head and thoracic mesenchyme (arrowhead bottom panel). Arrow in bottom panel: expression in the first branchial arch. *Gpc-3* transcripts are in ventral and posterior endoderm underlying the developing forebrain (arrow top and bottom panels). Other expression sites: presomitic mesoderm (pm), somites (s), lateral plate mesoderm. *Gpc-4* is expressed in forebrain (fb) and in primitive streak (ps). Expression of *gpc-4* in the anterior forebrain encompasses the ANR and responding neural cells (section bottom panel and inset; arrow in inset points to ANR). Line in bottom panel: level of the section in the inset. Note *gpc-6* expression in the head and trunk mesenchyme. *Other abbreviations:* A, anterior; P, posterior; n, node; h, heart; mb, midbrain; hb, hindbrain.

transcripts are present in the primitive streak and in the adjacent extraembryonic region (Fig. 2A). *Gpc-6* expression is most intense in the primitive streak and reduced anteriorly. In contrast, *gpc-1* and *gpc-2* transcripts are more uniformly distributed embryonically, with *gpc-1* additionally expressed extraembryonically in the chorion and allantois (Fig. 2A). *Gpc-5* is not expressed at this stage (Fig. 2A).

By two to seven somites (E8.5), *gpc-5* is still not expressed (data not shown). In contrast, *gpc-2* and *gpc-6* are expressed from anterior to posterior in the brain (Fig. 2B). *Gpc-1* is not expressed in the neural tube, except for the optic vesicle neuroectoderm (Fig. 2B bottom panel). Its expression occurs in the head and thoracic mesenchyme underlying the neural tube, with a sharp boundary in the region rostral to the hindbrain (Fig. 2B arrowhead in bottom panel). *Gpc-3* transcripts are not found in the neural tube, but start to be selectively present in endodermal tissues underlying the developing brain (Fig. 2B arrows in both top and bottom panels). Interestingly, Shh is produced by the prechordal endomesoderm, a signalling centre also ventral to the developing brain [21]. *Gpc-4* becomes further restricted to two distinct domains: anteriorly to the head region and posteriorly caudal to the node (Fig. 2B top panel). In agreement with Ybot-Gonzalez et al. [15], the anterior *gpc-4* expression encompasses the expression domains of *fgf-8* in the anterior neural ridge (ANR) signalling centre (Fig. 2B bottom panel, arrow in inset and Supplementary Fig. 1) and *bf-1* in the anterior neuroectoderm (Fig. 2B bottom panel, inset and Supplementary Fig. 1). Thus, during early brain patterning, *glypican* genes can be simultaneously expressed in cells producing regulatory signals and in cells responding to them.

Expression of *glypican* genes persists during neurogenesis. Shortly after neural tube closure (E9.5) *gpc-2* and *gpc-6* remain widely expressed within the developing brain (Fig. 3, data not shown). At this developmental stage *gpc-1* transcripts become apparent in the developing neural tube (Fig. 3, data not shown). *Gpc-4* transcripts are abundant in the neuroepithelium of the developing telencephalon in both dorsal and ventral regions (Fig. 3), and in the adjacent non-neural ectoderm (Fig. 3 inset bottom panel) and [15]. A novel site of expression appears in the midbrain (Fig. 3 top panel). At this stage, G*pc-3* transcripts appear across the ventral neural tube, and are most abundant in the developing diencephalon (Fig. 3 bottom panel). These structures correspond to ventral brain regions whose pat-

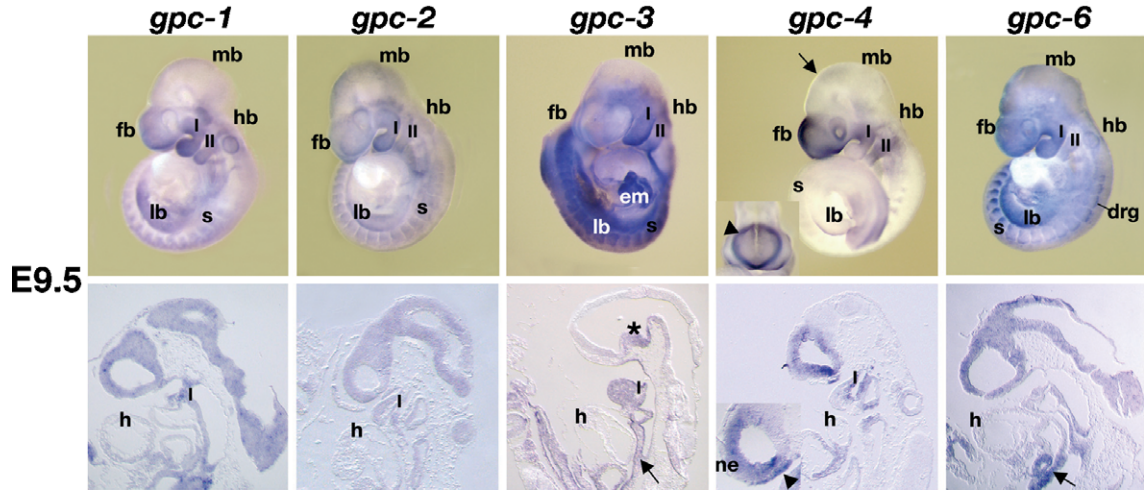

Fig. 3. Expression of *glypicans* after neural tube closure at E9.5. Top panels: whole mount in situ hybridisation using *gpc-1* to *gpc-6* probes; bottom panels: in situ hybridisation on paraffin sections using *gpc-1* to *gpc-6* probes. *Gpc-1*, *gpc-2* and *gpc-6* are widely expressed within the developing brain. Other expression domains are the first and second branchial arches (I and II), limb buds (lb), somites (s). *Gpc-6* is also present in dorsal root ganglia (drg), gut (arrow bottom panel) and at lower levels in head and body mesenchyme (bottom panel see also [11]. *Gpc-3* transcripts span from the ventral forebrain (fb) to the anterior hindbrain (hb; asterisks bottom panel and inset; ne, neuroectoderm). *Gpc-3* transcripts in somites (s), developing limb buds (lb), branchial arches (I and II) and developing gut (top panel and arrow bottom panel). *Gpc-4* transcripts are abundant in dorsal and ventral neuroepithelium of the developing telencephalon. Inset: *gpc-4* expression in the neuroectoderm at E9.5 (arrowhead). A novel site of expression appears in the midbrain (mb; arrow top panel). Inset in bottom panel shows *gpc-4* expression in sagittal section at the level of forebrain. Note *gpc-4* expression in the neuroectoderm (ne) and adjacent ectodermal tissue (arrowhead). Other domains of *gpc-4* expression: proximal region of first and second branchial arches, heart (h), somites, limb buds and tail bud (asterisk top panel). *Other abbreviations:* em, extraembryonic membrane.

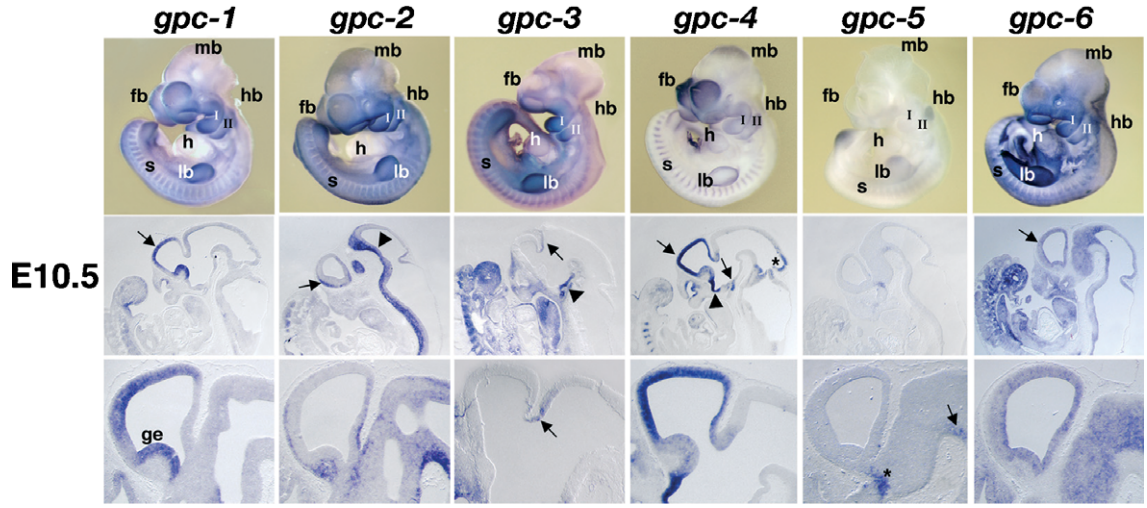

Fig. 4. Expression of *gpc-1* to *gpc-6* at E10.5. Whole mount in situ hybridisation (top panels) and on paraffin sagittal sections. Bottom panels: enlargement at the level of fore-midbrains. *Gpc-1* expression in the developing dorso-anterior telencephalon (arrow middle panel) and ventral telencephalic ganglionic eminences (ge). *Gpc-2* is expressed in differentiating neurons (arrow and arrowhead middle and bottom panels). *Gpc-3* transcripts are at the telencephalic–diencephalic junction. Parasagittal sections show *gpc-3* in lamina terminalis (arrow middle and bottom panels) and hypothalamus (arrowhead middle panel). *Gpc-4* remains expressed in the telencephalon (top panel and arrow middle panel) and midbrain (top panel and asterisk middle panel). Arrowhead in bottom panel points to *gpc-4* in hypothalamus and adjacent to the optic stalk. *Gpc-5* is expressed in the striatum primordium and ventral diencephalic wall (middle panel; arrow and arrowhead bottom panel, respectively). *Gpc-5* is also present in the developing limb bud (lb). Expression of *gpc-6* in the brain is similar to E9.5. Arrow in the middle *gpc-6* panel points to forebrain. *Gpc-1*, *gpc-2*, *gpc-3*, *gpc-4* and *gpc-6* are also expressed in limb buds (lb), first and second branchial arches (I and II), somites (s) mandibular and maxillary processes. *Other abbreviations:* fb, forebrain; mb, midbrain; hb, hindbrain; h, heart.

terning and regionalization is controlled by FGFs and Shh [21].

One day later (E10.5), expression of *gpc-6* in the CNS remains unchanged whereas that of *gpc-1* becomes highest in the developing dorso-anterior telencephalon (Fig. 4 arrow middle panel) and ventral telencephalic ganglionic eminences (Fig. 4 middle and bottom panel). Interestingly, *gpc-2* transcripts are restricted to most differentiated neural

cell types of E10.5 embryos (Fig. 4 arrow and arrowhead middle and bottom panel). This is in agreement with expression of *gpc-2* in post-mitotic neurons of older mouse embryos [13]. *Gpc-5* transcripts appear in the striatum primordium and ventral diencephalic wall (Fig. 4 bottom panels asterisk and arrowhead respectively; [12] and *gpc-4* remains in the developing telencephalon (Fig. 4 and arrow middle panel) and [15]). Other sites of its expression are the hypothalamus and the adjacent optic stalk (Fig. 4 arrowhead middle panel). *Gpc-1* and *gpc-4* expression in the telencephalic neuroepithelium partially overlaps the distribution of signalling molecule receptors such as *fgfr1* (Supplementary Fig. 1; [22]). *Gpc-3* expression covers the lamina terminalis and hypothalamus (Fig. 4).

Discussion

The *glypican* expression pattern described here shows that these signalling modulators are expressed in a dynamic and developmental stage specific manner. Loss-of-function mutations of *gpc-2* and *gpc-3* in mice resulted in no brain phenotype [23]. We show here that while *glypicans*, such as *gpc-3*, *gpc-4* and *gpc-5*, have specific expression patterns, others like *gpc-1*, *gpc-2* and *gpc-6* are more widely distributed. Therefore, it is possible that lack of one *glypican* gene is functionally compensated by others. Thus, our studies can indicate which compound mutants should be generated to give insight into glypican function in developing brain. Mutations in *gpc-3* and *gpc-4* genes are associated to the human congenital disorder *Simpson–Golabi–Behmel* syndrome (SGBS; [24]). The SGBS syndrome is characterized by general pre- and postnatal overgrowth [24]. A fraction of SGBS patients also show mental retardation, seizures and hydrocephaly [24,25]. It is important to note that expression domains of *gpc-3* and *gpc-4* also correlate with regions that play a role in the aetiology of the SGBS [26]. Therefore, combined loss-of-function mutations of these *glypicans* may also result in animal models to study human brain syndromes.

Our studies revealed that in some tissues sites of glypican expression coincide with the presence of signalling pathways that glypicans are known to be capable of modulating. For example they are progressively found in some signalling centres important for specification and patterning of the developing brain. *Gpc-3* is expressed in the middle region of telencephalon and diencephalon that is a source of BMP and Wnt signals [27]. *Gpc-4* is in the AVE, ANR and in the anterior neuroectodermal cells adjacent to the ANR. The AVE is a source of Wnt, BMP and signal antagonists [28,29]. The ANR is the most rostral junction between neural and non-neural ectoderm. It forms a key-signalling centre that regulates forebrain patterning [21], and produces signals such as FGFs and Wnt inhibitors [30,31]. Embryological studies have shown that Gpc-4 modulates FGF signalling during forebrain patterning in *Xenopus* embryos [17]. Moreover, the *Drosophila* glypican gene Dally controls cell division in the larva brain by modulating morphogens [32]. Interestingly, *glypicans* are also expressed during development of other embryonic structures, such as limb bud, kidney and *Drosophila* imaginal disc, where they are known to regulate the action of different signalling molecules [4,8]. Analysis of glypican functions by gene targeting in mouse will reveal their requirement during brain morphogenesis. Thus our study, by showing novel sites of *glypican* gene expression during key brain patterning events, may provide insights into the analysis of mechanisms for tight control of morphogenetic events.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.185

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