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DISTINCT BUT REDUNDANT EXPRESSION OF THE *Frizzled* Wnt RECEPTOR GENES AT SIGNALING CENTERS OF THE DEVELOPING MOUSE BRAIN

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Abstract—The establishment of the regional subdivisions of the vertebrate CNS is accomplished through the activity of different neuroepithelial organizing centers. The wingless/int (Wnt) family of secreted glycoproteins, among other factors, plays a crucial role in signaling from these centers. Wnt1 secreted from the boundary between the mid- and hindbrain, for instance, controls the development of this brain region and of associated neuronal populations. Different Wnts secreted from the caudomedial pallium, the cortical hem, pattern the adjacent hippocampal field. The first step in Wnt signal transduction is binding of the Wnt ligand to its receptors, the seven-pass transmembrane Frizzled proteins. Inactivation of different *Frizzled* genes in mice have revealed an extensive functional redundancy between these receptors. In order to discriminate between a possible participation of different Frizzled receptors in the transduction of Wnt signals at the mid-/hindbrain boundary and the cortical hem, we have performed a detailed expression study of the 10 known murine *Frizzled* genes at crucial stages of mouse embryonic development. Our analysis reveals a highly dynamic yet distinct expression pattern of individual *Frizzled* genes in the anterior neural tube of the developing mouse embryo. The overlapping spatio-temporal expression of at least two and up to six *Frizzled* genes in any region of the developing mouse brain, however, also suggests a vast functional redundancy of the murine Frizzled receptors. This redundancy has to be taken into consideration for future analyses of Frizzled receptor function at these signaling centers in the mouse.

Key words: Fzd, mid-/hindbrain boundary, isthmic organizer, cortical hem.

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Abbreviations: Acc., accession; A/P, anterior–posterior; bp, base pairs; CRD, cysteine-rich domain; D/V, dorsal–ventral; E, embryonic day; Fgf, fibroblast growth factor; Fzd, Frizzled (receptor); hFZD, human Frizzled; ISH, *in situ* hybridization; IsO, isthmic organizer; LOF, loss-of-function; Lrp, low-density-lipoprotein-related protein; mFzd, mouse Frizzled (receptor); MHB, mid-/hindbrain boundary; MZ, marginal zone; PBS, phosphate-buffered saline; PCR, polymerase-chain-reaction; Ror2/Ryk, orphan tyrosine kinase-related receptors; r1, rhombomere 1; SVZ, subventricular zone; VZ, ventricular zone; Wnt, wingless/int (protein); ZLI, zona limitans intrathalamica.

During the development of the vertebrate CNS, the relatively simple and two-dimensional neural plate is patterned into the numerous three-dimensional morphological and functional subdivisions of the neural tube. The establishment of these regional subdivisions is orchestrated by different organizing centers, which are characterized by the release of signaling molecules and the concomitant localized expression of distinct transcription factors (reviewed by Wurst and Bally-Cuif, 2001; Echevarria et al., 2003). In early neural development, a prominent signaling center is established at the junction of the midbrain and the hindbrain, also called the mid-/hindbrain boundary (MHB) or isthmic organizer (IsO). Several transcription and secreted factors are expressed in specific patterns at the MHB (reviewed by Wurst and Bally-Cuif, 2001; Prakash and Wurst, 2004). Among these secreted factors is *Wnt1*, which is expressed in a ring encircling the neural tube at the rostral border of the MHB in the caudal midbrain, as well as in the dorsal and ventral midline of the mesencephalon (Wilkinson et al., 1987; Davis and Joyner, 1988; Parr et al., 1993). Loss-of-function (LOF) experiments have demonstrated that wingless/int (Wnt) 1 signaling from the MHB is crucial for the development of the posterior midbrain and anterior hindbrain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992; Danielian and McMahon, 1996; Lee et al., 1997). More recent data have shown that Wnt1 controls the proliferation of neural precursors in the dorsal midbrain and the specification of dopaminergic neurons in the ventral midbrain (Panhuysen et al., 2004; Prakash et al., 2006). At later stages of neural development, Wnt signaling is implicated in the development of the dorsomedial telencephalon (reviewed by Sur and Rubenstein, 2005; Mallamaci and Stoykova, 2006). Based on the specific expression of several *Wnt* genes (including *Wnt2b*, *Wnt3a*, *Wnt5a*, *Wnt7b* and *Wnt8b*) (Grove et al., 1998; Lee et al., 2000; Garda et al., 2002) at the dorsomedial margin of the telencephalic hemispheres, this region was postulated as a signaling center and termed “cortical hem” (Grove et al., 1998). Subsequent LOF studies have indeed confirmed the prominent role of Wnt signaling from the cortical hem in the development of the adjacent hippocampus and choroid plexus (Galceran et al., 2000; Lee et al., 2000; Machon et al., 2003).

Wnts are secreted lipid-modified glycoproteins involved in many aspects of CNS development, including neural induction, patterning, cell fate specification, cell proliferation, migration, axon guidance, synaptogenesis and in the maintenance of the adult stem cell niche (reviewed by

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Cadigan and Nusse, 1997; Stern, 2001; Ciani and Salinas, 2005; Bovolenta et al., 2006). Wnt signals are transduced by at least three different intracellular signaling pathways, known as the canonical Wnt/β-catenin pathway, the Wnt/Ca^{2+} pathway and the planar cell polarity (PCP) pathway (reviewed by Cadigan and Liu, 2006; Gordon and Nusse, 2006). Common to all three pathways is the binding of the secreted Wnt ligands to the Frizzled (Fzd) receptors. So far, 10 genes encoding the Fzd receptors have been identified in mammals (reviewed by Huang and Klein, 2004). The Fzd proteins share a common structure with an amino-terminal signal peptide, an extracellular cysteine-rich domain (CRD) and seven hydrophobic transmembrane domains (Bhanot et al., 1996; Wang et al., 1996; Huang and Klein, 2004). The CRD consists of 120–125 amino acids with 10 conserved cysteines, all of which form disulfide bonds, and is required for binding of the Wnt ligands (Bhanot et al., 1996; Dann et al., 2001). The carboxy-terminal part is not well conserved among the Fzd receptor family except from a highly conserved motif of six amino acids (KTXXXW) located two amino acids after the seventh transmembrane domain. This conserved motif is essential for the activation of the canonical Wnt/β-catenin pathway (Umbhauer et al., 2000).

The specific functions of different Fzd receptors in mammals have been addressed by a number of LOF experiments in the mouse (Ishikawa et al., 2001; Wang et al., 2001, 2002, 2006a; Lyuksyutova et al., 2003; Guo et al., 2004; Huang and Klein, 2004; Hsieh et al., 2005; Ranheim et al., 2005; Zhao et al., 2005). The phenotypes reported for the *Fzd* mutants appear to be relatively mild compared with the phenotypes of the Wnt/Wnt signaling LOF mutants. Furthermore, the inactivation of a specific *Fzd* receptor gene has so far not phenocopied the inactivation of a specific Wnt ligand. These findings suggest an extensive functional redundancy between the different Fzd receptors in the mouse, similar to the redundant functions of the *Drosophila* Fzd1 and Fzd2 receptors (Bhat, 1998; Bhanot et al., 1999; Chen and Struhl, 1999). The interaction of the Wnt ligands with their Fzd receptors is further complicated by the presence of co-receptors like the low-density-lipoprotein-related proteins (Lrp) 5/6, the Kremen 1/2 proteins and the orphan tyrosine kinase-related receptors Ryk and Ror1/2 (Tamai et al., 2000; Mao et al., 2002; Oishi et al., 2003; Bovolenta et al., 2006). Wnt inhibitors such as Dickkopf (Dkk) and soluble Fzd-related proteins (Sfrp) additionally modulate the outcome of Wnt signaling in the vertebrate embryo (Rattner et al., 1997; Glinka et al., 1998).

The functional redundancy of Fzd receptors also relies on the overlapping expression of the corresponding *Fzd* genes. Such spatial redundancies have already been described for the expression of some murine *Fzd* genes during somitogenesis, in telencephalic development and at later stages of mouse embryogenesis (Wang et al., 1996; Borello et al., 1999; Kim et al., 2001). To predict distinct or redundant functions of the mouse (m) Frizzled receptors (mFzd) at important developmental stages, however, a precise analysis of the expression patterns of all 10 *mFzd* genes needs to be performed. Therefore, we focused our *mFzd* expression study on three crucial stages of Wnt function during mouse embryonic brain development. These are firstly, the patterning of adjacent mesencephalic and rhombencephalic tissues by signals from the IsO or MHB (including Wnt1) around embryonic days (E) 9.5 and E10.5 (McMahon and Bradley, 1990; Parr et al., 1993; Wurst and Bally-Cuif, 2001; Prakash and Wurst, 2004), and secondly, the patterning of the dorsomedial telencephalon or medial pallium by signals emanating from the cortical hem (including Wnt3a, Wnt2b, Wnt5a, Wnt7b and Wnt8b) around E12.5 (Parr et al., 1993; Grove et al., 1998; Garda et al., 2002; Sur and Rubenstein, 2005; Mallamaci and Stoykova, 2006). In order to detect even low to very low expression levels of the 10 *mFzd* genes, a more sensitive method by means of radioactive *in situ* hybridization (ISH) was used in the present work.

EXPERIMENTAL PROCEDURES

Animals

Outbred CD-1 mice were purchased from Charles River (Kisslegg, Germany). Animal treatment was conducted under federal guidelines for the use and care of laboratory animals and was approved by the GSF Institutional Animal Care and Use Committee. The number of animals used was kept to a minimum, and animals were killed by cervical dislocation to minimize their suffering. Collection of embryonic stages was done from timed-pregnant females as indicated in the text. Noon of the day of vaginal plug detection was designated E0.5. Embryos were staged according to Theiler (1989). At least three embryos of each stage were analyzed for each riboprobe.

ISH

Embryos were dissected in ice-cold phosphate-buffered saline (PBS), pH 7.4, and immersion-fixed overnight in 4% paraformaldehyde in PBS. Embryos were dehydrated in a graded series of ethanol (70%, 96%, 100%), cleared in xylene and embedded in paraffin. Embryos were sectioned at 8 μm in sagittal or coronal planes at the level of the midbrain or forebrain. Serial sections were processed for radioactive (^{35}S) ISH according to a modified version of Dagerlind et al. (1992). A detailed version of the protocol is given at http://www.empress.har.mrc.ac.uk/EMPReSS/servlet/EMPReSS.Frameset. Sections were counterstained with Cresyl Violet (0.5%, Sigma, Munich, Germany) according to standard procedures.

Riboprobes

Gene-specific primers were designed for each mouse (m) *Fzd* cDNA and polymerase-chain-reaction (PCR) amplification was performed using a cDNA that was reverse transcribed from mouse E10.5 total RNA according to the manufacturer's instructions (Advantage RT-for-PCR Kit, Clontech, Mountain View, CA, USA). Primer sequences and amplification conditions are available upon request. All *mFzd* probes were located in the 3′ part of the coding sequence. The *mFzd* cDNA fragments used were: *mFzd1* (base pairs (bp) 1236–2205, accession (Acc.) No. NM_021457; Borello et al., 1999), *mFzd2* (bp 959–1915, Acc. No. NM_020510; Malik and Shivdasani, 2000), *mFzd3* (bp 866–2050, Acc. No. NM_021458; Wang et al., 1996), *mFzd4* (bp 1161–2161, Acc. No. NM_008055; Wang et al., 1996), *mFzd5* (bp 1466–2405, Acc. No. NM_022721; Wang et al., 1996), *mFzd6* (bp 1436–2605, Acc. No. NM_008056;