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# Frizzled-10 promotes sensory neuron development in *Xenopus* embryos

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ABSTRACT

Formation of the vertebrate nervous system requires coordinated cell–cell interactions, intracellular signalling events, gene transcription, and morphogenetic cell movements. Wnt signalling has been involved in regulating a wide variety of biological processes such as embryonic patterning, cell proliferation, cell polarity, motility, and the specification of cell fate. Wnt ligands associate with their receptors, members of the frizzled family (Fz). In *Xenopus*, five members of the frizzled family are expressed in the early nervous system. We have investigated the role of *Xenopus* frizzled-10 (Fz10) in neural development. We show that Fz10 is expressed in the dorsal neural ectoderm and neural folds in the region where primary sensory neurons develop. Fz10 mediates canonical Wnt signalling and interacts with Wnt1 and Wnt8 but not Wnt3a as shown in synergy assays. We find that Fz10 is required for the late stages of sensory neuron differentiation. Overexpression of Fz10 in *Xenopus* leads to an increase in the number of sensory neurons. Loss of Fz10 function using morpholinos inhibits the development of sensory neurons in *Xenopus* at later stages of neurogenesis and this can be rescued by co-injection of modified Fz10B and β-catenin. In mouse P19 cells induced by retinoic acid to undergo neural differentiation, overexpression of *Xenopus* Fz10 leads to an increase in the number of neurons generated while siRNA knockdown of endogenous mouse Fz10 inhibits neurogenesis. Thus we propose Fz10 mediates Wnt1 signalling to determine sensory neural differentiation in *Xenopus* in vivo and in mouse cell culture.

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## Introduction

Formation of the vertebrate nervous system requires temporal and spatial coordination of multiple processes, including neural induction, cell proliferation, commitment to lineage, regulated cell cycle withdrawal, and neuron-specific gene expression accompanying differentiation. The efforts to understand exactly how these signals work have focused on molecules that can modify patterns of gene expression (Purves et al., 2001; Seo et al., 2005b).

During neurulation, the neural plate mediolateral axis will later form the dorso–ventral axis along which will occur the diversification of the major neural cell types: Floor plate cells at the ventral midline, motor neurons at the ventrolateral position, and ventral interneurons at more dorsal locations. Cells in the dorsal regions give rise to neural crest cells and to roof plate cells and dorsal sensory neurons.

The process of neural development involves cooperation between different classes of signals. Neural induction requires a sequence of signals in a specific order. In the dorsal ectoderm, blockade of BMP signalling by BMP antagonists such a noggin, chordin, and follistatin elicits the activation of early pan-neural markers such as Sox2 and Sox3 (Reversade et al., 2005). Following initial neuralization, neuronal differentiation and patterning along the anterior–posterior axis then take place (Papalopulu and Kintner, 1996).

Neurogenesis comprises at least five processes: proliferation of stem cells and progenitor cells, neuronal differentiation, migration, integration into neural circuitary, and survival of cells (Scholzke and Schwaninger, 2007). In *Xenopus*, as with other vertebrates, primary neurogenesis occurs as a cascade of events taking place after expression of pan-neural genes such as Sox2 and N-CAM. The neural progenitors proliferate to ensure a sufficiently large pool of cells is available to generate all the cell types required for the correct development of the nervous system. Subsets of these cells express proneural genes such as XASH3 and Neurogenin, which define domains of neuronal competence within the neural plate and subsequently induce neuronal differentiation genes such as NeuroD (Chitnis and Kintner, 1995; Sasai, 1998; Seo et al., 2005a). In *Xenopus*, primary neurons are important for early tadpole behavior. Committed neural progenitor cells expressing proneural genes arise from the deep layer of the neuroectoderm (Chalmers et al., 2002; Hartenstein, 1989; Seo et al., 2005b). Primary neurons are observed in three longitudinal domains on either side of the dorsal midline and later in a dorso–ventral pattern corresponding to motoneurons, interneurons, and sensory neurons in a ventral to dorsal order. Neurogenesis within these domains is regulated by basic helix-loop-helix (bHLH) proneural genes. The earliest proneural gene expressed is Neurogenin-related-1 (Ngnr-1) which induces later bHLH factors such as NeuroD.

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Ngnr-1 also activates the Z-finger transcription factor MyT1 and the Notch ligand Delta1 stimulating lateral inhibition, which restricts the number of neural cells specified to become neurons. MyT1 renders neuronal precursors resistant to Notch signalling. They then undergo neuronal differentiation, as seen by β-tubulin expression (Bellefroid et al., 1996; Cayuso and Marti, 2005; Lee and Jessell, 1999; Tanabe and Jessell, 1996).

Wnt signalling is important for many aspects of development including neural differentiation (Ciani and Salinas, 2005). Frizzleds are seven-transmembrane domain proteins that act as the receptors for Wnt signalling. In *Xenopus* five frizzleds have been identified as expressed in the nervous system including Fz2, Fz3, Fz4, Fz7, and Fz10 (Deardorff and Klein, 1999; Moriwaki et al., 2000; Shi and Boucaut, 2000; Wheeler and Hoppler, 1999). Fz10 expression is seen in the neural ectoderm and in the neural fold at the early neurula stage. During later neurula stages the neural tube expression increases in the midbrain region (Moriwaki et al., 2000; Wheeler and Hoppler, 1999).

Canonical Wnt signalling is important during neurogenesis. Wnt1 and Wnt3a have been shown in mouse and chick to play an important role in growth control in the neural tube (Dickinson et al., 1994; Ikeya et al., 1997; Megason and McMahon, 2002). In *Xenopus* Xwnt1 and Xwnt3a are expressed in the dorsal neural tube and have been shown to be involved in patterning the neuroectoderm along its dorso–ventral axis (Saint-Jeannet et al., 1997). Overexpression of GSK3β in *Xenopus* neural tube inhibits N-tubulin expression in the most lateral stripe and dominant negative (dn) forms of GSK3β, which mimic a gain of function in canonical signalling, produce an increase in the number of N-tubulin positive cells in the lateral stripe (Marcus et al., 1998). Patterning of the lateral neural plate is mediated by Wnt signals that establish the posteriolateral domain, where dnWnt8 injection blocks Pax3, Krox20, En2 expression, and the development of Rohon-Beard cells (Bang et al., 1999). The function of canonical Wnt signalling in increasing the number of neurons has also been demonstrated in *in vitro* studies (Lyu et al., 2003; Patapoutian and Reichardt, 2000; Smolich and Papkoff, 1994; St-Arnaud et al., 1989; Yang et al., 1998).

The Fz10 expression pattern strongly suggests that it may play important roles in the development of neural tissues, especially in the formation of dorsal neural structures such as sensory neurons (Moriwaki et al., 2000; Wheeler and Hoppler, 1999). We show here that Fz10 is important for neurogenesis during *Xenopus* development and also during retinoic acid (RA) mediated differentiation of mouse P19 cells into neural cells.

## Materials and methods

Note: We previously reported the cloning and expression of *Xenopus* frizzled-9 (Wheeler and Hoppler, 1999). Subsequent sequence analysis of frizzled-9 and frizzled-10 in other species clearly showed *Xenopus* Fz9 to be in fact the orthologue of Fz10B (our unpublished results and Moriwaki et al. (2000)).

### *Embryo manipulations and microinjections*

*Xenopus* laevis eggs were fertilized in vitro and incubated in 0.1X MMR solution and de-jellied using 2% L-cysteine (Fluka). Staging of embryos was according to the normal table of Niewkoop and Faber (Niewkoop, 1994).

Capped RNAs encoding for Fz10B, Wnt1, Wnt8, β-catenin, Wnt3a, Wnt7b, and Fz10B SDM were generated using SP6 mMESSAGE-Mmachine™ kit (Ambion); antisense morpholino oligonucleotides (MO) were synthesized by Gene Tools and tested by *in vitro* translation using the TNT coupled reticulocyte lysate system (Promega). The Fz10B MO used was 5′-GCGGTAACAACTCTCGGCTCC-ATTG-3′. A standard morpholino oligo was injected as control. In vitro site-directed mutagenesis for Fz10B was performed following Stratagene modified protocol, as follows: 5 µl of 10× reaction buffer, 1 µl of WT dsDNA template (5–50 mg), 1 µl forward primer (20 mM; 5-TTGGGCGGCAATGGACC**AC**G**T**GT**C**GTTACCGCGCTCCTTCTC-3), 1 µl reverse primer (20 mM; 5-AACCCGCCGTTACCTGGT**TG**C**A**CA**G**-CAATGGCGCGAGGAAGAG-3), 0.7 µl of dNTP mix (10 mM), 40.8 µl Sigma $H_2O$, and 0.5 µl *Pfu* DNA polymerase. Then the following 18-cycle conditions were followed: 94 °C 30 s, 94 °C 30 s, 55 °C 1 min, 68 °C 9 min. The reaction was digested with 1 µl *Dpn I* (Roche) and incubated at 37 °C for 2 h. The mRNA or MO was co-injected with lacZ (500 pg). Microinjection was carried out in 3% MMR containing 3% Ficoll PM400 (Sigma). One or both blastomeres were injected into the animal pole of two-cell stage embryos or twice into the ventral or dorsal marginal zone of four-cell stage embryos. β-Galactosidase activity was detected by colorimetric reaction using Red-Gal (Sigma) as substrate. Embryos were staged and fixed for 1 h at room temperature in MEMFA. Animal cap experiments were carried out as previously described (Saint-Jeannet et al., 1997). Briefly, embryos were injected at one-cell stage in the animal pole with 500 pg mRNA for noggin alone or in combination with Wnts, frizzleds, and/or MOs at concentrations indicated in the figure legends, incubated in 3% Ficoll, and then transferred to filtered sterilized 1× MMR. At stage 6 embryos were devitellinized and at stage 9 embryos were transferred to 1% FBS/1× MMR and animal caps for each injection were cut then incubated in 0.5× MMR/gentamicin at 18 °C until their siblings reached stage 18. Total RNA was extracted using the RNeasy® Micro Kit (Qiagen, catalog no. 74004). RT-PCR was done as previously described (Abu-Elmagd et al., 2006).

### *In situ hybridization and histology*

Single or double in situ hybridisation was carried out as previously described (Harrison et al., 2004; Tomlinson et al., 2008). Sense and antisense probes were synthesised for each marker. In situ probes used in this study were kind gifts of Prof. Yoshiki Sasai (Sox2), Dr. Malcolm Whitman (N-CAM), Prof. Nancy Papalopulu (N-tubulin, ElrC, and En2), and Dr. Michael Sargent (Slug). For histological examination embryos were fixed in MEMFA for 2 days, washed twice in 0.1% Tween-20/PBS then 70% ethanol for 15 min each, followed by 85% and 95% ethanol for 30 min, three times in 100% ethanol for 30 min each, and twice in 100% xylene for 30 min at room temperature. Then embryos were washed in 1:1 xylene/paraffin for 45 min at 60 °C. Finally embryos were washed three times in 100% Fibrowax (VWR international) at 60 °C and placed in 100% paraffin and allowed to solidify. Sections (15 µm thick) were obtained in a microtome (American Optical Corporation). Frozen sectioning for double in situ embryos was done by incubating embryos in 30% sucrose/filtered sterilized PBS overnight at 4 °C and then washing them twice in OCT (Sakura). The final embedding was done by immersing of OCT capsules in absolute isopropanol and freezing it with liquid nitrogen. Sections (15 µm) were obtained (Cryostat, Leica), rinsed twice with 1× PBS, and mounted with Mowiol 4-8 (Calbiochem).

### *Cell culture*

Mouse embryonic carcinoma cell line P19 was purchased from ATCC. Cells were grown as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen) and antibiotics (100 units/ml penicillin and 100 units/ml streptomycin) (Invitrogen) in a 5% $CO_2$-humidified chamber at 37 °C. Cells were passaged using 0.05% trypsin–EDTA (Invitrogen). P19 cell differentiation was carried out as described (Jones-Villeneuve et al., 1982; McBurney et al., 1982). P19 cells ($1 \times 10^5$) were seeded into non-coated bacteria culture plates with fresh medium in the presence of 1 × 10–6 M all-*trans*-retinoic acid (RA) (Sigma) for 2 days. RA medium was then removed by centrifugation

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