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TGFβ ligands promote the initiation of retinal ganglion cell dendrites *in vitro* and *in vivo*

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Each type of neuron develops a unique morphology critical to its function, but almost all start with the basic plan of one long axon and multiple short, branched dendrites. Though extrinsic signals are known to direct many steps in the development of neuronal structure, little is understood about the initiation of processes, particularly dendrites. We find that *Xenopus* retinal ganglion cells (RGCs) explanted early will extend axons and not dendrites in dissociated cultures. If RGCs develop longer *in vivo* prior to culturing, many now extend dendrite-like processes *in vitro*, suggesting that an extrinsic factor is required to stimulate dendrite initiation. Members of the transforming growth factor beta (TGFβ) superfamily, bone morphogenetic protein 2 (BMP2), and growth and differentiation factor 11 (GDF11), can signal cultured RGCs to form dendrites. Furthermore, TGFβ ligands have an endogenous role: blocking BMP/GDF signaling with a secreted antagonist or inhibitory receptors reduces the number of primary dendrites extended *in vivo*.

Keywords: TGFβ; BMP; GDF; *Xenopus*; Dendrite development

Introduction

Neurons are complex structures designed to receive and integrate many inputs, and to send information over long distances. Often elaborate dendritic arbors accomplish this former task, though the signals directing their development are largely unknown. Typically, several primary dendrites initiate from one side of the cell body, extend in a specific direction, branch extensively, and eventually stop growing and synapse with axon terminals. Recent evidence suggests that similar to axon growth, all of these steps are under the control of both intrinsic and extrinsic mechanisms (reviewed in Goldberg, 2004; Jan and Jan, 2003; McFarlane, 2000a,b).

Many types of neurons when grown in culture will extend a single axon and multiple dendrites, as *in vivo*. In many cases, it is unclear whether this is an intrinsic property of the cell, or if it is a response to factors either added to the media or coming from a glial feeder layer used to promote survival. Certainly, extrinsic factors are necessary for some neurons to extend neurites (i.e. axons and/or dendrites). For instance, when the dependence on growth factors for survival is bypassed by expression of the anti-apoptotic factor Bcl-2, rat retinal ganglion cells (RGCs) grown *in vitro* do not produce any neurites (Goldberg et al., 2002a). Similarly, sympathetic neurons grown without serum or glia will extend axons, but not dendrites in culture (Tropea et al., 1988). Interestingly, treatment with certain bone morphogenetic proteins (BMPs), members of the transforming growth factor beta (TGFβ) superfamily, will induce sympathetic neurons to initiate dendrites *in vitro* (Beck et al., 2001; Guo et al., 1998; Lein et al., 1995). However, an *in vivo* role for BMPs in dendrite initiation has not been described. Furthermore, the extrinsic signals required for RGCs to extend dendrites are unknown. Here we have examined the role of TGFβ family ligands in prompting RGCs to initiate dendrites both *in vitro* and *in vivo*.

A RGC is adapted to transmit visual impulses from the eye to the brain: multiple short branched dendrites receive information from amacrine and bipolar cell processes in the inner plexiform layer (IPL), and a single long axon conducts action potentials through the optic nerve to a target in the brain. Analysis of mice mutant for BMP ligands or receptors have identified critical roles for BMPs in early growth and patterning of the eye and induction of the lens (Dudley et al., 1995; Furuta and Hogan, 1998; Liu et al., 2003; Luo et al., 1995; Murali et al., 2005; Wawersik et al., 1999; Wordinger and Clark, 2007). Growth and differentiation factors (GDFs) are a closely related family of TGFβ ligands, even dimerizing with BMPs in some cases (Butler and Dodd, 2003), and recent studies indicate that GDFs are also important in early eye development (Hanel and Hensey, 2006; Kim et al., 2005). However, we know little about possible later functions for BMP/GDF signaling in the morphological differentiation of retinal neurons, largely because of the severe defects that often arise

Abbreviations: ActRIIB, activin receptor IIB; BMP, bone morphogenetic protein; GCL, ganglion cell layer; GDF, growth and differentiation factor; INL, inner nuclear layer; IPL, inner plexiform layer; NAA, neurofilament associated antigen; ONL, outer nuclear layer; RGC, retinal ganglion cell; TGFβ, transforming growth factor β.

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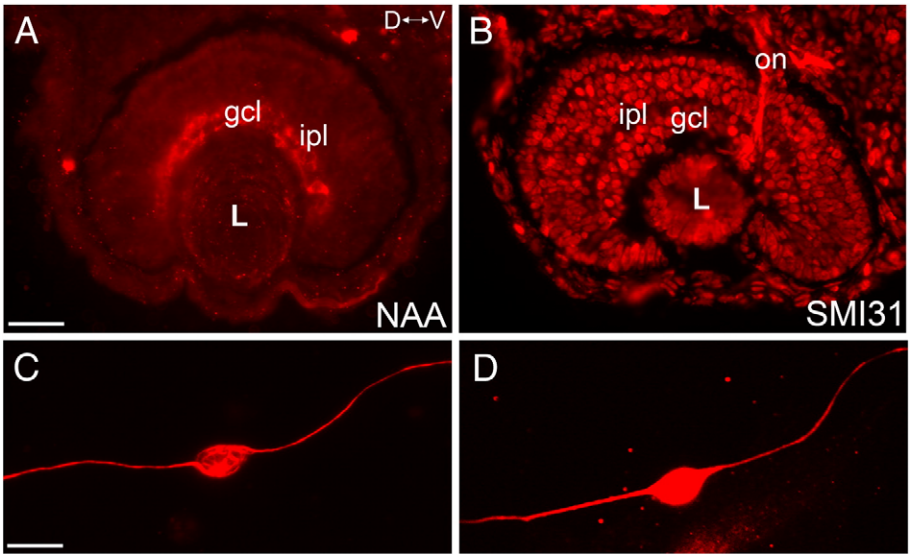

Fig. 1. Cultured *Xenopus* RGCs extend axons but not dendrites. (A, B) Cross-sections of stage 40 *Xenopus* retinas immunolabeled with an antibody against NAA (3A10; A) or SMI31 (B). The NAA antibody specifically labels RGCs and their axons (data not shown) in the retina. SMI31 labels cell bodies of all retinal neurons and the axons of RGCs, which form the optic nerve. (C, D) Cultured *Xenopus* RGCs extending two axons after stage 24 eyebuds were dissected, dissociated, and grown on poly-L-ornithine/laminin coated coverslips. RGCs are immunolabeled with the NAA antibody (C) or SMI31 (D). Scale bar in panel A is 50 μm for panels A, B, and in panel C is 10 μm for panels C, D. D, dorsal; gcl, ganglion cell layer; ipl, inner plexiform layer; L, lens; on, optic nerve; V, ventral.

following the early and complete loss of one or more of these ligands or their receptors. Nonetheless, there is evidence to support such later roles. The addition of BMP2, BMP13, or GDF8 increased neurite number, length, and branching of cultured purified postnatal rat RGCs (Kerrison et al., 2005), and BMP7 promoted axon growth from chicken retinal explants (Carri et al., 1998). Also, ventral RGC axons guide aberrantly within the retinas of BMPRIb null mice (Liu et al., 2003), and the BMP receptor II (BMPRII) is expressed by developing RGCs during the period of early dendritogenesis in frog, chick, and mouse (Belecky-Adams and Adler, 2001; Hocking and McFarlane, 2007; Liu et al., 2003).

We use RGCs in embryos of the frog *Xenopus laevis* as a model to study dendrite growth for several reasons: the visual system is functionally mature within 3 days, *Xenopus* retinal cells can be cultured in a minimal media without additional growth factors, and gene expression can be manipulated within the retina on a single cell level at any stage of development (Chen et al., 2007; Holt et al., 1990; Webber et al., 2005). In this study, we examine dendrite initiation by RGCs, using both *in vitro* and *in vivo* methods. First, we show that RGCs do not extend dendrites by default, but likely require an external signal. Second, BMP2 stimulates RGCs to initiate dendrites *in vitro*, and expression of a BMP antagonist reduces dendrite initiation *in vivo*. Third, another TGFβ ligand, GDF11, promotes RGC dendrite growth in culture, and *gdf11* mRNA is present in the retina. Finally, our data strongly argue that GDF and BMP signals act together to promote dendrite initiation *in vivo*: the inhibition of both BMP and GDF signaling by co-expression of two dominant negative receptors reduced the number of primary dendrites extended by RGCs *in situ*, whereas each receptor alone had no effect. This is one of only a few studies to identify factors required to promote dendrite initiation.

Results

Dendrite initiation is not a default property of Xenopus RGCs

Xenopus RGCs *in vivo* typically extend a single long axon and multiple shorter, branched dendrites (Holt, 1989; Sakaguchi et al., 1984). The first RGCs initiate axons at stage 28, while dendrite growth begins at stage 30/31 (Holt, 1989; Sakaguchi et al., 1984). By stage 40, most RGC axons have reached their target in the tectum, a substantial dendritic arbor has elaborated, and the visual system is functional. How much of the morphological development of a RGC is an intrinsic property of the cell? In order to gain insight into this question, we cultured retinal cells dissociated from stage 24 embryos, when the first RGCs are born and have not yet extended processes (Holt, 1989). The cells were grown at low density in basal media for two days, at which point RGCs in sister embryos would have long axons and substantial dendritic arbors. RGCs were identified on the basis of morphology (i.e. phase bright, large cell body with at least one

Fig. 2. RGCs dissected from later stage embryos extend dendrite-like processes *in vitro*. Eyes from stage 24–35/36 embryos were dissociated and cultured for two days on a poly-L-ornithine/laminin substrate. Cultures were fixed and labeled with NAA or SMI31 antibodies and the presence of dendrite-like processes on isolated RGCs was assessed. (A–D) RGCs dissected from stage 32 embryos extending dendrite-like processes (arrowheads) viewed in phase micrographs (A, C), and with matching images of NAA (B) or SMI31 (D) immunoreactivity. Significant numbers of RGCs dissected from stage 28 and later stage embryos will extend dendrite-like processes (arrowheads) that are identified based on morphology, as seen with phase optics (A, C), and by lack of immunoreactivity for the axonal markers NAA and SMI31 (arrows mark labeled axons in panels B, D). Scale bar in panel A is 10 μm. (E) Graph of the percentage of RGCs *in vitro* that extend dendrite-like processes, as identified by a lack of NAA immunoreactivity, vs. embryo age at time of dissection. n = the number of independent experiments. Numbers in brackets are the total numbers of cells analyzed from each age group. Error bars represent the standard error of the mean (S.E.M.). *$p<0.05$, **$p<0.01$, ***$p<0.001$, One-way ANOVA, Student–Newman–Keuls post hoc test.