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Developing neurites from mouse basal forebrain gonadotropin-releasing hormone neurons use Sonic hedgehog to modulate their growth

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ABSTRACT

Hypothalamic gonadotropin-releasing hormone (GnRH) neurons are required for fertility in all mammalian species studied to date. GnRH neuron cell bodies reside in the basal forebrain, and most extend long neurites in the caudal direction to terminate at the median eminence (ME), the site of hormone secretion.

Using *in vitro* neurite growth assays, histological methods, and genetic deletion strategies in mice we have analysed the role of the morphogen and neurite growth and guidance molecule, Sonic hedgehog (Shh), in the growth of GnRH neurites to their target. Immunohistochemistry revealed that Shh was present in the basal forebrain, the preoptic area (POA) and mediobasal hypothalamus (MBH) at gestational day 14.5 (GD 14.5), a time when GnRH neurites grow towards the ME. Furthermore, *in situ* hybridization revealed that mRNA encoding the Shh receptor, Smoothened (Smo), was present in GnRH neurons from GD 15.5, when the first GnRH neurites are extending towards the MBH. *In vitro* neurite growth assays using hypothalamic explants from GD 15.5 fetuses in 3-D collagen gels showed that Shh was able to significantly stimulate GnRH neurite outgrowth. Finally, genetic deletion of Smo specifically from GnRH neurons *in vivo*, using Cre-*loxP* technology, resulted in a significant decrease in GnRH neurites innervating the ME.

These experiments demonstrate that GnRH neurites use Shh for their neurite development, provide further understanding of the mechanisms by which GnRH nerve terminals arrive at their site of hormone secretion, and identify an additional hypothalamic neuronal population for which Shh/Smo signaling is developmentally important.

1. Background

Gonadotropin-releasing hormone (GnRH) neurons are the main neuronal regulators of fertility (Kalra and Kalra, 1983; Schally et al., 1971). GnRH neurons originate in the olfactory placode (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989), where they can be detected as early as gestational day 9 (GD 9) in the mouse. GnRH neurons then migrate caudally to populate the basal forebrain, and from there extend their neurites towards the median eminence (ME) beginning about GD 12.5 for the earliest arriving cells (Livne et al., 1993; Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Once at the ME, GnRH neurite terminals release GnRH peptide hormone into the pituitary-portal vasculature (Goldsmith and Ganong, 1975). The molecular interactions that modulate GnRH neuron motility, and directed migration have been extensively studied; and a clear picture of the process, as well as its relation to human brain development and infertility mutations is being revealed (Cariboni et al., 2007; Giacobini, 2015; Giacobini et al., 2008; Wierman et al., 2011; Wray, 2010).

The cell bodies of GnRH neurons are scattered along and just lateral to the ventral midline of the basal forebrain, and most extend their processes to the ME along a similar plane (Goldsmith and Ganong, 1975; Gross, 1976; Hoffman et al., 1978). During development, GnRH neurons respond to environmental cues to extend their neurites correctly towards the ME. However, by contrast with studies of GnRH neuron cell body migration, studies describing the growth and guidance of GnRH neurites during development are few (Cronin et al., 2004; Fiorini and Jasoni, 2010; Gibson et al., 2000; Gill et al., 2004; Gill and Tsai, 2006; Low et al., 2012). Basic fibroblast growth factor (FGF2) has been reported to act as a chemoattractant to guide GnRH neurites towards the ME (Gibson et al., 2000; Gill et al., 2004; Gill and Tsai, 2006) and brain-derived neurotrophic factor (BDNF) was shown to promote the growth of GnRH neurites during development (Cronin et al., 2004). More recently, both kisspeptin (Kiss1) and Netrin-1 have been shown to regulate GnRH neurite growth *via* phospholipase C signaling and DCC

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calcium-dependent signaling, respectively (Fiorini and Jasoni, 2010; Low et al., 2012). Like most neurons, the development of GnRH neurites requires a combination of factors for proper neurite growth, guidance and termination. Though a number of factors have been identified, understanding remains incomplete. In this study, we add Sonic hedgehog (Shh) to the list of molecules known to be involved in modulating the growth of neurites from developing GnRH neurons.

Shh is a crucial developmental signaling molecule (Echelard et al., 1993; Ericson et al., 1995; Grindley et al., 1997; Marigo et al., 1996; Yamada et al., 1993; Roelink et al., 1994), and is highly expressed along the ventral midline of the nervous system, including the basal forebrain where developing GnRH neurons can be found (Suzuki-Hirano et al., 2011; Tekki-Kessaris et al., 2001). Recent studies have shown that Shh acts as a diffusible factor to stimulate neurite outgrowth and/or to guide neurites from non-GnRH neurons during development (Bourikas et al., 2005; Kolpak et al., 2005a; Okada et al., 2006). Given that Shh is expressed in the basal forebrain during the time of GnRH neuronal development, we hypothesised that Shh may be involved in GnRH neurite growth/guidance towards the ME.

To determine whether this was a tenable hypothesis, we first examined the spatial and temporal relationship between Shh protein expression and GnRH neuron cell bodies and neurites, and found that Shh was in the right place at the right time to affect GnRH neurite growth. To evaluate further the possibility that GnRH neurons might be affected directly by Shh we used *in situ* hybridization to show that GnRH neurons expressed mRNA encoding the Shh receptor *Smoothened* (*Smo*). The effect of Shh on GnRH neurite outgrowth was then investigated *in vitro* using a 3D collagen gel assay, in which Shh showed neurite growth-promoting activity on GnRH neurons. Finally, we deleted *Smo*, from GnRH neurons using Cre-loxP recombination, and found that the density of GnRH nerve terminals at the ME was signficantly reduced. The data obtained from these experiments indicate that Shh stimulates GnRH neurite growth *via* Smo signaling, and suggest that Shh acts in concert with previously identified GnRH neurite growth regulators to ensure successful innervation of the ME.

2. Results

2.1. GnRH neurons and their neurites co-localize with Shh in the basal forebrain

Dual-label immunohistochemistry was performed to examine the relative spatial distribution of Shh protein and GnRH neurons in the developing brain. Shh was found to be expressed in two hypothalamic nuclei in the basal forebrain at GD14.5 (Fig. 1). Shh was expressed in the POA (Fig. 1A), where many GnRH cell bodies extending neurites were also found intermingled with Shh-expressing cells (Fig. 1A).

Shh was also expressed in the mediobasal hypothalamus in the ventral aspect of the ventromedial hypothalamic nucleus (VMH), where a patch of Shh immunoreactivity could be seen extending medially from the perimeter of the third ventricle, where immunoreactivity was quite intense, to nearly the lateral edge of the forebrain, where immunoreactivity was diminished (Fig. 1B). Some GnRH neurites could be seen approaching the ME at this age (Fig. 1C), but within this region they do not appear to colocalize spatially with Shh.

These results indicate that GnRH neurons and their neurites in the POA appear to come into contact with Shh at a time and place when GnRH neurons are actively extending neurites.

2.2. GnRH neurons express Smo coincident with GnRH neurite growth

To evaluate whether GnRH neurons have the capacity to respond to Shh at this time, we used *in situ* hybridization to determine whether they express mRNA encoding *Smo*. *Smo* transcripts could be detected in a subpopulation of approximately 60% of POA GnRH neurons (Fig. 2A–C).

2.3. Shh stimulates GnRH neurite growth in vitro

The co-localization studies above supported our hypothesis that Shh may have the ability to affect the growth of neurites from POA GnRH neurons. To test this hypothesis a 3-dimensional (3D) collagen gel assay (Rosoff et al., 2004), which has been used previously by our group and others (Fiorini and Jasoni, 2010; Low et al., 2012; Piper et al., 2009; Rosoff et al., 2004; Wang et al., 2017), was employed. GD15.5 POA explants were used, because this is the developmental time when the largest proportion of GnRH neurons are actively extending their neurites, and when GnRH neurons express *Smo* (Fig. 2) (Gill and Tsai, 2006).

GnRH neurite outgrowth was increased significantly in the presence of Shh (0.7 μg/mL) after 24 h when compared with POA explants in either control or 2.5 μg/mL Shh conditions (vehicle: 19.7 μm ± 2.7; low Shh: 35.0 μm ± 8.4, $p < 0.05$) (Fig. 3). At a low concentration of Shh (0.7 μg/mL) GnRH neurites were approximately twice as long as those in the vehicle-treated group (vehicle: 19.7 μm ± 2.7; low Shh: 35.0 μm ± 8.4, $p < 0.05$) and could be seen clearly extending at greater length outside the margins of the explant compared with controls (Fig. 3A, B, D). This suggests that Shh stimulates the growth of GnRH neurites from the forebrain explants, which is consistent with previous observations with retinal ganglion cells (Kolpak et al., 2005a). By contrast, no change in GnRH neurite length was observed in explants treated with a high (2.5 μg/mL) concentration of Shh (vehicle: 19.7 μm ± 2.7; high Shh: 17.4 μm ± 4.0) and neurites appeared very similar in length to untreated controls (Fig. 3A, C, D). This suggests that high levels of Shh may be unable to affect the growth of GnRH neurites, which has been seen previously with commissural neurons from the embryonic spinal cord (Yam et al., 2009). Thus, low but not high concentrations of Shh stimulate the outgrowth of neurites from GnRH neurons *in vitro*.

2.4. GnRH neurite density was reduced in GnRH-Smo KO mice

To further investigate the effect of Shh on the development of GnRH neurons *in vivo*, we used a cre-loxP deletion strategy to remove *Smo* specifically from GnRH neurons, rendering them unable to respond to Shh *via* Smo during development (Fig. 4A). First, the excision of the Smo gene was verified by PCR (Fig. 4B). In addition, the expression of *Smo* in GnRH neurons was examined by dual immunohistochemistry/*in situ* hybridization. GnRH neurons from GnRH-Smo mice were never observed to show evidence of *Smo in situ* hybridization signal (Fig. 2D–F). Finally, tissue samples from both the basal forebrain, containing numerous GnRH neurons, and the cerebral cortex, which is devoid of GnRH neurons, at GD16.5 were collected. In the presence of Cre, an excision band was observed only in the basal forebrain, but not the cortex (Fig. 4B). This result confirmed that the *Smo* gene was only excised from the brain region where Cre-expressing GnRH neurons are located.

GnRH neurite density was then compared between WT and GnRH-Smo KO mice at GD 18.5, a stage at which the bundles of GnRH neurites can be found at and near the ME (Gill et al., 2008), and in adult. GnRH neurite density at the level of the ME was determined by measuring fluorescence intensity, a proxy indicator for the presence of GnRH-containing nerve fibers (Fig. 5A). GnRH neurite density was significantly decreased by more than 30% in GnRH-Smo KO mice at GD 18.5 (WT 100% ± 25%, $n = 6$; GnRH-Smo KO 67% ± 17%, $n = 8$, $p < 0.05$) (Fig. 5B). By contrast, in adulthood, the density of GnRH nerve fibers at the ME was not different between wild-type and GnRH-Smo deletion mice. (WT 100% ± 35%, $n = 5$; GnRH-Smo KO 88.4% ± 37%, $n = 10$) These results suggest that the absence of Shh-Smo signaling in GnRH neurons impairs their ability to extend neurites to their target on their normal developmental schedule.