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Patterning Axon Targeting of Olfactory Receptor Neurons by Coupled Hedgehog Signaling at Two Distinct Steps

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SUMMARY

We present evidence for a coupled two-step action of Hedgehog signaling in patterning axon targeting of *Drosophila* olfactory receptor neurons (ORNs). In the first step, differential Hedgehog pathway activity in peripheral sensory organ precursors creates ORN populations with different levels of the Patched receptor. Different Patched levels in ORNs then determine axonal responsiveness to target-derived Hedgehog in the brain: only ORN axons that do not express high levels of Patched are responsive to and require a second step of Hedgehog signaling for target selection. Hedgehog signaling in the imaginal sensory organ precursors thus confers differential ORN responsiveness to Hedgehog-mediated axon targeting in the brain. This mechanism contributes to the spatial coordination of ORN cell bodies in the periphery and their glomerular targets in the brain. Such coupled two-step signaling may be more generally used to coordinate other spatially and temporally segregated developmental events.

INTRODUCTION

From insects to mammals, olfactory receptor neurons (ORNs) expressing a given odorant receptor project their axons to a common glomerulus, thereby creating a spatial map for odor processing (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Gao et al., 2000; Vosshall et al., 2000). Although ORNs that express a given receptor are distributed widely across the olfactory epithelium, there is a certain degree of spatial correspondence between ORN cell bodies in the periphery and their glomerular targets in the brain. In mice, there is a coarse topographic correspondence of ORNs along the dorsomedial-ventrolateral axis in the nasal epithelium and their glomerular targets along the dorsal-ventral axis of the olfactory bulb (Miyamichi et al., 2005). Axon-axon interactions via Semaphorin-3F and its receptors contribute to the establishment of this topography (Takeuchi et al., 2010). In flies, ORNs belonging to distinct types of sensillae target their axons to different coarse domains in the antennal lobe (Couto et al., 2005). Here, we show that Hedgehog signaling acts via a coupled two-step mechanism to contribute to this spatial coordination in *Drosophila*.

The Hedgehog (Hh) protein acts by binding to and inactivating the Patched (Ptc) receptor, relieving Ptc inhibition of the 7-transmembrane protein Smoothened (Smo). Active Smo transduces the Hh signal to the transcription factor Cubitus interruptus (Ci), which activates transcription of Hh target genes including *ptc* itself (Hooper and Scott, 2005) (Figure 1A). In the *Drosophila* wing disc, Hh is produced and secreted from the posterior compartment, and induces high-level Ptc expression at the anterior-posterior compartment boundary. High Ptc at the boundary in turn sequesters and prevents most of Hh from moving more anteriorly. Meanwhile, in the posterior compartment, the transcription factor Engrailed (En) represses *ci* expression, thus accounting for the lack of Ptc expression in the posterior compartment despite the presence of Hh. The consequence of this Hh signaling loop establishes different Ptc expression levels in different compartments (Hooper and Scott, 2005). The transmembrane proteins Interference hedgehog (Ihog) and Brother of Ihog (Boi) function with Ptc as essential coreceptors involved in binding to and transduction of the Hh signal (Yao et al., 2006; Zheng et al., 2010). In addition to this canonical pathway resulting in the regulation of transcription for developmental patterning, a vertebrate Hedgehog homolog, Sonic Hedgehog (Shh), has also been proposed to act as an axon guidance molecule for spinal commissural neurons (Charron et al., 2003; Bourikas et al., 2005; Okada et al., 2006; Yam et al., 2009). It is unclear whether axon guidance function for Hh signaling is a vertebrate innovation or reflects an evolutionarily conserved function.

During our studies of *Drosophila* olfactory system development, we uncovered a mechanism whereby Hh signaling is used in two distinct but coupled steps. We found that Hh signaling in the larval antennal disc and early pupal antenna creates two populations of ORNs with different Ptc levels. Smo and Ihog are cell-autonomously required in only a subset of ORN classes for their axon targeting. Surprisingly, only

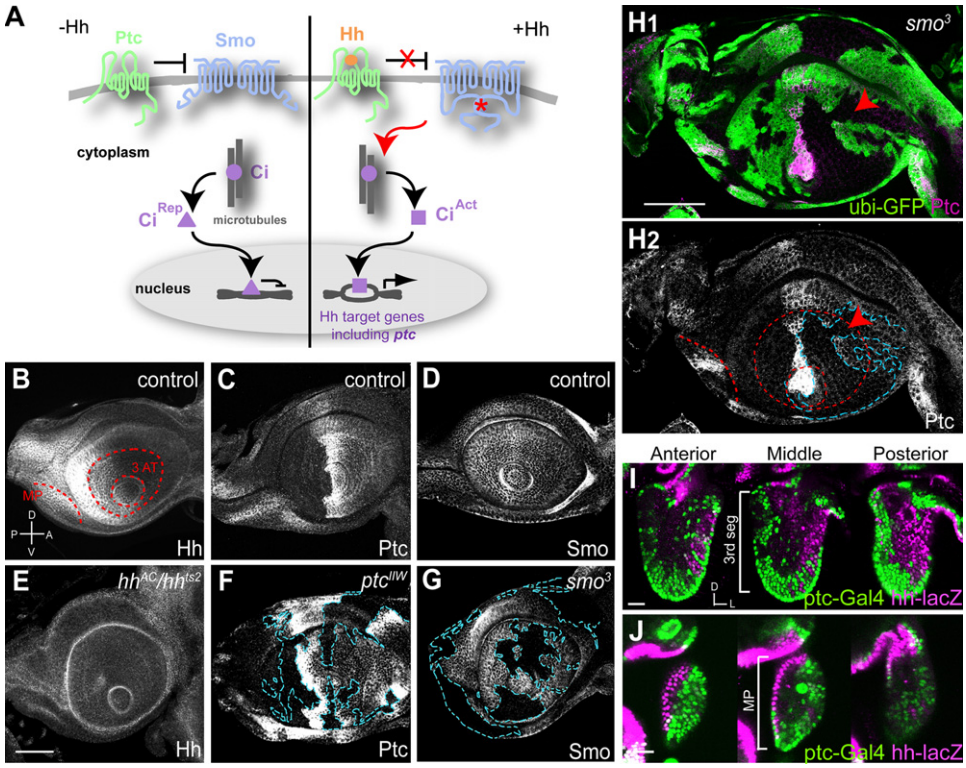

Figure 1. Distribution of Hh, Ptc, and Smo in the Developing Olfactory System

(A) The classic Hh signaling pathway. Left, in the absence of Hedgehog (Hh), Patched (Ptc) inhibits Smoothened (Smo) activity. In the absence of Smo activity, Cubitus interruptus (Ci) is processed to a repressor form (Ci^{Rep}). Right, Hh acts by binding to Ptc and relieving its inhibition of Smo. Active Smo (asterisk) transduces a signal that leads to the maturation of Ci to an activator form (Ci^{Act}). Among genes activated by Ci^{Act} is *ptc* itself.

(B–D) In wild-type control late-third instar larval antennal discs, Hh protein is found in the posterior compartment (B). Ptc protein is highly expressed in the anterior-posterior compartment border, expressed at a medium level in the anterior, and undetectable in the posterior compartment (C). Smo is ubiquitously expressed, with a slightly higher level in the posterior compartment (D).

(E–G) Antennal discs from late-third instar lavae. Hh protein is greatly diminished in hh^{AC}/hh^{ts2} discs from larvae shifted to restrictive temperature 4 hr before dissection (E). Ptc and Smo proteins are greatly diminished in clones homozygous for ptc^{IIW} (F) or smo^{3} (G), respectively. Clone boundaries (blue dashed lines) were determined by loss of the clone marker GFP (see H_1).

(H) Ptc protein is reduced in smo^{3} clones, shown in magenta with green clone marker (H_1) or alone (H_2). Even in the most anterior part of the anterior compartment, there is a reduction of Ptc protein level in smo^{3} clones (arrowheads).

A, anterior; P, posterior; D, dorsal; V, ventral. Red dashed lines in (B) and (H_2) mark the precursor of third segment of antenna (3 AT) and maxillary palp (MP).

(I and J) Cells in the antenna (I) and maxillary palp (J) at 36 hr after puparium formation (APF) labeled by *ptc-Gal4*-driven nuclear GFP (green) and *hh*-nuclear *lacZ* (magenta). Three single confocal sections at anterior, middle and posterior of the right antenna and maxillary palp are shown. Dorsal (D) is up and lateral (L) is right. *Ptc-Gal4* and *hh-lacZ* label complementary populations of cells that are largely spatially segregated, with some intermingling at the border.

The scale bars represent 50 μm (B–H) or 20 μm (I and J). Full genotypes for this and subsequent figures are described in Table S2. Figure S1 provides additional data on Smo, Ptc, Hh, Ihog, and Boi expression in the pupal antenna and maxillary palp, and Smo, Ptc, and Ihog proteins in ORN axons.

low-Ptc ORNs require Smo and Ihog; high-Ptc ORNs do not require Smo and Ihog for their axon targeting. Further genetic analyses, including tissue-specific and temporally regulated loss- and gain-of-function studies of several Hh pathway components, strongly support a coupled two-step model: early Hh signaling in the sensory organ precursors confers differential ORN responsiveness to Hh in the brain, such that only axons of low-Ptc ORN classes respond to later signaling by brain-derived Hh. This coupled two-step mechanism contributes to the spatial coordination of ORN cell bodies in the periphery and their axon targets in the brain, and suggests a general paradigm for coordinating spatially and temporally segregated developmental events.

RESULTS

Hh Signaling Establishes Differential Ptc Protein Levels in ORNs

Drosophila ORNs reside in the third antennal segment and in the maxillary palp (Stocker, 2001). Both structures derive from the larval antennal disc (Figure 1B). We find that Hedgehog (Hh) signaling patterns the larval antennal disc along the anterior-posterior axis. In late third instar larvae, Hh protein is enriched in the posterior compartment of the antennal disc (Figures 1B and 1E), consistent with a previous study based on expression of the *hh-lacZ* enhancer trap (Lee et al., 1992). The level of Ptc protein is highest at the compartment border and moderate in