Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum

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Purkinje cells (PCs) are the projection neurons of the cerebellar cortex. They receive two major types of synaptic input – that from the inferior olive via climbing fibres and that from the granule neurons via parallel fibres. The precursors of granule neurons proliferate at the surface of the developing cerebellum in the external granule layer (EGL), which persists until postnatal day 14 in the mouse [1]. PCs are thought to provide trophic support for granule neurons [2,3] and to stimulate the proliferation of cells in the EGL [4], but the signalling molecules that mediate these cell–cell interactions have not been identified. I show here that PCs in the developing mouse cerebellum express the gene encoding the morphogen Sonic hedgehog (Shh) and that dividing cells in the EGL express *Patched* (*Ptc*) and *Gli1*, two target genes of which expression is upregulated in response to Hedgehog signalling (see [5] and references therein). Treatment of developing mice with hybridoma cells that secrete neutralizing anti-Shh antibodies [6] disrupted cerebellar development and reduced bromodeoxyuridine (BrdU) incorporation in the EGL of neonatal mice, whereas treatment of dissociated granule neuron cultures with recombinant Shh stimulated BrdU incorporation. These results suggest that PC-derived Shh normally promotes the proliferation of granule neuron precursors in the EGL.

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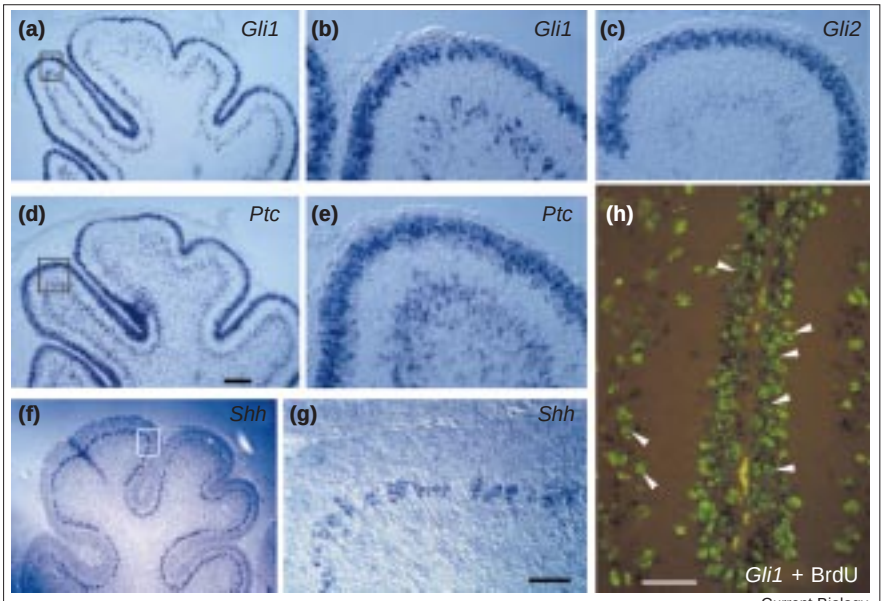
Results and discussion

Shh is an extracellular signalling molecule that plays a key role in tissue patterning, cell-fate determination, and cell proliferation in the developing vertebrate embryo (reviewed in [7]). To determine whether the Hedgehog signalling cascade is activated in the developing cerebellum, I performed *in situ* hybridization with *Ptc* and *Gli1* probes on cryosections of postnatal murine cerebellum. Cells expressing the genes *Ptc*, and *Gli1* were located in the EGL and in the PC layer (Figure 1a,b,d,e). I also examined the expression of *Gli2* and *Gli3* because the transcription factors encoded by these genes have also been implicated in Hedgehog signalling [8]. *Gli2* was found to be expressed in a similar pattern to *Gli1* (Figure 1c). *Gli3* was only expressed in the EGL, and at very low levels (data not shown). *Ptc* and *Gli* genes were expressed in the outer layer of the EGL, where proliferating cells are located, suggesting that the genes are expressed mainly in dividing precursor cells. To confirm this, I labelled cells in S phase with BrdU *in vivo* and performed *in situ* hybridization for *Gli1* expression and immunocytochemistry for BrdU incorporation. As shown in Figure 1h, the region of BrdU staining in the EGL co-localized with the region of *Gli1* expression, and many BrdU$^+$ cells clearly expressed *Gli1*. Many BrdU$^+$ cells in the EGL also expressed *Ptc* (data not shown). The BrdU staining also co-localized with *Gli1* and *Ptc* expression in the PC layer of the cerebellum (Figure 1h). Although I have not identified the *Ptc*- and *Gli1*-expressing cells in the PC layer, the cells have small cell bodies and are not PCs. Taken together, these findings suggest that proliferating cells in the EGL might be direct targets of Hedgehog signalling.

Next, *in situ* hybridization was performed to study the expression of *Hedgehog* genes in the developing cerebellum. As shown in Figure 1f, *Shh* was expressed in a single layer of cells in the PC layer where PCs, identified by their large cell bodies, were clearly labelled (Figure 1g). The expression of *Indian hedgehog* and *Desert hedgehog*, two additional mammalian *Hedgehog* genes [9], was not detected in the cerebellum (data not shown and [10]). As described by Traiffort *et al.* [10], I found that *Shh*, *Ptc* and *Gli1* were also expressed in the adult cerebellum: *Shh* was expressed in PCs, whereas *Ptc* and *Gli1* were expressed in other cells in the PC layer, presumably Bergmann glia (data not shown). The functional significance of Shh signalling in the adult cerebellum is unknown.

To determine whether Shh plays a part in normal EGL development, I injected hybridoma cells that secrete neutralizing anti-Shh monoclonal antibodies (5E1 cells; [6]) into the brains of postnatal day 1 (P1) mice. As a control, I injected hybridoma cells that secrete a monoclonal anti-Thy-1 antibody (OX-7; [11]) of the same immunoglobulin G (IgG) subclass into the brains of their littermates. I then analyzed *Ptc* expression in the cerebellum 3 days later. *Ptc* expression was reduced in both the PC layer and the EGL of anti-Shh-treated animals compared with uninjected or anti-Thy1-treated littermates

Figure 1



In situ hybridization for *Gli*, *Ptc* and *Shh* expression in the postnatal cerebellum. (a,b) *Gli1*, (c) *Gli2* and (d,e) *Ptc* expression in sagittal sections of postnatal day 2 (P2) cerebellum. *Gli2* and *Ptc* are expressed in the EGL and PC layer. (f,g) *Shh* expression in P5 cerebellum (horizontal section). *Shh* is expressed in a single layer of cells in the PC layer. (b,e,g) Higher magnifications of boxed insets in (a,d,f). (h) *In situ* hybridization for *Gli1* expression followed by immunocytochemistry for BrdU in P1 cerebellum. To label cells in S phase, animals were injected with BrdU and 2 h later their tissues were fixed and processed for *in situ* hybridization followed by immunocytochemistry. The BrdU staining (green) overlaps with the *in situ* hybridization signal for *Gli1* (black) in the EGL (centre of the panel) and the PC layer. The white arrowheads indicate examples of $BrdU^+$ cells that express *Gli1*. For (a,d,f), scale bar represents 200 μm and for (b,c,e,g,h), 50 μm.

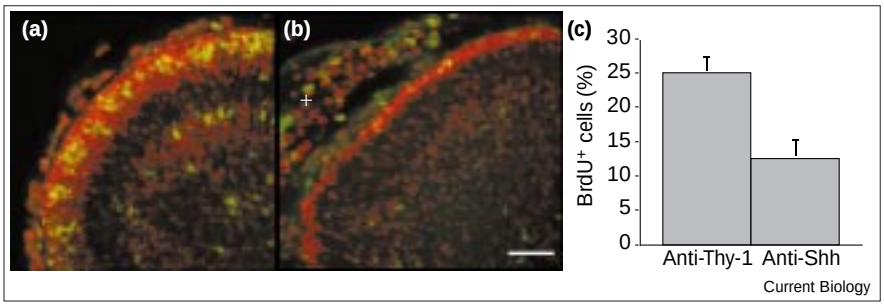
(Figure 2). The EGL was also thinner, and the folia were not as deep in the anti-Shh-treated animals compared with controls (Figure 2).

The reduction in the depth of the folia and the thinning of the EGL in the anti-Shh-treated animals is reminiscent of the disruption in cerebellar development that is observed when dividing cells in the EGL are eliminated by X-irradiation [12] and is consistent with the possibility that the antibody treatment reduces proliferation and/or survival of cells in the EGL. To determine whether treatment with anti-Shh antibodies increased the number of dying cells in the EGL, I stained cerebellar sections with propidium iodide to visualize nuclei and examined the EGL for the presence of pyknotic nuclei. I could not detect an increase in the number of pyknotic nuclei in the EGL of the antibody-treated mice (data not shown). To determine whether the treatment reduced proliferation in the EGL, hybridoma-treated animals were given two injections of BrdU 2 hours apart, and the cerebellum was stained with anti-BrdU antibodies 2 hours after the last injection. Compared with animals injected with anti-Thy-1-secreting

Figure 2

Intracranial injection of hybridoma cells secreting anti-Shh antibodies disrupts cerebellar development in neonatal mice. *In situ* hybridization for *Ptc* expression in sagittal sections of the cerebellum of P4 mice that were (a) uninjected or injected intracranially 3 days earlier with hybridoma cells secreting (b) anti-Thy-1 antibodies or (c) anti-Shh antibodies. *Ptc* expression is reduced in the EGL (arrows) and the PC layer of anti-Shh-treated animals. In addition, the depth of the folia (indicated by the asterisk) is reduced in anti-Shh-treated animals compared with uninjected or anti-Thy-1-treated littermates. The hybridoma cells (arrowheads) are present along the edge of the cerebellum. The anti-Shh-secreting hybridoma cells also express *Ptc*. The experiment was performed on two litters, with similar results in all of the anti-Shh-treated mice ($n = 7$), whereas all of the anti-Thy-1-treated mice ($n = 4$) were indistinguishable from uninjected mice ($n = 2$). Scale bar represents 200 μm.

Figure 3



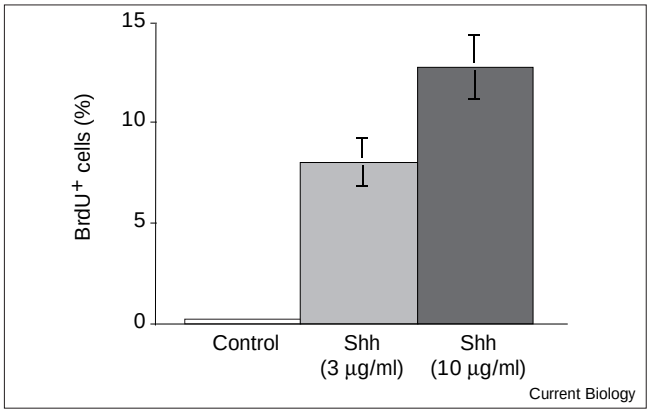
Treatment with cells secreting anti-Shh antibodies reduces BrdU incorporation in the EGL. P1 mice were injected intracranially with hybridoma cells secreting (a) anti-Thy-1 antibodies or (b) anti-Shh antibodies, and 4 days later the mice were given two injections of BrdU 2 h apart. The animals were sacrificed 2 h after the last injection and their cerebella were processed for immunocytochemistry with anti-BrdU antibodies (green); nuclei were stained with propidium iodide (red). The EGL is thinner and BrdU incorporation is reduced in the anti-Shh-treated animals compared with the anti-Thy-1-treated animals. The + symbol in (b) indicates hybridoma cells that are adjacent to the EGL. In (a,b), the sections are mid-sagittal and the scale bar represents 25 μm. (c) The proportion of $BrdU^+$ cells in the EGL is reduced in anti-Shh-treated mice. The total cell number and the number of $BrdU^+$ cells in at least three regions of the EGL were counted (400–1200 cells per animal). Each bar represents the average ± SEM (n = 3 mice per group).

hybridoma cells, anti-Shh-treated animals showed a dramatic reduction in the proportion of cells in the EGL that incorporated BrdU (Figure 3). To determine whether Shh can promote EGL cell proliferation *in vitro*, I treated cultures of mixed P8 cerebellar cells with a recombinant amino-terminal active fragment of Shh (Shh-N) for 2 days and labelled dividing cells with BrdU. As shown in Figure 4, Shh increased BrdU incorporation more than 10-fold. The $BrdU^+$ cells were small neuron-like cells with processes; this suggests that they were EGL cells, which represent the great majority of the cells in the cultures.

Focal loss of PCs, induced by transgenic expression of an attenuated form of diptheria toxin under the control of a PC-specific promoter, results in a focal reduction of proliferation in the EGL directly overlying the region of PC loss [4]. The findings suggest that PCs normally stimulate proliferation in the EGL, possibly through signalling molecules that act over short distances. Shh is a good candidate for such a PC-derived short-range mitogen; its post-translational processing results in the covalent addition of cholesterol to the carboxyl terminus of Shh-N, which serves to tether the fragment to the surface of Shh-expressing cells [13,14]. In addition, a palmitoylated form of Shh-N has also been identified [15]. Although Shh can apparently act over long distances, it acts mainly over short distances to induce tissue patterning [7]. My findings that *Shh* is expressed in PCs in the developing cerebellum and that *Ptc* and *Gli1* are expressed by dividing cells in the EGL, suggest that dividing cells in the EGL are direct targets of Shh signalling. The findings that anti-Shh antibodies disrupt cerebellar development and reduce *Ptc* expression and BrdU incorporation in the EGL *in vivo*, and that treatment with recombinant Shh-N increases BrdU incorporation in cultures of EGL cells suggest that Shh from PCs is one of the signals that promotes proliferation of granule neuron precursors in the EGL. During revision of this manuscript, Wechsler-Reya and Scott [16] reported that Shh is mitogenic for granule neuron precursors in cerebellar explant and dissociated cultures and that blocking Shh signalling *in vivo* reduces proliferation in the EGL, which is in agreement with my findings.

Shh is unlikely to be the only factor that regulates the proliferation of EGL cells. There is evidence that bFGF also plays a part in stimulating EGL proliferation *in vivo*: treatment with bFGF increases proliferation in the EGL [17], and treatment with anti-bFGF monoclonal antibodies reduces proliferation in the EGL [18]. Although purified granule neuron precursor cells can stimulate themselves to divide as long as they are cultured at high density as reaggregates [19], it is possible that proliferation in the EGL

Figure 4



Recombinant Shh increases BrdU incorporation in cultures of mixed cerebellar cells. Dissociated cultures of cerebellar cells from P8 mice were cultured for 2 days in the presence of the myristoylated form of the amino-terminal active fragment of Shh (Shh-N). The cultures were pulsed with BrdU (10 μM) for an additional 14 h and the proportion of $BrdU^+$ cells was determined. Each bar represents the average ± SEM, where n = 5 for control and n = 3 for each dose of Shh-N.

requires PC-derived signals to overcome inhibitory signals from other cell types. For example, Bergmann glia have been shown to inhibit proliferation and promote differentiation of granule neuron precursors *in vitro* [19].

The Shh signalling pathway might also play a role in the development of medulloblastomas, a rare type of cerebellar tumour. Humans and mice that are heterozygous for loss-of-function mutations of the *Ptc* gene have a high incidence of medulloblastoma (reviewed in [5]). In addition, mutations in *Ptc* and *Smo*, a transmembrane protein that is required for Hh signalling [20, 21], have been identified in a number of spontaneous medulloblastomas and the expression of Shh target genes such as *Ptc* and *Gli1* is derepressed in a number of these medulloblastomas (see [5] and references therein).

My colleague and I showed previously that *Shh* is expressed in the retinal ganglion cell layer of the developing retina, that *Ptc* is expressed in the neuroblast layer of the retina, and that Shh is a mitogen for retinal precursor cells [22]. My present findings in the cerebellum show a striking parallel with those in the retina. In both organs *Shh* is expressed by the first-born projection neurons – PCs in the cerebellum [1] and retinal ganglion cells in the retina [23] – and promotes the proliferation of neural precursor cells. Taken together, these findings suggest a common developmental mechanism in which Shh from first-born projection neurons stimulates the division of the neural precursor cells that give rise to later-born neurons.

Supplementary material
Additional methodological details are published with this paper on the internet.

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