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Making a grade: Sonic Hedgehog signalling and the control of neural cell fate

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My grandfather once told me that you should never do anything that might win you a medal. This advice came from his time in the military and although he was a man whose opinion I respected greatly, this was one piece of advice that I never thought would be relevant to me as a scientist. In my defence, it was never my intention or ambition to win a medal: no one could have been more surprised and delighted than I was when I received the email from EMBO informing me that I had won the Gold Medal.

Indeed, looking back, I can honestly say it was never my intention or ambition to become a research scientist. Although one or two of my current colleagues appear to have had well-structured career plans before they could walk, read or say 'tenure committee decision' I did not. Similar to many scientists I suspect, I accidentally fell into research. I enjoyed and did reasonably well in science classes at school, so it was obvious to choose a science subject when thinking about university. I ended up at Warwick University studying Microbiology and Virology, a course that involved a great deal of molecular biology. The elegance and complexity of bacterial and viral life and the way in which studying these organisms at a molecular level seemed to resolve the complexity to simple, straightforward explanations of function captivated me. An intercalated year spent working at Amersham International in Cardiff (now GE Healthcare) reinforced this view and whetted my appetite for some proper research. Back at Warwick for my final year, I was particularly struck by how much had been learned about the mechanism at work in eukaryotic cells from the study of viruses and viral infections.

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Signalling, the start

With this in mind, I decided to look for a suitable PhD position. Certainly my university studies and experience at Amersham contributed to this decision, but it is also important to note that this was 1992, the depth of the recession in the UK. Seeing my friends lose their jobs and struggle to make ends meet was also a strong motivator. A safe academic job seemed preferable to the uncertainties of life in the 'real world'. I was fortunate to gain a place in Ian Kerr's lab at the Lincolns Inn Fields laboratories of the Imperial Cancer Research Fund (now Cancer Research UK). I was doubly fortunate to arrive in the lab and institute at just the right time. Ian had a long-standing interest in unravelling how interferon (IFN) initiated an antiviral state in cells. Together with George Stark's group, which had occupied an adjoining space in the institute, they had embarked on a somatic cell genetic approach to identify genes required for the IFN response (Stark, 1997). This was beginning to come to fruition as I joined the lab. Between them, the labs had screened for and isolated several mutant cell lines that were unresponsive to IFNα/β and/or IFNγ. By identifying the mutated gene in one of these lines, Sandra Pellegrini had already shown that the tyrosine kinase Tyk2 was a component of the IFN signal-transduction pathway (Pellegrini et al, 1989; Velazquez et al, 1992). When I joined the lab, I was set to work with an experienced postdoc, Mathias Müller, to help find the defective genes in two further mutants. One of the cell lines turned out to be deficient in a STAT transcription factor that forms part of the IFN-regulated transcriptional complex (Müller et al, 1993a). For the other cell line, I was given the job of testing a series of candidate genes. On the basis of our hunches, one candidate was the Tyk2-related kinase JAK1. Meanwhile, Mathias was busy with more complicated experiments using cDNA libraries to screen for clones that restored IFN responses in the mutant cells, in case none of our guesses paid off. My task was simple. It involved transfecting mutant cells with candidate genes, exposing them to IFN, preparing RNA and then using RNase protection assays to test whether the induction of IFN-dependent gene expression had been re-established. The assay was usually finished late on the fifth day of the experiment and ordinarily the assays would be exposed to autoradiographic film overnight, ready to be analysed the next morning. But we were always impatient. So instead of overnight, we would set up the exposure and then nip around the corner to the George pub for an hour. After a pint or two, Mathias and I

would return and run the film through the developer. After such a short exposure, the bands on the film were very faint, but if you held it up to the light at just the right angle you could make them out. I remember doing this with the assay containing Jak1 and hardly believing my eyes when I saw weak bands indicating that the mutants had indeed regained an IFN response (Müller *et al*, 1993b). The excitement and exhilaration of this experience hooked me into research. These rare moments when you see a result that nobody else has seen before, when for a short period you are the only person to have this knowledge, still make science rewarding and compelling.

These studies, together with elegant biochemical and molecular experiments from the labs of Darnell, Ihle and co-workers in the USA, were the foundation of what is now referred to as the JAK/STAT signalling pathway (Darnell *et al*, 1994; Ihle, 1995). It was an exciting time. Hardly a week seemed to pass without the JAKs and STATs being implicated in another signal-transduction pathway and the mutant cell lines proved an invaluable resource. We were able to use them to show that the cytokine IL-6 used the JAK/STAT pathway. They were the ideal tools for molecular structural studies of the JAK and STAT proteins and for dissecting the mechanism of signal transduction (Guschin *et al*, 1995; Briscoe *et al*, 1996; Kohlhuber *et al*, 1997). Ian was a challenging but purposeful supervisor, always focused and supportive and I could not have asked for more generous and helpful colleagues in the lab. Moreover, the entire atmosphere at ICRF was vibrant and stimulating. To me it seemed like the centre of the signal-transduction world; down the corridor Richard Treisman's lab was dissecting the MAP kinase pathway, whereas a floor below Julian Downward's lab was identifying new Ras effectors and Peter Parker's lab was analysing PKC signalling. This atmosphere was further enhanced when Paul Nurse and a group of developmental biologists, including Phil Ingham, David Ish-Horowitz and Julian Lewis, moved to the institute. The seminars from these groups were my first exposure to embryology, introducing me to the type of questions that developmental biologists would like to tackle. The images of embryos were more aesthetically pleasing than the graphs and blots I was accustomed to and the questions the researchers asked seemed to me to be more profound. I began to realize that this was a subject I wanted to pursue. Specifically, having spent the previous few years studying the mechanisms of signal transduction in tissue culture cells, I decided I wanted to analyse a signal-transduction mechanism *in vivo* during embryogenesis.

Moving into morphogens

Once again, having made a decision, I found myself in a fortunate situation when Tom Jessell offered me a postdoc position in his lab at Columbia Medical School in New York City. Tom is interested in understanding the molecular mechanisms that direct the assembly of neuronal circuits in the spinal cord and how the organization of these circuits coordinates muscle movement and behaviour (Jessell, 2000). A first step in this process is the production of the appropriate neuronal subtypes in the right place and time in the forming neural tube.

The question of how different types of neurons are produced in their correct positions in the spinal cord is a specific example of a general and fundamental problem in developmental biology. How do cells know where they are within a tissue and how is this information translated so that they form the appropriate structures for their positions? The framework that has emerged over the last century to answer this question involves the concept of positional information and signalling gradients. Lewis Wolpert best encapsulated this idea in a highly influential paper in 1969 (Wolpert 1969, 1996) in which he introduced what is now called the French Flag Model. This is a general mechanism to divide a field of cells into three equal partitions such as the red, white and blue of the French tricolour. In the model, a fixed part of the tissue is postulated to correspond to an organizer that produces a signal. The signal propagates through the rest of the tissue to establish a gradient. Cells within the tissue respond and interpret the graded signal in a quantitative manner. Cells therefore ascertain their distance from the organizer according to the concentration of the signal (Figure 1A). The beauty of this model is that the abstract concept of positional information is replaced by a tangible biochemical coordinate
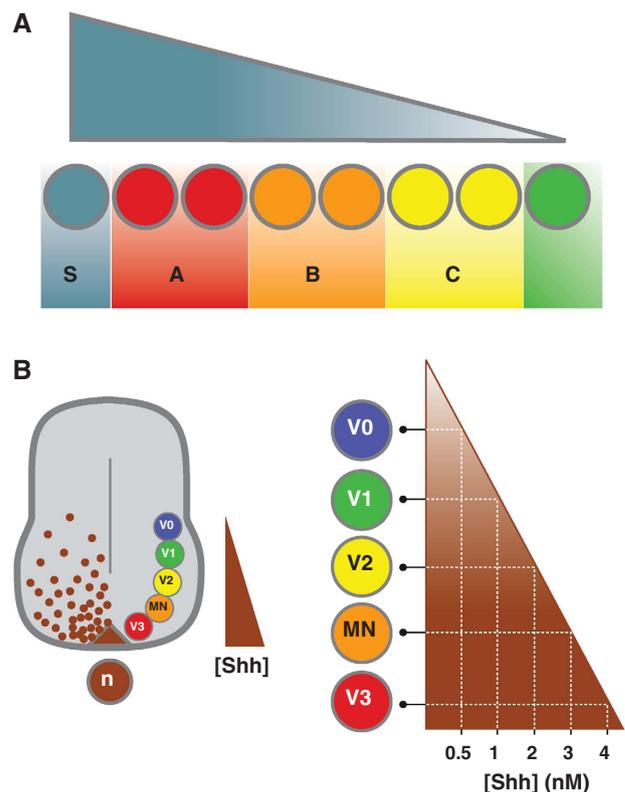

Figure 1 Sonic Hedgehog acts in a graded manner to pattern the ventral neural tube. (**A**) A morphogen can pattern a developing tissue. A secreted signal (blue) is produced from a localized source (S) and spreads through the tissue to establish a gradient. Cells respond to different concentrations of the signal by regulating different sets of genes (red, orange and yellow). This induces distinct cell fates (A, B and C) at different distances from S (adapted from the French Flag Model in Wolpert, 1969). (**B**) Shh protein (brown) is produced from the notochord (n) and floor plate at the ventral midline of the neural tube. Shh spreads dorsally establishing a gradient that controls the generation of distinct neuronal subtypes (interneurons (V0–V3) and motor neurons (MN)). *In vitro*, different concentrations of Shh are sufficient to induce the distinct neuronal subtypes. The concentration of Shh necessary to induce a specific subtype corresponds to its position of generation *in vivo*.

system that defines the location of a cell with respect to a fixed point within the tissue. The term morphogen was adopted to define signals that function in this way. This definition emphasizes two characteristics of a morphogen: it must function in a concentration-dependent manner to induce different responses at different thresholds and it must spread through a tissue to act at a distance from its source. The model also raises several additional questions that have to be answered for a complete understanding of how a morphogen works. How does the signal spread through the tissue to establish a gradient? How is the extracellular gradient perceived by responding cells, specifically how is the quantitative information transduced across the membrane and through the signalling pathway to control differential gene expression? How is the continuous graded information interpreted to generate discrete, all-or-none changes in gene expression that must underpin the switches in cell type produced at different concentrations of signal?

These issues are of particular relevance in the spinal cord where several distinct classes of neurons including motor neurons (MNs) and a number of interneurons involved in relaying sensory information and coordinating motor output are generated (Jessell, 2000). Each neuronal population arises from blocks of proliferating progenitors that are arrayed in a stereotypic order along the dorsal–ventral (DV) axis of the neural tube. Experiments that dated back to the 1920s indicated that in the ventral half of the spinal cord the pattern of neuronal generation is directed by cues emanating from the floor plate, a population of cells that reside at the ventral midline of the neural tube, and the notochord (reviewed in Placzek *et al*, 1991). A couple of years before I arrived in New York, the Jessell lab and several other labs cloned vertebrate homologues of the *Drosophila* gene Hedgehog, which encode secreted signalling proteins (Echelard *et al*, 1993; Krauss *et al*, 1993; Riddle *et al*, 1993; Chang *et al*, 1994; Roelink *et al*, 1994). It turned out that one of them, Sonic Hedgehog (Shh), was expressed in the notochord and floor plate and corresponds to the instructive cue that provides DV polarity to the ventral neural tube (Figure 1B). Moreover, the initial studies strongly hinted that Shh patterned the neural tube in a concentration-dependent manner, as predicted if it was a morphogen (Marti *et al*, 1995; Roelink *et al*, 1995). Whether Shh satisfied the exacting criteria necessary to qualify it as a morphogen and how cells interpreted the signal to specify distinct neuronal cell types remained to be determined.

These were the questions that excited me on arriving in the Jessell lab. However, having no previous experience in developmental biology and never having laid hands on an embryo before, I teamed up with a skilled developmental biologist, Johan Ericson. We worked closely together. Johan showed extreme patience during the first few months as I tried to learn the experimental techniques that were much more delicate than ones I used previously. He also showed a similar level of patience, but for a much longer period of time, on the Bronx public golf course where in response to his drives straight up the middle of the fairway, I would inevitably hack my way through the rough, zigzagging slowly towards the green. My golf never improved, but nonetheless, the scientific partnership with Johan continued with long discussions about which experiments to do and arguments over what the results meant. These were interspersed by sage and

timely advice from Tom offering piercing insight and ensuring we kept our focus.

The idea that Shh was necessary for correct formation of MNs and other ventral neuronal subtypes *in vivo* was supported by loss-of-function studies (Chiang *et al*, 1996; Ericson *et al*, 1996). To further understand how Shh controlled neuronal subtype identity, we carried out experiments using explants of naïve neural tissue. The results indicated that exposure to varying concentrations of Shh protein induced distinct neuronal subtypes characteristic of the ventral neural tube (Ericson *et al*, 1997a, b). Progressive 2- to 3-fold changes in Shh concentration led to the generation of the different neuronal subtypes and there was a good correlation between the concentration of Shh necessary to induce each neuronal subtype and their position of generation *in vivo* (Figure 1B). Thus, the induction of neurons generated in more ventral regions of the neural tube required correspondingly higher Shh concentrations. In addition to being necessary for their development then, Shh was sufficient to induce ventral interneurons and MNs in neural tissue. Furthermore, these experiments supported the idea that Shh functions as a morphogen with cells in the ventral neural tube being exposed to ventral^{HIGH}–dorsal^{LOW} gradient of Shh, emanating from the ventrally located notochord and floor plate.

Coding neural cell fate

These findings highlighted the question of how positional identity is imposed on progenitor cells and how this determines neuronal subtype identity. A series of studies over the next few years suggested that a group of transcription factors, predominately homeodomain proteins, were important intermediaries in the process (Ericson *et al*, 1997a, b; Briscoe *et al*, 1999, 2000; Sander *et al*, 2000; Novitch *et al*, 2001; Vallstedt *et al*, 2001). These transcription factors exhibit distinct patterns of expression along the DV axis of the neural tube (Figure 2). On the basis of their mode of regulation by Shh signalling, we subdivided them into two groups, termed class I and II proteins. The threshold responses of the proteins to graded Shh signalling were defined using cultures of chick explants. This provided evidence that the expression of each class I protein is repressed at distinct thresholds of Shh activity. Consequently, their ventral limits of expression are determined by Shh signalling. Conversely, neural expression of the class II proteins depends on Shh signalling, so their dorsal boundaries of expression are defined by graded Shh signaling. The combined expression profiles of both classes of proteins defined five domains of progenitors within the ventral neural tube, each of which generated a distinct neuronal subtype. Thus, the profile of homeodomain protein expression appeared to correspond to a transcriptional code that assigns positional identity to progenitors that prefigured the neuronal subtype generated by each domain.

The idea that the progenitor transcription factor code determined the fate of differentiating postmitotic neurons was supported by gain- and loss-of-function experiments in chick and mouse embryos (Ericson *et al*, 1997a, b; Briscoe *et al*, 1999, 2000; Sander *et al*, 2000; Vallstedt *et al*, 2001). Forced expression of a class I or II protein in the neural tube changed the position in which individual neuronal subtypes were generated in a manner predicted by the normal expression profile of the class I and II proteins. Conversely, the
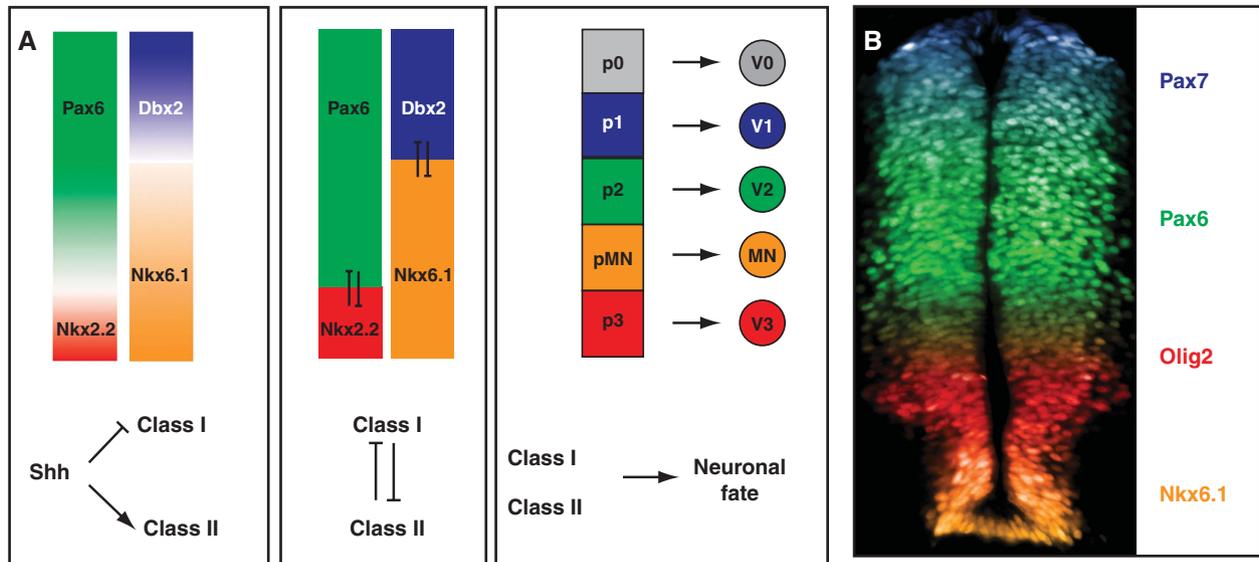
Figure 2 Graded Shh signalling acts by controlling the expression of a set of transcription factors in neural progenitors. (**A**) Shh signalling results in the repression of class I proteins such as Pax6 and Dbx2 and is required for the activation of class II proteins such as Nkx2.2 and Nkx6.1. Selective cross-repressive interactions between pairs of class I and II proteins generate discrete switches in gene expression. The combinatorial expression of class I and II proteins defines five domains of progenitors in the ventral neural tube. These progenitor domains are arrayed along the dorsal–ventral axis and each domain generates one of the distinct neuronal subtypes characteristic of the ventral neural tube. (**B**) The expression profile of the transcription factors Pax7, Pax6, Olig2 and Nkx6.1 represent examples of the spatially restricted expression patterns that delineate distinct progenitor domains in the neural tube.

targeted inactivation in mice of individual progenitor transcription factors resulted in predictable switches of neuronal fate. In these experiments, we were enabled by a generous supply of mutant embryos from several labs, in particular Lori Sussel and John Rubenstein in San Francisco and Pen Rashbass and Veronica Van Heyningen in Edinburgh.

These experiments also revealed the presence of selective cross-repressive interactions between pairs of class I and II proteins expressed in adjacent progenitor domains (Figure 2A). This first became apparent for the class II protein Nkx2.2 and the class I protein Pax6 (Ericson *et al*, 1997a, b; Briscoe *et al*, 2000). The observation that Nkx2.2 expanded dorsally in embryos lacking Pax6, resulted in another of those infrequent but exciting scientific moments. It suggested how the system could be operating, further underscored when gain-of-function experiments indicated a mutual cross-repression between Pax6 and Nkx2.2. Subsequently similar observations were extended to other pairs of class I and II proteins (Briscoe *et al*, 2000; Vallstedt *et al*, 2001). Taken together, the data indicated that cross-repression between pairs of class I and II proteins established the DV boundaries of gene expression, thereby defining the positions at which distinct neuronal subtypes are generated. The cross-repressive interactions also provided a plausible explanation for the switch-like response of genes to the gradient of Shh. Such a mechanism accounting for the conversion of a graded signal into discrete all or none changes in gene expression is essential for the function of a morphogen. Moreover, the principle of cross-repressive interactions observed in the neural tube resembled mechanisms involved in other developing tissues, such as the anterioposterior patterning of the *Drosophila* embryo (Small and Levine, 1991). Thus, it may represent a general strategy for the regional allocation of cell fate in response to graded inductive signals.

Patching a direct link

Although the ability of Shh to induce distinct neuronal subtypes in a concentration-dependent manner suggested that Shh acted directly at long range to control gene expression, no direct *in vivo* observation of a gradient of Shh protein had been made. It was possible, therefore, that Shh exerted its long-range effect by inducing an intermediary signal to relay positional information to the neural tube. To test the range of Shh signalling, I worked with Yu Chen in Gary Struhl's lab, handily located on the floor above the Jessell lab. They had previously shown, with a series of exquisite genetic experiments in *Drosophila*, that Patched (Ptc) was the receptor for Hh and binding of Hh to Ptc restricted the movement of Hh through tissue (Chen and Struhl, 1996). They went on to construct mutated forms of Ptc that acted as dominant inhibitors of signalling in both *Drosophila* and vertebrates. Mosaic expression of the vertebrate version of the mutant Ptc construct in the neural tube inhibited Shh signalling in transfected cells and resulted in the cell-autonomous inhibition of the cell types normally found in the ventral neural tube (Briscoe *et al*, 2001). This indicated that Shh acted directly at long range to control gene expression and cell fate in the neural tube. Moreover, I noticed that the blockade of signalling resulted in more dorsally positioned cells responding as if exposed to a higher concentration of Shh. This indicated that, similar to Hh in *Drosophila*, a feedback mechanism limited the spread of Shh in the neural tube; consequently, blocking signalling increased the range of Shh. These conclusions were further supported by work from Andrew McMahon's lab, including the recent direct demonstration of a gradient of Shh in the neural tube (Gritli-Linde *et al*, 2001; Jeong and McMahon, 2005; Chamberlain *et al*, 2008). Collectively, the studies argued strongly against signal

relay models and indicated that Shh functions over a distance in the neural tube. Together with evidence of its concentration-dependent activity, this confirmed that Shh signalling, in the neural tube, meets the strict definition of a morphogen.

These studies established graded Shh signalling in the neural tube as an experimentally tractable system for understanding the molecular mechanism of morphogen action. This prompted the question of how cells convey the external concentration of Shh through the signal-transduction pathway to regulate differential gene expression in the nucleus. It is this 'information processing' question that I was keen to tackle as I set up my own lab and, despite several interesting and fruitful sidetracks, this is still the main occupation of the lab. In another fortunate coincidence of timing, as I was looking for an independent position, I learnt that NIMR wanted to recruit several new group leaders. The collegiate atmosphere and the support of senior members of the institute immediately impressed me and I jumped at the opportunity when offered a position. However, what was not clear to me until the day I started, was that the temporary space I had been allocated was at the very top of the building underneath the roof, inaccessible by elevator and barely heated. Although I was used to the cold from winters in New York, it was a shock to my first student who arrived in my lab, during a cold spell, straight from her home on the Mediterranean island of Crete. In spite of this, she stayed and within a few months we moved into new lab space on a more salubrious floor of the institute complete with its own thermostat. Since then the environment and my colleagues have been wonderful; the exchange of ideas, the rigorous questioning during internal seminars and the generous sharing of reagents and resources make NIMR a fantastic and stimulating place to work.

The Gli-tzy ways of Shh signalling

We focused on the question of how graded information from Shh signalling is perceived and transmitted in responding cells. Intracellular Shh signalling depends on two transmembrane proteins: Ptc1, already mentioned, the receptor which binds Hh proteins, and Smoothened (Smo), which is responsible for transducing Hh signals intracellularly (for reviews, see Ingham and McMahon, 2001; Varjosalo and Taipale, 2008). In the absence of Shh, Ptc1 inhibits Smo activity, and binding of Shh to Ptc1 releases this inhibition allowing intracellular signal transduction. The exact mechanism of signal transmission downstream of Smo remains unclear and is the subject of much interest. However, the evidence suggests that the signal concludes with the regulation of a family of zinc-finger containing transcriptional effectors known as Gli proteins (Gli1, 2 and 3). All three Gli genes are expressed in the neural tube and several studies had begun to examine the functions they have in neural tube patterning (Jacob and Briscoe, 2003; Ruiz i Altaba et al, 2003). Michael Matise in Alex Joyner's lab had shown that a targeted deletion of Gli2 in mouse embryos (Matise et al, 1998) led to a failure in the generation of the floor plate and the adjacent domain of V3 neuron progenitors: these are the cell types induced by highest concentrations of Shh. Concomitant with the loss of these cell types, there was a ventral expansion in the production of neighbouring MNs, whereas neuronal classes located dorsal to MNs were

unaffected. A compound mutant lacking both Gli1 and Gli2 had more severe defects than Gli2−/− mutants (Park et al, 2000); however, these embryos still produced MNs and interneurons dorsal to MNs. This suggested that Gli2, with partially redundant assistance from Gli1, is required for specifying the cell types that require the highest levels of Shh signalling. The data did, however, leave open the question of the role of Gli3 and whether Gli activity was involved in controlling all responses to Shh.

Despina Stamataki, the PhD student from Crete, having acclimatized to the weather, collaborated with Johan Ericson (now back in Stockholm) and his lab to test the involvement of Gli activity and the function of Gli3 in neural patterning. Despina used a truncated version of Gli3 that lacked the transcriptional activation domains but retained its inhibitory function to block all Gli transcriptional activation in the chick neural tube. Similar to the results with the mutant Ptc1 construct that inhibited signalling, the truncated Gli3 caused a ventral-to-dorsal shift in progenitor cell identity and a concomitant failure to generate MNs and ventral interneurons (Persson et al, 2002; Meyer and Roelink, 2003). This confirmed the central importance of Gli activity in the provision of positional information to cells responding to graded Shh signalling. Gli3 had been proposed to function primarily as an inhibitor of Shh signalling in the neural tube. Supporting this idea, in the absence of Gli3, progenitor domains located in the intermediate region of the neural tube expanded dorsally, concomitant with a switch in the identity of the neurons generated in this region. This phenotype was corrected in mice bearing a targeted allele of Gli3, made in Uli Ruther's lab. This allele encodes only a truncated isoform of Gli3 (Böse et al, 2002), equivalent to proteolytically processed Gli3. Moreover, abolishing Gli3 function in Shh−/− embryos partially restored the expression of several ventral progenitor transcription factors that are normally lost in Shh mutant embryos (Litingtung and Chiang, 2000). This supported the idea that only repressor activity of Gli3 is required in the neural tube.

Although ventral cell types were generated in the Shh;Gli3 double mutants, the patterning is somewhat disrupted, in particular the strict DV organization characteristic of the normal neural tube is less evident (Litingtung and Chiang, 2000). This suggested two things. First, the induction of most ventral cell types can take place in the absence of Shh signalling, as long as the repressive activity of Gli3 is removed. Second, other extrinsic signals might provide positional information, albeit less accurately, to the ventral neural tube when Shh signalling is removed. Thus, without transcriptional input from Gli proteins, cells lack the positional information provided by Shh signalling, nevertheless, the cross-repressive interactions between progenitor transcription factors remain. Therefore, within individual progenitors, stochastic bias or other external signals might determine gene expression. In this situation, the stochastic biases or the imprecision of other external signals means that neighbouring cells could adopt different positional identities resulting in a neural tube consisting of intermixed cell identities. In this context, it is interesting to note that BMP signalling, which emanates from the dorsal pole of the neural tube, influences the response of ventral neural progenitors to Shh. Exposure of neural plate explants to a fixed concentration of Shh in the presence of BMPs resulted in a ventral-to-dorsal shift in

progenitor and neuronal subtype identity (Liem *et al*, 2000). Conversely, BMP inhibitory proteins ventralized the response of neural plate cells to a set Shh concentration (Liem *et al*, 2000). BMP signalling could therefore have a function in establishing DV patterning and might have a significant effect on gene expression in *Shh;Gli3* double-mutant mice.

The conclusion that Gli proteins function downstream of Shh to control ventral patterning through transcriptional regulation of target genes led to an attractive model to explain Shh morphogen activity. In this model, graded Shh signalling evokes a gradient of Gli activity by progressively inhibiting Gli repressor activity and potentiating Gli activator function (Jacob and Briscoe, 2003). To test this model Despina, together with Fausto Ulloa, made a series of dominant active Gli constructs each producing a different level of transcriptional activity. Consistent with the model, gain-of-function experiments with these constructs suggested that progressive changes in the level of Gli activity were sufficient to emulate the patterning activity of graded Shh signalling (Stamataki *et al*, 2005). This implied that during neural tube development a gradient of Gli transcriptional activity is produced that mirrors the gradient of Shh signalling. Thus, the level of Gli activity produced in a responding cell would be proportional to the concentration of Shh to which it is exposed.

In this model, the 2- to 3-fold differences in extracellular Shh concentration, which is sufficient to switch gene expression profiles, are transduced by similar small differences in the level of Gli activity. This predicts that little, if any, amplification of the signal occurs during signal transduction. Of note, analyses of other morphogen signalling pathways (Shimizu and Gurdon, 1999; Stathopoulos and Levine, 2002) had led to similar conclusions. For example, a series of elegant studies by John Gurdon and colleagues examining gene induction by Activin during mesoderm specification concluded that a three-fold difference in the absolute number of receptors occupied by Activin are relayed to a three-fold difference in the level of nuclear SMAD2 transcription and this is sufficient to discriminate between the induction of two genes, Xbra and Xgsc (Dyson and Gurdon, 1998; Shimizu and Gurdon, 1999). Thus, it appeared that a common feature of morphogens is that differences in signal strength are relayed directly, without amplification, to the nucleus. This mechanism contrasts with the type of signal-transduction strategy I was used to thinking about from my graduate student days, such as those involving kinase cascades, which amplify the signal during intracellular transmission so that small extracellular differences result in substantial downstream differences in the signal.

Taking some time

Although consistent with the data, the model was clearly a simplification. It relied on a rather static view of neural development that ignored the dynamic nature of the response of cells to Shh signalling. This was highlighted by studies of Shh signalling in the developing limb indicating that the duration of Shh signalling, in addition to the concentration of Shh, influenced patterning (Ahn and Joyner, 2004; Harfe *et al*, 2004). Hence when a new postdoc, Eric Dessaud, started in the lab he wanted to assess the influence of duration on the interpretation of Shh signalling in the neural tube. He revisited the technique of using explants of naïve

neural tissue to ask how the response of cells to Shh developed over time (Dessaud *et al*, 2007). He found that the induction of one of the progenitor transcription factors Nkx2.2, which requires higher concentrations of Shh than the MN progenitor marker Olig2, also took longer to be induced than Olig2. Moreover, the concentrations of Shh that induced Nkx2.2 produced a transient expression of Olig2. This *in vitro* response to Shh was paralleled *in vivo* by the sequential onset of Olig2 and Nkx2.2 expression.

To investigate the reason for the temporal dependence of the response, Eric analysed the output of the Shh signal-transduction pathway by adapting a reporter assay of Gli activity for use in explants that Fausto Ulloa had originally established. This allowed him to measure the level of Gli activity induced by defined concentrations of Shh at specific times (Figure 3A). The results of these experiments indicated that the sensitivity of cells to Shh signalling progressively decreased. Cells first appeared to be highly sensitive to exposure to Shh ligand. Consequently, low concentrations of Shh were sufficient to produce high levels of Gli activity. With increasing time, cells became desensitized to Shh signalling; thus, the concentration of Shh necessary to achieve the highest levels of Gli activity increased. As a result, different concentrations of Shh generate an intracellular signal for different periods of time, such that the duration of signalling is proportional to Shh concentration. These results led us to propose that a 'temporal adaptation' mechanism transforms the extracellular concentration of the Shh morphogen into time-limited periods of signal transduction, such that the duration of signalling is proportional to ligand concentration (Dessaud *et al*, 2007, 2008).

These results posed the question of how cells convert extracellular concentration into proportional periods of signal transduction. Luckily, the function of Ptc1 immediately suggested an explanation for the gradual desensitization of cells to ongoing Shh signalling (Figure 3B). Ptc1, as well as being the Shh receptor and negative regulator of the pathway, is a transcriptional target of Gli proteins (Goodrich *et al*, 1996; Marigo and Tabin, 1996). In response to Shh signalling, cells steadily upregulate *Ptc1*, as well as other inhibitors of Shh signalling, conferring a negative feedback loop to the pathway. This means that increasing concentrations of Shh are necessary to block the inhibitory activity of accumulating Ptc1. Consistent with this, inhibition of Ptc1 with siRNAs resulted in a low concentration of Shh being sufficient to induce the expression of markers normally associated only with higher concentrations of ligand (Dessaud *et al*, 2007). This revealed a crucial cell-autonomous role for Ptc1 in the interpretation of graded Shh signalling complementing the previous studies that indicated a role for Ptc1 in controlling the spread of ligand (Chen and Struhl, 1996; Jeong and McMahon, 2005).

In lab meetings, as we considered and discussed these data, it began to dawn on me that what we were talking about had much in common with the mechanism of bacterial chemotaxis I had learnt 15 years previously as an undergraduate (Wadhams and Armitage, 2004). Chemotaxis also relies on a negative feedback loop that controls the duration of intracellular signalling to sense an external gradient. Indeed, the gradual adaptation of cells to extracellular signals had been proposed to permit the sensing and transduction of concentration ranges of signals in several situations
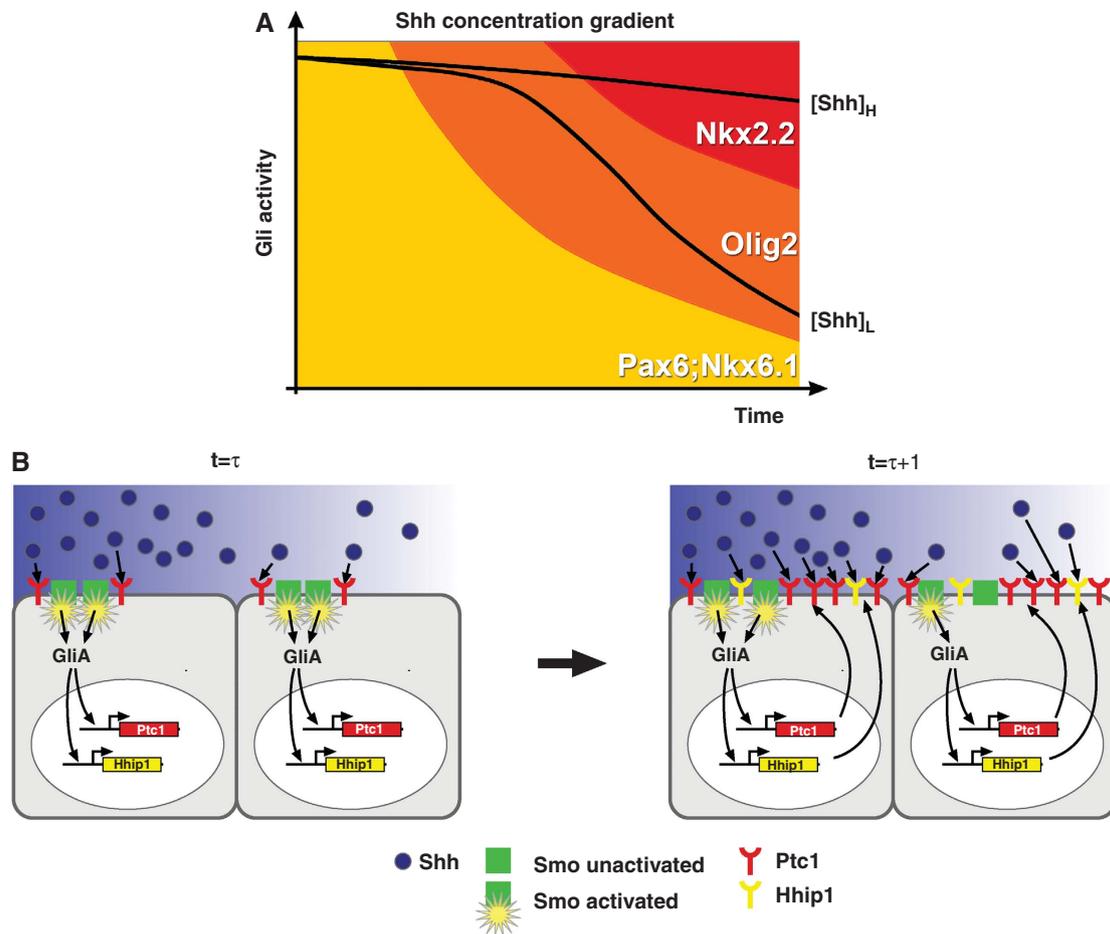
Figure 3 A temporal adaptation mechanism interprets graded Shh signalling. (**A**) Cells exposed to a high concentration of Shh ($[Shh]_H$) sustain high levels of Gli activity for a prolonged period of time and induce Nkx2.2. By contrast, although exposure to a lower concentration of Shh ($[Shh]_L$) is initially able to induce high levels of Gli activity, these levels are not maintained and the cells express Olig2. The decrease in Gli activity indicates that cells adapt to the concentration of Shh over time. (**B**) Upregulation of Ptc1, and possibly other negative regulators of the pathway such as Hhip1, contributes to the gradual adaptation of cells to ongoing exposure to Shh. Initially ($t = \tau$) the level of Ptc1 is low, thus even a low concentration of Shh is sufficient to bind the available Ptc1 and initiate high levels of signalling. The transcriptional upregulation of *Ptc1* by Shh signalling results in the gradual accumulation of Ptc1 protein in responding cells ($t = \tau + 1$). If the cells are exposed to a high concentration of Shh, sufficient ligand will be available to bind the induced Ptc1; signal transduction will therefore be maintained. By contrast, in cells exposed to low concentration of Shh, unliganded Ptc1 will begin to build up; this will inhibit signal transduction.

(Matthews and Reisert, 2003). The transduction of a concentration of ligand into a corresponding duration of intracellular signalling offers an alternative to the established mechanisms of morphogen signalling: a mechanism that is strikingly similar to the 'sequential cell context' hypothesis proposed by Pages and Kerridge (2000) several years ago. Moreover, the adaptation mechanism explains why both the amount and the duration of Shh exposure are important for the cellular response. It may be applicable in other tissues such as the limb bud, which are also patterned by both the time and concentration of Shh signalling (Harfe *et al*, 2004). It also seems satisfying that evolution appears to have derived comparable strategies for measuring concentrations of external factors in very different situations and for very different purposes.

The model, together with studies from other systems, suggests that the conventional definition of a morphogen needs revising. In the strict interpretation of the French Flag Model, responding cells of the tissue are assumed to be passive recipients of the positional information supplied by the graded signal (Jaeger and Reinitz, 2006; Jaeger *et al*,

2008). This does not fit the case for Shh signalling in the neural tube. The response of cells to Shh signalling, conspicuously the upregulation of Ptc1, is fundamental to the generation of the morphogen response. In addition, the regulatory interactions between the transcription factors that are transcriptionally controlled by Shh in progenitors are essential for generating the appropriate pattern of neurogenesis in the neural tube. This lends support to the view that positional information in the ventral neural tube is, in part, an emergent property that relies on both a gradient of ligand and the response of the target cells. Thus, signal and tissue collaborate to produce a morphogen. Experimental findings for other morphogens and tissues have also led to modifications and elaborations to the conventional morphogen definition (Jaeger and Reinitz, 2006, Jaeger *et al*, 2008).

The model also raises further questions that we need to address. What are the relative contributions of duration and level of Shh–Gli signalling for specification of each of the progenitor domains that appear to depend on Shh signalling? What are the relevant genomic targets of Shh signalling and how do different durations or amounts of Gli activity control

differential gene expression? What are the molecular mechanisms that connect and regulate the responding genes in progenitors? How do the responding genes specify neuronal subtype identity? A range of experimental approaches—genetic, molecular, imaging and modeling—will be required. In particular, the data highlight the importance of methods that provide an ongoing measure of the activity of key components of the signalling pathway and methods that manipulate the duration of activity of these components. In addition to DV patterning, Shh signalling also influences other properties of neural cells such as survival and proliferation. How Shh achieves this and how growth and patterning of the neural tube are integrated remains poorly understood. However, the reagents and techniques now available should now allow these questions to be addressed. Finally, how does this relate to other signalling pathways? Several other secreted molecules are important for the patterning of the neural tube and there appears to be a significant degree of cross-talk between the pathways these activate. In most cases, details of the mechanisms and the contribution these make to neural development remain to be determined. Conversely, is the temporal adaptation mechanism outlined here relevant to other graded signals or does the interpretation of different morphogens rely on distinct strategies?

Although I started out with a bold ambition to answer fundamental questions about animal development, as is often the case in science, I have mainly succeeded in finding new problems and raising more questions. Questions that I know will keep me and others engaged, frustrated and amused for some time to come. For me, one of the greatest pleasures has been the opportunity of working with bright people and I have been fortunate to work with more than my fair share of the brightest. I hope this continues. And although I accidentally broke my grandfather's tenet to avoid medals, I'm pretty sure he would have overlooked the rule just this once.

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