CNS Regeneration: A Morphogen's Tale

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ABSTRACT: **Tissue regeneration will soon become an avenue for repair of damaged or diseased tissues as stem cell niches have been found in almost every organ of the vertebrate body including the CNS. In addition, different animals display an array of regenerative capabilities that are currently being researched to dissect the molecular mechanisms involved. This review concentrates on the different ways in which CNS tissues such as brain, spinal cord and retina can regenerate or display neurogenic potential and how these abilities are modulated by morphogens.** © 2005 Wiley Periodicals, Inc.
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INTRODUCTION

It has been thought for a long time that the adult central nervous system in most vertebrates is incapable of regeneration after injury. However, innovative research has challenged this concept in the recent years. Advances in our understanding of the molecular physiology of the CNS, the development of techniques to isolate and manipulate neural stem cells, and the increasing knowledge of the roles that different morphogens play in regeneration have shed new light into the possibilities of morphological and functional recovery of CNS injuries, something that a few years ago seemed unreachable (for reviews see Temple, 2001; Gould and Gross, 2002; Grugo et al., 2002; Nottebohm, 2002; Baizabal et al., 2003; Goh et al., 2003; Lie et al., 2004). However the field of regenerative neurobiology is only in its early stages, and despite the effort that several research groups are devoting to this exciting enterprise, we are still far from having a thorough understanding of the molecular mechanisms that drive regeneration of the CNS.

In the present review, we discuss some of the groundbreaking discoveries that have been made in this field, as well as the current state of knowledge on the roles that various morphogens play in the regenerative process of the CNS, and the avenues that remain unexplored. For practical purposes, we will consider a broad definition of regeneration as the morphological, physiological and functional recovery of lost or injured tissue, which in the case of the CNS involves neurogenesis, re-growth of axons, and the functional re-establishment of synapses. It should be clear, however, that this constitutes the ultimate goal of the process, and the extent of regeneration that most vertebrates can achieve is not that extensive and depends on the size of the injury and the age of the animal among other factors. Therefore, the knowledge acquired from each of the studies discussed in this review should be regarded as contributions to different aspects of this global process, the understanding of which will be crucial in the development of strategies to treat neurodegenerative diseases.

We have focused on several well-studied morphogens that have shown potential in this area; however

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this does not represent an exhaustive list of all the factors that could affect CNS regeneration.

Regeneration in the Brain

In the adult brain certain specific regions such as the subventricular zone (SVZ), the hippocampus, and the cortex undergo neurogenesis. The function of neurogenesis in these areas is not fully decrypted, but recent studies suggest various roles for it, including regeneration (Nakatomi et al., 2002) and the formation of new networks implicated in learning and memory (Gould et al., 1999; Kempermann, 2002; van Praag et al., 2002). In the border of the lateral ventricle, the subventricular zone (SVZ), (Reynolds and Weiss, 1992), as well as in the dentate gyrus area of the hippocampus (Palmer et al., 1997), neural stem cells and progenitors are still present during adulthood. The loss of progenitor cells in the SVZ due to a high dose of tritiated thymidine produces regeneration of neural progenitors by quiescent stem cells (Morshead et al., 1994). Regeneration is not limited to the SVZ cells. Constant neuronal cell death takes place in the olfactory bulb, and it was observed that the lateral corner of the SVZ continuously sends new neuroblasts and astrocytes towards that area to replace them (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Kato et al., 2001). Thus, the neurogenesis that occurs in this region would aim to restore the neuronal number, but so far there is no demonstration that the newly generated neurons take the place and the role of the dead ones.

The appearance of new neurons in the hippocampus and the cortex during adulthood seems to be linked to the memory process, and probably to the establishment of new networks respectively (in the associative cortex) to support learning and memory (Gould et al., 1999; Kempermann, 2002). Interestingly, in patients suffering from Alzheimer's disease, an increased number of newly generated cells was observed in the granule cell layer as well as in the CA1 region of Ammon's horn, where extensive cell death occurs during this disease (Jin et al., 2004). Neuronal regeneration was also observed in the hippocampus after local ischemia (Yoshimura et al., 2001; Nakatomi et al., 2002) or after kainic acid injection (Yoshimura et al., 2001). Indeed, certain types of ischemia can lead to neuronal cell death in the CA1 region of the hippocampus and as a result alter memory (Fig. 1). Nonetheless, it was shown that 28 days post-ischemia, several new neurons reappear in the lesion area and those neurons differentiate into the right phenotype and in the appropriate location (Nakatomi et al., 2002; Fig. 1). The cortex is also able to support regeneration in particular cases. The death of isolated neurons in the cortex can lead to the generation of new neurons that are able to reconnect to the original targets (Magavi et al., 2000). Indeed, the induction of cortico-thalamic neuronal cell death in layer VI, results in the stimulation of cell division that ends in the generation of new neurons, as attested by the co-localization of BrdU and markers of early and late stages of neuronal differentiation. Retrograde tracing from the hypothalamic region revealed that the newly generated neurons reestablished connections to the appropriate targets (Magavi et al., 2000). As seen from this study, the potential for regeneration is not limited to the areas normally undergoing neurogenesis during adulthood. Additional works revealed other non-neurogenic areas that are able to partially regenerate. For instance, no new formation of neurons is observed in the striatum. However, ischemia produced in this area induces cell proliferation in the SVZ followed by a migration of the newly generated cells towards the striatum (Arvidsson et al., 2002; Parent et al., 2002). Thirty five days after injury, close to 70% of the newborn cells differentiated into neurons that express specific striatal markers (Parent et al., 2002). A lesser differentiation process was observed in the study of Arvidsson et al. (2002), where there was a significant neurogenic response in the first two weeks after a stroke, but 80% or more of the new neurons died within 6 weeks, possibly due to an unfavorable environment. However, about half of the surviving newly formed neurons differentiated into striatal neurons. Altogether, these results show that stem and progenitor cells residing in the adult brain are still able to supply new glia and neurons throughout life, to either integrate into an existing neuronal network or to repair a damaged area.

Regeneration in the Spinal Cord

The spinal cord can also be regenerated in certain adult vertebrates such as fish and urodele amphibians, which can achieve considerable functional recovery; however most other vertebrates loose this potential during development. The urodele spinal cord is not only able to re-grow and connect neuronal axons, but also to replenish lost neurons and glia. The source of this regeneration seems to be the population of ependymal cells that line the central canal and extend processes to the surface of the pia mater. When a urodele's tail is amputated or the spinal cord is transected, these cells proliferate and form an ependymal tube that later differentiates into neurons and glia, to reconstitute a functional spinal cord within 4 to 6 weeks (Egar and Singer, 1972; Benraiss et al., 1999;

Echeverri and Tanaka, 2002; Ferretti et al., 2003). Despite the outstanding regenerative capacity of these animals, that makes them an ideal model system to study regeneration of the spinal cord, the molecular mechanisms that drive this process have been elusive.

Anuran amphibians have also been used as models in spinal cord regeneration studies (Forehand and Farel, 1982) however, in contrast to urodeles, their regenerative capacity decreases with development and is lost after metamorphosis (Beattie et al., 1990; reviewed by Chernoff et al., 2002; Ferretti, 2004).

Chick embryos, on the other hand, can only regenerate the spinal cord until embryonic day 13 (Shimizu et al., 1990; Ferretti et al., 2003), which coincides with the onset of myelination, and if this is delayed, the regenerative period can be extended (Keirstead et al., 1992). However it is not clear if neurogenesis takes place in this process or if the functional restoration is due to the re-growth of axons of existing neurons (reviewed by Ferretti, 2004).

In adult mammals, the ependymal cells that line the central canal can form neurospheres (colonies formed by neural stem cells [NSCs] *in vitro* and differentiate into neurons and glia *in vitro*) (Weiss et al., 1996). These cells increase their proliferation after spinal cord injury, but gliogenesis and not neurogenesis has been observed from these cells *in vivo* (Johansson et al., 1999; Horner et al 2000). On the other hand, embryonic rat spinal cord transplanted into neonatal rats in which the lower thoracic segments of the spinal cord had been resected, integrated into the host spinal cord and re-established neuronal connections, leading to a functional recovery in those animals (Iwashita et al., 1994). Also, grafting of hNT neurons (which are derived from a human embryonic carcinoma) or committed Sprague-Dawley rat spinal neuronal precursors into previously ischemic spinal cord segments of rats resulted in a successful recovery of motor function in a spastic paraplegia model. Three months after implantation, 1-2% of the grafted neurons survived, integrated and displayed a mature phenotype (Marsala et al., 2004). These studies indicate the potential of different transplantation methods for the recovery of function after spinal cord injury.

Regeneration in the Retina

The retina is another part of the CNS and constitutes a vital component of the eye, since it is able to capture light and processes it into electrical signals that are eventually transported to the brain via the optic nerve. Several organisms have devised ways to assure visual competence. Fish for example, upon retinal damage, can activate intrinsic stem cells located in the inner retina to proliferate and form a regenerative blastema that will eventually differentiate into the retinal cells that were lost. Salamanders regenerate their retina via transdifferentiation of the retinal pigmented epithelium (RPE). The RPE cells lose their characteristics of origin, proliferate, and form a neuroepithelium which will later differentiate into the various retina cell types (reviewed in Del Rio-Tsonis and Tsonis, 2003; Haynes and Del Rio-Tsonis, 2004; Hitchcock et al., 2004; Moshiri et al., 2004; Tsonis and Del Rio-Tsonis, 2004). Adult chickens and rodents can partially regenerate their retina if damaged via transdifferentiation of Muller glial cells into only limited types of neurons (Fischer and Reh 2001; Ooto et al., 2004). Embryonic chicks, on the other hand, are able to regenerate all the different retinal cell layers upon removal of the retina by two mechanisms. One involves transdifferentiation of the RPE and the other requires the activation of retinal stem/progenitors cells located in the anterior region of the eye. These activities can only take place at a specific time in development and under the influence of growth factors (Park and Hollenberg, 1989; 1991; Spence et al. 2004).

In this context, any environmental factor that is able to enhance or stop the regenerative process may aid in the development of strategies to repair a damaged area of the CNS. Here we discuss studies that suggest a potential to manipulate adult neural regeneration through the regulation of specific morphogens. The roles of the factors known to influence this process are developed in each "morphogen section" below.

It is important to point out that several events involved in CNS regeneration are regulated by these morphogens including cell proliferation, activation of progenitor cells, specification of cell fates, patterning of tissues, and even transdifferentiation of specific cell types. Some of these processes are also involved in the development of neural structures, and even though this does not mean that regeneration will recruit the same mechanisms that regulate development, their study in the developmental context aids in the understanding of the roles that different factors might play in regeneration. Thus, the potential role of morphogens in regeneration from the developmental findings will be briefly discussed as well.

ROLE OF GROWTH FACTORS

Several growth factor families have been identified in vertebrates that play crucial roles in development of different tissues and organs, including the CNS. Here we discuss the role of such families in the regulation of neural regeneration.

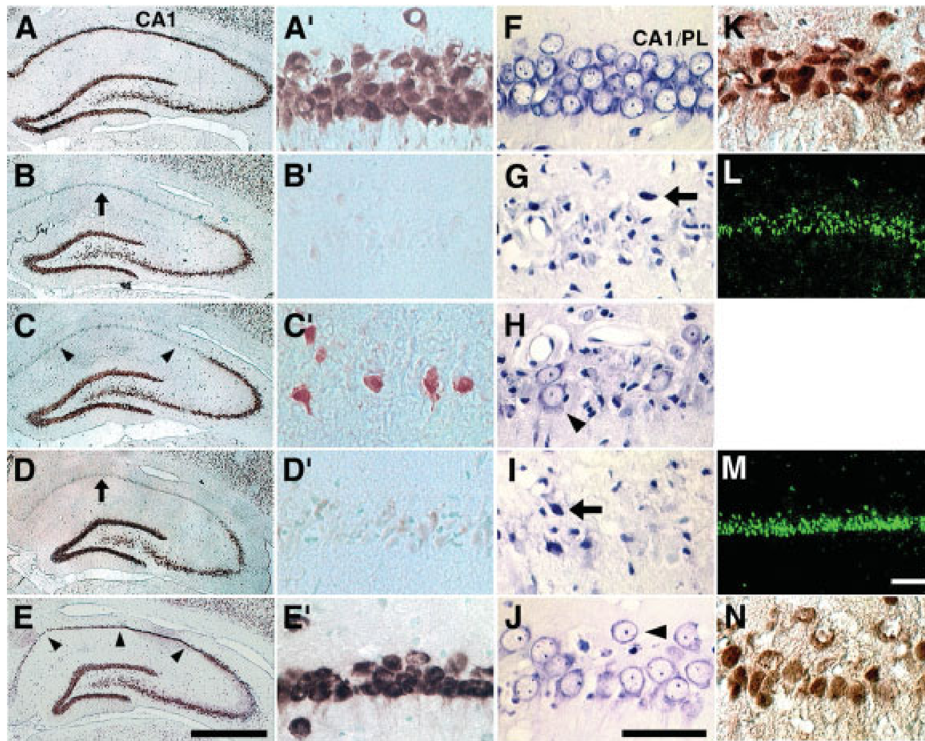

Figure 1 Hippocampal CA1 Pyramidal Neurons degenerate following Ischemia and eventually regenerate via recruitment of neural progenitors. Growth factors markedly augment this regenerative ability. (A–E′) NeuN (neuronal marker) and cresyl violet (F–J) staining (a histological stain for nervous tissue) of the CA1 pyramidal layer (CA1/PL) in intact (A, A′, and F), untreated/ishemic (B, B′, and G, at 7 days after ischemia; C, C′, and H, at 28 days after ischemia), and growth factor-treated animals (D, D′, and I, at 7 days after ischemia; E, E′, and J, at 28 days after ischemia). (A′– E′) are higher magnification views of the CA1 regions shown in (A–E) (arrows/arrowheads). (G–J) Cells with pyknotic morphology are indicated by arrows and cells with intact morphology are indicated with arrowheads. Expression of SCIP, a transcriptional factor expressed in adult CA1 neurons, in the intact CA1 region and in the growth factor treated tissue 27 days after ischemia (K and N respectively). TUNEL (L and M) staining of CA1 neurons, indicating levels of cell death 7 days after ischemia in untreated and growth factor treated hippocampus respectively. Scale bar for (A–E) shown in E equals 1 mm; for (A′–E′), (F–K), and (N) shown in J equals 50 μm; for (L) and (M) shown in M equals 100 μm. (Courtesy of Dr. Masato Nakafuku. Reprinted from Cell, 110, Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M., Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors, Pages 429–441, Copyright 2002, with permission from Elsevier).

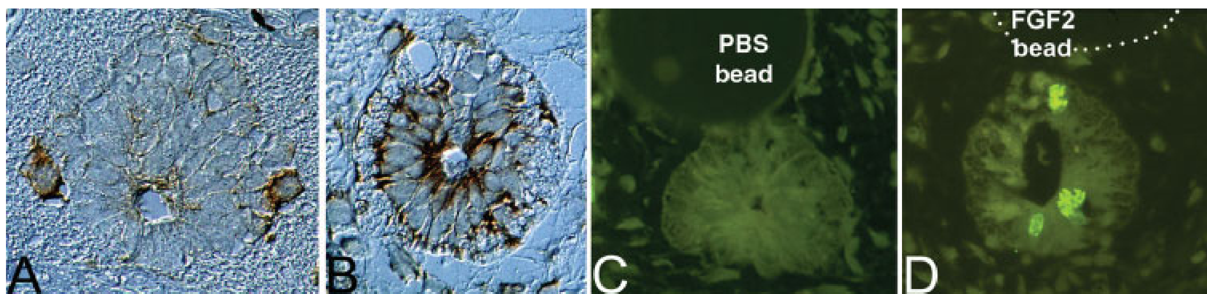

Figure 2

The infusion of EGF into the lateral ventricle of normal mice lead to the induction of proliferation in the SVZ, resulting in a migration of the newly generated cells into the striatum, the cortex, and the septum. Seven weeks after the treatment, 25% of the newly generated cells survived and differentiated mainly into astrocytes (28%) but also into neurons (3%) (Craig et al., 1996). This experiment showed for the first time that exogenous factors can induce the formation of new neurons in the brain, including areas normally devoid of neurogenesis during adulthood. Interestingly, the infusion of EGF failed to reproduce similar results in rats (Kuhn et al., 1997). In this system EGF stimulated SVZ progenitor proliferation, but no new neurons were formed and only glial cells were observed in the striatum. Moreover, EGF infusion decreased the number of neurons generated in the olfactory bulb and increased the appearance of glial cells. The reason for the difference between these two experiments could either be due to the animal species or to the bioavailability factor.

On the other hand, experiments performed by Fallon et al. (2000) revealed that the activation of the EGF receptor (EGFR) can induce new cells with the appearance of neurons in rats after injury, suggesting that the activation of the EGF pathway can indeed lead to neurogenesis. The infusion of TGFα, a ligand of the EGFR, into the telencephalon of rats in which the substantia nigra had been depleted of dopaminergic neurons by treatment with 6-hydroxydopamine, showed massive migration of newborn cells into the striatum that later differentiated into neurons and astrocytes. These experiments revealed that the endogenous progenitor population can be stimulated to potentially regenerate a lesioned area of the brain and that the EGFR signaling activation is effective both for the stimulation of proliferation of neural progenitors and for the differentiation of glia and neurons. Nonetheless, the mode of administration seems to be critical for the efficiency of the treatment. This approach may have implications in the generation of dopaminergic neurons in animal models of Parkinson's disease or in neuronal replacement after ischemic damage. Indeed, as discussed in the introduction, a study by Nakatomi et al. (2002) has shown that ischemia in the hippocampus area leads to the death of numerous neurons of the CA1 region immediately after the hypoxic condition (Fig. 1B, B′, G, L). Interestingly, an endogenous process of regeneration is observed 28 days after ischemia (Fig. 1C, C′, H). Infusion of FGF-2 and EGF between 2 and 5 days post-ischemia does not prevent neuronal loss, but dramatically enhances the process of regeneration (Fig. 1D, D′, I, M, E, E′, J, N). The neuronal recovery represented around 40% of the initial neuronal number. Moreover, they observed formation of new synapses and integration of the newly formed neurons into the brain circuitry, as assayed by electron microscopy, retrograde labeling and electrophysiological studies. Furthermore, the growth factor treatment was able to restore brain functions such as spatial learning and memory that were affected by the ischemic damage. This functional recovery was demonstrated using the Morris Water-Maze task, in which the rats learn to navigate to a platform submerged in a water pool by using extra-maze cues and memorize its position, being able to reach it in increasingly shorter times. Ischemic animals showed severe impairments in the acquisition of new memory, which was remarkably improved by the growth factor treatment.

An increase in regeneration efficiency by EGF stimulation was also observed in the striatum after ischemic damage (Teramoto et al., 2003). In this study, EGF and albumin administration into the lateral ventricle two days after ischemia (a time when EGFRs are up-regulated) increased the replacement of neurons by around 100 fold.

EGF, either alone or in combination with FGF-2, has also been used to induce *in vitro* proliferation of multipotent cells from different brain areas, (Roy et al., 2000; Kukekov et al., 1999; Arsenijevic et al., 2001) and from human spinal cord (Walder and Ferretti, 2004).

From all these experiments, it appears that EGF is a key regeneration-inducing factor in various areas of the brain. However, there are other factors that can

Figure 2 Expression and effects of FGF-2 on urodele spinal cord regeneration. (A-B) Expression of FGF-2 protein in cross sections of normal and regenerating *Pleurodeles waltl* spinal cord. In normal spinal cord, FGF-2 staining (brown) is restricted to a subset of neurons, and the ependymal cells are negative (A). Following tail amputation, FGF-2 expression is significantly up-regulated in the ependymal tube (B) from which the new cord will form. (B-C) Effect of adding exogenous FGF-2 on cell proliferation, detected by expression of phosphorylated histone 3, a marker of mitosis, in the regenerating spinal cord. Mitotic figures are infrequently observed when PBS beads are implanted (C), but their number significantly increases ($p < 0.05$) in the proximity of FGF-2-soaked beads (D). (Courtesy of Dr. Patrizia Ferretti; Modified from Zhang et al., 2000).

enhance or inhibit the degree of neurogenesis and their modulation could be an important tool to optimize this phenomenon. Some of those factors are listed below and in the specific sections dedicated to each morphogen.

Brain derived neurotrophic factor (BNDF) can also enhance the production of neurons (Zigova et al., 1998; Benraiss et al., 2001). Several studies have shown that BDNF induces the differentiation of progenitors derived from the ganglionic eminences *in vitro* (Ahmed et al., 1995; Arsenijevic and Weiss, 1998). The infusion or the over-expression of BDNF into the lateral ventricle (Zigova et al., 1998; Benraiss et al., 2001) produced an increase in newly generated neurons in the olfactory bulb. Newly generated neurons were also observed in the striatum, but in a much lesser extent (Benraiss et al., 2001). These experiments suggest that BDNF may enhance neurogenesis and even promote neurogenesis in non-neurogenic areas.

IGF-I has also been shown to enhance neurogenesis *in vitro* (Arsenijevic and Weiss, 1998; Shimazaki et al., 1999; Aberg et al., 2003) and to participate in the neurogenic process in the olfactory bulb (Vicario-Abejon et al., 2003). Peripheral infusion of IGF-I stimulated cell proliferation of hippocampal progenitors in the dentate granule cell layer (Aberg et al., 2000). Interestingly, IGF-I treatment preferentially increased the generation of neurons instead of astrocytes in this area. Therefore this factor, known to play a key role in organ and body growth (Le Roith et al., 2001), may also be required endogenously during neurogenesis or neuronal regeneration, and may be an important player in the design of a regenerative strategy for the CNS.

In regards to the role of Fibroblast Growth Factors (FGFs) in the brain, Santa-Olalla and Covarrubias (1999) showed that in dorsal and ventral mesencephalon cultures, FGF-2 supports the survival of EGF-responsive neural precursors, and that in these cells, FGF-2 treatment up-regulates the expression of the EGFR. In other studies, infusion of FGF-2 into the lateral ventricle of adult rats failed to produce new neurons in the striatum, but slightly increased the number of neurons in the olfactory bulb (Kuhn et al. 1997).

During vertebrate spinal cord development, FGFs promote the development of the posterior part of the CNS by maintaining the proliferative state of neural progenitors. Loss and gain of function experiments in chick embryos showed that FGF signaling maintains the expression of cyclin D2 in the caudal neural epithelium, favoring proliferation of neural progenitors over neuronal differentiation (Lobjois et al., 2004). Zhang et al. (2000) studied the role of FGF-2 in adult spinal cord regeneration in *Pleurodeles waltl* after tail resection. This urodele has the capacity of spontaneously regenerating the tail. They found that FGF-2 is expressed in a subset of neurons in the normal spinal cord but is hardly detectable in ependymal cells (Fig. 2A). However, amputation of the tail results in an induction of the presence of this growth factor in ependymal cells, and later in regeneration in some newborn neurons (Fig. 2B). Moreover, exogenous administration of FGF-2 in the regenerating tail resulted in an increase in proliferation of the ependymal cells, which are the source of regeneration of the spinal cord in these animals (Fig. 2C, D).

During eye development, FGFs are able to induce neural retina differentiation and to even switch the fate of retinal pigmented epithelial cells (RPE) into neural phenotypes (Pittack et al., 1991; Guillemot and Cepko, 1992; Pittack et al., 1997). Moreover, when the retinal neuroepithelium is surgically removed in chick embryos at stage E4, exogenous FGF-2 is able to induce regeneration of the neural retina by transdifferentiation of RPE cells and also by the induction of neurogenesis from stem/progenitor cells present in the ciliary marginal zone/ciliary body (CMZ/CB) of the eye (Park and Hollenberg, 1989, 1991; Spence et al., 2004; Fig. 3B). Likewise, in *Xenopus laevis* tadpoles, RPE explants can be induced to transdifferentiate into neural retina *in vitro* when FGF-2 is added to the culture medium (Sakaguchi et al., 1997).

In the post-hatched chicken retina, populations of Müller glia cells can be activated in response to neurotoxic-injury and they can dedifferentiate, proliferate, become progenitor-like, and some even differentiate into certain retinal cell types (amacrine or bipolar); however in the non-damaged retina, an intraocular injection of a cocktail of insulin and FGF-2 is enough to produce similar effects over these glial cells (Fischer et al., 2002; reviewed by Moshiri et al. 2004; Fischer, 2005). Moreover, the combination of insulin and FGF-2 increased the regeneration of ganglion cells in retinas that had been damaged by treatment with kainate and colchicine, which specifically damage that cell type (Fischer and Reh, 2002). The ciliary body of the eye, which is not a neural tissue, can also be stimulated by certain growth factors to produce neurons in vivo. Intraocular injections of insulin, EGF or FGF-2 were able to induce proliferation and neurogenesis from cells in the non-pigmented epithelium of this zone in post-hatched chickens, and the effect of each of these factors was region-specific (Fischer and Reh, 2003; reviewed by Moshiri et al., 2004; Fischer, 2005). FGF-2, EGF or both growth factors have also been used to induce proliferation/differentiation *in vitro* of retinal stem/

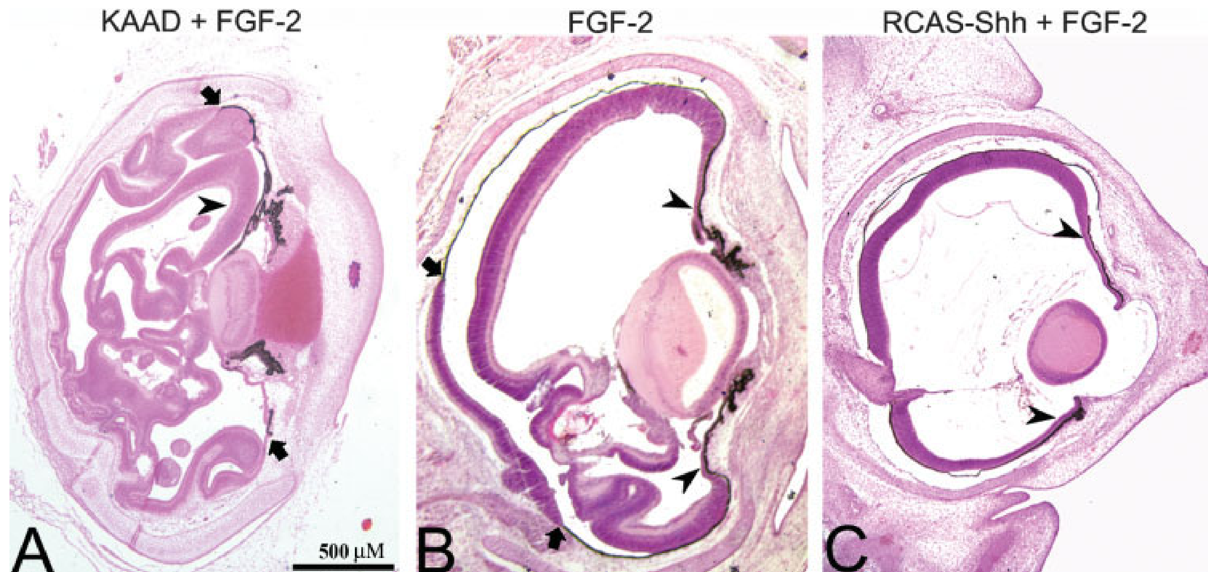

Figure 3 Retina regeneration in embryonic chick eyes can be modulated by the Hedgehog pathway. Retina regenerates via two sources in the embryonic chick eye as long as a source of FGF-2 is available (A–C). (B) Regenerated retina 7 days post-retinectomy, where retina has regenerated via transdifferentiation of the retinal pigmented epithelium (RPE) in the posterior region of the eye and from the activation of retinal stem/progenitor cells present in the anterior region of the eye. Note that when the RPE transdifferentiates to retina, it is not replenished. (A) When the Hh pathway is inhibited with cyclopamine/KAAD, the transdifferentiation domain is expanded from the posterior of the eye, where the FGF source is most concentrated, towards the anterior of the eye. Here most of the newly generated retina is missing RPE, a sign that this retina came from transdifferentiation. (C) When Shh is over-expressed via an RCAS virus, transdifferentiation is inhibited and most of the regeneration that takes place is due to the activation of stem/progenitor cells from the anterior region of the eye. Arrows denote transdifferentiation domain, while arrowheads denote the regenerated domain from activation of stem/progenitor cells. Scale bar shown in A equals 500 μM and applies to A, B, and C. (Modified from Spence et al., 2004).

progenitors cells from embryonic rats (James et al., 2004), of stem cells from the ciliary body of adult mice (Tropepe et al., 2000), rats (Ahmad et al., 2000), and humans (Coles et al., 2004).

These findings put together, suggest that FGFs are crucial factors in neural regeneration in the vertebrate CNS, inducing neurogenesis in stem/progenitor cells or inducing transdifferentiation of certain cell types. Given the complexity of the FGF signaling mechanism which involves different isoforms of the protein interacting with different receptors and activating a variety of intracellular signaling cascades, further investigation on the molecular mechanisms underlying this process need to be pursued.

ROLE OF RETINOIC ACID

Retinoic acid (RA) has been extensively studied as a key factor in the development of different vertebrate tissues and organs, because of its ability to promote differentiation and its role in the specification of positional information and therefore in tissue patterning. It was the first morphogen to be identified in vertebrates, and several studies have been performed dealing with its function in limb (reviewed by Tsonis, 1996; Maden and Hind, 2003), lens (Tsonis et al, 2000), and lung tissue regeneration (Massaro and Massaro, 1997; Hind and Maden, 2004).

RA is obtained from the diet in the form of retinylesters or β-carotene, and in the cytoplasm it is converted to retinoic acid, at which point it can enter the nucleus and bind to its receptors RAR (retinoic acid receptor) and RXR (retinoid X receptor). Different isoforms of these receptors exist, which in turn heterodimerize and recognize consensus sequences known as retinoic acid response elements ultimately regulating gene expression. There are two isomers of RA: all-trans RA and 9-cis RA that bind to different forms of the receptor.

RA is present at high levels in the developing nervous system, and its suppression leads to several

abnormalities including the absence of posterior hindbrain, abnormal dorso-ventral patterning of the spinal cord and lack of neurite outgrowth from the spinal cord to the periphery (Maden and Hind, 2003).

In the adult brain, RA has been shown to be involved in synaptic plasticity in the hippocampus; important in the acquisition of spatial and relational memory (Chiang et al., 1998; Misner et al., 2001; Etchamendy et al., 2001; Cocco et al., 2002); in the maturation of learned behaviors, as is the case of song learning in birds (Denisenko-Nehrbass et al., 2000; Denisenko-Nehrbass and Mello, 2001); and in the control of dopamine signaling in mesolimbic and mesostriatal neurons as well as in the survival of nigrostriatal dopaminergic neurons (McCaffery and Drager, 1994; Krezel et al., 1998; reviewed by Mey and McCaffery, 2004). Recent studies have shown that long term exposure of adult mice to clinical doses of 13-cis RA, a drug that is commonly used in the treatment of acne, reduced cell proliferation in the SVZ, and more markedly in the hippocampus (Crandall et al., 2004). This decline in cell division came with a lesser generation of neurons and, more importantly, with a decrease of spatial memory learning. These and previous data discussed, clearly show that several factors can either stimulate or inhibit neurogenesis during adulthood and may serve to promote or stop regeneration in damaged brain areas.

The role of RA on spinal cord regeneration has been studied using adult newt spinal cord explants which are able to extend neurites in culture conditions. Retinoic acid treatment in those explants can increase not only the length but also the number of neurites extending from them. Moreover, when a newt limb is amputated, the regenerating limb has to be re-innervated in order to be functional; thus, the regenerating blastema must produce an environment that is permissive for axon growth and provides guidance for those axons to reach their appropriate target. When spinal cord explants are co-cultured with regenerating blastemas, they are induced to extend more axons than when cultured alone, but that effect is reversed if the blastemas are treated with citral, an inhibitor of RA synthesis, suggesting that RA from the blastema has a synergistic or stimulatory role in its neurotrophic activity (Prince and Carlone, 2003).

Maden and Hind (2003) proposed an interesting hypothesis to explain the inability of the adult mammalian spinal cord to extend neurites in culture. They suggested that during the transition from embryo to adulthood, some of the transducers of the RA signal might be shut down permanently by transcriptional inactivation. The receptor responsible for transducing the RA signal in dissociated embryonic neurons is RARβ2, since it is up-regulated after RA addition, and an agonist of this receptor added to the cultures induces extensive neurite outgrowth (Corcoran et al., 2000). In addition, this receptor is up-regulated in embryonic spinal cord explants, which are able to extend neurites, but not in adult explants. Transfection of the RAR-β2 gene into adult mouse and rat spinal cord explants induced the formation of neurites in 70% of the cases, suggesting once again the importance of the RA pathway in the stimulation of axonal regeneration of neurons (Corcoran et al., 2002).

Retinoic Acid has also been implicated in the process of retina regeneration in rodents. When Müller glia cells are activated by toxic injury, an intraocular injection of RA increases the differentiation of proliferating cells into bipolar cells, suggesting that this morphogen does not increase proliferation in response to injury, but collaborates in the establishment of cell fates in the regenerate (Ooto et al., 2004). Nonetheless, the study of the molecular mechanisms underlying retina regeneration is only in its beginnings.

ROLE OF BONE MORPHOGENETIC PROTEINS

The Bone Morphogenetic Protein (BMP) signaling pathway is known to be involved in multiple developmental processes in vertebrates, including induction of proliferation and differentiation in the nervous system as well as in many different tissues and organs, triggering cell death, maintaining the proliferative state of neural progenitors and promoting neuronal survival (reviewed by Mehler et al., 1997; Kawabata et al., 1998; Peters, 2002).

The BMPs are members of the Transforming Growth Factor-β (TGF-β) superfamily. Secreted proteins like noggin and chordin regulate their activity by binding to them and preventing their interaction with the receptors. BMPs promote heterodimerization of a type I receptor (either type Ia or Ib) with a type II receptor. These receptors are serine-threonine kinases that phosphorylate Smad proteins, which can then dimerize and enter the nucleus to activate transcription of specific target genes (reviewed by Kawabata et al., 1998). The differential expression of type Ia and Ib receptors seems to be responsible for the different roles of BMPs during neural tube development, inducing a proliferative state of dorsal neural progenitors when high levels of type Ia receptors are expressed, and driving the cells to differentiation or apoptosis at a later stage, when the expression levels of type Ib receptors are higher (reviewed by Panchision and McKay, 2002).

In the adult vertebrate brain, BMP-4 is synthesized in the SVZ by GFAP positive cells (Lim et al., 2000) and the administration of BMP-4 to SVZ cells in vitro markedly inhibits neurogenesis. Additionally, BMP-7 over-expression in the lateral ventricle reduces proliferation in the SVZ cells by 2 fold, whereas opposite actions are observed with the administration of the BMP antagonist noggin. Lastly, SVZ progenitor cells, which normally differentiate preferentially into glial cells when transplanted into the striatum, can generate neurons if noggin is missexpressed by striatal cells (Lim et al., 2000). These experiments show that BMPs and their antagonists play an important role on the regulation of adult neurogenesis in the brain and the manipulation of their expression may help to regenerate neurons in damaged areas.

In the adult rat spinal cord, BMP-7 is expressed at low levels and only in glial cells. However its expression is up-regulated following traumatic injury, expanding its domain to motor neurons as well (Setoguchi et al., 2001). This effect could be due to a role of BMP-7 in protecting the cells from undergoing apoptosis. Also, considering the anti-neurogenic activity of this molecule and its role in inducing gliogenesis from neural precursors, it is possible that the increase on the expression levels aids in the scaring process. Thus, manipulation of the BMP pathway could be a potential tool to regulate regeneration in the adult mammalian spinal cord as well. Further research is needed however to address this issue, including the analysis of which BMP receptors are present in the injured tissue and the expression of other molecules that affect the pathway.

The tail of *Xenopus* tadpoles constitutes a good model to study spinal cord regeneration and the mechanisms behind the developmental loss of this capacity. These animals are able to regenerate their tail including the spinal cord, except for a refractory period between stages 45 and 47 in which healing takes place without regeneration (Beck et al., 2003). In these animals, expression of a constitutively active form of BMP-R1a results in the recovery of the regenerative capacity during the otherwise refractory period, and transgenic animals expressing a dominant negative form of the receptor or noggin, present decreased regeneration (Beck et al., 2003). The difference on the effect of BMP in the previously discussed experiments could be due to the expression of different types of BMP receptors in the described systems or to a dissimilar response in each animal model.

BMP molecules are also crucial for normal retinal development in vertebrates. BMP-7 knockout mice present severe defects in eye development while BMP-R1b knockout mice show defects in axon guidance as well as a significant increase in apoptosis at the end of the neurogenic period (Liu et al., 2003a). Lens-specific expression of Noggin in mice inhibits the formation of the ciliary epithelium, which normally expresses BMP-4 and BMP-7. Co-expression of BMP-7 can rescue the defects in the ciliary epithelium caused by ectopic Noggin (Zhao et al., 2002). These results suggest that the BMP pathway may play a role in establishing and possibly maintaining the retinal stem/progenitor cell population in the ciliary epithelium. On the other hand, in developing chick eyes, interfering with the BMP pathway by over-expressing noggin can either result in micropthalmia if manipulated at early stages, or it can result in abnormal formation of ventral structures, including transdifferentiation of the RPE into optic stalk-like tissue if manipulated at later stages of eye development (Adler and Belecky-Adams, 2002). In another study, BMP-4 reduced the proliferation of Muller glia cells in response to neurotoxic damage in post-natal chickens. This could be due to a direct effect of BMP-4 on these cells, or to a neuroprotective role of this protein, since it is able to reduce the number of apoptotic cells after the toxic treatment (Fischer et al., 2004). In all, these studies suggest that BMP signaling is essential for proper eye and retina development and could play critical roles during retina regeneration considering it is important in three of the main tissue sources of retina regeneration which include the ciliary epithelium, the RPE and Müller glia cells.

ROLE OF THE WNT PATHWAY

Wnt proteins are a large family of diffusible factors that are crucial for the patterning of axes, the control of proliferation, and specification of cell fates. In nervous tissues, these molecules act as conserved axon guidance cues, and are also able to remodel axons and act as retrograde signals during synapse formation in the Cerebellum (reviewed by Zou, 2004).

Wnt proteins bind to a membrane receptor, prizzled (PZ), leading to the activation of downstream effectors that ultimately promote the accumulation of β-catenin in the cytoplasm. Stabilized β-catenin translocates to the nucleus, where it binds to sequence-specific transcription factors to regulate gene expression. This canonical Wnt pathway is not the only mechanism by which Wnts exert their effects, and to further complicate the analysis of the function of these molecules, there are at least 19 Wnt factors and 10 prizzled receptors, as well as a few regulatory proteins identified in vertebrates, each of them exhibiting character-

istic and many times overlapping patterns of expression in different organs at different developmental times (See the Wnt web site by the Nusse laboratory at www.stanford.edu/~rnusse).

In the developing mouse brain, expression of a stabilized form of β-catenin is able to expand the neural precursor population in vivo (Chenn et al., 2002). Deficiency in Wnt-1 produces a phenotype that lacks the midbrain region, whereas deficiency in Wnt-3a or inhibition of the Wnt canonical pathway alters hippocampal development (McMahon et al., 1990, Lee et al., 2000, Kapsimali et al., 2004). On the other hand, studies performed in vitro with neural stem cell cultures, show a negative effect of Wnt-3a on the regeneration of neurospheres, driving differentiation of these cells into MAP-2 positive neuronal cells instead (Muroyama et al., 2004). This discrepancy could be due to an interaction with other signaling molecules in the *in vivo* context, regulation by the cellular environment, or redundancy of different Wnt molecules of overlapping expression.

During mouse spinal cord development, Wnt genes are primarily expressed in the ventricular zone, which remains mitotically active while other regions undergo differentiation (Parr et al., 1993). Furthermore, over-expression of Wnt-1, Wnt-3a and β-catenin increase cellular proliferation in this zone (Dickinson et al., 1994; Megason and McMahon, 2002). It is interesting to point out that in urodeles, Wnt expression is maintained in the CNS throughout adulthood. Wnt-5a, Wnt-5b, Wnt-7a and Wnt-10a are expressed in overlapping but not identical domains along the antero-posterior axis of the brain and spinal cord, each of them exhibiting gradual variations along that axis. It has been suggested that the sustained expression of Wnt in the adult urodele CNS might explain in part the maintenance of the morphogenetic potential of the neural cells and thus the outstanding regenerative capacities in these animals (Caubit, et al., 1997).

Several of the known Wnt ligands and their frizzled receptors are expressed in the retina with Wnt-2b (previously Wnt 13), frizzled-4, and LEF-1 specifically expressed in the anterior margin of the developing chick, mouse and zebrafish eye (Jasoni, 1999; Dorsky et al., 2002; Fuhrmann et al., 2003; Kubo et al., 2003; Liu et al., 2003b). Over-expression of Wnt-2b during chick retina development results in an increase in retinal stem/progenitor cells present in the anterior margin of the eye, while an inhibition of the Wnt pathway, by inhibiting the interaction of β-catenin and LEF-1, increased differentiation of the retinal stem/progenitor cells in this anterior region along limited retinal lineages (Kubo et al., 2003). These results suggest that the Wnt pathway plays a role in controlling stem/progenitor cell proliferation and delaying differentiation during retina development. Despite all the accumulated evidence, especially from developmental studies suggesting a role of Wnt genes in the control of neurogenesis, which make them major candidates to play a part in regeneration of neural tissues after damage, no conclusive studies have been performed linking these morphogens with regeneration of the CNS particularly during adulthood.

ROLE OF THE HEDGEHOG PATHWAY

Hedgehog (Hh) molecules have been implicated in proliferation, differentiation, morphogenesis and survival in many different systems. Hedgehog secreted proteins including Sonic (Shh), Indian (Ihh) and Dessert (Dhh) signal via their transmembrane receptor Patched (Ptc) which in the absence of a ligand, blocks the function of Smoothened (Smo), another transmembrane protein. When Hh molecules bind to Ptc, this block is released and Smo initiates a signaling cascade that eventually results in the activation of Gli proteins, which can translocate into the nucleus and control transcription of target genes (reviewed in Lum and Beachy, 2004).

Shh is a classic morphogen controlling the polarity of the CNS during early development, defending the ventral phenotype of the neural tube and inducing the formation of specific neurons (Echelard et al., 1993; Marti et al., 1995; Roelink et al., 1995; Ericson et al., 1995; Chiang et al. 1996; Patten and Placzek, 2000). It is essential for the correct specification of the midline in the brain (Macdonald et al., 1995; Chiang et al., 1996; Rallu et al., 2002), and when Hh signaling is inhibited, the midline fuses creating cyclopic monsters. Hh has been implicated in the specification of proximal-distal polarity as well (Chiang et al., 1996; Macdonald et al., 1995; Ekker et al., 1995).

During brain development, Hh signaling also regulates ventral structures (Chiang et al., 1996; Kohtz et al., 1998; Gaiano et al., 1999; Gunhaga et al., 2000; Rallu et al., 2002; Fuccillo et al., 2004), and the specification of oligodendrocytes (Lu et al., 2000; Nery et al., 2001). Recently it was discovered that the identities of the thalamus and the prethalamus depend on Shh signaling (Keicker and Lumdsen, 2004), and that it plays a major role during the formation of the cerebellum, where granule cell precursors depend on Shh to proliferate (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Corrales et al. 2004).

During adulthood, Ptc is present in neural progenitors of the hippocampus (Lai et al., 2003). *In vitro* stimulation of these progenitors with Shh increases cell proliferation markedly, while missexpression of Shh in vivo results in a 3 fold increase in cell proliferation (Lai et al., 2003). Similar results are observed in the hippocampus and the subventricular zone (SVZ) when hedgehog agonists are administrated orally (Machold et al., 2003), whereas the administration of Shh antagonist, cyclopamine, provokes a decrease in cell proliferation in the hippocampus (Lai et al., 2003). In addition, an upregulation of Ptc is observed in the striatum showing that the Shh pathway may be activated in the parenchyma of the adult brain. On the other hand, a Smo/Cre-floxed mice under the control of the nestin promoter, show that at P15 there are less cells in the SVZ, in the olfactory bulb, as well as in the dentate gyrus (Machold et al., 2003). In these animals, the number of SVZ neurospheres (colonies formed by neural stem cells [NSCs] in vitro) is markedly reduced. Interestingly, the size and the renewal ability (for a secondary passage) of the mutated spheres are similar to wild type spheres, suggesting that Shh is necessary for the maintenance of NSCs in their niche. Altogether, these experiments show that Shh is an important regulator of adult brain neurogenesis and therefore could potentially play an important role during repair and regeneration.

Shh is also important in the specification of ventral neural progenitors, in the control of cell proliferation and in the establishment of cell fates in the embryonic spinal cord (Briscoe et al., 2000; Jessell, 2000; Lee and Pfaff, 2001; Lobjois et al., 2004; Park and Appel, 2004), however the role of Shh in regeneration of the adult spinal cord is still elusive. Injection of Shh into demyelinated lesions in adult rat spinal cords promoted proliferation of nestin-positive cells, possibly derived from neural precursor cells (Bambakidis et al., 2003). In a different study, Shh was added to implanted oligodendrocyte precursors in rats that underwent incomplete spinal cord contusions, improving their effect on axonal conduction and spinal cord function as well as inducing endogenous proliferation of nestin-positive cells (Bambakidis and Miller, 2004).

During development of the eye, a source of Shh emanates from the eye primordium itself in chick and may play a role in the establishment of the dorso-ventral patterning of the eye during the transition from the optic vesicle to the optic cup (Zhang and Yang, 2001a). Hh signaling is also required for the regulation of differentiation and the proper lamination and organization of the retina and the differentiation of the optic disc and stalk (Levine et al. 1997; Neumann and Nusslein-Volhard, 2000; Stenkamp et al., 2000; Zhang and Yang, 2001b; Wang et al., 2002; Dakubo et al., 2003; Stenkamp and Frey, 2003; Dakubo and Wallace, 2004; Shkumatava et al., 2004), and it is essential for the differentiation of the RPE and the proximo-distal patterning of the eye (Perron et al., 2003).

As explained before, embryonic chicks can completely regenerate their retinas if removed, by transdifferentiation of the RPE and by the activation of retinal stem/progenitor cells located in the anterior region of the eye. These activities require a source of FGF-2 (Fig. 3 B). In addition, the transdifferentiation domain is limited to the area close to the FGF-2 source, however if the Hh pathway is inhibited during this process, the transdifferentiation domain expands to encompass most of the eye (Fig. 3A). On the other hand, if Shh is over-expressed, the RPE phenotype is maintained and transdifferentiation is inhibited. At the same time, activation of stem/progenitor cells from the anterior region of the eye is enhanced, and this activation can take place even in the absence of FGF-2 (Spence et al., 2004; Fig. 3C).

It is clear that the Hh pathway can modulate the type of retina regeneration that takes place in the embryonic chick eye and it does this in close contact with the FGF pathway (Fig. 3). Over-expressing Shh reduces FGF-2 induced signaling in the RPE as evident from the decrease of ERK activity. In addition, inhibiting the Hh pathway in the absence of FGF-2 is not sufficient for tansdifferentiation to take place. Interestingly, Hh induced regeneration from the anterior margin is inhibited in the presence of FGFR-specific inhibitors, indicating that even though Hh can activate these stem/progenitor cells in a FGF-2 independent manner, it still requires the activation of the FGF signaling cascade (Spence et al. 2004). This activity resembles the one displayed by oligodendrocyte during in vitro differentiation, where neocortical precursors can differentiate to oligodenrocytes precursors in the presence of either FGF-2 or Shh, however, the Hh-stimulated differentiation is dependent on a constitutive activity of FGFRs that maintain a basal level of MAPK (Kessaris et al., 2004).

The mitogenic effect of Shh on the stem/progenitor population of cells from the retina and anterior region of the eye has been observed in the postnatal chick, mice and Xenopus (Jensen and Wallace, 1997, Levine et al., 1997; Wang et al., 2002; Amato et al., 2004; Dakubo and Wallace, 2004; Moshiri and Reh, 2004; Moshiri et al., 2004). As a matter of fact, a Ptc −/+ mouse model where the Hh pathway is activated, has shown the appearance of a progenitor-like region in the anterior region of the eye, never seen in

rodent eyes before, as well as proliferating cells in their retinas throughout the first postnatal week. When these mice are crossed with retinal degeneration background mice (pro23his rhodopsin transgenic), this progenitor-like region proliferates and differentiates to photoreceptors, the lost cell type in this retinal degeneration mouse model (Moshiri and Reh, 2004).

Muller glia cells which express Ptc-1 have been shown to respond to Hh signaling by proliferating, differentiating and establishing/ maintaining the laminar organization in the developing retina (Jensen and Wallace, 1997; Wang et al. 2002; Amato et al., 2004; Dakubo and Wallace, 2004; Shkumatava et al., 2004). It would be interesting to know how the Hh pathway influences Muller glia responses to injury including dedifferentiation.

CONCLUSIONS

The central nervous system of vertebrates does have a certain potential for regeneration, which can be manipulated through the control of different signaling pathways. The brain, the spinal cord and the retina use different sources of cells to replace lost neurons, with varying success depending on the animal model, its developmental stage and the type and size of the lesion. In this context, molecules that have been known for some time to act as morphogens, establishing positional information and regulating cell fate during development through their specific patterns of expression, are now being analyzed from a different perspective as important inducers or modulators of the regenerative potential of the central nervous system. It is important to point out though that this is only one aspect of the complex process of regeneration, and that there are also intrinsic factors that are likely to affect this process, as well as other categories of extrinsic factors including molecules involved in cell-cell interactions, hormones, or different neurotrophic factors that might not act as morphogens. It is also important to emphasize the complexity of the signaling mechanisms of each of these morphogens if they are to be used in the manipulation of regeneration, especially since they can affect each part of the CNS in different ways at different time points. In addition, there is certainly not only one signal involved in the process, but a combination of them. To complicate things further, it has been shown that the pathways by which the different morphogens exert their actions interact with each other at different levels, however, the exact way in which this takes place remains to be elucidated for most of them. These difficulties should be (and certainly are) seen by investigators as an incentive to try to unravel the mechanisms that govern the regenerative process, for the outcome of such research is bound to have an important impact, not only in the development of treatments for degenerative diseases or injuries of the central nervous system, but also for the advancement of knowledge in the areas of neurobiology, developmental biology, regeneration and even functional biochemistry. The rate of progress in neuronal regeneration in the last few years has been exceptional, however there is still a lot to be done in this novel and exciting field.

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