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Growth Hormone and Kynesitherapy for Brain Injury Recovery

Jesús Devesa^{1,2}, Pablo Devesa^{1,2}, Pedro Reimunde² and Víctor Arce¹

¹*Department of Physiology, School of Medicine, Santiago de Compostela*

²*Medical Center "Proyecto Foltra", Teo
Spain*

1. Introduction

Acquired brain injury is a medical and social reality of growing magnitude and extraordinary severity requiring an increasingly specialised response to the extent permitted by technological advances and research. Although there are no accurate or reliable statistical data regarding the number of people affected by brain injury, scientific opinion maintains that brain injury represents one of the largest health problems in developed countries, both in terms of the number of deaths caused and the high number of people left suffering from some sort of functional and cognitive disability as a result of the sequelae caused by the damage occurred in the brain.

The most frequent causes of acquired brain injury are pre/perinatal hypoxia/ischemia or post-natal infections (for example, meningitis), cranial traumas, stroke. One of the first consequences of acquired brain injury is the loss of consciousness; the duration and degree of it are one of the most significant indicators of the severity of the injury. After the progressive recovery of the level of consciousness and the spatial orientation, most patients suffer a wide range of cognitive and motor sequelae; the nature and severity of them depend on the location and extent of brain damage, and the age of the patient as well.

The multiple functional and social impairments caused by the brain injury and the motor impairments affecting memory, speech or behaviour require a multi-disciplinary treatment approach from the critical-acute stage, calling for the highest level of specialisation until the patient can readjust back into the community. There is consensus among specialists regarding the damaged brain's ability to recover part of its functions spontaneously, a process that may take several months or even years. Experts also agree on the need for early neurorehabilitation to improve these natural mechanisms and achieve the best possible functional and social recovery, considering the complexity of the sequelae (motor, cognitive, emotional) caused in varying combinations by brain injury. However, access to rehabilitation facilities specialized in brain injury is marked by the shortage of public or private resources, with a huge difference in the availability of such facilities among the different countries. This shortage, scarcity or non-existence of public or private rehabilitation facilities in many countries, together with the enormous cost of these services offered by private centres, make access to rehabilitation and recovery difficult or even impossible for many people. Therefore, as a result of not seeing the benefit of potential recovery and social integration, this leads to a greater dependence and burden on the family. Therefore, acquired brain injury is a major public health, and yet

medical science has little to offer for the persistent symptoms that prevent many of these individuals from fully re-entering society.

Until few years ago it was thought that recovery from a brain injury would occur through the reinforcement of the mechanisms of neural plasticity. Establishing new synaptic connections between surviving neurons would partially allow to recover lost functions. There are evidences that environmental enrichment induces epigenetic changes that facilitate synaptogenesis and memory in models of brain plasticity. On this basis special units for brain recovery should be the closest correspondence for an enriched environment for patients with brain injury. Anything that can be done to optimize the hospital and rehabilitation environment should be beneficial. No study was shown to what extent the beneficial effect is due to specific rehabilitation strategies, to the time spent in physiotherapy and occupational therapy, and a non-specific effect of a more stimulating environment with competent staff that can encourage and support the patients and family members, but all these factors are likely to be important. In all, it is likely that admission to an acute rehabilitation unit would benefit the recovery after a brain damage (Johansson, 2011).

Today we know that apart of brain plasticity, the development of any brain injury quickly leads to enhanced proliferation of neural stem cells. From the damaged cerebral areas a number of cytokines would be released for activating the migration and differentiation of newborn cells. It has been recently identified a P2Y-like receptor GPR17 that is a sensor of brain damage and a new target for brain repair (Lecca et al., 2008). Upon brain injury, the extracellular concentrations of nucleotides and cysteinyl-leukotrienes (cysLTs), two families of endogenous signaling molecules, are markedly increased at the site of damage, suggesting that they may act as "danger signals" to alert responses to tissue damage and start repair. In brain telencephalon, GPR17, a recently orphanized receptor for both uracil nucleotides and cysLTs (e.g., UDP-glucose and LTD4), is normally present on neurons and on a subset of parenchymal quiescent oligodendrocyte precursor cells. Induction of brain injury using an established focal ischemia model in the rodent induces profound spatiotemporal-dependent changes of GPR17. In the lesioned area, an early and transient up-regulation of GPR17 in neurons expressing the cellular stress marker *Heat shock protein 70* (Hsp70, *Heat shock protein 70*) is observed. Magnetic resonance imaging in living mice showed that the *in vivo* pharmacological or biotechnological *knock down* of GPR17 markedly prevents brain infarct evolution, suggesting GPR17 as a mediator of neuronal death at this early ischemic stage. At later times after ischemia, GPR17 immuno-labeling appeared on microglia/macrophages infiltrating the lesioned area to indicate that GPR17 may also acts as a player in the remodeling of brain circuitries by microglia. At this later stage, parenchymal GPR17+ oligodendrocyte progenitors started proliferating in the periinjured area, suggesting initiation of remyelination. The *in vitro* exposure of cortical pre-oligodendrocytes to the GPR17 endogenous ligands UDP-glucose and LTD4 promoted the expression of myelin basic protein, confirming progression toward mature oligodendrocytes. Thus, GPR17 may act as a "sensor" that is activated upon brain injury on several embryonic distinct cell types, and may play a key role in both inducing neural death inside the ischemic core and in orchestrating the local/remodeling repair response (Lecca et al., 2008).

According to these concepts two different mechanisms are involved in trying to repair brain damage: 1) quick proliferation of neural precursors, and 2) development of neural plasticity. Both are independent but complementary of each other. Neural stem cells proliferation and brain plasticity require the intervention of neurotrophic factors. This concept is showed in Figure 1.

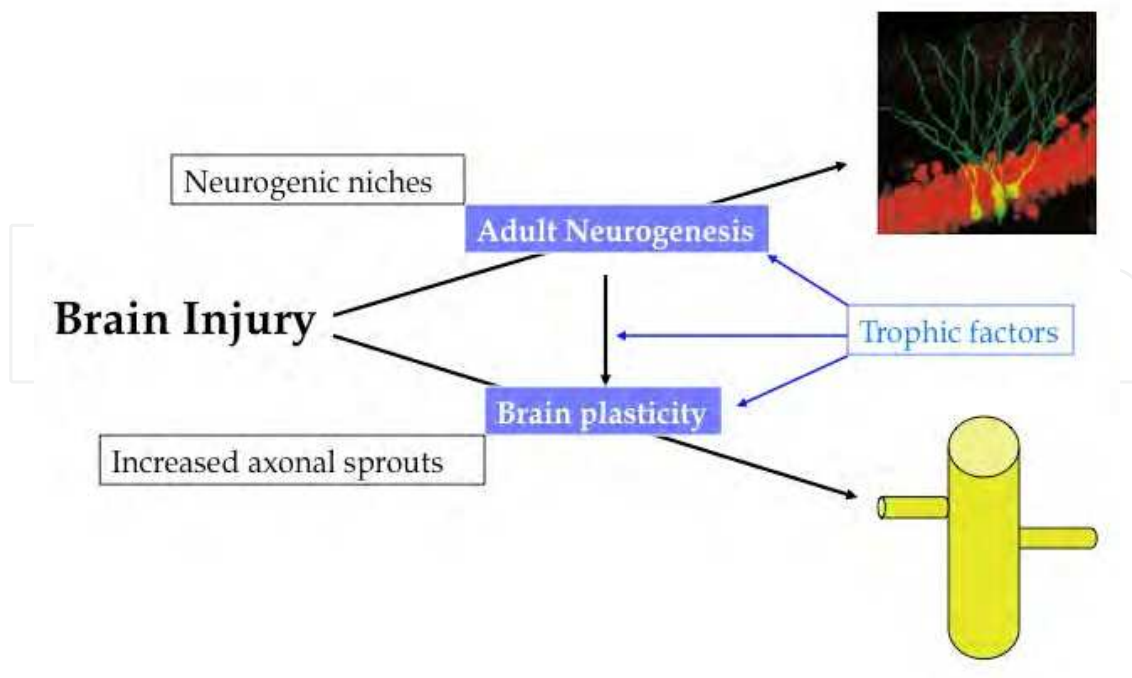


Fig. 1. Physiological responses to a brain injury. After a brain injury adult neurogenesis starts in neurogenic niches. Later, adequate rehabilitation and rich environment facilitate the development of brain plasticity. Both responses require the intervention of neurotrophic factors.

The knowledge of these and other events occurring after brain injury led to investigate how to enhance and improve mechanisms involved in brain repair.

In this chapter we will analyze current concepts about brain stem cells proliferation for brain repair and the effect of administering a neurotrophic and neuroprotective agent, growth hormone together with specific kinesitherapy, on the recovery of different kind of patients with acquired brain injury.

2. Adult neurogenesis

The complexity and specialization of neural functions increase as animal species ascend in the zoological scale. It is clear that the degree of specialization achieved, with so many and so complex interactions and functions, requires a long period of time for morphogenesis, in which not only cell proliferation and differentiation occurs, but also apoptosis must take place in a perfectly scheduled sequential expression of many different genes; later, after birth, the morphogenetic development of central nervous system is modulated by learning phenomena and the environment. However, this classical concept about neural development establishing that no new neurons are formed after birth has been changed when it was reported that new neurons are produced daily in several niches of the adult brain in many mammal species, including humans (Kuhn et al., 1996; Eriksson et al., 1998; Kempermann et al., 2004). This production is particularly important in the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus of the hippocampus, where neural progenitors reside primarily in the subgranular layers (Gould et al., 1999; Fukuda et al., 2003; Kronenberg et al., 2003).

The paradigm about a central nervous system unable to replace the daily loss of neurons began to be questioned in the mid-60's. However, the pioneer studies carried out in these years were not taken into account mainly due to technical and methodological issues (Altman & Gas, 1965; Kaplan & Hinds, 1977). Later, the notorious advance of the immunohistochemistry, cytology and molecular biology changed the dominant paradigm. Researchers, as Goldman (Goldman & Nottebohm, 1983) suggested that the generation of new neurons, neurogenesis, could be a normal phenomenon in the adult canary brain. The same group demonstrated later that the new formed neurons were incorporated into functional circuits (Paton et al., 1984). In the early 90's, cells with properties of neural precursors were isolated in the striatum of mice (Reynolds & Weiss, 1992). Soon the subventricular zone was identified as a niche where neural precursors were formed (Lois & Alvarez-Buylla, 1993). Lastly, at the end of 90's, it was described that adult neurogenesis is a phenomenon that also occurs in higher primates, including humans (Eriksson et al., 1998; Gould et al., 1999), a concept now widely accepted.

2.1 Where adult neurogenesis occurs and what is its functional significance?

Constitutive neurogenesis takes place in the rodent adult mammalian brain, and particularly in the subventricular zone. This zone harbors a population of stem cells that proliferate and give rise to neurons and glial cells. Neuroblasts formed here migrate long distances along the rostral migratory stream toward the olfactory bulb, where they differentiate into GABA and dopaminergic interneurons, involved in odor discrimination (Betarbet et al. 1996; Gheusi et al., 2000). Following cerebral injuries, such as ischemia, epileptogenesis, or focal neuronal degeneration, neurogenesis increases in the subventricular zone and the newly formed neurons are able to repopulate damaged areas (Parent, 2002; Romanko et al., 2004; Zhang et al., 2004). Apart of the subventricular zone and the subgranular zone of the dentate gyrus, recent data indicate that adult neurogenesis can also occur in other brain areas. It is thought that in the adult mammal brain physiological anti-neurogenic influences can be removed in pathological conditions or after any specific injury. This has been recently demonstrated in a model of unilateral vestibular neurectomy (UVN) that mimics human pathology in adult cats (Dutheil et al., 2011). UVN promoted an intense reactive cell proliferation in the deafferented vestibular nuclei located in the brainstem. The new cells survived up to one month, differentiated into glial cells - microglia or astrocytes - or GABAergic neurons, so highlighting a GABAergic neurogenesis. Surprisingly, post-UVN reactive cell proliferation contributed successfully to fine restoration of vestibular posturo-locomotor functions. Moreover, following brain injury, glia outside known neurogenic niches acquire or reactivate stem cell potential as part of reactive gliosis (Robel et al., 2011). A comparison of molecular pathways activated after injury with those involved in the normal neural stem cell niches highlights strategies that could overcome the inhibition of neurogenesis outside the stem cell niche and instruct parenchymal glia towards a neurogenic fate. This new view on reactive glia therefore suggests a widespread endogenous source of cells with stem cell potential, which might potentially be harnessed for local repair strategies (Robel et al., 2011).

Evenmore interesting is the fact that in the dentate gyrus of the adult hippocampus a continuous incorporation of new neurons exists. These new neurons slowly integrate into the existing dentate gyrus network: immature adult-born neurons appear to function as pattern integrators of temporally adjacent events, thereby enhancing pattern separation for events separated in time; whereas maturing adult-born neurons it is likely that contribute to

pattern separation by being more amenable to learning new information, leading to dedicated groups of granule cells, the principal projection neurons of the dentate gyrus, that respond to experienced environments (Aimone et al., 2010). This continuous neurogenesis is important for hippocampal function. At different stages in its maturation, each new neuron has different properties and, at any given time, the dentate gyrus population consists of excitatory granule cells of many different ages. The youngest, apparently more excitable in the network, could complement the pattern separation function of matures by adding a degree of similarity between events experienced close in time. Several models suggest that directing plasticity towards maturing neurons can preserve the representations of old memories in the dentate gyrus while maintaining its capacity to learn new information (Aimone et al. 2010).

Thus, although the role of this continuous adult neurogenesis remains to be fully established, a number of data suggest that it contributes to promote brain repair after injuries (Abdipranoto et al., 2008; Abdipranoto-Cowley et al., 2009). On the other side, it has been suggested that neurogenesis may be impaired in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, and this might contribute to the pathogenesis of these chronic neurodegenerative disorders (Abdipranoto et al., 2008; Yu et al., 2008).

2.2 How adult neurogenesis is regulated?

The knowledge about how adult neurogenesis is regulated may provide adequate therapeutic tools for trying to repair the brain after an injury. However this is a difficult task, because many factors seem to be involved, directly or indirectly, in this process. We will analyze here the role of some of these putative neurogenic factors for trying later to establish then a relationship between them and the effects of growth hormone on neurogenesis.

Since some years ago we know that adult neurogenesis can be modulated by several physiological processes including learning, exercise, environmental enrichment, or stress (Kempermann et al., 1997; Nilsson et al., 1999; van Praag et al., 1999; Trejo et al., 2001; Lledo et al., 2006; Zhao et al., 2008). Many, if not all, of these effects are related to signals mediated by hormones or growth factors.

2.2.1 Gonadal steroids and adult neurogenesis

In adult songbirds, integration and survival of new neurons in the vocal control nucleus is modulated by the gonadal steroids testosterone and estradiol (Nordeen EJ & Nordeen KW, 1989; Rasika et al., 1994; Hidalgo et al., 1995; Johnson & Bottjer, 1995). In mammals, estrogen has been implicated in hippocampal function, with sex differences observed in long-term potentiation (Maren et al., 1994) and performance of hippocampal-dependent tasks (Roof & Havens, 1992; Roof et al., 1993; Galea et al., 1996). During proestrus, a time when estrogen levels are high, cell proliferation in the subgranular zone of the dentate gyrus increases, compared with estrus and diestrus, when estrogen levels are lower (Tanapat et al, 1999). Estradiol exerts extensive influence on brain development and is a powerful modulator of hippocampal structure and function. Surprisingly, the remarkable increase in ovarian hormones existing during the first and third trimester of pregnancy has no effect on cell proliferation in the subgranular zone of the dentate gyrus, suggesting that concomitants changes in other factors, perhaps glucocorticoids, may counterbalance the positive regulation of cell proliferation by estradiol.

There is a sex difference in rates of cell genesis in the developing hippocampus of the laboratory rats, most likely occurring because of the effects of estradiol on brain during critical periods of neural development (Bowers et al., 2010). Males generate more new cells than females. A recent study shows that exogenous estradiol treatment promotes cell proliferation and survival in the neonatal female but not the male hippocampus, whereas antagonizing endogenous estradiol synthesis or action reduces cell proliferation in the male but not in the female hippocampus (Bowers et al., 2010). Moreover, in the adult female hippocampus, estradiol stimulates cell proliferation and survival and increases dendritic synapse density, while the adult male hippocampus is insensitive to the spinogenesis or cell genesis inducing effects of estradiol treatment. However, inhibiting aromatase activity or blocking estrogen receptor binding reduces cell proliferation in the developing male but not in the female hippocampus (Bowers et al., 2010). Both the amount of endogenous estradiol and aromatase activity in the developing hippocampus are very low compared to the hypothalamus and do not appear to be sexually dimorphic (Konkle & McCarthy, 2010), suggesting that hippocampal sensitivity to estradiol is high and differs between sexes.

The possibility exists that the effects of estradiol are not mediated directly at the hippocampus but, instead, were secondary to changes in other brain areas projecting to the hippocampus (Bowers et al., 2010). Cholinergic neurons of the medial septum/diagonal band of Broca are essential for estradiol-induced spinogenesis in adult CA1 hippocampus (Lam & Leranth, 2003) and cholinergic input modulates maturation and integration of adult born dentate gyrus granule cells (Campbell et al., 2010). Gonadally intact males release more acetylcholine into the hippocampus than females during locomotor tasks and this sex difference is organized by estradiol during development (Mitsushima et al., 2009a; Mitsushima et al., 2009b). The cholinergic system matures relatively early and more new septal cholinergic neurons are born in males during a brief period of gestation but the sex difference does not persist into adulthood (Schaevitz & Berger-Sweeney, 2005). Nonetheless, it is possible that the effects observed in the study of Bowers et al. are the results of estradiol-induced acetylcholine release into the neonatal hippocampus during the early postnatal period. It is also possible that estradiol is acting outside the central nervous system (Bowers et al., 2010). At this time it is important to remark that acetylcholine is a powerful inducer of pituitary growth hormone release (Devesa et al., 1992).

With regard to progesterone, it seems that enhances the survival of newborn neurons, rather than its proliferation, via the Src-ERK and PI3K pathways (Zhang et al., 2010). In fact, progesterone attenuates the estradiol-induced enhancement of cell proliferation (Galea et al., 2006).

2.2.2 Adrenal steroids and adult neurogenesis

Antidepressants increase adult hippocampal neurogenesis in animal models, an effect already described by Elizabeth Gould soon after discovering that adult neurogenesis occurs in non human primates (Gould et al. 1999); however, the underlying molecular mechanisms responsible for this effect of antidepressants were not known. Recent studies have suggested that glucocorticoids are involved in the neurogenic action of antidepressants (David et al., 2009; Huang & Herbert 2006). Glucocorticoids release is the main response of the organism to acute and chronic stress. Chronic exposure to stress results in a reduction of hippocampal neurogenesis and of hippocampal volume.

The antidepressant-induced changes in neurogenesis are dependent on the glucocorticoid receptor. Specifically, the selective serotonin reuptake inhibitor antidepressant, sertraline,

increases neuronal differentiation and promotes neuronal maturation of human hippocampal progenitor cells via a glucocorticoid receptor-dependent mechanism that is associated with glucocorticoid receptor phosphorylation via protein kinase A signaling, therefore increasing cytosolic cAMP levels. Interestingly, this effect is only observed when sertraline is present during the proliferation phase, and it is accompanied by exit of cells from the cell cycle, as shown by reduced proliferation and increased glucocorticoid receptor-dependent expression of the cyclin-dependent kinase 2 inhibitors, p27^{Kip1} and p57^{Kip2} (Anacker et al., 2011). These effects of antidepressants are only seen in the presence of glucocorticoids; however, the molecular processes that lead to increased neuronal differentiation are activated directly by antidepressants alone and do not require glucocorticoids (Anacker et al., 2011). That is, regulation of neurogenesis by antidepressants is complex; it involves different glucocorticoid receptor-dependent mechanisms that lead to enhanced cell proliferation without changes in neuronal differentiation, or enhanced neuronal differentiation in the presence of decreased cell proliferation.

The fact that antidepressants induce adult neurogenesis via glucocorticoid receptor-dependent mechanism is not in contradiction with previous studies demonstrating that adrenal steroids inhibit adult neurogenesis by suppressing cell proliferation in the hippocampal subgranular zone (Lenington et al., 2003). Aged rats and monkeys exhibit diminished cell proliferation in the subgranular zone as well as elevated levels of circulating glucocorticoids (Kuhn et al., 1996; Sapolsky, 1992). Removal of adrenal steroids by adrenalectomy increases cell proliferation in the subgranular zone in both aged and young adult (Cameron & McKay, 1999). Moreover, the number of new subgranular zone cells in adrenalectomized aged rats was threefold higher than the number in young control rats, indicating that adrenalectomized aged rats have rates of proliferation that surpass those normally found in young adults (Cameron & McKay, 1999). Moreover, a study on human post-mortem brain tissue has found that antidepressants increase the number of neural progenitor cells in patients with major depression to levels above those present in controls, and depressed patients are generally characterized by elevated endogenous levels of glucocorticoids (Boldrini et al., 2009). In the study of Anacker et al. (Anacker et al., 2011) the cell cycle-promoting genes, CCND1 and HDM2, were upregulated only by sertraline and dexamethasone co-treatment, the only condition which increases cell proliferation. In contrast, dexamethasone increased expression of the cell cycle-inhibiting genes, FOXO1 and GADD45B, which may explain the reduced cell proliferation and reduced neuronal differentiation with this treatment.

Experience has shown that therapy using music for therapeutic purposes has certain effects on neuropsychiatric disorders (both functional and organic disorders). However, the mechanisms of action underlying music therapy remain unknown, and scientific clarification has not advanced. The results of past studies have clarified that music influences and affects cranial nerves in humans from fetus to adult. The effects of music at a cellular level have not been clarified, and the mechanisms of action for the effects of music on the brain have not been elucidated. It has been proposed that listening to music facilitates the neurogenesis, the regeneration and repair of cerebral nerves by adjusting the secretion of steroid hormones, ultimately leading to cerebral plasticity. Music affects levels of such steroids as cortisol, testosterone and oestradiol, and it is likely that music also affects the receptor genes related to these substances, and related proteins (Fukui & Toyoshima, 2008).

2.2.3 Pituitary hormones and adult neurogenesis

Prolactin is a hormone that increases during the pregnancy and also at postpartum, signaling lactation. The study of Shingo et al. (Shingo et al., 2003) showed that neurogenesis rates increase in the subventricular zone during pregnancy by 65% and again after delivery. In addition to observing cell proliferation, this study tracked integration of new neurons in the olfactory bulb, indicating that olfactory discrimination is critical for recognition and rearing of offspring. A doubling of olfactory interneurons may thereby enhance olfactory function following pregnancy, providing the mother with enhanced olfactory capability (Shingo et al., 2003). A similar mechanism has been recently described in male mice. Paternal-adult offspring recognition behavior in mice is dependent on postnatal offspring interaction and is associated with increased neurogenesis in the paternal olfactory bulb and hippocampus. Newly generated paternal olfactory interneurons are preferentially activated by adult offspring odors, but disrupting prolactin signaling abolishes increased paternal neurogenesis and adult offspring recognition (Mak & Weiss, 2010).

Prolactin is a regulator of the stress response and stimulator of neurogenesis in the subventricular zone, but also protects neurogenesis in the dentate gyrus of chronically stressed mice and promotes neuronal fate (Torner et al. 2009). Neural stem and progenitor cells express the prolactin receptor and prolactin signals in these cells via ERK 1/2, however *in vitro* studies did not observe any effect of prolactin on these neural precursors proliferation, differentiation or survival, suggesting that prolactin action on *in vivo* neurogenesis occurs via an indirect mechanism (Wagner et al. 2009).

The effects of growth hormone on adult neurogenesis will be widely analyzed later in this chapter.

Recently it has been described that Oxytocin, but not Vasopressin, stimulates adult neurogenesis in the hippocampus of rats, even in animals subjected to glucocorticoid administration or cold water swim stress, indicating that the hormone stimulates neuronal growth and may protect against the suppressive effects of stress hormones on hippocampal plasticity (Leuner et al., 2011).

2.2.4 Growth factors and adult neurogenesis

When dissociated from the adult subventricular zone, neural stem cells require either epidermal growth factor (EGF) or basic fibroblast growth factor (FGF2) for self-renewal and long-term survival in culture (Reynolds & Weiss, 1992; Kuhn et al., 1997). Analysis of EGF and FGF2 responsiveness in the developing telencephalon indicates that early growth factor choice is temporally regulated (Tropepe et al., 1999; Maric et al., 2003). In the adult, the vast majority of subventricular zone cells expressing EGFR also express FGFR1 supporting the finding that most EGF-responsive cells can also be stimulated by FGF2 (Gritti et al., 1999). However, EGF and FGF2 appear to differ in their mechanisms of support, with EGF promoting faster expansion of the stem cell-like pool (symmetric division) compared to FGF2 (Gritti et al., 1999). This may be the result of differential control of cell cycle length by each growth factor, with SVZ stem-like cells cycling faster in the presence of EGF (Gritti et al., 1999). Alternatively, since the subventricular zone has two subsets of mitotically active cells, the neural stem cells (a relatively quiescent population with a cell cycle length up to 28 days) (Morshead & van der Kooy, 1992; Morshead et al., 1994), and the transitory amplifying progenitor (TAP) cells (cell cycle length approximately 12 h), these two growth factors may preferentially target one cell type. The latter appears to be supported by the work of Kuhn et al. (Kuhn et al., 1997) and Doetsch et al. (Doetsch et al., 2002). Kuhn et al.

(Kuhn et al., 1997) found that intracerebroventricular infusion of FGF2 into the lateral ventricle resulted in increased numbers of new neurons in the olfactory bulbe, while EGF infusion reduced the number of neurons reaching the olfactory bulbe, but substantially increased generation of astrocytes in the olfactory bulbe and the neighboring striatum. Extension of this study by Doetsch et al. (Doetsch et al., 2002) suggests that TAP cells are EGF receptive and these cells become invasive and glia-like, diverting neurogenesis to gliogenesis.

Recent studies using long-term ventricular infusion of EGF demonstrate intense cell proliferation around the ventricular wall, implicating the presence of EGF-reactive cells also outside the classical neurogenic lateral niche. Intraventricular injection of EGF induces within minutes CREB and ERK phosphorylation in astrocyte-like progenitor cells (type B cells) and EGF receptor-expressing transit-amplifying progenitor cells-both in the striatal and septal ventricular walls (Gampe et al., 2011). EGF infusion for 6 days induced continued CREB and ERK activation in nestin+ cells paralleled by intense periventricular cell proliferation. In addition, the ependyma became EGF receptor-immunoreactive, revealed intense CREB phosphorylation and underwent partial de-differentiation. These results demonstrate that intraventricular application of EGF induces CREB and ERK phosphorylation along the entire ventricular walls and thus permits a direct identification of EGF-responsive cell types. They further support the notion that not only the striatal ventricular wall where the subependymal zone is located but also the septal ventricular wall carries latent potential for the formation of neurons and glial cells (Gampe et al., 2011). Basal activity of CREB is required for the mitogenic signaling of EGF in neural stem cells at a level between ERK activation and SRE-mediated transcriptional activation (SRE: serum response element, a promoter sequence regulating c-fos gene expression) (Iguchi et al., 2011).

Interestingly, recent findings have shown that cells derived from subventricular zone Type-B cells (neural stem cells in the subventricular zone) actively respond to EGF stimulation becoming highly migratory and proliferative. A subpopulation of these EGF-activated cells expresses markers of oligodendrocyte precursor cells (OPCs). When EGF administration is removed, subventricular zone-derived OPCs differentiate into myelinating and pre-myelinating oligodendrocytes in the white matter tracts of corpus callosum, fimbria fornix and striatum. In the presence of a demyelinating lesion, OPCs derived from EGF-stimulated subventricular zone progenitors contribute to myelin repair. Given their high migratory potential and their ability to differentiate into myelin-forming cells, subventricular zone neural stem cells represent an important endogenous source of OPCs for preserving the oligodendrocyte population in the white matter and for the repair of demyelinating injuries (Gonzalez-Perez & Alvarez-Buylla, 2011). Of interest here is the fact that growth hormone induces EGF and EGFR expression in many territories (Pan et al., 2011) and activates EGFR by tyrosine phosphorylation as an essential element leading to MAP kinase activation and gene expression (Yamauchi et al., 1998).

Another growth factor involved in neurogenesis is the hematopoietic growth factor erythropoietin (EPO). mRNA and protein of EPO and its receptor (EPOR) are detected in a number of brain areas during brain development as well as *in vitro* in neurons, astrocytes, oligodendrocytes, microglia and cerebral endothelial cells. Expression of EPO and EPOR in the adult brain is stress-responsive and is regulated by oxygen supply; both are upregulated after hypoxia or ischemia. Other stimuli such as hypoglycemia and IGF-I activate hypoxia-inducible factor and lead to increased expression of EPO (Byts & Sirén, 2009). The tissue protective functions EPO are independent of its action on erythropoiesis. Peripherally

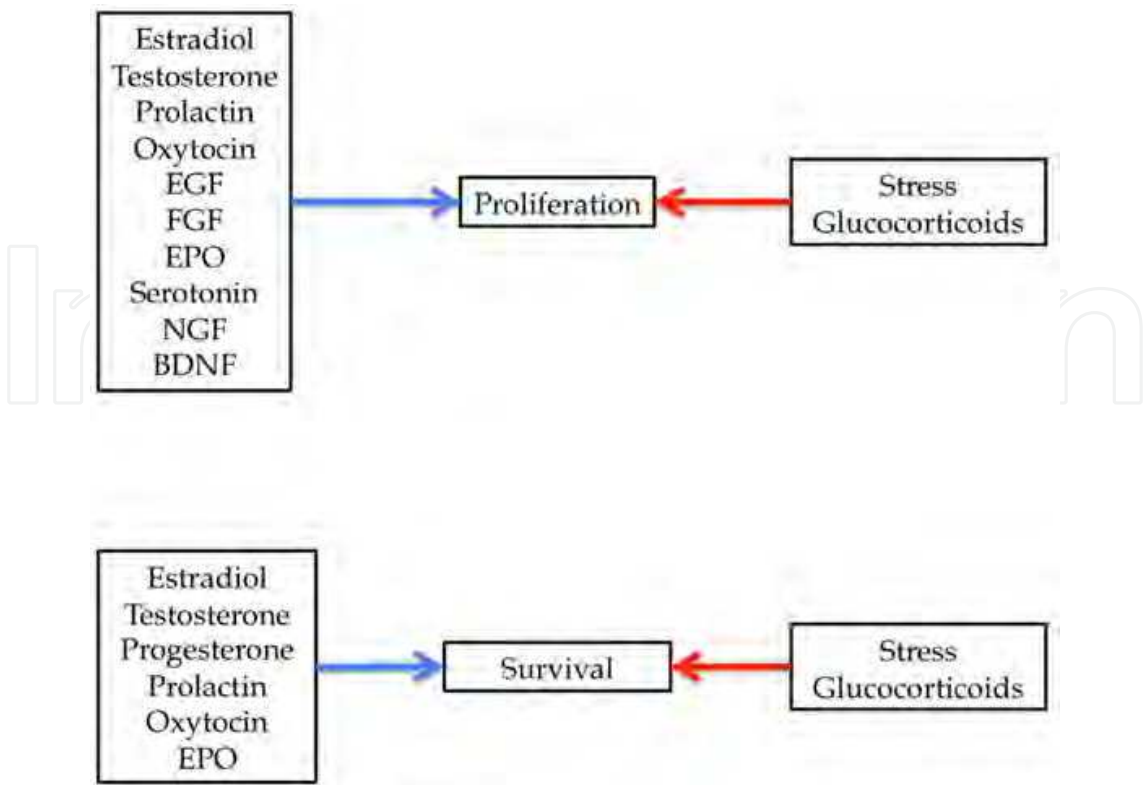
administered EPO crosses the blood-brain barrier and activates in the brain anti-apoptotic, anti-oxidant and anti-inflammatory signaling in neurons, glial and cerebrovascular endothelial cells and stimulates angiogenesis and neurogenesis. These mechanisms underlie its potent tissue protective effects in experimental models of stroke, cerebral hemorrhage, traumatic brain injury, neuroinflammatory and neurodegenerative disease (Byts & Sirén, 2009). Brain specific blockade of EPOR leads to deficits in neural cell proliferation and neuronal survival in the embryonic brain and in post-stroke neurogenesis in the adult brain (Chen et al., 2007; Tsai et al., 2006). Moreover, *in vivo* and *in vitro* data indicate that EPO amplifies stroke-induced oligodendrogenesis that could facilitate axonal remyelination and lead to functional recovery after stroke (L. Zhang et al., 2010). Of interest here is that growth hormone induces EPO release from kidneys.

Brain injuries such as ischemia affect adult neurogenesis in adult rodents as both global and focal ischemic insults enhance the proliferation of progenitor cells residing in neurogenic niches. The ischemic insult increases the number of progenitor cells in monkey subgranular and subventricular zones, and causes gliogenesis in the ischemia prone hippocampal CA1 sector (Tonchev, 2011). The analysis of the expression at protein level of a panel of potential regulatory molecules, including neurotrophic factors and their receptors revealed that a fraction of mitotic progenitors were positive for the neurotrophin receptor Tyrosine kinase B (TrkB), while immature neurons expressed the neurotrophin receptor Tyrosine kinase A (TrkA). Astroglia, ependymal cells and blood vessels in subventricular zone were positive for distinctive sets of ligands/receptors indicating that a network of neurotrophic signals operating in an autocrine or paracrine manner may regulate neurogenesis in adult primate subventricular zone (Tonchev, 2011). The analysis of microglial and astroglial proliferation in postischemic hippocampal CA1 sector showed that proliferating postischemic microglia in adult monkey CA1 sector express the neurotrophin receptor TrkA, while activated astrocytes were labeled for nerve growth factor (NGF), ligand for TrkA, and TrkB, a receptor for brain derived neurotrophic factor (BDNF). These results implicate NGF and BDNF as regulators of postischemic glial proliferation in adult primate hippocampus (Tonchev, 2011). Figure 2 summarizes how these hormones and growth factors act on adult neurogenesis.

2.2.5 Growth hormone/IGF-I system and adult neurogenesis

GH is a pleiotropic hormone expressed not only in the pituitary but in almost any tissue (Devesa et al., 2010b). Thus, far beyond of its classical actions on body growth and intermediate metabolism, GH exerts an important role in the regulation of cell proliferation and survival in several tissues, including the CNS (Costoya et al., 1999; Sanders et al., 2009; McLenachan et al., 2009; Aberg et al., 2009).

The hypothesis that Growth Hormone (GH) and IGF-I play a role on brain repair after an injury has been postulated years ago. The existence of GH expression within the CNS has been reported by several authors, however its physiological role and, in particular, its possible contribution in the reparation of neurologic injuries remain poorly understood despite of that the positive effects of GH treatment on adult neurogenesis have been demonstrated in laboratory animals (McLanachan et al., 2009; Christophidis et al., 2009; Svensson et al., 2008; P. Devesa et al., 2011), and recent data from our group and others (Devesa et al., 2009; High et al., 2010; Reimunde et al., 2010; Reimunde et al., 2011; Devesa et al., 2011) suggest that the hormone may play a similar role in humans. GH-driven neurogenesis may also depend on the local production of GH (P. Devesa et al., 2011), the so called peripheral GH system that may be activated under both physiological and pathologic conditions (Devesa et al., 2010b).



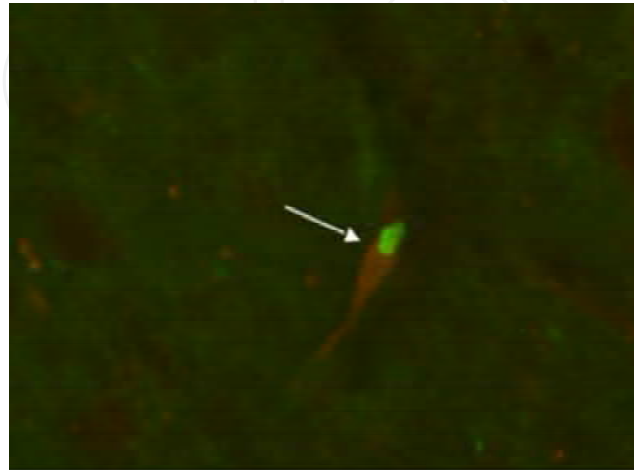
Blue lines indicate stimulation of neural precursors cells proliferation and/or survival, while red lines indicate inhibition.

Fig. 2. Factors involved in adult neurogenesis control.

In keeping with previous reports, we have found that hippocampal cells express GH under basal conditions and, more interestingly, the number of GH-expressing cells seems to increase after brain injury induced by kainate acid administration in rats (P. Devesa et al., 2011). Furthermore, using a double-labeling immunofluorescence we were able to notice that almost all GH-positive cells also showed BrdU immunoreactivity, thus suggesting the existence of a strong correlation between GH expression and cell proliferation (P. Devesa, 2011) (Figure 3). Interestingly, data from Katakowski et al. (Katakowski et al., 2003) demonstrate that the activation of PI3K/Akt signal transduction pathway mediates the migration of neuroblasts to damaged brain areas after stroke, most likely for inducing regeneration. PI3K/Akt is a key signaling pathway for the intracellular effects of GH (Costoya et al., 1999). Moreover, the group of Scheepens demonstrated that the expression of GH and its receptor is strongly upregulated after brain injury and specifically associated with stressed neurons and glia (Scheepens et al., 2000). More recently, the same group demonstrated that during recovery from an ischemic brain injury, a cerebral growth hormone axis is activated; the level of GHR immunoreactivity in the ipsilateral SVZ was significantly increased 5 days after injury vs. the contralateral SVZ, coinciding both spatially and temporally with injury-induced neurogenesis. The population of GHR immunopositive cells in the ipsilateral SVZ at this time was found to include proliferating cells, neural progenitor cells and post-proliferative migratory neuroblasts (Christophidis et al., 2009).

As indicated before, several lines of evidence support a role for GH in neurogenesis. The GH receptor is expressed in regions of the brain in which neurogenesis occurs during embryonic brain development (García-Aragón et al., 1992; Turnley et al., 2002) and in neurogenic

regions of the postnatal rat brain (Lobie et al., 1993). Growth hormone itself is also found in cells of the ventricular zone during embryonic neurogenesis (Turnley et al., 2002), and is produced endogenously within the postnatal hippocampus (Donahue et al., 2002; Donahue et al., 2006; Sun et al., 2005a; Sun et al., 2005b). Interestingly, GH gene expression within the hippocampus is increased by some factors known to increase neurogenesis (Parent, 2003), including learning (Donahue et al., 2002) and estrogen (Donahue et al., 2006).



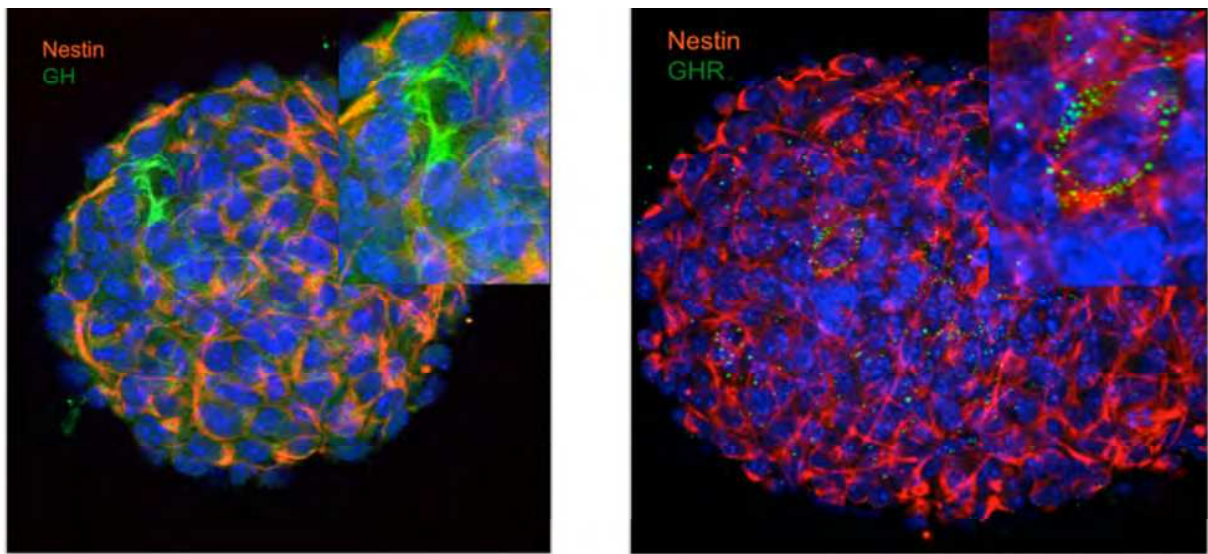
The detection of irBrdU in the nucleus of the cell (green) indicated by the arrow demonstrates that it is a newly born cell in rat hippocampal CA3 area showing irGH (brown), after brain injury induced by kainate acid administration and GH treatment, in its cytoplasm. Newly born neurons formed in the subgranular layer of the dentate gyrus migrate to the CA3 zone after commencing to mature in the granular area. Thus, it is feasible that the detection of irGH in CA3 cells may be related to a trophic and survival role of the hormone. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

Fig. 3. Immunofluorescence (60x). Colocalization of GH and BrdU in a newly born hippocampal cell.

However, the role of GH in the hippocampal dentate gyrus has been related to neuronal survival rather than generation, as altered hippocampal GH levels have no effect on cell proliferation but do affect the survival of immature neurons (Sun et al., 2005b; Sun et al., 2007; Lichtenwalner et al., 2006). Studies of the effects of GH on embryonic rat cerebral cortical (Ajo et al., 2003) and hippocampal (Byts et al., 2008) neuronal cultures found that it induces the proliferation and differentiation of these cells. Overall, these findings indicate that GH may facilitate the proliferation, differentiation and survival of new neurons in response to brain injury. Further studies will reveal whether or not GH is "called" for brain repair after GPR17 activation.

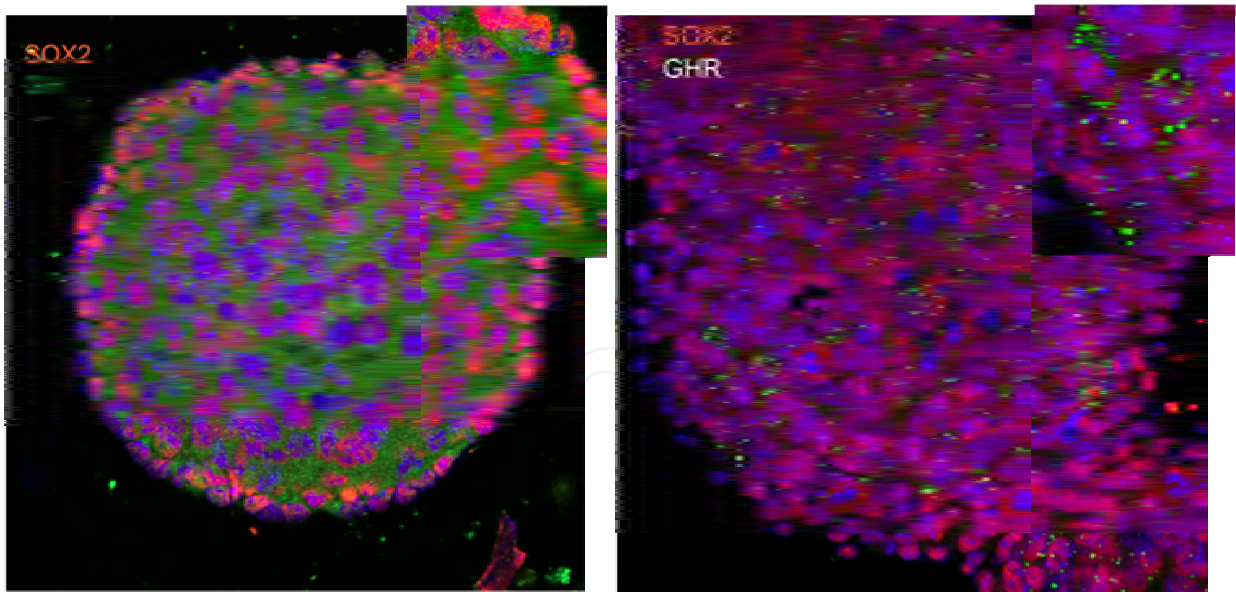
On these bases, we explored a potential role for GH and its receptor on the proliferation, differentiation and survival of neural stem cells (NSCs) *in vitro*. NSCs were isolated from the SGZ of the DG from 9 days old C57/BL6 mice and cultured as neurospheres.

Western blot and immunofluorescence studies showed that neurospheres derived from these NSCs were demonstrated to express both GH and its receptor either in conditions of proliferation and differentiation showing colocalization with nestin (Figure 4) and SOX2 (Figure 5), markers of undifferentiated cells or cells in development. Migrating cells from neurospheres also showed irGHR (Figure 6). In all, these results indicate that GH and its receptor seem to be needed for the physiological neurogenesis.



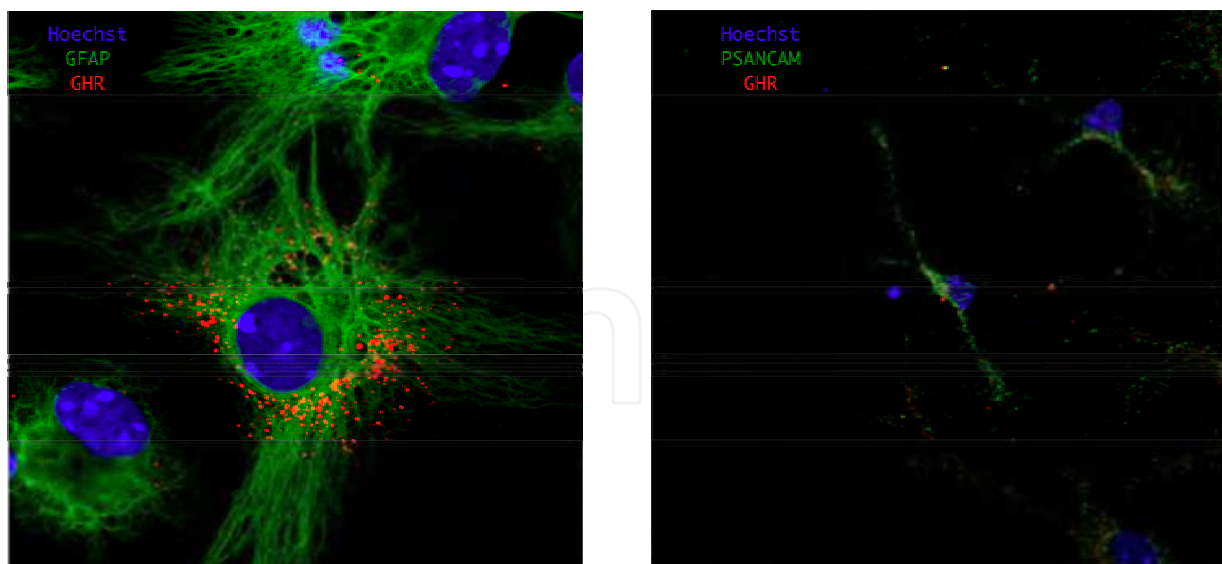
Neurospheres derived from NSCs obtained from the dentate gyrus of 9days old mice in proliferation medium showing irGH (left, green) and irGHR (right, green dots). The detection of ir for Nestin (red) indicates that cells in neurospheres are undifferentiated or in development. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

Fig. 4. Confocal microscopy. Colocalization of GH and GHR with Nestin in mice neurospheres.



Neurospheres derived from NSCs obtained from the dentate gyrus of 9days old mice in proliferation medium showing irGH (left, green) and irGHR (right, green dots). The detection of ir for SOX2 (red) indicates that cells in neurospheres are undifferentiated or in development. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

Fig. 5. Confocal microscopy. Colocalization of GH and GHR with SOX2 in mice neurospheres.



Detection of irGHR (red dots) in different cells migrating from neurospheres formed from NSCs obtained from the dentate gyrus of 9days old mice. The detection of irGFAP (left, green) indicates that these cells are astrocytes, while the detection of irPSANCAM (right, green) indicates that this cell is a migrating neuron. Cells nuclei are stained in blue. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

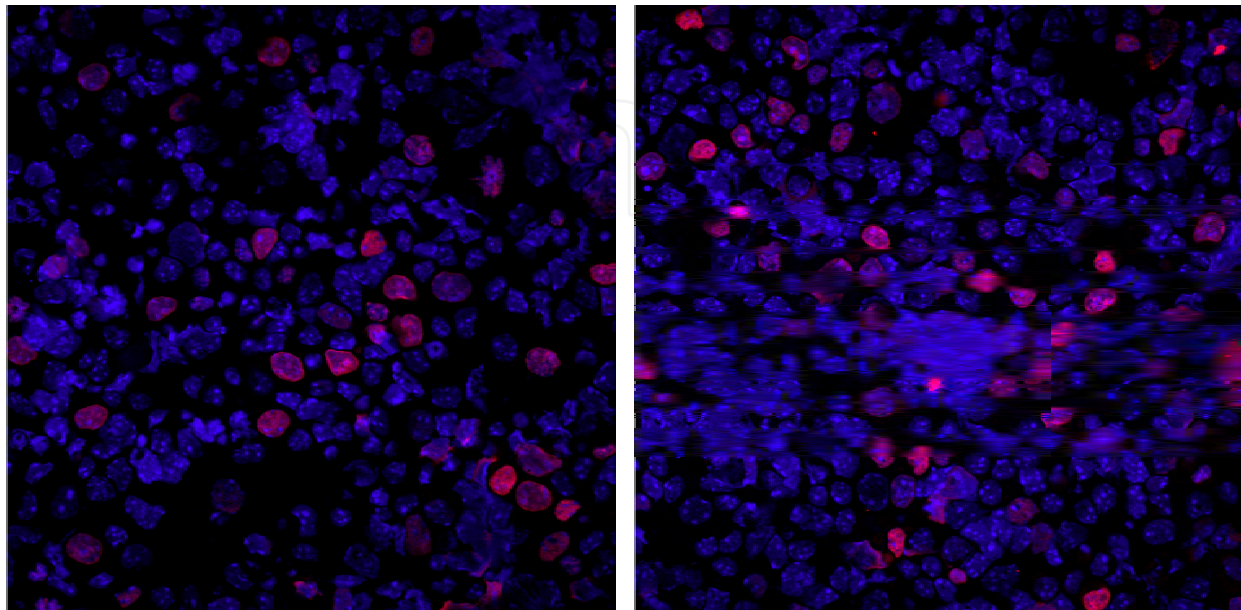
Fig. 6. Confocal microscopy. Detection of GHR in cells migrating from mice neurospheres.

However, from these studies we could not be able for establishing the exact role that the GH–GHR system plays on this mechanism. This is why we explored what would happen when adding the hormone to the culture media either in conditions of proliferation and differentiation.

As reported in other studies (Christophidis et al., 2009) treating NSCs with GH in the presence of BrdU significantly increased the proportion of cells incorporating BrdU almost doubling it (Figure 7). That is, despite of the fact that NSCs express the hormone, the addition of exogenous GH increased stem cells proliferation. This effect is similar to that we observed in animals with kainate acid-induced brain injury (P. Devesa et al., 2011).

It has been suggested that the GHR may actually play a greater role in the survival, migration or differentiation of neurons generated in response to hypoxia/ischemia than in proliferation (Christophidis et al., 2009). Consistent with a role for GHR in survival, GH protects both mature neurons (Mödersheim et al., 2007; Byts et al., 2008; Silva et al., 2003) and primary neurospheres derived from embryonic mouse NSCs (van Marle et al., 2005) from death *in vitro*. Also, the survival of newborn neurons in the subgranular zone of adult rat dentate gyrus is impaired as a result of GH deficiency (Lichtenwalner et al., 2006), and elevated GH levels within the hippocampus reduce apoptosis (Sun et al., 2007). A role for GH in neuronal differentiation is supported by a recent study that found that physiological concentrations of human GH stimulate neurite initiation and arborization of embryonic hippocampal neurons by activating the PI3K/Akt signalling pathway (Byts et al., 2008). Other studies have found that high concentrations of human GH reduce neuronal proliferation but enhance differentiation of these cells instead (Ajo et al., 2003; Lyuh et al., 2007). Indeed, it appears that GH promotes proliferation of neural cells at the expense of their differentiation, as neurosphere cultures derived from GHR *knockout* mice proliferate less than those of *wt* mice due to their inability to respond to autocrine GH, yet they exhibit

accelerated neuronal differentiation (McLenachan et al., 2008). Interestingly, treatment of neurospheres derived from newborn or adult mouse neural stem cells with 100 ng/mL rat GH, which stimulates proliferation (Christophidis et al., 2009), significantly reduced neuronal differentiation (Turnley et al., 2002; Scott et al., 2006).



GH treatment increased the number of proliferating stem cells in proliferation conditions, as irBrdU (red) shows. Left: control, saline treated cells. Right: GH treated cells. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

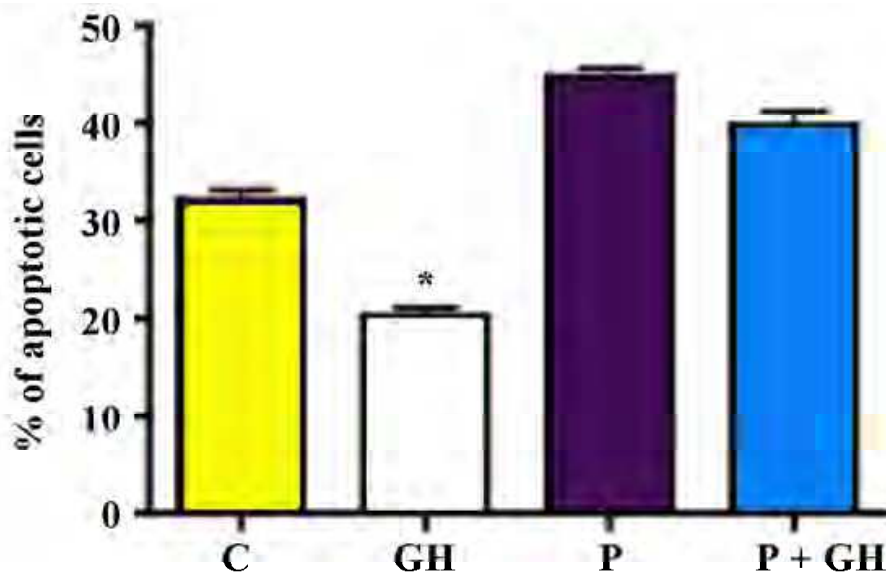
Fig. 7. *In vitro* effects of GH treatment on proliferating neural stem cells.

Overall, it appears that the effect of GH on neuronal differentiation depends upon the concentration of GH and proliferative potential of the cells used.

We also analyzed the role of GH on NSCs survival. In basal conditions there is a physiological rate of apoptosis that is significantly reduced by adding GH to the culture media in differentiation conditions. The antiapoptotic role of the hormone was clearly demonstrated by treating the cells with the GH agonist pegvisomant. This drug binds to the GHR but is unable to induce any kind of intracellular response because inhibits the dimerization of the GHR needed for intracellular signalling. In these conditions, basal apoptosis was significantly increased indicating that the autocrine interplay GH–GHR is key for NSCs survival. Moreover, treating the cells with GH did not modify the increased apoptosis elicited by pegvisomant (Figure 8).

GH effects are mediated via the GHR, which is a member of the cytokine receptor superfamily. The critical step in initiating GH signaling is the activation of receptor-associated Janus kinase 2 (JAK2), which induces cross-phosphorylation of tyrosine residues in the kinase domain of JAK2 and GHR. Phosphorylated residues on JAK2 and GHR form docking sites for the members of the STAT family of transcription factors (Perrini et al., 2008). Phosphorylation of the STATs by JAK2 results in their dissociation from the receptor and translocation to the nucleus, with subsequent binding to DNA and regulation of gene expression. Among the target genes, GH regulates the expression of suppressor of cytokine signaling (SOCS), a family of negative regulators that terminate the GH signaling cascade (Hansen et al., 1999). SOCS proteins bind to phosphotyrosine residues on the GHR or JAK2

and suppress GH signaling by inhibiting JAK2 activity, competing with STATs for binding to the GHR, or inducing degradation of the GHR complex.



GH administration significantly reduced basal apoptosis in cultured NSCs cells (* $p < 0.01$ vs. control). Treatment with pegvisomant abolished the antiapoptotic effect of GH. C: control; P: pegvisomant. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

Fig. 8. *In vitro* effects of GH treatment on the survival of mice neural stem cells.

The RAS/mitogen-activated protein kinase (MAPK) pathway has also been shown to be activated by GH. The GHR-JAK2 complex has been shown to recruit the adapter protein SHC, resulting in SHC tyrosine phosphorylation and binding to GRB2, and activation of RAS, RAF, MAPK/extracellular-regulated protein kinase (MEK), and ERK-1/2 (Vanderkuur et al., 1997). Alternatively, it has been suggested that GH might also regulate the activation of ERK by a SRC-dependent, JAK2-independent mechanism which involves phospholipase D (Zhu et al., 2002). This JAK2-independent mechanism has been recently demonstrated *in vivo* (Barclay et al., 2010). Tyrosine phosphorylation of a GRB2-binding site in the epidermal growth factor receptor could also be involved in GH-mediated MAPK activation (Yamauchi et al., 1997).

GH signaling via SHC and ERK can be interrupted after blocking insulin receptor substrate-1 (IRS-1) (Wang et al., 2009). IRS-1 is a docking protein tyrosine phosphorylated in response to insulin, IGF-I, GH, and other cytokines. IRS-1 greatly enhances GH-induced ERK.

GH has also been shown to stimulate the PI3K pathway through JAK2-mediated tyrosine phosphorylation of the insulin receptor substrates (IRS-1 to IRS-3), leading to their association with PI3K regulatory subunits (Zhu et al., 2001). In addition, direct binding of the p85 and p85 β subunits to phosphotyrosine residues in the carboxyl terminus domain of the GHR has also been demonstrated (Moutoussamy et al., 1998).

As stated before, GH stimulation of PI3K is linked to the stimulation of the antiapoptotic serine protein kinase B or Akt (Costoya et al., 1999). Akt activation has been shown to be dependent on the presence of the JAK2-binding region of GHR, and to promote cell survival through the inhibition of the proapoptotic protein caspase 3 (Sanders et al., 2006). The role of PI3K/Akt on cell survival is well known after the pioneer study of our group (Costoya et al.,

1999). Inhibiting PI3K/Akt with a potent inhibitor of phosphoinositide 3-kinases led to a significant increase in apoptosis in NSCs, however GH treatment was able to clearly decrease the rate of apoptosis observed by blocking PI3K/Akt signaling. This indicates that the hormone is able for signaling through other different pathways acting on cell survival (P. Devesa, 2011). We then studied the effects of inhibiting another cell survival pathway such as ERK. Inhibiting ERK phosphorylation significantly increased NSCs apoptosis in differentiation conditions, but again the treatment with GH was able to revert the proapoptotic effect of blocking ERK signaling.

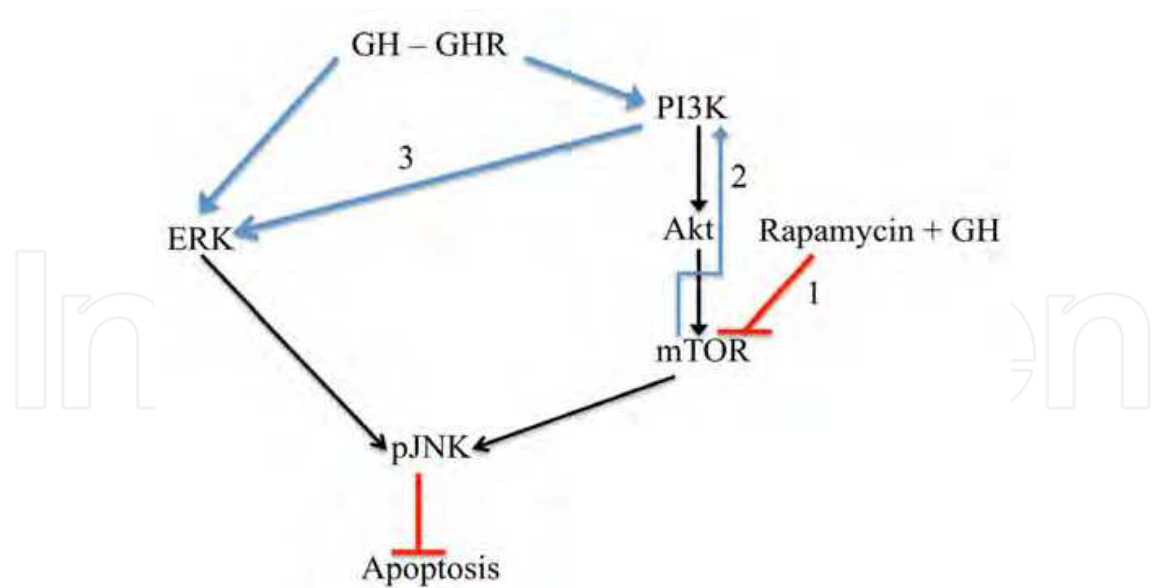
Since GH was able to revert the propapoptotic effects of the inhibition of both PI3K/Akt and ERK, we decided to study signalling pathways located upstream, specifically mTOR. This is a master regulator of cell mass and metabolism, which is in part regulated by growth factor signalling through the canonical RTK (receptor tyrosine kinase)-PI3K/Akt axis and by nutrient (through class III PI3Ks), hypoxia or AMP.

Rapamycin is an allosteric mTOR inhibitor. As expected, treating NSCs with rapamycin led to a significant increase in apoptosis, but once more this effect on cellular death was reverted when GH was added together with rapamycin. This seems to be surprising given the importance of mTOR in signalling cell survival, and again indicated that the hormone uses different pathways for promoting cell survival. However, evidence exists showing that mTOR inhibition can lead to pathway reactivation: abrogation of the negative-feedback loop which is normally initiated by the direct substrate p70S6K (p70 S6 kinase) on insulin receptor substrate proteins can lead to strong PI3K/Akt pathway reactivation, most likely producing ERK pathway reactivation in a PI3K/Akt dependent manner (Carracedo et al., 2008). For a better understanding of such a concept see the scheme shown in Figure 9.

Thus, while PI3K/Akt inhibition would allow GH to act through ERK pathway, ERK inhibition would not impede PI3K/Akt to be the survival-signalling pathway. Of interest here is the fact that both kind of inhibitions only were reverted when exogenous GH was added. This means that the autocrine production of the hormone by NSCs is not enough for blocking intense cell death signals.

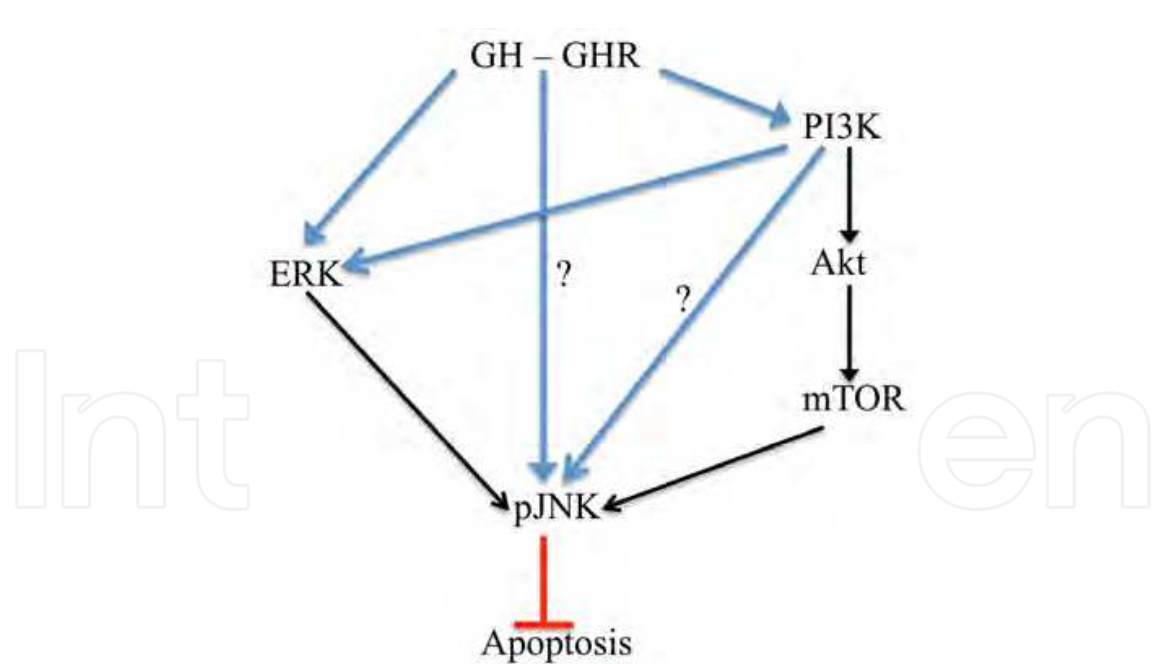
We then blocked simultaneously ERK and mTOR activation. Once more the intense apoptosis observed after pharmacological blockade of these pathways was reverted when GH was added together with them. The only explanations for this result is that the hormone may act directly at a upper level in the stream of signaling pathways for cell survival or the effect is due to the overactivation of PI3K occurred as a result of blocking those two other signaling pathways. To test the first possibility we used SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase (Bennett et al., 2001). That is we were acting at the terminal level in the stream. In this case the clear increase in apoptosis produced by the inhibitor could not be reverted by administering GH together with the drug. It suggests either a direct effect of the hormone on JNK activation for cell survival or a direct effect of overactivated PI3K on JNK (as postulated by Zhu et al., 1998).

In summary, our results (P. Devesa, 2011), together with other studies previously described, demonstrate that GH is able for promoting NSCs proliferation, and also plays a key role on the survival of these cells. With regard to the antiapoptotic role of GH, it seems to be exerted through different signaling pathways: PI3K/Akt, ERK and pJNK (Figure 10). Since culture media contain insulin our results do not allow us to conclude whether the effect of GH on pJNK is a direct effect or it results from the known crosstalk between GH and insulin at the cellular level (Xu & Messina, 2009) or from the overactivation of PI3K directly enhancing JNK activity (Zhu et al., 1998). In any case GH effects a very strong effect on the survival of NSCs.



As showed in the scheme, rapamycin treatment inhibits mTOR, thus leading to a feedback activation of the PI3K/ Akt pathway [2]. Physiologically this would lead to increased ERK activation [3], but this not occurs unless exogenous GH is given together with rapamycin [1]. Red lines indicate inhibition; blue lines indicate activation. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

Fig. 9. Schematic representation of the cell survival signaling pathways studied.



The scheme shows how GH may act on cell survival in NSCs according to the results we obtained. The possibility of a direct effect of the hormone on JNK phosphorylation could not be demonstrated here; another possibility is that the activation of JNK occurs because of overactivation of PI3K after mTOR and ERK blockade. Red line indicates inhibition; blue lines indicate activation. Taken from Pablo Devesa, Doctoral thesis, 2011. University of Santiago de Compostela, Spain.

Fig. 10. Schematic representation about the putative role of GH in NSCs survival.

A number of studies indicate that the neurobiological consequences of the decline in GH/IGF-I, that occurs physiologically during aging, include decreased neurogenesis in the dentate gyrus of the hippocampus (Lichtenwalner et al., 2001; Lichtenwalner et al., 2006), where IGF-I appears to affect primarily the survival of newborn neurons, but also may influence the maturation and differentiation of newborn cells (Aberg et al., 2000; Darnaudery et al., 2006).

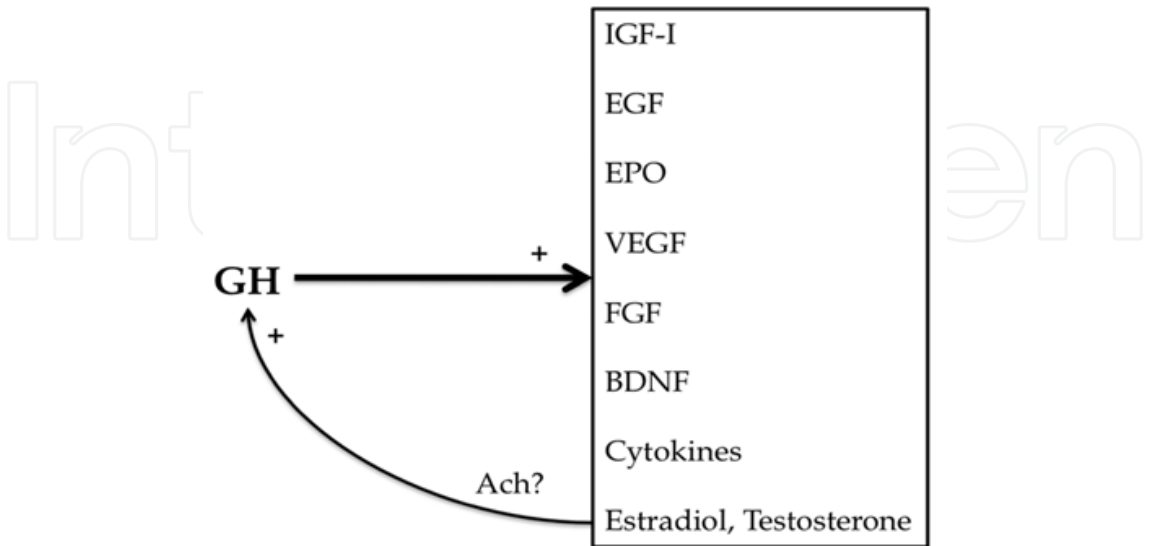
Despite that neurogenesis is dramatically reduced during senescence, newborn granule cells in the aged dentate gyrus retain the capacity for participation in functional hippocampal networks (Marrone et al., 2011); this is in agreement with the aforementioned hypothesis, suggesting that it is the age-associated lack of neurotrophic factors (GH/IGF-I?) the cause of decreased neurogenesis. In fact, growth hormone prevents neuronal loss in the aged rat hippocampus (Azcoitia et al., 2005).

GH induces IGF-I expression in liver and other tissues; however it is not the only factor responsible for it. In fact, there are a number of pathological situations in which a clear divergence exists between GH production and plasma levels of IGF-I. This is the case, for instance, of Anorexia Nervosa; in these patients, while large burst of pituitary GH are released into the blood, IGF-I plasma levels are consistently lower than normal. The opposite situation can be observed in obese children; GH secretion is deficient in these children, but plasma IGF-I levels are normal and growth velocity is above the mean for age (Devesa et al., 1992). The common link between these two situations seems to be plasma glucose levels, low in Anorexia Nervosa and showing a tendency to hyperglycemia in obesity. Thus, it has been postulated that glucose is needed for the hepatic production of IGF-I; more particularly a product of intracellular metabolism of glucose, since 2-deoxyglucose (who can not be metabolized) is unable to induce hepatic IGF-I expression. Therefore, although GH usually is the factor responsible for the hepatic production of IGF-I, in terms of neural effects both hormones may follow different paths. In fact, studies in rats submitted to moderate and severe hypoxia showed that the spatial distribution of the neuroprotection conveyed by growth hormone correlates with the spatial distribution of the constitutive neural growth hormone receptor, but not with the neuroprotection offered by IGF-I treatment in this model. These results suggest that some of the neuroprotective effects of growth hormone are mediated directly through the growth hormone receptor and do not involve IGF-I induction (Scheepens et al., 2001).

The effects of the GH/IGF-I axis on cell turnover in the adult brain probably are not limited to neuronal progenitors, since IGF-I can promote proliferation of oligodendrocyte progenitor cells (OPCs) and differentiation and survival of oligodendrocytes (McMorris & McKinnon, 1996; Mason et al., 2000; Aberg et al., 2007; Pang et al., 2007), an effect also induced by EGF (Gonzalez-Perez & Alvarez-Buylla, 2011) whose expression and that of its receptor may also be induced by GH (Pan et al., 2011). Thus, it is likely that the aging-related decline in GH/IGF-I and dependent changes in oligodendrocyte genesis and/or maturation may contribute to impaired remyelination in the central nervous system of aged individuals (Gilson & Blakemore, 1993; Shields et al., 1999; Franklin et al., 2002; Sim et al., 2002) and to a decline in normal cognitive function. In fact, GH has positive cognitive effects when given to GH-deficient patients.

In all, the system GH/IGF-I exerts both neuroprotective and regenerative effects at the CNS. In line with this a recent study in human patients describes that a high serum IGF-I during the rehabilitation phase of stroke correlates to better recovery of long-term function (Aberg et al., 2011).

It is likely that some of the positive effects of GH at central level are not directly exerted by the hormone but they are mediated trough the induction and release of a number of neurotrophic factors, as shown in Figure 11.



Some of the neurotrophic effects of GH *in vivo* could be mediated and/or enhanced through the induction of the expression and release of a number of neurotrophic factors. GH induces the expression of IGF-I, EGF, EPO; VEGF and FGF. The GH-effects on the induction of BDNF expression have not been tested yet but it is likely that they occur, directly or indirectly. GH induces an acute and strong release of neutrophils from the bone marrow, then allowing the release of neurotrophic cytokines. GH has trophic effects on the gonads, thus facilitating estradiol and testosterone production. In turn, it is likely that the neurotrophic effects of these sexual steroids may be partially due to a positive effect on the pituitary GH release mediated by Ach and noradrenaline (Devesa et al., 1992).

Fig. 11. GH induction of the expression and/or release of a number of factors with known neurotrophic activity.

3. Effects of GH treatment combined with kynesitherapy on the recovery of patients with brain injuries

3.1 Cerebral palsy children

Cerebral palsy (CP) is a catastrophic acquired disease, occurring during development of the fetal or infant brain. It mainly affects the motor control centres of the developing brain, but can also affect cognitive functions, and is usually accompanied by a cohort of symptoms including lack of communication, epilepsy, and alterations in behavior.

Major causes for CP include abnormal intrauterine developments, due to fetal-maternal infections, asphyxia before birth, hypoxia during delivery, brain trauma during labor and delivery, and complications in the perinatal period. Apart from these, prematurity is responsible for 40%–50% of cases of CP. Periventricular leucomalacia (PVL) and parenchymal venous infarction complicating germinal matrix/intraventricular hemorrhage have long been recognized as the two significant white matter diseases responsible for the majority of cases of cerebral palsy in survivors of preterm birth.

However, in more recent studies using magnetic resonance imaging to assess the preterm brain, two new appearances have been documented, adding to the spectrum of white matter

disease of prematurity: punctate white matter lesions, and diffuse excessive high signal intensity. These appear to be more common than PVL but less significant in terms of their impact on individual neurodevelopment. They may, however, be associated with later cognitive and behavioral disorders known to be common following preterm birth.

Most CP children often have poor linear growth during childhood, resulting in a diminished final adult height. However the number of studies in which it has been reported whether or not GH secretion is impaired in CP is quite limited. These studies reflect that GH provocative testing induced a GH deficient secretion. In a recent study it was indicated that diminished circulating IGF-1 and GH concentrations may explain why children with CP are smaller than normally growing children (Ali et al., 2007a). On the other hand, osteopenia is a common finding in children with CP, and seems to be associated with decreased IGF-1 and IGFBP3 plasma levels, usual markers of deficient GH secretion. The large percentage of CP children with GH deficiency (GHD) has been reported to be noteworthy. However, given the complexity of GH neuroregulation (Devesa et al., 1992) it seems to be logical that severe brain damage may affect a number of neurotransmitter pathways involved in GH control, thus affecting the normal secretion of the hormone.

Other possible causes of decreased growth in CP include psychosocial deprivation and suboptimal nutritional status, but these are also involved in subnormal GH secretion (Devesa et al., 1992).

We studied whether GH secretion was affected in 46 CP children (28 males, 18 females; aged 3 to 11 years old). Our results indicated that 70% of the patients seemed to have deficient GH secretion (Devesa et al., 2010a), therefore they were candidates to benefit from GH replacement therapy.

In few studies have the benefits of GH-replacement therapy in children with CP been reported, and most of these studies only reflect the increased growth observed during the treatment period with the hormone (Coniglio & Stevenson, 1995; Shim et al., 2004; Ali et al., 2007b).

Neuropsychological assessments have demonstrated that GHD is associated with reduced cognitive performance; specifically, in the majority of studies it has been found that GHD can lead to clinically relevant changes in memory, processing speed, attention, vocabulary, perceptual speed, spatial learning, and in reaction time tests. Cognitive dysfunction appears to be specifically related to GH deficiency; this hypothesis is supported by the positive correlations between serum IGF-I concentration and IQ, whereas poorer emotional well-being and reduced perceptual-motor performance are attributed to other pituitary hormone deficiencies.

In a recent study we described (Devesa et al., 2011) the positive effects of GH therapy together with psychomotor and cognitive stimulation in 11 children (7 males, 4 females; aged 3 to 7 years old) with CP and GHD. Cognitive performances in the children studied were assessed by using the Battelle Developmental Inventory Screening Test (BDIST) (Newborg et al., 1988). Tests were performed at admission, two months after commencing psychomotor and cognitive stimulation and two months after adding GH replacement therapy to psychomotor and cognitive stimulation. Before admission all these CP children had been intensively stimulated, most of them since they were 1 year old. Psychomotor and cognitive stimulation were adapted to the specific needs of each patient. Psychomotor stimulation involved tasks aimed at improving tonic-postural control, laterality, breathing and relaxation, static and dynamic balance, motor coordination and dissociation, body image, oculomotor coordination, spatial and temporal orientation, and gross and fine motor

skills. Cognitive stimulation involved tasks directed at improving interaction with the environment, communication, attention, perception, memory, reasoning, and concept learning. These therapies were carried out for 45 minutes per day, 5 days per week during 4 months. Recombinant human GH was given subcutaneously, 30 $\mu\text{g}/\text{kg}/\text{day}$, 5 days/week, during 2 months (after 2 months of psychomotor and cognitive stimulation).

Patients did not improve their psychomotor and cognitive status during the pretreatment period, during which only psychomotor and cognitive stimulation were performed. However, significant improvements in all BDIST domains were observed when GH was administered together with psychomotor and cognitive stimulation; specifically, patients improved in personal and social skills, adaptive behavior, gross motor skills and total psychomotor abilities, receptive and total communication, cognitive skills, and in the total score of the scale ($p < 0.01$), and in fine motor skills and expressive communication ($p < 0.02$). As expected, plasma IGF-1 and IGFBP3 significantly increased after GH treatment.

These results led us to conclude that the combined therapy involving GH replacement and psychomotor and cognitive stimulation is useful for the appropriate neurodevelopment of children with CP and GHD (Devesa et al., 2011). Since these children had received an intensive neurostimulation without significant improvements before being treated with GH, it seems to be clear that the hormone, and/or IGF-1 was the main factor responsible for the results obtained. Moreover, since exogenous GH combines with locally produced GH for repairing brain injuries (P. Devesa et al., 2011) it is feasible to assume that GH treatment may be used too in CP children without GHD.

In another study (Reimunde et al., 2011), we assessed the effects of growth hormone treatment (30 $\mu\text{g}/\text{kg}/\text{day}$) combined with physical rehabilitation in the recovery of gross motor function in children with GHD and CP (four males and six females, mean age 5.63 ± 2.32 years) as compared with that observed in a similar population of CP children (five males, five females, mean age 5.9 ± 2.18 years) without growth hormone deficiency treated only with physical rehabilitation for two months. The Gross Motor Function Measure (GMFM-88) and Modified Ashworth Scale were performed before commencing the treatment and after completion thereof. The GMFM-88 is a scale constructed for evaluation of change in gross motor function in children with cerebral palsy and consists of 88 items grouped into five dimensions, ie, dimension A (lying and rolling, 17 items), dimension B (sitting, 20 items), dimension C (crawling and kneeling, 14 items), dimension D (standing, 13 items) and dimension E (walking, running, and jumping, 24 items).

Scores for each dimension are expressed as a percentage of the maximum score for that dimension, adding the scores for all dimensions, and dividing by 5 to obtain the total score. The reliability, validity, and responsiveness of the GMFM-88 scores are documented for children with cerebral palsy (Palisano et al., 2000). The Modified Ashworth Scale is used for measuring spasticity in spastic patients (Bohannon & Smith MB, 1987).

In children with CP and GHD, Dimension A ($p < 0.02$), dimension B ($p < 0.02$), and dimension C ($p < 0.02$) of the GMFM-88, and the total score of the test ($p < 0.01$) significantly improved after the treatment; dimension D and dimension E did not increase, and four of five spastic patients showed a reduction in spasticity. However, in children with cerebral palsy and without growth hormone deficiency, only the total score of the test improved significantly after the treatment period. Plasma IGF-I values (previously low in GHD CP children) were similar in both groups at the end of the treatment period, indicating that growth hormone replacement therapy was responsible for the large differences observed between both groups in response to physical rehabilitation.

According to these results it is likely that GH administration plays a key role in recovering from a brain injury, independently or not that a GH deficiency exists. In this regard, voluntary physical exercise is known to be a factor increasing neurogenesis and also enhancing learning and memory (Beckinstein et al., 2011). However few positive effects are obtained in CP children undergoing exhaustive daily physical work, as our data also reflect. Exercise is a powerful stimulus for endogenous growth hormone release, most likely through enhanced central NA tone (Devesa et al., 1992). It has been demonstrated that inhibiting PI3K/Akt signaling, one of the pathways by which GH acts (Costoya et al., 1999), blocks exercise-mediated enhancement of adult neurogenesis and synaptic plasticity in rats (Brüel-Jungferman et al., 2009). The possibility exists that the lack of significant improvements in CP children submitted to intensive physical work is due to an impaired GH secretion, as it seems to occur whenever a brain injury exists, independently that current provocative tests do not reflect the existence of a GH deficiency.

As described before, blindness is a common finding in CP children. We studied 20 children with CP occurring as a consequence of prematurity leading to Periventricular Leukomalacia (11 males, 9 females, aged $2,05 \pm 1,43$ years; mean \pm SD of the mean) with critical impairment of vision and marked pallor of the optic nerve. They were treated with GH and visual stimulation performed with a tachistoscope (repetitive white light flashes, 100-150 ms, carried out in 10 phases lasting 1 min each one, 80 flashes/min; 5 days/week). Stimulation was performed in a dark isolated room. Visual evoked potentials (VEP) were recorded before commencing the treatment and after finishing it. Treatment lasted for 5 ± 2.65 months. The rationale for interrupting visual stimulation and GH treatment was based on clinical observations (for example, visual interaction with the environment, looking at objects or relatives, taking objects with the hands...).

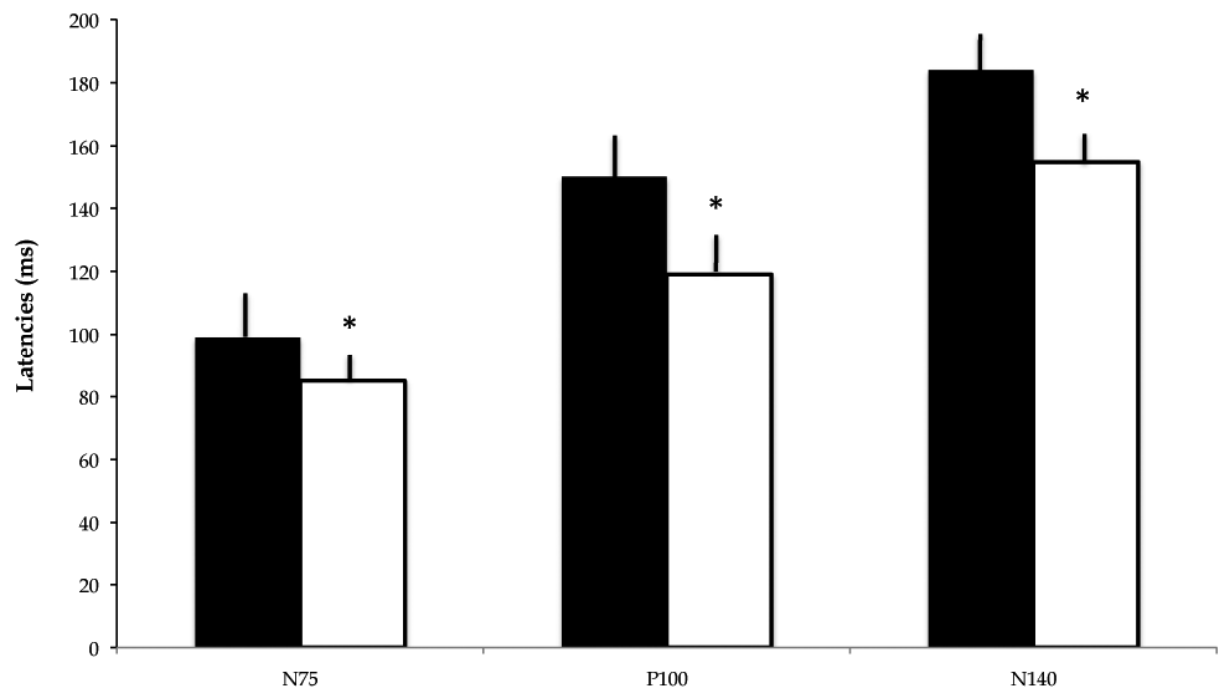
Results from this study (unpublished data) show that the children we studied presented severe visual deficiencies, characterized by a delayed conduction from the retina to the occipital cortex, as VEP showed, but these were corrected at the end of treatment period, as Figure 12 shows. Latencies were significantly decreased, while no significant changes were observed in amplitudes.

Delayed conduction from the retina to the occipital cortex means a deficient myelination. This may be related to a delayed maturation of the CNS, but it is unlikely that this was the reason in these CP children since they suffered brain injuries produced by prematurity leading to Periventricular Leukomalacia in which cerebral white matter is consistently affected. Moreover, some of these children were older than 2 years old, a period of time from which it is considered that a brain damage is already established and significant improvements are unlikely to appear. Injuries involving the optic radiations, such as it happens in Periventricular Leukomalacia, have a bad prognosis (Hoyt, 2003) and most of these children are expected to remain visually handicapped.

We utilized a tachistoscope as a stimulation method of both visual hemifields, but it is unlikely that visual stimulation alone could be responsible for the clinical and VEP changes observed after the treatment. Despite the fact that there was not a control group in our study and this could lead to misinterpretating the results obtained, most of these children had been receiving intense stimulation previously without achieving significant results.

A recent study describe that plasma levels of GH/IGF-I influence glial turnover in the white matter (Hua et al., 2009). It is not clear whether the maintenance of Oligodendrocyte Precursor Cells (OPCs) and oligodendrocyte turnover in the adult brain serves normal function or only provides a rapidly recruitable population of cells for myelin repair

following damage. Proliferation of oligodendrocyte precursors and recruitment of new, myelinating oligodendrocytes from immature precursors contribute to myelin repair following demyelinating lesions. Following demyelination, proliferation of OPCs and commitment, differentiation and survival of adult-born oligodendrocytes all appear to be targets of regulation by inflammatory cytokines and growth factors.



The figure shows changes observed in the latencies of Visual Evoked Potential waves registered in CP children before (black bars) and after (white bars) the treatment with GH and visual stimulation. * $p < 0.005$ vs. pretreatment.

Fig. 12. Latencies of Visual Evoked Potential waves in blind CP children.

The GH/IGF-I system appears to play a particularly critical role in myelin repair. IGF expression is induced in multiple models of demyelination and is increased during remyelination (Fushimi & Sharabe, 2004). Treatment with IGF-I or overexpression of IGF-I in transgenic mice inhibits oligodendrocyte death during demyelination and/or enhances remyelination following demyelinating lesions (McMorris & McKinnon, 1996; Mason et al., 2000; Kumar et al., 2007).

The possibility exists that remyelination observed in our study, as showed by the improvements in VEPs latencies, might be due to increased plasma IGF-I values. This does not exclude a direct effect of GH on myelination. As described before, GH induces EGF and EGF-R expression, factors recently involved on central nervous system remyelination (Gonzalez-Perez & Alvarez-Buylla, 2011).

Thus, it is feasible to assume that GH treatment played a significant role in the visual recovery elicited by specific visual stimulation.

Case report

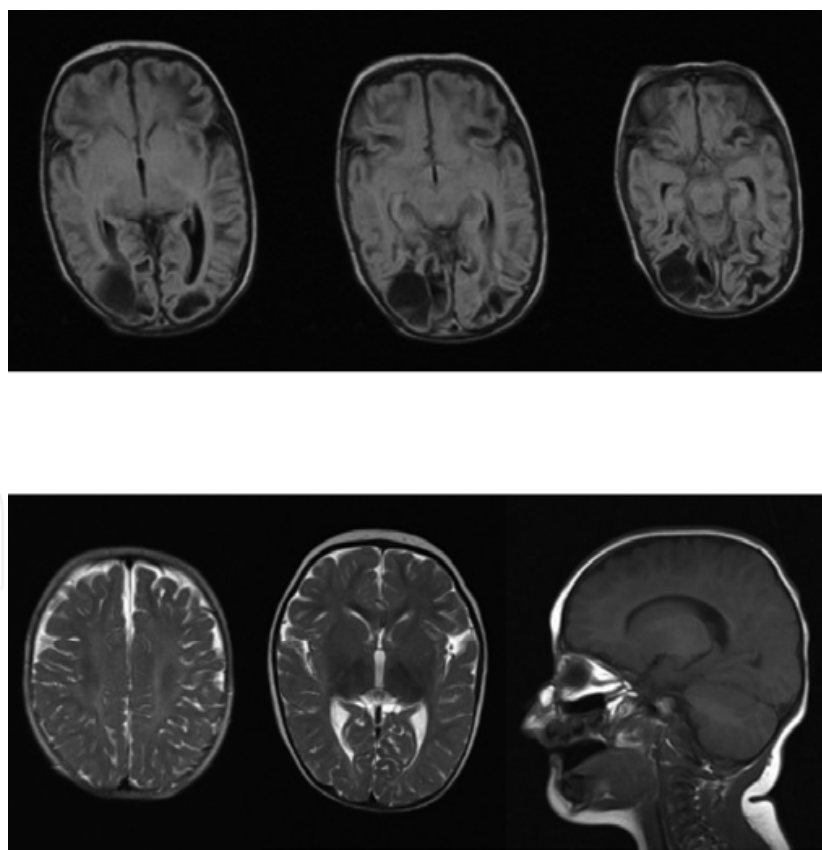
At age 3 weeks a CP baby was admitted for rehabilitation in our Center. He suffered a massive bleeding *in utero* that produced a 20-minute cardiac arrest. A MRI carried out at age

2 weeks showed a severe encephalopathy with cystic cavities in both occipital lobes. VEP and auditory evoked potentials were isoelectric without any signs of conduction. Prognosis could not be worst: blind, deaf, dumb and spastic tetraparesia.

After obtaining signed informed consent from the parents we commenced an intensive rehabilitation with him. Visual stimulation, auditory stimulation, sensorial and physical stimulation and GH treatment (0.04 mg/kg/day). Three months later the child was showing signs of positive evolution. There was electrical conduction from the retina to the cortex, he responded to auditory stimuli and spasticity was decreased. GH treatment lasted for 4 months, but physical and sensorial stimulation continued for 12 months. At this time the child was absolutely normal as functional physical and cognitive tests revealed. Currently, at age 17 months, he walks, he speaks, he laughs, he plays, he is like any other child of his age. No sequelae have been observed and the treatment did not produce any kind of adverse effects.

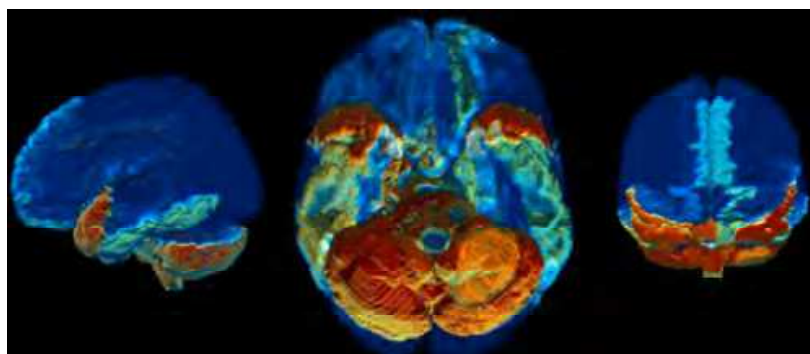
A new MRI taken when he was 1 year old showed that the brain was completely regenerated. Cystic cavities disappeared and occipital lobes were fully functional. Voxels based morphometry comparing both MRI indicated that a number of brain areas experienced a significant regeneration; this was particularly significant in thalamus.

Figures 13 and 14 respectively show MRI and voxels based morphometry studies in this child.



Upper images were taken at age 2-weeks. Notice the cavities affecting occipital lobes. Lower images were taken at age 1-year; no occipital cysts exist.

Fig. 13. Some images of MRI studies in the child described as a Case report.



These images were obtained after comparing MRI studies carried out at 1-year interval in the child described as a Case report. The intensity of red colours is associated to greater regeneration.

Fig. 14. Voxels based morphometry.

To our knowledge this is the first description about complete human brain regeneration demonstrated with brain images. Brain plasticity may be responsible for achieving important functional recoveries in patients after a brain injury. In our case these recoveries were found not only by clinical and specific physical and cognitive tests, but they have been demonstrated with brain images. It is clear that the age at which we commenced the treatment of this child had a significant influence on the results obtained; brain development continues for 1 year after birth, approximately, but it also clear that physical and cognitive stimulation alone are not able for achieving a complete and functional recovery of the brain. Since the system GH/IGF-I has been involved in playing a very important role during embryonic brain development it seems that the treatment with the hormone was the main factor responsible for results achieved in this study. This supports our previous postulate about commencing GH treatments as soon as possible after a brain injury (Devesa et al., 2010a).

3.2 Traumatic brain injury

Traumatic brain injury (TBI) is an important health problem and a leading cause of death and disability worldwide, mainly in people under 40. Specifically, TBI is an important identified risk factor for cognitive deficits, such as attention, concentration, learning, memory, conceptual thinking, problem-solving or language (Vogenthaler, 1987). Recent studies have demonstrated that hypopituitarism, and particularly growth hormone deficiency (GHD), is common among survivors of TBI tested several months or years following head trauma (Popovic, 2005a; Popovic et al., 2005b). The subjects at risk are those who have suffered moderate-to-severe head trauma, although mild intensity trauma or even minor head injuries may precede hypopituitarism (Popovic et al., 2005b). Complex pituitary deficits are found in 35–40% of TBI patients; severe GHD occurs in 10–15% and partial GHD in 15% of TBI patients (Popovic et al., 2005b).

Neuropsychological assessments have demonstrated that GHD is associated with reduced cognitive performance; specifically, most of the studies indicate that GHD can lead to clinically relevant changes in memory, processing speed, attention, vocabulary, perceptual speed, spatial learning and in reaction time tests as well (van Dam, 2006; Falletti et al., 2006; Nieves-Martinez et al., 2010).

Cognitive dysfunction appears to be specifically related to GH deficiency; this hypothesis is supported by the positive correlations between serum IGF-I concentration and IQ. Thus, it

has been shown that cognitive disorders secondary to GHD may be reversed by GH replacement (Maruff & Falletti, 2005).

We studied (Reimunde et al., 2010) 20 male adult patients with TBI suffered in the previous 10 years. Main brain injuries occurred because of frontal contusions affecting brain frontal lobes. Eleven of them presented GHD (mean age: 53.36 ± 17.35 years), while GH secretion seemed to be normal in the other nine patients (mean age: 47.12 ± 14.55 years).

All patients received daily cognitive rehabilitation, 1 hour per day, 5 days per week during 3 months. The contents of cognitive rehabilitation were aimed to improve the spatial, temporal and personal orientation, visual, auditory and tactile discrimination and perception, body recognition, planning and execution of motor acts, all memory types and processes, oral and writing language, calculation and executive function tasks. GHD patients were given human GH (0.5 mg/day during 20 days and then 1 mg/day, 5 days per week, for 3 months). Control patients were given placebo.

To assess different cognitive abilities we used a widely recognized neuropsychological test battery, the Wechsler Adults Intelligence Scale (WAIS) (Wechsler, 1990). This assessment battery involves several sections that specifically measure multiple cognitive functions. WAIS test was performed before commencing the treatment and after 3 months of treatment in all patients.

Results from our study demonstrate that individuals treated only with cognitive rehabilitation improved, specifically, their attentional functioning. However, the group treated with GH and cognitive rehabilitation improved their attentional functioning too, but moreover showed specific improvements in memory, understanding, associative thinking skills, information management and storing, learning, visual-motor dexterity and speed of execution.

In addition, the GHD group reached greater improvements in aspects related with memory, understanding, associative thinking skills and information management and storing information than the control patients.

These data correlated with previous results showing that GH-deficient subjects treated with GH experience significant improvements in concentration and attention, different memory modalities and learning.

It has been described that memory performance is weakly related to the mean daily GH dose, but there was a stronger positive correlation between the increase in mental performance and the GH-induced rise in serum IGF-I; this phenomenon can be explained by the large inter-individual differences in GH sensitivity (Deijen et al., 1998). However, in our study, no significant differences between plasma IGF-I levels in both groups of patients were observed at the end of the treatment period. Thus, it is unlikely that the IGF-I rise could be responsible for the results here obtained.

In conclusion, our study demonstrated, for the first time, the utility of a combination therapy involving GH replacement and cognitive rehabilitation in the recovery of cognitive impairments in patients who had suffered a TBI and a subsequent GHD. Our data show that the combination therapy has significantly greater effects than those achieved by carrying out only cognitive rehabilitation in patients with cognitive impairments secondary to a TBI without GHD. GH administration seems to be responsible for the results obtained. On these bases, we concluded that it would be interesting to test whether GH administration to no GHD patients would improve cognitive functions in a number of neurological disorders (Reimunde et al., 2010).

4. Conclusions

Throughout this chapter we have reviewed the evidence showing that adult neurogenesis is a physiological mechanism to repair brain damage. We analyzed a number of factors directly involved in this process.

We demonstrated that both GH and its receptor are expressed in neural precursors in mice, and we showed that after a brain injury, in rats, the treatment with the hormone increases the number of newly born stem cells produced in response to the damage. We demonstrated that GH is present in the cytoplasm of these newly born cells, most likely for facilitating their migration from neurogenic niches. Our data in cell cultures obtained from mice stem cells demonstrate that the hormone plays a key role for neural cells survival.

In human patients with brain injury and GH-deficiency, we demonstrated that GH treatment together with physical and cognitive stimulation is responsible for the recovery of motor and cognitive abilities. GH treatment also appears to play a key role for remyelination of the visual pathways in children with cerebral palsy.

For the first time we showed here that an early treatment with the hormone can recover a brain injury in the first months of the life.

Although not presented here, GH treatment may repair central neurogenic dysphagias (Devesa et al., 2009; Reimunde et al., 2011, submitted for publication).

By analyzing the role of many factors positively involved on adult neurogenesis, we postulate that some of the positive effects of GH at the central level may be due to GH-induced expression of a number of neurotrophic factors and/or to enhanced release of some of them.

We conclude that GH treatment is a powerful tool for being used in a number of brain injuries, including neurodegenerative non-genetical diseases (for example, multiple sclerosis, Alzheimer, etc). It seems that there is no need that GH-deficiency exists for the hormone being useful.

GH treatments have to be well scheduled and short in time. The positive effects of the hormone remain in the time.

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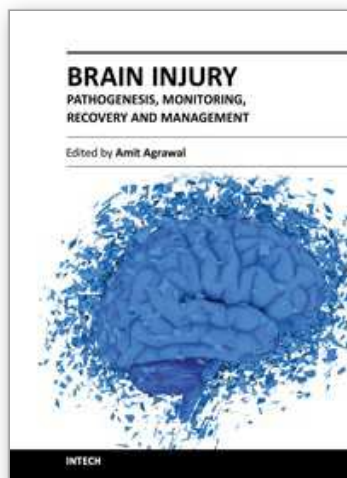
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The present two volume book "Brain Injury" is distinctive in its presentation and includes a wealth of updated information on many aspects in the field of brain injury. The Book is devoted to the pathogenesis of brain injury, concepts in cerebral blood flow and metabolism, investigative approaches and monitoring of brain injured, different protective mechanisms and recovery and management approach to these individuals, functional and endocrine aspects of brain injuries, approaches to rehabilitation of brain injured and preventive aspects of traumatic brain injuries. The collective contribution from experts in brain injury research area would be successfully conveyed to the readers and readers will find this book to be a valuable guide to further develop their understanding about brain injury.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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