



Research report

Early growth hormone (GH) treatment promotes relevant motor functional improvement after severe frontal cortex lesion in adult rats

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HIGHLIGHTS

- We induced a severe frontal motor cortex ablation in adult rats.
- We examined the effects of GH treatment and rehabilitation on brain recovery.
- Early administration of GH led to significant motor recovery.
- Nestin immunoreactivity appeared in the contralateral motor cortex of GH-treated injured rats.
- GH plus rehabilitation increased neurogenesis and brain plasticity in injured rats.

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ABSTRACT

A number of studies, in animals and humans, describe the positive effects of the growth hormone (GH) treatment combined with rehabilitation on brain reparation after brain injury. We examined the effect of GH treatment and rehabilitation in adult rats with severe frontal motor cortex ablation. Thirty-five male rats were trained in the paw-reaching-for-food task and the preferred forelimb was recorded. Under anesthesia, the motor cortex contralateral to the preferred forelimb was aspirated or sham-operated. Animals were then treated with GH (0.15 mg/kg/day, s.c) or vehicle during 5 days, commencing immediately or 6 days post-lesion. Rehabilitation was applied at short- and long-term after GH treatment. Behavioral data were analyzed by ANOVA following Bonferroni *post hoc* test. After sacrifice, immunohistochemical detection of glial fibrillary acid protein (GFAP) and nestin were undertaken in the brain of all groups.

Animal group treated with GH immediately after the lesion, but not any other group, showed a significant improvement of the motor impairment induced by the motor lesion, and their performances in the motor test were no different from sham-operated controls.

GFAP immunolabeling and nestin immunoreactivity were observed in the perilesional area in all injured animals; nestin immunoreactivity was higher in GH-treated injured rats (mainly in animals GH-treated 6 days post-lesion). GFAP immunoreactivity was similar among injured rats. Interestingly, nestin re-expression was detected in the contralateral undamaged motor cortex only in GH-treated injured rats, being higher in animals GH-treated immediately after the lesion than in animals GH-treated 6 days post-lesion.

Early GH treatment induces significant recovery of the motor impairment produced by frontal cortical ablation. GH effects include increased neurogenesis for reparation (perilesional area) and for increased brain plasticity (contralateral motor area).

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1. Introduction

Severe lesions of the motor cortex produce devastating effects on the normal operation of the motor activity, affecting both the planning and organization of voluntary movements and to the execution of these. This is due to the limited capacity of the adult brain for self-repair after neuronal loss caused by trauma or anoxia/ischemia. Traumatic alterations of axonal wirings, as it occurs in cortical lesions, immediately leads to a permanent functional impairment which produces behavioral deficits and have several anatomic consequences [1–5].

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Studies carried out for obtaining a certain degree of adequate axonal rewiring necessary for the reconstruction of damaged cortical circuitry and restoration of lost brain function have been made by transplanting embryonic tissue into the damaged cortex of adult rats. These studies have shown successful survival and establishment of reciprocal connections between the host and grafted tissue [4–13], leading to behavioral graft-dependent recovery [4,14–17].

Experimental frontal motor cortex lesion in adult rats leads to the appearance of important alterations in the forelimb skills to obtain food, motor asymmetries and difficulties for moving on irregular surfaces [18].

In line with other studies [14–16], we demonstrated that fine motor skills can be recovered after grafting of the frontal cortex lesion in adult rats with homotopic fetal cortex or fetal amygdaloid grafts, indicating that functional recovery depends on grafting but is only evident when the animal is obliged to use the affected limb [4,17]. We also demonstrated that transplants of encapsulated astrocytes in alginate spheres induce a long-term improvement of motor lesion deficits induced by frontal motor cortex lesion [19]. Moreover, our data indicated that grafted neurons receive functionally effective contacts from the adjacent motor cortex and then restore, at least partially, previously damaged circuits [13,20].

While from these and other studies it seems to be clear that precursor neural cell transplants may contribute to recover an injured brain in rats, a number of ethical, methodological and health issues make impossible for now to apply this knowledge to human people with traumatic brain injury. Therefore we sought for new and different experimental approaches, such as to study the effects of growth hormone (GH) administration combined with rehabilitation in rats in which a severe frontal cortex lesion had been induced.

A number of hormones play an important role in the recovery of brain injuries acting either on neurogenesis and/or neural plasticity. Among them the growth hormone–insulin-like growth factor-1 (GH-IGF-I) system seems to play a pivotal role in inducing adult neurogenesis and increasing brain plasticity [21]. Many observations support a role for GH in development and function of the brain [22]. GH and IGF-I are expressed in the brain [23–25], and both hormones can cross the blood–brain barrier [25]. The GH receptor (GHR) and the IGF-I receptor (IGF-IR) are widely expressed in several zones of rodent and human brain [26–31]; particularly GH, GHR and IGF-IR are expressed in hippocampal neural progenitors, acting on the proliferation and differentiation of these neural stem cells [32,33]. Exogenously applied GH and prolactin (PRL) promote the proliferation and migration of neural stem cells derived from fetal human forebrain in the absence of epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) [34]. Thus, besides its major role in several metabolic processes, the GH-IGF-I system has multiple and important neurotrophic effects both in the central and peripheral nervous system [21,25,35]. According to this, GHR expression is increased in the subventricular zone after focal ischemia [36], and GH has been demonstrated to increase cell proliferation in the hippocampus of adult hypophysectomized rats [37]. Similarly, IGF-I increases cell proliferation in hippocampal cells [32,38], and its expression is increased in the affected brain hemisphere after an ischemic injury [39,40]. Recently, it has been demonstrated that the addition of exogenous GH significantly increased the expansion rate in long-term neurosphere cultures derived from wild-type mice and the same study detected a doubling in the frequency of stem cell-derived colonies for up to 120 days following a 7-day intracerebroventricular infusion of GH, suggesting that GH activates populations of resident stem and progenitor cells [41]. No studies have been carried out for analyzing whether GH treatment might contribute to brain repair after a severe brain injury in experimental models, but we recently demonstrated, in rats, that exogenous GH administration promotes the proliferation of hippocampal neural

precursors after brain injury induced by kainate administration [42].

On these bases, this study was designed to investigate whether GH treatment combined with rehabilitation might collaborate to functional motor recovery after inducing a severe frontal cortex lesion in adult rats. Our results demonstrate that GH treatment administered immediately after the lesion allows a significant improvement of motor impairments despite of the severity of the frontal cortex lesion.

2. Material and methods

Thirty-five male Wistar rats (Charles River Laboratories, Spain) aged 2.5 months old, were housed under conditions of controlled temperature (18–20°C) and natural light/dark, at least 4 days before beginning experiments. Rats were fed with a normal chow diet and water *ad libitum* except when the paw-reaching-for-food task was carried out; at this time animals were maintained in the 86–88% of its initial weight.

All experiments and procedures involved in this study were approved by the University of Salamanca Ethics Committee, and were conducted in accordance with the animal care guidelines of the European Communities Council (86/609/EEC) and Spanish normatives (Real Decreto 1201/2005); very effort was made to minimize the suffering and number of animals used.

2.1. Experimental design and behavior test

The experimental design consisted in the following phases:

1.1) *Presurgical phase* – Animals were trained in the paw-reaching-for-food task and the preferred forelimb was recorded.

1.2) *Induction of frontal cortex lesion* – Anaesthetized animals were lesioned by aspiration in the motor cortex contralateral to the preferred forelimb or sham-operated. The effectiveness of the lesion was then verified at day 6 or 7 post-lesion.

1.3) *Treatment with GH or vehicle and rehabilitation* (forced use of the affected paw). Evaluation of the paw-reaching-for-food responses.

1.4) *Sacrifice and removal of the brain for immunohistochemistry studies of the perilesional area and contralateral frontal motor cortex*.

These study phases are schematically shown in Fig. 1.

Surgical procedures and sacrifice were carried out under deep anesthesia with Equithesin (20 mg/kg intraperitoneally).

1.1) *Presurgical phase* – Four days after the arrival of animals they were trained for paw-reaching-for-food task, a specific motor test in which animals are conditioned to perform high precision motor movements of extension and flexion of the forelimb fingers for obtaining food. This test for fine motor skills has been adapted from that developed by Whishaw et al. [1], and has been widely used in previous studies from our group [4,17,19]. Before carrying out the test, animals were housed individually and food was restricted until their body weight decreased to 86–88% of their previous body weight. The design of the test cage has been described in previous studies of our laboratory [4,17,19].

In the paw-reaching-for-food test, rats were required to extend a forelimb through the hole, grasp and retrieve a pellet from the groove, take it to the mouth and eat it. Each time an animal succeeded in eating a pellet without dropping it was counted as a successful response (Fig. 2). Dropping the pellet after grasping it was counted as an unsuccessful response.

Each experimental animal was placed in the test cage in individual sessions lasting 3 minutes during 10 days (in the presurgical phase) for quantifying the number of successful and unsuccessful responses.

During the presurgical phase it was established which was the preferred forelimb (right or left) of each animal and the total number of responses (successful and unsuccessful) with both forelimbs, and the percentage of successful responses with the preferred forelimb with regard to the total number of responses with both paws.

Apart of recording the preferred forelimb this motor test was used in the presurgical phase for training the animals and after inducing the lesion (for testing the efficiency of it) and during the rehabilitative therapy as well (Fig. 1).

1.2) *Induction of frontal cortex lesion* – Each rat was placed in a stereotaxic apparatus and the skull exposed at the level of bregma. Animals were divided at random into two groups. One group ($n=25$) was subjected to a unilateral frontal cortex lesion. The other group ($n=10$) was sham-operated. Lesions were made at the coordinates indicated by Neafsey et al. [43] to remove the forelimb area of the motor cortex. A section of the skull was removed unilaterally from 1 to 4 mm anterior to bregma, and 1 to 3.5 mm lateral to the midline. Corpus callosum established the ventral limits of the lesion. A schematic representation of the lesion induced is shown in Fig. 3.

Lesions were made by aspiration in the hemisphere contralateral to the preferred paw determined in the presurgical phase. Under visual guidance using an operating microscope, meninges were removed, and a glass pipette connected to an aspiration pump was gently introduced into the cortex to remove the tissue. Care was taken to spare the white matter underlying the cortex. Lesions were severe and homogenous in size and localization. They were restricted to the primary and secondary motor cortex areas (M1 and M2). After surgery, the skin was sutured.

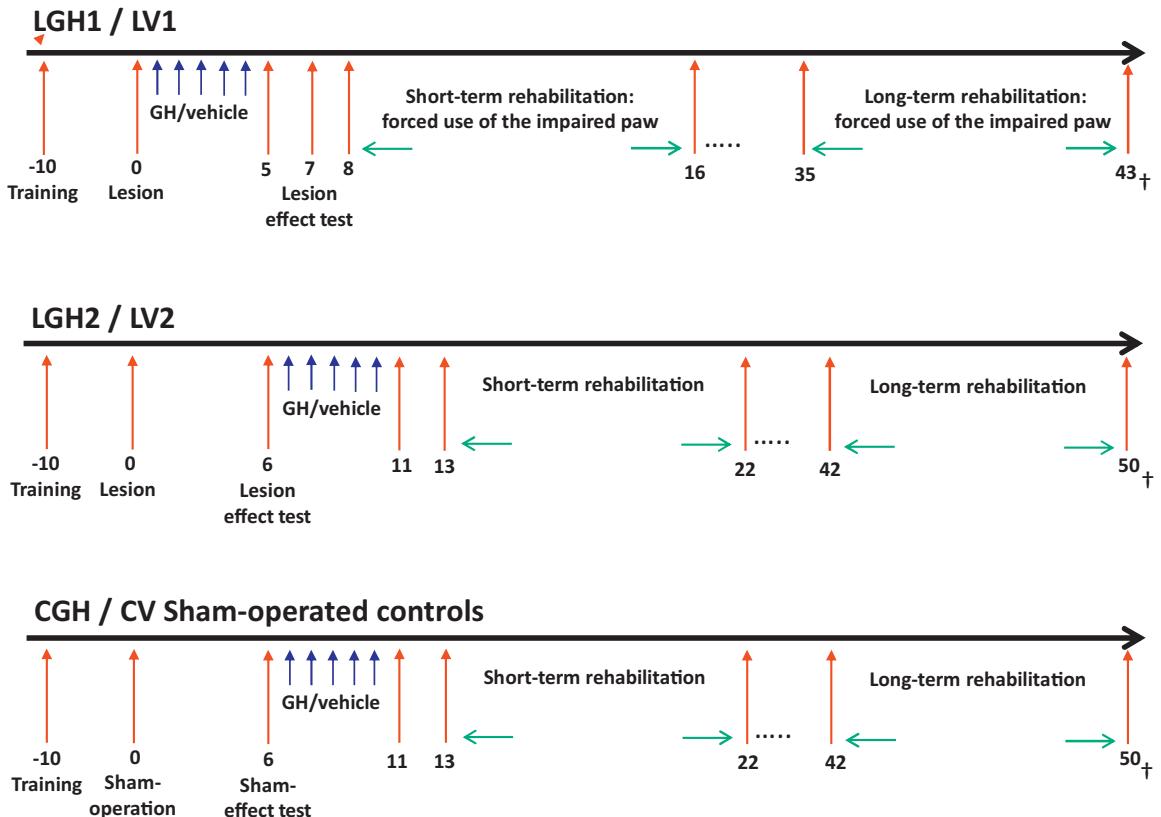


Fig. 1. Schematic diagram of the experimental design. The treatment with GH or vehicle started immediately after the lesion (LGH1 and LV1 groups, respectively) or 6 days post-lesion (LGH2 and LV2 groups, respectively). Sham-operated controls were treated with GH or vehicle 6 days after the lesion (CGH and CV, respectively). Day 0 indicates the day of the lesion. After the lesion (on day 6 or 7), the effectiveness of the injury was evaluated in the paw reaching test. The rehabilitation therapy was applied at short-term and at long-term after the lesion. It consisted in daily sessions of 3 min, with the forced use of the impaired paw, during 9 consecutive days. Time scale: days.

Control animals were subjected to the same surgical process in the contralateral hemisphere to the preferred forelimb except the lesion itself.

After surgery, the paw-reaching-for-food task established whether the lesion had been effective: animals began to use their non preferred paw for reaching food or the percentage of successful responses was significantly decreased with regard to previous values in the presurgical phase.

1.3) Treatment with GH or vehicle and rehabilitation – In two groups of injured animals, rhGH (Saizen, Merck; 0.15 mg/kg/day, subcutaneously) was administered during 5 consecutive days commencing immediately after the frontal lesion (Lesion Group treated early with GH: LGH1, $n=7$) or 6 days after the lesion had been induced (Lesion Group treated with GH 6 days after injury: Group LGH2, $n=8$). Two other groups of lesion animals were given vehicle (PBS 0.1 M, pH 7.4) immediately after the lesion (Lesion Group treated early with vehicle: Group LV1, $n=5$) or 6 days post-lesion (Lesion Group treated with vehicle 6 days after injury: Group LV2, $n=5$). For control purposes, two groups of sham-operated animals received GH (Control Group treated with GH: Group CGH, $n=5$) or vehicle (Control Group treated with vehicle: Group CV, $n=5$) during 5 days, 6 days after sham operation. One of the rats in Group LV1 died after surgery, therefore at the time of statistical analysis only the four animals that completed the

experiment were considered in this Group LV1. GH/vehicle treatments are shown in Fig. 1.

Rehabilitative therapy consisted in inducing the obliged use of the forelimb affected by the frontal motor cortex lesion (preferred forelimb) by attaching a removable plaster bracelet, which prevented reaching food but not other movements, to the forearm of the non preferred paw (as established in the presurgical phase), so that animals could reach food only with the paw contralateral to the lesion (impaired paw) (Fig. 4). The animals wore the bracelet only during the test and not continuously. Rehabilitative therapy was carried out during 9 consecutive days in daily sessions of 3 minutes. This kind of rehabilitative therapy, applied to all experimental groups (including sham-operated controls), was performed during two different time periods after cortical lesion and GH or vehicle treatment: 1.3.1) Short-term rehabilitation, 2 days after finishing GH or vehicle administration (commencing on day 8 post-lesion in groups LV1 and LGH1, and on day 13 post-lesion in groups LV2 and LGH2), within the critical period for maximal efficiency of rehabilitative therapy [44]; and 1.3.2) Long-term rehabilitation, 30 days after finishing treatment with GH or vehicle. During the period of time comprised between short- and long-term rehabilitation, the animals were kept in their cages and food was available *ad libitum*. These procedures are shown in Fig. 1.



Fig. 2. Consecutive photographs illustrating a rat in the test cage performing a successful response in the paw-reaching-for-food task, during the training in the preoperative phase. The design of the test cage prevented use of the tongue to retrieve food pellets or to rake the pellets.

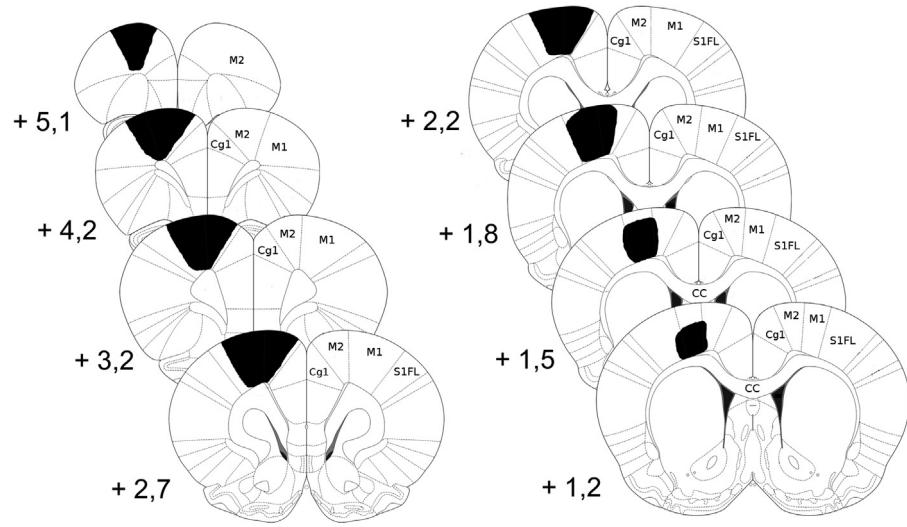


Fig. 3. Schematic representation of brain coronal sections showing an example of a cortical lesion by aspiration. Areas of cortical ablation are outlined in black. Coordinates are relative to bregma (in millimeters). In all rats, excepting sham-operated animals, the secondary motor cortex (M2) and primary motor cortex (M1) were damaged. Cg1, cingulate cortex, area 1. S1FL, primary somatosensory cortex, forelimb. CC, Corpus callosum.

1.4) Sacrifice and removal of the brain for immunohistochemistry studies of the perilesional area and contralateral frontal motor cortex.

After completion of the long-term rehabilitation therapy, the presence of the glial fibrillary acid protein (GFAP, marker of astrocytes) and nestin (a marker of neural precursors) was examined by immunohistochemistry in the perilesional area and the intact contralateral frontal motor cortex in all groups. Eight weeks post-lesion, animals were sacrificed under deep anesthesia with Equithesin and transcardially perfused with 2% dextrane in 0.1 M sodium phosphate buffer (PB), pH 7.4, followed by a fixative solution (4% paraformaldehyde solution in the same buffer).

Brains were removed, placed in fixative solution and then stored in cryoprotectant solution at -18 °C before use. Brains were sliced in a freezing microtome; two rostral-caudal sets of 40 µm-thick coronal sections were taken throughout the brain. One set of sections was processed using a free-floating immunohistochemistry method for nestin. Another set was processed for GFAP. Sections were first placed at room temperature in 0.3% hydrogen peroxide in Tris-phosphate buffer saline (TPBS), pH 7.4, for 12 min to inactivate endogenous peroxidase activity. Sections underwent several TPBS washes and then were incubated in a block solution to prevent non-specific protein binding. The block solution included 0.2% Triton X-100 and 3% normal horse serum in TPBS. Sections were incubated in primary antibody solution (TPBS with 1.5% horse normal serum), overnight, at room temperature. The primary antibodies were: anti-Nestin (clone Rat-401, 1:90, Chemicon) or anti-GFAP (polyclonal rabbit antibody, Dako, 1:100). Following primary antibody incubation, sections were rinsed several times in TPBS and then placed in the secondary antibody (1:200 biotinylated anti-mouse or anti-rabbit, IgG; Vector Laboratories) in TPBS. After this incubation, sections were rinsed several times in TPBS and incubated at room temperature for 2 h in a horseradish peroxidase-avidin complex (ABC kit, Vector Laboratories). Immunoreactivity was then visualized using

3-3' diaminobenzidine tetrahydrochloride. Specificity of antibody binding was verified with tissue sections incubated without primary antibody (no primary controls). Each run of immunocytochemical processing included tissue from all groups to decrease the contribution of batch effects to the variability in the immunohistochemistry staining. Sections were examined and photographed with a Leica DM microscope. Observations were predominantly restricted to the motor cortex.

2.2. Data analysis

Statistical analysis was performed by using the Statview program.

Fine motor skills results were analyzed by two-way (group and session) analysis of variance (ANOVA). When ANOVA showed a significant difference among groups ($p < 0.05$), partial ANOVA comparing the different groups in each session was made. To compare individual means Bonferroni *post hoc* test ($p < 0.003$) was used.

3. Results

3.1. Paw-reaching-for-food task

Results from this fine motor skills test during all the experimental phases are shown in Fig. 5.

Mean percentages of successful responses obtained with the preferred paw with regard to the total number of responses is shown in Fig. 5A, while the total number of responses (successful plus unsuccessful) obtained with both paws during the different phases of the experiment is shown in Fig. 5B.

3.1.1. Presurgical phase

In this phase the ability for taking food pellets from the groove and eating them was similar in all the rats. All rats displayed a stable strategy, using a single paw to reach for pellets from the third to the last session, so that a spontaneous limb preference was established during the training.

In all animals, limb preference was established by the end of the third session, and the paw used was then considered the preferred paw. When the percentage of attempts using the right or left paw was between 85 and 100%, a rat was classified as right- or left-handed. It was considered that animals were well trained when the percentage of successful responses was higher than 60% during three consecutive sessions.

For distributing animals into the different experimental groups the mean of results obtained in the three last sessions of this phase was taken. Therefore both the percentage of successful responses and the total number of responses were similar in all experimental



Fig. 4. Photography of a rat during the rehabilitative therapy. The rat wears a removable plaster bracelet (arrow) fitted onto the non-preferred paw, to force the use of the impaired paw affected by the motor cortical lesion, in the paw reaching test.

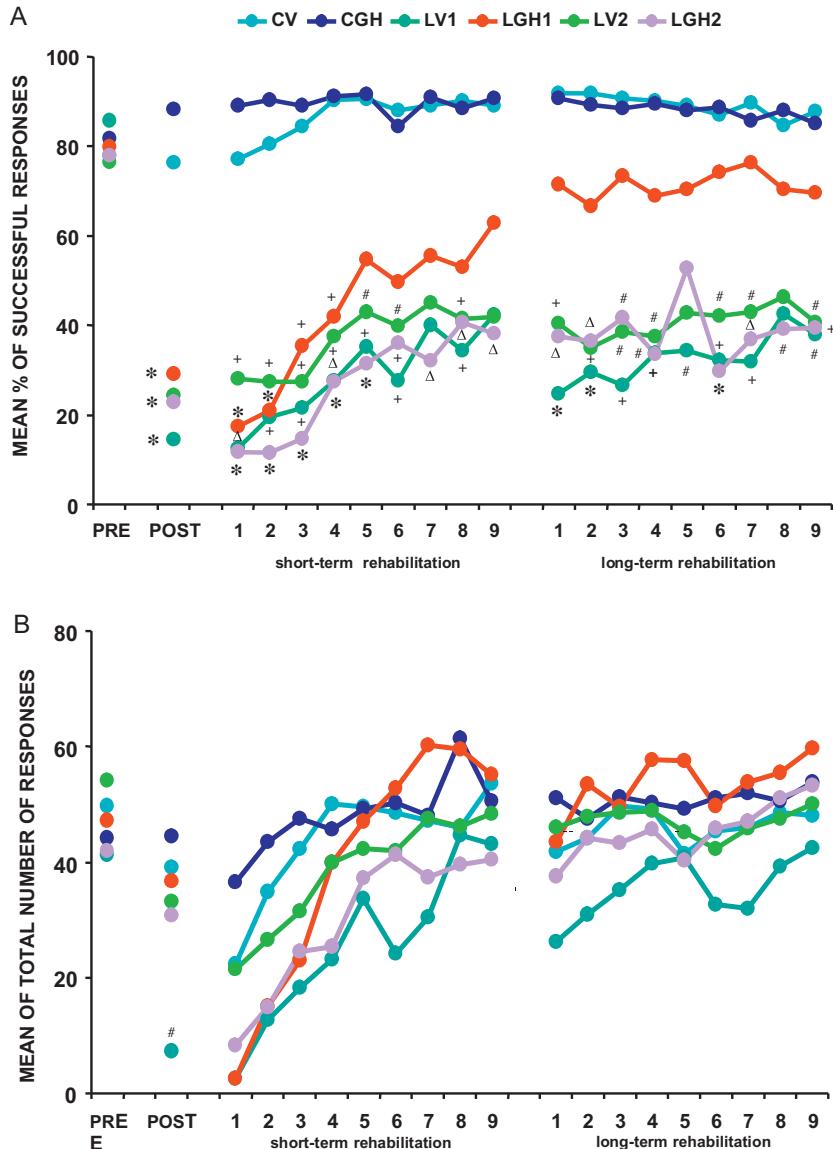


Fig. 5. Results obtained in the paw-reaching-for-food task with the preferred paw (impaired paw) at the preoperative phase (PRE), post-lesion (POST) and rehabilitative therapies. A) Mean of the percentages of successful responses (successful responses/total number of responses). B) Mean of the total number of responses (successful and unsuccessful with both paws). The rehabilitative therapies (short- and long-term rehabilitation), consisted in the forced use of the impaired paw, in daily session of 3 min, during 9 consecutive days. LGH1/LV1, Animals treated with GH or vehicle immediately after the lesion, respectively. LGH2/LV2, Animals treated with GH or vehicle 6 days post-lesion, respectively. Significance levels are done compared from sham-operated control Groups (CV and CGH). * $p < 0.0001$, $\Delta p < 0.0005$, + $p < 0.001$, # $p < 0.003$ (Bonferroni's test). x-axis indicates days (1–9).

groups ($F_{5,29} = 0.955$ and $F_{5,29} = 1.884$, Fig. 5A and B, PRE, respectively).

3.1.2. Postsurgical phase

The effectiveness of the lesion was tested before commencing short-term rehabilitation (as indicated before one rat in Group LV1 died in this phase). ANOVA showed that very significant differences existed between groups ($F_{5,28} = 15.885$, $p \leq 0.0001$); as Fig. 5A shows, while the percentage of successful responses was preserved in sham-operated control groups (CGH and CV), these responses significantly decreased in all injured animals (Bonferroni *post hoc* test, $p \leq 0.0001$) as compared to those obtained in this phase in control groups and those in the presurgical phase. Successful responses were similar in all injured animals (Fig. 5A, POST). Some of these injured animals changed their preferred paw, while others continued to use the preferred paw but the number of successful responses clearly decreased.

With regard to the total number of responses, ANOVA showed significant differences between groups ($F_{5,28} = 2.70$, $p \leq 0.05$). Bonferroni *post hoc* test indicated that animals from group LV1 decreased the number of total responses in relation to control groups (CGH and CV; Fig. 5B, POST).

1.3) Short-term rehabilitation (Fig. 5) – Two days after finishing GH or vehicle administration, a rehabilitative therapy during 9 consecutive days in daily sessions of 3 min, was carried out. It consisted in the forced use of the impaired paw (preferred paw). Two-way (group \times session) ANOVA showed significant differences between groups ($F_{5,28} = 11.80$; $p \leq 0.0001$). In addition, given that the number of successful responses was increasing along the rehabilitation in all groups, a significant session-effect was observed ($F_{8,224} = 10.12$; $p \leq 0.0001$). On the contrary, the interaction group \times session was not significant ($F_{40,224} = 1.10$). Partial ANOVA tests showed significant ($p \leq 0.001$) differences among groups in all rehabilitative sessions (Fig. 5A). As expected,

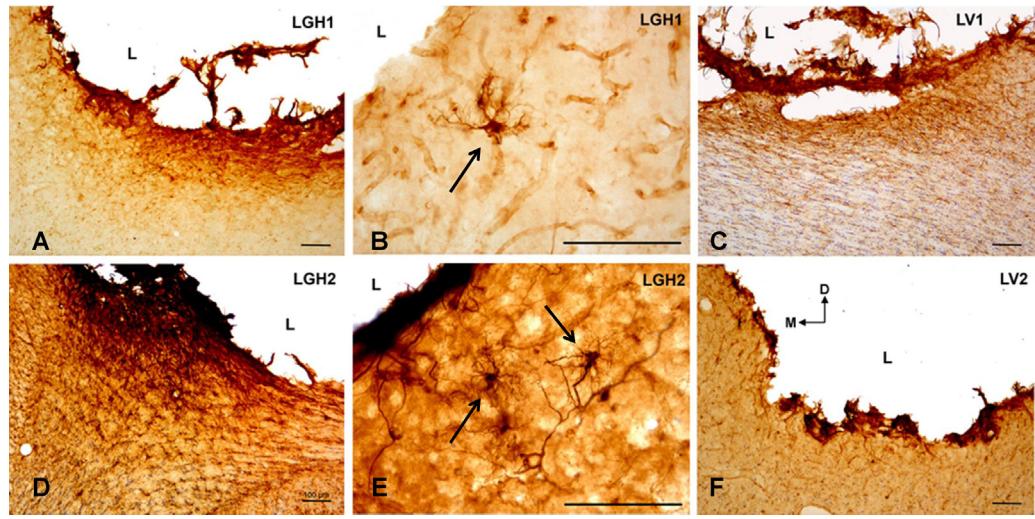


Fig. 6. Nestin immunolabeling in coronal sections of the perilesional area of GH/vehicle treated rats, at 8 weeks post-lesion. A: Section of a rat treated with GH immediately after the lesion (LGH1). B: Reactive astrocyte expressing nestin localized in the boundary of the cortical lesion in a rat of the LGH1 group (arrow). C: Section of an animal treated with vehicle immediately after the lesion (LV1). D: Section of a rat treated with GH 6 days after lesion (LGH2). E: Reactive astrocytes expressing nestin in the perilesional area of a rat of the LGH2 group (arrows). F: Section of an animal treated with vehicle after 6 days of the lesion (LV2). Note the increased expression of nestin in GH-treated animals than in rats given vehicle. In turn, LGH2 rats showed more perilesional nestin expression than LGH1 animals. L, lesion cavity. D, Dorsal. M, Medial. Scale bar = 100 μ m.

the number of successful responses in both control groups was not changed during short-term rehabilitation. However, interestingly, Bonferroni *post hoc* test revealed that the number of successful responses in animals treated with GH immediately after the lesion (group LGH1), increased from values significantly lower than in control sham-operated groups ($p \leq 0.001$), to values similar to those in control groups after 5 days of rehabilitation, showing a clear trend to increase and be normalized at the end of this rehabilitative period (Fig. 5A). This significant improvement was not observed in any other group of animals with motor cortex lesion.

Total number of responses was similar in all the experimental groups (Fig. 5B), therefore no significant differences were observed among them ($F_{5,28} = 1.21$). However, since all groups progressively increased the number of total responses along the rehabilitative sessions a clear session-effect ($F_{8,224} = 28.52$, $p \leq 0.0001$) and an

interaction group \times session ($F_{40,224} = 1.59$, $p \leq 0.05$) were detected (Fig. 5B, short-term rehabilitation).

1.4 Long-term rehabilitation (Fig. 5) – Thirty days after being treated with GH or vehicle a new period of rehabilitative therapy, similar to that carried out during short-term rehabilitation was performed in all animals (as depicted in Fig. 1). Again the animals were forced to use the paw affected by the cortical lesion. Session-group global ANOVA demonstrated that significant differences existed among groups ($F_{5,28} = 7.10$, $p \leq 0.0002$). However, no session-effect ($F_{8,224} = 0.86$) or interaction group-session ($F_{40,224} = 1.15$) were observed. Bonferroni *post hoc* test revealed that in animals given GH immediately after the cortical lesion (LGH1), a clear improvement of the motor deficit produced by the lesion existed, being the percentage of successful responses, in all nine sessions, similar to that in control animals (Fig. 5A, long-term

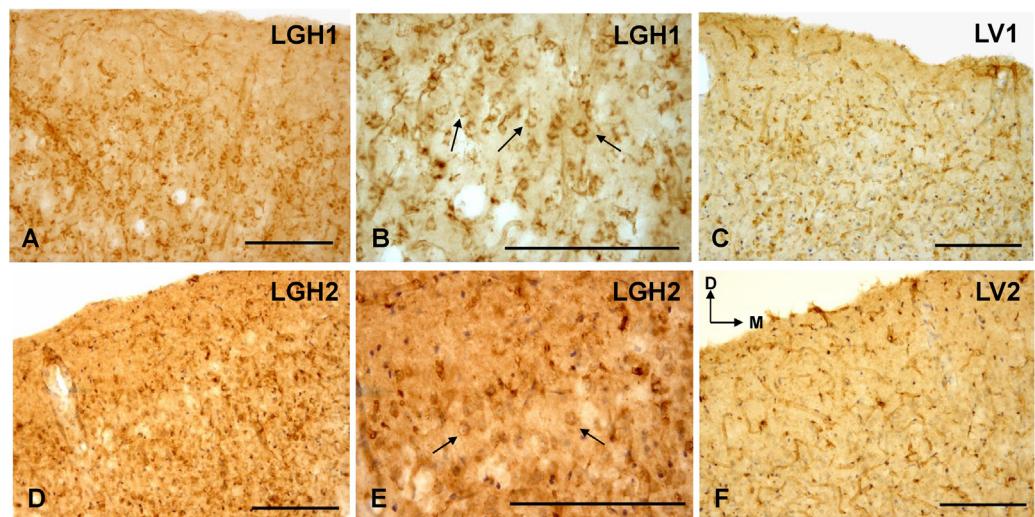


Fig. 7. Nestin immunoreactivity in coronal sections of the homotopic undamaged motor cortex in GH or vehicle treated animals, 8 weeks after lesion. A: Immunopositive nestin cells located at the layers II/III of the undamaged motor cortex, in a rat treated with GH immediately after the lesion (LGH1). B: Magnification of A. Arrows point out some cells re-expressing nestin. C: Section of an animal treated with vehicle immediately after the lesion (LV1). D: Nestin immunolabeling in an animal treated with GH 6 days after lesion (LGH2). E: Magnification of D. Arrows point out some nestin immunolabeled cells. F: Section of a rat treated with vehicle 6 days post-lesion (LV2). Note that nestin re-expression was higher in LGH1 than in LGH2 animals. No nestin immunoreactivity was detected in vehicle-treated animals (LV1 and LV2). L, lesion cavity. D, Dorsal. M, Medial. Scale bar = 100 μ m.

rehabilitation). This was not observed in any other group of injured animals (LV1, LV2 and LGH2).

With regard to the total number of responses (Fig. 5B, long-term rehabilitation), global ANOVA showed that no significant differences existed among groups ($F_{5,28} = 0.59$), and that there was not any interaction group \times session ($F_{40,224} = 0.79$), but a significant session-effect ($F_{8,224} = 4.36$, $p \leq 0.0001$) was observed.

3.2. Immunohistochemical results

2.1) Nestin – In control animals, given GH or vehicle (CGH and CV groups), nestin detection was restricted to the vascular endothelium and the ependimal cells. However, in lesion animals, independently of GH or vehicle treatment, a re-expression of nestin was detected in the perilesional area. This immunoreactivity for nestin was localized in fibrillar structures and in some different cellular phenotypes, mostly reactive astrocytes. Nestin labeling in the perilesional area was similar in animals given GH immediately after the lesion (LGH1) and in injured animals treated with vehicle (LV1 and LV2), but clearly increased in animals being treated with GH 6 days after the lesion (LGH2). This is shown in Fig. 6.

Interestingly, nestin immunoreactivity was detected in the homotopic motor cortex of the intact hemisphere in lesion animals treated with GH (LGH1 and LGH2) but not in lesion animals treated with vehicle (LV1 and LV2) (Fig. 7). Nestin immunoreactivity was more evident in LGH1 than in LGH2 animals. Nestin re-expression in this area appeared to be localized in synaptic terminals in neurons of layers II/III of this contralateral frontal motor cortex (Fig. 8). No nestin re-expression was detected in control animals (CGH and CV) (data not shown).

2.2) Glial Fibrillary Acid Protein (GFAP) – In all groups, GFAP immunopositive astrocytes were detected scattered throughout the cortex, mainly in cortical layers I, II/III and VI. In injured animals, at eight weeks post-lesion, an intense GFAP immunoreactivity was observed in the perilesional area, both in fibrillar structures and in cells with astrocytes morphology. This morphology corresponded to that present in reactive astrocytes. GFAP immunoreactivity was similar in injured animals treated with GH or vehicle (Fig. 9).

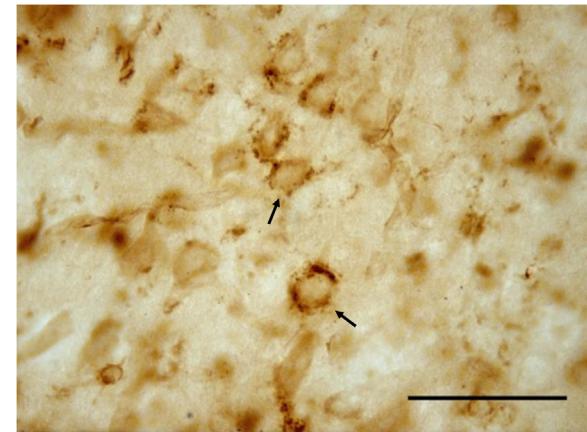


Fig. 8. High magnification of nestin immunoreactivity in the homotopic undamaged motor cortex of a rat given GH immediately after injury. Nestin re-expression outline cell bodies of unlabeled neurons (arrows) located in layers II/III of the undamaged motor cortex. Scale bar = 50 μ m.

4. Discussion

This study describes, for the first time, the effects of a combined treatment with GH and rehabilitative therapy in an experimental model of motor lesion that induces a marked deficit in fine motor skills. Our results demonstrate that an early treatment with GH, together with rehabilitative therapy, is able for developing compensatory mechanisms in the contralateral hemisphere that allow the functional recovery of the motor deficits produced by the frontal cortex lesion.

In agreement with previous studies from others and our group, our data show that a lesion of the frontal cortex produces an impaired performance of the paw-reaching-for-food-task [1–4,17,45–47].

It is well known that the impairment induced by the cortical lesion leads the animal to use the forepaw ipsilateral to the lesion, a response seen immediately after the injury. Lesion is considered to

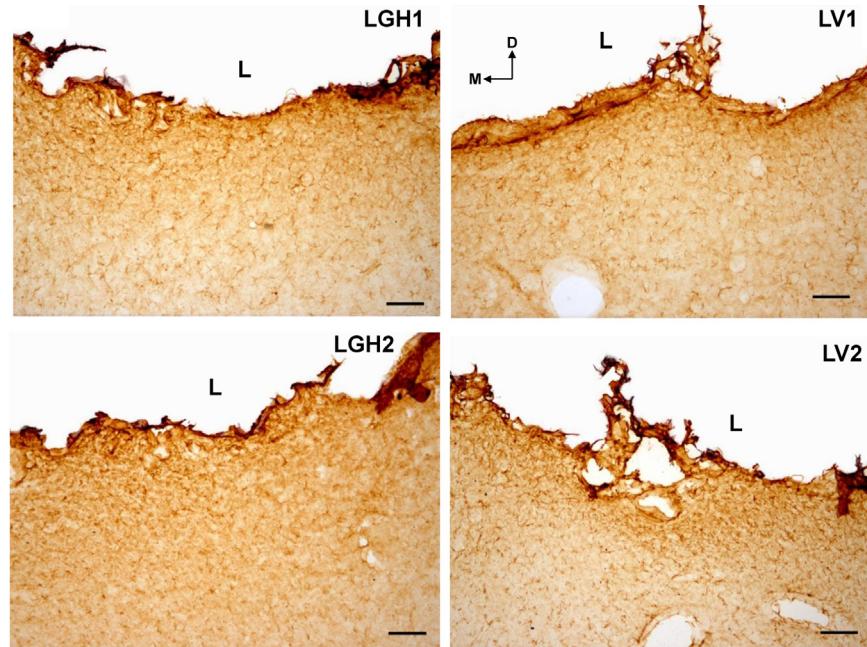


Fig. 9. Immunoreactivity for GFAP in coronal sections of the perilesional area of GH/vehicle treated rats, at 8 weeks post-lesion. LGH1/LV1, Animals treated with GH or vehicle immediately after the lesion, respectively. LGH2/LV2, Animals treated with GH or vehicle 6 days post-lesion, respectively. Note that astrogliosis in the form of upregulated GFAP expression was similar in all injured rats, independently of GH or vehicle treatment. L, lesion cavity. D, Dorsal. M, Medial. Scale bar = 100 μ m.

have been effective if animals change its preferred paw or notably reduce the number of successful responses when using the preferred paw ($\geq 30\%$ decrease). In this situation, lesion animals are able to grasp pellets but are usually unable to bring them to the mouth, dropping them before completing the response. That is, the lesion does not affect movement of the whole limb but affects the functional ability of the paw [4]. Consequently, the number of successful responses of the paw-reaching-for-food-task significantly decreased after the cortical lesion.

Previous studies in rats demonstrated that injured brains display heightened sensitivity to rehabilitative experience early after the lesion but declines with time [44]. The critical period of time for achieving maximal efficiency during rehabilitative therapies has been established between 5 and 14 days after injury. Rehabilitative therapies initiated during these days have been shown enhanced dendritic growth in the undamaged motor cortex [44]. That is, compensatory mechanisms of neural plasticity have been developed for reaching a certain degree of functional recovery.

In this study, rehabilitative therapies were commenced during this critical period of time for recovery (short-term rehabilitation), although on different days depending on the time at which GH treatment was initiated (day 8 post-lesion in groups LV1 and LGH1, and day 13 post-lesion in groups LV2 and LGH2). Despite of it, recovery was found only in animals in which GH treatment commenced immediately after the cortical motor lesion. The lack of positive results in animals given vehicle or in those in which GH treatment commenced 6-day post-surgery can be explained because the lesion induced by cortical aspiration is the most severe leading to enduring forelimbs impairments. After this kind of lesion, neural plasticity compensatory mechanisms do not appear in the contralateral cortex [48]. This agree with behavioral results obtained in our study in animals given vehicle or in which GH treatment commenced 6-days after cortical aspiration, but not with those in animals given GH immediately after surgery. A quick and progressive functional recovery was observed in this group of animals (LGH1), so that their responses to the fine motor skills test was not different from those in control groups after 5 days of rehabilitative therapy. The only possible explanation for these results has to be attributed to an effect produced by the early administration of GH.

The hypothesis that the system GH/IGF-I plays a role on brain repair after an injury has been postulated years ago [49]. However, most of studies carried out basically analyzed the role of IGF-1 [32,38,50]. We did not analyze plasma IGF-I values after GH treatment, but a number of data support a role for GH itself in brain repair after an injury. GH receptor is expressed in regions of the brain in which neurogenesis occurs during embryonic brain development [51,52], and in neurogenic regions of the postnatal rat brain [53]. Growth hormone itself is also found in cells of the ventricular zone during embryonic neurogenesis [52], and is produced endogenously within the postnatal hippocampus [54–57]. GH gene expression within the hippocampus is increased by some factors known to increase neurogenesis [58], including learning and estrogen [54,55].

Studies of the effects of GH on embryonic rat cerebral cortical and hippocampal neuronal cultures found that it induces the proliferation and differentiation of these cells [59,60]. Moreover, there is a population of neural stem cells which are activated by GH infusion, and which give rise to neurons in mice. These stem cells are activated by voluntary exercise in a GH-dependent manner and local synthesis of GH occurs in the hippocampus in response to a memory task [22]. As previously indicated, data from our group demonstrated that early GH administration leads to an attempt of repairing the brain damage produced by kainate administration in rats, as shown by the significantly increased proliferation of hippocampal neural precursors observed in GH-treated animals [42].

A similar effect of GH has been shown in a number of zones in the intact adult rat brain [61].

Nestin is a class VI intermediate filament protein expressed in different tissues [62]. Among neural cells, nestin expression seems to be limited to neural progenitor cells in the developing brain; after these cells differentiate, nestin expression is replaced by the expression of neuronal or glial specific markers [63–66]. Therefore, detection of nestin positive cells in the adult brain had to be restricted to the neurogenic niches, particularly the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus [67,68]. However, we detected nestin positive cells in the perilesional area of injured rats and in the intact contralateral frontal motor cortex of injured animals treated with GH but not with vehicle.

The finding of nestin positive cells in the perilesional area is not a surprising finding. Nestin re-expression has been reported following cerebral injury, mainly surrounding the lesion and being still apparent 28 days post-injury, suggesting that nestin may be involved in brain repair [69]. In that study, most of these nestin positive cells were shown to be astrocytes, but newly-formed cells detected in the subventricular zone prior to injury were readily detected in the perilesional area 3 days post-ablation, concomitant with nestin in this area, supporting a potential role for nestin re-expression in brain repair [69]. Interestingly, we found an intense nestin expression 8 weeks after the injury, while in the study by Douen et al. [69], only a weak apparent nestin immunoreactivity was observed 28 days post-injury. Since our objective was to examine whether GH treatment was able to induce functional recovery in injured animals, we could not sacrifice them before finalizing the behavioral study (43/50 days post-surgery), a period of time too long for detecting bromodeoxyuridine (BrdU) immunoreactivity. This is the reason by which we did not inject BrdU (a marker of cell division) after the lesion; therefore, we can not know whether these nestin positive cells are newly-formed neurons migrating from classic neurogenic niches or they indicate a local proliferation of nestin-expressing Class I cells in response to the cortical injury, triggered to divide by signals arising from injured cells [70].

Our study does not provide any clear explanation to the fact that nestin expression in the perilesional area was higher in animals given GH 6-days after the lesion than in any other group of injured rats, specifically rats in which GH was given immediately after surgery. Immunohistochemical data are not quantitative data, but results shown here seem to be clear about the fact that late administration of GH increased the number of nestin positive cells and fibrillar structures in the perilesional area. As stated before, we demonstrated that GH administration increases neural precursor proliferation after kainate-induced brain injury in rats [42]; therefore, both groups of lesion animals given GH had to exhibit a similar perilesional increased nestin immunoreactivity in relation to that observed in animals given vehicle. We do not know whether the severity of the lesion and/or the timing of GH administration led to the differences observed. In addition, the fact that a functional recovery was seen soon after the lesion in animals given GH immediately post-surgery could have contributed to these differences in the nestin re-expression between both groups of GH-treated rats.

With regard to the detection of intense GFAP immunoreactivity in the perilesional area in all injured rats it is clearly related to the reactive scar formation. It is well known that after a brain lesion, astrocytes respond with hypertrophy and proliferation that depend on the severity of the lesion. The roles played by reactive astrocytes after a brain lesion are not well understood. Potential protective effects could be provided by glutamate uptake and neuromodulation [71,72], while potentially detrimental effects might be caused by the release of pro-inflammatory cytokines and cytotoxic radicals [72,73]. Our results show that the perilesional area of both GH or vehicle treated animals, exhibit reactive

astrogliosis with increased expression of GFAP. This post-lesion GFAP upregulation is identical regardless the animals are treated with GH or not, suggesting that the lesion was homogenously severe. Furthermore, it has been shown that animals with a cortical lesion by contusion experiment a beneficial effect by the reactive astrocytes in moderate lesions, an effect that is not observed in severe lesions [74]. In our study, the administration of GH produced beneficial effects when the treatment was administered immediately after the lesion (a severe one) was induced, which is the main finding of the present study. We cannot discard the possibility that both beneficial and detrimental effects of reactive astrocytes could be involved in this process. Further studies should be necessary to elucidate this issue.

Interestingly, as described above, animals treated with GH immediately after surgery, but not other lesion animals, soon improved motor deficits. Using the affected paw by the motor cortex lesion, they were able for achieving a mean percentage of successful responses in the fine motor skills test similar to that observed in control animals. This was described before in a number of studies in which embryonic tissue was transplanted into the damaged cortex of adult rats [4–17]. However, our study is the first in describing that early GH administration together with rehabilitative therapy leads to behavioral results similar to those reported after embryonic tissue grafts [4–17].

Interestingly, we found a significant number of nestin positive cells in the intact contralateral motor cortex of lesion animals treated with GH, but not in animals given vehicle or in sham-operated controls. This has not been reported before. The number of these nestin positive cells was higher in animals given GH immediately after surgery than in those receiving the hormone 6-days after the lesion. Thus, the expression of nestin in the contralateral motor cortex appears to be related both to GH treatment and functional recovery. Cells expressing nestin had the phenotype of neurons, but this nestin immunoreactivity was detected in synaptic terminals. Therefore, the possibility exists that these neurons were already present and commenced to express nestin after GH treatment and rehabilitative therapy.

However and according to the results here obtained, it seems not to be possible that only the combination of GH and rehabilitative therapy could give arise to the appearance of nestin positive cells in the contralateral motor cortex of lesion animals, since this was not observed in sham-operated rats receiving the same combined treatment. Thus, most likely, the existence of the cortical lesion, and the resulting inflammation, led to the release of cytokines able for inducing changes responsible for this response. In fact, in our previous study, GH treatment only induced increased proliferation of neural precursor cells in injured rats, but not in intact animals [42]. One of these changes could be an overexpression of the GH receptor in the contralateral cortex. We did not study such a possibility but many studies reported that GHR is up-regulated after different brain injuries [36,49,75–77]. Therefore, an increased GHR expression in the contralateral motor cortex would facilitate the effect of GH and rehabilitation on the expression of nestin in this motor area.

Recently, a Class III nestin-expressing neurons have been found in the normal adult rodent and human basal forebrain [70]. The role of these cells remains unknown, but in view of their selective distribution in the cholinergic basal forebrain and related regions of the brain that are involved in higher cognitive functions such as attention, learning and memory, it has been speculated that cell cycle and/or plasticity-related events may be implicated in the expression of nestin by these Class III cells [70]. However, in that study there were not evidences indicating that Class III cells had re-entered the cell cycle and divided, nor had they arisen from the division of another cell [70]. Despite of it, the endogenous repopulation of degenerating pyramidal cells in the CA fields of

the hippocampus following an ischemic insult has been demonstrated [78]. Thus, we can not discard that such a possibility did also occur for explaining the appearance of nestin positive cells in the contralateral motor cortex of lesion animals treated with GH and rehabilitative therapy. Another possibility, before pointed by Hendrickson et al. [70], is that at any given time a subset of neurons is remodeling their cytoskeletons, because of the role they play in higher brain function, and that the expression of nestin by these cells reflects this remodeling. If this was the explanation for the detection of nestin positive cells in the contralateral motor cortex in our study, it is likely that GH and rehabilitative therapy had to be responsible for this effect addressed to increasing brain plasticity. Further experiments will clarify which are the mechanisms responsible for these results and the role that signals released by injured cells play on a putative GHR upregulation and the role of this GHR and/or IGF-I on the development of brain plasticity after the injury.

5. Conclusions

For the first time our study provides evidence demonstrating very important effects of early GH administration for brain repair after a severe injury. The positive effect of GH needs to be accompanied by a parallel rehabilitative therapy to be shown. Despite of the fact that late GH administration together with rehabilitative therapy was not traduced in significant motor improvements, our data do not allow us to exclude that benefits also could have been reached if GH administration and rehabilitation had lasted longer. Further studies in a larger number of animals will clarify this possibility already observed in human patients [79,80].

Competing interest

No competing financial interests exist.

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