Growth hormone responsive neural precursor cells reside within the adult mammalian brain

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The detection of growth hormone (GH) and its receptor in germinal regions of the mammalian brain prompted our investigation of GH and its role in the regulation of endogenous neural precursor cell activity. Here we report that the addition of exogenous GH significantly increased the expansion rate in long-term neurosphere cultures derived from wild-type mice, while neurospheres derived from GH null mice exhibited a reduced expansion rate. We also detected a doubling in the frequency of large (i.e. stem cell-derived) colonies for up to 120 days following a 7-day intracerebroventricular infusion of GH suggesting the activation of endogenous stem cells. Moreover, gamma irradiation induced the ablation of normally quiescent stem cells in GH-infused mice, resulting in a decline in olfactory bulb neurogenesis. These results suggest that GH activates populations of resident stem and progenitor cells, and therefore may represent a novel therapeutic target for age-related neurodegeneration and associated cognitive decline.

It is now clear that the adult mammalian brain contains populations of endogenous neural stem1–2 and progenitor cells (together termed precursor cells) that have the ability to replace lost populations of cells under normal conditions3 and can become activated after injury4–5. Stem cells are best defined by their ability to proliferate, self-renew over an extended period of time, and generate a large number of differentiated progeny6–7. While this functional definition is accurate, it unfortunately restricts investigators to a retrospective analysis. This degree of uncertainty has made the identification of neural stem cells (NSCs) a controversial area of research since their discovery4. Coincident with the investigation of NSC biology using functional assays, was the pioneering work of Alvarez-Buylla and colleagues8–9 which elegantly described the cytoarchitecture and cellular hierarchy of the adult subventricular zone (SVZ) of the lateral ventricle; one of two locations within the adult mammalian brain known to contain NSCs and their progeny. As recently reviewed by Kreigstein and Alvarez-Buylla10, NSCs in this region (termed Type B cells) proliferate to produce transient amplifying cells (Type C cells) that in turn generate migratory neuroblasts (Type A cells). It is these Type A cells that ultimately repopulate lost populations of interneurons in the olfactory bulb (OB) via the rostral migratory stream (RMS).

There is now a growing list of NSC markers that have been reported to localize with Type B cells such as glial fibrillary acidic protein (GFAP)11–12, Nestin13, CD13314 and platelet derived growth factor receptor alpha (PDGFRα)15–16. Unfortunately, as these markers are not found exclusively on NSCs, investigators continue to use a multifaceted approach, combining the use of these markers with functional studies to more confidently identify neural stem and progenitor cells in vivo17. Indeed, previous studies have used the neurosphere assay to assess stem and progenitor cell numbers and have shown that both type B and C cells have the ability to form neurospheres18–20 making it difficult to discriminate between the two populations. Fortunately, an in vivo culture technique, the neural colony forming cell assay (N-CFCA), has recently been developed whereby colony size enables the discrimination between NSC- and progenitor-derived colonies21. Studies employing this new technique have reinforced the hypothesis that Type C cells do not appear to possess the extensive self-renewal capabilities typically observed in populations of NSCs22–25.
In addition to phenotypic identification, understanding how NSCs and progenitor populations are activated is of considerable importance. While best known for its role in regulating somatic growth and metabolic processes, there is substantial evidence to suggest that growth hormone (GH) plays an important role in the development and repair of the mammalian central nervous system. For example, growth hormone receptor GHR null (GHR$^{-/-}$) and Suppressor of Cytokine Signaling-2 knockout mice, which represent loss and gain of GHR function respectively, display altered brain size, cortical architecture, and neuron and glial cell number. Moreover, GH administration improves cognitive deficits in GH-deficient rodents, and acts as a neuroprotective agent in aged animals.

Prior work describing the widespread expression of GH and GHR in the perinatal and adult rat brain, the ability of GH to cross the blood brain barrier, and its ability to act as a neuroprotective agent when infused directly into the ventricles of rodents after stroke suggests GH acts directly on neural cells. Building on these findings, more recent in vitro studies have demonstrated that the addition of exogenous GH increases the frequency of both rodent and human-derived neurospheres. Moreover, neurospheres generated from GHR$^{-/-}$ mice appear smaller, contain fewer proliferating cells, and exhibit reduced self-renewal. These results clearly demonstrate that GH is acting on neural precursors, but do not directly address whether a functional GHR is found on NSCs. Although, the detection of GHR and GHR-immunoreactive (GHR$^{\text{ir}}$) cells in germinal regions of the adult brain highly enriched in precursor cells and our recent observation of an absence of exercise-dependent enhancement of NSC number in the SVZ of adult GHR$^{-/-}$ animals further suggests the GH/GHR pathway plays a direct role in activating endogenous NSCs. Accordingly, we sought to directly investigate whether a functional GHR is present on resident neural stem and progenitor cells in the adult mouse brain and determine whether it is able to regulate the activity of these cells.

**Results**

GHR$^{\text{ir}}$ cells exhibiting stem cell properties are present in germinial regions of the mouse brain. Consistent with prior studies in the rat using in situ hybridization, GHR$^{\text{ir}}$ cells (i.e. those immunoreactive for sc-20747Ab, which labels the intracellular portion of the receptor) were detected both in the adult SVZ surrounding the lateral ventricles and in the dorsolateral corner of the lateral ventricles. Using double-label immunocytochemistry we detected a population of GHR$^{\text{ir}}$ cells within the SVZ that localized with markers typically found on endogenous stem/progenitor cells, namely CD133 (Fig. 1A–C) and GFAP (Fig. 1D–F). GHR$^{\text{ir}}$ cells also localized with the mitotic cell marker Ki67 (Fig. 1G–I), the immature neuronal marker doublecortin (Dcx; Fig. 1J–L) and PSA-NCAM (polysialic acid-neuronal cell adhesion molecule, Fig. 1M–O), a known marker of migrating neuroblasts destined for the OB.

As these results suggest that a GHR is found on a subpopulation of neural precursor cells, we next employed two functional assays; the neurosphere assay and repair of the mammalian central nervous system. For example, growth hormone receptor GHR null (GHR$^{-/-}$) and Suppressor of Cytokine Signaling-2 knockout mice, which represent loss and gain of GHR function respectively, display altered brain size, cortical architecture, and neuron and glial cell number. Moreover, GH administration improves cognitive deficits in GH-deficient rodents, and acts as a neuroprotective agent in aged animals.

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Deletion of GHR in vivo reduces the number of NSCs in vitro. As GHR stimulation increased the number of NSCs in vitro, we next determined whether deletion of the receptor in vivo resulted in the generation of fewer NSCs. Accordingly, long-term neurosphere cultures were generated from adult GHR+/− mice (which lack a functional GHR due to the targeted deletion of the mouse GHR/GHBP gene20), and littermate controls. GHR deletion resulted in significantly decreased slope in the neurosphere assay as compared to cultures from littermate controls (Fig. 3C; p<0.01). Furthermore, when cells from these mice were harvested at passage 5 and cultured in the N-CFCA, significantly fewer NSC-derived colonies were detected from GHR+/− cultures compared to controls (Fig. 3D; p<0.01). Of note, neurosphere-derived cells harvested from GHR+/− mice also generated significantly fewer progenitor cell-derived colonies as compared to littermate controls (p<0.01).

Fewer endogenous stem and progenitor cells are present in GHR+/− mice. To determine whether GHR+/− mice have fewer endogenous stem and progenitor cells, we next cultured cells harvested from the PVR of adult GHR+/− mice and cultured them directly in the N-CFCA without an intermediate neurosphere culture step. We found that significantly fewer NSC-derived (p<0.01) and progenitor cell-derived (p<0.05) colonies were generated from GHR+/− mice than from littermate controls (Fig. 4A). A significant reduction in the number of progenitor cell-derived colonies was not unexpected here, given our previous observation of a reduction in the number of primary neurospheres from adult GHR+/− mice compared to controls.

Acute infusion of GH results in a long-term increase in NSC number. To complement the loss-of-function data, we next infused GH (5 ng/hour) directly into the lumen of the lateral ventricle of WT mice for 7 days, and then harvested PVR tissue 3 days after completion of the infusion, and cultured the resulting single cell suspension directly in the N-CFCA. Consistent with the in vitro actions of exogenous GH (Fig. 3A and B), GH infusion resulted in a significant increase in the number of NSC-derived colonies (p<0.01) with no significant change in the number of progenitor cell-derived colonies (p>0.05) as compared to saline-infused controls (Fig. 4B).
To determine whether the GH-dependent activation of endogenous precursor cells was transient, as observed with the ICV infusion of basic fibroblast growth factor (bFGF) (Fig. 4C), we harvested and cultured PVR tissue from GH-infused mice over increasing survival periods following the 7-day infusion. Surprisingly, we observed a significantly greater number of large colonies at 21 (p < 0.01), 48 (p < 0.01), 60 (p < 0.05), and 120 days (p < 0.01) after pump placement (comparing to saline-infused controls), demonstrating a unique long-term effect of GH in the activation of endogenous NSCs (Fig. 4D).

Endogenous NSCs are activated in the SVZ following GH infusion. It is possible that the long-term increase in the number of large (i.e. NSC-derived) colonies following GH infusion reflects a peculiarity of the N-CFCA, and not a bona fide increase in the number of endogenous NSCs. We therefore adopted an in vivo ablation/regeneration approach to distinguish between these two possibilities. Ablation of the dividing cells within the SVZ can be achieved either by the infusion of anti-mitotic agents directly into the ventricle or by exposing the brain to ionizing or x-irradiation. Given their quiescent nature, NSCs are considered to be largely spared by this process as evidenced by efficient regeneration of the region post-irradiation. We reasoned that if GH infusion was activating normally quiescent NSCs to divide, then exposure to ionizing radiation after GH infusion would ablate the GH-responsive dividing NSCs, resulting in an incomplete repopulation of the region.

Accordingly, GH- and saline-infused mice were exposed to a single dose of ionizing radiation (3.5 Gy) 14 days after pump removal, and then sacrificed at increasing chase periods. We tracked the repopulation of all dividing cells in the region of the ventricles harvested in a typical SVZ dissection (i.e. 12.5 mm to 10.9 mm in relation to Bregma) by administering a single injection of BrdU (45 mg/kg body weight) to GH- and saline-infused mice 2 hours prior to sacrifice. Compared to saline-infused controls, significantly fewer BrdU-immunoreactive (BrdU−) cells were detected in the SVZ of GH-infused mice at 1-, 2- and 3-week (p < 0.01) survival time points (Fig. 5A), reflecting an incomplete repopulation, presumably due to the irradiation-induced loss of NSCs activated in response to GH infusion.

To more directly demonstrate that a single 3.5 Gy dose of irradiation spared the NSC population, a cohort of wild-type animals was irradiated 5 hours prior to sacrifice and compared to...
non-irradiated animals. When PVR tissue was harvested from these mice and cultured directly in the N-CFCA, we observed a significant (p<0.01) decrease in the number of progenitor cell-derived colonies (Fig. 5B). However, consistent with the sparing of a relatively quiescent population of NSCs, we failed to observe a change in the number of NSC-derived colonies between control and irradiated animals (Fig. 5B; 127.3±22.9 vs. 129.3±5.5, respectively).

Therefore, to complement our in vivo finding of an incomplete repopulation of BrdU" cells in the SVZ of GH-infused mice (Fig. 5A), we next repeated the ablation/repopulation approach using the reappearance of neurosphere-forming cells as an additional in vitro readout of SVZ repopulation. We included two additional cohorts of mice to provide support for our hypothesis that the incomplete repopulation effect was due to the GH-induced activation (and subsequent radiation-induced ablation) of endogenous NSCs. One cohort received a 7-day ICV infusion of EGF (20 ng/ml; a treatment we previously demonstrated stimulates endogenous progenitor cell proliferation, leaving NSC number unchanged) 14 days prior to irradiation to demonstrate that ablation of mitotically-active progenitor cells does not have the same detrimental effect on SVZ repopulation. A second cohort of mice received an additional dose of irradiation (3.5 Gy) 2 days prior to normal irradiation. These "double-dose" mice were employed based on the observation by Morshad and colleagues that normally quiescent NSCs (which were activated in response to the initial ablation of dividing cells) could be ablated by subjecting the mice to a second "kill" 2 days following the initial ablation2. Having demonstrated (Fig. 5A) that a single dose of ionizing radiation was sufficient to ablate dividing cells, largely sparing and then activating quiescent NSCs (as evidenced by the previously reported efficient repopulation of the region2,51 and our work here, see Fig. 5B), we reasoned that the "double-dose" approach of NSC ablation would mimic GH-infused animals, where a substantial proportion of NSCs were GH-activated prior to irradiation.

As expected from previous reports52,53 the number of neurosphere-forming cells in saline-infused mice was reduced to approximately 60% of normal (i.e. the number of cells present in non-irradiated (T0) animals), 24 hours following irradiation, rebounding to supraneural levels in the period following ablation (Fig. 5C). Interestingly, although EGF-infused mice generated significantly more neurospheres immediately prior to irradiation (T0, p<0.01), and displayed a more rapid SVZ repopulation post-irradiation compared to controls (1 vs. 3 weeks, respectively), no significant difference in the number of neurospheres generated by 6 weeks post-irradiation was observed in the EGF-infused mice compared to the controls, reflecting complete repopulation of the region. In contrast, the repopulation of the SVZ in both "double-dose" and GH-infused mice was
neurospheres at T0 (2672±79, p = 0.002). At 24 hours post-irradiation, PVR tissue from GH-infused (300±44, p = 0.002) and Double (Dbl) dose (236±9, p = 0.0002) mice generated significantly fewer neurospheres than controls. By 1 week post-irradiation the number of neurospheres generated from both GH and Dbl dose mice remained significantly reduced (p = 0.0004 and 0.007, respectively) as compared to control (1318±56). In contrast, EGF-infused mice generated significantly more neurospheres compared to controls at both T0 (p = 0.003) or 1 week post-irradiation (2358±72, p<0.001). By 6 weeks, the number of neurospheres generated from EGF-infused (1797±29) and Dbl dose mice (1746±85) did not differ from controls (1416±157), while GH-infused mice continued to generate significantly fewer neurospheres (888±66, p<0.05). Significance relative to saline control. N=3 unless otherwise stated. *p<0.05, **p<0.01.

Severely compromised, with sphere-forming cells remaining significantly reduced in these cohorts, as compared to saline controls 3 weeks post-ablation (p<0.01) and pre-irradiation levels (p<0.01). Indeed, repopulation of GH-infused mice remained static between 3 and 6 weeks post-irradiation.

GH-induced increase in SVZ NSCs augments adult neurogenesis. Considering the known contribution of SVZ stem and progenitor cells to OB neurogenesis, we next investigated how GH infusion affected the frequency of GH-responsive cell types in the SVZ, and whether the GH-dependent increase in SVZ NSCs increased OB neurogenesis. Accordingly, brains were removed from GH-infused mice 10 and 21 days after the onset of a 7-day ICV infusion, sectioned (14 μm), and processed for double-label immunocytochemistry to detect GHR<sup>ve</sup> cells from the onset of the lateral ventricles to the level of the anterior commissure (i.e. +1.42 to 0.14 mm rostral to Bregma). We failed to detect any change in the overall frequency of GHR<sup>ve</sup> cells in the PVR surrounding the lateral ventricles or the dorsolateral corner of the ventricle at either 10- (1282±56) or 21-day (1084±17) survival times, compared to naïve mice (1098±47). However, the frequencies of four subsets of GHR<sup>ve</sup> cells (those expressing putative NSC antigens) did increase transiently at 10 days, returning to basal levels by 21 days. These included GHR<sup>ve</sup>/Ki67<sup>ve</sup> (72±6 to 111±6, p = 0.01, n = 3), GHR<sup>ve</sup>/Nestin<sup>ve</sup> (62±5 to 100±7, p<0.01, n = 3), and GHR<sup>ve</sup>/CD133<sup>ve</sup> (184±15 to 219±9, p<0.05, n = 3) cells in the PVR surrounding the ventricle, and GHR<sup>ve</sup>/PSA-NCAM<sup>ve</sup> (474±12 to 594±40, p<0.05, n = 3) cells in the dorsolateral corner of the ventricle.

Although these results suggest that GH infusion increases the frequency of migrating neuroblasts; to more accurately determine whether the GH infusion alters the number of new neurons being generated in the OB, and whether this is a NSC-related effect, two final experiments were performed. In the first experiment, GH-infused mice were given a single i.p. injection of BrdU (45 mg/kg body weight) 7 days after GH pump placement and sacrificed after a 28-day chase period, providing sufficient time for newly generated cells to migrate to the OB and differentiate into neurons. The number of newly generated cells reaching the OB (i.e. BrdU<sup>ve</sup>) and newly differentiated neurons (i.e. BrdU<sup>ve</sup>/NeuN<sup>ve</sup>) was determined by examining tissue sections through the entire OB. While GH did not alter the overall number of migrating cells reaching the OB (BrdU, Fig. 6A), it did significantly increase the number of new neurons (BrdU/NeuN, p<0.01, n = 3).

In the second experiment to determine whether radiation ablation of GH-activated NSCs would also result in a significant decline in OB neurogenesis, a cohort of mice received a 7-day ICV infusion of GH, followed by a single dose (3.5 Gy) of irradiation 7 days later. Twelve days after the end of the infusion, these mice were administered one i.p. injection of BrdU every 2 hours until five injections were given. Multiple injections of BrdU were employed to ensure a meaningful number could be detected following the irradiation-induced ablation of the majority of dividing cells. Twenty-eight
days after the irradiation, the mice were sacrificed. As illustrated in Figure 6B, and consistent with our observation of a significant increase in GHR+/−/PSA-NCAM+/− cells in the RMS following GH infusion, the number of BrdU+/− cells reaching the OB was significantly reduced (p<0.01, n = 3) as compared to saline-infused controls. Moreover, the number of newly generated neurons was also significantly reduced in GH-infused mice (p<0.05, n = 3). Taken together, these results demonstrate that in addition to its NSC-stimulatory effect, GH also regulates the number of new neurons destined for the OB in adult mice.

Discussion

In light of its importance in neural development36, its role in the exercise-induced increase in NSCs24 and our detection of GHR+/− cells in the PVR of the adult brain32,38, we hypothesized that a functional GHR is present on a population of NSCs, where it regulates the activity of these cells both in vitro and in vivo. Consistent with this hypothesis is our recent demonstration that while insufficient to generate neurospheres, GH functions as an autocrine mitogen, augmenting the proliferation of adult neurosphere cultures42. However, given only a minority of neurospheres are NSC-derived, these results do not address whether GHRs are present on NSCs, or whether they regulate NSC activity directly.

In contrast, our demonstration here of a reduced rate of expansion of long-term neurosphere cultures generated from GHR−/− mice and reduced numbers of NSC-derived colonies (as compared to WT littermates), together with our observation that GH increases the rate of expansion and the number of NSC-derived colonies generated from long-term neurosphere cultures, now provides direct evidence of a regulatory role for GH/GHR on adult NSCs in vitro. Furthermore, we have shown that GHR+/− cells in the PVR of adult mice localize with the NSC markers GFAP11,12 and CD133 (which is coexpressed with musashi and SOX-1/−2), and when sorted directly into neurosphere generating conditions, GHR+/− cells from the PVR exhibit the cardinal stem cell attributes of proliferation, self-renewal and the ability to generate differentiated progeny (Fig. 2). Taken together, these results demonstrate the presence of GHR on a population of cells in vivo, whose phenotypic and functional attributes are consistent with those of NSCs.

The inability of BrdU-label retention to discriminate NSCs from more restricted proliferative progenitors39, the absence of selective positive markers and sorting strategies to generate pure populations of viable NSC35, and the difficulty of apply ultrastructural approaches dictated our approach to next demonstrate a regulatory role for GH/GHR on endogenous NSCs. We began by showing that deletion of the receptor results in fewer NSC-derived colonies being detected (Fig. 4A), while the infusion of GH results in the activation and associated increase in the number of endogenous NSCs (Fig. 4B). Interestingly, as part of our infusion studies we unexpectedly found that in contrast to similar infusions of the mitogens EGF44 or bFGF (Fig. 4C), the short-term infusion of GH resulted in a significant and sustained elevation in the number of endogenous NSCs a response not previously observed with any growth factor. While outside of the scope of this study, further study is warranted to determine whether these unexpected effects reflect an alteration in the phenotype and/or function of these GH-responsive cells, and the physiological consequences of such a change.

To provide additional in vivo evidence of a functional role for GH in the regulation of endogenous NSCs we undertook a series of SVZ ablation/regeneration studies, using both in vivo (i.e. neurosphere and N-CFCA assays) and in vitro assays (immunocytochemistry) as readouts of stem and progenitor cell activity. Based on earlier studies2,52,53, we predicted that if GH was activating normally quiescent NSCs, these activated NSCs would be ablated by subsequent irradiation, resulting in fewer NSC-derived colonies, and a diminished regenerative response. Indeed, this was the case. Consistent with previous reports2,54, those animals whose endogenous stem cells were not activated (i.e. saline and EGF-infused mice) prior to irradiation exhibited a complete repopulation (to pre-irradiation levels) of the BrdU+/− (Fig. 5A) and neurosphere forming (Fig 5C) cells in the SVZ by 3 weeks post-irradiation. However, those animals in which quiescent NSCs were activated prior to irradiation (GH-infused, and “double dose” mice, failed to repopulate either BrdU+/− or neurosphere forming cells to pre-irradiation levels by 3 weeks. Of interest, the repopulation of “double dose” mice reached pre-irradiation levels, but took approximately double the time to complete, demonstrating that the radiation-induced depletion of the PVR in itself is not sufficient cause incomplete repopulation.

Finally, we examined the effect GH infusion would have on OB neurogenesis. This approach was based on the finding that SVZ NSCs (Type B cells) represent the ultimate source of newly-generated OB interneurons30. Consistent with GH activating Type B cells, we found that an acute ICV infusion of GH increased the number of new neurons in the OB, while the irradiation-induced ablation of GH-activated NSCs resulted in a significant reduction both in the number of BrdU+/− cells and newly generated neurons in the OB.

Taken together, these results suggest that GHRs are present on a population of endogenous NSCs, where they regulate the activity of these cells.
It has become apparent that stem cell numbers in a variety of tissues decline as part of the aging process, and are unable to maintain tissue homeostasis at youthful levels\textsuperscript{5,6,9}. It is plausible that activating a cohort of NSCs by GH infusion could lead to a long-term temporal extension of functional tissue homeostasis. This could be a powerful therapeutic in counteraging age-related decline in regenerative capacity and cognitive function, as this progressive loss also correlates with the age-dependent decline in GH secretion in rodents and humans\textsuperscript{2}. Independent of its potential therapeutic value, investigation into the mechanism by which NSC numbers are altered will undoubtedly increase our understanding of how the brain responds to its environment.

**Methods**

**Tissue processing.** Animals were treated in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and all experiments were approved by The University of Queensland Animal Ethics Committee. Adult (6–8 week old) male and female C57BL/6J mice were deeply anesthetized with sodium pentobarbitone (260 mg/kg), and then transcardially perfused with ice-cold 0.9% saline followed by 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were harvested, post-fixed, and cryoprotected, as previously described\textsuperscript{7}. Serial frontal sections (14 µm) were cut with a MICROM cryostat, mounted on SuperFrost Plus slides (SuperFrost Plus), dried at room temperature (RT) and stored at −20°C until processing. The section was washed up to the rostral extent to the OB to Bregma (+2.58 mm) for examination of the OB, and from the onset of the lateral ventricle (+1.42 mm) to the joining of the anterior commissure (+0.14 mm) for examination of the lateral ventricles.

In all cases, tissue sections were initially rinsed in PBS (3×5 minutes) before incubation in blocking solution (5% fetal bovine serum (JRH Biosciences, USA) plus 0.3% Triton X-100 (Sigma-Aldrich), diluted in PBS) before incubation with anti-GHR (SC-20747) and anti-Ki67 (1:1000; Abcam, AB6142), anti-glial fibrillary acidic protein (GFAP, 1:300; Novacastra, NCL-GFAP), anti-PDGFRa (1:300; Abcam, AB69506) anti-PSA-NCAM (1:200; Chemicon, MAB5324), and anti-CD133 (1:100; Abcam, AB27699), diluted in blocking solution and incubated overnight at 4°C. Sections were washed three times with PBS and then incubated for 60 minutes (RT) with the appropriate secondary antibodies (1:300; Alexa Fluor goat anti-rabbit 488 for anti-GHR and 1:1000; Alexa Fluor goat anti-mouse 568 for remaining antibodies; Molecular Probes, USA) plus 4,6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich). Sections were then washed three times with PBS and once in D-h2O, before being coverslipped with fluorescent mounting medium (DakoCytomation, USA). Images were captured using a Zeiss Axioimager Z1 with Apotome and Axiocam MRm software (Zeiss, Germany).

**Intracerebroventricular (ICV) infusions.** Twelve hours prior to surgery, osmotic mini pumps (Alzet, #1007D; 7 day infusion at 0.5 µl/hour) were loaded with GH (10 µg/ml, recombinant rat, GroPept Australia), bFGF (20 µg/ml, human recombinant, Stem Cell Technologies, Canada), EGF (40 µg/ml, Stem Cell Technologies) or vehicle solution (0.9% sterile physiological saline, Sigma-Aldrich), and attached to the infusion cannula. Although the physiological concentration of GH in the cerebral spinal fluid of mice is unknown, a previous study\textsuperscript{28} carried out a single ICV infusion in rats of 20 µg/ml, thus we reasoned that a conservative dose of 5 ng/hour of GH should mimic endogenous levels in the mouse. Details of the infusion protocol are given in Blackmore\textsuperscript{19}.

**Rearrangement.** Mice were restrained within a plastic chamber and placed in a lead-shielded container leaving only the head exposed for irradiation. Single or “double-dose” irradiation was induced by exposure to a 260 source in a Gamma Cell 200 irradiator until a 3.5 Gy dose had been given. The PVR was harvested from GH-, EGF- and vehicle-injected animals at 24 hours, 1, 3-, 6- or 8-weeks post-irradiation and cultured in the neurosphere assay (described below). In addition, a second cohort of GH- and vehicle-injected mice received a single injection of BrdU (as above) 2 hours prior to sacrifice, after which the brain was removed, sectioned, and processed for the detection of BrdU\textsuperscript{+} cells. For OB experiments, owing to the irradiation-induced loss of dividing cells, cohorts of GH- and vehicle-injected mice received a total of five BrdU injections (as above) over a period of 10 hours, 14 days post-irradiation. These mice were irradiated 48-hours after the last BrdU injection and sacrificed 28 days post-irradiation.

**Primary neurosphere cultures.** Adult (6–8 week old) mice (C57Bl/6J or GHR\textsuperscript{−/−} on this background) were sacrificed by cervical dislocation, their brains removed immediately, and transferred to Petri dishes containing HEPES-buffered minimum essential medium (HEM). Full details of the protocol are given in Blackmore et al.\textsuperscript{19}.

**Neural colony forming cell assay.** Primary tissue was dissociated and the number of viable cells determined as the per primary neurosphere cultures (see above). Neurosphere-derived cells were cultured in 35 mm cell culture dishes with a 2 grid (Nunc, USA) at a density recommended for the mouse NeuroCult\textsuperscript{TM} Neural Colony Forming Cell Assay (Stem Cell Technologies). Cells were incubated for 21 DIV in 5% CO\textsubscript{2} with appropriate growth factors (either EGF alone or EGF + GH) being added every 7 days. After 21 DIV, the total number of colonies and the diameter of each colony was determined using an eyepiece graticule on an inverted Leica light microscope with phase contrast.

**Flow cytometry.** Single cell suspensions were generated from tissue and sorted for surface growth hormone binding protein (GHBP) expression using an anti-GHBP antibody (BETO 8041)\textsuperscript{48}. Murine GHBP consists of a ligand-binding domain that is identical to the extracellular portion of the GHR, together with a short C-terminal sequence coded by an alternate exon. For immunostaining, cell suspensions were incubated in NS basal medium with appropriate growth factors (either EGF alone or EGF + GH) being added every 7 days. After 21 DIV, the total number of colonies and the diameter of each colony was determined using an eyepiece graticule on an inverted Leica light microscope with phase contrast.

**Statistical analysis.** Factorial design analysis of variance (ANOVA) or Student’s two-tailed unpaired t-tests were used to analyze data as appropriate. Significant ANOVA values were followed by post hoc comparisons of individual means using the Tukey multiple comparisons test where appropriate. All values are expressed as mean ± SEM unless otherwise indicated with significance for all comparisons <0.05.

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