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Growth hormone (GH) is a survival rather than a proliferative factor for embryonic striatal neural precursor cells

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Objective: A possible role of GH during central nervous system (CNS) development has been suggested by the presence of this hormone and its receptor in brain areas before its production by the pituitary gland. Although several effects have been reported for GH, the specific role of this hormone during CNS development remains unclear. Here, we examined the effect of GH on proliferation, survival and neurosphere formation in primary cultures of striatal tissue from14-day-old (E14) mouse embryos.
Design: GH receptor gene expression was confirmed by RT-PCR. Primary cultures of embryonic striatal cells were treated with different doses of GH in serum free media, then the number of neurospheres was determined. To examine the GH effect on proliferation and survival of the striatal primary cultures, bromodeoxyuridine (BrdU) and TUNEL immunoreactivity was conducted.
Results: In the presence of the epidermal growth factor (EGF), GH increased the formation of neurospheres, with a maximal response at 10 ng/ml, higher doses were inhibitory. In absence of EGF, GH failed to stimulate neurosphere formation. Proliferation rate in the primary striatal cultures was inhibited by 24 or 48 h incubation with GH. However, in the absence of EGF, GH increased BrdU incorporation. GH treatment decreases the rate of apoptosis of nestin and GFAP positive cells in the primary striatal cultures, enhancing neurosphere formation.
Conclusions: Our *in vitro* data demonstrate that GH plays a survival role on the original population of embryonic striatal cells, improving Neural Precursor Cells (NPCs) expansion. We suggest that this GH action could be predominant during striatal neurodevelopment.

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1. Introduction

In the past few years, GH, the protein responsible of post-natal body growth and metabolism regulation has been described as an important neurotrophic factor during CNS development even before the synthesis of this hormone by the pituitary gland [1–4]. The presence of the GH receptor (GHR) in several brain regions during development [3,5], as well as the detection of local production of this hormone in different brain regions [6] have prompted to investigate the role of GH in the CNS. Indirect evidence of GH role in CNS development has been obtained from animal models where the synthesis of this hormone or its receptor has been deleted or blocked [2]. Absence or inactivity of GH in these animals generates different grades of CNS damage [7]. At the same time, alteration of GH signaling in transgenic or knockout animals modifies the distribution and composition of CNS cell population, suggesting an important role of this hormone in the fate of progenitors and neural stem cells (together called neural precursor cells or NPCs) [8].

In vitro evidence shows that GH is an embryonic mitogenic factor for NPCs from murine and human brain cortex [9–11]; the same effect was reported by McLenachan et al. [12] on NPCs from adult subventricular zone (SVZ). Interestingly, the mitogenic action of GH is still present in the adult brain, since infusion of this hormone stimulates an acute proliferative effect in different brain regions [13–15]. On the other hand, GH has been suggested to be a survival factor during brain development. This action has been clearly established in the chick neural retina, where GH neuroprotects retinal ganglion cells (RGCs) during the developmental waves of apoptosis that characterize RGC differentiation [16]. However, the same effect has not been clearly demonstrated in the CNS where the absence of the GHR has no effect on motoneuron survival of the spinal cord or brain stem [17]. In contrast, adult-onset deficiency of GH decreases survival of

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dentate granule neurons [18]. Therefore, GH exact role during CNS development is still a matter of interest.

In the basal ganglia circuitry, the striatum is a highly complex structure coordinating motor and cognitive functions. This brain region has medical significance since is the location of several neurodegenerative processes like Parkinson and Huntington's diseases. The striatum is originated from an anlage located in the ventral portion of each hemisphere of the developing telencephalon. Neuron colonization of the striatum comes from proliferation of NPCs localized at the SVZ through an early wave of neurogenesis that leads to striatal projecting neurons, which comprise nearly 90% of neurons in this structure. This early phase of neurogenesis peaks at about E14 in the rat embryo. A second phase of neurogenesis can be observed between E17 and P2, which is referred as late phase of striatal neurogenesis (Reviewed in [19]). Interestingly, the early phase of striatal neurogenesis coincides with the increase of GHR expression during embryogenesis [5,20], suggesting a possible role of this hormone in the regulation of this process.

Embryonic striatal NPCs can be isolated and propagated *in vitro* employing a chemically defined serum-free culture system [21]. In this assay, the majority of differentiated cell types die within a few days of culture but a small population of growth factor responsive cells undergo active proliferation in the presence of EGF. These cells give rise to colonies of undifferentiated cells called neurospheres which detach from the culture plate as they grow. The presence of NPCs in the neurosphere has been demonstrated by the ability of these cells to passage (self-renew) and differentiate into the three major CNS lineages; astrocytes, oligodendrocytes, and neurons [22]. Therefore, the neurospheres assay allows the isolation of NPCs under controlled conditions, without the influence of unknown factors present in the animal sera and facilitates the study of factors that regulate the biology of these cells.

There is cumulative evidence supporting the role of extrapituitary GH as a developmental growth factor [1]. Moreover, several biological actions have been attributed to GH on different neuronal cell types, including proliferation, migration, differentiation and survival [2]; however, the exact role of this hormone in the development of CNS remains unclear. In the present work we analyze the effect of GH on proliferation and survival of primary striatal cultures from E14 mouse embryos, as well as its influence in the generation of neurospheres. We think that this approach could better reflect the actual *in vivo* role of GH during CNS development.

2. Materials and methods

2.1. Mouse strains

BALB/c mice were raised in our animal facility. All experimental procedures were approved and conducted according to the Institutional Ethical Committee, in agreement with the national (Norma Oficial Mexicana, NOM-062-ZOO-2003) and international guidelines (The guide for the care and use of laboratory animals of the Institute of Laboratory Animal Resources, U.S. National Research Council) for the production, care and use of laboratory animals.

2.2. Primary neurosphere cultures

Embryos were removed from pregnant mice culled by cervical dislocation. The embryonic striatal germinal zone, which comprises both medial and lateral ganglionic eminences and the SVZ, was isolated with the aid of a dissecting microscope, then was subjected to 90 min incubation with a 0.05% trypsin-EDTA solution (Life Techonologies, Carlsbad, CA, USA) at 37 °C, in a humidified atmosphere of 5% carbon dioxide (CO_2). Further proteolysis was prevented by adding serum free media (SFM) composed of 1:1 mixture of DMEM/F12 (Dulbecco's Modified Eagle's Medium/F-12 nutrient, Life Techonologies, Carlsbad, CA,USA) supplemented with 20 ng/ml EGF (Sigma, St Louis, MO, USA), 25 μg/ml bovine insulin (Sigma), 10.42 μg/ml human apotransferrin (Sigma), 1 mM progesterone (Sigma), 1 mM sodium selenite (Sigma), 1 mM putrecsine (Sigma) [21]. Cells were centrifuged at 3000 rpm/5 min (1409 ×g). Finally, the cell pellet was resuspended in fresh SFM and seeded into uncoated 48-well plates at 5×10^4 cells/cm^2 in the same medium with or without different doses of recombinant human GH (Humatrope, Lilly, USA), and cultured for 6 days, allowing primary neurospheres to form. All cultures were maintained in a humidified incubator at 37 °C and 5% CO_2 [21,23].

2.3. Neurosphere counting

After 6 days in culture, the total number of primary neurospheres was counted in at least three wells (48 multiplate) per experimental condition, using a 10× objective of a bright field inverted microscope (Iroscope, México City). Results of the neurosphere counts were expressed as mean ± standard error of the mean (SEM).

2.4. BrdU proliferation assay

Proliferative effects of GH were assessed in primary striatal cultures using the BrdU labeling method. Cells were plated onto poly-L-lysine coated glass coverslips in 48 well plates at a density of 5×10^4 cells/well in SFM. Twenty four hours (24 h) later, the culture medium was replaced with fresh SFM containing 10 μM BrdU (Sigma) and different concentrations of GH. Following an incubation of 24 h or 48 h, cells were then washed, fixed with cold 4% PFA/PBS (paraformaldehyde + phosphate buffered solution) and processed for BrdU immunostaining. Each assay was carried out in duplicate in at least three independent experiments.

2.5. BrdU immunostaining

PFA fixed cells were post-fixed in ice-cold methanol during 30 min and subsequently washed with PBS. Thereafter fixed cells were incubated with 2 N HCl for 10 min at room temperature (RT), followed by three washes of 5 min with PBS. To block nonspecific staining, cells were incubated with 10% goat serum (Sigma) in PBS for 2 h at RT. Then, cells were incubated overnight at 4 °C with 1:500 mouse anti-BrdU antibody (Millipore, Billerica MA, USA) diluted in PBS + 1% goat serum. The next day, cells were washed and further incubated with 1:400 goat anti-mouse IgG (H + L) fluorescein conjugated secondary antibody (Chemicon, Temecula, CA, USA) for 2 h at RT. Following 3 × 5 min washes with PBS, cells were incubated with propidium iodide (PI) solution (eBioscience, San Diego, CA, USA) for 10 min at RT as a nuclear stain. Cells were washed again and coverslips were mounted with FluorSave Reagent (Calbiochem, San Diego, CA, USA). BrdU positive cells (BrdU$^+$) were counted and expressed as a proportion of the total number of cells (PI$^+$ cells). At least forty 40X fields were counted in two different coverslips (duplicate) per experiment, total of three independent experiments. Images were analyzed using the NIH ImageJ software (http://rsweb.nih.gov/ij).

2.6. Apoptosis assay

To asses any potential effect of GH on apoptosis of primary striatal cultures, we performed the method of terminal deoxynucleotidyl transferased UTP nick end-labeling (TUNEL). Cells (5×10^4 cells/well) in SFM were plated onto poly-L-lysine coated glass coverslips in 48 well plates. Twenty four hours later, the medium was replaced with fresh SFM with or without different doses of GH for additional 24 h, then cells were fixed overnight with 4% PFA and subsequently washed twice with PBS for 5 min at RT. Cells were postfixed in a cold methanol:acetic acid (2:1) solution for 5 min and washed once with PBS during 5 min. TUNEL labeling was performed with the ApoTag Red In Situ kit (Chemicon, Temecula, CA, USA), according to the manufacturer's

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