

# Neuronal repopulation into arcuate nucleus of hypothalamus

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## ABSTRACT

Recent evidences suggest that neurogenesis could occur in different zones of the central nervous system. *Objective:* with the purpose of analyzing neurogenesis, we have done a study in the arcuate nucleus of hypothalamus after administration of glutamate and epidermal growth factor (EGF) plus fibroblast growth factor-2 (FGF-2). *Material and Methods:* we have used the electron and light microscopy, immunocytochemistry and immunofluorescence studies and the organotypic cultures. The markers used were NeuN, neurogenin, Tuj 1, CD24, *lycopersicum sculetum*, DARPP32, vimentin, nestin, PSA-NCAM, caspase 9, BrdU and 3HT. We used rats (P4), the time of sacrifice was at 3 h, 6 h, 12 h, 36 h, 15 d, 30 d, 90 d and 120 d. This research has been divided in 3 experiments. *Experiment A*, we administrated BrdU (100 mg/kg) and we detected the cellular proliferation. *Experiment B*, we used glutamate (4 mg/g, sc), and we observed the cellular death. *Experiment C*, we used glutamate, BrdU and EGF+FGF-2 (5 ng/g, sc). *Results and conclusions:* the results obtained indicate that we can induce the neuronal regeneration of the arcuate nucleus by means of use of EGF and FGF-2, and we identify the third ventricle like a zone of neurogenesis.

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**Key words:** arcuate nucleus, neurogenesis, glutamate, growth factors.

## REPOBLACIÓN NEURONAL EN EL NÚCLEO ARCUATO DEL HIPOTÁLAMO

### RESUMEN

Evidencias recientes sugieren que la neurogénesis podría ocurrir en diferentes zonas del sistema nervioso central (SNC). *Objetivo:* con el propósito de analizar la neurogénesis, se realizó un estudio en el núcleo arcuato del hipotálamo después de administración de glutamato y factor de crecimiento epidérmico (FCE) más el factor de crecimiento de fibroblasto tipo 2 (FCF-2). *Material y métodos:* se utilizaron las técnicas de inmunocitoquímica, inmunofluorescencia, cultivos celulares y microscopía electrónica. Los marcadores usados fueron: NeuN, neurogenina, Tuj 1, CD24, *lycopersicum sculetum*, DARPP32, vimentina, nestina, PSA-NCAM, caspasa 9, BrdU y 3HT. Así como ratas posnatal 4 (P4), tiempo de sacrificio fue a las: 3, 6, 12, 36 hs, 15, 30, 90 y 120 días. Esta investigación fue dividida en 3 experimentos. *Experimento A*, administramos BrdU (100 mg/kg), para evaluar la proliferación celular. *Experimento B*, usamos glutamato (4 mg/g, sc), para observar la muerte celular. *Experimento C*, administramos glutamato, BrdU y FCE+FCF-2 (5 ng/g, sc). *Resultados y conclusiones:* los resultados obtenidos indican que podemos inducir la regeneración neuronal en el núcleo arcuato utilizando los factores de crecimiento FCE y FCF-2, e identificamos al tercer ventrículo como una zona de neurogénesis.

**Palabras clave:** núcleo arcuato, neurogénesis, glutamato, factores de crecimiento.

All mammals have replication cells in many organs and in some cases, notably the skin, blood, and gut, stem cells have been shown to exist throughout life, contributing to rapid cell replacement. Furthermore, neural progenitor cells (NPCs) can replicate in vertebrates throughout life<sup>1</sup>. Recent evidence shows that new neurons, in normal adult mammals ranging from rodents to primates, have been confined to the dentate gyrus and olfactory bulb<sup>2</sup>. These neurons are thought to derive from a population of progenitor cells, and it was shown by Reynolds and Weiss (1992) that NPCs taken from the adult brain can be propagated *in vitro*<sup>3</sup>. These cells have the capacity for self-renewal and can generate the major classes of central nervous system (CNS) cell types: neurons, astrocytes and oligodendrocytes<sup>4</sup>, and can be cultured from the adult subventricular zone (SVZ). Consistent with these findings, continuous generation of new neurons has been detected in other CNS regions<sup>5</sup>. Furthermore, it has been shown that various insults, for example, ischemia and hypoxia, stimulate the proliferation of endogenous progenitors either in known neurogenic sites<sup>6,7</sup> or in regions where neurogenesis normally does not occur<sup>8</sup>. To be able to manipulate the endogenous adult progenitor, we believe it is crucial to determine the extracellular signals that can stimulate cell division and regulate the fate of these NPCs<sup>9</sup>. The possibility that growth factors may also influence NPCs *in vivo* has been supported by findings in which intraventricular administration of EGF and FGF-2, expanded proliferative progenitors in the SVZ of adult mice<sup>10</sup>, and in the regeneration of hippocampal pyramidal neurons after ischemic brain injury<sup>11</sup>. Here we sought to augment the regenerative capacity of endogenous progenitors upon focusing on the arcuate nucleus (AN) and the third ventricle. In the development embryonic day 11 (E11), the rat neuroendocrine hypothalamus is generated predominantly from the third ventricular neuroepithelium in a "lateral early to medial late" pattern dictated perhaps by the medially receding third ventricle. The AN is generated between E12 and E17. It is composed of several neuronal subpopulations that collectively contain a number of neurotransmitters or modulatory substances (e.g., dopamine, acetylcholine, enkephalin, various members of the opiomelanocortin series, thyrotrophin releasing hormone [TRH], corticotrophin releasing hormone [CRH], growth hormone releasing hormone [GRH], gonadotrophin releasing hormone [GnRH] and soma-

tostatin); and has been implicated in a variety of functions including reproduction, affective physiology and ingestive behaviour<sup>12</sup>. The third ventricle is located in the diencephalon, and this zone has not been analyzed like a neurogenesis region in the adult brain. Since Olney's work (1969) it is well established that administration of monosodium glutamate (MSG) to neonates of different species of rodents causes selective destruction of a large number of neurons of the hypothalamic AN<sup>13</sup>. Although, neurotoxic effects of MSG have been shown in considerable studies, it remained unknown, however, whether regeneration of arcuate neurons occurs late after neuronal injury induced by the administration of different excitatory neurotoxins<sup>14</sup>.

## MATERIAL AND METHODS

**Animals:** male sprague-dawley rats (4-8-12 weeks of age) received a single subcutaneous injection of an aqueous solution of MSG (Sigma-Aldrich, 6-1626, St. Louis, MO, USA) at a dose of 4 mg/g on day 4 of life (postnatal 4, P4). To stimulate the proliferation of endogenous progenitors, we administered 5 ng/g by subcutaneous injection of epidermal growth factor (EGF, Sigma-Aldrich, St. Louis, MO, USA,) and basic fibroblast growth factor (FGF-2, Sigma-Aldrich, St. Louis, MO, USA), 12 h after glutamate injection, per brain for 10 d<sup>15</sup>. Neonatal and adult male rats were anesthetized deeply with thiopental (Roche, laboratories) and perfused transcardially with 0.9% saline, followed by either 100 ml of Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde, Sigma-Aldrich, St. Louis, MO, USA) for conventional electron microscopy or by 100 ml of 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) for immunohistochemistry. The rats were sacrificed 3 h, 6 h, 12 h, 10 d, 30 d and 90 d after glutamate injection.

**Electron microscopy:** transverse or sagittal 100 $\mu$ m sections were cut with a vibratome (Glassworx, instruments, NY, USA). The sections were post-fixed in 2% osmium for 2 h, rinsed dehydrated, and embedded in Araldite (Ducurpan, Fluka BioChemika, Ronkonkoma, NY, USA). To study the overall organization of the wall of the third ventricle, we cut serial 1.5  $\mu$ m semithin sections with a glass knife (ultramicrotome LKB ultratome III 8800, NY, USA) and stained then with 1% toluidine blue (Merck, Egaa, 64271 Darmstadt, Germany).

For the identification of individual cell types, ultrathin 0.05  $\mu$ m sections were cut with a diamond

knife, stained with lead citrate, and examined under a Jeol 100 CX electron microscope (Jeol, Tokyo, Japan). To determine the relationships among the different types of cells, we examined 50 serial ultrathin sections per third ventricle site. The classification of cell types was based on 1500 cells from sites throughout the third ventricle.

*[3H] Thymidine autoradiography:* five neonatal and five adult male rats received one 50  $\mu$ l injection of 1  $\mu$ Ci [3H] thymidine (specific activity 999 GBq/mmol, 27  $\mu$ Ci/mmol, Amersham) intraperitoneally and were sacrificed 1 h later; the brains were processed as for conventional electron microscopy. Serial 1.5- $\mu$ m-thick semithin sections were cut with a glass knife and mounted into slides, dipped in autoradiographic emulsion (Kodak NTB2, Chicago, Illinois, USA), exposed for 4 weeks at 4° C, developed in Kodak D-19 (Kodak, Chicago, Illinois, USA), and counterstained with 1% toluidine blue. A cell was considered labeled if twenty or more silver grains overlaid the nucleus and the same cell was labeled in six adjacent sections. Seventy-five [3H] thymidine-labeled cells identified in the semithin sections were selected for electron microscopic examination. Ultrathin sections were cut with a diamond knife and examined under a Jeol 100 CX electron microscope (Jeol, Tokyo, Japan) to determine which cell types incorporated [3H] thymidine.

*Labeling procedures for tissue sections.* Sections 60  $\mu$ m thick were cut on the vibratome and were processed for preembedding immunostains for lectin histochemistry, primary monoclonal antibodies anti IgG NeuN (1:100, Chemicon, Temecula, CA, USA), anti BrdU (1:200, Dako, Denmark), we have used the avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques<sup>16</sup>. Some sections were in addition stained (Nissl's stain) with the cellular stain cresyl violet (Sigma-Aldrich, St. Louis, MO, USA).

*5-bromo-2'-deoxyuridine (BrdU) administration and detection:* BrdU (Sigma-Aldrich, St. Louis, MO, USA) was given as single ip injection of 50 mg/kg in 0.9% saline or 1 mg/ml for 2 weeks through drinking water and detected with mouse anti-BrdU (1:200, Dako, Denmark). Double staining for Neurogenin/NeuN and BrdU was performed, we used the kit vision doublestain system K 1395 (Dako, Denmark).

*Cell count:* the proportion of the different cell types of the walls of the third ventricle and the AN was estimated by the next method. Individual cells were identified in serial ultrathin sections, and the number of the different types of cells was calculated. This method is based on serial section reconstructions providing an accurate determination of cell number that is not

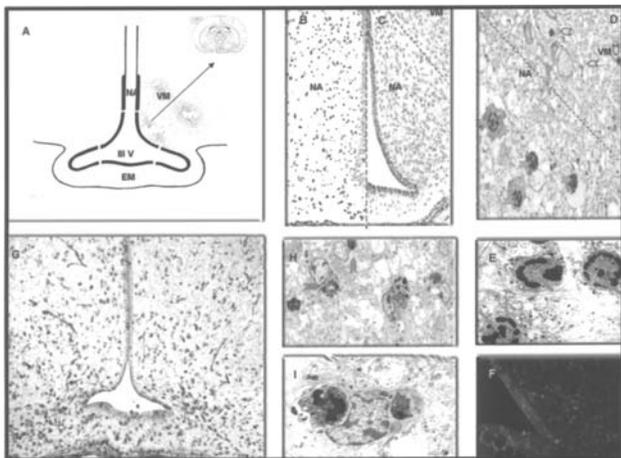
affected by section thickness or cell size. We will use to quantify the NeuN and TuJ1 neurons, the modified version of West's method<sup>17</sup>. Also, a microscope Axiophot Zeiss (Germany) will be used to estimate the total number of marked cells with anti-Parkin, NeuN, TuJ1, GFAP and Doublecortin. The cellular count will be carried out with the MS Excel program (Microsoft Office Excel, Professional Edition 2003, Redmond, WA, USA). An average will be determined for each experimental group and the data will be subjected to the analysis with the Stat View program and with the Student's t test or ANOVA, continued by the analysis Student-Newman-Keuls *post hoc* comparisons and P value.

## RESULTS

*Degeneration and cell death into AN after glutamate injury.* With this animal model we observed an extensive loss of neurons in the AN. Immunostaining for NeuN and neurogenin markers for mature neurons detected gradual loss of neurons 6-12 h after glutamate injury. The lesion was very prominent at 12 h and was characterized by the presence of numerous pyknotic nuclei. The percentage of cell death was of 90.81% (+/- 1.2%), and only very few surviving neurons were observed 9.19% (+/- 1%) at 12 h (figure 1 B, C). In semithin sections the pyknotic nucleus with cresyl violet show a high stain degree (figure 1D) and we observed apoptotic electron microscopy findings, such as chromatin condensation, nucleosomal fragmentation and perturbation of the nuclear envelope (figure 1E). During this initial period, most of the damaged arcuate neurons expressed apoptosis-inducing activated caspase-9 (figure 1F), 78.08% (+/- 0.98%) were stained with Nissl's stain at 12 h. Our study also revealed that following the lesion induced by MSG, especially between 24 h and 36 h post-lesion, the number of microglial cell was increased (figure 1 G, H, I).

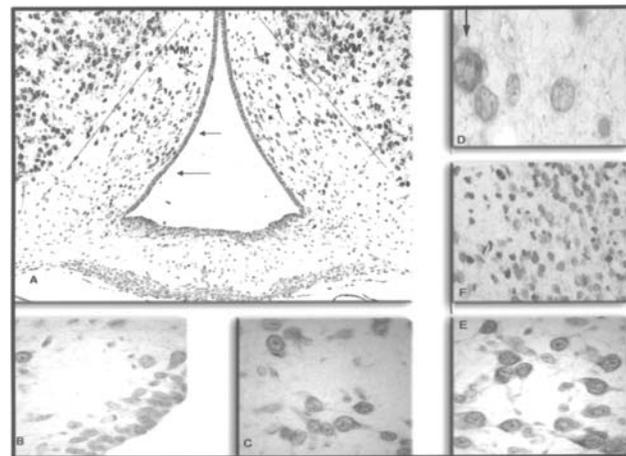
*Growth factor treatment.* EGF+FGF-2, which act as mitogens for adult progenitors were administered by a single subcutaneous injection for 10 days<sup>12</sup>. Despite such an extensive neuronal loss in the initial 2 weeks, we detected a small but significantly higher number of NeuN+ and neurogenin+ (figures 2A, B, C), on the 4th week we observed a neuronal repopulation into AN of 58% (+/- 2%) (figures 2 E, F).

Generation of new neurons. To determine whether neurons observed after GMS injury were produced by endogenous progenitors; BrdU was administered for 2 d (P4, P15). The analyses revealed marked effects of growth factors on the production of



**Figure 1.** A. Representative scheme of the different portions of the arcuate nucleus (NA). B. Vertical cuts of mediobasal hypothalamus of an animal (4d+12h). Nissl's stain (134x), animal tried with Glutamate (GMS), the lesion affects the entire territory of the arcuate nucleus AN. C. Animal with physiologic solution 0.9% saline, the territory of the AN is well defined and we can see normal nervous cells. D. Dorsomedial region of the AN of a rat (4d+12h) tried with GMS. Nissl's stain (536x). Intact neurons are observed (yellow arrows) in VM territory aside of the AN territory where we can observe apoptotic nucleus (orange arrows) (VM= Ventromedial). E. Apoptotic nucleus of an arcuate cell of a rat (4d+12h) tried with GMS (21,900x). The non loss of the nuclear membrane is observed, with dilations (blue), the chromatin condensation (yellow) and the internucleosomal fragmentation of the DNA (red). F. Ventrolateral portion of the AN of an animal (4d+12h) after administration of GMS. Immunofluorescence for caspase-9 (536x). Is evident the condensation of the chromatin in cells reactive to caspase-9. G. AN of a rat (4d+36h), after the administration of GMS. Immunohistochemistry for *lycopersicon esculentum* (134x). Globular and ameboid forms of the reactive microglia are observed disseminated by the whole territory of the AN. H. AN territory approx. 6 h after destruction showing different stages of apoptosis of a rat (4d+12h) (256x). Green arrow: an apoptosis in early karyorrhexis. Grey arrow: apoptotic cell in pyknosis. Yellow arrow: late karyorrhexis. White arrow: surviving neuron. I. Electron microscopy showing a fagocytosis (36 h after destruction) (21,900x).

new neurons (figure 2D). The number of BrdU cells detected in the AN was increased about 16.4 times by the treatment (82 cells and 14 cells in treated and untreated animals, respectively in 5 animals, 4 hypothalamus at 30 d). During P19 several BrdU+ cells coexpressed neuronal markers. At P34 many cells were detected in the AN and in the third ventricle, and some of them expressed the neuronal marker NeuN and neurogenin in growth factor-treated animals. The percentage of NeuN+ and neurogenin+ neurons among these BrdU+ cells in treated animals (2,298 cells of 3,955 cells a percentage of 58% at 30 d,  $p < 0.0001$ ) was much higher than that in untreated animals (98 cells of 3,869 cells, a percentage of 2.5% at 30 d,  $p > 0.5$ ). Thus, short-term growth factor-treatment appeared to stimulate not only proliferation



**Figure 2.** A. Immunohistochemistry for BrdU in the AN (134x). This photograph at P15 shows with arrows proliferation of new cells into the territory of the AN. (VM= Ventró Medial). B and C. Comparative photographs P34 of medial basal territory with NeuN (256x). B, rats treated with GMS, only with 2.5% of neuronal repopulation. C, rats treated with GMS+EGF+bFGF with 58% of neuronal repopulation. D. Doublestain immunohistochemistry with BrdU+NeuN in P34 (536x). The arrow shows a new neuron. E. Doublestain immunohistochemistry of neurogenin+BrdU of P94 (134x). Proliferation is observed with the incorporation of new neurons in the AN. F. Dorsomedial territory of the AN showing an immunohistochemistry in P94 with NeuN+ (256x). Mature and functional neurons incorporate in the AN.

of endogenous progenitors, but also their differentiation into neurons (table 1).

**Table 1.** New neurons into the arcuate nucleus.

Control animals	Untreated animals (Glutamate+BrdU)	Treated animals (Glutamate+BrdU+Growth factors)
16 neurons of 943 neurons	98 neurons of 869 neurons	2298 neurons of 3955 neurons
30 days	30 days	30 days

Multiple cell types incorporate [3H] thymidine in the AN and third ventricle. To determine which cells were dividing, [3H] thymidine was injected and animals were sacrificed 1 h later. Astrocytes, oligodendrocytes, new neurons and labeled ependymal cells were observed, specifically tanycytes located in the wall of the third ventricle.

## DISCUSSION

We determined the effects of growth factors on the proliferation and differentiation of neural progenitor cells in the third ventricle and into the AN. Here we have demonstrated an important regenerative capacity of

endogenous neural progenitors in the adult brain AN of hypothalamus undergoing an extensive degeneration following glutamate toxicity. We have shown that subcutaneous administration of growth factors can recruit endogenous progenitors *in situ*, thereby inducing neuronal repopulation into the AN after glutamate administration. Thus, the treatment led to 63% (+/- 2.5%) recovery of the total number of NeuN+ cells lost by GMS lesion (90 d+4 d). We also noticed that in the control animals (intact brain), the proliferative response in the walls of the third ventricle was moderate. When we administered glutamate to damaged animals, and then EGF+FGF-2, after 10 d we proved neuronal repopulation in the AN of 62% in animals sacrificed at 90 d. So, this study shows that we can induce neuronal repopulation into the AN. We think that adult progenitors present in distinct regions may have distinct intrinsic properties. And, distinct environmental cues may instruct endogenous progenitors to differentiate into specific neuronal subtypes depending on their locations. The viability of signaling factors in local environments may be limiting the behavior of endogenous progenitors. Thus, multiple inhibitory and stimulatory signals are probably involved in controlling the rate of neurogenesis, depending on the location and type of lesions. Elucidation of the molecular mechanisms of the actions of these signals will be essential for further understanding of the regenerative capacity of the adult CNS.

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