

Molecular signaling of 5-HT_{1A} and presence of serotonergic cells in the fetal cerebral cortex

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ABSTRACT

Early appearance of the serotonergic system in the fetal brain and the various effects of serotonin (5-HT) on brain morphogenesis, have given support to a neurotrophic role of serotonin. This function of serotonin is accomplished through a system of serotonin nerve terminals in the target regions that involves various 5-HT receptors. In visual, auditory and somatosensory cortex an early and intense serotonergic innervation is particularly important. The neuronal somata of these terminals are normally located in the mesencephalon and they have not been observed in the maturing cerebral cortex, neither in the adult brain. By using immunolabeling techniques, fluorescence and confocal microscopy, we observe the presence of both, 5-HT terminals and 5-HT cells in mesencephalon (Me, E17) and in the neopallium (Np, E13-E16) cocultures. Cells immunopositive to 5-HT and to tryptophan-5-hydroxylase are also observed in the Np on day 12 of culture. These results concerning the unexpected presence of serotonergic cells in the fetal cerebral cortex are interesting and may be of importance in corticogenesis. As it happens with other elements of the serotonergic system, the presence of these phenotypically serotonergic cells in the early cerebral cortex may be transitory and probably supporting cortex maturation processes. The molecular signaling path of the 5-HT_{1A} receptor has also been identified.

Keywords: Serotonergic System; Serotonin Receptor 5-HT_{1A}; Molecular Signaling Paths

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1. INTRODUCTION

The early appearance of the serotonergic system in the fetal brain [1-3] together with the well known effects of serotonin (5-HT) during the morphogenesis of the central nervous system (CNS) [4] support that these early functions of 5-HT are exerted through a system of axon terminals in the target regions of the fetal brain, derived of neuronal somata exclusively located in the brainstem. A series of studies support 5-HT as an early appearing molecule (E11) during CNS development in the laboratory rat which has a neurotrophic role in advance to its neurotransmitter and neuromodulator role in the adult brain. Both 5-HT and its specific receptors bound to ionic channels or to metabotropic cascades have, at these early periods of brain development, various functions [5, 6]. We obtained in our laboratory complementary information on the functional role of 5-HT in neuroblastic structures like the axonal growth cones from rat E17 encephala, and the presence of the 5-HT_{1A} and 5-HT_{1B} receptors [3,7]; the 5-HT synthetic path as a well as a 5-HT releasing system K⁺ and Ca⁺⁺ dependent and a Na⁺ dependent uptake system [3,8].

A transitory increase in the expression of SERT and 5-HT nerve terminals in sensory areas of the cerebral cortex, has also been described [9,10]. The early appearance of serotonergic terminals in the maturing cerebral cortex is particularly intense especially in the sensory areas: visual, somatosensory and auditory [9,11,12]. Mazer *et al.*, [13] reported that experimental decrease of serotonergic signals alters the normal development and maturation of the cerebral cortex, decreasing the synaptic density with a consecutive damage of cognitive development. It is also known that 5-HT has a regulatory role on various cortical areas through axonic terminals of

serotonergic neurons located in the mesencephalic raphe nuclei, and not observed, to our knowledge, in the developing cerebral cortex. Lebrand [14] proposed that regulation of corticogenesis by 5-HT, takes place through thalamo-cortical fibers, supported by the presence of 5-HT_{1A} and 5-HT_{1B} receptors and the serotonin transporter (SERT) in this system, also shown the presence of serotonergic receptors in fetal rat brain [15].

There are other types of early appearance neurons in the growing cerebral cortex like Cajal-Retzius in the marginal zone, related to cell migration and to the inside-out cell development pattern of the cortex [16]. As well as gabaergic and tyrosine hydroxylase (TH) immunopositive neurons in the rat dorsolateral and medial cortex [17] prenatally and postnatally [15]. Probably their expression is epigenetically regulated, like in the peripheral nervous system [19,20], so that cortical neurons may transiently express some enzyme molecules like TH [18]. Here we propose that the same may happen with the serotonin synthesizing enzyme Tryptophan-5-hydroxylase (T5-H), and other key 5-HT markers. So, in the present work we describe the presence of 5-HT cells in the fetal neopallium in coculture with the mesencephalon, and in also not cultured cortices in the same developmental stage, and besides the molecular signaling path of the 5-HT_{1A} receptor, which is presented in the fetal cortical target area.

2. METHODS

Experimental Animals: Nulliparous female Wistar rats, 200 - 300 g body weight (bw), were used. Kept during two weeks before experiments under standard conditions: 12 hours light periods; 20°C ± 1.5°C temperature and 55% - 65%, humidity; fed water and Purina Chaw pellets ad-libitum. 250 g bw males were employed for mating and the presence of a vaginal plug was taken as the beginning of gestation. Crown-rump length was assessed to confirm gestational age. At E13, E16 or E17, a cesarean section was practiced under pentobarbital anesthesia (40 mg/Kg, ip), then the fetal encephala were dissected out and kept in a Petri dish in PBS at 4°C. The experimental protocol was approved by The Internal Committee for the Use of Experimental Animals, according to the Official Mexican Norm (NPM-062-ZOO-1999) and to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 8023, 1978).

Organotypic Cultures: The Stoppini method was generally followed, briefly: the E-17 encephala were included in low melting point agarose (8°C - 17°C, Sigma, 6% in PBS) [21]. 300 µm slices were obtained with a Vibratome (Pellco101, series 1000) in the area of the mesencephalon (Me), following the coordinates of Foster and Lavdas, where serotonergic cells are located [22,23] (**Figures 1(a)** and **(b)**). Neopallium (Np) fragments (0.5

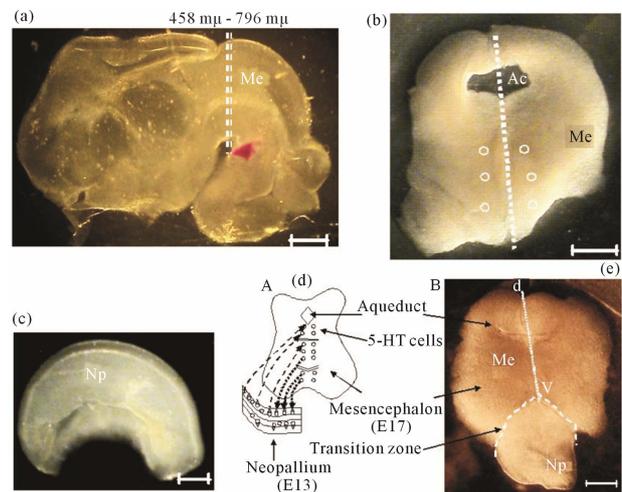


Figure 1. Microdissection procedure to obtain fetal encephalon (E17). (a) Lateral view of fetal rat encephalon. h, Hemispheres; Me, mesencephalon (E17). Dotted lines indicate the cross section to obtain a 300 µm mesencephalic slices (Foster 1998); (b) A 300 µm thick slice of mesencephalon that is used for the culture. Ac, aqueduct; dotted line, dorso-ventral axis (d-v); open circles, location of 5-HT cells. Scale bar = 1 mm; (c) Lateral view of a fetal hemisphere, the neopallium (Np, E13 or E16) is dissected and separated in sections of 0.5 to 1 mm that later are used in coculture. Scale bar = 1 mm; (d) Model proposed for the organotypic culture. Shows mesencephalon in contact to the neopallium, fiber communication paths (.....) from Me to Np; or (- - -) from Np to Me; (e) Actual Me section and Np (E13 or E16), after 3 days in culture. (- - -) transition zone between both regions; Dorso-ventral axis of Me (d-v). Scale bar = 1 mm.

to 1.0 mm) from E13 or E16 encephala were dissected out (**Figure 1(c)**). Slices were set on culture wells on a permeable membrane (0.4 µm pore, Falcon), close to each other with 1.2 ml culture medium: Sigma MEM-Eagle, added with 24% of inactivated horse serum (By Products); 0.2 mM Hepes (Sigma); 2% glutamax (Gibco); 0.07% gentamicin (Sigma). Both regions were put in close contact (**Figures 1(d)** and **(e)**). Cultures were incubated at 37°C in 5% CO₂ (Hotpack, Phil., PA, USA) during 12 days and 50% of medium was changed every 48 hours.

Immunolabeling and Microscopic Fluorescence: Tissues were washed in cold phosphate buffer saline (PBS), pH 7.4 and fixed with 4% paraformaldehyde. Immunolabeling was carried out with polyclonal antibodies for 5-HT_{1A}, 5-HT, serotonin transporter (SERT), MAPK, ERK1 or Vimentin (1:1000), or with monoclonal antibodies for T5-H, GFAP, Giα-1, MAPKK, Enolase or NeuN. Fluorescent secondary antibodies (1:500) were used for FITC (Jackson IR), Fluorescein (Pierce), Alexa 568 (Molecular Probes) and Lisamine-Rhodamine (LRSC, Jackson IR). Epifluorometry (Carl Zeiss, axio-scope) or Confocal microscopy (Leica, TCS SP2) were

performed to identify and locate the various labeled molecules. Appropriate negative controls omitting the primary antibodies were included in each experiment.

3. RESULTS

In **Figure 2(a)** there is a control that confirms the presence of 5-HT cells in the Me area in coculture. **Figure 2(b)** shows immunopositive cells for 5-HT in the Np. The rate limiting enzyme T5H, (**Figure 2(c)**) is in a not cultured Np. In **Figure 2(d)**, 5HT_{1A} Immunopositive cells also in the Np are shown.

Immunopositive cells to T5H and 5-HT coexisting in the same cell were also observed in coculture or in not cultured Np (**Figures 3(a)** and **(b)**). Neuronal markers Enolase or NeuN were positive together with 5-HT, in culture or in not cultured Np (**Figures 4 (a)** and **(b)**), SERT colocalizes with T5H or 5-HT in cells of the Np (**Figures 5(a)** and **(b)**), GFAP label also did coexist with the serotonin marker (T5H) in cells of cultured or in not cultured Np (**Figures 6(a)** and **(b)**), GFAP coexist with 5-HT in cells of not cultured Np (**Figure 6(c)**). The signaling molecules (Gi α -1, MAPK, MAPKK and ERK1) also do colocalize with their receptor 5-HT_{1A}, in the cocultured as well as in not cultured Np (**Figures 7-11**).

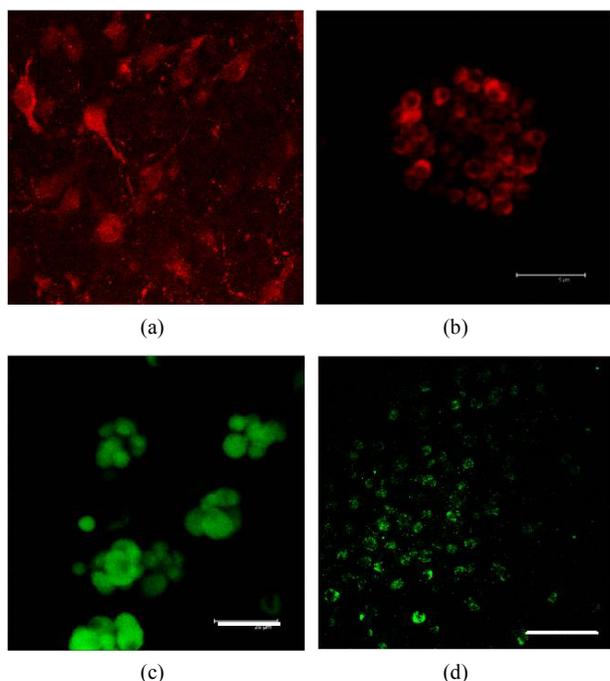


Figure 2. (a) Control preparation that shows the presence in the Me of 5-HT immunopositive cells in coculture. Scale bar = 75 μ m; (b) 5-HT Immunopositive cells in the Np in coculture. Scale bar = 5 μ m; (c) T5H positive cells in a not cultured Np. Scale bar = 20 μ m; (d) 5-HT_{1A} positive cells in the Np in coculture. Scale bar = 75 μ m. Confocal microscopy.

4. DISCUSSION

There are cells with a neurochemical phenotype that appears early in the primitive cerebral cortex, like gabaergic neurons probably implicated in the processes of corticogenesis [17,24]. Catecholaminergic cells also appear in the prenatal period probably implied too in neurogenesis [18,25,26]. Very early in the rombencephalon area (E11-E12) serotonergic neurons are already presented, they will form later the mesencephalic raphe nuclei, also observed in some early hypothalamic nuclei

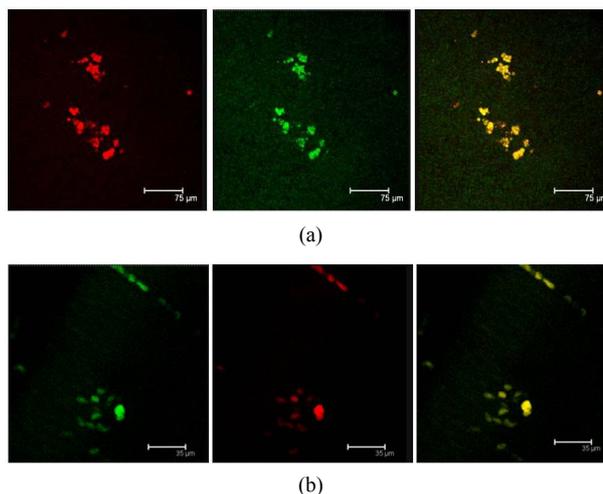


Figure 3. (a) Immunopositive 5-HT label (red) coexists with T5H label (green) and both labels in the same cell (yellow) in the Np in coculture. Scale bar = 75 μ m; (b) Coexistence of T5H (green) and 5-HT (red) antibody labels in the same cells (yellow) in a not cultured Np. Scale bar = 35 μ m. Confocal microscopy.

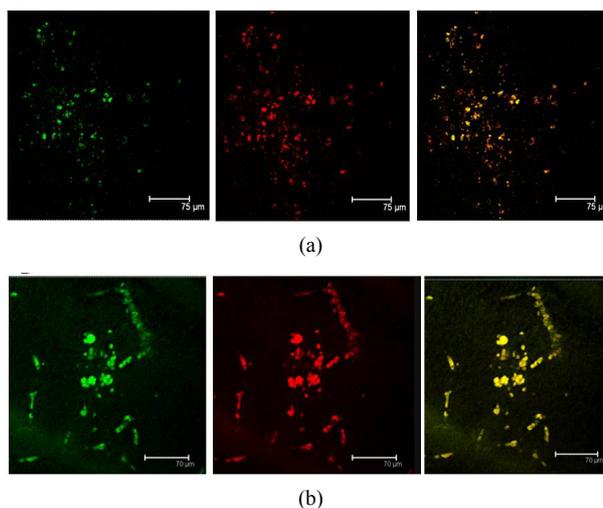


Figure 4. (a) 5-HT immunopositive cells (red) also positive to NeuN (green) label, in the Np in coculture. Both labels coexisting in the same cells, are shown in yellow. Scale bar = 75 μ m; (b) Enolase (green) and 5-HT (red) coexist (yellow) in a not cultured Np. Scale bar = 70 μ m. Confocal microscopy.

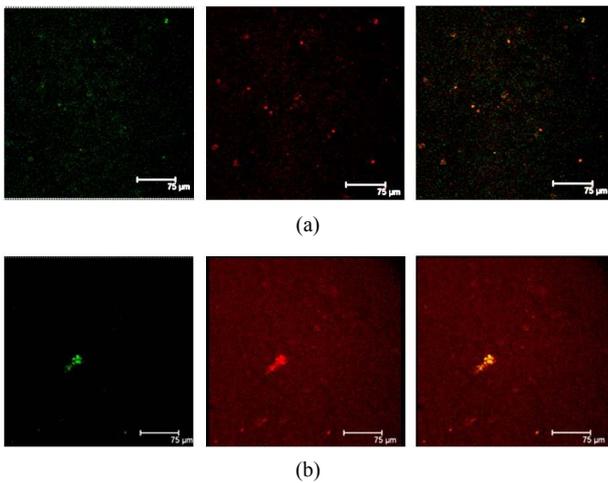


Figure 5. (a) T5H (green) or SERT (red) antibody labels do coexist in cells (yellow) of the Np in coculture. Scale bar = 75 μm; (b) 5-HT (green); antibody label does also coexist with SERT (red) in the same cells (yellow), in the Np in coculture. Scale bar = 75 μm. Confocal microscopy.

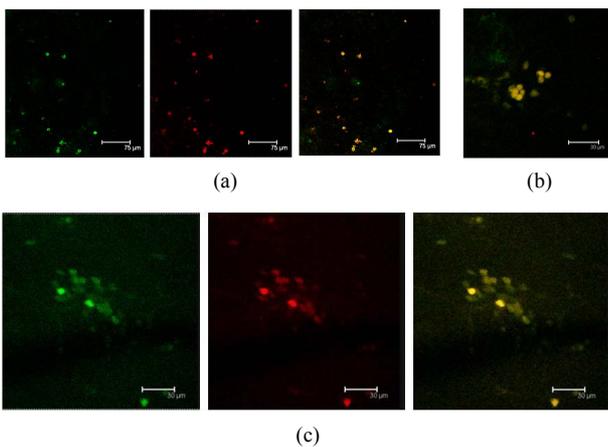


Figure 6. (a) GFAP positive cells (green) show also the T5H antibody labels (red) and coexistence of both (yellow) in the Np in coculture. Scale bar = 75 μm; (b) GFAP antibody labels (green) and T5H (red), can be seen coexisting (yellow) in cells of not cultured Np. Scale bar = 30 μm; (c) Glial cells, with GFAP positive mark (green), show also the 5-HT antibody labels (red), in a not cultured Np. Scale bar = 30 μm. Confocal microscopy.

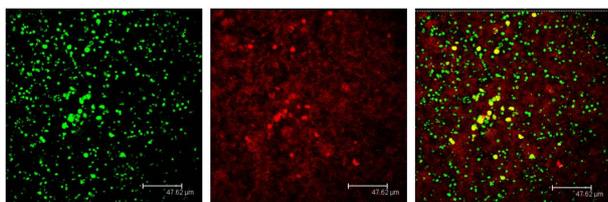


Figure 7. Cells immunopositive to the 5HT_{1A} antibody (green), show the Giα-1 antibody labels (red) coexisting in cells of the Np in coculture (yellow). Scale bar = 47 μm. Confocal microscopy.

[14]. However, the early serotonergic cells do differ with

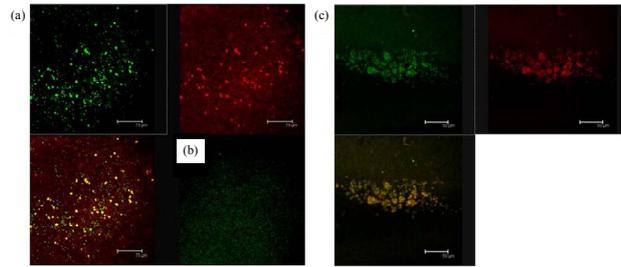


Figure 8. Cells in (a), shows the antibody labels for the 5HT_{1A} receptor (green) and MAPK (red) coexisting (yellow) in cells of the Np in coculture. Scale bar = 75 μm; In (b) a representative negative control, without the primary antibody is shown; (c) 5HT_{1A} labelled cells (green) and MAPK (red) coexisting (yellow) in cells of a not cultured Np (yellow). Scale bar = 50 μm. Confocal microscopy.

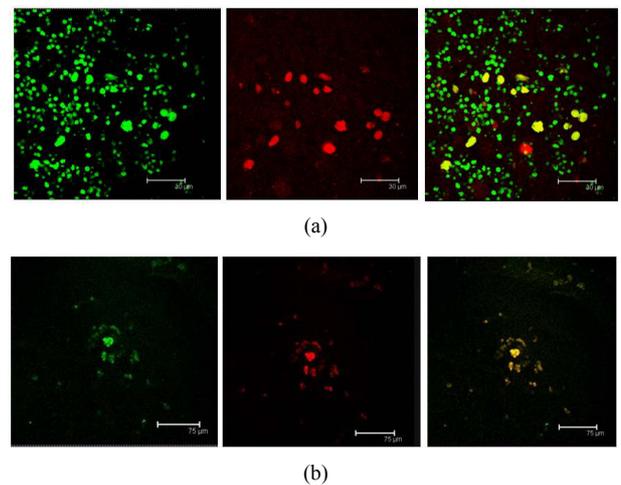


Figure 9. (a) MAPKK immunopositive cells (red) and 5HT_{1A} receptor labelled cells (green) show coexistence in cells of the Np in coculture (yellow). Scale bar = 30 μm; (b) The same sequence in a not cultured Np. Scale bar = 75 μm. Confocal microscopy.

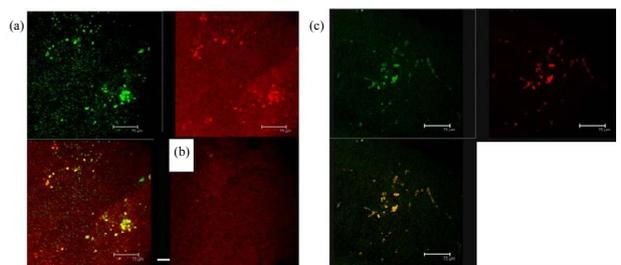


Figure 10. (a) 5HT_{1A} labelled cells (green) and ERK-1 (red) coexistence of both labels in cells of the Np in coculture. Scale bar = 75 μm; (b) A representative Negative control without primary antibody; (c) 5HT_{1A} positive cells (green) and ERK-1 (red) show coexistence of both labels (yellow) in cells of not cultured Np. Scale bar = 75 μm. Confocal microscopy.

the catecholaminergic cells in that they have not been observed in the early cerebral cortex (neopallium) of the fetal brain, neither in the adult cortex.

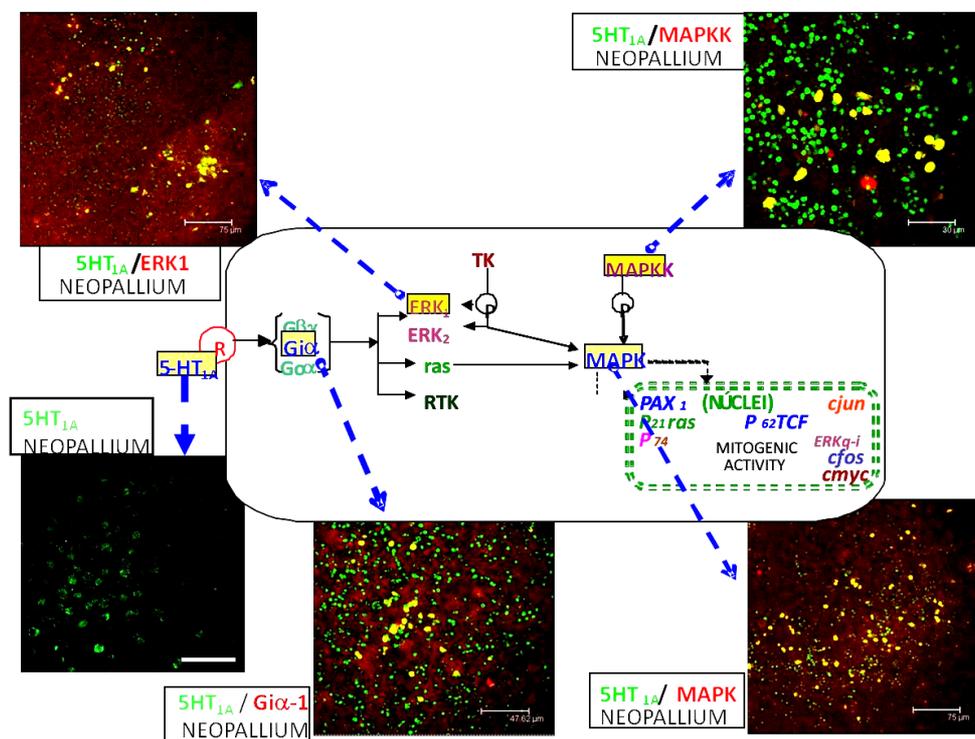


Figure 11. 5-HT_{1A} serotonin receptor signaling path is shown in this scheme in a theoretical fetal Np target cell, observed at 12 days of coculture with the fetal Me. Within squares the signaling molecules, coexisting with the 5-HT_{1A} receptor label, are shown.

Early serotonergic cells appearing at the mesencephalic area, are the sources of a wide axonal system afferent to multiple brain regions including the maturing cerebral cortex, particularly the sensory cortex, or areas like the marginal zone, septal, piriform and the cortical plate. Up to now it is accepted that regulatory serotonergic messages are displayed through this neuronal axon fiber system, widely distributed [13,27-29]. These elements of the serotonergic system can increase their expression and function during some periods of cortical development [11] as it is the case of SERT, the serotonin transporter, in the visual and in the somatosensory cortices [7,12,30].

Considering the preceding reference framework, the observations made in the present work may take relevance concerning the unexpected presence of serotonergic cells in the rat fetal neopallium in the culture conditions here described, with the 5-HT label coexisting in the same cells with T5-H, the main enzyme in the serotonin synthesizing path. Their presence has been also found in the fetal Np (E16) not cultivated (*in situ*), suggesting that they appear earlier. It is also interesting that serotonin markers did also coexist with Vimentin and with GFAP, strongly suggesting that young and mature neuroglia are also expressing the serotonergic phenotype in the maturing cortex. The morphology of the cells, excludes the possibility of serotonergic terminals.

In conclusion it seems possible that as it happens with

serotonergic fibers that augment significantly during certain periods of corticogenesis and after some time they decrease to normal, the serotonergic cells here described in the fetal neopallium in culture or *in situ*, could transiently express the serotonergic phenotype that would possibly allow them to participate directly in the structuration processes of the cerebral cortex, to disappear afterwards, as it happens with the mentioned TH positive cells [18,31].

Results of the present study also show the presence of a molecular signaling system that coexists with 5-HT_{1A} receptors in cells of the Np after 12 days in cocultures and also *in situ*. So it may well be that these molecules (Giα-1, ERK1, MAPK and MAPKK) are involved in a signaling cascade initiated by the serotonergic input on the 5-HT_{1A} receptor.

It is also known that elements of the serotonergic system may increase their expression during some periods of cortical development [11] as it is the case of SERT in the visual and somatosensory cortex [7,12,30,32]. The presence of SERT in the Np both *in-situ* or cultured, would reinforce the hypothesis that there are serotonin recognizing target cells in the fetal Np. We are currently examining how long their presence could last and how could we study their possible functional role.

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