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"POSTNATAL CELL PROLIFERATION IN THE HIPPOCAMPUS IS MODULATED BY DOPAMINERGIC SYSTEMS IN THE RAT: EFFECT OF PERINATAL ASPHYXIA"

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THESIS FOR THE DEGREE OF MASTER IN BIOMEDICAL SCIENCES WITH MENTION IN NEUROSCIENCES

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A mis padres Nivia y Fernando,
y hermanos Paloma, Gabriel y Leo.

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ABSTRACT

Postnatal neurogenesis has been observed in brain regions, such as dentate gyrus of the hippocampus and subventricular zone of telencephalon. Postnatal neurogenesis is an active phenomenon modulated by several factors including monoamine transmission. Mesencephalic dopamine (DA) neurons have been observed to project to the hippocampus, although a detailed map of that projection is still lacking, and the modulation of neurogenesis by DA is still controversial. Neurocircuitries of the basal ganglia and hippocampus are vulnerable to global anoxia/ischemia occurring at the neonatal stage.

We have investigated here on the DA modulation of postnatal cell proliferation and neurogenesis in hippocampus using organotypic cultures from control and asphyxia-exposed rats. Tissue from hippocampus, substantia nigra (SN) and/or ventral tegmental area (VTA) was taken at P1-3 for preparing organotypic cultures, plated on a coverslip and cultured for 22-24 days. The cultures were treated with 5-bromo-2'deoxyuridine (BrdU, 10μM), a marker of mitosis, and apomorphine (10mM), a DA agonist, for 48h. At P22-24, the cultures were fixed and treated for immunocytochemistry, using selective antibodies for tyrosine hydroxylase, microtubule-associated protein-2, and BrdU, evaluated with confocal microscopy.

We found that: (i) DA neurons project to hippocampus, under *in vivo* and *in vitro* conditions. (ii) Postnatal hippocampal cell proliferation occurs under *in vivo* and *in vitro* conditions, both in control and asphyxia-exposed animals. In hippocampus monocultures cell proliferation was increased in tissue from asphyxia-exposed animals when compared to the control condition. However, cell proliferation was only increased in hippocampus/VTA control cultures. (iii) Apomorphine treatment increased cell proliferation in hippocampal monocultures from both control and asphyxia-exposed animals.

This study demonstrates the modulation of postnatal neurogenesis taking place in hippocampus by DA, which is vulnerable to perinatal asphyxia.

ABBREVIATIONS

6-OHDA, 6-hydroxydopamine
AM, calcein-acetoxymethyl ester
APO, apomorphine
AS, asphyxia-exposed
BDNF, brain-derived neurotrophic factor
bFGF, basic fibroblast growth factor
BrdU, 5-bromo-2'deoxyuridine
BSA, bovine serum albumin
CCK, cholecystokinin
CNS, central nervous system
CNTF, ciliary neurotrophic factor
CS, controls
DA, dopamine
DAPI, 4,6 diamino-2-phenylindole
DIV, day *in vitro*
DG, dentate gyrus
EthD-1, ethidium-homodimer
G, gestation day
GDNF, glial cell line-derived neurotrophic factor
HCl, hydrochloride acid
H, monocultures of hippocampus
H/SN, co-cultures of hippocampus and SN
H/VTA, co-cultures of hippocampus and VTA
IHC, immunohistochemistry
MAP-2, microtubule-associated protein-2
MPTP, 1-methyl-4-phenyl-1,2,3,5-tetrahydropyridine
N, normal
NA, numerical aperture
NGF, nerve growth factor
NGS, normal goat serum
P, day after birth
PBS, phosphate-buffered saline
PF, paraformaldehyde
RRF, retrorubral field
SGZ, subgranular zone
SN, substantia nigra
SVZ, subventricular zone
TBS, Tris buffered saline
TH, tyrosine hydroxylase
VTA, ventral tegmental area

INTRODUCTION

As a therapist, I have experienced the difficulties of treating children with neurological and neuropsychiatric deficits. For 10 years, I have been involved in their rehabilitation, implying working with their neurological sequelae and unspecific pharmacological treatments, dealing with the daily frustration of the lack of understanding of the involved pathophysiological mechanisms and of the therapeutic rationality to which the children are exposed. Along to the rehabilitation process it is possible to observe changes in behavioural, motor and cognitive skills, improving the social participation and integration of the children, despite their damaged brain. Despite their handicaps, they are able to learn and establish a social contact and collaboration. Caregivers and educators, who are in daily contact with the children, also report these observations. The professionals are able to testify the children evolution and progress together with the clinical treatment.

This thesis addresses three important statements. *First*, *postnatal neurogenesis does occur*. At the clinical stage, it is known that learning and memory processes can take place, becoming a cornerstone for neuronal plasticity and for the rehabilitation process, probably involving hippocampal neurocircuitries. *Second*, *dopamine (DA) neurotransmission is a target for therapeutic interventions,* the cornerstone of the pharmacological treatment. Indeed, the indirect DA agonist methylphenidate (Ritalin®) is the only available pharmacological treatment for attention deficits, while DA antagonists are widely used for more severe cases of behavioural disorders. Most children I deal with have been prescribed with one or several of these drugs, while I roughly know about their mechanism of actions, I have learnt that the treatment affects the children behaviour, also inducing more or less severe side effects. Finally, *third, perinatal asphyxia* is a feature shared by most of the patients.

Postnatal cell proliferation and neurogenesis

Postnatal neurogenesis is an active phenomenon that occurs throughout life of individuals, encompassing proliferation and fate specification of neural progenitors, followed by subsequent differentiation, maturation, direction-path finding and functional integration into the existing neuronal circuitry (see Conover and Notti 2008, Ehninger and Kempermann 2008). Adult neurogenesis has been described in different vertebrates, from fishes (Costa et al.

2010), reptiles (Perez-Canellas and Garcia-Verdugo 1996), birds (Goldman 1998), primates (Gould et al. 1997) and humans (Eriksson et al. 1998).

Postnatal neurogenesis has been described in two specific regions of the mammalian brain: the subventricular zone (SVZ), on the wall of the lateral ventricles, and in the subgranular zone (SGZ) of dentate gyrus (DG) of hippocampus (see Deierborg et al. 2009, Landgren and Curtis 2011). There is evidence showing neurogenesis in other brains regions, such as neocortex (see Gould 2007), but that evidence is not conclusive.

Neuroblasts generated in the SVZ can migrate through the rostral migratory stream to the olfactory bulb, to be differentiated and integrated into the neuronal networks of the olfactory system (Curtis et al. 2007). Neuroblasts generated in the DG migrate short distances into the adjacent granular layer, where they develop as granule neurons, to be integrated into neuronal networks involved in learning and memory processes (Collin et al. 2005, Gould 2007, Deierborg et al. 2009, Lee and Son 2009, Dranovsky et al. 2011).

It has been shown that postnatal neurogenesis is modulated by several factors (see von Bohlen et al. 2011), including basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) (Otha et al. 2000, Reuss and Unsicker 2000, Guo et al. 2002, Yang et al. 2008). Several neurotransmitters have also been involved in postnatal neurogenesis, including glutamate, GABA, serotonin and DA (Mori et al. 2008, see O´Keeffe et al. 2009, Lee and Son 2009, Pathania et al. 2009, Platel et al. 2010).

There are evidences that multisensory stimulation, social interaction (see Dranovsky et al. 2011), exploration and physical activity, as well as learning and memory increase neurogenesis in the DG, while stress (see Paizanis et al. 2007), drug abuse and alcoholism decrease neurogenesis (see Nixon 2006). Furthermore, neurogenesis has been found to be increased by brain injuries like hypoxia-ischemia, suggesting a compensatory mechanism for repairing damaged neurocircuitries (see Morales et al. 2005, 2008).

Dopaminergic systems

There are several DA systems in central nervous system (CNS), i.e. mesostriatal, mesolimbic, mesocortical, tuberoinfundibular systems and olfactory bulb, playing a relevant role in a large

amount of behavioural programmes, including sensory motor integration, reward, learning and goal-directed motor responses (see Bjorklund and Dunnett 2007).

The development of DA neurons is a complex multi-step process. It involves events occurring at prenatal stages, such as cell fate specification, differentiation and migration, and postnatal events, including neurite growth, guidance, synapse formation and pruning. Mesencephalic DA neurons are organised in several anatomically and functionally distinct subgroups, e.g. A9 (substantia nigra *pars compacta*, SNc), A10 (ventral tegmental area, VTA) and A8 (retrorubral field, RRF), according to Dahlstrom & Fuxe nomeclature (1964) and Ungerstedt (1971) (see Bentivoglio and Morelli 2005, Roeper 2013). Neurons from SNc send axons to the dorsal striatum (see Bentivoglio and Morelli 2005, Pathania et al. 2010), and its dysfunction or neurodegeneration is associated with parkinsonism and Parkinson´s disease. Neurons from VTA and RRF project to the ventromedial striatum, nucleus accumbens and prefrontal cortex, regions associated to emotion and reward (see Lisman and Grace 2005, van den Heuvel and Pasterkamp 2008). Both, SN and VTA project to the neurogenic niches in SVZ and SGZ establishing anatomical and functional connections (Descarries et al. 1987, see Backer et al. 2004, Höglinger et al. 2008, Platel et al. 2010, Roeper 2013).

Hippocampus receives a strategic input from mesencephalic DA neurons. Anatomical studies using retrograde and anterograde labelling have described the presence of tyrosine hydroxylase (a marker of DA neurons, TH) positive projections into the hippocampus, originating in medial SN and VTA (Verney et al. 1985), showing different patterns of distribution (see Goldsmith and Joyce 1994). DA axons reach the hippocampal formation at early postnatal ages, the ventral junction prosubiculum-CA1 field being the main target area (Verney et al. 1985). Furthermore, the entorhinal cortex is also a target for mesencephalic DA neurons (Hökfelt et al. 1974, see Bentivoglio and Morelli 2005, Roeper 2013), and the entorhinal cortex, including the amygdalo-piriform transition, provides the main input to the hippocampus via the perforant pathway (Winter et al. 1993, van den Heuvel and Pasterkamp 2008).

Two families of DA receptors, D1 and D2, have been described in the CNS (see Seeman 1980). The D1 family (D1 and D5 receptors) activates the enzyme adenylyl cyclase through a G_s coupled protein, while the D2 family (D2, D3 and D4 receptors) inhibits the same enzyme through a G_i coupled protein. A nonuniformly distribution of DA receptors

families, have been described in hippocampus (see Goldsmith and Joyce 1994), but a dense amount of D5 (a member of the D1 family receptors), has been particularly described in that region (Goldsmith and Joyce 1994, see Bentivoglio and Morelli 2005). The D2 family of receptors has limited expression in the *lacunosum moleculare* of the subiculum and CA1 fields (Goldsmith and Joyce 1994).

Thus, the uneven distribution of DA projections and DA receptors in the hippocampus could be related to distinct forms of anatomical organization. However, a mismatch between innervation and receptor expression would suggest a complex organization.

DA receptors and neurogenesis

Stimulation of D2 receptor promotes proliferation of neural progenitors in SVZ (O´Keeffe et al. 2009) and DG, probably via receptors located on GABA neurons (Platel et al. 2010). Activation of D3 receptor increases cell proliferation in the embryonic germinal zones (Ohtani et al. 2003) and in cells derived from the postnatal SVZ (Backer et al. 2004, Coronas et al. 2004, Hoglinger et al. 2004). Furthermore, it has been reported that antagonism of DA receptors, or lesion of DA neurons of the VTA/SN with 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,5-tetrahydropyridine (MPTP), induce a transient decrease of cell proliferation in SVZ and DG of adult rodents (Backer et al. 2004, Hoglinger et al. 2004). It has also been shown that astrocytes express DA receptors (Bal et al. 1994), which, when treated with non sellective DA receptor agonists like apomorphine (APO) (see Luo 2009), synthesise and release growth and neurotrophic factors, such as bFGF (Reuss and Unsicker 2000, Luo 2009) involved in neurogenesis (Otha et al. 2000, Guo et al. 2002, Yang et al. 2008). Thus, DA receptors play probably a modulatory role in postnatal cell proliferation (Bal et al. 1994, Díaz et al. 1997, Kippin et at. 2005, Mori et al. 2008, Mu et al. 2011).

Perinatal asphyxia

Perinatal asphyxia is major cause of death and neurological injury of newborn babies, frequently associated with difficult or prolonged delivery. Its international incidence is 2-6/1000 term births in developed countries, but it has been demonstrated that that incidence is increased several times in low-income countries (Lawn et al. 2005). Perinatal asphyxia still occurs with great incidence, despite improvements of perinatal care (see Herrera-Marschitz et

al. 2011). After asphyxia, infants can suffer from long-term neurological sequelae, their severity depending upon the extent of the insult. Severe asphyxia has been linked to cerebral palsy, mental retardation, and epilepsy, while mild-severe asphyxia has been associated with attention deficits and hyperactivity in children and adolescents (Mañeru et al. 2001, 2003), and increased risk for low IQ score (Odd et al. 2009). Indeed, obstetric complications are risk factors for several psychiatric diseases, including schizophrenia. The mechanisms by which these complications affect development are largely unknown.

At present, there is not any accepted therapeutic strategy for significantly preventing the long-term effects produced by perinatal asphyxia, apart from hypothermia, still a controversial subject (see Herrera-Marschitz et al. 2011), therefore it is important to further study on the mechanisms by which perinatal asphyxia can lead to long-term deficits.

Perinatal asphyxia, DA transmission and neurogenesis

There is a regional vulnerability to the effect of perinatal asphyxia (Serrano et al. 2007). Neurocircuitries of the basal ganglia and hippocampus are particularly vulnerable to global anoxia/ischemia occurring at the neonatal stage (see Morales et al. 2005, 2008, 2010; Klawitter et al. 2005, 2006, 2007; Herrera-Marschitz et al. 2011; Allende-Castro et al. 2012)

Previous work confirmed that vulnerability, assayed with immunohistochemistry (IHC) (Dell'Anna et al. 1997, Morales et al. 2003, 2008, Klawitter et al. 2005) and molecular biology (Anderson et al. 1995, Gross et al. 2000, 2005), *in vivo* (Dell'Anna et al. 1995, 1997), *in vitro* (Morales et al. 2003, Klawitter et al. 2005, 2006, 2007), and *ex vivo* (Bustamante et al. 2003) biochemistry, confirming the idea of regional vulnerability, related to (i) the severity of the insult; (ii) the metabolic imbalance during the re-oxygenation period, and (iii) the developmental stage of the affected region.

Bustamante et al. (2003, 2007) reported a decrease in DA levels in neostriatum, and nucleus accumbens several months after severe asphyxia, and with organotypic cultures, it was demonstrated that the impairment implied a decrease in the number of DA cell bodies, neurite atrophy and connectivity (Morales et al. 2003, Klawitter et al. 2007), in agreement with previous studies using IHC (Andersson et al. 1995).

Several compensatory mechanisms have been suggested to be triggered for protecting the CNS from delayed cell death, including neurogenesis (Morales et al. 2008) and

neuritogenesis (Morales et al. 2010). In the hippocampus, asphyxia induces neurite and branching atrophy, affecting synaptophysin and PSD95 expression, pre- and postsynaptic markers respectively, found to be decreased at P30 in tissue from asphyxia-exposed animals (Rojas-Mancilla et al. 2013).

The role of DA systems in hippocampal cell proliferation is still controversial and its alteration following perinatal asphyxia has not been characterized yet. The issue is relevant, because indirect DA agonists are used for treating attention deficit hyperactivity disorder, a disorder strongly associated with perinatal asphyxia (Robertson and Perlman 2006, see Konrad and Eickhoff 2010), among other pathologies.

Thus, in the present thesis, we have investigated the modulation of postnatal cell proliferation in hippocampus by mesencephalic DA systems, in control and asphyxia-exposed rats, with organotypic cultures.

HYPOTHESIS

Postnatal cell proliferation in rat hippocampus is modulated by DA systems in normal and asphyxia–exposed animals.

OBJECTIVES

General objective:

To investigate whether DA systems modulate cell proliferation taking place in hippocampus, using organotypic cultures from control and asphyxia-exposed rats.

Specific objectives:

1. To evaluate cell viability and connectivity in monocultures of hippocampus (H) and co-cultures (H/SN or H/VTA) from control and asphyxia-exposed rats.
2. To evaluate the dopaminergic innervation of hippocampus in co-cultures (H/SN and H/VTA) from control and asphyxia-exposed rats, focusing on neurite length and branching assayed with IHC.
3. To evaluate and compare cell proliferation in monocultures (H) and co-cultures (H/SN or H/VTA) from control and asphyxia-exposed rats, assayed by IHC against BrdU, MAP-2 and TH at DIV 22-24.
4. To evaluate the effect of APO on cell proliferation in H monocultures of control and asphyxia-exposed rats, assayed with IHC against BrdU at DIV 22-24.

MATERIAL AND METHODS

Experimental models

Organotypic cultures were prepared from neonatal *Rattus norvegicus* (Wistar) rats from a local colony at the *Programme of Molecular & Clinical Pharmacology, ICBM*. Animals were treated according with protocols approved by a Local Ethics Committee for experimentation with laboratory animals at the Medical Faculty, University of Chile (Protocol CBA# 0389 FMUCH).

Oestral cycle determination

The oestral cycle was determined periodically using a pipette smear technique (see Herrera-Marschitz et al. 2011), to perform programmed mating of rats at pro-oestrum stage. Gestation day 1 (G1) corresponds to the day when sperm cells are detected in the vaginal smear. Pregnant rats are then placed in individual boxes until delivery, further examined by clinical abdominal palpation to predict foetal size and time of birth.

Perinatal asphyxia

Pregnant Wistar rats within the last day of gestation (G22) were euthanized by neck dislocation and hysterectomized. One or two pups were removed immediately and used as caesarean-delivered controls (CS), and the remaining foetuses-containing uterine horns were immersed in a water bath at 37°C for 21 min (AS). Following asphyxia, the uterine horns were incised and the pups were removed, stimulated to breathe and after 55 min observation period were evaluated with a Apgar scale for rats, as described below (Dell'Anna et al. 1995). Thereafter, the neonates were given to surrogate dams, pending further experiments. The original model of perinatal asphyxia was established by B. Bjelke, K. Andersson and M. Herrera-Marschitz and col. (1991, 1992, 1993) at the Karolinska Institutet, Stockholm, Sweden, and is performed in Chile according to Morales et al. (2003).

Apgar scale for rats

The Apgar scale for rats evaluates the severity of the asphyxia insult, assessed 55 min after asphyxia. Parameters considered are detailed in Table 1. The Apgar scale revealed a severe

insult induced by perinatal asphyxia, with survival rate below 65%. Surviving rats showed a decreased respiratory frequency (less than 30 event/min), positive gasping, no vocalizations and lack of spontaneous movements and pink-blue skin color, which contrasted to that observed in the caesarean delivered controls. Thus, apart of the respective controls, only severed asphyctic pups were used to prepare organotypic cultures (Table 1).

Table 1. Apgar scale for rat parameters.

Parameters(*)	Control (CS, caesarean delivered control pups (0 min asphyxia) (n= 72; m=72)	Asphyxia-exposed pups (AS, >21 min asphyxia (n=72; m=72)
Survival (Yes/No; %)	100%	65%
Skin colour (%): Pink, pink-blue Blue, Blue-pink	 100% -	 58% 43%
Movements (4, 3, 2, 1, 0) (**)	4	1
Vocalizations (Yes/No) (%)	98%	10%
Gasping (No/yes) (%) (***)	0.4%	55%
Respiratory frequency (events/min)	77±1	28±1

(*) % refers to the corresponding litter (m)
(**) 4 indicates coordinated and spontaneous movements, 0 indicates no movement.
(***) Gasping forced reflex inspirations. In control rats gasping is absent after 1 min.

Organotypic cultures

Different rat series of both sexes were used for preparing organotypic cultures, one to three days after birth (P1-3) (see Klawitter et al. 2007)

The brains were rapidly removed under sterile conditions and stored in a Petri dish containing Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Life Technologies AB, Täby, Sweden). Coronal sections were cut with a microslicer (DTK-2000, Dosaka CO, Japan) (300µm thick) and stored in cold DMEM. Samples from SN, VTA and hippocampus were dissected and placed on a coverslip (Nunc Thermanox Coverslips; Nunc, Naperville, IL, USA) containing a spread layer of chicken plasma (25µL) and further coagulated by bovine thrombin (20µL/350µL DMEM, Sigma, St. Louis, MO, USA). The standard thrombin solution contained 1000 NIH units in 750µL of H_2O.

The coverslips were then transferred to sterile Nunc flat CT-tubes containing 750µL of culture medium [Basal Medium Eagle 50%, Hanks Balanced Salt Solution 25% and horse serum 25% (GIBCO BRL), glucose 0.5%, L-glutamine 0.5 mM (Sigma), and penicillin/streptomycin 1% (GIBCO BRL)]. H monocultures, H/SN and H/VTA co-cultures were grown at 35°C, and 10% CO_2 in a Cell Incubator (Model TC2323, ShelLab, USA), using a roller device exposing the cultures to gaseous and water phases every minute. At DIV 3, the medium was changed to a serum-free medium [Neurobasal-A medium supplemented with B27 2% (GIBCO BRL), glucose 5mM, L-glutamine 2.5mM (Sigma)]. The medium was changed every 3-4 days. At DIV 20-22, the mitotic marker BrdU (10µM) (Sigma, USA) was added to the culture medium for 48 hours and one group of monoculture was also treated with APO (1µM) for 48h. Thereafter cultures were fixed with formalin solution (4% paraformaldehyde, PF; Sigma, in 0.1M phosphate-buffered saline, PBS, pH 7.4) for 45 min at 4ºC, rinsed and stored in PBS at 4ºC pending further experiments (see Fig 1 and 2)

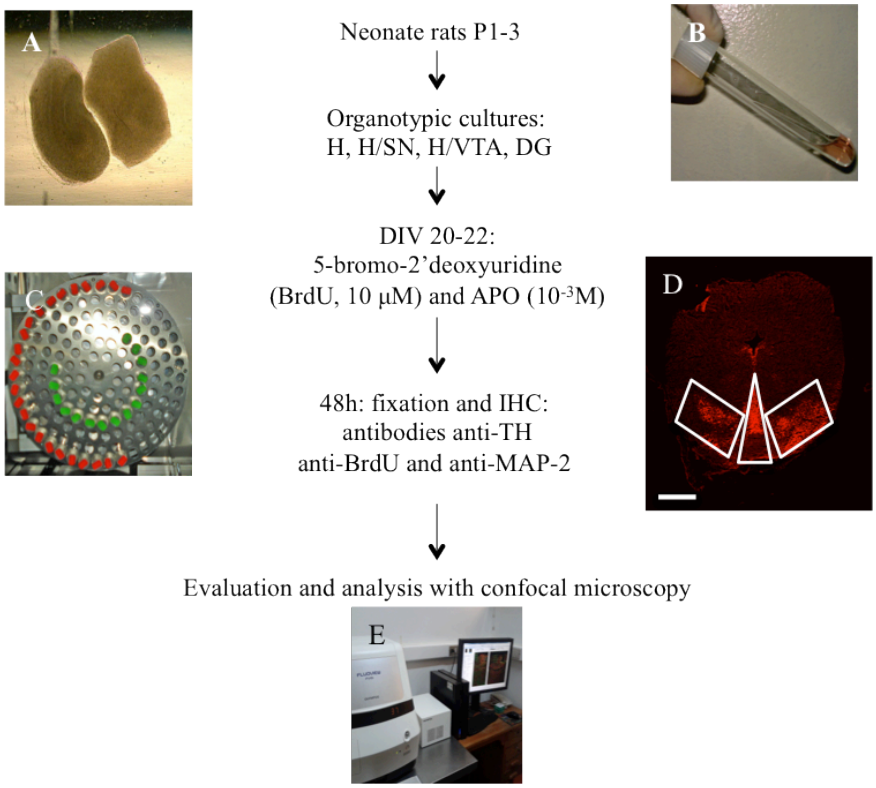

Figure 1: Metodology. Tissue from hippocampus (H) and mesencephalon (SN, VTA) was taken from control and asphyxia-exposed rats at P1-3 for preparing mono (H) or co-cultures (H/SN, H/VTA). A representative microphotograph of a H/SN co-culture at DIV 3 is shown in A. Samples were cultured for 22-24 days on sterile Nunc flat CT-tubes (B), in a Roller device (C) within the arena of a Cell Incubator (ShelLab, Oregon, USA). The culture medium was changed every 3-4 days. BrdU and APO were administered at DIV 20-22. At DIV 22-24 the cultures were fixed with a formalin solution and treated for immunocytochemistry using antibodies against TH, BrdU and MAP-2. A confocal microscope unit (E) was used for analysis and evaluation. In D, an *in vivo* coronal section from a control mesencephalon (P1) was treated for TH-immunocytochemistry for showing the SN (rhomboid) and VTA (triangle).

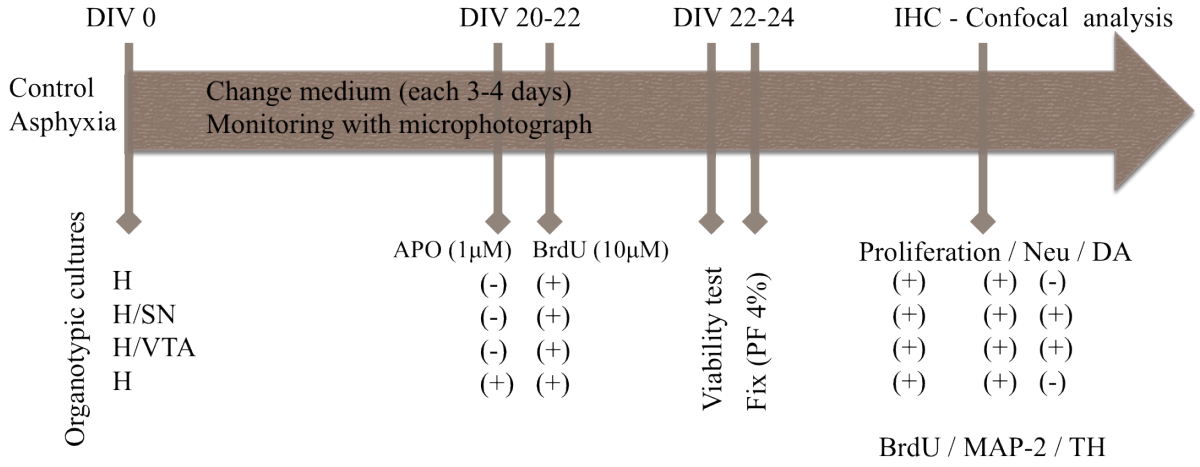

Figure 2: Experimental design. Hippocampus (H), H/SN, H/VTA organotypic cultures from control and asphyxia-exposed rats were cultured for 22-24 days on sterile Nunc flat CT-tubes, in a Roller device within the arena of a Cell Incubator. The culture medium was changed every 3-4 days. At DIV 20-22, APO- (1μM) and BrdU-treatment (10μM) were administered for 48 hrs. At DIV 22-24, viability test was applied to one group of tubes and the other group were fixed with a formalin solution (PF 4% in PBS) and treated for IHC using antibodies against TH, BrdU and MAP-2. Microphotographs were taken with confocal microscopy and analysed using ImageJ software.

In vitro monitoring

The progression of the cultures was monitored by using an inverted microscope (NIKON T100), directly from the culture tubes, at DIV 1, 3, 11, 17 and 22-24, using Hoffman optic, taking representative microphotographs at different days, using different magnification (see Fig 3).

In vitro cell viability

Some organotypic cultures (one case from each experimental series) were analysed for cell viability at DIV 22-24, using the LIVE/DEAD Viability/Cytotoxicity kit L3224 (Molecular Probes, Eugene, OR, USA), implying ethidium-homodimer (EthD-1) and calcein-acetoxymethyl ester (AM) for labelling dead and alive cells, respectively, according to the instructions of the manufacturer. Alive cells were distinguished by a green fluorescence produced by the action of intracellular esterase activity that separates the AM group, retaining the calcein dye within live cells. Dying cells were identified by a red fluorescence produced when EthD-1 enters the cells with damaged membranes and binds to nucleic acids. EthD-1 positive cells (red fluorescence) were counted in 4-5 selected regions of 1.13mm^2 (~0.02mm^3) of the organotypic culture at DIV 22-24 considering the full-labelled area, according to Klawitter et al. (2007) (see Fig 4).

Immunohistochemistry

Tissue from formalin perfused brains or formalin fixed cultures were treated in a similar manner. The fixed tissue was rinsed with TBS (3x5 min). DNA was denature with HCl 1N (30min on thermoregulated bath at 37ºC) and, after rinsing cycles, were permeabilized with 1% Triton X-100 in TBS 1x (10 min). Then, the tissue was preincubated in blocking solution (6.5% normal goat serum (NGS), 2% bovine serum albumin (BSA) and 0.5% Triton X-100 in TBS 1x) for 1h and incubated for 24h with antibody against-BrdU (ABCAM ab81106, rat; 1:500 dilution in blocking solution at 4ºC), in order to detect cell proliferation. After rinsing cycles with 1% NGS in TBS 1x (3x5), a secondary antibody (goat against rat, Alexafluor 488, 1:500 dilution in 1% NGS in TBS 1x) was added and maintained for 2h. Finally, the tissue was rinsed with TBS 1x (5x5) and 0.5% Triton X-100 in TBS 1x, for 10 min.

For visualizing neuronal and DA neuronal phenotype, the tissue was preincubated in blocking solution 2 (2% horse serum, 2% BSA and 0.5% Triton X-100 in TBS 1x) for 1h and incubated with antibody against-MAP-2 (ABCAM ab5392, chicken, 1:750 dilution in blocking solution 2) and antibody against-TH (ABCAM ab113, sheep, 1:1000 in blocking solution 2) respectively, over night on 4ºC. After rinsing cycles with 0.5% horse serum in TBS 1x (3x5) the tissue was incubated with goat against-chicken (Alexafluor 594, 1:400), goat against-sheep (Alexafluor 647,1:400) secondary antibodies and DAPI (4,6 diamino-2-phenylindole; Invitrogen 1:10000 for nuclear labelling) in 0.5% horse serum in TBS 1x, for 2h. Then, the tissue was rinsed, mounted with hydrophilic resin (Fluoromount) and examined with confocal microscopy (Olympus-fv10i).

Quantification of TH positive cells and neurites (soma, length and branching)

Three parameters were quantified: (1) number of TH positive cells/mm^3 (DAPI positive nuclei/TH positive soma), (2) neurite number and length (maximum length of TH positive processes, μm) and (3) TH-branching, as described in Klawitter et al. (2007).

Microphotographs (5-6) were taken of SN or VTA areas showing DAPI and TH labelling in the field of a confocal-inverted Olympus-fv10i microscope with a 60x objective lens (NA 1,2). The area inspected was 0.04 mm^2. The thickness (Z axis) was measured for each case.

The number of DA neurons was estimated by counting TH positive somas (including DAPI labelling), expressed as cells/mm^3, in cultures from control and asphyxia-exposed conditions (n= 6-9) using ImageJ software.

Neurite number was estimated by counting each primary, secondary and terciary projections observed in X, Y, Z confocal planes from each TH positive neurons. Neuritic length was estimated by measuring each process/neuron with the ImageJ software. The total length (μm) was the summatory of all neurites´s length observed for each neuron.

Quantification of BrdU positive cell proliferation

Cell proliferation was evaluated by counting BrdU positive cells/mm^3 in microphotographs of organotypic culture fixed at DIV 22-24 (0.04 mm^2; n=5-6), taken with a 60x objective lens

(confocal-inverted Olympus-fv10i microscope; NA 1,2). The thickness (Z axis) was estimated for each case.

Statistical analysis

The values are expressed as the means ± SEM. Data have been analysed with parametric (Student's t-test) or non-parametric (Mann-Whitney U test) post-hoc tests depending upon data distribution, using the GraphPad Prism software. A $p<0.05$ level was critical for statistically significant differences.

RESULTS

Perinatal asphyxia.

Table 1 shows the parameters evaluated by the Apgar scale, indicating that all asphyxia-exposed animals used in this study suffered of a severe perinatal insult (n=72), which contrasted with that observed in caesarean delivered controls (n=72).

In vitro evaluation of cell viability and connectivity, in monocultures of hippocampus (H) and co-cultures (H/SN or H/VTA) from control and asphyxia-exposed rats.

(a) Monitoring of organotypic cultures

Each organotypic culture was monitored and photographed at DIV 1, 3, 7, 11, 17 and 22-24, assessing their development on the coverslip. At DIV 3, it was observed that tissue from co-cultures had incipient contacts between the structures, becoming largely fused and integrated at DIV 11 (see Fig 3). At DIV 1, thickness of organotypic cultures was 300μm, decreasing to ~45μm at DIV 22-24, measured at the stage of the confocal microscope (Z- axis).

(b) Cell viability in organotypic cultures

At DIV 22-24, the viability test was applied to one sample from each mono and co-culture series. Many alive cells (green fluorescence) were observed in both mono and co-cultures. Only a few death (red fluorescence) cells were observed on each culture. The amount of death cells/mm^3 did not differ among mono- and co-cultures, either from control or asphyxia-exposed animals, or following treatment with APO (see Fig 4).

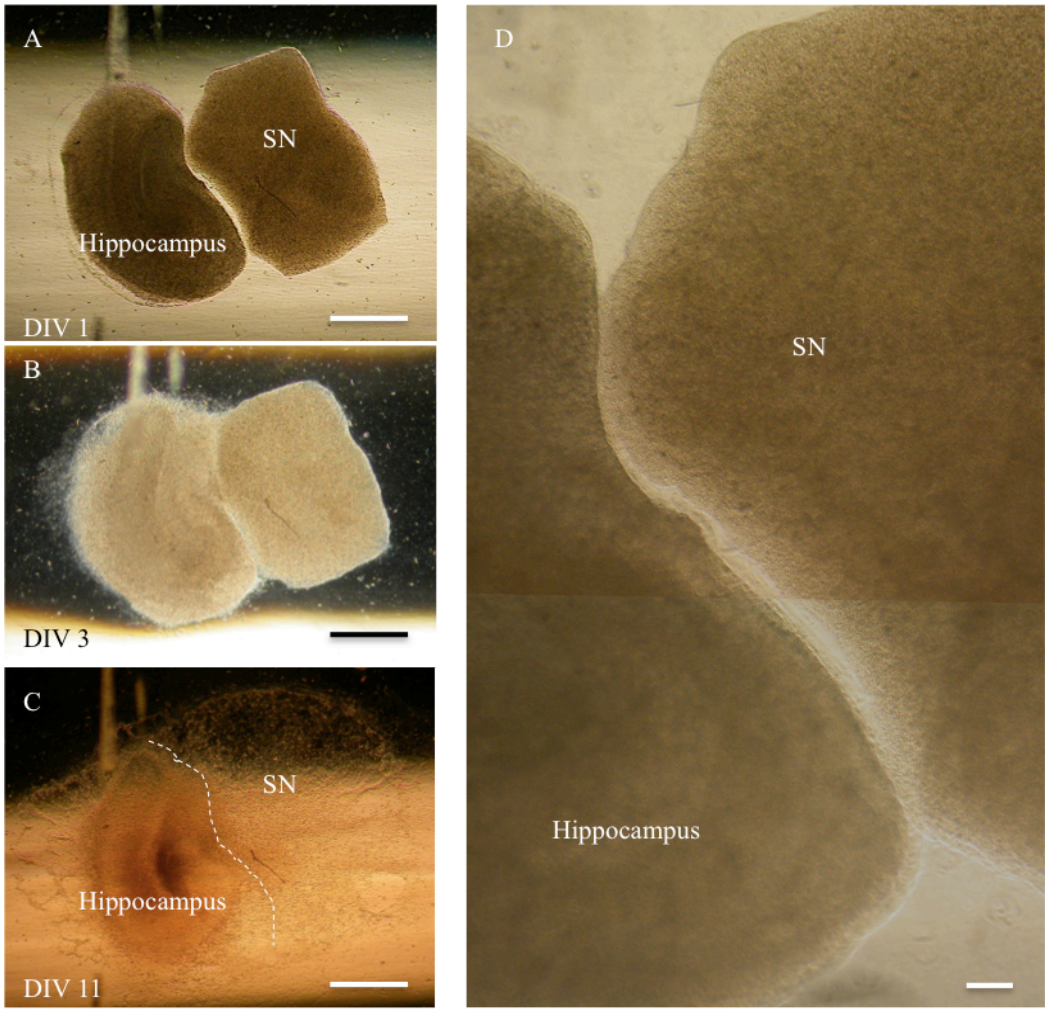

Figure 3: Monitoring organotypic cultures from H/SN. A-C: The progression of co-cultures is shown at DIV 1, 3 and 11 (magnification 2x, scale bar: 1000µm). D: Magnification of the apposition between H and SN at DIV 1 (20x, scale bar: 100µm).

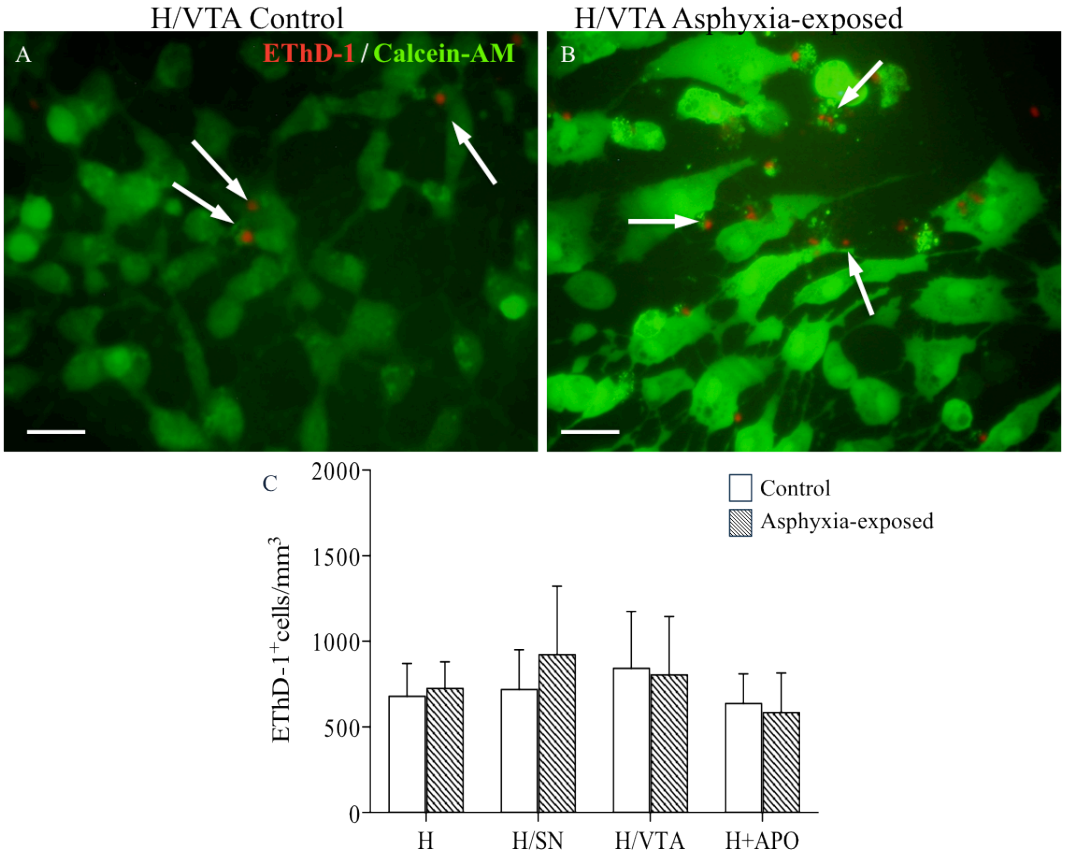

Figure 4: Viability test. At DIV 22-24, viability test was randomly applied to mono and co-cultures, in order to evaluate life/death cells. Alive cells (green fluorescence) were observed in both mono and co-cultures, greatly over numbering dead cells (red fluorescence, white arrows). Representative H/VTA co-culture microphotographs (40x, scale bar: 10µm) from control (A) and asphyxia-exposed (B) rats are shown. Quantification of EThD-1 positive cells (dead cells) in hippocampal (H), H/SN, H/VTA co-cultures, and apomorphine-treated hippocampus from control (open bars, n=4) and asphyxia exposed (dashed bars, n=4) rats (C). No significant differences in the number of EThD-1 positive cells/mm^3 between the samples were found (comparisons, Student's t-test, $p<0.05$).

***In vivo* and *in vitro* evaluation of DA innervation of hippocampus from control and asphyxia-exposed rats, focusing on neurite length and branching.**

In vivo: In control animals, many TH positive cells and fibres were observed in brain assessed at P1 and 1 month of age, although differences in the amount and selectivity of fibre distribution were observed when comparing both ages. Indeed, the TH positive labelling of hippocampus and neocortical regions was strong and widely spread at P1, with many fibres seen in all layers of neocortex, but also in presubiculum and subiculum. At 1 month, TH fibres were concentrated in the *stratum oriens* and DG of hippocampus (see Fig 5).

In vitro: In organotypic cultures from control animals, TH positive cells with different morphological features were observed in both VTA and SN (monopolar, bipolar, stellate). No differences were observed in the number of TH positive cells/mm^3, when comparing co-cultures from control and asphyxia-exposed rats in (H/VTA or H/SN) (see Fig 8). TH positive projections to the hippocampus were observed in co-cultures from H/VTA and H/SN of both control and asphyxia-exposed animals (see Fig 6 and 7). No differences were observed among the number of neurites of DA cells in H/SN and H/VTA cultures from control and asphyxia-exposed animals [control H/SN, 3.9±0.8 (range 2-9; n=10); asphyctic H/SN, 3.4±1.13 (range 1-15; n=12); control H/VTA, 3.7±0.9 (range 1-8; n=7); asphyctic H/VTA, 3.0±0.47 (range 1-8; n=23)] (see Fig 8)

The length of primary, secondary and tertiary neurites of each TH positive neuron was added and expressed as total neurite length. Significant differences in total length were found in H/SN, when comparing cultures from control versus asphyxia-exposed animals (see Fig 8F). The range of the total length was 21- >500μm (n=10) in cultures from control, versus 7- >280μm (n= 12) in cultures from asphyxia-exposed animals. In H/VTA co-cultures, the total neurite length was larger in cultures from asphyxia-exposed (23- >590μm; n= 23) compared to that from control (19- >360μm; n=7) rats, although the differences did not reach the statistically significant level. Only in selective co-culture cases double-labelled cells ($BrdU^+/TH^+$) were observed, probably indicating proliferation of DA neurons (data not shown).

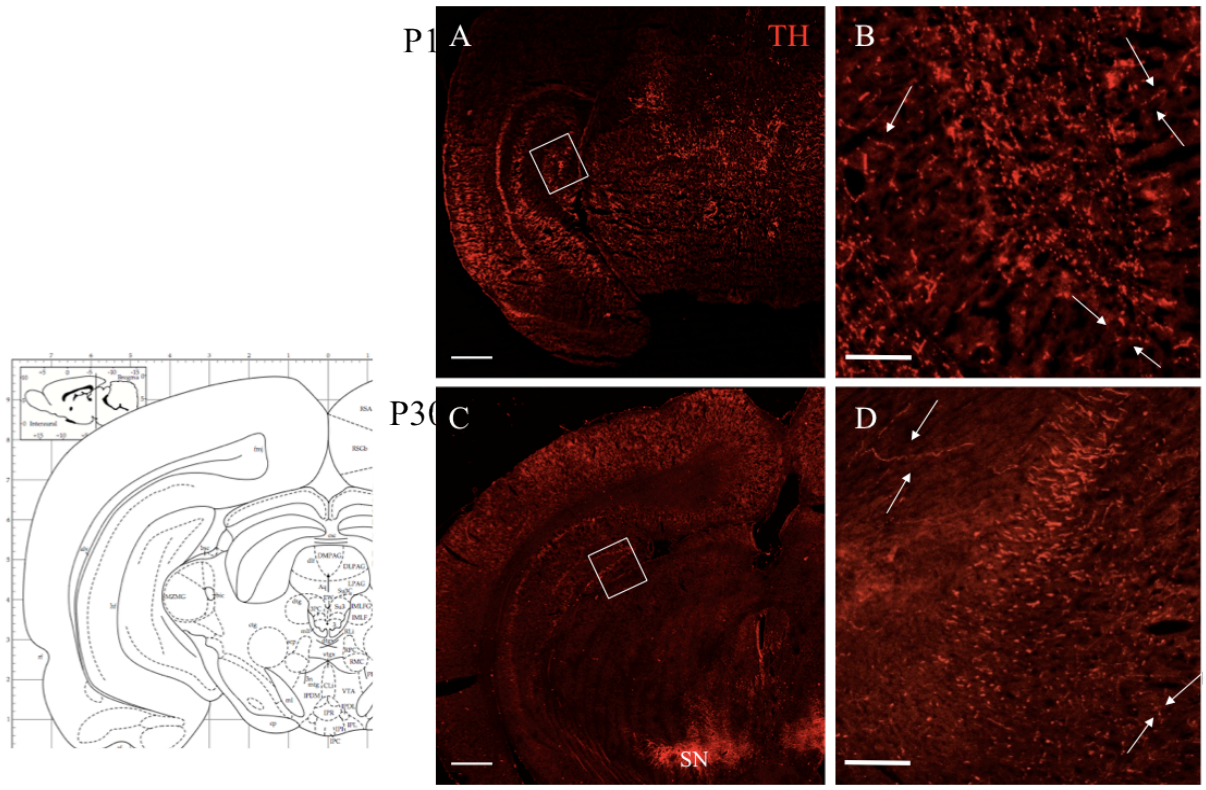

Figure 5: TH-IHC in neonatal and adult brain rats *in vivo*. Microphotograph of mesencephalic control brain at P1 (A, scale bar: 1000µm. B, scale bar: 10 µm) and P30 (C, scale bar: 1000µm. D, scale bar: 10µm) show TH positive cells (white arrow heads) and projections (red) concentrated in the *stratum oriens* (rectangle in A and C) White rectangles in A and C show areas magnificated in B and D, respectively, revealing TH positive projections into the hippocampus (white arrows).

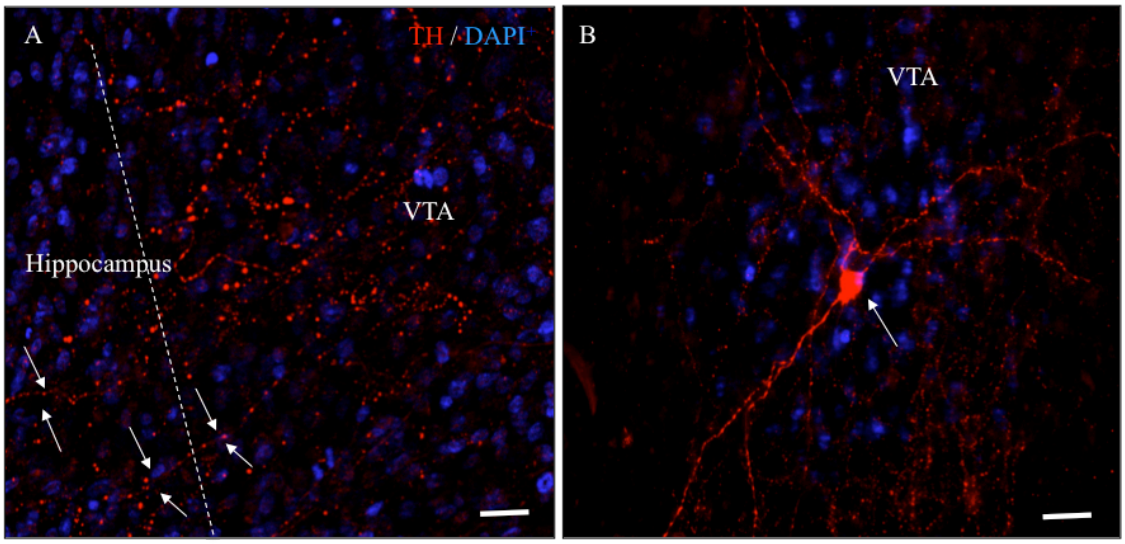

Figure 6: DA neurons of VTA projecting to hippocampus in H/VTA cultures. Microphotographs showing TH positive (red) and DAPI (blue) labelling in H/VTA co-cultures. A: DA projections (red) into hippocampus (white arrows). Dotted line indicates the border between VTA and H. B: Magnification of a DA neuron (red) in VTA. Scale bar: 10µm.

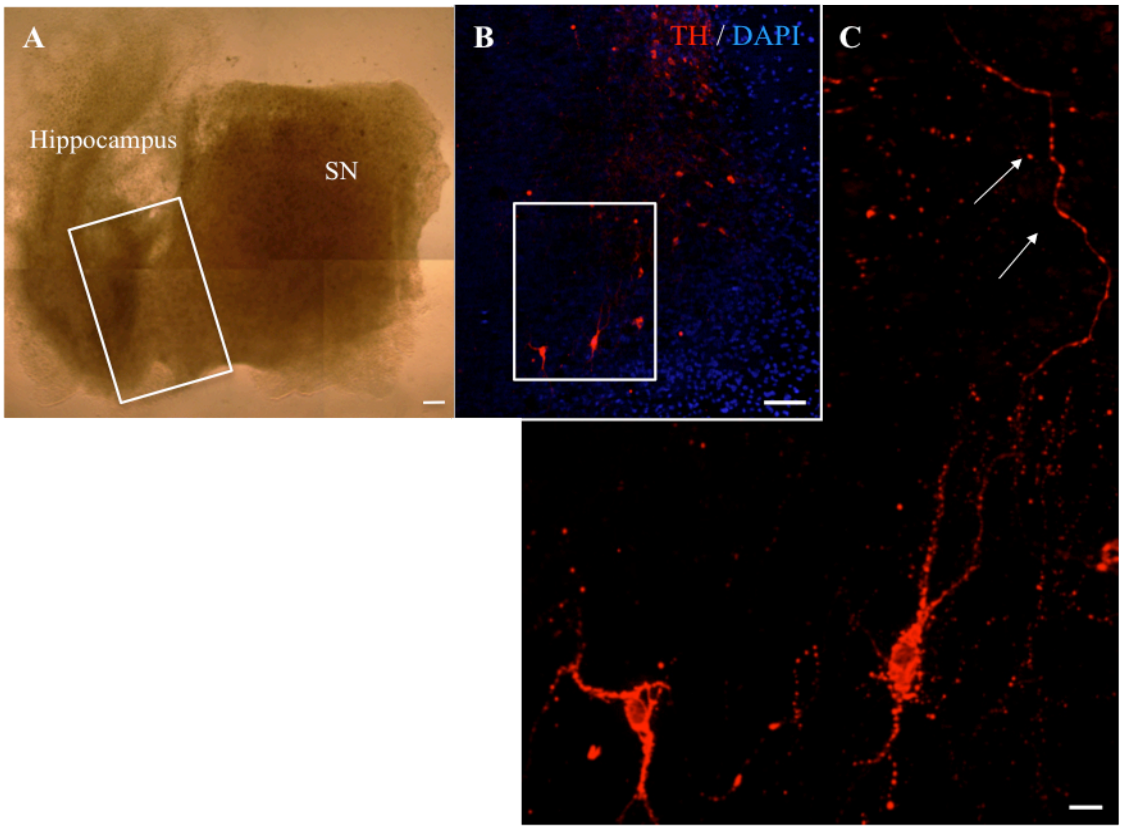

Figure 7: DA neurons in SN projecting to hippocampus in H/SN cultures. A: H/SN co-culture of at DIV 3 (scale bar: 500µm). White rectangle in A indicates an area where SN and DG merge (B), labelled for TH (red) and DAPI (blue, a nuclear marker) IHC (scale bar: 100µm). The white square in B is magnified in C, showing a TH^+ neuron projecting to the DG (Scale bar: 10µm).

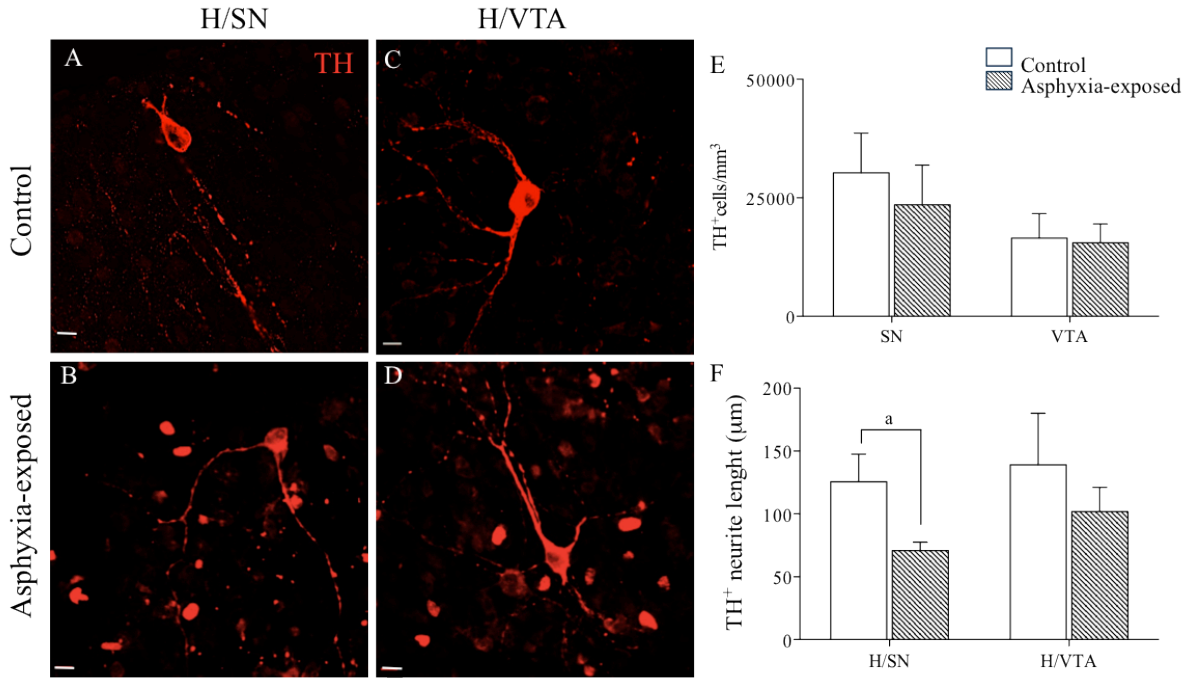

Figure 8: DA neurons in co-cultures. Microphotographs were obtained by confocal microscopy showing fluorescent proteins tagged to antibodies against TH (red) in SN (A, control; B, asphyxia-exposed cultures) and VTA (C, control; D, asphyxia-exposed cultures) (scale bars, 10µm). E: Quantification of number of TH^+ somas. F: Quantification of projection length. White columns, control cultures; hatched columns, asphyxia-exposed cultures. [a]control versus asphyxia-condition ($p<0.05$, Student's t-test).

***In vivo* and *in vitro* evaluation of cell proliferation, from control and asphyxia-exposed rats.**

In vivo: Double-labelled (MAP-2^+/$BrdU^+$) cells were observed in brain niches of cell proliferation (SVZ, DG) at P1 and adult (1 month) stages of control animals (data no shown).

In vitro: In cultures (mono and co-cultures) double IHC ($BrdU^+$/MAP-2^+) was performed at DIV 22-24. Cell proliferation ($BrdU^+$ cells/mm^3) was observed in mono and co-cultures of both control and asphyxia-exposed animals. The cells presented differences in size and in the shape of their nuclei (round, elongated, small and large nuclei), probably indicating different cell phenotypes (see Fig 9). No differences were observed among the experimental groups. However, quantitative analysis revealed that there was a significant increase of cell proliferation in hippocampus when co-cultivated with VTA ($p<0.05$), but not when cultivated with SN, but only in cultures from control rats (see Fig 9G). In H monocultures from asphyxia-exposed animals, there was a significant increase of cell proliferation compared to that observed in the controls ($p<0.05$). The quantitative analysis disclosed that in H monocultures the average of $BrdU^+$ was ~40.000 cells/mm^3 (n=6) in control, but ~140.000 cells/mm^3 (n=9) in tissue from asphyxia-exposed animals ($p<0.05$). No such increase was observed in H/SN co-cultures, cell proliferation being the same whether evaluated in cultures from asphyxia-exposed or control animals (see Fig 9G).

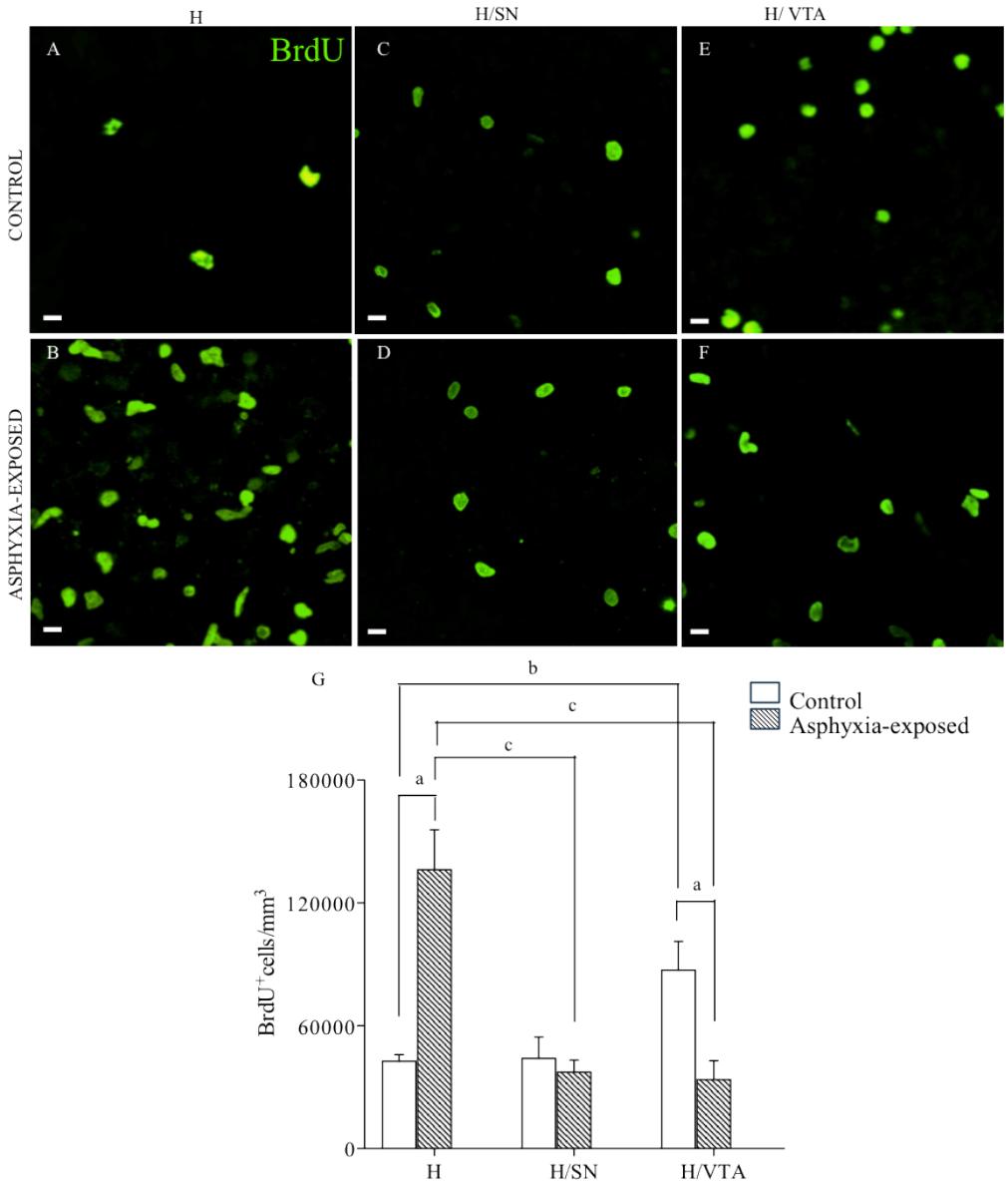

Figure 9: Hippocampus cell proliferation in mono and co-cultures. Microphotographs show $BrdU^+$ nuclei (green) in H monocultures (A, B) and co-cultures (C, D; H/SN. E, F; H/VTA) from control (A, C, E) and asphyxia-exposed (B, D, F) animals. Scale bar: 10µm. G: Quantification of $BrdU^+cells/mm^3$ in hippocampus of monocultures (control, n=6; asphyxia, n=9); H/SN (control, n=9; asphyxia-exposed, n=9), and H/VTA co-cultures (control, n=9; asphyxia-exposed, n=8). White columns, control; hatched columns, asphyxia-exposed samples. [a]control versus asphyxia-condition; [b]mono versus co-cultures from control animals; [c]mono versus co-cultures from asphyxia-exposed animals ($p<0.05$; Student ‘s t-test).

Evaluation of cell proliferation in H monocultures from control and asphyxia-exposed rats: Effect of apomorphine.

At DIV 20-22, one group of H monoculture was treated together with APO (1μM)+BrdU (10μM) for 48h. Another H monoculture group was treated with BrdU only, to be used as a control to the effect of the APO treatment. Representative microphotographs of H monocultures are shown in Fig 10. Quantitative analysis revealed that there was a significant increase in the number of $BrdU^+$ cells in APO-treated-H monocultures from control animals, compared to the non-treated condition. Under the basal condition, a significantly larger number of $BrdU^+$ cell/mm^3 was observed in H monocultures from asphyxia-exposed animals, which, however, was not further increased by APO treatment (see Fig 10E).

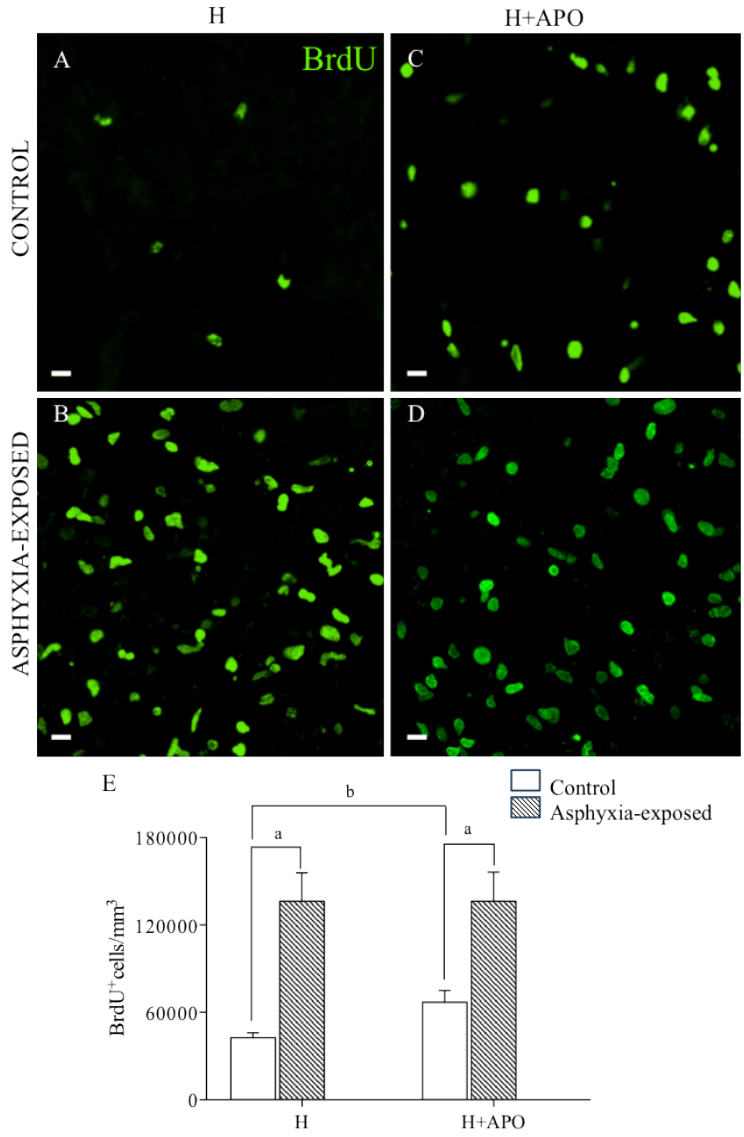

Figure 10: Apomorphine effect in hippocampus cell proliferation. Microphotographs were obtained by confocal microscopy showing fluorescent proteins tagged to antibodies against the mitotic marker BrdU (green) in H monocultures of control (A, n=6; C; n=6) and asphyxia-exposed (B, n=9; D; n=8) animals, following vehicle (A, B) or APO-treatment (C, D). Scale bar: 10µm. E: Quantification of $BrdU^{+}cells/mm^{3}$. White columns, control; hatched columns, asphyxia-exposed condition. [a]Control versus asphyxia-exposed condition; [b]Vehicle versus apomorphine treatment in cultures from control animals ($p<0.05$; Student's t-test).

DISCUSSION

In the present thesis, we investigated on the modulation of postnatal cell proliferation in hippocampus by mesencephalic dopaminergic systems in control and asphyxia-exposed rats using organotypic co-cultures. We found that: (i) DA neurons project to hippocampus, under *in vivo* and *in vitro* conditions. (ii) Postnatal cell proliferation occurred in hippocampus under *in vivo* (data no shown) and *in vitro* conditions, both in control and asphyxia-exposed animals. In hippocampus monocultures cell proliferation was increased in tissue from asphyxia-exposed animals when compared to the control condition. However, cell proliferation was only increased in hippocampus/VTA control cultures and (iii) it was further enhanced by the dopamine agonist APO, compared to the control condition

Perinatal asphyxia is a severe medical problem leading to long-term neurological sequelae whenever the babies survive the insult. Perinatal asphyxia can impair normal neurodevelopment, or even can be associated with neuropsychiatric deficits with a delayed onset as reported by Odd et al. (2009). The pathophysiology underlying these long-term consequences is not fully understood, and at present there is not any consensus about how to prevent these long-term consequences.

In the present study we have used an animal model that has provided to be very useful for investigating the issue (Bjelke et al. 1991, Andersson et al. 1992, Herrera-Marschitz et al. 1993), and for proposing clinically relevant therapeutic interventions (Herrera-Marschitz et al. 2011). Thus, severe perinatal asphyxia was induced in ready to be delivered pups and for preparing the organotypic cultures (see Klawitter et al. 2007), making possible to address the specific issue of postnatal cell proliferation and neurogenesis in hippocampus and its modulation by DA systems.

We restricted the study to the effects produced by severe asphyxia, characterised by an Apgar scale indicating ~50% of death, and delayed recovery, implying cardiovascular and respiratory deficits prolonging the metabolic deficit. Several laboratories have reported that there is a regional vulnerability to the insult, mainly affecting hippocampus (see Morales et al. 2003, 2005, 2007, 2008) and DA circuitries (see Klawitter et al. 2007), with delayed neuronal

death (Dell'Anna 1997), but also postnatal neurogenesis (Morales et al. 2008, 2010, see von Bohlen und Halbach 2011), mainly in DG of hippocampus.

We selected severe asphyxiated pups and the corresponding controls for preparing organotypic cultures at P1-3, focusing on hippocampus and mesencephalic tissue, which is known to contain DA neurons projecting to basal ganglia and mesolimbic regions, respectively (Ungerstedt 1971). Hippocampus was cultured alone, as a monoculture, or together with SN or VTA, as co-cultures for approximately one month. *In vitro* cell viability was assayed with a LIVE/DEAD kit, revealing that the cultures progressed equally well whether prepared as mono or co-culture, from asphyxia-exposed or control animals, at least in terms of cell viability.

The monoculture condition provided a unique opportunity to evaluate the effect of DA availability on markers affecting the neurocircuitries of hippocampus, mainly cell proliferation and postnatal neurogenesis (Morales et al. 2007, 2010), which can indicate a compensatory mechanism for the delayed-apoptotic-like cell death, also observed to occur in hippocampus following asphyxia (Morales et al. 2005).

It was found that cell proliferation evaluated by BrdU IHC was increased >2-fold in the H monoculture from asphyxia-exposed, as compared to that from control animals, as previously reported (Morales et al. 2008), where a similar increase was shown. A third of this proliferation corresponded to neurogenesis. This result suggests that the perinatal insult leads to cell proliferation, with both a neuronal and glial phenotype, as already reported by other authors (Keilhoff et al. 2010). In co-cultures with VTA, hippocampal cell proliferation was increased in the control, but not in the asphyxia condition. No changes were observed in H/SN co-cultures, either from control or asphyxia-exposed animals. This result supports the idea of the heterogeneity of mesencephalic DA systems, in term of co-localization, trophic factors and neuropeptides, as well as electrophysiological and behavioural properties (Roeper, 2013). Indeed, cholecystokinin (CCK) has only been observed in DA neurons of the VTA (see Gomez-Urquijo et al. 1999), perhaps modulating cell signalling for glial and/or neuronal progenitors. Furthermore, VTA, but not SN, has been reported to project to limbic regions, including nucleus accumbens, amygdala, hippocampus, and prefrontal cortex. Anatomical studies using retrogade and anterograde labelling have described dopaminergic terminals in hippocampus mainly originating from VTA, and the retrorubral fields (Verney et al. 1985,

Goldsmith and Joyce 1994 Gasbarri et al. 1997), and, as show here DA terminals have been observed in the *stratum oriens*, dorsal subiculum and DG (see Fig. 4).

In the present rat model of perinatal asphyxia, it has been demonstrated a regional specific delayed cell death in hippocampus, together with an increase of pro- and anti-apoptotic proteins (Morales et al. 2005, 2007, 2008, Neira et al. 2013). Several compensatory mechanisms have been suggested to be triggered for protecting from delayed cell death, including neurogenesis (Yagita et al. 2001, Kee et al. 2001, Jin et al. 2001, Nakatomi et al. 2002, Daval et al. 2004, Bartley et al. 2005) and neuritogenesis (Biernaskie and Corbett 2001, Morales et al. 2010).

The D1/D2 DA agonist APO was found to induce cell proliferation in H monocultures from both control and asphyxia-exposed animals, including in cultures from asphyxia-exposed animals a significant increase of neurogenesis in DG, but not that in cultures from control animals (see Espina-Marchant, 2012). This is an exciting observation, perhaps promoting to a strategic therapeutic intervention, as supported by the clinical observation that the first choice for treating the attention deficit syndrome is the use of the indirect DA agonist methylphenidate (see Allende-Castro et al. 2012). APO has been shown to have a dose-dependent effect on hippocampal cell proliferation under *in vitro* and *in vivo* conditions (see Hiramoto et al. 2007, Mori et al. 2008).

The co-culture model allowed to study the effect of perinatal asphyxia on DA neurotransmission, as reported by Klawitter et al. (2007), using the same experimental model, but focusing on basal ganglia neurocircuitries. Indeed, the present study confirms the previous observation that there is a decrease in the number of DA cells, branching and length of DA neurites affecting the SN, but not the VTA of asphyxia-exposed animals (Klawitter et al. 2007).

The differential effect of perinatal asphyxia on the number of DA neurons in SN and VTA, can be explained by neurodevelopmental features. Indeed, at delivery the brain of rats has the same number of DA cell bodies as in adulthood, and DA fibres start to invade the neostriatum before birth (Seiger and Olson 1973). The nigrostriatal DA system has an earlier maturity than the mesolimbic systems, and the mesocortical system achieves maturity at pre-postpubertal periods. Thus, a metabolic insult occurring during the perinatal period will affect normal neurocircuitries formation.

In the present study, it was observed that TH positive projections penetrated into the hippocampus, covering a wide area of the parenchyma, in both, H/SN and H/VTA and in control and asphyxia-exposed conditions. It was further observed a significant decrease in the length of TH positive projection in H/SN co-cultures, but not in H/VTA co-cultures of asphyxia-exposed animals, supporting the idea that DA neurons from SN are more vulnerable to metabolic insults occurring at birth (see Herrera-Marschitz et al. 2011).

It has been reported that DA neurites contact the progenitor cells in the subgranular zone of DG (Höglinger et al. 2004), forming funtional synapses (Winter et al. 1993, Backer et al. 2004, Lisman and Greace 2005, Höglinger et al. 2008, Platel et al. 2010). It has been proposed that DA modulates the proliferation of neural precursor cells in subgranular zone of DG (Höglinger et al. 2004, Domínguez-Escribá et al. 2006, Park and Enikolopov 2010). In agreement, the number of neuronal precursor cells is reduced in neurogenic regions of individuals with Parkinson's disease, a disorder characterized by DA depletion resulting from degeneration of DA neurons from SN (Höglinger et al. 2004). Despite emerging studies revealing the role of DA in the early phases of adult neurogenesis, DA regulation of functional integration of newborn neurons has yet to be determined.

This study opens a field of research on the role of DA systems in the modulation of postnatal neurogenesis taking place in hippocampus.

The conclusions of this thesis are: (i) Postnatal cell proliferation does occur; (ii) DA neurotransmission modulates postnatal cell proliferation, and finally, (iii) perinatal asphyxia interferes with the modulation of dopamine of postnatal cell proliferation

Learning and memory processes are cornerstones for neuronal plasticity and for the rehabilitation process, probably involving hippocampal neurocircuitries, which is a target for pharmacological treatment implying DA agonist. Indeed, methylphenidate (Ritalin®) is the only pharmacological treatment for attention deficits, while DA antagonists are widely used for more severe cases of behavioural disorders (Robertson and Pearlman 2006). Most children attending to neurorehabilitation centres are prescribed with one or several of these drugs, without consideration to their mechanism of actions, but affecting learning and children behaviour, and also inducing various side effects. It is my hope that the present study will contribute to the treatment and well being of my dear patients.

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