UNIVERSITAT DE BARCELONA

Brain effects of fetal growth restriction and their prevention in an animal model

Míriam Illa Armengol

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DOCTORAL THESIS

Programa de Doctorat en Medicina

Universitat de Barcelona

Brain effects of fetal growth restriction and their prevention in an animal model

Míriam ILLA ARMENGOL

supervised by

Eduard GRATACÓS SOLSONA

Francesc FIGUERAS RETUERTA

Fetal and Perinatal Medicine Research Group

BCNatal – IDIBAPS – University of Barcelona

May 2017

- Universitat de Barcelona
- Divisió de Ciències de la Salud
- Facultat de Medicina
- Departament d'Obstetrícia i Ginecologia, Pediatria, Radiologia i Medicina Física.
- Programa de Doctorat de Medicina RD 778/1998

The thesis entitled "Brain effects of fetal growth restriction and their prevention in an animal model" is submitted by Míriam Illa Armengol for the Ph.D. degree of Doctor in Medicine of the University of Barcelona, including the mention of "International Doctor" under the direction of Eduard Gratacós Solsona, Professor of Obstetrics and Gynecology at Barcelona University, and Francesc Figueras Retuerta, Associate Professor of Obstetrics and Gynecology at Barcelona University.

The co-directors declare that Míriam Illa Armengol has conducted under their supervision the studies presented in this thesis at the Fetal Medicine Research Center, BCNatal - Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Déu), Institut Clínic de Ginecologia, Obstetrícia i Neonatologia, in the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), in the Fundació Clínic of Barcelona and in the Institut de Recerca Fundació Sant Joan de Déu. The Thesis has been structured following the normative for Ph.D. theses as a compendium of publications for the degree of Doctor in Medicine. The studies included in the thesis belong to the same research line leading to three papers already

published or submitted for publication in international peer-reviewed journals. The mentioned studies are ready to be presented to the Tribunal:

1. Miriam Illa, Elisenda Eixarch, Emma Muñoz-Moreno, Dafnis Batalle, Rocío Leal-Campanario, Agnès Gruart, José María Delgado-García, Francesc Figueras, Eduard Gratacós. **Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models.** Fetal Diagnosis and Therapy. 2017 Jan 5

State: published

Impact factor 2015/2016: 2.7; Quartile: 1st

2. Miriam Illa, Elisenda Eixarch, Dafnis Batalle, Ariadna Arbat-Plana, Emma Muñoz-Moreno, Francesc Figueras, Eduard Gratacos. **Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction.** PLoS ONE. 2013 Oct 15;8(10):e76453

State: published

Impact factor 2015/2016: 4.4; Quartile: 1st

3.- Miriam Illa, Verónica Brito, Laura Pla, Elisenda Eixarch, Ariadna Arbat-Plana, Dafnis Batalle, Emma Muñoz-Moreno, Fatima Crispi, Ester Udina, Francesc Figueras, Sílvia Ginés, Eduard Gratacós. **Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction.** Fetal Diagnosis and Therapy.

State: submitted, under review

Impact factor: 2.7; Quartile: 1st

The co-directors confirm that Míriam Illa Armengol substantially contributed to all the studies presented. She participated in the study design, underwent animal testing, pre- and post-processed the data, analyzed the results, wrote the papers and revised the final version of all the presented papers. The co-directors also confirm that none of the co-authors have used, or is going to use any of the articles here presented in another Ph.D. Thesis.

Barcelona, May 2017

Prof.Eduard Gratacós Solsona Dr.Francesc Figueras Retuerta

Míriam Illa Armengol

STRUCTURE OF THE THESIS

This Ph.D. Thesis is based on three projects aiming to characterize, in the setting of intrauterine growth restriction, the degree of neurodevelopmental problems depending on the severity of the prenatal insult, characterize the structural brain changes underlying long-term neurobehavioral and cognitive impairments and evaluate the neuroprotective effects of the environmental enrichment strategy. Each project has led to a publication accepted, or considered to be published, in international peer-reviewed journals. Two articles have been already published in first quartile international journals and one has already been submitted also in an international peer-reviewed journal.

Project 1: Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models

2017, Fetal Diagnosis and Therapy

Project 2: Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction

2013, PLoS ONE

Project 3: Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction

2017, Fetal Diagnosis and Therapy submitted

The presentation of the Thesis is structured with a general introduction followed by a general overview of the methodology used in each project and a summary of the global methodology used for the three projects is also given prior to the presentation of the results. After that, specific conclusions from each project will be given including a general discussion as well. Then, a summary of the thesis in Catalan is given. As additional documents, the ethics committee documents and a copy of the already published papers or the submitted work are also given at the end.

“the genius is composed of two percent talent

and ninety-eight percent of persevering application”

Ludwing van Beethoven

TABLE OF CONTENTS

ABBREVIATIONS

ADHD: attention deficit hyperactive disorder

CNS: central nervous system

DI: discriminatory index

DTI: diffusion tensor imaging

DS: dendritic spine

DWI: diffusion-weighted images

EE: environmental enrichment

FA: fractional anisotropy

FGR: fetal growth restriction

GM: gray matter

IUGR: intrauterine growth restriction

L: left

MDEFT: modified driven equilibrium Fourier transform

MRI: magnetic resonance imaging

OFBT: open field behavioral test

ORT: object recognition task

PBS: phosphate-buffered saline

PFA: paraformaldehyde

PNNs: perineural nets

PU: placental insufficiency

R: right

RARE: rapid acquisition with relaxation enhancement

t-IUGR: treated intrauterine growth restriction

UN: undernutrition

VBA: voxel-based analysis

WFA: Wisteria Floribunda histochemistry

WM: white matter

1. INTRODUCTION

1.1. Neurodevelopmental problems of prenatal origin

Neurological disorders are one of the most serious perinatal complications affecting approximately 3/1000 newborns (Anon 2001). It is currently considered that even in more than 70% of cases the damage already existed before the onset of labor (Jacobsson & Hagberg 2004). Brain damage of perinatal origin includes a broad spectrum of entities. While severe cases are clinically manifested as hypoxic-ischemic encephalopathy, intraventricular hemorrhage, periventricular leukomalacia and cerebral palsy, the most prevalent abnormalities derived from brain damage of prenatal origin manifests as subtle neurological abnormalities. Although the paucity of apparent brain abnormalities underlying these milder cases, a broad spectrum of subtle neurological abnormalities is still detected during the follow-up of that babies, including neurobehavioral and cognitive disorders.

Many factors have been described to affect the developing brain contributing to a broad spectrum of functional and behavioral disorders that manifest throughout life (Mwaniki et al. 2012). One of the most reported causes of neurodevelopmental impairment is prematurity (delivery before 37 weeks of gestation) (Blencowe et al. 2013). The risk is even higher in those preterm newborns who have also suffered from prenatal insults during fetal life (Leviton et al. 2013). Chronic hypoxia due to placental insufficiency and prenatal undernutrition are probably the two major causes worldwide of an adverse intrauterine environment having an impact on neurodevelopment. Clinically, both situations manifest as an intrauterine growth restriction (IUGR) (Borowicz & Reynolds 2010; Baschat & Hecher 2004), a situation defined as a significant reduction in fetal growth resulting in a birth weight below the 10th percentile.

This condition has been estimated to affect 7-10% of all pregnancies (Kady & Gardosi 2004) and around 50% of them have been estimated to develop some degree of neurological damage (Fouron et al. 2001). Although this risk is higher in those IUGR neonates being born prematurely, this association even persists in IUGR newborns born at term (Bassan et al. 2011). The association between IUGR and short- term neurodevelopmental dysfunctions has been extensively described. In the neonatal period, neurodevelopmental dysfunctions have been reported, being attention, habituation, regulation of the state, motor and social interactive competencies the most affected (Figueras et al. 2009; Feldman & Eidelman 2006). Long-term follow-up studies have reported that even milder forms of fetal impaired growth, have an increased risk of neurobehavioral and cognitive disabilities during childhood and adolescent periods. Several reports have linked IUGR with reduced cognitive skills (Leitner et al. 2007; Morsing et al. 2011), impaired memory, learning difficulties (Rodrigues et al. 2006; Tideman et al. 2007), poor academic performance (Synnes et al. 2010; Larroque et al. 2001), inattention (Geva et al. 2006a; MJ et al. 2003) and reduced psychosocial function (Geva et al. 2006b; Alati et al. 2009; Lund et al. 2012) and also an increased risk of attention-deficit hyperactivity disorder (ADHD)(Heinonen et al. 2010). Moreover, most of these studies also have shown that severity of these dysfunctions in children and adulthood depends on the severity of IUGR and clinical expression of that disabilities (behavioral and cognitive difficulties) seem to be even more evident over time. These functional impairments seem to be secondary to a disruption of specific basic brain processes compromising short-term memory, attention, and anxiety, but its neuroanatomical correlations have not yet been fully characterized.

1.2. Pathophysiological mechanisms of brain damage

As previously mentioned chronic hypoxia and undernutrition are the most relevant clinical situations worldwide related to adverse neurodevelopment in-utero (Baschat & Hecher 2004; Borowicz & Reynolds 2010). Both situations alter normal intrauterine environment inducing a reduction of oxygen and/or nutrients. Whereas placental insufficiency causes an impaired uteroplacental blood flow decreasing the supply of both oxygen and nutrients to the fetus (Meschia G: Placenta respiratory gas exchange and fetal oxygenation, in "Robert Creasy, Robert Resnik, Jay Iams, Charles Lockwood 2009"), undernutrition mostly affect nutrient supply to the fetus (Borowicz & Reynolds 2010). However, the specific mechanism underlying neurodevelopmental problems related to placental insufficiency and undernutrition are not fully described.

In order to elucidate the mechanism and the specific structural brain changes underlying prenatal insults, it is important to bear in mind that prenatal insults are interfering upon an organ that it is under development. In this regard, human brain development is a complex and dynamic process that starts during early gestation and continues after birth. The complexity is based on the integration of different processes, including proliferation, differentiation, migration and organization of neurons, followed by the formation and maturation of synaptic contacts, myelination and finally establishment of neural networks and connectivity that is crucial for correct central nervous system (CNS) (Stiles & Jernigan 2010; Bourgeois 1997). Synapse formation is also a complex process itself requiring presynaptic and postsynaptic element coupling and adequate branching to achieve proper localization and function of a synapse

(Scheiffele 2003). Axons terminate in synapses that mediate the transfer and the storage of information. The synapse thus represents the central functional element of the nervous system. Depending on the moment of presentation and the severity of the insults, different neurodevelopmental events would be affected sculpting the complex neural substrates that ultimately would define brain function in later life.

Classically, systemic fetal hypoxia is widely considered to be a critical factor which affects adversely the fetal brain (Pasternak & Gorey 1998). Basic research has given important information regarding the structural brain changes underlying fetal hypoxia pointing out how the consequences could be different depending on the onset, severity, and extent of the hypoxic insult itself. Animal models of acute hypoxia in early pregnancy have been related to a reduction in neuronal numbers, mostly affecting the Purkinje cells in the cerebellum, pyramidal cells in the hippocampus and cortical neurons. White matter (WM) damage also resulted in diffuse injury and cystic lesions in the periventricular area (Rees et al. 1999). Late in pregnancy, these pattern of alterations persisted in acute hypoxia, although less marked compared with early presentation of the prenatal insults. This appeared to be due to the differential vulnerability of neurons and especially immature oligodendrocytes to hypoxemia at different moments of development (Loeliger et al. 2003).

On the contrary, mild chronic insults, those occurring in placental insufficiency, resulted in different structural effects. Chronic hypoxia during fetal life results in a delayed axonal myelination in the CNS (Nitsos & Rees 1990), that seems to be restored at the long-term period (Tolcos et al. 2011). Indeed, even after postnatal restoration of myelination abnormalities, behavioral deficits

seemed to persist at the long-term period (Reid et al. 2012). All this evidence suggested that in spite of myelination reaches normal levels postnatally, early transient myelination defects might affect the normal functional development of the CNS and may also induce subtle structural changes that last up to long-term period. On the other side, it is increasingly recognized that gray matter (GM) is also injured in the context of mild chronic insults. Neurons seem to survive to chronic and mild intrauterine compromises, although some populations may still be affected (Rees et al. 2011). Hippocampal and cerebral cortex neurons seemed to be specially vulnerable to IUGR with a reduction in number of cells in both areas (Liu et al. 2011; Tashima et al. 2001). Apart from that, recent evidence is now suggesting that one of the major mechanism of GM damage due to chronic insults would be mediated by an alteration at the level of neuronal connectivity. Indeed, changes in dendritic spine (DS) density and morphology along with changes in synaptic receptors have been described after chronic placental insufficiency in the surviving neurons at the neonatal period in a guinea pig model (Dieni & Rees 2003; Piorkowska et al. 2014). Unfortunately, changes in synapsis and neuronal connectivity have not been explored at the long-term period and whether it is restored as WM or not is still unknown.

Intrauterine nutrient restriction alone (undernutrition) was described to play an important role in brain damage, particularly when it persists over time. Studies that evaluated the role of malnutrition or nutrient imbalance in the prenatal period have observed mildly impaired neurodevelopmental problems involving anxiety, memory and learning difficulties (Reyes-castro et al. 2012; Akitake et al. 2015; Keenan et al. 2013). However, few studies assessed the structural effects on the developing brain due to maternal undernutrition. These

studies observed structural changes mostly affecting neurons from the limbic system (Morgane et al., 2002).

To sum up, it is still unclear which is the pathophysiological mechanism and the long-term brain consequences of chronic hypoxia and undernutrition, both occurring in IUGR. A deeper understanding of that would allow us to test potential neuroprotective therapies aiming to mitigate the related long-term neurobehavioral and cognitive disabilities. For all these reasons, Project 1 aimed to add new insights into the pathophysiological mechanism underlying brain damage describing long-term neurodevelopmental consequences in animal models of placental insufficiency and undernutrition.

1.3. Advanced structural evaluation of brain development

Consequences of mild and chronic prenatal hypoxia and undernutrition have been associated with disruption of the normal brain neurodevelopment rather than gross tissue destruction (Rees et al. 2011). Therefore, our ability to characterize the structural brain changes underlying chronic and mild prenatal insults would depend on the use of specific techniques capable of identifying these subtle brain changes.

Whereas histology techniques have several serious drawbacks, including invasiveness, labor-intensive and could not examine the entire brain, brain imaging techniques are noninvasive, provides high-resolution three-dimensional structural evaluation, and requires less time to characterize the entire brain anatomy (Mori & Zhang 2006; Lodygensky et al. 2010). Of the available brain imaging techniques, magnetic resonance imaging (MRI) have been established as a promising tool for the evaluation of the human brain development in normal and even in pathological situations. As a first step, conventional MRI gave initially evidence of the structural brain changes underlying IUGR. During fetal and neonatal period, conventional MRI evidenced a decreased volume in cortical GM (Tolsa et al. 2004), in the hippocampus (Lodygensky et al. 2008) and differences in cortical development (Dubois et al. 2008), persisting that changes even beyond the childhood period (de Bie et al. 2011; Martinussen et al. 2005; Martinussen et al. 2009). However, with the significant advance of MRI in the recent years, the structural brain changes underlying the neurobehavioral and cognitive delays due to IUGR are now being elucidated in a higher detail. Overall, high-resolution MRI gives us important information regarding microstructural changes affecting the WM and GM and also information

regarding brain connectivity by the assessment of the brain networks (Lodygensky et al. 2010). Diffusion MRI (dMRI) is a noninvasive approach based on the measurement of the diffusion of water molecules in tissues (Basser & Pierpaoli 1996), which provides indirect information about brain microstructure. This technique has been used to assess brain reorganization in response to brain injury in both developing and adult brain (Neil et al. 2002; Nucifora et al. 2007). In addition, it has also been used in animal models demonstrating changes in diffusivity parameters in neonatal period after acute hypoxia (Derrick et al. 2007; Drobyshevsky et al. 2007a; Drobyshevsky et al. 2007b), but also after chronic hypoxia in an IUGR rabbit model (Eixarch et al. 2012). Aside from water diffusion parameters, dMRI allows us to non-invasively reconstruct WM tracks applying diffusion tensor technique (DTI). DTI allows us to evaluate important information regarding the number, structure and the organization of WM tracks among brain regions regulating specific brain functions (Nucifora et al. 2007). Finally, using anatomical and dMRI acquisitions, it is also possible to obtain and study the structural brain networks, a technique known as “connectomics” (Bullmore & Sporns 2009). With this regard, network features have been introduced as a useful tool to identify brain organization and elucidate its level of complexity in prenatal conditions such as IUGR (Batalle et al. 2012). Thus, with the significant advance of MRI techniques are now giving us evidence that neuronal microstructural changes (Lodygensky et al. 2010; Sizonenko et al. 2007) along with disrupted brain network organization may play an important role in the setting of IUGR, at least during first years after birth. In this regard, altered brain network architecture has been suggested to underpin the wide variety of neurobehavioral disabilities described

to last beyond infancy after IUGR (Fischi-Gómez et al. 2014; Batalle et al. 2012; Batalle et al. 2014; Muñoz-Moreno et al. 2016). Project 2 and 3 from this thesis used high-resolution brain imaging techniques in order to characterize the neuroanatomical correspondence of neurobehavior and cognitive impairments at the long-term period in an animal model of IUGR.

Understanding the histological substrate of brain injury seen on MRI would provide further insights into the mechanisms of injury during brain development. Standard histological assessment has not been able to show the long-term structural brain changes underlying brain injury of IUGR. However, more specific techniques focused on neuronal connectivity could better reflect the structural changes in IUGR. Actually, changes in neuronal connectivity and synapsis, including changes in the axonal and dendrite development, have been suggested to be the histological basis of brain changes assessed by DTI studies (van den Heuvel et al. 2016; Dean et al. 2013). Therefore, in the context of IUGR, long-term structural changes at the cellular level might be assessed by evaluating key markers involved in neuronal connectivity and synapsis. Synapse formation is complex and involves successful completion of many processes, including neurogenesis, axon and dendrite migration, arborization, and pre- and postsynaptic element coupling. An interruption in any of these steps could lead to aberrant neuronal communication. Connectivity markers that have been used include the evaluation of dendritic morphology, dendritic spine (DS) density and evaluation of pre and postsynaptic receptors and proteins involved in the synaptic transmission. Several cognitive disorders such as ADHD, autism, intellectual disability, and fragile X syndrome have been related to abnormalities in dendritic spines, especially the number of spines and their

maturity (Penzes et al. 2011). Likewise, changes in DS density have been described at the neonatal period in a guinea pig and sheep models after chronic and acute prenatal insults (Piorkowska et al. 2014; Dieni & Rees 2003; McClendon et al. 2014; Dean et al. 2013).

Apart from that markers, perineural nets (PNNs), a specialized extracellular matrix component that enwraps neurons in the CNS, have been described to play an important role in formation, maintenance, and function of synapses (Wang & Fawcett 2012). Therefore, similarly to DS, PNNs have been used as a marker of synaptic connectivity within neurons (Dzyubenko et al. 2016). Recently, alterations in PNNs have been described in specific brain diseases such as Alzheimer, schizophrenia, and epilepsy (Dzyubenko et al. 2016; Cabungcal et al. 2013). Although the recent interest in that specific histological marker for the assessment of brain disorders, the pattern of alterations in the PNNs related to IUGR has not been yet evaluated. Considering that hippocampus has a well-known role in memory formation and cognition in animals and humans (Eichenbaum 2004; Nakashiba et al. 2009; Deng et al. 2010) and seems to be especially vulnerable to IUGR condition (Mallard et al. 1999; Mallard et al. 2000; Lodygensky et al. 2008). In Project 3 we aimed to investigate alterations in the development of the hippocampus including neuronal connectivity and their correlation with neurological sequelae associated with IUGR.

1.4. Environmental enrichment strategy in IUGR

IUGR might have long lasting consequences, and currently, breastfeeding has been demonstrated to be one of the more effective strategies to partially ameliorate the long-term neurodevelopmental sequelae of IUGR (Rao et al. 2002). Apart from the breastfeeding, some other therapies are now arising as promising strategies to overcome brain diseases, such as environmental enrichment strategy. Environmental enrichment (EE) strategy has consistently been demonstrated to exerts beneficial effects on the CNS by improving complex cognitive functions (Rampon et al. 2000) and animal's emotional and stress reactivity (Chapillon et al. 2002; Fares et al. 2013; Rosenzweig 1996). This functional improvement has been accompanied by changes in neuronal connectivity including increased dendritic arborization, the number of DS, synaptic density and postsynaptic thickening, particularly in the hippocampus (Fares et al. 2013; Rampon et al. 2000; Rampon & Tsien 2000).

Environmental enrichment strategy is based on stimulation of the brain by its physical and social surroundings, at the level of sensory, motor, cognitive and social areas. There is a strong research tradition in developmental psychobiology to support the notion that "enriching" the postnatal environment can accelerate development and facilitate recovery of function after early brain damage (Rosenzweig 1996; Johnston 2009), including focal stroke (Janssen et al. 2010). In the context of human brain development, a large number of studies have also demonstrated the existence of time windows in early postnatal life during which neural circuits display a heightened sensitivity from the external environment inputs (Johnston 2009; Meaney & Aitken 1985). As the immature brain is susceptible to environmental stimuli, EE has been proposed as a

potential strategy applied during first years of infancy period. Recently, early stimulation based on playing and reading has been postulated as a reliable strategy to improve neurobehavioral childhood disabilities especially in low-income countries and in children at risk of developing secondary impairments (Maulik & Darmstadt 2009). In the context of IUGR, a NIDCAP strategy (a strategy based on giving physical and emotional support to the premature infant during Neonatal intensive care unit admission) has also demonstrated to induce neurobehavioral and structural improvement in severe IUGR preterm infants (Als et al. 2012). No structural and functional effects of environmental enrichment strategy at the long-term period in the context of IUGR born at term have been evaluated. Going in this line, in Project 3 we sought to evaluate the effects at functional and structural level of environmental enrichment strategy at long-term period in the context of IUGR in an animal model of placental insufficiency.

Finally, although this strategy has been well defined and characterized in animal research, the evidence of the real extent to which EE in animal models is relevant for humans remains to be evaluated. In this regard, epidemiological evidence shows that lifestyle, including occupation, leisure activities, and physical exercise, has a direct effect on the risk of cognitive decline. Indeed, higher level and variety of mental and physical activity is associated with a lower cognitive decline and a reduced risk for dementia (Kramer et al. 2006; Kramer & Erickson 2007).

1.5. Animal models of IUGR

Notwithstanding their obvious limitations, animal models are still required in order to identify the causative mechanisms underlying brain damage of prenatal origin and to test, in a first step, potential neuroprotective therapies. The major limitation of animal research is that reproducing the features of the human condition to be studied is challenging and could limit the potential transferability of the results obtained to the human. To model prenatal human brain damage some considerations should be taken into consideration regarding the animal species and the methodology used.

Regarding animal species, intrinsic physiological characteristics in placentation and brain maturation need to be considered for each species in comparison to humans. Firstly, the human placenta is hemochorial, meaning that maternal blood contacts directly with the chorion. Secondly, human brain maturation is characterized to start during mid-gestation and continue during the first years of life, that is perinatal brain maturation (Ballesteros et al. 1993). Ideally, the selected animal species needs to present a similar placentation and a similar proportion of brain development should occur in utero. Apart from that, the insult should be delivered in utero at an equivalent stage of development identified to be vulnerable in humans. Rabbit species fulfills most of these criteria. First, the rabbit has a discoid, villous, and hemochorial placenta (Carter 2007) and have a perinatal brain development initiating WM myelination during fetal life (van Marthens et al. 1975). This contrast with sheep that have an epitheliochorial placenta (Carter 2007) and have a prenatal brain development, presenting most of the white-matter tracts myelinated at the time of birth (Carter 2007). Other animals that have been extensively used as a model for placental

insufficiency (Schröder 2003; Vuguin 2007) and brain damage (Rees & Inder 2005) are rodents. Unlike rabbit, rats have a postnatal brain development, starting the myelination postnatally (Finlay 2008), so the paucity of white matter during the prenatal period does not allow replicating human neurological lesions of prenatal origin (Rees & Inder 2005). Other advantages of rabbits in comparison to rodents is that rabbit present a relatively long gestation, allowing for intrauterine manipulations at different developmental stages and also fetus size that is bigger which makes easier to monitor fetal effects of the intrauterine insults (Eixarch et al. 2011). All these evidence suggested us that rabbit could be a suitable animal to explore brain damage of prenatal origin and was selected for being used in the three projects of this thesis.

Another issue to consider in basic research regarding brain damage is the methodology used in reproducing the prenatal insult (IUGR). The most used methods in reproducing IUGR so far have been based either on maternal food restriction or surgical reduction of placenta blood supply (Schröder 2003; Vuguin 2007). Nutrient manipulation during pregnancy has been an established model of growth restriction in animals. Different strategies have been used such as global nutrient restriction, isocaloric low-protein diet, low-iron deficiency and overnutrition at different points during pregnancy (Vuguin 2007). Depending on the onset type and length of the nutritional manipulation different effects have been described. Overall, most of these strategies induce birth weight reduction with no effect on fetal mortality (Eixarch et al. 2011; Vuguin 2007), probably due to the absence of fetal oxygen reduction. On the contrary, surgical methods based on the selective ligature of the uteroplacental vessels that irrigate the placenta (Bassan et al. 2000; Bassan et al. 2009; Eixarch et al. 2009) induce a

restriction of nutrients and oxygen supply and demonstrated to reproduce major features of human placenta insufficiency in terms of IUGR induction, fetal mortality, cardiovascular Doppler changes and neurobehavioral impairments during the neonatal period (Eixarch et al. 2011; Eixarch et al. 2012). Moreover, by using this model different degrees of growth restriction could be achieved by modulating the timing and the proportion of vessels ligated (Eixarch et al. 2009), which contrast with methods based on uterine artery ligation or uteroplacental embolization that results in non-predictable reductions of blood supply (Lang et al. 2003).

In this thesis, we induced IUGR following two different schemes: maternal food restriction aiming to reproduce undernutrition and uteroplacental vessels ligation as a placental insufficiency condition. Both models were used in the Project 1 and uteroplacental vessel ligation model (placental insufficiency) was used in Project 2 and Project 3.

2. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Main hypothesis

Intrauterine growth restriction produces subtle structural brain changes that underlie the long-term neurobehavioral and cognitive impairments. These neurodevelopmental consequences could be ameliorated applying a strategy based on environmental enrichment during the early postnatal period.

Specific hypothesis

1. The severity of functional impairments on anxiety, short-term memory and learning at long-term is related to severity of IUGR as demonstrated in two animal models based on placental insufficiency and maternal food restriction.
2. The degree of changes in brain connectivity assessed by diffusion MRI underlying functional impairments at long-term period is related to severity of IUGR as demonstrated in two animal models based on placental insufficiency and maternal food restriction.
3. Altered brain microstructure in specific brain areas and a reduced global and specific brain networks assessed by diffusion MRI correlates with functional impairments on anxiety, short-term memory and learning in an animal model of IUGR based on placental insufficiency.
4. At the cellular level, changes in dendritic spine and perineural nets density in the hippocampus underlies functional impairments on anxiety, short-term memory and learning in an animal model of IUGR based on placental insufficiency.

5. Early environmental enrichment strategy ameliorates structural and functional impairments that persist at the long-term period in an animal of IUGR based on placental insufficiency.

OBJECTIVES

Main objective

To characterize structural brain changes underlying long-term neurobehavioral and cognitive impairments in an animal model of intrauterine growth restriction and to evaluate the neuroprotective effects of a postnatal strategy based on an environmental enrichment strategy

Specific objectives

1. To compare functional impairments at long-term on anxiety, short-term memory and learning in two animal models of IUGR based on placental insufficiency and maternal food restriction
2. To describe the brain connectivity changes underlying functional impairments at long-term period in two animal models of IUGR (placental insufficiency and maternal food restriction) by using diffusion MRI
3. To explore the correlation between diffusion and network parameters obtained by means of diffusion MRI and functional impairments on anxiety, short-term memory and learning in an animal model of IUGR based on placental insufficiency
4. To explore structural changes at cellular level by evaluating dendritic spine and perineural nets density in the hippocampus and to evaluate whether these changes underlie functional impairments on anxiety, short-term memory and learning in an animal model of IUGR based on placental insufficiency
5. To evaluate structural and functional improvements at long-term period after early environmental enrichment strategy in an animal of IUGR based on placental insufficiency

3. METHODS

In order to achieve the main and specific objectives, the three different projects were planned and performed as explained below. All projects were performed by using animal models. Animal handling and all the procedures were performed following all applicable regulations and guidelines of the Animal Experimental Ethics Committee of the University of Barcelona, and all efforts were made to minimize suffering. Previous to animal manipulation and experimentation, an approval expedited by the Animal Experimental Ethics Committee of the University of Barcelona was obtained for each experiment. For more information, see Appendix I, where ethics committee documents have been included.

3.1. Project 1: Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models

Study design: Controlled laboratory study

Study population:

- Two cohorts of pregnant New-Zealand rabbits were included in two different IUGR induction protocols:

    a. A cohort of pregnant rabbits was included in the surgical placental underperfusion model (PU) induced at 25 days of gestation

    b. The other cohort was included in the undernutrition protocol (UN) starting at the 22 days of gestation

- From both models, four different groups were obtained:

    a. Growth restricted rabbits obtained from the PU

    b. Growth restricted rabbits obtained from the UN

    c. Control rabbits obtained from PU

    d. Control rabbits obtained from UN

Interventions:

- Prenatal induction of IUGR at 22 or 25 days of gestation following the two IURG protocols: PU or UN
- Cesarean section at 30 days of gestation in the PU model or vaginal delivery at 31 days of gestation in the UN model
- Neurobehavioral evaluation at first postnatal day (+1P) and at long-term period (+60-70th postnatal days)
- Sacrifice and samples collected at the 70th postnatal days

• Magnetic resonance imaging (MRI) acquisition, diffusion MRI and connectome analysis of excised and fixed brains at the 70th postnatal days

The methodology of the study and flow chart are summarized in Figure 1.

Measures:

a. Survival and growth parameters

b. Functional data:

i. At the neonatal period: general motor skills, reflexes, and olfactory sensitivity

ii. At the long-term period:

- From the Open Field Behavioral Test (OFBT): latency of time of leaving the starting familiar point and the time spent in the internal area (seconds)

- From Object Recognition Task (ORT): Discriminatory Index (DI)

- From Skinner test: percentage of learning (%)

c. Brain network analysis:

i. Global features: Average strength, global efficiency, and local efficiency

ii. Correlation between global features and functional evaluation at long-term period

Outcome variables: Fetal survival, neonatal weight, neurobehavioral evaluation scores during neonatal period, latency of time in leaving the familiar starting point, time in the internal area, DI, percentage of learning, average

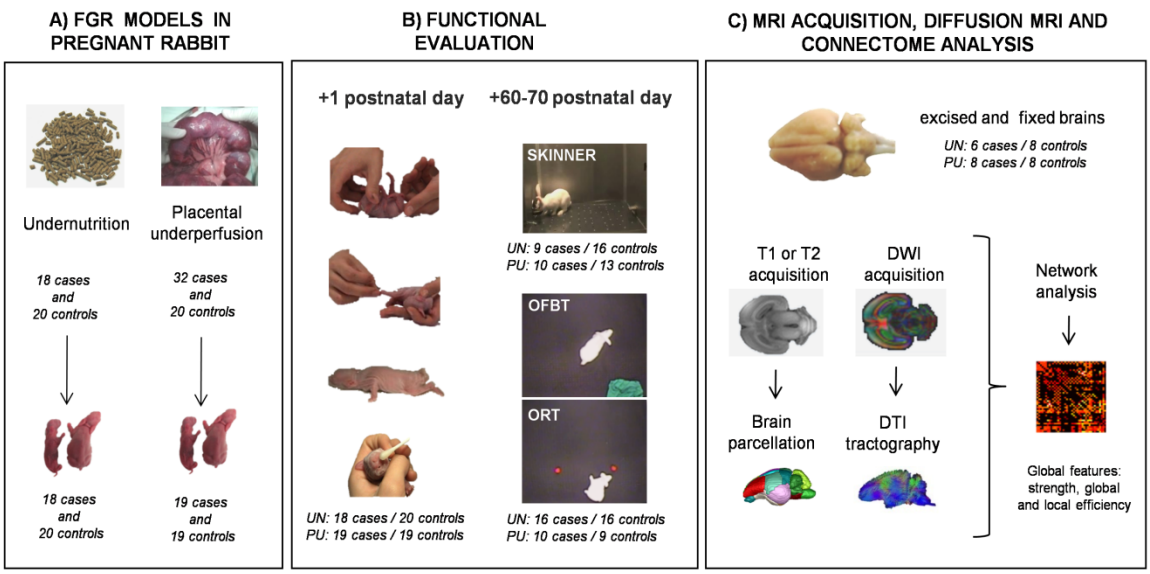


Figure 1: Study design, methods and flow chart of the animals included in the first project

A) Illustrative images and scheme of fetal growth restriction (FGR) induction models and number of animals derived from both models.
B) Illustrative images of neurobehavioral tests applied and number of animals evaluated.
C) Illustrative images of MRI acquisition, processing and connectome analysis.
Abbreviations: UN= undernutrition; PU= placental underperfusion; OFBT= open Field Behavioral test; ORT= object Recognition Task.

strength, global and local efficiency from global networks analysis and the correlation between brain networks features and functional data.

3.2. Project 2: Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction

Study design: Controlled laboratory study

Study population: New-Zealand rabbits at 25 days gestation were included in the surgical protocol (PU) and two different groups were obtained:

a. Growth restricted rabbits

b. Control rabbits

Interventions:

- Prenatal induction of IUGR at 25 days of gestation (PU)
- Cesarean section at 30 days of gestation
- Neurobehavioral evaluation at long-term period (+60-70th postnatal days)
- Sacrifice and samples collected at 70th postnatal days
- Magnetic resonance imaging (MRI) acquisition, diffusion MRI, tractography analysis in excised and fixed brains at 70th postnatal days

The methodology of the study and flow chart are summarized in Figure 2.

Measures:

a. Survival and growth parameters

b. Functional data at long-term period:

   i. From the OFBT: latency of time of leaving the starting familiar point (seconds), total squares crossed (number), total time exploring (seconds), velocity of travelling (cm/s), external squares

crossed (number), time in external squares (seconds), internal squares crossed (number), time in internal squares (seconds), grooming and rearing (number). Moreover, a Spearman correlation between birth weight and these functional variables were included.

ii. From ORT: Time exploring right and left object in both Familiarization and Testing phases and Discriminatory Index (DI)

c. MRI analysis:

i. Diffusion analysis (Voxel-based analysis): Fractional anisotropy (FA), coefficients of linearity, planarity and sphericity

ii. Correlation between diffusion parameters (voxel-based analysis) and functional evaluation

iii. Quantitative tractography metrics: the number of fibers in the whole brain; the number of fibers in the anxiety and memory circuitry, including a bilateral analysis and also including a right and left analysis, correlation of ratio of fibers from both circuits with neurobehavioral variables and finally measurement of mean FA in fibers involved within each circuit. Moreover, a Spearman correlation between birth weight and the ratio of fibers were included.

Outcome variables: Fetal survival, neonatal weight, latency of time of leaving the starting familiar point, total squares crossed, total time exploring, velocity of travelling, external squares crossed, time in external squares, internal squares crossed, time in internal squares, grooming and rearing, time exploring right and left object in both Familiarization and Testing phases, DI, FA, coefficients of

linearity, planarity and sphericity, number of fibers for the whole brain and number of fibers in the anxiety and memory circuitry, FA values of fiber tracts within each circuit, correlation analysis from diffusion MRI or connectivity parameters with functional variables, Spearman correlation between birth weight and OFBT variables and between birth weight and the ratio of fibers from tractrography analysis.

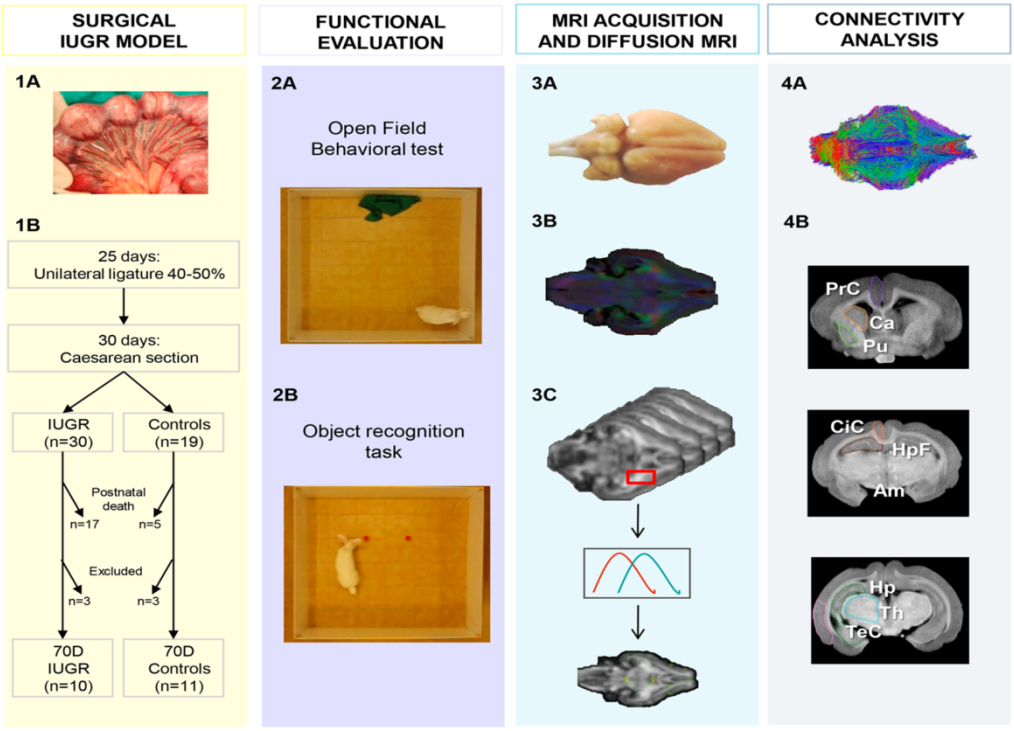


Figure 2: Study design, methods and flow chart of the animals included in the second project

PANEL 1: (A) Illustrative image of unilateral ligation of 40–50% of uteroplacental vessels at 25 days of pregnancy, (B) Scheme of surgical procedures and study groups.

PANEL 2: Illustrative pictures of neurobehavioral and cognitive evaluation in the Open Field Behavioral Test (A) and Object Recognition Task (B).

PANEL 3: MRI acquisitions: (A) Fixed brains were scanned to obtain high resolution T1 weighted images and diffusion-weighted images. After masking brain volume, (B) global analysis was performed to obtain average DTI parameters (FA, linearity, planarity and sphericity coefficients). (C) Then voxel-based analysis of diffusion-related parameters was performed by elastic registration to a reference FA map.

PANEL 4: (A) Illustrative image of tractography used for connectivity analysis. It was performed by measuring the ratio of fibers involved in anxiety and short-term memory networks over the total number of fibers reconstructed. (B) Manual delineation of brain regions involved in anxiety, attention and memory networks in coronal slices including prefrontal cortex (PrC), striatum (Ca + Pu), cingulate cortex (CiC), temporal cortex (TeC), thalamus (Th), amygdala (Am), hippocampus (Hp) and hippocampus formation (HpF).

3.3. Project 3: Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction

Study design: Controlled laboratory study

Study population: New-Zealand rabbits at 25 days of gestation were included in the surgical protocol (PU) obtaining growth restricted rabbits (IUGR) and control rabbits. After breastfeeding period (>30 postnatal days), a subgroup of IUGR animals was housed in an enriched environment (t-IUGR). At the long-term period three different groups were obtained:

a. Growth restricted rabbits (IUGR)

b. Treated growth restricted rabbits (t-IUGR)

c. Control rabbits

Interventions:

- Prenatal induction of IUGR at 25 days of gestation (PU)
- Cesarean section at 30 days of gestation
- Environmental enrichment strategy in a subgroup of restricted animals after the breastfeeding period (>30th postnatal days) up to the sacrifice of the animals (70th postnatal days)
- Neurobehavioral evaluation at first postnatal day (+1P) and at long-term period (+60-70th postnatal days)
- Sacrifice and samples collected at 70th postnatal days
- Histology assessment: Dendritic spine density and Perineural nets at 70th postnatal days

• Magnetic resonance imaging (MRI) acquisition, diffusion MRI and connectome analysis in excised and fixed brains at 70th postnatal days

The methodology of the study and flow chart are summarized in Figure 3.

Measures:

a. Survival and growth parameters

b. Functional data:

i. At the neonatal period: general motor skills, reflexes, and olfactory sensitivity

ii. At the long-term period:

- From the OFBT: time spent in the internal area (seconds)

- From ORT: Discriminatory Index (DI)

- From Skinner test: percentage of learning (%)

c. Brain network analysis:

i. Global features: Average strength, global efficiency, and local efficiency

ii. Regional analysis in hippocampus: hippocampal volume (mm^3), median Fractional anisotropy (FA) from the hippocampal regions and FA of the reconstructed streamlines crossing hippocampal regions

d. Histology assessment:

i. Dendritic spine (DS) density evaluation at CA1 of the dorsal hippocampus (number /µm)

ii. Perineural nets (PNNs) immunoreactivity at CA3 of the hippocampus (contact/μm^2)

Outcome variables: Fetal survival, neonatal weight, neurobehavioral evaluation scores during the neonatal period, time in the internal area, DI, percentage of learning, density of DS, PNNs immunoreactivity, average strength, global and local efficiency from global networks analysis and hippocampal volume, median Fractional anisotropy (FA) from the hippocampal regions and FA of the reconstructed streamlines crossing hippocampal regions.

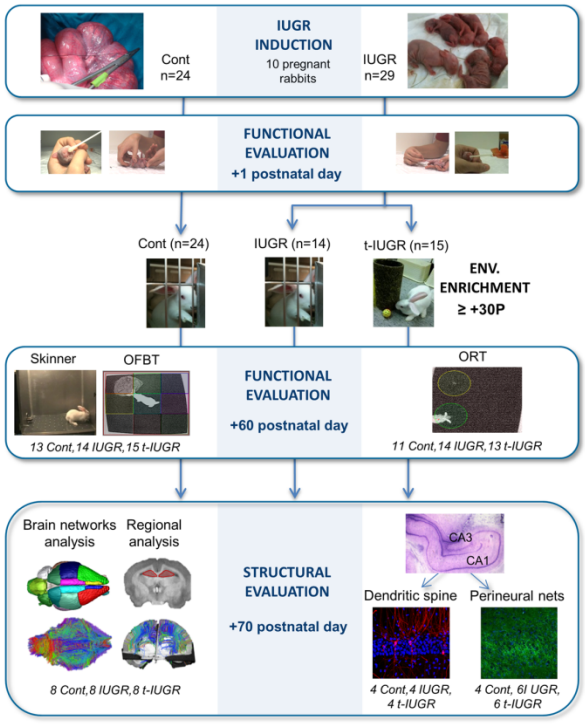


Figure 3 **Graphical representation of the study design and methods**

A) Illustrative images and scheme of IUGR induction. After the breastfeeding period (>30 postnatal day), a cohort of IUGR animals were randomized into the environmental enrichment protocol (t-IUGR) whereas the rest were housed conventionally.
B) Illustrative images of neurobehavioral tests applied. At postnatal day +1, tone, spontaneous locomotion, reflex motor activity, coordination of suck and swallow and motor responses to olfactory stimuli were assessed. At postnatal day +60 and +70P, Skinner test, OFBT and ORT were applied.
C) Magnetic resonance imaging. Fixed brains were scanned, obtaining anatomical and diffusion-weighted images. FA brain networks were extracted and global graph theory features were applied. Regional analysis was done with mean FA of hippocampus and mean FA of fibbers crossing it.
D) Histology. Connectivity characteristics of hippocampal regions were assessed including dendritic spine density and perineural nets evaluation.
Abbreviations: Cont: control; IUGR: intrauterine growth restriction; t-IUGR:Treated intrauterine growth restriction animals; OFBT: open field behavioral test; ORT: object recognition task.

3.4. Description of the research methodology

3.4.1. IUGR induction protocols

As mentioned previously, for the first project IUGR was induced following two different schemes: i. maternal food restriction aiming to reproduce undernutrition (UN) and ii. uteroplacental vessels ligation as a placental insufficiency or placental underperfusion (PU) condition. For the rest two projects, only uteroplacental vessel ligation aiming to reproduce placental insufficiency or placental underperfusion (PU) condition model was used.

a) Placental insufficiency or placental underperfusion (PU) induction:

Uteroplacental vessel ligation was performed following a previously described protocol (Eixarch et al. 2009). New Zealand pregnant rabbits provided by a certified breeder were housed for 1 week before surgery in separate cages on a reversed 12/12 h light cycle, with free access to water and standard chow. At 25 days of pregnancy, progesterone 0.9 mg/kg was administered intramuscularly for tocolysis prior to surgery. A peripheral ear venous catheter was placed and antibiotic prophylaxis (Penicillin G 300.000 UI) was administered. Ketamine 35 mg/kg and Xylazine 5mg/kg were given intramuscularly for anesthetic induction. Inhaled anesthesia was maintained with a mixture of 1-5% isoflurane and 1- 1.5 L/min oxygen. Maternal heart rate, oxygen saturation, central temperature and blood pressure were monitored during the procedure (Pluto Veterinary Medical Monitor, Bionics corp.). An abdominal midline laparotomy was performed and both uterine horns were exteriorized. Gestational sacs of both horns were counted and numbered and each fetus was identified taking into account the fetal position within the

bicornuate uterus. The fetus at the ovarian end was considered to be the first fetus. At random, one horn was assigned as the case horn and the other horn was considered as the control horn (no procedure was performed). In the case horn, part of the uteroplacental vessels of all gestational sacs was ligated in a proportion of 40-50%. Ligatures were performed with silk sutures (4/0). The exteriorized sacs were continuously rinsed with warm Ringer lactate solution. After the procedure, the abdomen was closed in two layers with a single suture of silk (3/0). Animals were kept under a warming blanket until they awoke and became active, and received subcutaneous Buprenorphine (Buprex 0.05mg/kg) every day for 48 h, as postoperative analgesia. The animals were again housed and their well-being was controlled daily.

b) Undernutrition (UN) model:

A cohort of New Zealand pregnant rabbits provided by a certified breeder was housed for 1 week before the IUGR induction in separate cages on a reversed 12/12 h light cycle, with free access to water and standard chow. At 22 days of pregnancy, a reduction of the 70% of the basal food intake was performed, which corresponds to a final administration to approximately 45 g/day of the standard chow specifically designed for the pregnant and lactating rabbit mother (2030 Teklad Global Rabbit Diet). Basal food reduction was kept up to the delivery (Matsuoka et al. 2006). As a control for the UN model, we included three pregnant rabbits that were fed ad libitum.

3.4.2. Delivery and postnatal care

Delivery was achieved at 30 gestational days in the PU model after a cesarean section was performed, whereas vaginal delivery was allowed or

induced with oxytocin at 31 days of pregnancy. Pups from both models were weighed and identified with a subcutaneous chip. The PU model pups were housed and breastfed by a surrogate mother, whereas pups from the UN model were fed by their own mother, with a maximum of eight pups in both models. Pups were housed until the 30th postnatal day when they were weaned. Thereafter, both groups of rabbits were housed in groups of three with a reversed 12/12-hour light cycle and free access to water and food.

3.4.3. Environmental enrichment

After weaning (>30th postnatal days), animals were housed in standard conditions, except for a subgroup of IUGR animals (n=15) that were housed following an EE strategy (t-IUGR group). The designed EE protocol was based on previous knowledge of behavioral needs and data available from enrichment studies in rabbits (Baumans & Van Loo 2013). The implemented strategy aimed to increase the animal sensory, physical, cognitive and social stimulation. For that purpose, the animals were housed in larger cages (150 x 70 x 40cm) in comparison to the standard ones (75 x 70 x 40cm). Inside both types of cages, an upper platform allowing the animal to lookout was placed, as a basic environmental refinement. However, only inside the t-IUGR animals cage different inanimate objects (wooden bridge, colored balls, bricks) and different flavors of the food were placed. Every three days per week the inanimate objects and the type of food were changed in order to induce novelty and cognitive stimulation. In addition, social stimulation was induced by placing the animals in a big room for one hour twice per week, allowing them to freely

explore the environment and to interact with a researcher (M.I., L.P.). This protocol was kept during 30 days up to the sacrifice of the animals.

3.4.4. Neurobehavioral evaluation

a) Functional evaluation during neonatal period:

A cohort of pups from Projects 1 and 3 was evaluated during the first postnatal day (+1P). At that moment, neurobehavioral evaluation was performed following the methodology previously described (Derrick et al. 2007; Tan et al. 2005), evaluating general motor skills, reflexes, and olfactory sensitivity. Concretely, this test includes the assessment of tone, spontaneous locomotion, reflexes, coordination of suck and swallow and olfactory sensitivity. For each animal, the testing was videotaped and most of the variables were scored on a scale of 0 to 3 (0 = worst and 3 = best), except for tone that was scored (0-4) according to Ashworth scale (Damiano et al. 2002), by two blinded observers (M.I., L.P.). Tone was assessed by active flexion and extension of the forelimbs and hindlimbs (0: No increase in tone, 1: Slight increase in tone when limb is moved, 2: Marked increase in tone but limb is easily flexed, 3: Increase in tone, passive movement difficult, 4: Limb rigid in flexion or extension). Posture was assessed observing the animal posture while performing the test (0: Lays supine; 1: Lays on the side; 2: Cannot maintain prone position, wobbly; 3: The prone position with legs coiled). Duration of the movement was evaluated during 1 minute (0: No movement, 1: activity <20 seconds, 2: activity 20-40 seconds, 3: activity 40-60 seconds). During these minute of testing, circular motion by assessing the range of movement and jumping, locomotion on a flat surface was assessed by grading the amount of spontaneous

movement of the head, trunk, and limbs and lineal movement were evaluated. The lineal movement was assessed counting the number of times the animal crossed a perpendicular line when walking straight. Moreover, when the animal walks in straight line, the mean of the shortest fore– hind paw distance was registered (fore-hind paw distance). The righting reflex was assessed when the pups were placed on their backs and the number of times turned prone from the supine position in 10 tries was registered. Suck and swallow were assessed by the introduction of formula (Lactadiet with omega 3; Royal Animal, S.C.P.) into the pup's mouth with a plastic pipette. Finally, olfaction was tested by measuring the aversive response (time in seconds the animal moves the nose away from the aversive stimulus) to a cotton swab soaked with pure ethanol that was placed close to the pups' nose.

b) Functional evaluation at long-term period:

Between postnatal days 60^{th} and 70^{th}, evaluation of learning, anxiety, and memory were performed in the animals coming from all the three projects.

Concerning the learning evaluation, a Skinner box was constructed as detailed in Leal-Campanario et al. (box for operant conditioning and instrumental learning for rabbits, 2012. Inscription number in Spain: P2001231369), and the protocol was adapted from the methodology previously described (Zworykinas et al. 1997) with food reward reinforcement and a continuous reinforcement schedule. One week before starting the evaluation, rabbits were food deprived (~ 20 g/day of food chow) to increase their motivation to get the food reward. After observing a 10–15% reduction in their basal weight, the first shaping phase was started. This phase lasted 5 days, and any advancement toward the feeder bar was rewarded. After 2 days of rest, the

training phase lasting 5 more days was performed, and a reward was given only when the animal specifically pressed the lever. In this phase, the learning criterion was considered to be when the animal pressed the lever and went directly toward the food dispenser to obtain the reward at least three times in one session. All sessions lasted 10 min and were recorded and evaluated later by blinded examiners (M.I., L.P.).

After the Skinner test, the animals were allowed to rest for 2 days before continuing with the open field behavioral test (OFBT) and the object recognition task (ORT), respectively. The OFBT evaluates locomotion and exploratory activities that compete against fear, anxiety, and attention (Bouët et al. 2003; Kowalska et al. n.d.; Walsh & Cummins 1975). The ORT evaluates declarative short-term memory, specifically recognition (Olton & Feustle 1981), as well as attention capacity (Cowan et al. 1999) and is based on the tendency of rodents to explore new stimuli for a longer time compared to familiar stimuli (Dere et al. 2006; Ennaceur & Aggleton 1997; Mumby 2001). Both tests were adapted for application in rabbits. For that purpose, a square arena (140 cm x 140 cm) surrounded by opaque plastic walls (height 40 cm) was specifically created. First, we evaluated the OFBT with their first contact with the novel environment. As we sought to evaluate any degree of anxiety, we decided not to habituate the animals to the novel area as suggested previously (Treit et al. 1993). After the OFBT, the animals were removed from the arena and in 30 to 60 minutes were again placed in the arena to evaluate the ORT. Both tests were applied between 10 am to 5 pm and after each session, the exploring area was cleaned with a 10% ethanol in order to erase any olfactory cue. The room was insulated

from sound and with full overhead illumination. To minimize interference due to human contact, each session was videotaped and later evaluated.

The OFBT was designed and used in accordance with the procedure previously described (Walsh & Cummins 1975). In Project 2, the testing area was divided into 36 squares of 23x23 cm, the 4 central squares were considered as the internal area and the remaining squares were defined as the peripheral area. In Projects 1 and 3, the testing area was divided into 9 squares, 8 as a peripheral and one as the internal square. For testing, the rabbits were taken out of their cage wrapped with a cloth and placed close to one of the lateral walls (starting point) and behavior was assessed during 10 minutes. Multiple parameters were recorded including latency of leaving the starting point (seconds), the number of squares explored (internal or external), total time spent in internal and peripheral areas (seconds) and other general activities such as the number of rearing and grooming. The ORT was performed, being adapted from the original description (Ennaceur & Delacour 1988) including some modifications in the stimulus used. Instead of using visual stimulus, odour-based stimulus was used by means of placing pieces of fruit (apple or orange) inside perforated plastic boxes, since olfactory sensitivity is highly developed in rabbits (Ennaceur 2010). This is in agreement with the notion that the type of stimulus presented must be one in which the sensory perception of the species chosen is adequate (Ennaceur 2010). The test was divided into two consecutive phases. First, two boxes containing the same odour-based stimuli (apple) were presented to the animal during 5 minutes. This constituted the Familiarization phase. The rabbit was then returned to its cage for a 30-minute retention interval. Then, one of the objects was removed and replaced by a

novel stimulus (orange) and the animal was again placed in the area with the novel and familiar objects for 5 minutes more in the Testing phase. Exploration of the object was considered when the rabbit showed sniffing, touching and having moving vibrissae while directing the nose towards the object at a distance of less than 1 cm. Cumulative time (seconds) exploring each object in the two sessions was recorded (right and left objects in the Familiarization phase, whereas novel and the familiar objects in the Testing phase). Finally, the discrimination index (DI), which represents the ability to discriminate the novel from the familiar object, was calculated as follows:

$$DI = \frac{\text{time exploring novel object} - \text{time exploring the familiar one}}{\text{time exploring novel object} + \text{time exploring the familiar one}}$$

A preserved memory was considered with DI > 0, whereas a DI ≤ 0 indicated problems in short-term memory. Animals that did not explore one object in the Familiarization phase and at least one time both objects in the Testing phase were excluded from the analysis, as previously suggested (de Bruin & Pouzet 2006). In the Project 1 and 3 all the recorded videos from OFBT and ORT were evaluated by using a video tracking software (SMART Software Tracking System from Panlab, from Panlab Harvard Apparatus, UK). In the Project 2 the analysis was done by two blinded observers (M.I. and A.A.) without the use of that software.

3.4.5. Sample collection

After the long-term functional tests, the rabbits were anesthetized with ketamine 35 mg/kg and xylazine 5 mg/kg given intramuscularly and were

sacrificed with an endovenous overdose of sodium pentobarbital (200 mg/kg). Immediately, brains were fixed through an intravenous perfusion (heart or common carotid) with phosphate-buffered saline (PBS) followed by paraformaldehyde (PFA) or formalin. Brains from Project 1 and 3 (except brains included in the Dendritic spine protocol) were perfused by 10% buffered formalin through a cardiac catheterization. After that, brains were removed and placed in 10% buffered formalin solution overnight. The different fixative protocol was carried out in those brains included in the DS evaluation (Project 3). In that brains, heart perfusion was done with 2% of PFA followed by 10 minutes immersion in that solution. Finally, brains from the Project 2 were perfused through common carotid arteries catheterization by PBS followed by 4% paraformaldehyde PBS. Finally, the brains were dissected and fixed in 4% paraformaldehyde PBS for 48 h.

3.4.6. Magnetic resonance evaluation

A subset of the animals that were functionally evaluated at the long-term period was randomly selected to perform magnetic resonance imaging (MRI).

a) Magnetic resonance acquisition

MRI was performed using a 7T animal MRI scanner (BrukerBioSpin MRI GmbH). Due to technical issues, high-resolution three-dimensional acquisition was obtained following two different schemes:

- In the UN animals coming from Project 1 and in all the animals included in Project 2, T1-weighted were obtained from brain samples by a modified driven equilibrium Fourier transform (MDEFT) 3D sequence with the following parameters: Time of Echo (TE) = 3.5 ms,

Time of Repetition (TR) = 4000 ms, 0.7 mm slice thickness with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 184 x 188 and Field of View (FoV) of 28 x 28 mm^2, resulting in a voxel dimension of 0.15 x 0.15 x 0.7 mm^3. Any potential tissue alteration, mainly significant tissue loss that could alter the results of further image-based analysis, was considered as exclusion criteria. Moreover, within this group of animals, different diffusion-weighted images (DWI) were acquired covering different gradient directions.

- From animals included in the Project 2, DWI was acquired using a standard diffusion sequence covering 126 gradient directions with a b-value of 3000 s/mm^2 together with a reference (b = 0) image. Other experimental parameters were: TE = 26 ms, TR= 250 ms, slice thickness = 0.7 mm with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 40 x 40, FoV of 28 x 28 mm^2, resulting in a voxel dimension of 0.7 x 0.7 x 0.7 mm^3. The total scan time for both acquisitions was 13 h 56 m 40 s.
- This was different for the UN brains included in Project 1, where diffusion-weighted images (DWI) were acquired using a diffusion sequence covering 30 gradient directions with a b-value of 3000 s/mm^2 together with a baseline (b = 0 s/mm^2) image. Other experimental parameters were: TE = 26 ms, TR = 250 ms, 0.7 mm slice thickness with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 40 × 40, FoV of 28

× 28 mm^2, resulting in a voxel dimension of 0.7 × 0.7 × 0.7 mm^3. The total scan time for both acquisitions was 4 h 51 min.

- In the PU animals coming from Project 1 and all the animals included in Project 3, high-resolution three-dimensional T2-weighted images were obtained by a rapid acquisition with relaxation enhancement (RARE) sequence. After that, DWI was acquired using a diffusion sequence covering 30 gradient directions with a b-value of 3000 s/mm^2 together with a baseline (b = 0 s/mm^2) image. Other experimental parameters were: TE = 26 ms, TR = 250 ms, 0.7 mm slice thickness with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 40 × 40, FoV of 28 × 28 mm^2, resulting in a voxel dimension of 0.7 × 0.7 × 0.7 mm^3. The total scan time for both acquisitions was 6 h 58 min.

b) MRI preprocessing:

As a first step, each brain (for all the projects) was segmented from the background by means of customized software implemented in Matlab 2011a (The MathworksInc, Natick, MA, USA) similar to what has been described previously (Eixarch et al. 2012). Tensor model of diffusion MRI was estimated at each voxel inside the brain mask (Fillard et al. 2007). Based on the tensor model, a set of measures describing the diffusion were computed: fractional anisotropy (FA) and the coefficients of linearity, planarity, and sphericity (Basser & Pierpaoli 1996). Linearity, planarity and sphericity coefficients describe the shape of the diffusion. High values of linearity indicate that diffusion occurs mainly in one direction, which mainly involves the presence of fiber tracts; high

planarity indicates that diffusion is performed mostly in one plane, which could be related to crossing fibers; and high values of sphericity are related to isotropic diffusion (Westin et al. 2002). Only in the Project 2, the orientation diffusion function (ODF) of each voxel was also reconstructed following a Q-Ball approach (Descoteaux et al. 2007). The ODF of each voxel was used to reconstruct fiber tracts by means of the deterministic tractography algorithm implemented in MedINRIA 1.9 (Toussaint et al. 2007) (Inria Sophia Antipolis website, available at www-sop.inria.fr/asclepios/ software/MedINRIA/. Accessed 2013 September 1). In the Projects 1 and 3, Diffusion Toolkit (http://trackvis.org/dtk/; Date last accessed: August 2015) was used to estimate the diffusion tensor image (DTI) and perform tractography, considering a fractional anisotropy (FA) threshold of 0.1.

c) MRI analysis in Projects 1 and 3:

c.1. Brain parcellation

Only in the animals from Project 1 and 3, automatic brain parcellation of the subjects' brain was performed using the New Zealand Rabbit MRI atlas (Muñoz-Moreno et al. 2013). The atlas was defined considering a T1 template, so in the animals with a RARE acquisition (PU animals coming from Project 1 and all the animals included in Project 3) a previous step was required by modifying image intensity in order to simulate RARE acquisition contrast. Then, elastic registration was performed between the correspondent atlas template (T1 or RARE-adapted) to each subject's brain using a consistent block matching algorithm (Tristan A & Arribasi J 2007). The elastic transformation was applied to the ROI labels, obtaining a parcellation of each brain in 60 ROIs.

Coherence between the T1- and RARE-based parcellation was evaluated in the Project 1 by scanning one subject using both modalities. Parcellation obtained from both images was compared, observing similar results in both cases (global Dice Coefficient = 0.97) (Muñoz-Moreno et al. 2013). In order to align the labels obtained for each subject in the T1 or T2 volumes to its corresponding DWI, affine registration between T1 or T2 and the baseline diffusion image was performed with IRTK (www.doc.ic.ac.uk/~dr/software/; date last accessed: August 2015) (Studholme et al. 1999). Discrete values of the labels were preserved by nearest neighbor interpolation in both transformations. ROIs comprising only white matter (WM) tissue were discarded, leaving a total of 44 regions for each subject (see Table 1), each of them considered as a brain network node.

c.2. Network extraction and analysis

Brain network of each subject was extracted by means of an in-house algorithm as previously described (Batalle et al. 2014), defining a network edge eij between two nodes if there is at least one streamline starting in one node and ending in the other one. In order to assign weights to each edge eij, we considered the average fractional anisotropy (FA) along with all the fibers connecting each pair of regions i and j (Batalle et al. 2014). Hence, FA-weighted (FA-w) were obtained from each subject. Graph theory network features characterizing the global functioning of each network were computed using the Brain Connectivity Toolbox (Rubinov & Sporns 2010). Particularly, we assessed infrastructure (average strength), integration (weighted global efficiency) and segregation (weighted local efficiency) of each weighted network. In the Project

3: median FA from the hippocampal regions (as defined by the previous parcellation) was estimated for each hemisphere, as well as the median FA of the reconstructed streamlines crossing hippocampal regions.

Table 1: Regions of interest used as nodes in the structural brain networks.

ID	Label	Name	ID	Label	Name
1	FCx-L	Frontal cortex L	23	Len-L	Lenticular nucleus L
2	FCx-R	Frontal cortex R	24	Len-R	Lenticular nucleus R
3	MFCx-L	Medial frontal cortex L	25	Th-L	Thalamus L
4	MFCx-R	Medial frontal cortex R	26	Th-R	Thalamus R
5	CiCx-L	Cingulate cortex L	27	Am-L	Amygdala L
6	CiCx-R	Cingulate cortex R	28	Am-R	Amygdala R
7	PiCx-L	Piriform cortex L	29	OlB-L	Olfactory bulb L
8	PiCx-R	Piriform cortex R	30	OlB-R	Olfactory bulb R
9	ECx-L	Entorhinal cortex L	31	Hc-L	Hipopocampus L
10	ECx-R	Entorhinal cortex R	32	Hc-R	Hipopocampus R
11	PaCx-L	Parietal cortex L	33	FB-L	Forebrain L
12	PaCx-R	Parietal cortex R	34	FB-R	Forebrain R
13	OcCx-L	Occipital cortex L	35	CeH-L	Cerebellar hemisphere L
14	OcCx-R	Occipital cortex R	36	CeH-R	Cerebellar hemisphere R
15	InCx-L	Insular cortex L	37	Ht	Hypothalamus
16	InCx-R	Insular cortex R	38	Ve	Vermis
17	TeCx-L	Temporal cortex L	39	BF	Basal forebrain
18	TeCx-R	Temporal cortex R	40	De	Diencephalon
19	Cl-L	Claustrum L	41	Me	Mesencephalon
20	Cl-R	Claustrum R	42	Po	Pons
21	Cau-L	Caudate nucleus L	43	MO	Medulla oblongata
22	Cau-R	Caudate nucleus R	44	Spt	Septal nuclei

Abbreviations: R: right, L: Left

d) MRI analysis in Project 2:

d.1. VBA:

Only in the animals included in the Project 2, voxel-based analysis (VBA) was evaluated in order to identify regional changes in diffusion-related parameters. This analysis consists of the normalization of all the volumes to a reference volume and the comparison of the values at the same voxel of all the normalized volumes, thus identifying statistically significant differences. Registration of the DWI volumes to the reference was performed by means of a block matching algorithm, based on a DTI-specific metric (Muñoz-Moreno & Martin-Fernandez 2009). Moreover, to preserve the coherence between DTI orientation information and the transformed volumes, the Preservation of Principal Direction (PPD) algorithm was applied (Alexander et al. 2001). In order to compensate for possible misregistrations and reduce noise effects, the registered volumes were smoothed. This smoothing also reduces the effective number of multiple comparisons in the statistical testing, thereby improving statistical power (Lee et al. 2009). Van Hecke et al. (Van Hecke et al. 2010) stated that anisotropic smoothing leads to more accurate VBA results, since it preserves the edges between different kinds of tissues, reducing the partial volume effects. For this reason, we applied an anisotropic Wiener filter (Martin-Fernandez et al. 2007) to the registered volumes. Once the images are aligned to the reference, it can be assumed that voxels in the same location in all the registered images belong to the same structure, and therefore, they can be compared. Voxel-wise t-test was performed, thereby obtaining voxels with a statistically significant different distribution of diffusion-related parameters including FA and linearity, planarity and sphericity coefficients, between controls and IUGR. The main goal of the use of VBA in this study was to explore and suggest potential relationships on all possible structural changes underlying the

functional impairments in our IUGR model. Consequently, we decided to set a threshold of p=0.01 and we deliberately decided not to perform multiple comparisons correction. In addition to the analysis of differences in DTI parameters between cases and controls, the Spearman correlation between diffusion parameters and functional outcomes at each voxel was also calculated to identify which regions were related to the changes observed in the neurobehavioral and cognitive evaluation. Since VBA requires the definition of a reference brain, the results may be biased by this choice. In order to avoid this bias and to increase the reliability of the results obtained, the VBA procedure was repeated using each subject as the template, and only the regions where differences were consistently noted in all the templates were considered. In this way, the variability produced by the arbitrary choice of the reference template is discarded.

d.2. Connectivity analysis:

Connectivity analysis within specific brain areas involved in anxiety, attention and short-term memory were evaluated by evaluating reconstructed fibers tracts that crossed specific brain areas. These fibers tracts were obtained from the reconstruction of each voxel ODF and a deterministic tractography using MedIndria software. Depending on the brain areas included in the analysis we defined two main brain networks:

– Anxiety and attention network. The selection of areas was based on previous evidence that regulation of attention and emotional reactivity depends on the correct interaction between brainstem, limbic and cortical systems (Duncan et al. 1996; Merker 2007). Within the limbic system, the amygdala and

hippocampus were included because of their role in fear and anxiety (Butler et al. 2012). In addition, several cortical areas (frontal, temporal, cingulate cortices) and deep gray nuclei (striatum and thalamus) were selected due to their relation with attention and emotion (Butler et al. 2012; Haber & Calzavara 2009; Toft 1999; Torta & Cauda 2011; Tromp et al. 2012). Moreover, some of these brain areas have been identified as components of the Papez circuit which has been proposed to play a major role in emotion (Papez 1995). Given this evidence, we arbitrarily defined the ‘‘anxiety and attentional network’’ as all those WM fibers passing through the amígdala and the hippocampus formation, and which additionally passed through at least one of the following structures: striatum, thalamus, prefrontal cortex, temporal cortex or cingulate cortex.

– Short-term memory network. Brain areas proposed to be involved in short-term memory were selected. Although the exact type of memory encoded remains under debate, there is universal agreement that the hippocampus (Squire 1992; Vanelzakker et al. 2008), and especially the hippocampal formation (Battaglia & Pennartz 2011; Zola-Morgan & Squire 1990), have important roles in declarative memory. In addition, memory based on olfactory recognition depends of the temporal lobe, mainly of the perirhinal cortex (Otto & Eichenbaum 1992) and performance of the ORT has been proposed to rely on the correct interaction within the perirhinal-hippocampal-medial prefrontal network (Brown et al. 2010; Delatour & Witter 2002; Powell et al. 2004). Finally, recent evidence has suggested the involvement of the thalamus in the regulation of short-term memory (Watanabe & Funahashi 2012). Based on these data, we arbitrarily defined the ‘‘short-term memory network’’ as all those WM fibers passing through the hippocampal formation and which additionally

passed through at least one of the following structures: hippocampus, thalamus, prefrontal or temporal cortices.

Manual delineation of GM structures was performed on T1 weighted images including multiple cortical areas (prefrontal, cingulate, temporal), putamen, caudate nucleus, thalamus, amygdala, hippocampus and hippocampal formation (Figure 2, PANEL 4). Combining these regions with previously calculated tractography, WM fiber tracts involved in the two networks of short-term memory and anxiety were extracted. The measurement of connectivity within each network was assessed applying two different quantitative tractography metrics: 1) number of fibers within proposed networks corrected by the total number of fibers in each brain and 2) measurement of mean FA in fibers involved in the proposed networks. For both networks, we analyzed global circuit connectivity considering both right and left hemisphere fibers together (bilateral analysis), and specific right and left circuit connectivity, considering each hemisphere separately (right and left analysis). In addition, correlation between ratio of fibers and mean FA with functional test scores was also analyzed adjusting for gender.

3.4.6. Histology assessment

Only the animals included in the last project (Project 3) were histologically evaluated at the long-term period after the functional and MRI evaluation were performed. Histological techniques applied were: dendritic spine (DS) and Perineural nets (PNNs) evaluation.

a) Dendritic spine evaluation:

15 to 20 of basal dendrites from each subject's hemisphere were selected to be evaluated from CA1 of the dorsal hippocampus using the Helios Gene Gun System (Bio-Rad) (Grutzendler et al. 2003). CA1 was selected for this analysis, as it has been described to be the hippocampal area that receives the major input connections (Takács et al. 2012; Spruston 2008). The density of DS (number of spines / µm) was then calculated for each group. Briefly, a suspension containing 3 mg of DiI (Molecular Probes, Invitrogen) dissolved in 100 µl of methylene chloride (Sigma- Aldrich) and mixed with 50 mg of tungsten particles (1.7 mm diameter; Bio-Rad) was spread on a glass slide and air-dried. The mixture was resuspended in 3.5 ml distilled water and sonicated. Subsequently, the mixture was drawn into Tefzel tubing (Bio-Rad), and then removed to allow tube drying during 5 minutes under a nitrogen flow gas. Then, the tube was cut into 13-mm pieces to be used as gene gun cartridges. Particles were delivered to the hippocampus using a modification of the gun to enhance accuracy by restricting the target area (O'Brien et al. 2001). Dye-coated particles were delivered in the hippocampus shooting over 150-µm coronal sections at 80 psi through a membrane filter of 3 µm pore size and 8 × 10 pores/ cm2 (Millipore). Sections were stored at room temperature in PBS for 3 hours protected from light and then incubated with DAPI, and mounted in Mowiol to be analyzed. DiI-labeled pyramidal neurons from CA1 of the dorsal hippocampus were imaged using a Leica Confocal SP5 with a ×63 oil-immersion objective. Conditions such as pinhole size (1 AU) and frame averaging (4 frames per z-step) were held constant throughout the study. Confocal Z-stacks were taken with a digital zoom of 5, a Z-step of 0.5 µm, and at 1.024 × 1.024 pixel resolution, yielding an image with pixel dimensions of

49.25 × 49.25 µm. 2 or 3 basal dendrites of various neurons were selected for the analysis of spine density according to the criteria described in Brito et al 2014 (Brito et al. 2014): (a) segments with no overlap with other branches that would obscure visualization of spines and (b) segments either “parallel” to or “at acute angles” relative to the coronal surface of the section to avoid ambiguous identification of spines. Only spines arising from the lateral surfaces of the dendrites were included in the study; spines located on the top or bottom of the dendrite surface were ignored. Given that spine density increases as a function of the distance from the soma, reaching a plateau 45 µm away from the soma, we selected dendritic segments of basal dendrites 45 µm away from the cell body.

b) Perineural nets evaluation:

PNNs expression was analyzed using lectin histochemistry Wisteria Floribunda (WFA) -binding and quantifying the average density of immunolabeling (contact/μm^2) from CA3 of the hippocampus. Similarly to what has been observed in previous works (Hylin et al. 2013), CA3 area from the hippocampus was preferred to analyze PNNs since the greatest amount of WFA staining was observed in comparison to the CA1 area. Briefly, the frozen block that contains basal ganglia was embedded in Tissue-Tek, serially cut in 20- µm-thick transverse sections with a cryostat, and collected onto gelatin-coated glass slides. All sections were first blocked with 2% normal bovine serum for 1 h, followed by overnight incubation at 4°C with Wisteria Floribunda Lectin (1:20, Sigma). After washes, immunoreactive sites were revealed by using species-specific secondary antibodies conjugated to Streptavidin 488 Alexa Fluor (1:200, Invitrogen). After incubation, the sections were thoroughly

washed, counterstained with Hoechst 33258 (1:1000, Thermofischer), mounted on slides, and cover-slipped with Fluoromount-G (Sigma). Labeled neurons were localized in CA3 zone, a region of interest (ROI) was manually selected and images were acquired with a scanning confocal microscope (Leica Confocal SP5, 40×/1.3 Oil DIC M27). Image analysis and processing were performed by means of imageJ software. Three representative serial sections from each animal in CA3 area were used and a constant threshold was applied to obtain an estimated average density of immunolabelling (contact / μm^2).

3.4.8. Statistics

For quantitative variables, normality was assessed by Shapiro-Wilk Test and homoscedasticity by Levene's Test. Results were expressed as mean and standard deviation (SD) for normal variables; whereas median and interquartile rates (IQR) were used in non-normal variables. In the neonatal data, normal-distributed quantitative variables were analyzed by t-test, while non-normal distributed variables were analyzed by the non-parametric Kruskall-wallis test. For categorical variables, chi-squared test was used. In the long-term period data, statistical comparisons between groups were performed by general linear models (GLM) and were adjusted by gender. Interaction of group (controls and cases) and gender was first included into the model, but as it did not show any significant effect were excluded from the final model. Log transformation was performed before GLM analysis if the null hypothesis in Shapiro-Wilk or in Levene's Test was rejected. Significance was declared at $p < 0.05$ (uncorrected). Software packages used in this thesis included SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and STATA13.0.

Additionally, in order to evaluate functional impairments between both models in Project 1, the mean difference (mean result in controls minus mean result in cases) and its 95% confidence interval (CI) were calculated for each model and for each functional variable. Also in this Project, the association of network features with functional results was performed by means of a partial correlation or GLM, as needed (all the analysis adjusted by gender).

Regarding the VBA approach from the Project 2, registered and smoothed volumes of FA, linearity, planarity and sphericity coefficients were used to obtain volumetric maps of t-statistics, showing the voxels that presented a significant difference between groups (uncorrected p=0.01). In addition, a correlation volume (r) was also calculated for each functional item, expressing positive and negative Spearman correlations between FA, linearity, planarity and sphericity coefficients and neurobehavioral and cognitive outcomes and between birth weight and functional variables from OFBT and with the ratio of fibers in memory and anxiety circuitry. All the correlations were analyzed adjusting for gender. Image analysis, processing, and regression analysis were performed by means of an in-house software implemented in Matlab 2011a (The MathworksInc, Natick, MA, USA).

4. RESULTS

4.1. Project 1: Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models

- The results of this project have been published in "Fetal Diagnosis and Therapy" journal and cited as: "Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models. Illa M, Eixarch E, Muñoz-Moreno E, Batalle D, Leal-Campanario R, Gruart A, Delgado-García JM, Figueras F, Gratacós E. Fetal Diagn Ther. 2017 Jan 5. doi: 10.1159/000454859".
- And presented in the following congresses:
    - 12th World Congress in Fetal Medicine 2013. Marbella, Spain. June 2013. Presented as a Poster. Authors: Illa M, Eixarch E, Batalle D, Muñoz-Moreno E, Arbat-Plana A, Figueras F, Gratacos E. Title: Animal models of intrauterine growth restriction: comparison of the neurobehavioral consequences in neonatal and long-term period.
    - 24th World Congress on Ultrasound in Obstetrics and Gynecology (ISUOG), Barcelona, Spain. September 2014. Presented as an Oral communication. Authors: Illa M, Eixarch E, Muñoz-Moreno M, Batalle D, Figueras F, Gratacos E. Title: Different effects in neurodevelopment after IUGR: hiponutrition and surgical rabbit models.

4.1.1. Study population

A total of 52 fetuses were included (20 controls and 32 ligated fetuses) in the PU model, 38 of which were alive at delivery (19 controls and 19 ligated fetuses), while a total of 38 fetuses were included in the UN group (20 controls and 18 cases), all of them being alive at delivery. All the animals were attempted to be functionally evaluated at the long-term period. However, due to technical problems, Skinner tests and OFBT were not available in all the animals from both models, including 23 in the PU and 25 animals in UN model in the Skinner test, and 19 animals from the PU model and 32 animals from UN in the OFBT. ORT was attempted in all animals with a successful OFBT test. However, only 17 animals from the PU and 23 animals from the UN model were suitable to be included in the analyses, since they explored at least one object in the familiarization phase and at least one time both objects in the testing phase (de Bruin & Pouzet 2006). After sacrifice, a subsample of fixed brains was selected to be scanned (16 animals from the PU and 14 from the UN group) and included in the MRI evaluation.

4.1.2. Survival and growth parameters

Stillbirth was statistically higher in cases coming from the PU model compared to their respective controls (44 vs. 5%, $p < 0.001$). No stillbirth was observed in the UN model. Postnatal mortality rate did not differ between cases and controls in both models (42 vs. 32%, $p = 0.55$ in PU; 6 vs. 5%, $p = 0.94$ in UN, cases vs. controls, respectively). Regarding birth weight, both models had a similar effect, observing a significant birth weight decrease in cases compared to their respective controls (see Figure 4). The degree of growth restriction induced by both models was similar (birth weight in PU: 30.23 g (SD 12.08);

birth weight in UN: 51.92 g (SD 7.57)), as both FGR's birth weights corresponded to their 10th percentile derived from normal birth weight distribution (10th percentile from PU: 33 g; 10th percentile from UN: 53 g).

At the long-term period evaluation, no differences in weight (1,444 g (SD 136) vs. 1,589 g (SD 376), p = 0.24 in PU; 1,378 g (SD 101) vs. 1,442 g (SD 111), p = 0.17 in UN, cases vs. controls, respectively) and gender distribution (percent of females: 55 vs. 62%, p = 0.73 in PU; 33 vs. 31% in UN, cases vs. controls, respectively) were observed in these models.

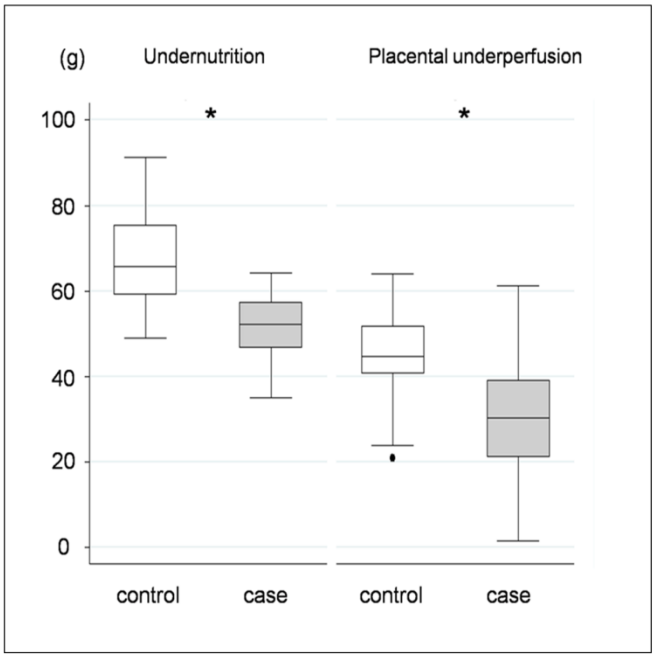


Figure 4: Birth weight differences in the study groups.

Birth weight (g) in controls and cases for both models.
* p < 0.05, statistical significance.

4.1.3. Functional data

At the neonatal period, cases from both models showed poorer results in almost all the parameters (see Table 2). These differences were more

pronounced in the PU model, as shown in Figure 5, where mean difference and its 95% CI for each functional variable were higher in the PU model.

Table 2: Functional results at the neonatal period in study groups.

Neonatal period		Control	Case	*p*
Tone (score)	*UN (n=38)*	4 (0)	4 (0)	*1.000*
	PU (n=38)	4 (0)	3 (2)	*<0.001*
Posture (score)	*UN (n=38)*	3 (0)	3 (0)	*0.357*
	PU (n=38)	3 (0)	3 (1)	*0.023*
Duration (score)	*UN (n=38)*	3 (1)	3 (1)	*0.638*
	PU (n=38)	3 (0)	3 (1)	*0.007*
Circular motion (score)	*UN (n=38)*	3 (1)	2 (1)	*0.231*
	PU (n=38)	2 (1)	1 (2)	*<0.001*
Locomotion (score)	*UN (n=38)*	3 (0)	3 (1)	*0.243*
	PU (n=38)	3 (0)	2 (2)	*<0.001*
Lineal movement (number)	*UN (n=38)*	3 (3)	1 (3)	*0.033*
	PU (n=38)	3 (2)	0 (1)	*<0.001*
Fore-hindpaw distance (cm)	*UN (n=38)*	3 (5)	3 (7)	*0.024*
	PU (n=38)	3 (2)	10 (5)	*<0.001*
Righting reflex (number)	*UN (n=38)*	10 (0)	9.5 (2)	*0.644*
	PU (n=38)	10 (0)	8 (5)	*0.039*
Suck&Swallow (score)	*UN (n=38)*	3 (0)	3 (0)	*0.357*
	PU (n=38)	3 (0)	3 (2)	*0.008*
Smelling test (seconds)	*UN (n=38)*	5 (2)	8 (10)	*0.551*
	PU (n=38)	2 (4)	3 (4)	*0.099*

Results are median and interquartile range (median (IQR)). Statistical comparisons between controls vs. cases for each model were performed by general linear models (GLM) after log-transformation of the variable was performed.
Abbreviations: UN= undernutrition; PU= placental underperfusion.

At the long-term period, all the animals that reached that period did not present any motor abnormality that could have interfered in the execution of the neurobehavioral tasks. Skinner test results showed a lower proportion of cases from the PU model reaching the learning criteria when compared with their controls (30 vs. 77%, p = 0.03, cases vs. controls, respectively), whereas no differences were observed in the UN model (44 vs. 56%, p = 0.56, cases vs. controls, respectively). Regarding OFBT results, cases from both models

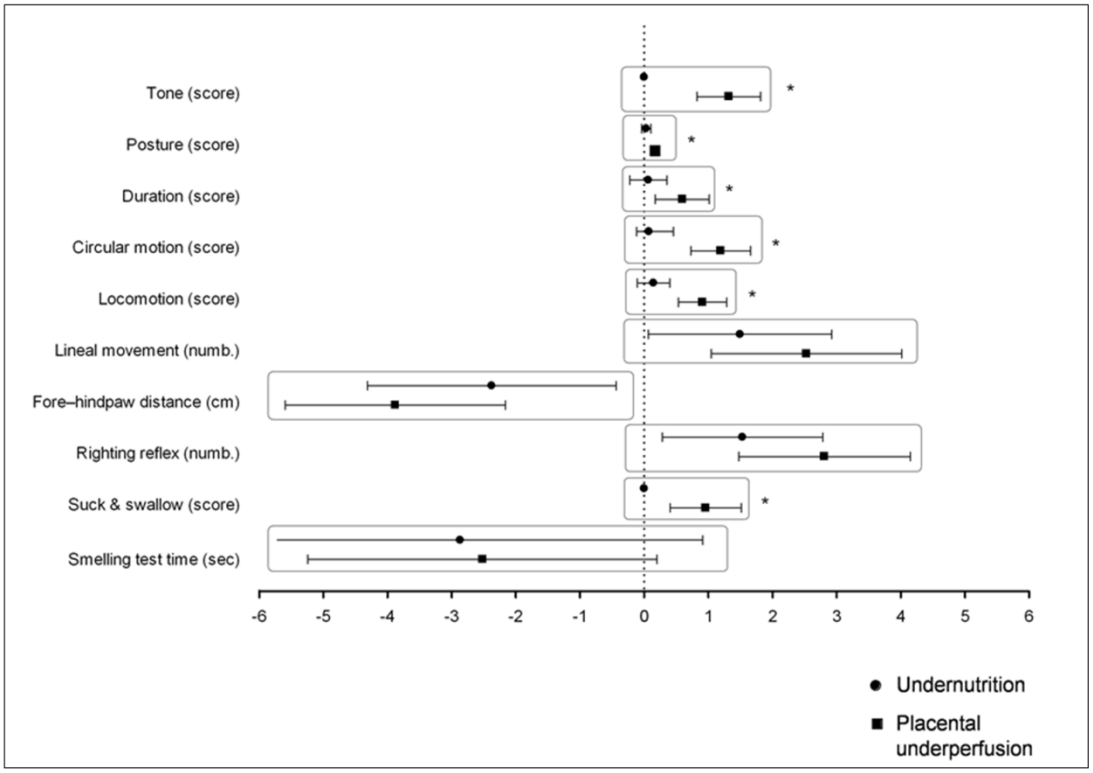


Figure 5: Mean difference of neonatal functional performance in both models.

Mean difference (mean result in controls minus mean result in cases) and its 95 confidence interval between controls and cases for each model and for each functional log-transformed variable at the neonatal period.
* $p < 0.05$, statistical significance.

presented a significantly increased latency of leaving the familiar starting point and a reduced number of external and internal boxes explored. When ORT was assessed, a decreased DI was observed in cases compared to their respective controls in both models (see Table 3). Again, these differences at the long-term period were more pronounced in the PU model, as shown in Figure 6, where mean difference and its 95% CI for each functional variable were higher in the PU model compared with the UN model.

4.1.4. Brain network results

Table 3: Functional results at the long-term period in study groups and models.

Skinner		**Control (n=29)**	**Cases (n=19)**	***p***
Learning (%)	*UN (n= 25)*	56%	44%	*0. 557*
	PU (n= 23)	77%	30%	*0.032*
OFBT		**Control (n=25)**	**Case (n=26)**	***p***
Latency (sec)†	*UN (n= 32)*	64 (171)	334 (319)	*0.013*
	PU (n= 19)	59 (62)	192 (249)	*<0.001*
Internal boxes (numb)†	*UN (n= 32)*	11 (19)	5 (8)	*0.014*
	PU (n= 19)	13 (10)	4 (5)	*<0.001*
External boxes (numb)†	*UN (n= 32)*	277 (177)	40 (66)	*<0.001*
	PU (n= 19)	60 (37)	36 (33)	*0.020*
Total boxes explored (numb)†	*UN (n= 32)*	301 (161)	53 (78)	*0.001*
	PU (n= 19)	85 (53)	41 (35)	*0.012*
ORT		**Control (n=18)**	**Cases (n=22)**	***p***
Discriminatory index	*UN (n= 23)*	0.3 (0.3)	0.0 (0.3)	*0.080*
	PU (n= 17)	0.3 (0.2)	-0.1 (0.3)	*<0.001*

Results are median and interquartile range (median (IQR)). Statistical comparisons between controls vs. cases for each model were performed by general linear models (GLM). In non-normal variables (†), log-transformation of these variables were performed prior to GLM.
Abbreviations: OFBT: open field behavioral test; ORT: object recognition task; UN= undernutrition; PU= placental underperfusion; Sec: seconds; Numb: number.

Overall, animals with FGR presented a significant decrease in brain network parameters when compared with their respective controls in both models at the long-term period. Regarding global and local efficiencies, cases presented decreased values, although these differences were only statistically significant in the PU model (Figure 7). In addition, significant correlations were observed between global network features and neurobehavioral results, especially in the OFBT variables (see Table 4).

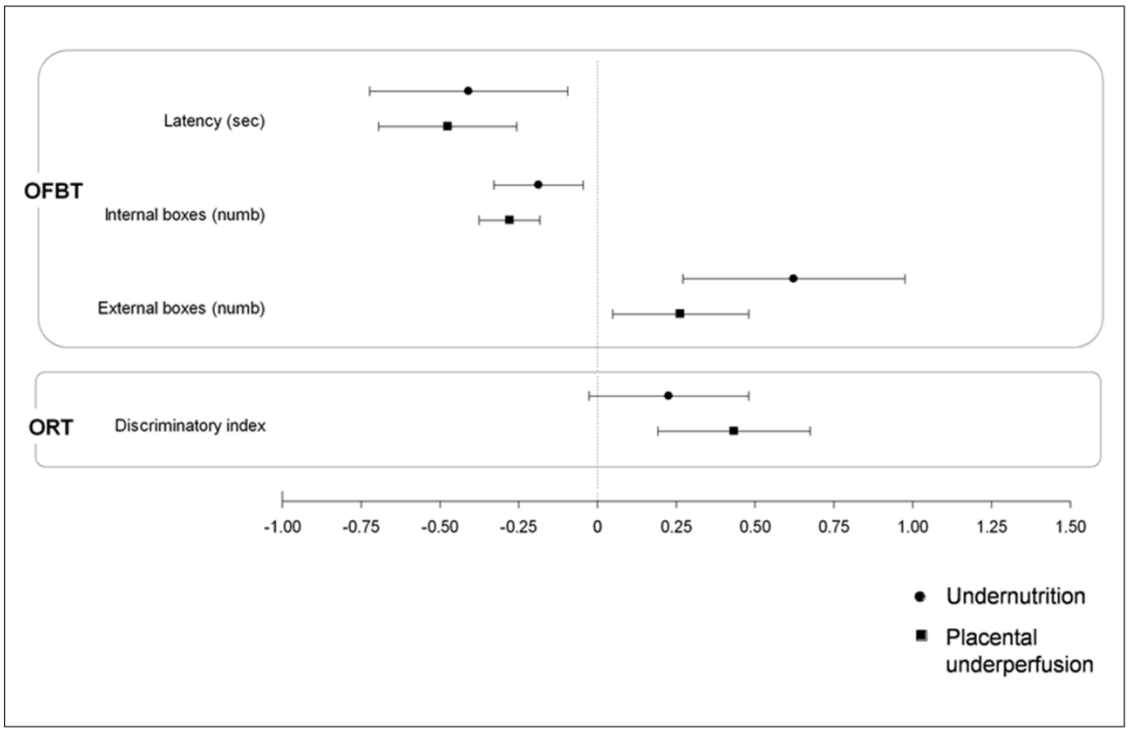


Figure 6: Mean difference of long-term functional performance in both models.

Mean difference (mean result in controls minus mean result in cases) and its 95% confidence interval between controls and cases for each model and for each functional log transformed variable at the long-term period.
Abbreviations: OFBT, open field behavioral test; ORT, object recognition task. * $p < 0.05$, statistical significance.

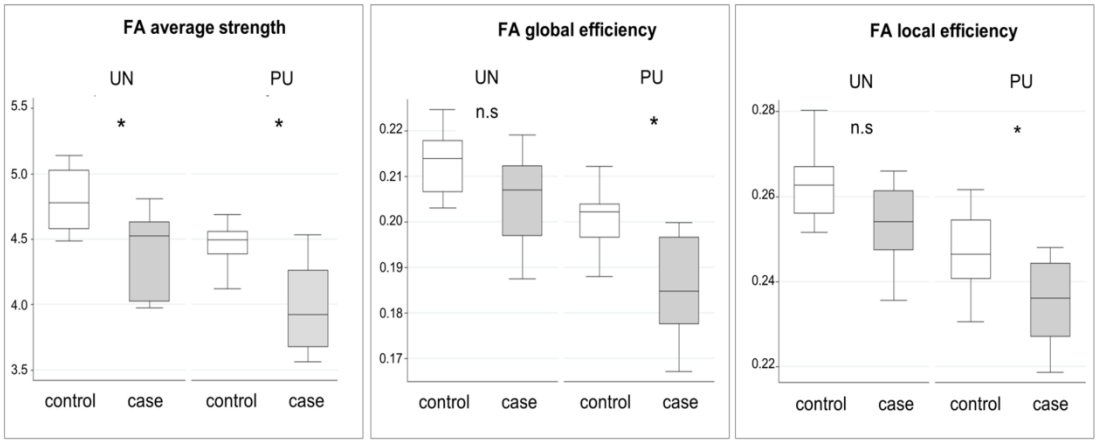


Figure 7: Fractional anisotropy (FA)-weighted network features.

FA-weighted network features in controls and cases for both models, including average strength, and global and local efficiency of weighted FA network.
Abbreviation: UN, undernutrition; PU, placental underperfusion; ns, not significant. * $p < 0.05$, statistical significance.

Table 4: Mean correlation coefficients between neurobehavioral items results and FA-weighted network features.

		Average strength	Global efficiency	Local efficiency
Latency	*UN*	**-0.82****	**-0.73****	**-0.64***
	PU	**-0.50***	-0.43	-0.42
Internal boxes	***UN***	0.58*	0.65*	0.61*
	PU	**0.64***	**0.63***	**0.66***
External boxes	*UN*	**0.80****	**0.83****	**0.77****
	PU	0.45	0.39	0.38
Total boxes	***UN***	**0.78***	**0.82****	**0.76***
	PU	0.44	0.39	0.38
DI	*UN*	0.14	0.12	0.03
	PU	0.47	0.50	0.38
% learning	***UN***	0.32	0.24	0.33
	PU	0.38	0.38	0.25

Association of network features with functional results was performed by means of a partial correlation in quantitative variables or GLM in categorical variables.
Abbreviations: OFBT: open field behavioral test; ORT: object recognition task; DI: discriminatory index; UN: undernutrition; PU: placental underperfusion. * $p<0.05$. ** $p<0.01$

4.2. Project 2: Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction

- The results of this project have been published in "Plos One" journal and cited as" Long-term functional outcomes and correlation with regional brain connectivity by MRI diffusion tractography metrics in a near-term rabbit model of intrauterine growth restriction. Illa M, Eixarch E, Batalle D, Arbat-Plana A, Muñoz-Moreno E, Figueras F, Gratacos E. PLoS One. 2013 Oct 15;8(10):e76453. doi: 10.1371/journal.pone.0076453".
- And presented in the following congresses:
    - 10th World Congress in Fetal Medicine in 10th World congress in Fetal Medicine. Malta. June 2011. Presented as an Oral communication. Authors: Illa M, Eixarch E, Batalle D, Arbat A, Acosta-Rojas R, Figueras F, Gratacos E. Title: Fetal growth restriction: Evaluation of the fetal rabbit as a model to evaluate neurostructural and neurodevelopmental changes.
    - 43rd European Brain and Behaviour Society Meeting. Sevilla, España. September 2011. Presented as a Poster. Authors: Illa M, Eixarch E, Batalle D, Arbat A, Acosta-Rojas R, Figueras F, Gratacos E. Title: Neonatal and long-term neurodevelopment and neurostructure in a rabbit model of fetal growth restriction.
    - 22nd World Congress on Ultrasound in Obstetrics and Gynecology (ISUOG), Copenhagen, Denmark. September 2012. Presented as an

Oral poster. Authors: Illa M, Eixarch E, Batalle D, Muñoz-Moreno E, Arbat-Plana A, Figueras F, Gratacos E. Title: Short and long-term impact of intrauterine growth restriction on neurobehavior, white matter diffusion and connectivity in a rabbit model.

4.2.1. Study population

A total of 69 fetuses were included at the time of the PU induction (23 controls and 47 cases), 49 of which were alive at delivery (19 controls and 30 cases). Postnatally, 5 controls and 17 cases died within the first week of life, thus, 14 controls and 13 cases reached the long-term period. Of the 27 animals that were functionally evaluated at the long-term period, 6 animals (3 controls and 3 cases) were excluded from the final analysis due to gross tissue abnormalities resulting from sample extraction or manipulation observed in the standard MRI acquisition, with 21 animals in the final sample (11 controls and 10 cases). Regarding the Object Recognition Task, 7 cases and 8 controls fulfilled the previously established criteria (de Bruin & Pouzet 2006). After sacrifice, all the fixed brains (11 controls and 10 cases) were scanned and included in the MRI evaluation.

4.2.2. Survival and growth parameters

Overall, both the fetal and neonatal mortality rate was higher in cases (stillbirth 17.4% vs. 36.2%, $p = 0.08$ and neonatal mortality 26.3% vs. 56.7%, $p = 0.01$, controls vs. cases respectively). The birth weight was significantly lower in cases compared to controls (49.54 g (SD 5.85) vs. 38.34 g (SD 5.36), $p \leq 0.001$). Nevertheless, these differences were not observed at the 70th

postnatal day (2747 g (SD 190) vs. 2626 g (SD 489), p = 0.41). Neither were any differences found in the time of postnatal evaluation (71 (IQR 3) vs. 70 (IQR 4) postnatal days, p= 0.099) nor in gender distribution (63.6% vs. 50% females, p = 0.425).

4.2.3. Functional data at long-term period

During postnatal period, no gross motor abnormalities such as paresia or spasticity were observed in either group. In the OFBT, IUGR rabbits presented reduced exploratory activities, with a significantly increased latency of leaving the starting point and a trend to present reduced speed while exploring and less rearing. In addition, cases showed a significant reduction in time spent in the internal area as well as a reduction in the number of areas crossed in both the internal and external areas (Table 5). Regarding the ORT, no differences were found in the time exploring right and left objects between groups in the Familiarization phase (right object: 9.50 s (SD 5.31) vs. 7.85 s (SD 0.04), p = 0.585; left object: 6.00 s (IQR 6.75) vs. 2.00 s (IQR 11.00), p =0.69, controls vs. cases respectively). On the contrary, in the Testing phase controls spent significantly less time exploring the familiar object compared to cases (3.63 s (SD 1.92) vs. 6.71 s (SD 1.80), p= 0.011, controls vs. cases, respectively). Interestingly, significantly decreased DI was observed in cases as well as a decreased proportion of rabbits achieving learning criteria (Figure 8). Additionally, we explored the relationship between birth weight and the neurobehavioral and cognitive measures, and as expected, we observed a

Table 5: Open field behavioral results in study groups adjusted by gender.

	Controls (n=11)	Cases (n=10)	*p*
Latency of leaving the starting point, seconds†	3.0 (29.0)	59.0 (217.5)	*0.036*
Total squares crossed, number†	113.0 (26.0)	74.5 (54.0)	*0.272*
Total time exploring, seconds	424.5 (106.3)	330.1 (149.5)	*0.139*
Velocity of travelling (total squares/total time)	0.3 (0.1)	0.2 (0.1)	*0.395*
External squares crossed, number	101.2 (37.3)	65.2 (35.4)	*0.034*
Time in external squares, seconds†	578.0 (16.0)	598.0 (8.0)	*0.017*
Internal squares crossed, number	9.2 (4.5)	3.3 (3.2)	*0.004*
Time in internal squares, seconds†	22.0 (16.0)	2.0 (8.0)	*0.083*
Grooming, number†	1.0 (2.0)	0.0 (0.0)	*0.268*
Rearing, number	23.3 (10.3)	15.7 (11.2)	*0.165*

Results are mean and standard deviation (mean (SD)) in normal variables, with median and interquartile range (median (IQR)) in non-normal variables †.

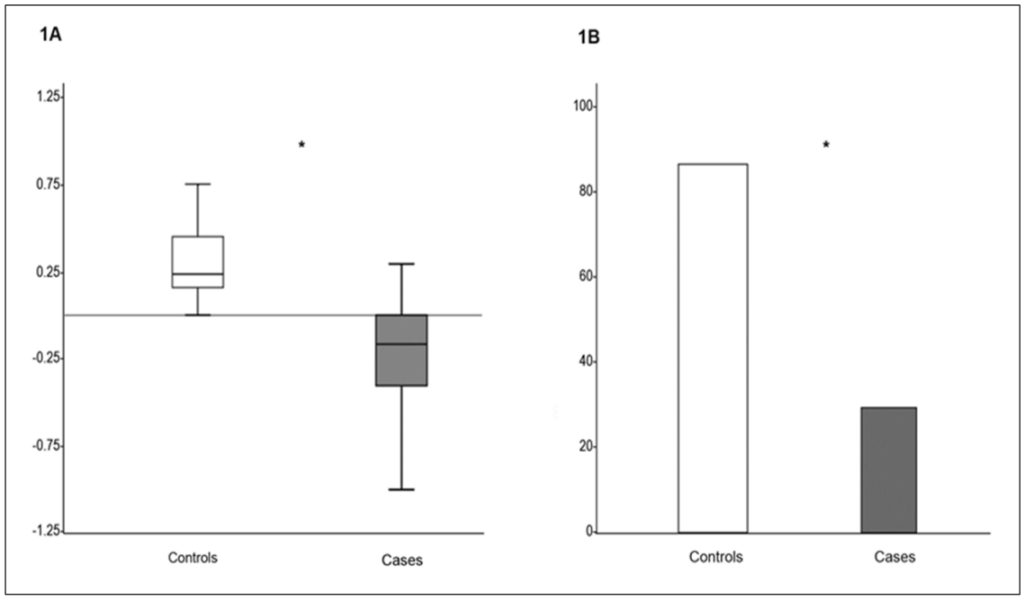


Figure 8: Discriminatory index results and percentage of learning in study groups.

(A) Discriminatory index values of the study group (p=0.013, adjusted for gender); (B) Percentage of controls and cases that reached the learning criteria (p= 0.03, adjusted for gender).

significant correlation with almost all the parameters (Table 6). Concordance between the two functional tests from each blinded observer (M.I. and A.A.) was

explored using the interclass correlation coefficient which demonstrated good reliability (mean: 0.941).

Table 6: Spearman correlation between birth weight and neurobehavior variables.

variables	rho	*p*
Latency of leaving the starting point, seconds	-0.52	*0.02*
Total squares crossed, number	0.48	*0.03*
Total time exploring, seconds	0.16	*0.51*
External squares crossed, number	0.49	*0.03*
Time in external squares, seconds	-0.58	*0.01*
Internal squares crossed, number	0.40	*0.08*
Time in internal squares, seconds	0.53	*0.02*
Grooming, number	0.07	*0.76*
Rearing, number	0.20	*0.41*
Time exploring familiar object, seconds	-0.45	*0.18*
Time exploring familiar object, seconds	0.55	*0.04*
Discriminatory index	0.66	*0.01*

4.2.4. MRI analysis

a) Regional analysis: Voxel-based analysis

When VBA analysis was applied, statistically significant differences were found in FA distribution with a decreased FA in cases compared to controls in multiple structures including cortical regions (insular and temporal) and subventricular WM. The coefficient of linearity was also lower in cases in multiple areas including cortical regions (insular, temporal, prefrontal, and occipital), thalamus, superior colliculus, hippocampal formation and fimbria of

the hippocampus. The coefficient of planarity showed increased values in the occipital cortex and thalamus in IUGR rabbits but decreased values in the insular cortex and cerebellar hemispheres. Finally, an increased coefficient of sphericity was observed in insular cortex and subventricular WM (Figure 9).

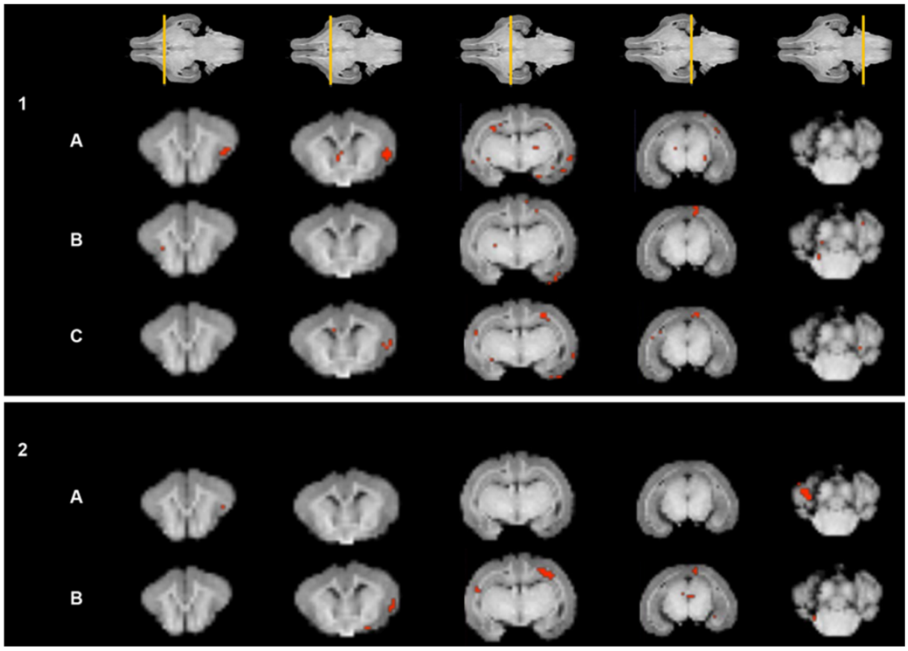


Figure 9: Fractional anisotropy, linearity, planarity and sphericity coefficients: regions showing statistically significant differences (p<0.01) between cases and controls.

Coronal slices of the 3D reference image displayed contain representative anatomical structures for specific coefficients. Slice locations are shown in the T1 weighted images at the top. PANEL 1: Representative anatomical regions showing a significant decrease in linearity (A) and planarity (B) coefficients and in fractional anisotropy (C) in cases compared to controls. PANEL 2: Representative anatomical regions showing a significant increase in planarity (A) and sphericity (B) coefficients in cases compared to controls.

b) Correlation between MRI diffusion and neurobehavioral and cognitive outcomes

The FA map shows correlations between functional variables, especially for the Open Field Test, and multiple brain areas (Figure 10 and Table 7).

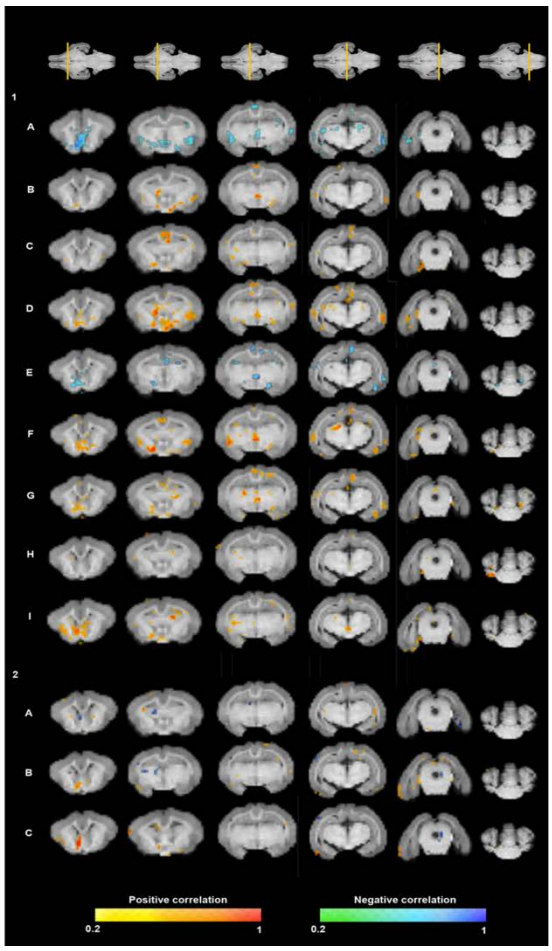


Figure 10: Correlation maps between neurobehavioral and cognitive test items and fractional anisotropy values.

Coronal slices (from anterior to posterior) of the 3D reference image. Colormap highlights the areas where the correlation coefficient is higher than 0.2. Spearman correlation $p<0.001$.

PANEL 1: (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, (I) Rearing. PANEL 2: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.

Regarding the GM structures, FA changes in the hippocampus and hippocampal formation and in the cingulate and temporal cortex were correlated to more neurobehavioral domains; followed by the prefrontal cortex, thalamus and putamen nucleus. Interestingly, the amygdala presented a significant correlation with two of the variables that are strongly related to anxiety (number of squares crossed and time spent in the internal area). Within the WM structures, the anterior commissure and corona radiata areas showed more correlations with neurobehavioral and cognitive domains. All these findings were supported by similar changes in linearity, sphericity and planarity coefficients (Figure 11, Figure 12 and Figure 13).

Table 7: Significant correlations (p<0.01) between functional variables and FA

	Positive correlation	Negative correlation
	Open Field Behavioral Test	
A		Temporal and cingulate cortices, putamen, thalamus, claustrum, anterior commissure
B	Cingulate cortex, claustrum, hippocampus, corpus callosum, anterior commissure, lateral lemniscus	
C	Cingulate, prefrontal and occipital cortices, hippocampal formation, corona radiata	
D	Prefrontal, temporal, cingulate and insular cortices, putamen, thalamus, claustrum, lateral lemniscus	
E		Cingulate cortex and hippocampus
F	Cingulate cortex, thalamus, amygdala, hippocampus, claustrum, superior colliculus, lateral lemniscus	
G	Cingulate and occipital cortices, amygdala, anterior comissure	
H	Caudate nucleus, cerebellar hemisphere	
I	Temporal cortex, hippocampus, thalamus, claustrum	
	Object Recognition Task	
A	Occipital cortex, corona radiata	
B	Occipital cortex, anterior comissure	
C	Cingulate cortex, anterior comissure	

Open Field Behavioral Test items:(A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, (I) Rearing; Object Recognition Task items: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.

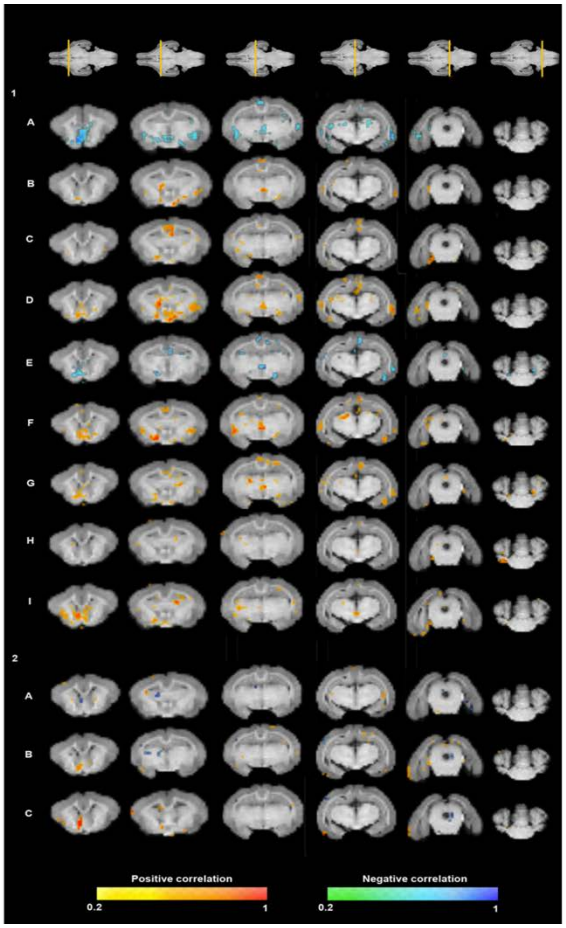


Figure 11: Correlation maps between neurobehavioral and cognitive test items and linearity coefficient, sphericity and planarity, respectively.

Coronal slices (from anterior to posterior) of the 3D reference image are displayed. Colormap highlights the areas where each correlation coefficient is higher than 0.2. Spearman correlation p<0.001. PANEL 1: (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, and (I) Rearing. PANEL 2: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.

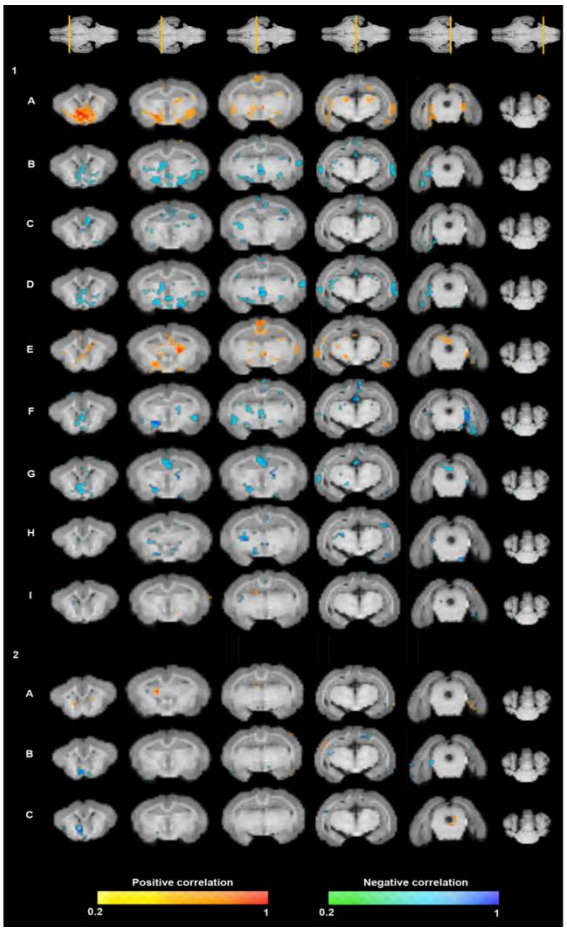


Figure 12: Correlation maps between neurobehavioral and cognitive test items and linearity coefficient, sphericity and planarity, respectively.

Coronal slices (from anterior to posterior) of the 3D reference image are displayed. Colormap highlights the areas where each correlation coefficient is higher than 0.2. Spearman correlation p<0.001. PANEL 1: (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, and (I) Rearing. PANEL 2: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.

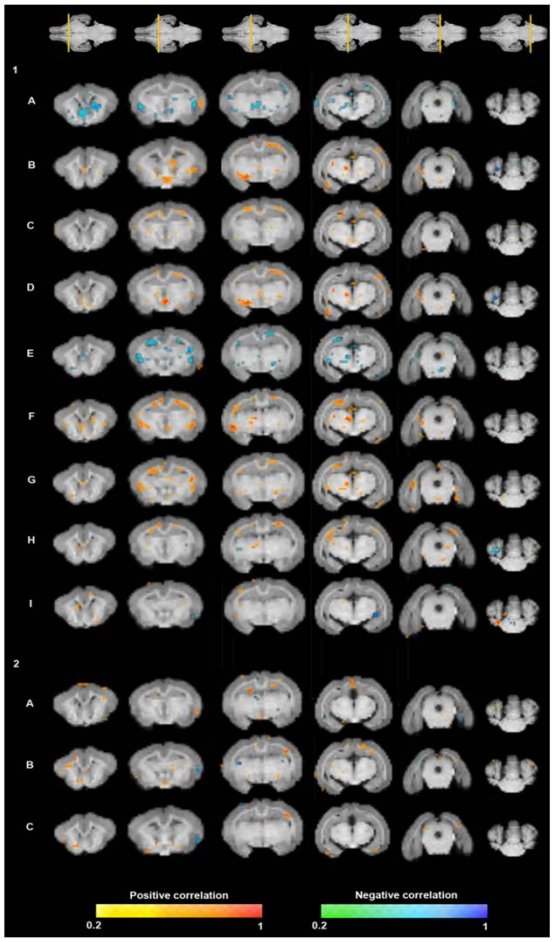


Figure 13: Correlation maps between neurobehavioral and cognitive test items and linearity coefficient, sphericity and planarity, respectively.

Coronal slices (from anterior to posterior) of the 3D reference image are displayed. Colormap highlights the areas where each correlation coefficient is higher than 0.2. Spearman correlation p<0.001. PANEL 1: (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, and (I) Rearing. PANEL 2: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.

c) Quantitative tractography metrics: Connectivity analysis

Analysis of the total number of WM fiber tracts reconstructed for the whole brain did not differ between groups (14775 (SD 2332) vs. 13921 (SD 2148), p = 0.371, controls vs. cases). Nevertheless, the evaluation of the percentage of fibers involved in a specific network, the cases showed a trend to present a lower ratio of fibers in both networks; being statistically significant in the left hemisphere for both networks (Figure 14 and 15). Table 8 depicts the mean correlation coefficients between the percentage of fibers and functional test results. Regarding the anxiety network, the left hemisphere was significantly correlated to nearly all the variables in the OFBT, whereas in the memory network any variable achieved statistical significance. Finally, we did

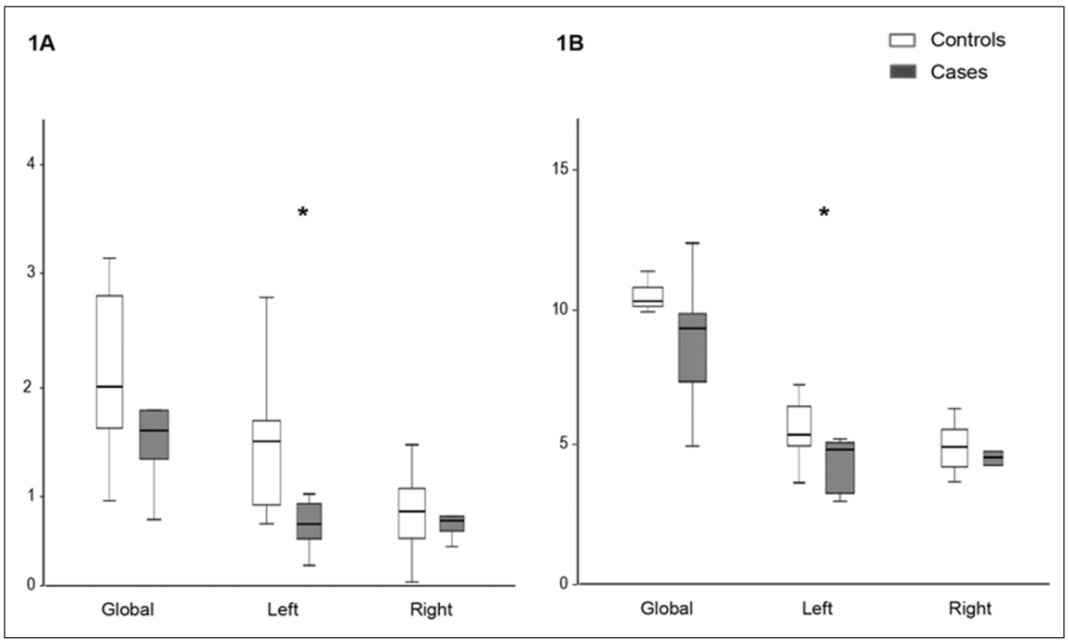


Figure 14: Ratio of fibers involved in the anxiety or memory circuit over the total number of fibers.

(A) Ratio of fibers adjusted for gender in anxiety network; in global analysis (p= 0.10), in left (p= 0.01) and right hemisphere (p=0.59 †); (B) Ratio of fibers adjusted for gender in memory network; in global analysis (p= 0.08), in left (p= 0.03 †) and right hemisphere (p=0.92 †). † Non-normal variables.

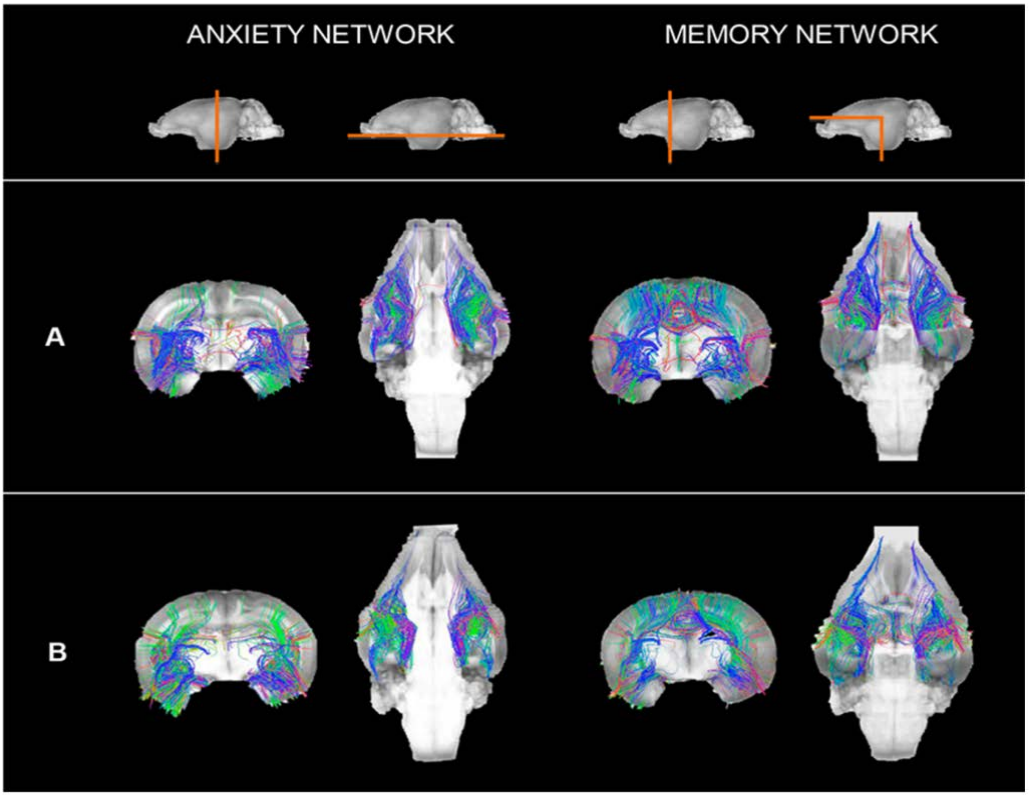


Figure 15: Reconstructed fibers in anxiety and memory networks in the experimental groups.

Coronal and axial views of anxiety and memory networks of one control (A) and one case (B). Reconstructed fibers are overlapped to the 3D reconstrucción of T1 weighted images.

Table 8: Mean correlation coefficients between ratio of fibres in anxiety and short-term memory networks and neurobehavioral and cognitive test items results (Spearman's correlation) adjusted by gender.

	Global	Left Hemisphere	Right hemisphere
Anxiety network			
A	-0.30	**-0.50***	-0.06
B	0.30	**0.56****	-0.05
C	-0.01	0.13	-0.01
D	0.30	**0.55***	-0.05
E	-0.43	**-0.49***	-0.10
F	0.35	**0.52***	0.10
G	0.43	**0.48***	0.10
H	0.05	-0.05	0.24
I	0.18	0.18	0.22
Memory network			
A	-0.51	-0.31	-0.42
B	0.18	0.34	-0.45
C	0.50	0.46	0.11

Open Field Behavioral Test items: (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, (I) Rearing. Object Recognition Task items: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index. *<p 0.05. **<p 0.01

not observe significant differences in the mean FA in the two networks, although there was a trend to presenting a lower FA in cases compared to controls, especially in the anxiety network (Table 9). Again, we explored the relationship between birth weight and the ratio of fibers in both networks observing significant correlations in almost all ratios for both networks (Table 10).

Table 9: Fractional anisotropy values of fiber tracts within each network adjusted by gender.

	Controls (n=11)	Cases (n=10)	*p*
Anxiety network			
Global	0.2539 (0.0161)	0.2448 (0.0250)	*0.319*
Left	**0.2512 (0.0164)**	**0.2478 (0.0332)**	***0.759***
Right	0.2519 (0.0408)	0.2391 (0.0283)	*0.367*
Memory network			
Global †	0.2310 (0.0112)	0.2197 (0.0181)	*0.128*
Left	**0.2322 (0.0118)**	**0.2203 (0.0216)**	***0.162***
Right †	0.2233 (0.0273)	0.2219 (0.0128)	*0.128*

Results are mean and standard deviation (mean (SD)) in normal variables, with median and interquartile range (median (IQR)) in non-normal variables †.

Table 10: Spearman correlation between birth weight and ratios of fibers.

variables	rho	*p*
Global anxiety network	0.46	*0.04*
Left hemisphere in anxiety network	0.12	*0.62*
Right hemisphere in anxiety network	0.54	*0.01*
Global memory network	0.68	*0.00*
Left hemisphere in memory network	0.31	*0.18*
Right hemisphere in memory network	0.42	*0.06*

4.3. Project 3: Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction

- The results of this project have been submitted to the "Fetal Diagnosis and Therapy" journal. Authors: Miriam Illa, Verónica Brito, Laura Pla, Elisenda Eixarch, Ariadna Arbat-Plana, Dafnis Batalle, Emma Muñoz-Moreno, Fatima Crispi, Ester Udina, Francesc Figueras, Sílvia Ginés, Eduard Gratacós.
- And presented in the following congresses:

  - 14th World Congress in Fetal Medicine, Creta, Greece. June 2015. Presented as an Oral communication. Authors: Illa M, Eixarch E, Muñoz-Moreno E, Batalle D, Pla L, Figueras F, Gratacos E. Title: Impact of Environmental Enrichment in neurodevelopment in an animal model of IUGR.

  - 4th International Fetal Growth Conference, Barcelona, Spain. September 2015. Presented as an Oral Poster. Authors: Illa M, Eixarch E, Muñoz-Moreno E, Batallé D, Pla L, Figueras F, Gratacos E. Title: Impact of postnatal environmental enrichment in neurodevelopment in an animal model of IUGR.

  **** Awarded as the "Best oral poster presentation" from the conference.***

  - 26th World Congress on Ultrasound in Obstetrics and Gynecology (ISUOG), Rome, Italy. September 2016. Presented as an Oral poster. Authors: Illa M, Brito V, Eixarch E, Pla L, Muñoz-Moreno E, Serrano G,

Figueras F, Ginés S, Gratacos E. Title: Long-term functional impairment and their structural correspondence of intrauterine growth restriction.

- Part of these results was included and presented at 26th World Congress on Ultrasound in Obstetrics and Gynecology (ISUOG), Rome, Italy. September 2016. Presented as an Oral communication. Authors: Illa M, Brito V, Eixarch E, Pla L, Muñoz-Moreno E, Serrano G, Figueras F, Ginés S, Gratacos E. Title: Survival and neurodevelopment effects of Lactoferrin, Docosahexaenoic acid and Environmental enrichment in an IUGR animal model.

**** This work was awarded with the "Young Investigator" award.***

4.3.1. Study population

A total of 243 fetuses were included at the time of the PU induction (60 controls, 183 cases), 141 of which were alive at delivery (55 controls and 86 cases). Postnatally, 42 controls and 57 cases died within the first week of life, thus, 13 controls and 29 cases reached the long-term period (14 IUGR and 15 t-IUGR). All the animals were attempted to be functionally evaluated at the long-term period. However, due to the high number of animals eligible to be evaluated in the control group (n=24), only a subsample of the controls was included in this analysis (n= 13). The rest of the IUGR were included in the functional evaluation (IUGR: n= 14; t-IUGR: n=15). Regarding ORT, only 11 controls, 14 IUGR, and 13 t-IUGR fulfilled the ORT's established criteria as previously suggested (de Bruin & Pouzet 2006). After sacrifice, 8 animals from each group were scanned and included in the MRI evaluation. Regarding histology assessment, 4 brains were included in the DS evaluation for each

group, whereas 16 brains (controls: n= 4; IUGR: n= 6; t-IUGR: n=6) were randomly selected to evaluate the PNNs.

4.3.2. Survival and growth parameters

Stillbirth was higher in non-treated IUGR compared with controls (55% vs. 8%, $p<0.001$), with no statistical differences when comparing t-IUGR and IUGR (45% vs. 55%, $p=0.125$). Birth weight was significantly lower in IUGR than in controls (33.6g (SD 1.3) vs. 46.7g (SD 1.3), $p<0.001$), without any significant difference between IUGR groups (IUGR 34.3g (SD 2.4)) and t-IUGR (33.6g (SD 1.3), $p=0.871$). At +60P, no differences between groups were observed either in weight or in gender distribution.

4.3.3. Functional data

At the neonatal period, non-treated IUGR pups showed poorer results in almost all the neurodevelopmental parameters assessed when compared to controls, whereas no significant differences were observed when comparing IUGR and t-IUGR groups (Table 11).

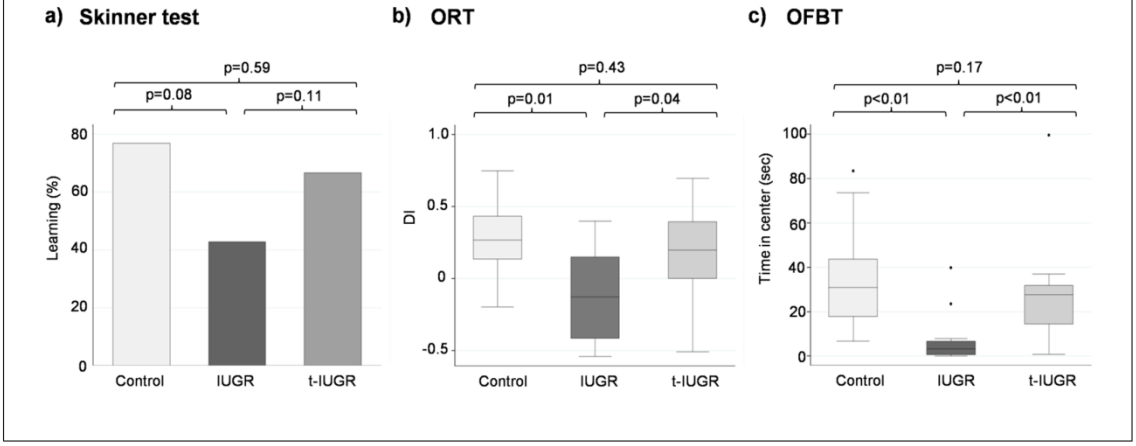
At the long-term period, non-treated IUGR animals presented functional impairments compared to controls, showing a trend to present reduced learning skills although not being statistically significant, significant memory impairment and a higher degree of anxiety (lower DI and less time exploring the internal area). Of note, t-IUGR animals presented an improvement in memory and anxiety trait when compared with IUGR subjects (Figure 16).

Table 11: Functional results at neonatal period in study groups.

variables	Control n=24	IUGR n=14	t-IUGR n=15	*p* *	*p* **
Tone (score)†	4 (0)	4 (3)	2 (1)	*<0.001*	*0.234*
Posture (score)†	3 (0)	3 (1)	3 (0)	*<0.001*	*0.530*
Duration (score)†	3 (0)	3 (1)	3 (2)	*0.007*	*0.756*
Circular motion (score)	2.4 (0.6)	1.4 (1.0)	0.9 (0.8)	*0.003*	*0.123*
Locomotion (score)†	3 (0)	2 (1)	3 (2)	*0.001*	*0.697*
Lineal movement (numb)†	3 (0)	0 (2)	0 (1)	*0.004*	*0.792*
Righting reflex (number)†	10 (0)	9.5 (5)	7 (2)	*0.062*	*0.280*
Suck & Swallow (score)†	3 (0)	3 (2)	2 (2)	*0.020*	*0.556*
Smelling test (seconds)†	2 (4)	5 (6)	3 (1)	*0.082*	*0.203*

Results are mean and standard deviation or median and interquartile range (median (IQR))†. Statistical comparisons between groups were performed by Kruskall-Wallis between control and IUGR groups (*) and between IUGR and t-IUGR (**).
Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals

Figure 16: Functional evaluation at long-term period

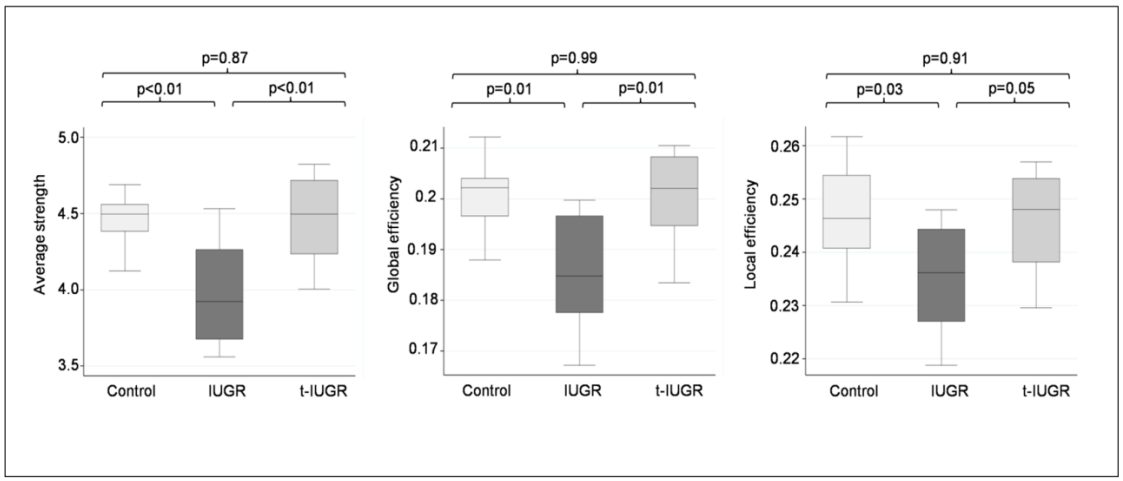


A) Percentage of learning in the study groups (controls, IUGR and t-IUGR) obtained from the Skinner test.
B) Discriminatory index in the study groups (controls, IUGR and t-IUGR) from the ORT.
C) Time spent in the internal area in the study groups (controls, IUGR and t-IUGR) from the OFBT.
Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals; OFBT: open field behavioral test; ORT: object recognition task. * p< 0.05 statistical significance.

4.3.4. Brain network analysis

Analysis of global network features evidenced a significant decrease in average strength, global and local efficiencies in non-treated IUGR when compared to controls. A significant increase with respect to IUGR was observed in all these variables in the IUGR group in which therapy was applied (Figure 17). Regional analysis revealed no significant differences in the brain volume of the hippocampus within the different groups (Table 12). Analysis of regional FA parameters showed reduced median FA in both the left hippocampus region and the fibers crossing it in non-treated IUGR animals with respect to controls. Interestingly, when compared with IUGR group, t-IUGR animals showed a significant increase in these parameters with similar values to the control group (Figure 18).

Figure 17: Global FA network features



Global fractional anisotropy (FA) network features in the study groups (controls, IUGR and t-IUGR). Networks features included average strength, global and local efficiency of weighted FA network. Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals. * p< 0.05 statistical significance.

4.3.5. Histology assessment

Non-treated IUGR animals presented a significant decrease in DS density when compared to controls, with a significant increase in the t-IUGR animals compared to IUGR (Figure 19a). Similarly, non-treated IUGR animals

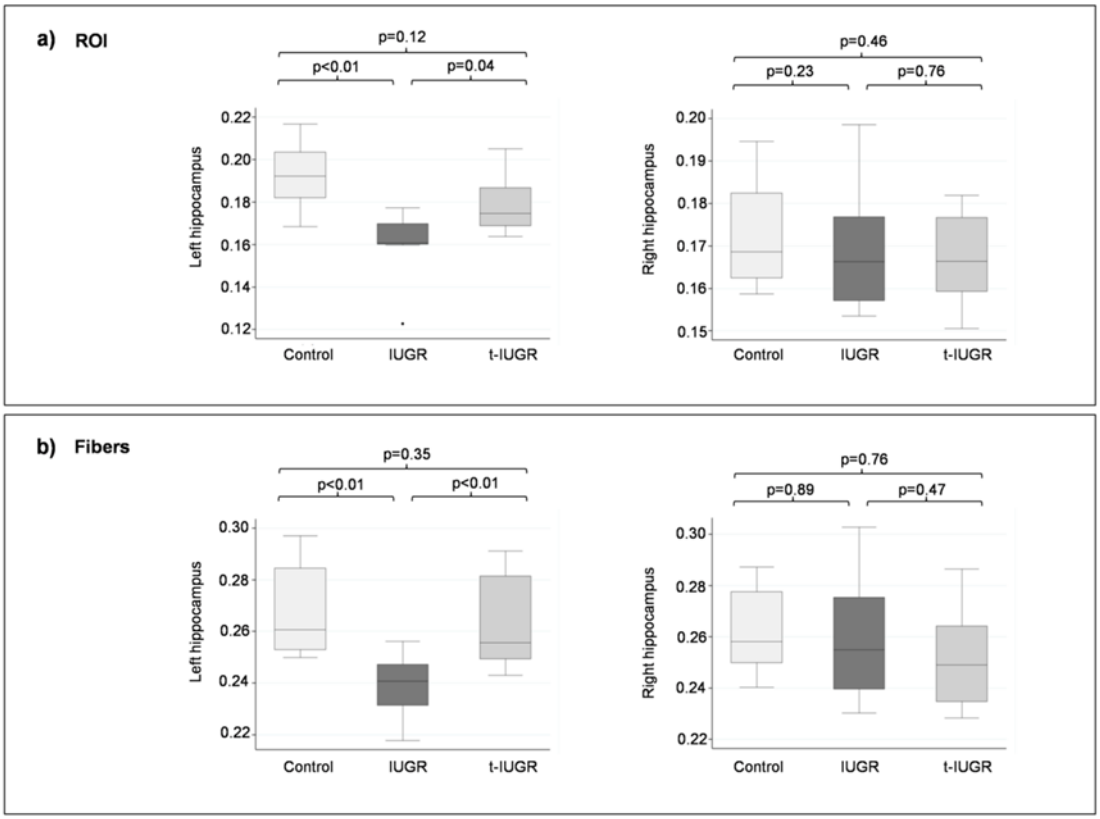
presented a significant decrease in PNNs immunoreactivity when compared to controls, with trends to increase and normalize to control levels if the therapy has been applied (Figure 19b).

Table 12: Hippocampus volume (mm3)

	Control n=8	IUGR n=8	t-IUGR n=8	*p* *	*p* **
Left	1238 (12)	1111 (268)	1187 (79)	*0.146*	*0.408*
Right	1201 (116)	1086 (224)	1185 (83)	*0.115*	*0.391*

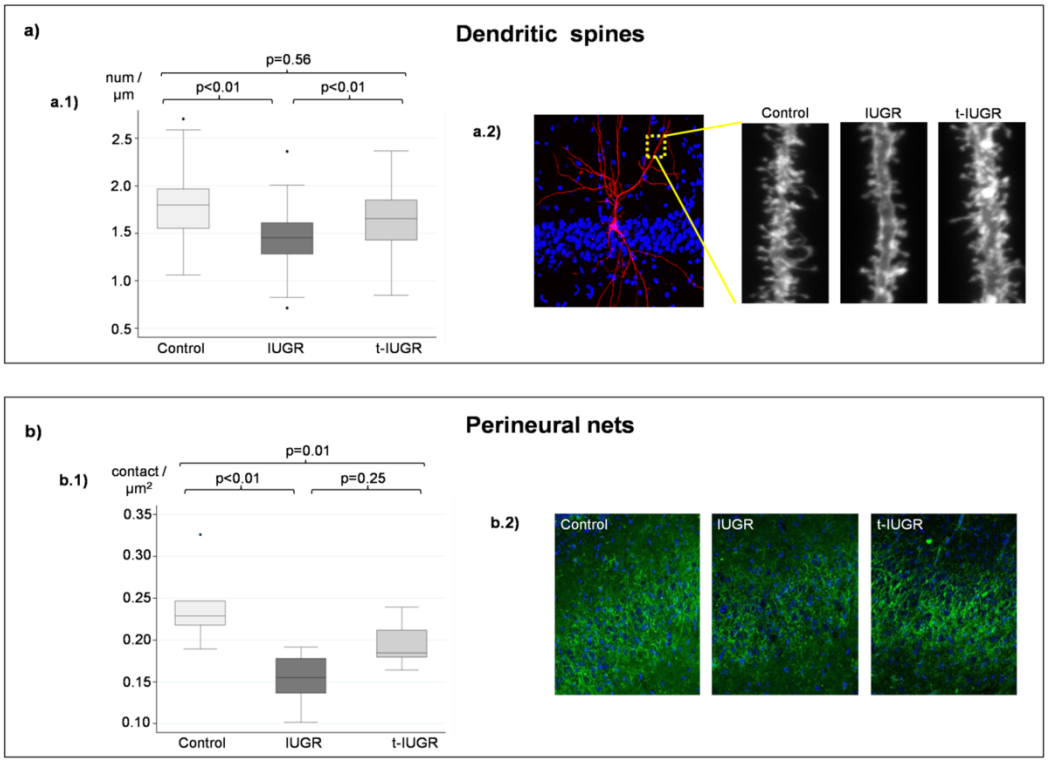
Results are median and interquartile range (median (IQR)). Statistical comparisons between groups were performed by Kruskall-Wallis between control and IUGR groups (*) and between IUGR and t-IUGR (**).
Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals

Figure 18: MRI regional Hippocampus



Median FA from the hippocampal regions and from the reconstructed fibers crossing hippocampal regions were evaluated in the study groups (controls, IUGR, and t-IUGR).
Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals. * p< 0.05 statistical significance.

Figure 19: Histology



A) Dendritic spine analysis:

A.1 Density of dendritic spine from basal dendrites of CA1 pyramidal neurons from the dorsal hippocampus in the study groups (controls, IUGR and t-IUGR).

A.2 Illustrative images of the dendritic spine density in the study groups (controls, IUGR and t-IUGR).

B) Perineural nets analysis:

A.1 Average density of immunolabelling from hippocampus CA3 zone in the study groups (controls, IUGR and t-IUGR).

A.2 Illustrative images of the immunolabelling of PNNs in the study groups (controls, IUGR and t-IUGR).

* p< 0.05 statistical significance

5.DISCUSSION

5.1. Project 1: Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models

The results from that Project showed that FGR models induced functional impairments in the neonatal and the long-term periods that correlate with structural changes observed by network analysis. Interestingly, these differences were more pronounced in the PU model, suggesting a link between severity of the prenatal insult and the degree of the neurodevelopmental consequences later in life.

Perinatal data

Regarding perinatal results, both models induced a reduction of birth weight, but only the PU model was related to an increased fetal and early postnatal mortality, reproducing severe forms of human FGR (Kady & Gardosi 2004). The same findings were observed in previous animal studies, in which PU was associated with changes in cardiovascular Doppler parameters, leading to increased fetal mortality (Eixarch et al. 2011). This contrasted with the UN models, based either on global nutrient reduction or low-protein diet, which was associated with birth weight reduction with no significant increase in fetal mortality (Vuguin 2007; Eixarch et al. 2011; Akitake et al. 2015).

Functional results

Neurobehavioral data confirm previous studies showing that both FGR models correlate with neonatal and long-term neurobehavioral impairments,

especially for PU cases. During the neonatal period, clinical studies have described neurobehavioral problems related to FGR, including psychomotor delays and cerebral palsy in the most severe cases (Baschat 2014; O'Callaghan et al. 2011), or subtler neurocognitive difficulties in less severe forms (Figueras et al. 2011; Cruz-Martínez et al. 2011). Along with this line, important motor (Derrick et al. 2004) and olfactory problems (Drobyshevsky et al. 2006) were observed in a severe and acute hypoxic-ischemic model in pregnant rabbits, whereas weaker functional disturbances were observed in less severe and chronic PU exposure (Eixarch et al. 2012). At the end of the spectrum, moderate nutrient restriction in pregnant mice has been related to subtle neurobehavioral impairment, such as delayed development of physical and coordinated movements (Akitake et al. 2015; Belluscio et al. 2014). At the long-term period, reports on infants having suffered from FGR showed neurocognitive difficulties (Levine et al. 2015) that were even more prevalent in those cases with evident signs of placental insufficiency (Murray et al. 2015). In basic research, FGR animal models including UN, a low-protein diet, and PU showed higher degrees of anxiety, reduced social interaction, and depression-related behaviors (Belluscio et al. 2014; Robinson et al. 2005), as well as learning, short-term memory, and attention problems (Akitake et al. 2015; Reyes-Castro et al. 2012; Valadares et al. 2010; Delcour et al. 2012a; Delcour et al. 2012b). Our results support the notion that severity and type of insult during the prenatal period results in a differential effect on neurobehavior, with more remarkable changes in the PU model.

Brain network results

In addition, this study provides new evidence on brain reorganization underlying neurobehavioral and cognition impairments in both models. The global reduction in FA-weighted average strength in both models supported the idea that FGR has an impaired network infrastructure. These results are in line with previous results in a rabbit model, in which average degree of structural brain networks was also decreased (Batalle et al. 2014). However, altered regional organization evidenced by means of reduced global and local efficiencies was only found in the PU model, demonstrating a more severe effect at this level. Because FA has been related to axonal packing, neuronal density, and myelination of fiber tracts (Sen & Basser 2005), these results suggested that altered network connectivity could be mainly associated with less mature connections. These results are in line with previous studies in humans (Fischi-Gómez et al. 2014) and animal models (Batalle et al. 2014), showing significantly reduced FA weighted network efficiencies in FGR at the long-term period.

This study demonstrates that sustained intrauterine exposure to placental underperfusion or undernutrition results in functional disturbances and correlates with brain network reorganization. The severity of neurodevelopmental impairment and its association with structural brain reorganization seem to be related to the degree of the prenatal insult, with more remarkable effects in the placental underperfusion model. The present study adds new evidence regarding neurodevelopmental problems of prenatal origin and improves the understanding of brain programming due to prenatal insults associated with neurobehavioral dysfunctions in FGR. Moreover, it demonstrates the feasibility of using brain network features from diffusion MRI

as biomarkers to assess and monitor potential treatments using different experimental models.

Strengths and limitations

The main strength of this study is the evaluation of neurodevelopmental consequences in two models of FGR by using the same animal species during the same period. There are a high number of studies describing neurodevelopmental problems in FGR by using different animal species that have provided an undoubted value. However, the rabbit model may have some advantages over the rodent model, as it closely resembles humans in terms of timing of perinatal brain white matter maturation as compared to rats (Derrick et al. 2007). As in humans, brain maturation begins in the intrauterine period and continues during the postnatal period. Apart from that, the suitability of PU and UN in rabbits to reproduce human features of FGR has been established (Eixarch et al. 2009; López-Tello et al. 2015; Derrick et al. 2004). Finally, another strength of this study is the fact that both models followed the same evaluation protocol in terms of functional test and brain connectivity assessment, offering the possibility to compare the two models.

Limitations of the study include methodological differences between the designs of both models. First, animals from the PU group were delivered at 30 days of pregnancy (near term) by cesarean section, whereas animals from the UN group were allowed to deliver vaginally at 31 days' gestation. In reality, these differences make our results more transferable to clinics, as severe FGR cases tend to be delivered earlier during pregnancy by means of a cesarean section, whereas less severe cases, which seems to be more accurately

reproduced by the UN model, usually are delivered near term by vaginal delivery. Depending on the method of delivery, fetal oxytocin exposure was different. In addition, the difference in the time of birth between the two models has a direct impact on the weight at birth. This difference was evident in the birth weight of the control animals, where controls in the PU model were smaller than the controls in the UN model. Finally, the difference in the rearing of the pup could also have an important effect on later neurodevelopment observed in both models. Animals coming from the PU model were fed by a surrogate, whereas animals from the UN model were fed by their mother, who had, however, been undernourished. In order to limit bias due to these design differences, structural and functional differences were assessed, comparing each FGR animal with their matched control, minimizing potential confounders between models.

Regarding the brain network extraction, we have applied a tractography method based on diffusion tensor imaging appropriate for the acquisition of the 30 gradient directions. It is known that this technique is less robust in fiber-crossing areas than techniques based on high-angular resolution diffusion imaging, leading to a lower number of recovered fiber trajectories. However, it has been shown that, from the point of view of case-control studies based on brain network analysis, diffusion tensor imaging-based tractography could reduce intersubject variability, being more sensitive to intergroup variance (Bastiani et al. 2012).

Conclusion

Overall, this work provides evidence demonstrating that chronic reduction of nutrients with or without a reduction of oxygen, even when started at later stages of pregnancy, still results in a real impact on brain programming. Data presented in this work strengthen the concept that poor nutrition during prenatal life has an impact on later neurobehavioral and cognitive development (Dauncey & Bicknell 1999). Moreover, this study also proves that chronic hypoxia added to undernutrition during the prenatal period has a more severe effect on functional and structural neurodevelopment, thus making the PU model suitable to study neurodevelopmental consequences of severe forms of FGR. On the contrary, the UN model can be of interest to study effects of less severe forms of FGR.

5.2. Project 2: Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction

This project aims to characterize simultaneously long-term neurobehavioral and cognitive dysfunctions and the related neuroanatomical changes in the near-term IUGR rabbit model using advanced imaging techniques. The IUGR model that was used in this project and in the third one was the PU model, as we had observed from the Project 1 that this was the model that presented a higher impact on brain neurodevelopment.

Long-term neurobehavior and cognitive results

Results from the OFBT showed that IUGR rabbits from the PU model presented a higher degree of anxiety expressed by reduced exploratory activities similar to what has been described in rat models after acute hypoxic-ischemic injury (Robinson et al. 2005). In addition, there was an increase in the time spent in the periphery and in the latency after leaving the starting point, a characteristic sign of anxiety in animals (Koob et al. 1993). Likewise, the IUGR rabbits presented decreased grooming activity. Although the interpretation of grooming behavior in rodents is complex, changes in the incidence of this particular behavior has been also related to altered levels of anxiety (Spruijt et al. 1992). Increased anxiety has been described in rats after perinatal hypoxic insult (Lubics et al. 2005), and in human adolescents and adults with a history of IUGR (Alati et al. 2009; Vasiliadis et al. 2010). Data derived from the ORT

have demonstrated that the IUGR rabbit model demonstrates short-term memory and attentional disorders similar to what has been reported in humans (Geva et al. 2006b). Our results are comparable to those obtained in rats after prenatal unilateral uterine artery occlusion (Delcour et al. 2012a; Delcour et al. 2012b). Overall, with the application of these two tests, we have demonstrated that the surgical model of IUGR in pregnant rabbits reproduces some of the cognitive and neuropsychological features described in IUGR children.

MRI regional analysis

Long-term structural changes were more remarkable in GM areas and included multiple cortical regions (insular, temporal, prefrontal, occipital cortices, and cerebellar hemisphere) and deep GM nuclei (thalamus and hippocampus). Interestingly, our findings of DTI changes in the prefrontal and entorhinal cortices and hippocampus are in line with previous evidence aimed at describing histology changes in the long-term period in the offspring of pregnant rats with IUGR after prenatal occlusion of the unilateral uterine artery. These histological changes include a decreased number of neurons, astrogliosis, an increase in GABAergic neurons and diffuse axonal degeneration (Delcour et al. 2012a; Delcour et al. 2012b). Changes in GM detected by DTI have been proposed to reflect changes in the dendritic architecture of pyramidal cells (Neil et al. 2002; Sizonenko et al. 2007) which could, in turn, suggest a connectivity impairment of these GM structures. Concerning WM, regional analysis of DTI parameters revealed significant differences with decreased FA and linearity and increased sphericity values in the fimbria of the hippocampus and in the subventricular WM in IUGR group. FA values are closely related to myelination

process, increasing its values in WM areas during brain maturation (Neil et al. 2002). Decreased values of FA in WM tracts have previously been described after mild hypoxic-ischemia injury and correlated to decreased myelin content, persisting these changes after the recovery period (Wang et al. 2009). Consistently with decreased FA, IUGR showed decreased linearity and increased sphericity coefficients that are related to less organized fiber tracts in WM bundles (Westin et al. 2002). Therefore, our results support the hypothesis that IUGR is related to an altered and delayed WM organization and maturation that persists even at the long-term period. It should be noted that WM changes seemed to be less pronounced in comparison with our previous findings in which structural brain changes in the neonatal period were assessed using the same animal model (Eixarch et al. 2012). One explanation for the few differences observed in WM structures could be derived for the voxel size used. It should be taken into account that a voxel size of 0.7 x 0.7 x 0.7 mm^3 may produce some partial volume effects which may hinder the presence of differences in some small brain areas, such as thin WM tracts. If these partial volume effects had been present, they would have resulted in a conservative bias, thus attenuating the existing differences and not affecting the validity of the differences observed. Aside from methodological limitations, the assignment of most of the diffusion changes observed to GM compared to WM may indicate that long-term brain plasticity throughout childhood and adolescence (Larvaron et al. 2007; Paus et al. 2001) is more efficient at correcting WM than GM deficits. In line with this notion, myelin content increases from the neonatal period up to young adulthood in an IUGR surgical guinea pig model (Tolcos et al. 2011). The same histological findings, together with a reduction in the

magnitude of differences with respect to controls in FA, have been reported in long-term as compared with neonatal measurements in rats (Wang et al. 2009). Regional changes in FA showed significant correlations mostly in GM structures with functional results, especially those related to the OFBT. With this test, the hippocampal complex, prefrontal, and cingulate cortices presented the highest number of correlations. Animal studies have demonstrated the important role of a normal functioning of the hippocampus in the regulation of anxiety (Daenen et al. 2001; Bannerman et al. 2004; Deacon et al. 2002). Concerning prefrontal and cingulate cortices, reduced volumes in children with ADHD (Emond et al. 2009) and healthy individuals (Spampinato et al. 2009), as well as histological changes in rodents (Miller et al. 2012) in these structures, have been associated with attention and anxiety traits. In addition, changes in diffusion MRI parameters of the amygdala were correlated to the number of squares crossed and the time spent in the internal area, two items strongly related to anxiety. These findings are in line with the reported role of the amygdala in the processing of fear and anxiety (Butler et al. 2012; Daenen et al. 2001). Concerning correlations with the ORT, within GM structures we observed a significant correlation between regional FA changes in the cingulate cortex and the ORT results. Several experimental studies in rodents have found that the cingulate cortex plays a key role in novelty detection, attention and memory in fearful situations (Vetere et al. 2011; Weible et al. 2012; Weible et al. 2009; Zhao & Zuo 2005), and any disruption in this structure could impair memory consolidation (Einarsson & Nader 2012). Taking this into account, although the ORT was conducted in the same arena in which the rabbit had previously performed the OFBT, persistence of any degree of anxiety while performing the

ORT could not be ruled out. This could impair memory consolidation in those animals with structural changes in the cingulate, such as our results suggest. This suggestion is in line with clinical studies that have postulated that short-term memory problems observed in IUGR children may be accounted for by a lack of sufficient attention rather than a deficit in processing the information per se (Geva et al. 2006a), impeding short-term memory function. Regarding WM and ORT results, the most consistent correlations, as they were observed in all the DTI parameters, were found in the anterior commissure and corona radiata. These tracts connect several brain areas that are engaged in memory and attention (Douaud et al. 2011; Hillary et al. 2011; Yin et al. 2011). Contrary to our original hypotheses, we did not observe any significant correlations between GM and ORT results in brain areas classically described to be involved in memory recognition, such as hippocampal formation, temporal lobe and prefrontal cortex (Squire 1992; Vanelzakker et al. 2008; Battaglia & Pennartz 2011; Otto & Eichenbaum 1992; Delatour & Witter 2002). These findings suggest that short-term memory impairment induced by IUGR as reflected in the ORT could depend more on the connectivity between relevant regions than on intrinsic changes in their GM. This notion is in line with previous findings supporting strong dependence of memory formation and on the integrity of the perirhinal-hippocampal-medial prefrontal network (Brown et al. 2010; Delatour & Witter 2002; Powell et al. 2004). In summary, these results partially confirm the hypotheses formulated in clinical studies on children and adolescents with IUGR, but provide new insight as to the specific structural anomalies underlying neurobehavioral and cognitive impairments.

Connectivity analysis

IUGR showed a decreased number of fibers in anxiety, attention and memory networks over the total number of fibers reconstructed. These differences were statistically significant in the left hemisphere, with a trend to decreased FA in both networks. Moreover, a significant correlation was observed between the ratio of fiber in the left hemisphere for anxiety network and functional results. Overall, our results are in line with previous MRI diffusion studies in patients with anxiety and attention disorders or memory impairments. Changes in connectivity within the prefrontal and anterior cingulate cortexes and the amygdala have been correlated to anxiety (Tromp et al. 2012; Kim & Whalen 2009; Modi et al. 2013). In addition, microstructural changes in the connectivity within the frontostriatal pathway and WM tracts connecting the amygdala and the prefrontal cortex have been described to be strongly related to the ADHD disorder in children and adolescents (de Zeeuw et al. 2011; Sarkar et al. 2012; Tamm et al. 2012; Wang et al. 2012a). Concerning the memory network, reduced FA has been observed in WM tracts connecting the temporal cortex and the hippocampus in children (Ortibus et al. 2012) and in the corona radiata in adults with mild traumatic injury (Hillary et al. 2011). In these studies, decreased FA was correlated to ORT results in children, and with attentional and memory impairment in adults. In addition, changes in parahippocampal WM that connect the entorhinal cortex with the hippocampus were correlated to declarative memory problems in elderly patients with mild cognitive impairment (Rogalsky 2010; Wang et al. 2012b). Most of the differences observed in our study were restricted to the left hemisphere. Differences observed in the left anxiety network are consistent with previous evidence suggesting left

hemisphere lateralization in fear-related anxiety processing (Hardee et al. 2008).

Altogether, the results of this study support the contention that altered connectivity patterns within regions involved in anxiety, attention, and memory are involved in the functional impairment associated with IUGR that persists up to the preadolescent period and suggests the importance of completing the normal programming of neuronal connectivity patterns for the achievement of normal neurodevelopment. The data reported demonstrating a decreased number of fibers in combination with more modest changes in FA. These results are different to those observed in the neonatal period (Eixarch et al. 2012) and support the idea that, in the long-term, structural changes are essentially related to the distribution rather than with the integrity of fibers. These findings are in line with previous evidence demonstrating that delayed myelination during critical developmental periods can be restored later (Tolcos et al. 2011), but will lead to long-term consequences in the patterns of connectivity, as has been consistently demonstrated in human and experimental studies (Hagmann et al. 2010; Salami et al. 2003).

Methodological considerations and limitations of the study

The methodology used to perform both VBA and connectivity analysis in this study deserves some discussion. With regard to connectivity analysis, we acknowledge that the networks defined in this study have not been fully validated, although we used consistent evidence from the literature demonstrating the involvement of all the selected regions in the functions of interest. In addition, we acknowledge that there are no standard or widely

validated approaches for quantifying tractography metrics in defined networks. Several studies have previously used this approach in human studies to characterize changes in brain structure and its neurobehavioral correlates in neurodevelopmental diseases such as ADHD (de Zeeuw et al. 2011), focal perinatal brain injury (Roze et al. 2011), and periventricular leucomalacia (Rha et al. 2011; Thomas et al. 2005). Only a few studies have used fiber count to assess the connectivity within specific brain areas (Rha et al. 2011; Thomas et al. 2005; Son et al. 2007) and these studies did not adjust for brain size or total number of fibers reconstructed. In the present study, we introduced this methodological change in order to counter the potential bias of differences in the total number of fibers reconstructed from case to case. Regarding VBA, the use of this approach implies weaker statistical power due to a large number of voxels tested (Lee et al. 2009), increasing type I error rate. This is partially compensated by the smoothing after registering the DTI volumes to the reference. By smoothing the DTI maps, the effective number of multiple comparisons in the statistical testing was reduced, thereby improving statistical power (Lee et al. 2009). We acknowledge that not correcting for multiple comparisons introduces a bias in the interpretation of results. However, as noted above, we intended to use this method in an exploratory fashion which allowed to suggest potential relationships. We would like to stress that confirmation of the relationships here suggested requires further studies with larger sample sizes. Another issue concerning VBA is that the method requires registration of all the subjects in the dataset to a template volume, and therefore the arbitrary choice of this template could bias the result (Lee et al. 2009). As described in the methodology section, this issue was addressed by repeating

the VBA considering each of the subjects as the reference. Finally, we did not include ADC data in the regional analysis since the fixation process decreases the water content in brain tissue, reducing absolute ADC values in a non-homogeneous and, therefore, non-predictable manner (Sun et al. 2003), especially in hypoxic tissue (Sun et al. 2005). From the point of view of the experimental design, the high mortality rate during the first postnatal week may have selected less severe cases for the long-term follow-up, thus attenuating the true impact of the condition. Despite this conservative bias, we were able to demonstrate structural and functional changes after IUGR. Finally, we acknowledge that our sample size may be underpowered to evaluate gender differences in the variables assessed. However, we decided to include gender as a potential confounder in our analysis since adjustment is recommended when biologically confounding is likely, as occurs in many neurobehavioral processes (Institute of Medicine (US) 2011).

Conclusions

In conclusion, PU model in a pregnant rabbit presented functional and neurostructural consequences that persist up to young adulthood. Diffusion MRI demonstrated differences in the specific brain regions involved in the regulation of anxiety, attention, and memory and in their related networks which were correlated to long-term functional impairments.

The study provides evidence of the type of structural changes involved in long-term neurodevelopmental anomalies associated with IUGR and support the potential value of methods based on diffusion quantitative metrics to assess changes associated with brain reorganization that is not demonstrable by

standard imaging techniques. Using the methodology described herein, further multi-scale studies could be performed in order to advance the understanding of the prenatal mechanisms underlying abnormal neurodevelopment to thereby target potential biomarkers based on diffusion MRI and connectivity analysis for early diagnosis and monitoring of the impact of interventional studies.

5.3. Project 3: Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction

To our knowledge, this is the first study using connectivity analysis at whole brain and cellular level to show an altered brain connectivity following IUGR that persists beyond adolescence. We hypothesize that these structural brain changes could underpin the neurobehavioral disabilities observed in our animal model. Additionally, we demonstrated that exposure to an enriched environment during early postnatal period ameliorates these effects on brain development after IUGR, partially recovering connectivity and neurobehavioral impairments.

Structural brain changes

In this study, advanced *ex vivo* MRI combined with histological markers of neuronal connectivity described changes in brain connectivity that persist up to the long-term period after IUGR. MRI results support previous findings in the rabbit model showing impaired global network infrastructure, integration and segregation evidenced by reduction in FA-weighted strength, global and local efficiencies (Batalle et al. 2014; Illa et al. 2017). Likewise, alterations in brain networks have been previously described in humans to persist in childhood and early adolescence (Fischi-Gomez et al. 2016; Fischi-Gómez et al. 2014; Muñoz-Moreno et al. 2016; Batalle et al. 2012). Apart from global changes, regional analysis of hippocampus was also explored due to its important role in memory and cognition in animals and humans (Eichenbaum 2004) and for their vulnerability to IUGR (Mallard et al. 2000). Regional analysis showed decreased

FA in the left hippocampus together with a reduction of median FA of fibers passing through the hippocampus. These results suggested the presence of less mature connections since FA has been related to axonal packing, neuronal density, and myelination of fiber tracts (Sen & Basser 2005). Predominant changes affecting one of the brain hemispheres is coherent with the idea that some neural functions tend to be more dominant in one hemisphere than in the other (Duboc et al. 2015). In particular, left hippocampus has been described to be related to memory and neurobehavioral impairments in the considered rabbit model (Illa et al. 2013) as well as in rodents (Shipton et al. 2014; Hu et al. 2010).

Regarding histological assessment, a significant reduction of DS and PNNs density in CA1 and CA3 hippocampal pyramidal neurons was observed. Both DS and PNNs have been involved in the regulation of synaptic connectivity and plasticity (Yuste 2011; Harris & Kater 1994; Dzyubenko et al. 2016). Our results showing decreased levels of DS in IUGR rabbits are in line with previously described studies on guinea pig and sheep model showing changes in DS density and morphology along with changes in synaptic receptors after acute and chronic intrauterine insults (Dean et al. 2013; Dieni & Rees 2003; McClendon et al. 2014; Piorkowska et al. 2014). On the contrary, although there is growing interest in the description of PNNs alterations related to specific brain diseases such as Alzheimer, schizophrenia, and epilepsy (Dzyubenko et al. 2016; Cabungcal et al. 2013), the pattern of alterations in the PNNs related to IUGR had not been previously evaluated. It has been described that normal completion of PNNs guarantees, in the adult brain, the stability of the

established neuronal connections (Wang & Fawcett 2012). Therefore, decreased PNNs density in the IUGR animals in CA3 suggests less consolidated connections in the hippocampus, which is coherent with the lower amount of DS found in CA1. Indeed, preliminary evidence suggests that reduction of synapses expressed as reduced DS is associated with reduced PNNs formation (Faissner et al. 2010). These changes at cellular level were related to MRI findings, especially with regional reduction of FA in the hippocampus and white matter tracts connection.

Environmental enrichment strategy

Our results demonstrate for the first time that an early postnatal strategy based on EE can improve behavioral performance and brain connectivity after IUGR. This is in agreement with previous basic research where the potential of EE as a non-invasive rehabilitation strategy has been established in rat models of hypoxic-ischemic neonatal injury (Jiménez et al. 2013) and prenatal exposition to alcohol (Hannigan & Berman 2000). Previous evidence have demonstrated the beneficial effects of EE in animal models as modulator of key sites of brain connectivity (Rampon & Tsien 2000; Rampon et al. 2000). Moreover, our data goes in line with clinical evidence showing that NIDCAP program (physical and emotional support to premature infant during neonatal intensive care unit admission) is related to neurobehavioral and structural improvement in severe IUGR preterm infants (Als et al. 2012). Together with positive effects on function, we also observed a recovery in brain connectivity with improved global network feature and increased DS density and PNNs. These changes at cellular level after EE have also been showed in a rat model

of neonatal hypoxia–ischemia with preserved DS (Jiménez et al. 2013) and in addiction based model with increased PNNs density (Slaker et al. 2016). The improvement at both behavioral and structural level is crucial to demonstrate the actual effect of EE therapy identifying those functions and regions more sensitive to its effects and to support its implementation in clinical conditions.

Strengths and limitations

This study has some strengths and limitations that merit comment. Despite the limitations of animal research, one of its major strengths is the potential to test therapies and the transferability of these results to humans. On one hand, rabbit brain shows a timing of perinatal brain white matter maturation closer to humans compared to other species (Derrick et al. 2004). Regarding the IUGR model, either perinatal results and the reported neonatal and long-term neurodevelopmental impairments are in good agreement with the literature for this model (Batalle et al. 2014; Eixarch et al. 2009; Illa et al. 2017; Illa et al., 2013) and also with clinical observations (Geva et al. 2006a; Geva et al. 2006b; Alati et al. 2009; Larroque et al. 2001; Tideman et al. 2007; Kady & Gardosi 2004). Regarding histological assessment, DS are plastic structures and they are constantly subjected to external inputs (Engert & Bonhoeffer 1999). However, the experimental setting reduces this variability. Indeed, other features of structural synaptic plasticity, such as dendritic spine morphology and distribution patterns, dendritic branching and length, or analyses of specific perineuronal net component of the extracellular may be of equal interest to be evaluated in the IUGR and may give additional insights in our results. Further studies should be considered to evaluate all these additional features of

structural synapsis and also evaluate them in other brain areas different from the hippocampus, as imaging studies of IUGR have revealed reduced volumes and diffusion MRI changes of other gray matter structures (Dean et al. 2013). Finally, due to sample size, we acknowledge that we were underpowered for some of the comparisons. In order to quantify the degree of the underpowerment of the reported variables, a supplementary table reporting the mean and risk differences, as appropriate, and its 95% confidence interval is provided (Table 13).

Conclusions

Hence, by combining dMRI with histological results we observed that IUGR may disrupt the normal pattern of brain development affecting special key sites for synaptic activity. These connectivity impairments either at global or at cellular level that persist up to the long-term period may explain, at least in part, the basis for the neurodevelopmental disorders associated with IUGR. Environmental enrichment at early postnatal period could ameliorate the effect of prenatal insults on neurodevelopment, with functional and structural changes that partially recovers normal conditions. Overall, our results reinforce the notion that environmental factors during critical periods of neurodevelopment could modify development and predispose the individual to lifelong health problems or enhance it. Further evaluation of EE effects in a clinical setting is needed to explore its real effects and also to determine the exact moment to apply such strategy in IUGR infants.

Table 13: Mean or risk difference in the outcome variables.

	Control vs. IUGR	t-IUGR vs. IUGR
Functional evaluation		
Skiner (% learning)	34.2% (-7.59 to 65.15)	24% (-16.01 to 57.09)
OFBT (time in center)	-27.7 (-42.38 to -13.06)	-24.6 (-34.7 to -14.4)
ORT (DI)	-0.18 (-0.41 to 0.06)	-0.08 (-0.08 to 0.19)
Global brain network analysis (MRI)		
Average strength	-0.48 (-0.79 to -0.18)	-0.49 (-0.84 to -0.14)
Global efficiency	-0.02 (-0.02 to -0.01)	-0.02 (-0.02 to -0.01)
Local efficiency	-0.01 (-0.02 to -0.00)	-0.01 (-0.02 to 0.00)
Regional MRI analysis (Hippocampus)		
Left volume	-27 (-49.11 to -4.89)	-7 (-69.18 to 55.18)
Right volume	-115 (-305.47 to 76.47)	-99 (-279.3 to 82.37)
Left ROI FA	-0.03 (-0.05 to -0.02)	-0.02 (-0.03 to -0.01)
Right ROI FA	-0.01 (-0.02 to 0.01)	-0.00 (-0.17 to 0.02)
Left FA fibers	-0.03 (-0.05 to -0.01)	-0.02 (-0.04 to -0.01)
Right FA fibers	-0.01 (-0.3 to 0.01)	0.00 (-0.02 to 0.21)
Histology assessment		
DS	-0.35 (-0.42 to -0.29)	-0.2 (-0.26 to -0.13)
PNNs	-0.09 (-0.15 to -0.03)	-0.04 (-0.08 to 0.00)

Mean or risk difference, as appropriate, and its 95% confidence interval (CI) for reported variables

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5.4. General discussion

This thesis provides evidence regarding the pathophysiological mechanisms and the neuroanatomical correspondence underlying neurobehavioral and cognitive disabilities described to last up to the adolescent period due to IUGR. A deeper understanding of the brain effects of IUGR allowed us to improve our knowledge of the neurodevelopmental consequences of the IUGR and also to select neuroprotective therapies aiming to mitigate the long-term neurobehavioral and cognitive disabilities. In this regard, environmental enrichment seemed to be a promising strategy capable of ameliorating the neurodevelopmental consequences of IUGR.

From Project 1, we aimed to compare the brain effects of probably the two more common situations causing IUGR: placental insufficiency and undernutrition. Both situations have been related to neurodevelopmental problems that persist up to long-term period. However, no previous work has characterized in the same study and using the same animal species the impact of these situations in brain neurodevelopment. Data derived from that project suggested that the severity of neurodevelopmental impairment and its association with structural brain reorganization seem to be related to the degree of the prenatal insult, with more remarkable effects in the placental underperfusion model.

From Project 2 and 3, we provide new insights regarding the neuroanatomical correspondence of the neurobehavioral and cognitive impairments described to last up to the adolescent period secondary to placental insufficiency. Concretely, in the second project, most of the observed diffusion changes were in the GM, including multiple cortical regions (insular,

temporal, prefrontal, occipital cortices, and cerebellar hemisphere) and in deep GM nuclei (thalamus and hippocampus), whereas fewer differences were observed affecting the WM (fimbria of the hippocampus and the subventricular WM). Rather than observing a direct damage of the WM, by using the tractography technique, a decreased in the number of fibers involved in specific networks related to anxiety and memory were observed, without observing differences in the total number of fibers from the brain. This observation supports the idea that, at the long-term, WM structural changes is essentially related to the distribution rather than with changes in the integrity of fibers at least from these two circuits. Additionally, a significant correlation between the diffusion MRI changes and functional results were observed. This observation ratified the role of these specific brain areas and networks for the function being evaluated and suggested us how even subtle structural changes affecting these key brain areas might lead to the neurobehavioral and cognitive impairments related to IUGR. Going in this line, Project 3 reinforces previous evidence suggesting how neuroanatomical correspondence of functional disabilities in IUGR at long-term period might be related to subtle structural brain changes rather than gross tissue destruction. For this purpose, in this project abnormalities in brain connectivity at the global and cellular level were explored in the PU model. Connectomics analysis evidenced altered brain network architecture at global level. The correspondence of the global connectivity alteration at cellular level was evidenced by evaluating key markers involved in neuronal connectivity and synapsis, such as the extracellular matrix and dendritic spine. All these results reinforce the concept suggesting that structural changes secondary to mild and chronic prenatal insults would be essentially

related to a disruption of the normal development and maturation pattern of neuronal circuits and connectivity. These structural changes would underpin the neurobehavioral disabilities that persist beyond adolescence.

Finally, also from Project 3, we described for the first time by using an animal model of IUGR that exposure to an enriched environment during early postnatal period could ameliorate the long-term effect of IUGR on neurodevelopment, observing functional recoveries that were correlated to structural improvements. This evidence goes in line with preliminary evidence in humans studies suggesting the effectiveness of a strategy based on early postnatal stimulation in children at risk of developing childhood disabilities. The strategy applied in the animal setting based on enriching the environmental could be transferred to human setting by applying proven strategies capable of inducing an early cognitive stimulation, such as play and reading. Although there is a lack of well-conducted studies in humans, the data derived from our research allow us to propose such strategies in children having suffered from IUGR due to the potentials benefits without any risk of negative side effects.

Overall, this thesis adds to the previous evidence new insights regarding the pathophysiological mechanisms underlying IUGR and gives strong evidence linking IUGR with altered brain connectivity as the basis for the neurological sequelae associated with IUGR. Additionally, this thesis gives preliminary evidence suggesting that a strategy based on physical, sensory, cognitive as well as social stimulation applied during early postnatal life, where brain plasticity is higher, would ameliorate the neurodevelopmental consequences of IUGR.

6. CONCLUSIONS

1. Sustained intrauterine exposure to placental underperfusion or undernutrition results in functional disturbances that correlate with structural brain changes at the level of brain network reorganization.

2. The severity of neurodevelopmental impairment and its association with structural brain reorganization seem to be related to the degree of the prenatal insult, with more remarkable effects in the placental underperfusion model.

3. Neurobehavioral and cognitive impairments that persist up to the long-term period due to intrauterine growth restriction are correlated to microstructural changes affecting specific GM brain areas and brain networks demonstrable by high-resolution magnetic resonance imaging diffusion techniques.

4. The histology correspondence of the altered brain connectivity related to intrauterine growth restriction corresponds to changes in the dendritic spine density and in the perineural nets in the hippocampus.

5. Environmental enrichment strategy applied during critical periods of neurodevelopment ameliorates the effects of intrauterine growth restriction on neurodevelopment, observing functional recoveries that were correlated to structural improvements.

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ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my thesis directors, Eduard Gratacós and Francesc Figueras. I owe them my interest in research, they trusted me, supported and facilitated every proposal I suggested allowing me to grow as a researcher, always under their guidance and advice.

Another sincere thanks to my co-worker and friend Elisenda Eixarch, who in a totally disinterested way has been my constant support both in my work and research career and in other very important aspects of my life.

My thanks also to all the co-authors of the articles presented in this thesis, from whom I have learned so much. Specially, thanks to Laura, who has accepted the challenge to continue this research in her thesis. I would also like to thank the rest of the research team that have accompanied and have given me support during all this time. Many of them have already started other ambitious projects in other countries, research groups or companies, although part of his/her support is embodied in this thesis. In this regard, a specially mention to Ariadna, Senda, Anna, Dafnis and Emma, that helped me so much especially in my early beginnings.

Finalment, no puc deixar de mencionar les més sinceres gràcies a tota la meva família. Al meu pare Agustí i Maria Teresa per ensenyar-me que la constància i persistència és la clau per l'èxit i que les coses quan es comencen, s'acaben. A la meva germana Montserrat per contagiar-me el seu entusiasme en totes les petites coses de la vida i per ensenyar-me a aixecar un i altre cop

després de cadascuna de les relliscades que poguis tenir a la vida. Als meus avis per ensenyar-me el valor del compromís. I finalment el més sincer agraïment al meu marit Xavi, i als meus dos fills, Roger i Arnau, perquè tot i les hores que aquest projecte us han robat, heu tingut una paciència i comprensió immesurable, imprescindible perquè pogués desenvolupar tot aquest treball de recerca. Gràcies Xavi per fer-me recordar dia a dia que puc comptar sempre amb tu.

APPENDIX I

PRESENTACIÓ

- Universitat de Barcelona
- Divisió de Ciències de la Salud
- Facultat de Medicina
- Departament d'Obstetrícia i Ginecologia, Pediatria, Radiologia i Medicina Física.
- Programa de Doctorat de Medicina RD 778/1998

Aquest treball de tesis titulat com "**Efectes cerebrals de la restricció del creixement intrauterí i la seva prevenció en un model animal**" és presentada per Míriam Illa Armengol per a optar pel grau de Doctor en medicina de la Universitat de Barcelona, incloent la menció de "Doctor Internacional", sota la direcció del Professor Eduard Gratacós Solsona i el Dr. Francesc Figueras Retuerta. La tesi s'ha estructurat seguint la normativa per a la tesi de doctorat com un compendi de publicacions.

Els projectes inclosos en aquesta tesis han estat publicats, o recentment submesos per ser avaluats, en revistes internacionals d'alt factor d'impacte. A continuació es detallen els diferents treballs generats, especificant els detalls de publicació. Els dos primers han estat publicats, mentre que el tercer i últim treball ha estat enviat a a una revista d'alt impacter per a possible publicació:

1. Míriam Illa, Elisenda Eixarch, Emma Muñoz-Moreno, Dafnis Batalle, Rocío Leal-Campanario, Agnès Gruart, José María Delgado-García, Francesc Figueras, Eduard Gratacós. **Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models.** Fetal Diagnosis and Therapy. 2017 Jan 5

2. Míriam Illa, Elisenda Eixarch, Dafnis Batalle, Ariadna Arbat-Plana, Emma Muñoz-Moreno, Francesc Figueras, Eduard Gratacos. **Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction.** PLoS ONE. 2013 Oct 15;8(10):e76453

3.- Míriam Illa, Verónica Brito, Laura Pla, Elisenda Eixarch, Ariadna Arbat-Plana, Dafnis Batalle, Emma Muñoz-Moreno, Fatima Crispi, Ester Udina, Francesc Figueras, Sílvia Ginés, Eduard Gratacós. **Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction.** Fetal diagnosis and Therapy.

GUIÓ:

1.- INTRODUCCIÓ

1.1. Problemes en el neurodesenvolpament d'origen prenatal

Els trastorns neurològics són una de les complicacions perinatals més greus que afecten aproximadament 3/1000 nounats (Anon 2001). Actualment, es considera que en més del 70% dels casos el problema ja existia abans de l'inici del part (Jacobsson & Hagberg 2004). El dany cerebral d'origen prenatal inclou un ampli espectre d'entitats. Mentre que els casos més greus es manifesten clínicament com una encefalopatia hipòxic-isquèmica, hemorràgia intraventricular, leucomalàcia periventricular i paràlisi cerebral, les anormalitats més comuns derivats de dany cerebral d'origen prenatal s'acostumen a manigestar com a abnormalitats neurològiques subtils.

S'han descrit múltiples factors que poden afectar al neurodesenvolupament del cervell (Mwaniki et al. 2012). La prematuritat és una de les causes més freqüents d'alteració en el neurodesenvolupament, i encara més si a més va acompanyat d'insults succeïts durant etapa prenatal (Blencowe et al. 2013; Levinton et al. 2013). La hipòxia crònica a causa de la insuficiència placentària i la desnutrició prenatal són probablement les dues causes més importants a nivel mundial amb impacte en el desenvolupament neurològic d'origen prenatal. Clínicament, les dues situacions es manifesten com una restricció del creixement intrauterí (RCIU) (Borowicz & Reynolds 2010; Baschat & Hecher 2004), situació que es caracteritza per una reducció significativa en la taxa de creixement del fetus reslultant en un pes en néixer per sota del percentil 10. La RCUI afecta al voltant de 7-10% de tots els embarassos (Kady & Gardosi 2004) i la meitat d'aquests casos s'han associat a algun grau de dany neurològic (Fouron et al. 2001). En el període neonatal,

s'han reportat alteracions a determinades àrees del neurodesenvolupament, sobretot afectant àrees relacionades amb l'atenció, habituació, la regulació de l'estat, motora així com alteració en les competències social (Figueres et al. 2009; Feldman & Eidelman 2006). Estudis de seguiment han observat com fins i tot les formes més lleus de RCIU presenten un major risc de discapacitats cognitives i de comportament neurològic evidenciables en etapa infantil. De forma concreta, s'ha relacionat la RCIU amb trastorns de la memòria, dificultats d'aprenentatge, baix rendiment acadèmic, falta d'atenció i alteració a nivel psicosocial (Rodrigues et al. 2006; Geva et al. 2006a; Geva et al. 2006b; Alati et al. 2009; Larroque et al. 2001; Leitner et al., 2007; McCarton et al., 1996; Morsing et al. 2011; MJ et al. 2003; Tideman et al. 2007; Lund et al. 2012; Synnes et al. 2010), així com també un major risc de trastorn per dèficit d'atenció amb hiperactivitat (TDAH) (Heinonen et al. 2010).

1.2. Mecanisme fisiopatològic del dany neurològic

Com s'ha indicat anteriorment, la insuficiència placentària i la desnutrició prenatal són probablement les dos situacions clíniques més rellevants a nivel mundial relacionades amb la gènesis de RCIU (Baschat & Hecher 2004; Borowicz & Reynolds 2010). Per un costat, la insuficiència placentària ocasiona una disminució del subministrament d'oxigen i nutrients al fetus (Meschia G: Placenta intercanvi de gas respiratori i l'oxigenació fetal, en "Robert Creasy, Robert Resnik, Jay Iams, Charles Lockwood 2009"), mentre que la desnutrició afecta sobretot el transport de nutrients al fetus (Borowicz & Reynolds 2010). L'impacte d'ambdues situacions en el cervell en formació no han estat del tot ben caracteritzat.

Un dels objectius principals d'aquesta tesis és la d'esbrinar el mecanisme a través del qual succeix el dany neurològic així com el de caracteritzar els canvis estructurals subjacents específics per cada una de les situacions. Abans però és important tenir en compte que els insults prenatals estan interferint en un òrgan que es troba en desenvolupament. En aquest sentit, val a dir que el desenvolupament del cervell humà és un procés complex i dinàmic que s'inicia en etapes primerenques durant la gestació i continua després del naixement. La complexitat es basa en la integració dels diferents processos, incloent la generació, la diferenciació, la migració i l'organització de les neurones, seguit de la formació i maduració de les sinapsis, la mielinització i, finalment, l'establiment de xarxes neuronals i la connectivitat (Stiles & Jernigan 2010; Bourgeois, 1997). A més, la formació de sinapsis és en sí mateix un procés complex (Scheiffele 2003). Depenent del moment en la presentació i la severitat dels insults, més o menys esdeveniments clau del desenvolupament neurològic es veuran afectats, ocasionant al final un major o menor grau d'incapacitat neurològica.

Clàssicament, la hipòxia fetal ha estat considerada com un dels factor crítics amb repercussions negatives al cervell fetal (Pasternak & Gorey 1998). En aquest sentit, treballs previs han aportat evidències molt importants sobre els canvis estructurals cerebrals secundaris a la hipòxia, determinat com la severitat de les conseqüències de la hipòxia fetal variaven en funció de l'inici, la gravetat i l'extensió de la pròpia lesió hipòxica. Models animals d'hipòxia aguda han observat com aquesta es relacionava amb una reducció en el nombre de neurones, afectant sobretot a les cèl·lules de Purkinje del cerebel, cèl·lules piramidals de l'hipocamp i neurones corticals. Tanmateix el dany en la

substància blanca també s'ha descrit, manifestant-se com a lesions quístiques difuses a la zona periventricular (Rees et al. 1999). La hipòxia aguda però presentada en moments més tardans de la gestació, s'ha relacionat amb un patró d'afectació similar, encara que en menor extensió (Loeliger et al. 2003).

Per altra banda, els insults crònics i lleus relacionats amb la insuficiència placentària s'han relacionat amb canvis estructurals més subtils. Referent la substància blanca, s'ha observat com el dany és evidenciable en etapa prenatal (Nitsos & Rees 1990), però com aquesta lesió aparentment es resol durant l'etapa postnatal (Tolcos et al. 2011). Tot i la suposada normalització de la lesió a nivel de la substància blanca a llarg plaç, encara persisteixen certes alteracions funcionals (Reid et al. 2012), suggerint per tant l'existència d'algun grau d'alteració estructural difícilment identificable que les justificaria. Referent a la matèria grisa, encara que la gran majoria de neurones semblen sobreviure als insults intrauterins crònics i lleus, algunes poblacions es poden veure afectades (Rees et al. 2011). Neurones de l'hipocamp i l'escorça cerebral semblen ser especialment vulnerables a la RCIU amb una reducció del recompte de cèl·lules en les dues àrees (Liu et al. 2011; Tashima et al. 2001). En aquest context, últimes evidències apuntent que part important de l'afectació a nivel de la matèria gris podria correspondre a un dany a nivell de la connectivitat neuronal. En models animals d'insuficiència placentària, s'han descrit en etapa neonatal canvis a nivel tant de la densitat com de la morfologia de les espines dendrítiques juntament amb canvis a nivells de determinats receptors que participen a nivel de la sinapsis (Dieni & Rees 2003; Piorkowska et al. 2014). Tot i la rellevància que suposa aquesta troballa, no hi ha estudis a llarg termini que hagin avaluat l'alteració a nivel de la connectivitat i sinapsis a

nivel neuronal secundària a insults lleus i crònics com a possible substrat estructural de l'alteració neuroconductual i cognitiva que peristeix a llarg plaç.

Finalment, la restricció de nutrients a nivel fetal (desnutrició) s'ha relacionat amb problemes emocionals (ansietat), de la cognició i aprenentatge (Reyes-Castro et al. 2012; Akitake et al. 2015; Keenan et al. 2013). Estudis estructurals han determinat que les neurones del sistema límbic semblen correspondre a la población neuronal amb especial sensibilitat a la desnutrició fetal (Morgane et al. 2002).

En resum, malgrat totes aquestes evidències, encara no està clar quin és l'impacte real en el neurodesenvolupament de les dues situacions clíniques més rellevants relacionades amb la RCIU (insuficiència placentària enfront de la desnutrició). Quedaria per establir el mecanisme fisiopatològic subjacent al dany cerebral secundari a la insuficiencia placentària i la desnutrició fetal i la seva correspondència estructural que persisteix fins al període a llarg termini. Per totes aquestes raons, el Projecte 1 intenta esclarir el mecanisme fisiopatològic subjacent a la RCIU comparant l'efecte de les conseqüències deletèries a llarg plaç en un model animal d'insuficiència placentària enfront un model animal amb desnutrició prenatal.

1.3 Avaluació estructural mitjançant tècniques avançades

La nostra capacitat de detecció dels canvis estructurals subjacents secundaris a procesos prenatals crònics i lleus és molt baixa donada l'absència de canvis estructurals importants fàcilment identificables mitjançant tècniques diagnòstiques habituals (Rees et al. 2011). Per tant, la correcta identificació de

la lesió subjacent a aquest tipus de dany dependrà de la utilització de tècniqus diagnòstiques capaces d'identificar aquests canvis subtils.

A diferència de les tècniques d'histologia, les quals tenen diversos inconvenients com la invasivitat, la no possibilitat d'automatització ni la possibilitat d'estudiar tot el cervell, les tècniques d'imatge ofereixen la possibilitat d'estudiar tot el cervell d'una manera no invasiva, amb possibilitat d'estudiar el cervell tridemensionalment i a alta ressolució (Mori & Zhang 2006; Lodygensky et al. 2010). De totes les tècniques d'imatge disponibles, la ressonància magnética (RM) s'ha establert com una eina prometedora per a l'avaluació del desenvolupament del cervell humà en condicions normals i en situacions de patologia. Fins i tot, mitjançant la RM convencional s'han detectat canvis estructurals del cervell secundaris a la RCIU, en forma d'una disminució del volum cortical (Tolsa et al. 2004), en l'hipocamp (Lodygensky et al. 2008) així com diferències en el patró de desenvolupament cortical (Dubois et al. 2008; de Bie et al. 2011; Martinussen et al. 2005; Martinussen et al. 2009). No obstant això, gràcies a l'avanç significatiu de la RM en els últims anys, els canvis estructurals subjacents a la lesió crònica i lleu secundària a la RCIU s'estan començant a caracteritzar a gran detall. De forma global, la RM d'alta resolució ens aporta informació molt important sobre canvis microestructurals que afecten tant la substància blanca com gris. A més, ens aporta informació sobre els canvi a nivell de de la connectivitat mitjançant l'avaluació de les xarxes cerebrals (Lodygensky et al. 2010). La MRI de difusió (dMRI) és un mètode no invasiu basat en la mesura de la difusió de les molècules d'aigua en els teixits (Basser & Pierpaoli 1996). L'avaluació de la difusió de les molècules de l'aigua en el teixit ens proporciona informació indirecta sobre la

microestructura cerebral. Aquest tipus de RM s'ha utilitzat per avaluar la reorganització del cervell en resposta a la lesió cerebral, tant en el cervell en formació com en cervell adult (Neil et al. 2002; Nucifora et al. 2007). Tanmateix, s'ha utilitzat en models animals de RCIU demostrant canvis durant etapa neonatal tant després d'hipòxia aguda (Derrick et al. 2007; Drobyshevsky et al. 2007), però també després d'episodis d'hipòxia crònica (Eixarch et al. 2012). A part de l'avaluació de la difusió de l'aigua en teixit, amb la dMRI es poden reconstruir tractes de substància blanc que participen en els diferents circuits neurnals oferint la possibilitat d'estudiar diferents paràmetres relatius a tractes de substància blanca, com el número de tractes, l'estructura i l'organització dels tractes en diferents regions cerebrals (Nucifora et al. 2007). Finalment, mitjançant la connectòmica és possible estudiar el grau d'organització i complexitat de les xarxes neuronals globals del cervell (Bullmore & Sporns 2009). Evidència preliminars apunten com la RCIU es relacionaria amb una alteració a nivell de les xarxes neuronals que persisteix en etapa infantil (Batalle et al. 2012). Per tant, gràcies a l'avanç significatiu de tècniques de RM s'estan començant a caracteritzar els canvis a nivell de la microestructura neuronal (Lodygensky et al. 2010; Sizonenko et al. 2007), juntament amb canvis a nivell de l'organització de la xarxa cerebral com a possibles substrats estructurals de la lesió cerebral, evidenciats ja en etapes neonatal precoç. En aquest contexte, l'alteració a nivell de l'arquitectura de les xarxes neuronals s'ha suggerit com al substrat subjacent a alteracions neuroconductuals i de cognició relacionades amb la RCIU que persisteix al llarg de la infància (Fischi-Gómez et al. 2014; Batalle et al. 2012; Batalle et al. 2014; Muñoz-Moreno et al.

2016). Els Projectes 2 i 3 d'aquesta tesis utilitzen RM d'alta resolució per tal d'avaluar tots aquests canvis.

La correlació histològica de les troballes vistes per RM aproximaria una mica més el nostre coneixement als verdaders mecanismes que participen en la gènesis de la lesió durant el desenvolupament del cervell. Tècniques histològiques convencionals no han estat capaces de demostrar-nos els canvis estructurals subjacents a la lesió crònica i lleu que persisteix a llarg plaç en la RCIU. Tècniques focalitzades en l'avaluació d'alteracions a nivell de la connectivitat cel.lular potser ens aporten informació rellevant. A nivell cel·lular, l'alteració a nivell de la connectivitat pot ser avaluada mitjançant l'avaluació de marcadors clau involucrats en la connectivitat neuronal i de sinapsis. Com es va assenyalar anteriorment, la formació de sinapsis és complexa i implica la finalització amb èxit de molts processos, incloent la neurogènesi, migració de dendrites, arborització, i l'acoblament pre- i postsinàptica entre l'axó i la dendrita. Una interrupció en qualsevol d'aquests passos podria donar lloc l'alteració a nivell de la connectivitat neuronal. De fet, canvis a nivell de la sinapsis ha estat suggerida com l'alteració cel.lular estructural subjacent a estudis amb DTI (van den Heuvel et al. 2016; Dean et al. 2013). Els marcardos més freqüentment utilitzats per estudiar aquesta connectivitat cel.lular inclouen: avaluació de la morfologia dendrítica, densitat d'espines dendrítiques (DS), així com l'avaluació de receptors i proteïnes pre i postsinàptiques implicats en la transmissió sinàptica. Un conjunt de patologies neurològiques s'han trobat relacionades amb canvis a nivell de les espines dendrítiques (DS), tals com TDAH, autisme, síndrome X fràgil, alteracions intel.lectuals (Penzes et al. 2011).

A part d'aquests marcadors, les xarxes perineurals (PNNS), un dels component de la matriu extracel·lular que envolten les neurones, s'han descrit com un element important en la formació, manteniment i funcionament de les sinapsis (Wang & Fawcett 2012). Per tant, de manera similar a les DS, les PNNS s'han utilitzat com un marcador de la connectivitat sinàptica (Dzyubenko et al. 2016). Estudis recents han utilitzat aquest marcador histològic havent-se descrit com part de les abnormalitats estructurals de determinades patologies neurològiques com l'Alzheimer, esquizofrènia i l'epil.lèpsia es trobarien relacionades amb alteracions a nivell de les PNNs (Dzyubenko et al. 2016; Cabungcal et al. 2013). L'estudi de la connectivitat neuronal podria ser especialment rellevant estudiar-ho en determinades àrees claus, com pot ser l'hipocamp. Es sap que aquesta estructura participa en la formació de la memòria i la cognició en animals i éssers humans (Eichenbaum 2004; Nakashiba et al. 2009; Deng et al. 2010), a part de ser una estructura especialment vulnerable a l'efecte de la RCIU (Mallard et al 1999; Mallard et al., 2000; Lodygensky et al. 2008). En el Projecte 3 investigem alteracions estructurals a nivell cel.lular en l'hipocamp utilitzant marcadors sinàptics i de la connectivitat neuronal, com les PNNs i les espines dendrítiques.

1.4 Efecte de l'estratègia d'enriquiment ambiental en la RCIU

Actualment, només la duració de la lactància materna ha demostrat presentar un efecte neuroprotector en el contexte de la RCIU a terme (Rao et al. 2002). A part de la lactància materna, actualment estan sorgint altres teràpies prometedores en el contexte de malalties neurològiques, com l'enriquiment ambiental. L'enriquiment ambiental estratègia (EE) ha demostrat

exercir un efecte beneficiós sobre el CNS mitjançant una millora de funcions cognitives complexes (Rampón et al. 2000) i de la reactivitat emocional i l'estrès en models animals (Chapillon et al. 2002; Tarifas et al. 2013; Rosenzweig 1996). Aquesta millora funcional ha estat acompanyada per canvis a nivell de la connectivitat neuronal, incloent l'augment de l'arborització dendrítica, nombre d'espines, densitat sinàptica particularment en l'hipocamp (Fares et al. 2013; Rampon et al. 2000; Rampon & Tsien 2000). En el context de la RCIU, l'estratègia anomenada NIDCAP (estratègia basada en donar suport físic i emocional en el nadó prematur durant el període d'ingrés hospitalari) també ha demostrat induir una milloria tant funcional com estructural en nens amb RCIU prematurs greus (Als et al. 2012). Per altra banda, encara avui dia no s'ha avaluat l'efecte a llarg plaç d'aquesta estratègia en el contexte de RCIU nascuts a terme.

L'estratègia d'enriquiment ambiental es basa en l'estimulació del cervell mitjançant una estimulació de l'entorn físic i social i a nivell de funcions sensorials, motores i cognitives. Existeix evidència sobre l'efecte beneficiós d'aquesta estratègia en accelerar el desenvolupament i facilitar la recuperació de la funció (Rosenzweig 1996; Johnston 2009), inclòs després d'un accident cerebrovascular focal (Janssen et al. 2010). A més s'ha desmostrat com l'efecte d'aquesta estratègia aplicada en etapes precoces del neurodesenvolupament podria mostrar una major sensibilitat i per tant susceptibilitat als estímuls ambientals (Johnston 2009; Meaney & Aitken 1985). En Projecte 3, s'ha avaluat els efectes a llarg termini de l'estratègia d'enriquiment ambiental aplicat en etapes precoces postnatals en el context de la RCIU en un model animal d'insuficiència placentària.

1.5 Models animals de RCIU

La investigació en models animals encara continua essent necessària. Tot i així, la principal limitació en la investigació amb animals és l'extrapolació dels resultats obtinguts a l'humà. L'extrapolació serà més o menys fàcil depenent de l'espècie utilitzada i del mètode emprat per recrear la condició humana a estudiar.

En el contexte de l'estudi de la RCIU és important elegir una espècie animal que presenti un patró de neurodesenvolupament i una placentació similar a l'humà. Una de les espècies que presenta bastanta similitud en l'humà pel que fa a la placentació i al grau de desenvolupament neurològic és el conill (Ballesteros et al. 1993). El conill presenta una placenta hemodichorial (Carter 2007) i un desenvolupament cerebrals perinatal (van Marthens et al. 1975). Això contrasta amb alters espècies, com l'ovella que presenta una placenta epitheliochorial (Carter 2007) i el desenvolupament cerebral és bàsicament durant el període prenatal; en canvi, els rosegadors tenen un desenvolupament postnatal (Vuguin 2007; Rees i Inder 2005; Finlay 2008; Rees i Inder 2005).

Una altra qüestió a tenir en compte en la investigació bàsica relacionada amb el dany cerebral és la metodologia utilitzada en la reproducció de l'insult prenatal (RCIU). Els mètodes més utilitzats en la reproducció de RCIU fins ara s'han basat en la restricció d'aliments a nivell matern o en la reducció quirúrgica del subministrament de sang a la placenta (Schröder 2003; Vuguin 2007). La reducció de la ingesta materna s'han relacionat amb un descens del pes dels nounats, encara que sense objectivar-se cap efecte en la mortalitat fetal (Eixarch et al. 2011; Vuguin 2007). Per contra, els mètodes quirúrgics basats

en la lligadura selectiva dels vasos uteroplacentarias que irriguen la placenta (Bassan et al. 2000; Bassan et al. 2009; Eixarch et al. 2009) ocasionen una restricció de nutrients i d'oxigen aconseguint d'aquesta manera reproduir les característiques principals de la insuficiència placentària a l'observar-se un increment de la mortalitat fetal, canvis a nivell del Doppler i trastorns neuroconductuals durant el període neonatal (Eixarch et al. 2011; Eixarch et al. 2012).

Per totes aquestes raons, en aquesta tesi l'espècies animal seleccionada és el conill. Per al primer projecte, la RCIU s'induïrà mitjançant de dos esquemes diferents: mitjançant la restricció d'aliments a la mare amb l'objectiu d'aconseguir desnutrició fetal i mitjançant la lligadura dels vasos uteroplacentaris per tal de recrear una insuficiència placentària. Per a la resta de projectes (Projecte 2 i 3) s'utilitzarà la lligadura dels vasos uterplacentaris como a mètode per recrear la RCIU.

2. HIPÒTESIS I OBJECTIUS

HIPÒTESIS

Hipòtesi principal

La restricció del creixement intrauterí produeix canvis subtils a nivell de l'estructura cerebral que es relacionen amb transtorns neuroconductuals i cognitius que persisteixen a llarg termini. Una estratègia basada en l'enriquiment ambiental durant etapes primerenques del desenvolupament postnatal podrien millorar les conseqüències deletèries a llarg plaç tant a nivell funcional com estructurals.

Hipòtesis específiques

1. La gravetat de les disfuncions funcionals a nivell de l'ansietat, memòria a curt termini i l'aprenentatge que persisteixen a llarg termini està relacionat amb la gravetat de la restricció de creixement evidenciable en dos models animals: un animal amb insuficiència placentària comparat amb un altre model basat en la restricció d'ingesta materna.

2. Els canvis cerebrals a nivell de la connectivitat que persisteixen a llarg termini estan relacionats amb la gravetat de RCIU com es demostra en els dos models animals: un animal amb insuficiència placentària comparat amb un altre model basat en la restricció d'ingesta materna.

3. Canvis subtils a nivell de la microestructura cerebral que afecten a determinades àrees cerebrals així com alteracions a nivell de les xarxes cerebrals avaluats tots aquests canvis mitjançant RM de difusió es correlaciona amb les disfuncions cerebrals a nivell de l'ansietat, memòria a curt termini i l'aprenentatge en un model animal d'insuficiència placentària.

4. Canvis a nivell de la densitat en espines dendrítiques i a nivell de les xarxes perineurals a l'hipocamp corresponen als canvis cel.lulars subjacents a les disfuncions funcionals a nivell de la l'ansietat, memòria a curt termini i l'aprenentatge en un model animal d'insuficiència placentària.

5. L'estratègia d'enriquiment ambiental precoç millora els efectes deleteris funcionals i estructurals en el neurodesenvolupament en un model animal d'insuficiència placentària**.**

OBJECTIUS

Objectiu principal

Caracteritzar els canvis estructurals subjacents a les alteracions neuroconductuals i de cognició que persisteixen a llarg termini secundàries a la restricció del creixement intrauterí i avaluar com una estratègia basada en l'enriquimient ambiental pot atenuar les conseqüències deletèries en el neurodesenvolupament de la RCIU.

Objectius específics

1. Comparar les deficiències funcionals a llarg termini a nivell de l'ansietat, la memòria a curt termini i l'aprenentatge en dos models animals de RCIU: un model basat en la insuficiència placentària i un altre basat en la restricció materna d'aliments

2. Descriure els canvis de connectivitat cerebral subjacents a les deficiències funcionals a llarg termini en els dos models animals de RCIU (insuficiència placentària i restricció d'aliments materna) mitjançant l'ús de RM de difusió

3. Explorar la correlació entre paràmetres de difusió i de xarxes obtingudes mitjançant RM de difusió i les disfuncions funcionals a nivell de l'ansietat, la memòria a curt termini i l'aprenentatge en un model animal de RCIU basat en la insuficiència placentària

4. Explorar els canvis estructurals a nivell cel·lular mitjançant l'anàlisis de la densitat d'espines dendrítiques i de la matriu extracel.lular a nivell de les xarxes perineurals a l'hipocamp en un model animal de RCIU basat en la insuficiència placentària

5. Avaluar la milloria estructural i funcional a llarg termini després de l'aplicació de l'estratègia d'enriquiment ambiental en un model animal de RCIU basat en la insuficiència placentària

3. MÈTODES

Per tal d'assolir els objectius presentats, s'han previst tres projectes diferents que explicarem i detallarem a continuació. Inicialment explicarem la metodologia concreta i específica per cadascun dels tres projectes. A continuació detallarem la metodologia comuna pels tres projectes. Tots els projectes es porten a terme mitjançant l'ús d'animals. Per aquest motiu s'han seguit les regles i directrius aplicades des de la Comissió d'Ètica d'Animal d'experimentació de la Universitat de Barcelona i s'han passat els comitès específics. Per obtenir més informació, vegeu l'apèndix I, on s'han inclòs els documents del comitè d'ètica.

3.1. Projecte 1: Efectes de la desnutrició i de la insuficiència placentària en el desenvolupament neurològic en dos models animals de restricció de creixement intrauterí en el conill

Es tracta d'un estudi de laboratori controlat en el que s'han inclòs dues cohorts de conilles gestants New Zealand en les que s'han aplicat dos protocols d'inducció de RCIU diferents:

a. Una cohort de conilles es va incloure en el protocol de lligadura quirúrgica de vasos uteroplacentaris, reproduint d'aquesta manera una hipoperfusió placentària quirúrgic (PU), als 25 dies de gestació

b. L'altra cohort es va incloure en el protocol de reducció de la ingesta materna, induint-se d'aquesta manera una desnutrició fetal (UN) als 22 dies de gestació

A partir dels dos models, es van obtenir quatre grups diferents:

a. conills amb RCIU procedents del model PU

b. conills amb RCIU procedents del model UN

c. controls procedents del model PU

d. controls procedents del model UN

Intervencions:

- Inducció de la RCIU als 22 o 25 dies de gestació: PU o UN
- Naixement de les cries: cesària als 30 dies de gestació en el model PU o part vaginal als 31 dies de gestació en el model UN
- Avaluació neuroconductual en el primer dia postnatal (+ 1P) i en el període a llarg termini (+ 60-70 dies després del part)
- Sacrifici i recollida de mostres
- Ressonància magnètica (RM) de difusió i anàlisi de conectòmica

La metodologia aplicada es resumeix en la Figura 1.

Mesures:

a. Paràmetres de supervivència i de creixement

b. Dades funcionals:

i. En el període neonatal: habilitats motores, els reflexos i la sensibilitat olfactiva

ii. En el període a llarg termini:

- Camp obert: latència de temps en deixar el punt de partida familiar i el temps a la zona interna (segons)

- Reconeixement d'objectes: índex discriminatori (DI)

- De la prova Skinner: percentatge d'aprenentatge

c. Anàlisi de xarxes cerebrals:

i. Característiques globals: *average strength, global efficiency* i *local efficiency*

ii. Correlació entre les característiques globals i l'avaluació funcional a llarg termini

3.2. Projecte 2: Resultats funcionals a llarg termini i correlació amb la connectivitat regional cerebral mitjançant la ressonància magnètica de difusió i Tractografia en un model de restricció de creixement intrauterí en conilla gestant

Es tracta d'un estudi de laboratori controlat en el que s'ha inclòs conilles gestants de 25 dies de gestació en les que se els hi va induir una insuficiència placentària mitjançant la lligadura quirúrgica de vasos uteroplacentaris (model PU) obtenint els següents dos grups:

a. conills amb RCIU

b. conills controls

Intervencions:

• Inducció prenatal de la RCIU als 25 dies de gestació (PU)

• Naixement de les cries mitjançant cesària als 30 dies de gestació

• Avaluació neuroconductual en el període a llarg termini (+ 60-70 dies després del part)

• Sacrifici i recollida de mostres

• Ressonància magnètica (RM) de difusió i anàlisi de tractografia

La metodologia aplicada es resumeix en la Figura 2.

Mesures:

a. Paràmetres de supervivència i de creixement

b. En el període a llarg termini:

i. Camp obert: latència de temps en deixar el punt de partida familiar, número total d'àrees explorades, temps total d'exploració, velocitat de moviment, número d'àrees externes explorades, temps explorant àrees externes, número d'àrees internes explorades, temps explorant àrees internes, acicalament, exploració en bipedestació. A més, s'inclou la correlació de Spearman entre el pes en néixer i variables funcionals.

ii. Reconeixement d' objectes: Temps d'exploració de l'objecte localitzat a la dreta i esquerre, tant en la fase de Familiarització i la fase d'Avaluació i càlcul de l'Índex discriminatori (DI)

c. L'anàlisi de ressonància magnètica:

i. Anàlisi de difusió (*Voxel bases analysis*): anisotropia fraccional (FA), coeficients de linealitat, planaritat i esfericitat

ii. Correlació entre els paràmetres de difusió i avaluació funcional

iii. Tractografia: nombre total de fibres en el cervell; nombre de fibres en els circuïts de l'ansietat i de la memòria, incloent anàlisi bilateral i anàlisis dret i esquerra, correlació entre el nombre de fibres amb variables neuroconductuals i, finalment, FA de les fibres per cada circuït. A més, s'inclou una correlació de Spearman entre el pes en néixer i la proporció de fibres.

3.3. Projecte 3: L'estimulació postnatal precoç millora anomalies en la connectivitat en un model animal de restricció de creixement intrauterí

Es tracta d'un estudi de laboratori controlat en el que s'ha inclòs conilles gestants de 25 dies de gestació en les que se els hi va induir una insuficiència placentària mitjançant la lligadura quirúrgica de vasos uteroplacentaris (model PU) obtinguent cries amb un creixement intruterí restringit (RCIU) i cries control. Posteriorment van ser alletades per conilles dides. Després de finalitzar el període de lactància (> 30 dies postnatals), un subgrup dels animals RCIU es van estabular en un ambient enriquit (t-RCIU), mentre que els restants animals es van estabular segons metodologia habitual i estàndard. D'aquesta manera, a llarg termini es van obtenir tres grups diferents:

a. conills amb RCIU

b. conills controls

c. conills amb RCIU tractats (t-RCIU)

Intervencions:

• Inducció prenatal de RCIU als 25 dies de gestació (PU)

• Naixement de les cries mitjançant cesària als 30 dies de gestació

• Aplicació de l'estratègia d'enrequiment ambiental en un subgrup d'animals amb RCIU

• Avaluació neuroconductual en el primer dia postnatal (+ 1P) i al període a llarg termini (+ 60-70 dies després del part)

• Sacrifici i recollida de mostres

• Avaluació Histològica: densitat d'espines dendrítiques i xarxes perineurals

• Ressonància magnètica (RM) de difusió i anàlisi de conectòmica

La metodologia aplicada es resumeixen a la Figura 3.

Mesures:

a. paràmetres de supervivència i de creixement

b. Dades funcionals:

i. En el període neonatal: en general les habilitats motores, els reflexos i la sensibilitat olfactiva

ii. En el període a llarg termini:

- Camp obert: latència de temps en deixar el punt de partida familiar i el temps a la zona interna

- Reconeixement d'objectes: índex discriminatori (DI)

- De la prova Skinner: percentatge d'aprenentatge

c. Anàlisi de xarxes cerebrals:

i. Característiques globals: *average strength, global efficiency* i *local efficiency*

ii. Anàlisi regional en l'hipocamp: volum de l'hipocamp, mitjana de FA de l'hipocamp i FA mitjana de les fibres que passen per l'hipocamp (anàlisis de la dreta i esquerre)

d. Anàlisis Histològica:

i. Densitat d'espines dendrítiques (DS) a nivell de CA1 de la zona dorsal de l'hipocamp.

ii. Immunoreactivitat de les xarxes perineurals (PNNs) a l'àrea CA3 de l'hipocamp

3.4. Descripció de la metodologia comuna

3.4.1. Protocols d'inducció de la RCIU

Com s'ha esmentar anteriorment, pel primer projecte la restricció de creixement es va induir mitjançant dos esquemes diferents: i. Un model d'hiponutrició (UN); ii. Un model d'insuficiència placentària (PU).

a) El model d'insuficiència placentària (PU) es basa en la lligadura quirúrgica dels vasos uteroplacentaris seguint un protocol prèviament descrit (Eixarch et al. 2009). Breument, als 25 dies de gestació prèvia administració de tocolisis amb progesterona, profilaxis antibiòtica amb Penicilina i inducció anestèsica amb Ketamina i Xilacina, es realitza una laparotomia mitjana. Durant la cirurgia, el manteniment anestèsic es realitza amb anestèsia inhalatòria amb Isofluorà. Després de realitzar una laparotomia mitjana s'exterioritzen els dos corns uterins i es compten el número de sacs gestacionals de cada corn. De forma aleatòria, es procedeix a la lligadura dels vasos uteroplacentaris que irrigen cada placenta dels sacs d'un dels corns. El corn contra lateral serveix per produir els animals controls. La lligadura quirúrgica es realitza amb Seda 4/0 i es lligen un 40-50% dels vasos que irrigen cada placenta.

b) El model d'hiponutrició es basa en la restricció d'aliments a la mare gestant. La reducció d'ingesta s'inicia als 22 dies de l'embaràs reduïnt-se el 70% de la ingesta d'aliments basal (el que correspon a administrar 45 g / dia). Es manté la reducció fins al moment del part (Matsuoka et al. 2006).

3.4.2. Part i seguiment postnatal

El naixement de les cries en el model de PU succeeix als 30 dies de gestació mitjançant una cesària. En el model de UN el naixement és als 31 dies de gestació mitjançant un part vaginal. En el model de UN, si arribats als 31

dies sense inici de treball de part, a les conilles gestants se'ls hi administra oxcitocina per desencadenar el part. Les cries procedents del model de PU són alletades per una conilla dida, mentre que les cries de UN són alletades per la pròpia mare. Cada camada com a màxim es limita a 8 cries. Es manté alletament matern fins els 30 de dies postntals. Un cop deslletades les cries s'estabulen en grups de tres.

3.4.3. L'enriquiment ambiental

Després del deslletament (> 30 dies postnatals), un subgrup d'animals amb restricció de creixement (IUGR) s'estabulen seguint una estratègia d'enrequiment ambiental (EE) (t-IUGR, n = 15). El protocol d'EE utilitzat es basa en estudis previs en el conills (Baumans i Van Loo 2013) en la que s'indueix una estimulació tant sensorial, físic, cognitiu i social. Amb aquesta finalitat, els animals van ser allotjats en gàbies més grans (150 x 70 x 40 cm), comparat amb les gàbies estàndards (75 x 70 x 40 cm). A més, dins de les gàbies dels animals amb l'EE es posen diferents objectes inanimats (pont de fusta, boles de colors, maons) i diferents sabors d'aliments que es canvien cada dos-tres dies. L'estimulació social s'indueix mitjançant la col·locació dels animals en una habitació gran durant una hora dos cops per setmana permetent explorar lliurement el medi ambient i facilitant la interacció amb un dels investigador (M.I., L.P.). Aquest protocol es va mantenir durant 30 dies, fins al sacrifici dels animals.

3.4.4. Avaluació neuroconductual

a) Avaluació funcional durant el període neonatal:

Una cohort de cries procedents dels Projectes 1 i 3 es van avaluar durant el primers dies postnatal (+1P). En aquest moment, l'avaluació neuroconductual es va realitzar seguint la metodologia descrita anteriorment (Derrick et al. 2007; Tan et al. 2005), avaluant habilitats motores generals, reflexes i sensibilitat olfactiva i a on es detalla la sistemàtica aplicada. Concretament en aquest test s'avalua to, locomoció, reflexes, coordinació de xuclar i empassar i sensibilitat olfactiva. Per a cada animal, la prova va ser gravada en vídeos. Cada variable puntua en una escala de 0 a 3 (0 = pitjor i 3 = millor), a excepció de to en la que l'avaluació (0-4) es va fer segons l'escala Ashworth (Damiano et al. 2002). Els avaluadors van analitzar cada exploració de forma off-line a doble cec (MI, LP).

b) Avaluació funcional en període a llarg termini:

Entre els dies 60 i 70 després del part, l'avaluació de l'aprenentatge, l'ansietat i la memòria es va realitzar en els animals procedents dels tres projectes. L'aprenentatge es va avaluar mitjançant el test d'Skinner. La caixa Skinner es va construir com es detalla en Leal-Campanar et al. (Caixa per al condicionament operant i aprenentatge instrumental per a conills, 2012. nombre d'inscripció a Espanya: P2001231369). El protocol aplicat va ser adaptat de la metodologia descrita anteriorment (Zworykinas et al. 1997) seguint un programa de reforçament continu positiu. Una setmana abans de començar l'avaluació, els conills són privats d'aliments (~ 20 g / dia de pinso) per tal d'augmentar la motivació per aconseguir el menjar. Després d'observar una reducció del 10-15% en el seu pes basal, s'inicïa la primera fase de modeldejat. Aquesta fase dura 5 dies i es basa en recompençar cada avançament que

realitza l'animal tant a la menjadora com a la palanca. Després de 2 dies de descans, es va dur a terme la fase pròpiament de testat amb una duració de també 5 dies més. El premi/recompença en aquesta fase només es dóna quan l'animal pressiona la palanca i presenta un moviment clarament dirigit cap a la menjadora. En aquesta fase, el criteri d'aprenentatge es considera quan s'observa aquesta conducta almenys tres vegades en una sessió. Totes les sessions duren 10 minuts i es registren i avaluen després per dos examinadors cecs (M.I., L.P.).

Després de la prova Skinner, els animals se'ls va permetre descansar durant 2 dies abans de continuar amb la prova de camp obert (OFBT) i la tasca de reconeixement d'objectes (ORT), respectivament. El OFBT avalua les activitats de locomoció i exploració que competeixen amb la por, l'ansietat i l'atenció (Bouet et al. 2003; Kowalska et al. N.d; Walsh & Cummins 1975). La ORT avalua la memòria declarativa a curt termini, específicament reconeixement (Olton & Feustle 1981), així com la capacitat d'atenció (Cowan et al. 1999) i es basa en la tendència dels rosegadors per explorar nous estímuls durant més temps en comparació amb estímuls familiars (Dere et al. 2006; Ennaceur & Aggleton 1997; Mumby 2001). Prèviament, les dues proves van ser adaptades per a la seva aplicació en conills. Per a aquest propòsit, es va dissenyar un camp obert (140 cm x 140 cm) envoltat per parets de plàstic opaques (alçada 40 cm). En primer lloc, es va avaluar l'OFBT. Per tal d'avaluar directament i sense interferències l'ansietat que pot experimentar l'animal en el contacte amb l'OFBT es va decidir no habituar els animals al camp obert (Treit et al. 1993). Després de l'OFBT, els animals es retiren del camp obert i en 30 a 60 minuts es tornen a col·locar al camp per avaluar l'ORT. Les dues proves es

van aplicar entre les 10 del matí a 5 pm i després de cada sessió el camp obert es neteja amb un 10% d'etanol. L'habitació està aïllada de so i a baixa il·luminació. Per minimitzar la interferència deguda al contacte humà, cada sessió és gravada en vídeo i avaluada posteriorment.

L'OFBT va ser dissenyat i utilitzat d'acord amb el procediment prèviament descrit (Walsh & Cummins 1975). En el projecte 2, l'àrea de prova es va dividir en 36 àrees de 23x23 cm considerant-se les 4 zones centrals com a àrea interna i els quadrats restants com a zona perifèrica. En els Projectes 1 i 3, el camp obert es va dividir en 9 àrees, 1 interna i 8 perifèriques. La prova s'inicia embolcallant als animals amb una roba que els hi és familiar i es deixen al punt de sortida (a prop d'una de les parets laterals). El test s'inicia en aquest moment i dura 10 minuts. S'enregistren múltiples paràmetres, tal com es detalla en cada projecte. L'ORT es va adaptar segons Ennaceur i Delacour et al. (Ennaceur & Delacour 1988). En lloc d'utilitzar un estímul visual, es va preferir utilitzar un estímul basat en olor mitjançant la col·locació de peces de fruita (poma o taronja) a l'interior de caixes de plàstic perforades, ja que la sensibilitat olfactiva està més desenvolupat en conills que no pas la visual (Ennaceur 2010). La prova es divideix en dues fases consecutives. En primer lloc, durant 5 minuts es deixa a l'animal perquè explori les dues caixes que s'han deixat a l'interior del camp obert. Aquestes caixes contenen el mateix estímul olfactiu (poma), constituint la fase primera fase, la fase de familiarització. Després, el conill es retorna a la seva gàbia durant 30 minuts. Llavors, un dels objectes es canvia i és reemplaçat per un nou estímul (taronja) i l'animal es torna a col·locar de nou al camp obert durant 5 minuts més (fase testat). Es considera que l'animal explora els objectes si s'hi apropa en <1cm, presenta moviment de

vibrisses i dirigeix directament el nas cap a les caixes. S'enregistra el temps que passa registrant ambós caixes en les dues fases (fase de familiarització i fase de testat) (segons). Finalment, es calcula l'índex de discriminació (DI), que representa la capacitat de discriminar els objectes nous:

$$DI = \frac{temps\ explorant\ l'objecte\ nou - temps\ explorant\ l'objecte\ familiar}{temps\ explorant\ l'objecte\ nou + temps\ explorant\ l'objecte\ familiar}$$

Una memòria conservada es considera quan el DI> 0, mentre que un DI ≤ 0 indica problemes en la memòria a curt termini. Els animals que no exploren un dels objectes en la fase de familiarització o cap dels objectes en la fase de testat almenys una vegada, seran exclosos de l'anàlisi (de Bruin & Pouzet 2006). En el Projecte 1 i 3, l'OFBT i l'ORT es van avaluar mitjançant l'ús d'un software específic (Panlab, Aparell Panlab Harvard, Regne Unit). En el Projecte 2, l'anàlisi va ser realitzat per dos observadors cecs (M. I. i A. A.) sense l'ús d'aquest software.

3.4.5. La recollida de mostres

Després de les proves funcionals a llarg termini, els conills es van anestesiar amb ketamina 35 mg / kg i xilazina 5 mg / kg per via intramuscular i es van sacrificar amb una sobredosi endovenosa de pentobarbital sòdic (200 mg / kg). Immediatament, els cervells es van fixar mitjançant una perfusió intravenosa (cor o caròtida comuna) amb solució salina tamponada amb fosfat (PBS) seguit de paraformaldehid (PFA) o formalina. Els cervells dels Projecte 1 i 3 (excepte els cervells inclosos en el protocol d'avaluació de la densitat d'espines dendrítiques) es van perfondre amb un 10% de formalina tamponada

a través d'un cateterisme cardíac. Després d'això, es van separar els cervells de l'òs i es van deixar en solució de formalina tamponada al 10% durant una nit. En els cervells del Projecte 3 inclosos en el protocol d'avaluació d'espines dendrítiques la perfusió via cor es va realitzar amb un 2% de PFA seguit de 10 minuts d'immersió en aquesta solució. Finalment, els cervells del Projecte 2 es van sotmetre a perfusió a través de les artèries caròtides comunes. Inicialment es va perfondre amb PBS seguit de paraformaldehid al 4%. Després es va separar el teixt cerebral de l'òs i es van deixar en immersió amb paraformaldehid al 4% durant 48 h.

3.4.6. Avaluació de ressonància magnètica

Un subconjunt dels animals que es van avaluar funcionalment en el període a llarg termini van ser seleccionats a l'atzar i van incloure's per la realització de la ressonància magnètica (RM).

a) Adquisició per ressonància magnètica

Es va utilitzar un escàner de ressonància magnètica animals 7T (MRI BrukerBioSpin GmbH). L'adquisició es va realitzar seguint dos esquemes diferents:

- En els animals de l'UN procedents de Projecte 1 i en tots els animals inclosos en el Projecte 2, es va realitzar una adquisició en T1 (MDEFT): Temps d'eco (TE) = 3,5 ms, Temps de repetició (TR) = 4000 ms, gruix de tall 0,7 mm, 70 talls coronals, matriu en el pla adquisició de 184 x 188 i camp de visió (FOV) de 28 x 28 mm^2, resultant en una dimensió del voxel de 0,15 x 0,15 x 0,7 mm^3. Les

imatges de difusió (DWI) es van adquirir cobrint diferents direccions de gradient.

- En el Projecte 2, la DWI es va adquirir utilitzant una seqüència de difusió estàndard cobrint 126 direccions de gradient amb un valor b de 3000 s / mm^2 juntament amb una referència (b = 0) de la imatge. Altres paràmetres experimentals van ser: ET = 26 ms, TR = 250 ms, gruix de tall = 0,7 mm, 70 talls coronals, matriu d'adquisició al mapa de 40 x 40, FOV de 28 x 28 mm^2, resultant una dimensió voxel de 0,7 x 0,7 x 0,7 mm^3. El temps d'exploració total d'ambdós adquisicions va ser de 13 h 56 m 40 s.
- En els animals UN del Projecte 1, es va utilizar una seqüència de difusió cobrint 30 direccions de gradient amb un valor b de 3000 s / mm^2 juntament amb una línia de base (b = 0 s / mm^2 imatge). Altres paràmetres experimentals van ser: ET = 26 ms, TR = 250 ms, gruix de tall 0,7 mm, 70 talls coronals, matriu en el pla adquisició de 40 × 40, FOV de 28 × 28 mm^2, el que resulta en una dimensió voxel de 0,7 × 0,7 × 0,7 mm^3. El temps d'exploració total de tots dos adquisicions va ser de 4 h 51 min.

- Els animals PU procedents de Projecte 1 i tots els animals inclosos en el Projecte 3, les imatges anatòmiques es van realitzar en seqüència T2 mitjançant un adquisició ràpida amb seqüència de relaxació (RARE). Després d'això, la DWI es van adquirir utilitzant una seqüència de difusió cobrint 30 direccions de gradient amb un valor b de 3000 s / mm^2 juntament amb una imatge de referència (b = 0 s / mm^2). Altres paràmetres experimentals van ser: ET = 26 ms, TR = 250 ms, gruix de tall 0,7 mm, 70 talls coronals, matriu

d'adquisició al mapa de 40 × 40, FOV de 28 × 28 mm^2, el que resulta en una dimensió voxel de 0,7 × 0,7 × 0,7 mm^3. El temps d'exploració total de tots dos adquisicions van ser de 6 h 58 min.

b) Preprocessament de la RM:

Com a primer pas, cada cervell va ser segmentat per mitjà d'un software implementat a Matlab 2011a (El MathworksInc, Natick, MA, EUA), similar al que s'ha descrit anteriorment (Eixarch et al. 2012). El Tensor de la RM de difusió es va estimar en cada voxel dins de cada màscara de cervell (Fillard et al. 2007). Posteriorment, es van calcular un seguit de mesures per tal d'avaluar la difusió: anisotropia fraccional (FA) i els coeficients de linealitat, planaritat i esfericitat (Basser & Pierpaoli 1996). Només en el Projecte 2, l'orientació (ODF) de cada voxel es va reconstruir seguint un enfocament Q-Ball (Descoteaux et al. 2007), mitjançant un algoritme determinista implementat en MedINRIA 1,9 (Toussaint et al. 2007) (pàgina web Inria Sophia Antipolis, disponible a www-sop.inria.fr/asclepios/ programari / MedINRIA /. Consultat el 2013 1 setembre). En els projectes 1 i 3, Difusió Toolkit (http://trackvis.org/dtk/; data de l'última entrada: August 2015) es va utilitzar per estimar la imatge de difusió (DTI), tenint en compte una anisotropia fraccional (FA) amb llindar de 0,1.

c) Anàlisi de la RM en els projectes 1 i 3:

c.1. Parcel·lació del cervell

Només els animals de Projecte 1 i 3, la parcel·lació del cervell es va realitzar de forma automàtica gràcies a l'atles digital de RM en conill (Muñoz-Moreno et al. 2013; Tristan A & Arribasi J 2007). La transformació d'elasticitat

va ser de aplicat a les etiquetes de ROI, l'obtenció d'una parcel·lació de cada cervell en 60 ROIs. La coherència entre la T1 i la parcel·lació basada en RARE es va avaluar en el projecte 1 mitjançant l'escaneig d'un subjecte usant les dues modalitats. La parcel·lació obtinguda a partir de les dues imatges es van comparar, observant uns resultats similars en els dos casos (Dice Coeficient = 0,97) (Muñoz-Moreno et al. 2013). Per tal d'alinear les etiquetes obtingudes per a cada subjecte en el T1 o T2, el registre entre la imatge de la difusió i la imatge en T1 o T2 es va realitzar amb IRTK (www.doc.ic.ac.uk/~dr/ programari /; data d'últim accés: August 2015) (Studholme et al 1999). Es van incloure un total de 44 regions per a cada subjecte (vegeu la Taula 1), cadascun d'ells considerats com un node de xarxa cerebral.

c.2. Extracció i anàlisi de les xarxes

La xarxa cerebral de cada subjecte es va extreure per mitjà d'un algoritme com s'ha descrit prèviament (Batalle et al. 2014), que defineix un Eij de xarxa entre dos nodes si hi ha almenys una connexió (tracte) a partir d'un node i acabant en un altre. Per tal d'assignar pesos a cada Eij vora, hem considerat la anisotropia fraccional mitjana (FA) al llarg de totes les fibres que connecten cada parell de regions d'i i j (Batalle et al. 2014). Es va obtenir la FA (FA-w) de cada subjecte i calculant les característiques de xarxa (Rubinov & Sporns 2010). En particular, es va avaluar la infraestructura, integració i segregació de cada xarxa. En el Projecte 3, la mitjana FA de les regions de l'hipocamp es va estimar per a cada hemisferi, així com la mitjana FA dels tractes reconstruïts que creuen les regions de l'hipocamp.

d) Anàlisi de la RM en el Projecte 2:

d.1. VBA:

Només en els animals inclosos en el projecte 2, es va avaluar l'anàlisi de difusió de cada voxel (VBA) per tal d'identificar canvis regionals en paràmetres relacionats amb la difusió. Aquest anàlisi consisteix en la normalització de tots els volums a un volum de referència amb posterior comparació dels valors obtinguts en el mateix voxel de tots els volums normalitzats. El registre dels volums de DWI a la referència es va dur a terme per mitjà d'un algoritme DTI-específica (Muñoz-Moreno & Martín-Fernández 2009; Alexander et al. 2001; Lee et al. 2009; Van Hecke et al. 2010; Martín-Fernández et al. 2007). Una vegada que les imatges estan alineades amb la referència, es pot suposar que els voxels en el mateix lloc en totes les imatges enregistrades pertanyen a la mateixa estructura, i per tant, es poden comparar. Posteriorment es van calcular diferents paràmetres de difusió, incloent la FA i els coeficients de linealitat, planaritat i esfericitat, entre els controls i la restricció del creixement intrauterí. L'objectiu principal de l'ús de VBA en aquest estudi va ser explorar i suggerir possibles relacions en tots els possibles canvis estructurals subjacents a les alteracions funcionals en el model de RCIU. En conseqüència, vam decidir establir un llindar de $p = 0,01$ i deliberadament vam decidir no realitzar la correcció per comparacions múltiples. A més es va calcular la correlació de Spearman entre els paràmetres de difusió i els resultats funcionals en cada voxel per identificar quines regions estaven relacionades amb els canvis observats en l'avaluació neuroconductual i cognitiva. Per tal d'evitar el biaix secundari al cervell “template” utilitzat per fer aquest anàlisis i per augmentar la fiabilitat dels resultats obtinguts, el procediment de VBA es va repetir utilitzant

cada subjecte com la plantilla, considerant només diferències significatives aquelles que es repetient en tots els registres.

d.2. Anàlisi de la connectivitat:

L'anàlisi de connectivitat dins de l'àrees específiques del cervell implicades en l'ansietat, l'atenció i la memòria a curt termini es van avaluar mitjançant l'avaluació de les fibres reconstituïdes que creuaven àrees específiques del cervell descrites com a àrees importants per la funció a estudiar (ansietat, memòria, atenció). Aquests tractes de fibres es van obtenir de la reconstrucció de cada voxel/ODF utilitzant el programa MedIndria. Depenent de les àrees cerebrals inclosos en l'anàlisi que hem definit dues xarxes principals del cervell:

- Circuït d'ansietat. La selecció de les àrees involucrades en aquesta funció es va basar en l'evidència prèvia qu es té que la regulació de l'atenció i la reactivitat emocional depèn en la interacció correcta entre el tronc cerebral, sistema límbic i àrees corticals (Duncan et al. 1996; Merker 2007). Dins del sistema límbic, l'amígdala i l'hipocamp es van incloure donat al seu paper reconegut en la por i l'ansietat (Butler et al. 2012). A més, es van seleccionar diverses àrees corticals (frontal, temporal, escorces cingulada) i ganglis de la base (cos estriat i el tàlem), per la seva relació amb l'atenció i l'emoció (Butler et al. 2012; Haver & Calzavara 2009; Toft 1999; Coca & Cauda 2011; Tromp et al. 2012). D'altra banda, algunes d'aquestes àrees del cervell han estat identificades com a components del circuït de Papez que ha estat proposat per a exercir un paper important en l'emoció (Papež 1995). De forma arbitrària vam definir el "circuït de l'ansietat" com aquell circuït que inclou totes aquelles fibres

de substància blanca que passa a través de l'amígdala i la formació de l'hipocamp, i que, a més, passa a través d'almenys una de les següents estructures: cos estriat, el tàlem, l'escorça prefrontal, escorça temporal o escorça cingulada.

- Circuït de memòria a curt termini. Es van seleccionar àrees del cervell proposades per participar en la memòria a curt termini. Per aquest motiu es va incloure: l'hipocamp (Squire, 1992; Vanelzakker et al. 2008), formació de l'hipocamp (Battaglia & Pennartz 2011; Zola-Morgan & Squire 1990), l'escorça perirrinal (Otto & Eichenbaum 1992) i tàlem (Watanabe & Funahashi 2012). De forma arbitrària es va definir el "circuït de la memòria" com aquell circuït que inclou totes aquelles fibres de substància blanca que passa a través de la formació de l'hipocamp i que, a més, passa a través d'almenys una de les següents estructures: hipocamp, tàlem, prefrontals o escorces temporals.

L'anàlisis d'aquests dos circuïts inclouen un procés de delineació manual de les àrees de matèria gris en la imatge anatòmica en T1, delineant àrees corticals (prefrontal, cingulada, temporal), putamen, nucli caudat, el tàlem, l'amígdala, l'hipocamp i formació de l'hipocamp (Figura 2, panell 4). Amb la combinació d'aquestes regions amb l'anàlisis de Tractografia es van extreure els tractes de substància blanca implicats en els dos circuïts. La mesura de la connectivitat dins de cada xarxa es va avaluar aplicant dos mètriques quantitatives diferents: 1) nombre de tractes dins de cada xarxes ajustades pel nombre total de fibres en cada cervell i 2) mitjana de la FA en els tractes que participen en ambdues xarxes. Per a ambdues xarxes, es va analitzar la connectivitat de cada circuït tenint en compte tant les fibres de l'hemisferi dret i

esquerre junts (anàlisi bilateral), o analitzant el dret i esquerra de forma seperada. A més, es va avaluar també la correlació de la relació de tractes i la mitjana de la seva FA amb els resultats funcionals ajustat per sexe.

3.4.6. Avaluació de la histologia

Només els animals inclosos en l'últim projecte (Projecte 3) van ser avaluats histològicament en el període a llarg termini després de realitzar l'avaluació funcional i la RM.

a) Avaluació de l'espina dendrítica:

15 a 20 de les dendrites basals de cada hemisferi es van avaluar a partir de la zona dorzal de CA1 de l'hipocamp mitjançant el sistema gene gun tot utilizant una pistola "gene gun" (Bio-Rad) (Grutzendler et al. 2003). La zona CA1 va ser seleccionada donat que es tracta de l'àrea de l'hipocamp que reb les principals connexions d'entrada (Takács et al 2012; Spruston 2008). Es va calcular la densitat de les espines dendrítiques (DS), és a dir el nombre d'espines per µm. Per veure la metodologia específica en la preparació de les mostres així com en els criteris utilitzats per avaluar-ho es remet al lector a publicacions prèvies (Brito et al. 2014).

b) Xarxes perineurals:

L'expressió de les xarxes perineurals (PNNs) es va analitzar utlitzant l'anticòs lectina Wisteria floribunda (WFA). Es va avaluar la densitat mitjana de tinció (contacte / µm2) a nivell de la zona CA3 de l'hipocamp. De manera similar al que s'ha observat en treballs previs (Hylin et al. 2013), es va decidir analitzar la densitat de tinció de les PNNs en aquesta àrea de l'hipocamp, ja que es va observar una major quantitat de tinció WFA en comparació amb

altres àrees com l'àrea CA1. Breument, el bloc congelat que conté ganglis basals es va fixar amb Tissue-Tek, es va tallar el bloc en seccions transversals 20?m de gruix amb un criostat, i es va recullir sobre un portaobjecte de vidre recobert de gelatina. Totes les seccions es van bloquejar primer amb sèrum boví al 2% durant 1 h, seguit d'una incubació durant la nit a 4 ° C amb l'anticòs Wisteria floribunda lectina (1:20, Sigma). Després dels rentats, es va afagir l'anticòs secundari conjugat amb estreptavidina 488 Alexa Fluor (1: 200, Invitrogen). Després de la incubació, es van fer rentats, i es va tenyir amb Hoechst 33.258 (1: 1000, Thermofischer), es va muntar en portaobjectes i amb Fluoromount-G (Sigma). Les neurones marcades es van localitzar a la zona CA3 mitjançant una delineació manual (ROI) i es van adquirir les imatges amb un microscopi confocal de rastreig (Leica confocal SP5, 40 × / 1,3 Oli DES M27). L'anàlisi i processament d'imatges es va realitza mitjançant el software ImageJ. De cada animal es van seleccionar tres imatges representatives i es van calcular la densitat mitjana d'immunomarcatge (contacte / μm2).

3.4.8. Estadística

Per a les variables quantitatives, la normalitat es va avaluar mitjançant la prova de Shapiro-Wilk i la homocedasticitat mitjançant el test de Levene. Els resultats s'expressen com la mitjana i la desviació estàndard (SD) per a les variables normals; mentre mediana i el rang interquartil (IQR) es van utilitzar en les variables no normals. En les dades neonatals, les variables quantitatives normals es van analitzar mitjançant la prova t-test, mentre que les variables distribuïdes no normals es van analitzar amb la prova no paramètrica de Kruskall-Wallis. Per a les variables categòriques, es va utilitzar la prova de chi-

quadrat. En les dades a llarg termini, les comparacions estadístiques entre grups es van realitzar mitjançant models lineals generals (GLM) i es van ajustar per gènere. La interacció de grup (casos i controls) i el gènere es va incloure per en el model, però ja que no es va mostrar cap efecte significatiu es van excloure del model final. En variables no-normals o en les que no es complia el criteri d'homogeneïtat de les variances, abans d'aplicar el GLM, es va aplicar el logaritme a aquestes variables. La significància s'ha declarat en $p < 0.05$ (sense corregir). S'ha utilitzat en aquest treball de tesis els paquets estadístics SPSS 19.0 (SPSS Inc., Chicago, IL, EUA) i STATA13.0.

Només en el Projecte 1, per tal de valorar la severitat entre els dos models (UN i PU), es va calcular la diferència de la mitjana (mitjana del resultat en els controls menys el resultat del RCIU) per cada variable funcional i per cada model, així com també es va calcular el seu intèrval de confiança del 95% (IC).

Només en el Projecte 2, amb l'anàlisis de Voxel based (VBA) es van obtenir mapes cerebrals volumètris demostrant diferències significatives (no corregida $p = 0,01$) per les variables de la difusió (FA, linealitat, coeficients de planaritat i esfericitat). A més, també es va calcular la correlació d'Spearman d'aquestes diferències amb les variables funcionals (r), expressant correlacions positives i negatives. Totes les correlacions es van realitzar ajustant per sexe. L'anàlisi d'imatges, el processament i la regressió es va realitzar mitjançant el software Matlab 2011a (El MathworksInc, Natick, MA, EUA).

4. RESULTATS

4.1. Projecte 1: Efectes de la desnutrició i de la insuficiència placentària en el desenvolupament neurològic en dos models animals de restricció de creixement intrauterí en el conill

- Els resultats d'aquest projecte han estat publicats a la revista "Fetal Diagnosis and therapy", l'any 2017.
- I han estat presentat en els següents congresos:

  - 12è Congrés Mundial de Medicina Fetal 2013. Marbella, Espanya; Juny de 2013. Presentat com un Poster. Autors: Illa M, Eixarch I, D Batalle, Muñoz-Moreno E, Arbat-Plana A, F Figueres, Gratacós E.

  - 24º Congrés Mundial ISUOG, Barcelona, Espanya; setembre de 2014. Presentat com una comunicació oral. Autors: Illa M, Eixarch I, Muñoz-Moreno M, Batalle D, F Figueres, Gratacós E.

4.1.1. Població d'estudi

Es van incloure un total de 52 fetus (20 controls i 32 RCIU) en el model de la PU, 38 dels quals eren vius en el part (19 controls i 19 RCIU), mentre que un total de 38 fetus es van incloure en el grup d'UN (20 controls i 18 RCIU), tots ells nascuts vius. Tots els animals van ser considerats aptes per ser avaluats a llarg termini, tot i això a causa de problemes tècnics, l'Skinner i l'OFBT no estaven disponibles en tots els animals d'ambdós models, pel que s'inclogueren 23 animals del model de PU i 25 del model de UN a la prova d'Skinner, i 19 animals del model de PU i 32 animals del model UN en el test d'OFBT. L'ORT

es va intentar en tots els animals avaluats en l'OFBT. No obstant això, només 17 animals del model de PU i 23 animals del model de UN varen ser adequats per a ser inclosos en l'anàlisi, ja que no cumplien els criteris preestablerts (de Bruin i Pouzet 2006). Després del sacrifici, 16 animals del model de PU i 14 del model d'UN van ser inclosos per ser avaluats mitjançant RM.

4.1.2. Paràmetres de supervivència i de creixement

La mort fetal va ser estadísticament major en els casos procedents del model de PU en comparació als seus respectius controls (44 vs 5%, p <0,001). No es va observar cap mort fetal en el model de l'UN. La taxa de mortalitat postnatal no va diferir entre els casos i controls en tots dos models (42 vs 32%, p = 0,55 en PU; 6 vs 5%, p = 0,94 a l'UN, casos enfront de controls, respectivament). Pel que fa al pes al néixer, tots dos models van tenir un efecte similar, observant una disminució del pes en néixer que va ser significatiu en comparació amb els seus respectius controls (vegeu la Figura 4). El grau de restricció del creixement induïda pels dos models va ser similar (pes en néixer en PU: 30,23 g (SD 12.08); pes en néixer a UN: 51,92 g (SD 7,57)). El pes al néixer dels dos models de RCIU es trobaven per sota del percentil 10 de creixement, segons corves normalitzades pels dos models (percentil 10 de PU: 33 g; percentil 10 de UN: 53 g).

A llarg plaç, no hi ha diferències en el pes (1444 g (SD 136) vs. 1589 g (SD 376), p = 0,24 en PU; 1378 g (SD 101) vs. 1442 g (SD 111), p = 0,17 en el model UN, casos enfront de controls, respectivament) ni en la distribució de gènere (% de les femelles: 55 vs 62%, p = 0,73 en PU ; 33 vs. 31% al model UN, casos enfront de controls, respectivament) en aquests models.

4.1.3. Dades funcionals

En el període neonatal, els casos de tots dos models van mostrar pitjors resultats en gairebé tots els paràmetres (vegeu la Taula 2). Aquestes diferències van ser més pronunciades en el model de PU, com es mostra a la Figura 5, on la diferència de mitjanes i el seu IC del 95% per a cada variable funcional van ser majors en el model de PU.

A llarg termini, els animals avaluats funcionalment no van presentar cap abnormalitat motora que podria haver interferit en l'execució de les tasques de comportament neurològic. En el test d'Skinner, una menor proporció dels RCIU del model PU van aconseguir criteris d'aprenentatge en comparació amb els seus controls (30 vs 77%, p = 0,03, RCIU enfront dels controls, respectivament), mentre que no es van observar diferències en el model de l'UN (44 vs 56%, p = 0,56, RCIU enfront de controls, respectivament). Pel que fa als resultats OFBT, els casos de tots dos models presentaren un augment significatiu de la latència en deixar el punt de partida familiar, així com exploraven menys, considerant tant les àrees internes i externes. Referent a l'ORT, els RCIU d'ambdós models presentaven una disminució del DI en comparació amb els seus respectius controls (vegeu la Taula 3). Un cop més, aquestes diferències van ser més pronunciats en el model de PU (Figura 6).

4.1.4. Resultats de l'anàlisis de xarxes cerebrals

Els RCIU van presentar una disminució significativa en l'anàlisis de les xarxes cerebrals en comparació amb els seus respectius controls, sobretot en el model PU (Figura 7). A més, es van observar correlacions significatives entre

les característiques de xarxa globals i els resultats neuroconductuals, especialment en les variables de l'OFBT (veure Taula 4).

4.2. Projecte 2: Resultats funcionals a llarg termini i correlació amb la connectivitat regional cerebral mitjançant la ressonància magnètica de difusió i Tractografia en un model de restricció de creixement intrauterí en conilla gestant

- Els resultats d'aquest projecte han estat publicats la revista PLoS ONE, l'any 2013.
- I s'han presentat en els següents congressos:

  - 10è Congrés Mundial de Medicina Fetal, Malta; Juny de 2011. Presentat com una comunicació oral. Autors: Illa M, Eixarch I, Batalle D, Arbat A, Acosta-Rojas R, Figueres F, Gratacos E.

  - 43rd European Brain and Behaviour Society Meeting, Sevilla, Espanya. Setembre de 2011. Presentat com un pòster. Autors: Illa M, Eixarch I, Batalle D, Arbat A, Acosta-Rojas R, Figueres F, Gratacos E.

  - 22 Congrés Mundial ISUOG, Copenhaguen, Dinamarca. Setembre de 2012. Presentat com un Oral poster. Autors: Illa M, Eixarch I, Batalle D, Muñoz-Moreno E, Arbat-Plana A, Figueres F, Gratacos E.

4.2.1. Població d'estudi

Un total de 69 fetus van ser inclosos en el moment de la inducció de la PU (23 controls i 47 RCIU), 49 dels quals estaven vius en el part (19 controls i 30 RCIU). Després del naixement, 5 controls i 17 casos es van morir dins de la

primera setmana de vida, per tant, 14 controls i 13 casos van aconseguir el període a llarg termini. Dels 27 animals que es van avaluar funcionalment, 6 animals (3 controls i 3 casos) van ser exclosos de la RM a causa d'anomalies identificades en el teixit secundàries al procés d'extracció i manipulació, quedant d'aquesta manerea un total de 21 animals en la mostra final (11 controls i 10 casos). Pel que fa al test ORT, 7 controls i 8 RCIU van complir els criteris prèviament establerts (de Bruin i Pouzet 2006). Després del sacrifici, 21 cervells fixats (11 controls i 10 casos) es van incloure en l'avaluació per RM.

4.2.2. Paràmetres de supervivència i de creixement

La taxa de mortalitat fetal i neonatal va ser major en els RCIU (mort fetal: 17,4% vs 36,2%, p = 0,08; mort neonatal: 26,3% vs 56,7%, p = 0,01, controls enfront casos respectivament). El pes al naixement va ser significativament menor en els casos en comparació amb els controls (49,54 g (SD 5,85) vs. 38,34 g (SD 5,36), p?0.001). A 70 dies postntatals no es van observar aquestes diferències en quan al pes (2747 g (SD 190) vs. 2626 g (SD 489), p = 0,41). Tampoc hi havia cap diferència en el moment de realitzar-se l'avaluació postnatal (71 (IQR 3) vs. 70 (IQR 4) dies postnatals, p = 0,099) ni en la distribució de gènere entre els grups (63,6% vs. 50% de dones, p = 0,425).

4.2.3. Avaluació funcional a llarg termini

En el seguiment postnatal, no es van observar abnormalitats motores com ara parèsia o espasticitat en cap dels grups que dificultessin l'execució dels tests funcionals. A l'OFBT, els animals amb RCIU van presentar una reducció significativa de les activitats exploratòries (augment de la latència en

deixar el punt de partida, velocitat reduïda i bipedestació). A més, els RCIU van presentar una reducció significativa en el temps que restaven a l'interior del camp obert i una reducció significativa del número d'`àrees explorades, tant internes com externes (Taula 5). Pel que fa a l'ORT, no es van trobar diferències en el temps de l'exploració dels objectes en la fase de familiarització (objecte dret: 9.50 s (SD 5,31) enfront de 7,85 s (SD 0,04), p = 0,585; objecte esquerra: 6.00 s (IQR 6,75) vs, 2,00 s (IQR 11,00), p = 0,69, controls enfront casos respectivament). Per contra, els controls en la fase de testat van passar significativament menys temps explorant l'objecte familiar en comparació amb els casos (3,63 s (SD 1,92) enfront de 6,71 s (SD 1,80), p = 0,011, controls enfront casos, respectivament). A més, el DI va ser significativament menor en els RCIU comparat amb els casos (Figura 8). Es va observar una correlació significativa en gairebé tots els paràmetres funcionals i el pes al naixement (Taula 6). La concordança entre les avaluacions realitzades pels dos avaluadors es va explorar utilitzant el coeficient de correlació intraclasse que va demostrar una bona fiabilitat (mitjana: 0.941).

4.2.4. Ressonància magnètica

a) Anàlisi regional: Voxel based analysis

Quan es va aplicar l'anàlisi VBA, es van trobar diferències estadísticament significatives en la distribució de FA, amb una FA disminuïda en els casos en comparació amb els controls en múltiples estructures, incloent regions corticals (ínsula i àrea temporal) i a nivell de la substància blanca subventricular. El coeficient de linealitat va ser també menor en els casos en múltiples àrees, incloent les regions corticals (ínsula, i àrees temporals,

prefrontals i occipitals), tàlem, colícul superior, formació de l'hipocamp i fímbria de hipocamp. El coeficient de planaritat va mostrar augment dels valors en l'escorça occipital i el tàlem en conills RCIU, però valors disminuïts en l'ínsula i en els hemisferis cerebel·losos. Finalment, es va observar un augment a nivell del coeficient d'esfericitat en l'ínsula i la substància blanca subventricular (Figura 9).

b) Correlació entre la difusió i els resultats funcionals

El mapa de FA mostra correlacions amb les variables funcionals, especialment a nivell de les variables de l'OFBT, en múltiples àrees del cervell (Figura 10 i Taula 7). Pel que fa a estructures de substància gris, la FA canvia a nivell de hipocamp i a nivell de la formació de l'hipocamp i en l'escorça del cingulat i del temporal, seguit per l'escorça prefrontal, el tàlem i putamen. A destecar que l'amígdala presenta una correlació significativa amb dues de les variables que estan fortament relacionades amb l'ansietat (nombre d'àrees explorades i el temps a nivell de la zona interna). Dins de les estructures de substància blanca, les comissures i corona radiata van mostrar més correlacions. Correlacions similars es varen observar en altres mesures de difusió (linealitat, esfericitat i planaritat) (Figura 11, Figura 12 i Figura 13).

c) Tractografia: anàlisi de connectivitat

L'anàlisi del nombre total de tractes de substància blanca reconstruïdes per tot el cervell no va diferir entre els grups (14,775 (SD 2332) vs. 13,921 (SD 2148), p = 0,371, controls enfront casos). No obstant això, els casos van mostrar una tendència a presentar una proporció menor de fibres incloses en

els dos circuïts avaluats; essent aquesta diferència estadísticament significativa en l'hemisferi esquerre per a les dues xarxes (Figura 14 i 15). La Taula 8 mostra els coeficients de correlació entre el percentatge de fibres i els resultats de les proves funcionals. Pel que fa al circuït de l'ansietat, l'hemisferi esquerre es va correlacionar significativament amb gairebé totes les variables de l'OFBT, mentre que el circuït de la memòria no va assolir cap significació estadística. Finalment, no es van observar diferències significatives en la mitjana FA dels dos circuïts, tot i que hi va haver una tendència a presentar una FA menor en els casos en comparació amb els controls, especialment en el circuït de l'ansietat (Taula 9). Un cop més, es va explorar la correlació entre el pes al néixer i la proporció de fibres en les dues xarxes observant correlacions significatives en els dos circuïts (Taula 10).

4.3. Projecte 3: L'estimulació postnatal precoç millora les anomalies en la connectivitat en un model animal de restricció de creixement intrauterí

- Els resultats d'aquest projecte s'han enviat per a valorar ser publicats a la revista Fetal Diagnosis and Therapy.
- I a més s'han presentat en els següents congressos:

  - 14è Congrés Mundial de Medicina Fetal, Creta, Grècia; Juny de 2015. Presentat com una comunicació oral. Autors: Illa M, Eixarch I, Muñoz-Moreno I, Batalle D, L Pla, Figueres F, Gratacós E.

- 4ª Conferència Internacional del creixement fetal, Barcelona, Espanya. Setembre de 2015. Presentat com un pòster Oral. Autors: Illa M, Eixarch I, Muñoz-Moreno I, D Batallé, Pla L, F Figueres, Gratacós E.

* *Treball guardonat com el "Millor poster presentat en la conferència".*

- 26è Congrés Mundial ISUOG, Roma, Itàlia; Setembre de 2016. S'ha presentat com un poster oral. Autors: Illa M, Brito V, Eixarch I, Pla L, Muñoz-Moreno I, Serrano g, Figueres F, Ginés S, Gratacos E.

- Part d'aquests resultats es van incloure i es va presentar en: 26è Congrés Mundial ISUOG, Roma, Itàlia; Setembre 2016. Presentat com una comunicació oral. Autors: Illa M, Brito V, Eixarch I, Pla L, Muñoz-Moreno I, Serrano g, Figueres F, Ginés S, Gratacos E.

* *Aquest treball va ser guardonat amb el premi "Jove Investigador".*

4.3.1. Població d'estudi

Un total de 243 fetus van ser inclosos en el moment de la inducció de RCIU (60 controls, 183 casos), dels quals 141 varen néixer vius (55 controls i 86 casos). Postnatalment, 42 controls i 57 casos van morir dins de la primera setmana de vida, aconseguint arribar a llarg plaç un total de 13 controls i 29 casos amb 14 casos sense tractament i 15 que reberen tractament (t-RCIU). En l'avaluació funcional a llarg plaç es van avaluar un total de 13 controls. La resta de RCIU es van incloure en l'avaluació funcional (RCIU: n = 14; t-RCIU: n = 15). Respecte l'ORT, només 11 controls, 14 RCIU i 13 t-RCIU cumpliren els criteris establerts (de Bruin i Pouzet 2006). Després s'inclogueren en la RM. Referent l'avaluació histològica, 4 cervells es van incloure en l'avaluació DS, mentre que

16 cervells (controls: n = 4; RCIU: n = 6; t-RCIU: n = 6) van incloure's a l'avaluació de les PNNS.

4.3.2. Paràmetres de supervivència i de creixement

La mort fetal va ser major en el grup de RCIU en comparació amb els controls (55% vs. de 8%, p <0,001), sense observar-se diferències entre RCIU i els RCIU amb tractament (45% vs 55%, p = 0,125). El pes al naixement va ser significativament menor en els RCIU que en els controls (33,6 g (SD 1,3) vs. 46,7 g (SD 1,3), p <0,001), sense diferències els dos grups de RCIU (RCIU 34,3 g (SD 2,4)) vs. t- RCIU (33,6 g (SD 1,3), p = 0,871). Als 60 dies de vida postnatal, no es van observar diferències entre els grups en el pes ni en la distribució dels sexes.

4.3.3. Dades funcionals

En etapa neonatal, els RCIU mostraren pitjors resultats en gairebé tots els paràmetres d'avaluació funcional en comparació amb els controls, sense observar diferències significatives en comparar els RCIU i els RCIU que serien tractats a llarg plaç (Taula 11).

A llarg termini, els animals amb RCIU van presentar alteracions funcionals en comparació amb els controls, mostrant una tendència a presentar un aprenentatge reduït tot i no ser estadísticament significatiu, i una alteració a nivell de la memòria i de l'ansietat de firma significativa (inferior DI i menys temps explorant l'àrea interna) en comparació amb els controls. Per contra, els animals RCIU tractats (t-RCIU) van presentar una millora en la memòria i l'ansietat en comparació amb els RCIU no tractats (Figura 16).

4.3.4. Anàlisi de les xarxes cerebrals

L'anàlisi de les característiques globals de xarxa evidencien una disminució significativa en l'"*average*" i en la "*eficiència global i local*" en els RCIU no tractats en comparació amb els controls. En els animals RCIU tractats (RCIU) es va observar un augment significatiu en totes aquestes variables (Figura 17). L'anàlisi regional no va revelar diferències significatives en el volum cerebral de l'hipocamp dins dels diferents grups (Taula 12). No obstant això, l'anàlisi de la FA en l'hipocamp i de la FA dels tractes que passen per l'hipocamp van ser significativament menors en els RCIU en comparació amb els controls. Per contra, els animals RCIU amb tractament (t-RCIU) van mostrar un increment significatiu en aquests paràmetres comparat amb el grup RCIU sense teràpia (Figura 18).

4.3.5. Avaluació histològica

Els animals RCIU sense teràpia presentaren una disminució significativa en la densitat de DS, en comparació amb els controls, mentre que el valor de DS augmentava de forma significativa en els animals RCIU tractats (t-RCIU) comparat amb els RCIU no tractats (Figura 19a). De la mateixa manera, els animals RCIU sense teràpia presentaren una disminució significativa en les PNNs en comparació amb els controls, amb una tendència a augmentar i normalitzar-se en els RCIU amb tractament (t-RCIU) comparat amb RCIU sense teràpia (Figura 19b).

5. DISCUSSIÓ

5.1. Projecte 1: Efectes de la desnutrició i de la insuficiència placentària en el desenvolupament neurològic en dos models animals de restricció de creixement intrauterí en el conill

Els resultats d'aquest projecte demostren com la RCIU ja ben sigui per restricció de nutrients aïlladament o en conjunció amb una reducció de l'oxigen indueixen alteracions funcionals en etapa neonatal i a llarg plaç i que es correlacionen amb canvis estructurals a nivell de les xarxes cerebrals. Aquestes diferències van ser més pronunciades en el model PU, el que suggereix una relació entre la severitat de l'estímul lesional prenatal i les conseqüències a nivell del desenvolupament neurològic.

Dades perinatals

Tots dos models van induir una reducció del pes en néixer, però només el model de la PU es va relacionar amb un augment de la mortalitat fetal i postnatal precoç, el que vindria aquest model a reproduir formes greus de la RCIU a l'humà (Kady & Gardosi 2004). Aquests resultats segueixen troballes prèvies en el mateix model de PU, en el que es descriu canvis a nivell de paràmetres Doppler (Eixarch et al. 2011). De la mateixa manera, estudis anteriors en models basats amb la UN objectiven una reducció en el pes al néixer sense objectivar-se un augment de la mortalitat neonatal (Vuguin 2007; Eixarch et al. 2011; Akitake et al. 2015).

Resultats funcionals

Dades neuroconductuals prèvies mostren com tots dos models de RCIU es correlacionen amb les alteracions neuroconductuals neonatals tant a curt com a llarg termini, especialment per als casos de PU. Durant el període neonatal, estudis clínics han relacionat problemes neuroconductuals amb la RCIU, incloguent tant retards psicomotors com la paràlisi cerebral en els casos més greus (Baschat 2014; O'Callaghan et al. 2011), i en canvi alteracions més subtils s'han descrit en formes menys greus de RCIU (Figueres et al. 2011; Creu-Martínez et al. 2011). Seguint aquesta línea, estudis amb animals també han trobat resultats similars. Alteracions tant a nivell motor com a nivell sensitiu s'han descrit en models d'hipoperfusió placentària severa i aguda (Derrick et al. 2004; Drobyshevsky et al. 2006), mentre que els transtorns funcionals són més lleus en models d'hipoperfusió placentària crònica (Eixarch et al. 2012). A l'altre extrem, la restricció de nutrients de forma moderada s'ha relacionat amb anomalies lleus (Akitake et al. 2015; Belluscio et al. 2014). La correlació entre les disfuncions funcionals amb la severitat de lesió prenatal també s'ha observat a llarg termini (Levine et al. 2015; Murray et al. 2015). Models animals previs incloguent tant models basats amb UN com amb models de PU van relacionar-se a llarg plaç amb un major grau d'ansietat, depressió i amb alteracions a nivell de la interacció social (Belluscio et al. 2014; Robinson et al. 2005), així com a nivell de l'aprenentatge, la memòria a curt termini i problemes d'atenció (Akitake et al. 2015; Reyes-Castro et al. 2012; Valadares et al. 2010; Delcour et al. 2012). D'aquesta manera, els nostres resultats, de forma similar al descrit a la literatura, donen suport a la idea que la gravetat i el tipus d'insult es troba relacionat amb les alteracions funcionals posteriors.

Resultats de la xarxa cerebral

L'estudi de les xarxes cerebrals demostren com ambdós models presenten una alteració a nivell de la infraestructura, sobretot en el model de PU. Aquesta alteració de la FA s'ha relacionat amb alteracions a nivell axonal, de la densitat neuronal, i la mielinització de les fibres (Sen & Basser 2005). D'aquesta manera aquesta troballa ens suggereix que les xarxes cerebrals en el RCIU estarien relacionades amb connexions menys madures. Aquests resultats estan en línia amb els estudis anteriors en els éssers humans (Fischi-Gómez et al. 2014) i en models animals (Batalle et al. 2014), que demostren com l'eficiència de les xarxes cerebrals a llarg plaç està reduïda en el contexte de RCIU. Aquesta alteració estructural a més es correlaciona amb els resultats funcionals. Tanmateix, la severitat en l'alteració en el neurodesenvolupament i la seva associació amb la reorganització del cervell sembla estar relacionada amb el grau de la lesió prenatal, amb efectes més notables en el model de hipoperfusió placentària (PU).

Avantatges i desavantatges

La principal avantatge d'aquest estudi és que correlaciona les conseqüències a llarg plaç de l'alteració en el neurodesenvolupament secundari a RCIU mitjançant l'ús de dos models animals en la mateixa espècie animal. L'espècie conill té certs avantatges sobre els rosegadors, ja que presenta un patró de maduració cerebral més pròxim a l'humà que no pas la rata-ratolí (Derrick et al. 2007). La principal limitació de l'estudi és la diferència en aspectes metodològics entre els dissenys de tots dos models. En primer lloc, els animals del model de PU van néixer als 30 dies d'embaràs mitjançant cesària, mentre que els animals del grup de l'UN se'ls va permetre tenir un part

vaginal als 31 dies de gestació. Tot i així, aquesta diferència fan els nostres resultats més transferibles a la pràctica clínica, ja casos de RCIU més greus es solen finalitzar abans i mitjançant la realització d'una cesària, mentre que els casos menys greus, en general neixen mitjançant un part vaginal. La diferència del temps al néixer però fa que els animals del model de PU pesin comparativament menys. Per últim, la diferència en l'alletament. El grup de UN va ser alimentat a través de la seva pròpia mare, mentre que el model de PU van ser alletats mitjançant una conilla dida. Amb la finalitat de limitar totes aquestes diferències, la comparació dels efectes de la RCIU en cada model es va realitzar amb els propis controls procedents de cada model. Per últim, pel que fa referència al mètode de tractografia se sap que per la tècnica utilitzada aquesta és menys robust en les zones de creuament de les fibres en comparació amb tècniques basades en imatges d'alta resolució angular. No obstant això, s'ha demostrat que la tècnica utilitzada podria reduir la variabilitat interindividual, sent més sensibles a la variància intergrup (Bastiani et al. 2012).

Conclusió

En general, aquest treball proporciona evidència que demostra que la reducció crònica de nutrients amb o sense reducció d'oxigen, fins i tot quan s'inicia en etapes posteriors de l'embaràs, té un impacte real en la programació del cervell. Les dades presentades en aquest treball reforcen el concepte que la mala nutrició durant la vida prenatal té un impacte en el desenvolupament neuroconductual i cognitiu (Dauncey & Bicknell 1999). A més, aquest estudi també demostra que la hipòxia crònica afegit a la desnutrició durant el període prenatal té un efecte més sever.

5.2. Projecte 2: Resultats funcionals a llarg termini i correlació amb la connectivitat regional cerebral mitjançant la ressonància magnètica de difusió i Tractografia en un model de restricció de creixement intrauterí en conilla gestant

Aquest projecte té com a objectiu caracteritzar les conseqüències funcionals i estructurals a llarg termini relacionat amb la RCIU en un model animal utilitzant tècniques avançades d'imatge.

Canvis funcionals a llarg termini

Els resultats de la OFBT possen de manifest que els conills amb RCIU presenten un major grau d'ansietat de forma similar al descrit en models de lesions agudes hipòxico-isquèmica en rata (Robinson et al. 2005; Koob et al. 1993; Lubics et al. 2005), i en els adolescents i adults humans amb antecedents RCIU (Alati et al. 2009; Vasiliadis et al. 2010). De la mateixa manera, els conills amb RCIU vàren presentar una disminució en conductes com l'acicalament, alteració que també s'ha relacionat amb nivells alterats de l'ansietat (Spruijt et al. 1992). Les dades derivades de l'ORT demostren que el model de conill RCIU presenta memòria a curt termini i trastorns d'atenció similar al que s'ha reportat en els éssers humans (Geva et al. 2006b). Els resultats obtinguts són comparables als obtinguts en rates després de l'oclusió unilateral de l'artèria uterina (Delcour et al 2012a; Delcour et al 2012b).

Anàlisi regional de la RM

El canvis estructurals a llarg termini més notables varen localitzar-se sobretot en la matèria gris, incloguent múltiples regions corticals (còrtex de la

ínsula, del temporal, del prefrontal i de l'occipitals així com també l'hemisferi cerebel•lós) i ganglis de la base (tàlem i hipocamp). De fet, la troballa d'alteració a nivel de l'escorça prefrontal i l'escorça entorrinal i l'hipocamp estan en línia amb resultats anteriors en què es descriu la susceptibilitat de les cries de rates embarassades amb restricció del creixement intrauterí després de l'oclusió prenatal de l'artèria uterina unilateral. Aquests canvis histològics inclouen una disminució del nombre de neurones, astrogliosis, un augment en les neurones GABAèrgiques i degeneració axonal difusa (Delcour et al 2012a; Delcour et al 2012b). L'alteració de DTI en la matèria gris s'ha relacionat amb canvis a nivell de l'arquitectura dendrítica de les cèl•lules piramidals (Neil et al. 2002; Sizonenko et al. 2007) que podria suggerir un deteriorament a nivell de la connectivitat d'aquestes estructures. Pel que fa a substància blanca, l'anàlisi regional de paràmetres DTI va revelar diferències significatives a nivell de l'esfericitat, de la FA i la linealitat a nivell de la fímbria de l'hipocamp i en la substància blanca subventricular en el grup de RCIU. Els valors de la FA estan estretament relacionats amb el procés de mielinització, objectivant-se un augment dels valors de la FA a mesura que el cervell va madurant en el període perinatal (Neil et al. 2002). Tanmateix, la disminució dels valors de FA en els tractes de substància blanca s'ha descrit prèviament després de la lesió hipòxic-isquèmia lleu i es correlaciona amb disminució del contingut de la mielina (Wang et al. 2009). Consistentment amb la disminució de la FA, els animals amb antecedent de RCIU van mostrar una disminució a nivell de la linealitat amb un augment a nivell de l'esfericitat que estan relacionats amb tractes de fibres menys organitzades en els tractes de substància blanca (Westin et al. 2002). Per tant, els nostres resultats donen suport a la hipòtesi

que la restricció del creixement intrauterí es relaciona amb una organització alterada a nivell de la substància blanca i amb el retràs a nivell de la maduració i que persisteix fins i tot en període llarg termini. Cal assenyalar que els canvis a nivell de la substància blanca són menys pronunciats en comparació amb les troballes en període neonatal obtingut en el mateix model animal (Eixarch et al. 2012). Una possible explicació podria ser secundària a la resolució de RM utilitzada. Una mida de vòxel de 0,7 x 0,7 x 0,7 mm^3 pot produir alguns efectes de volum parcial que poden obstaculitzar l'observació de diferències en algunes petites àrees del cervell. A banda de les limitacions metodològiques, la troballa d'un major número de diferències que afecten a la substància gris en comparació a la substància blanca pot indicar-nos que a llarg termini la plasticitat del cervell durant la infància i l'adolescència (Larvaron et al. 2007; Paus et al. 2001) és més eficient a corregir els dèficit a nivell de la substància blanca i menys en la substància gris. Això és similar a altres estudis (Tolcos et al. 2011; Wang et al. 2009). Els canvis regionals a nivell de la FA demostren correlacions significatives principalment en estructures de substància gris especialment relacionades amb l'ansietat, tal com l'hipocamp, còrtex prefrontal i cingulada (Daenen et al. 2001; Bannerman et al. 2004; Deacon et al. 2002; Emond et al. 2009; Spampinato et al. 2009; Miller et al. 2012). A més, els canvis en els paràmetres de difusió per RM de l'amígdala es van correlacionar amb el nombre d'àrees explorades i el temps a la zona interna en el test OFBT, dos variables estretament relacionades amb l'ansietat (Butler et al. 2012; Daenen et al. 2001). Pel que fa a les correlacions amb l'ORT, dins de les estructures de substància gris, es va observar una correlació significativa entre els canvis FA regionals a l'escorça cingulada i els resultats d'aquest test.

Diversos estudis experimentals en rosegadors han trobat que l'escorça cingulada juga un paper clau en la detecció de la novetat, l'atenció i la memòria en situacions de por (Vetere et al. 2011; Weible et al. 2012; Weible et al. 2009; Zhao & Zuo 2005), i qualsevol interrupció en aquesta estructura podria perjudicar la consolidació de la memòria (Einarsson i Nader 2012). Tenint en compte això, la persistència de d'ansietat mentre es realitza l'ORT no es pot descartar i podria d'aquesta manera interferir en la memòria. Aquest suggeriment està en línia amb els estudis clínics que han postulat que els problemes de memòria a curt termini observats en nens amb RCIU poden ser explicades per la manca de suficient atenció en lloc d'un dèficit en el processament de la informació en si mateixa (Geva et al., 2006a), impedint la funció de memòria a curt termini. Pel que fa a la substància blanca i els resultats del test ORT, les correlacions més consistents van ser a nivell de la comissura anterior i la corona radiata. Aquestes estructures de substància blanca tenen la funció de connectar diverses àrees del cervell que estan involucrades en la memòria i l'atenció (Douaud et al. 2011; Hillary et al. 2011; Yin et al. 2011). Contràriament a les hipòtesis originals, no es va observar cap correlació significativa entre determinades àrees de substància gris i l'ORT, com ara la formació de l'hipocamp, lòbul temporal i l'escorça prefrontal (Squire, 1992; Vanelzakker et al., 2008; Battaglia & Pennartz 2011; Otto & Eichenbaum 1992; Delatour & Witter, 2002). Aquestes troballes suggereixen que la pèrdua de memòria a curt termini induïda per RCIU podria dependre més de la connectivitat entre diferentes regions més que a canvis únics en determinades àrees de substància gris. Aquesta idea està d'acord amb els resultats anteriors en el que es referma la forta dependència de la formació de la memòria i de la

integritat de la xarxa prefrontal perirrinal-hipocamp-medial (Brown et al., 2010; Delatour & Witter 2002; Powell et al., 2004). En resum, aquests resultats confirmen parcialment les hipòtesis formulades en els estudis clínics en nens i adolescents amb RCIU, i a més proporcionen una nova visió sobre les anomalies estructurals subjacents a les alteracions neuroconductual i cognitives subjacents.

Anàlisi de la connectivitat

Els animals amb antecedent de RCIU van mostrar una disminució del nombre de fibres en les xarxes d'ansietat, atenció i memòria sobre el nombre total de fibres reconstituïdes. Aquestes diferències van ser estadísticament significatives en l'hemisferi esquerre, amb una tendència a la disminució de la seva FA en ambdues xarxes en l'hemisferi dret. A més, es va observar una correlació significativa entre la proporció de fibres en l'hemisferi esquerre en el circuït de l'ansietat i els resultats funcionals. Aquestes troballes estan en línia amb anteriors estudis de difusió en pacients amb trastorns d'ansietat i d'atenció o problemes de memòria. Canvis en la connectivitat a nivell del còrtex prefrontal i cingulat anterior i l'amígdala s'han correlacionat amb l'ansietat (Tromp et al. 2012; Kim & Whalen 2009; Modi et al. 2013) mentre que canvis microestructurals a nivell dels tractes de substància blanca fronto-estriatals que connecten l'amígdala i l'escorça prefrontal s'han descrit estar fortament relacionats amb el trastorn ADHD en nens i adolescents (de Zeeuw et al. 2011; Sarkar et al., 2012; Tamm et al. 2012; Wang et al. 2012a). Pel que fa a al circuït de memòria, la disminució de la FA s'ha observat en tractes substància blanca que connecten l'escorça temporal i l'hipocamp en nens (Ortibus et al. 2012) i en

la corona radiata en adults amb antecedent d'una lesió traumàtica lleu (Hillary et al. 2011). En aquests estudis, la disminució de la FA es va correlacionar amb els resultats de ORT en els nens, i amb atenció i deteriorament de la memòria en els adults. A més, els canvis a nivell de la substància blanca de l'hipocamp que connecta l'escorça entorrinal amb l'hipocamp es van correlacionar amb problemes de memòria declarativa en persones d'edat avançada (Rogalsky 2010; Wang et al. 2012b). La major part de les diferències observades en el nostre estudi es trobaven a l'hemisferi esquerre. Pel que fa al circuït de l'ansietat, l'hemisferi esquerre s'ha relacionat directament amb l'ansietat relacionada amb la por (Hardee et al., 2008).

En conjunt, els resultats d'aquest estudi donen suport a la afirmació que l'alteració a nivell de la connectivitat en regions implicades en l'ansietat, l'atenció i la memòria estan implicats en el deteriorament funcional associat amb la RCIU que persisteix fins al període pre-adolescents i suggereix la importància de completar la programació normal en la connectivitat per tal d'assolir un neurodesenvolupament normal. Les dades reportades mostren una disminució del nombre de fibres en combinació amb canvis més modestos en la FA. Aquests resultats són diferents als observats en el període neonatal (Eixarch et al. 2012), i donen suport a la idea que, a llarg termini, els canvis estructurals estan essencialment relacionades amb la distribució en lloc de presentar una alteració a nivell de la integritat de les fibres. Aquests resultats estan en línia amb anteriors treballs en els que s'objectiva com un retràs en la mielinització durant períodes crítics del desenvolupament poden ser restaurats al llarg del temps (Tolcos et al. 2011), encara que el patró de connectivitat roman alterat a llarg termini, com s'ha demostrat de manera consistent en

estudis humans i en estudis experimentals (Hagmann et al. 2010; Salami et al. 2003).

Consideracions metodològiques i limitacions de l'estudi

La metodologia utilitzada per dur a terme l'anàlisis de VBA i de la connectivitat mereixen una certa discussió. Pel que fa a l'anàlisi de la connectivitat, reconeixem que les xarxes definides en aquest estudi no han estat plenament validades. La definició de les xarxes s'ha realitzat segons evidència prèvia que demostra la implicació de totes les regions seleccionades en les funcions d'interès. Per altra banda, la mètrica utilitzada per quantificar l'anàlisis de tractografia no està estandarditzada, tot i que ha estat utilitzada prèviament en estudis en humans per caracteritzar els canvis en l'estructura cerebral en malalties del neurodesenvolupament, com ara ADHD (de Zeeuw et al. 2011), lesió perinatal cerebral focal (Roze et al. 2011) i leucomalàcia periventricular (Rha et al. 2011; Thomas et al., 2005). Només uns pocs estudis han utilitzat el nombre de fibres per avaluar la connectivitat dins d'àrees específiques del cervell (Rha et al. 2011; Thomas et al., 2005; Son et al., 2007). Pel que fa a l'anàlisis del VBA, l'ús d'aquest enfocament implica un poder estadístic més feble a causa de la gran quantitat de voxels avaluats (Lee et al. 2009). Això es compensa parcialment mitjançant tècniques de suavitzat després de registrar els volums DTI a la referència. Suavitzant els mapes DTI, s'aconsegueix reduir el nombre efectiu de comparacions múltiples i així millorar el poder estadístic (Lee et al., 2009). Una altra qüestió relativa al VBA és que el mètode requereix el registre de tots els subjectes en referència a un subjecte, i per tant l'elecció arbitrària d'aquest individu "referència" podria esbiaixar el

resultat (Lee et al. 2009). Aquest problema es va abordar repetint el VBA considerant cadascun dels subjectes com la referència. Finalment, no es van incloure les dades de ADC en l'anàlisi regional, ja que degut al procés de fixació es disminueix el contingut d'aigua en el teixit cerebral d'una manera no homogènia i, per tant, de forma impredictible (Sun et al. 2003), especialment en el teixit hipòxic (Sun et al. 2005). Des del punt de vista del disseny experimental, l'alta taxa de mortalitat durant la primera setmana després del part pot haver seleccionat els casos menys greus que aconsegueixen arribar al període "llarg termini", atenuant així el veritable impacte de la malaltia. Malgrat aquest biaix conservador, hem estat capaços de demostrar la presència de canvis estructurals i funcionals secundaris a la RCIU. Finalment, es va decidir incloure el gènere com un factor de confusió potencial en el nostre anàlisi ja que es recomana ajustar quan biològicament és plausible, com passa en molts processos neuroconductuals (Institute of Medicine (US) 2011).

Conclusions

En conclusió, el model animal utilitzat reprodueix les conseqüències funcionals i neurostructurals de la RCIU a curt termini que persisteixen fins a l'edat adulta. Mitjançant tècniques de difusió de RM s'evidencien alteracions en regions específiques del cervell implicades en la regulació de l'ansietat, l'atenció i la memòria i en les seves xarxes relacionades secundàries a la RCIU que es correlacionen amb els impediments funcionals a llarg termini. L'estudi proporciona evidència sobre els canvis estructurals subjacents a les anomalies del desenvolupament neurològic a llarg termini associats amb RCIU. Tanmateix, aporta evidència del valor potencial dels mètodes basats en difusió

per avaluar els canvis associats amb la reorganització del cervell que no són demostrables per tècniques d'imatge estàndard.

5.3. Projecte 3: L'estimulació postnatal precoç millora les anomalies en la connectivitat en un model animal de restricció de creixement intrauterí

Amb el tercer projecte hem demostrat com la RCIU presenta una connectivitat cerebral alterada tant a nivell global com a nivell cel•lular que persisteix més enllà de l'adolescència i que aquestes alteracions podrien substentar les discapacitats neuroconductuals observades en el nostre model animal. A més, hem demostrat com l'exposició a un ambient enriquit durant el període postnatal primerenc millora les disfuncions ocasionades per la RCIU.

Canvis estructurals del cervell

Les troballes obtingudes a través de la RM de difusió recolzen resultats anteriors obtinguts en el model animal de lligadura quirúrgica en el conill en el que es demostrava problemes a nivell de la infraestructura global de xarxa, amb alteració a nivell de la integració i la segregació (Batalle et al. 2014; Illa et al. 2017). Tanmateix, aquestes troballes ja havien estat descrites en estudis humans tant en etapa infantil com en l'adolescència (Fischi-Gómez et al 2016; Fischi-Gómez et al 2014; Muñoz-Moreno et al 2016; Batalle et al. 2012). A part dels canvis globals, en aquest projecte també s'ha realitzat un estudi regional a nivell de l'hipocamp donada la seva importància en determinades funcions com la memòria i la cognició tant en animals com en els humans (Eichenbaum 2004) així com per ser una àrea especialment vulnerable a la RCIU (Mallard et

al., 2000). L'anàlisi regional va mostrar tant una disminució de FA juntament amb una reducció de la mitjana de la FA de les fibres que passen a través de l'hipocamp, sobretot en l'hemisferi esquerre. Aquests resultats suggereixen la presència de connexions menys madures, ja que FA s'ha relacionat amb l'embalatge axonal, la densitat neuronal, i la mielinització de tractes de fibres (Sen & Basser 2005). La troballa que els canvis estiguin limitats en un dels dos hemisferis és coherent amb la idea que algunes funcions neuronals tendeixen a ser més dominants en un hemisferi que en l'altre (Duboc et al. 2015). En particular, l'hipocamp esquerre s'ha descrit que està relacionat amb la memòria i alteracions neuroconductuals en espècies animals, tant en el conill (Illa et al. 2013) com en rosegadors (Shipton et al. 2014; Hu et al. 2010).

Referent als resultats d'histologia es va observar una reducció significativa tant a nivell de la densitat d'espines (DS) com de les xarxes perineurals (PNNs) en CA1 i CA3 de les neurones piramidals de l'hipocamp, respectivament. Ambdós marcadors histològics (DS i PNNs) han estat involucrats en la regulació de la connectivitat sinàptica i la plasticitat (Yuste 2011; Harris & Kater 1994; Dzyubenko et al. 2016). De forma concreta, la troballa de la disminució a nivell de les DS van en línea amb resultats previs realitzats en fetus de conillet d'índies i d'ovelles després d'insults prenatals hipòxico-isquèmics tant aguds com crònics, en els que es van observar canvis a nivell de la DS i la morfologia juntament amb els canvis a nivell dels receptors sinàptics (Dean et al. 2013; Dieni & Rees 2003; McClendon et al. 2014; Piorkowska et al. 2014). Per altra banda, actualment hi ha un especial interès en l'avaluació de les PNNs en el contexte de determinades malalties específiques del cervell com l'Alzheimer, l'esquizofrènia i l'epilèpsia (Dzyubenko

et al. 2016; Cabungcal et al. 2013). Tot i la novetat d'aquest marcador histològic, encara no s'ha avaluat aquest marcador en el contexte de la RCIU. En condicions de normalitat, la PNNs sembla estar relacionada amb garantir en el cervell adult l'estabilitat de les connexions neuronals establerts (Wang & Fawcett 2012). Per tant, disminució de la densitat PNNs observada en els nostres animals amb RCIU en CA3 suggereix una alteració a nivell de la consolidació de les connexions a nivell de l'hipocamp, cosa que és coherent amb la disminució de la DS trobada en CA1. De fet, evidència preliminar suggereix que la reducció de les sinapsis expressat com la reducció de DS està associada amb una disminució a nivell de la formació de PNNs (Faissner et al. 2010).

Estratègia d'enriquiment ambiental

Els nostres resultats demostren per primera vegada que l'estratègia postnatal basat en l'estimulació precoç pot millorar el rendiment conductual i la connectivitat cerebral després de RCIU. Aquestes troballes van en línia amb resultats similars observats en estratègia de rehabilitació no invasiva en models de lesió neonatal hipoxico-isquèmica en rata (Jiménez et al. 2013) així com després d'exposició prenatal a l'alcohol (Hannigan i Berman 2000). Tanmateix, estudis amb animals han determinat el mecanisme a través del qual l'estratègia d'enrequiment ambiental actuaria, modulant la connectivitat cerebral (Rampon i Tsien 2000; Rampon et al. 2000). D'altra banda, les nostres dades van en línia amb l'evidència clínica que demostra com el programa NIDCAP (suport físic i emocional al nen prematur mentre el nen està ingressat a la unitat de cures intensives) està relacionada amb una milloria a nivell funcional i estructural (Als

et al. 2012). El nostre treball, a part de descriure un efectes positiu a nivell de la funció, també es va observar una recuperació de la connectivitat cerebral amb una millor funció de la xarxa i amb un augment a nivell de la densitat i de les PNNS. Aquests canvis a nivell cel•lular després d'EE també s'han demostrat a nivell de la DS (Jiménez et al. 2013) i de les PNNs en un model de rata de neonatal hipòxia-isquèmia i en un model basat en addicció (Slaker et al. 2016), respectivament.

Avantatges i desavantatges

Una de les principals limitacions de l'experimentació animal és la capacitat de transferibilitat a l'espècie humana. De tot les espècies animals disponibles, el conill presenta un patró de neurodesenvolupament similar al descrit en el humà en comparació amb altres espècies (Derrick et al. 2004). Pel que fa al model de RCIU, sembla que el model recrea la condició RCIU humana, en el que s'objectiva un increment de mortalitat associada així com els animals que arriben a llarg termini presenten alteracions funcionals similar al descrit en l'humà (Geva et al 2006a; Geva et al 2006b; Alati et al. 2009; Larroque et al., 2001; Tideman et al., 2007; Kady & Gardosi 2004). Pel que fa a l'avaluació histològica, cal tenir en ment que les espines dendrítiques són estructures amb elevada plasticitat i altament influenciables (Engert & Bonhoeffer 1999). No obstant això, l'entorn experimental redueix aquesta variabilitat. Per altra banda, altres característiques de la plasticitat sinàptica, com ara els patrons de morfologia etc..., així com anàlisi d'altres components presents a nivell de la matriu extracel.lular neuronal pot ser d'igual interès per ser avaluats en la RCIU. Tanmateix, l'estudi d'aquests marcadors histològics

serien interessants avaluar-los en altres àrees com per exemple en el còrtex (Dean et al. 2013). Finalment, causa de la grandària de la mostra, reconeixem que la potència estadística està limitada en algunes de les comparacions. Per tal de quantificar aquesta limitació en les variables, s'adjunta una taula on es mostra la diferència de mitjanes i dels riscos, segons s'escaigui, facilitant tanmateix el seu interval de confiança 95% (Taula 13).

Conclusions

Mitjançant la combinació de tècniques d'imatge avançada com és la RM de difusió amb anàlisis de la connectòmica conjuntament amb la utilització de marcadors histològics complexos s'ha pogut observar com la RCIU altera el patró normal de desenvolupament del cervell afectant llocs claus de l'activitat sinàptica. Aquestes deficiències a nivell de la connectivitat, ja sigui a nivell global o a nivell cel•lular que persisteixen fins al període a llarg termini poden explicar, almenys en part, la base dels trastorns del neurodesenvolupament associats amb RCIU. L'enriquiment ambiental en el període postnatal precoç podria alleujar l'efecte negatiu sobre el neurodesenvolupmanet d'insults presentats en etapes prenatals. En general, els nostres resultats reforcen la idea que determinats factors ambientals presentats en períodes crítics del desenvolupament neurològic interferirien en el neurodesenvolupament predisposant a l'individu a problemes de salut de per vida o fins i tot podrien millorar-la.

5.4. Discussió general

Aquesta tesi proporciona evidències sobre el mecanisme fisiopatològic i la correspondència neuroanatòmica subjacents als transtorns neuroconductuals i d'alteració cognitiva que persisteixen fins a l'adolescència secundària a la RCIU. Una comprensió més profunda dels efectes cerebrals de RCIU ens permet seleccionar teràpies amb capacitat potencial per millorar les disfuncions que persisteixn a llarg plaç. En aquest sentit, l'enriquiment ambiental aplicat en etapes precoces sembla ser una estratègia prometedora capaç de millorar les conseqüències del desenvolupament neurològic de RCIU.

Respecte el Projecte 1, l'objectiu principal ha estat el de comparar els efectes cerebrals de probablement les dues situacions més comunes relacionades amb la RCIU: la insuficiència placentària i la desnutrició. Ambdues situacions s'han relacionat amb problemes de desenvolupament neurològic que persisteixen fins al període a llarg termini. No obstant això, cap treball anterior ha avaluat l'impacte d'ambdues situacions en el mateix estudi tot utilitzant la mateixa espècie espècie. Les dades derivades d'aquest projecte suggereixen que la gravetat dels trastorns del neurodesenvolupament i la seva associació amb la reorganització estructural del cervell sembla estar relacionat amb el grau de la lesió prenatal, amb efectes més notables en el model de hipoperfusió placentària.

Referent als Projectes 2 i 3, s'ha pogut esclarir l'alteració estructural subjacent a l'alteració funcional que persisteix fins a l'etapa adolescent secundària a insuficiència placentària. Concretament, en el segon projecte, s'ha observat com la majoria dels canvis detectats per RM de difusió estaven adscrits a nivell de la substància gris, incloent múltiples regions corticals (ínsula, còrtex temporal, prefrontal i occipitals i a nivell de l'hemisferi

cerebel•lós) i nuclis de la base (tàlem i hipocamp), observant menys diferències a nivell de la substància blanca (fímbria de l'hipocamp i a nivell de la substància blanca subventricular). Per altra banda, mitjançant l'ús de la tècniques avançades de tractografia, es va observar una disminució en el nombre de fibres que participen en xarxes específiques relacionades amb l'ansietat i la memòria, sense observar diferències en el nombre total de fibres del cervell. Aquesta observació dóna suport a la idea que, a llarg termini, els canvis estructurals a nivell de substància blanca estan essencialment relacionats amb la distribució en lloc d'afectar la integritat de les fibres. A més, la correlació significativa entre els canvis de difusió de ressonància magnètica i els resultats funcionals observada ratificar el paper d'aquestes àrees específiques del cervell així com de les xarxes per a la funció que s'està avaluant i ens suggereix com, fins i tot, els canvis estructurals subtils que afecten aquestes àrees clau del cervell podrien conduir als transtorns neuroconductual i d'alteració cognitiva a causa de la RCIU. En aquesta línea, el Projecte 3 reforça evidència prèvia que suggereix com la correspondència neuroanatòmica de les discapacitats funcionals en la RCIU en el període a llarg termini podria estar relacionades amb canvis estructurals subtils del cervell. Per a aquest motiu, en aquest projecte l'alteració a nivell de la connectivitat es van explorar mitjançant l'anàlisis de xarxes cerebrals a nivell global gràcies a la RM de difusió. La correspondència de l'alteració de la connectivitat global a nivell cel•lular es va evidenciar mitjançant l'avaluació de marcadors clau involucrats en la connectivitat neuronal i la sinapsi, com ara la matriu extracel•lular i de l'espina dendrítica. Tots aquests resultats van en línia amb les evidències anteriors que suggereixen que els canvis estructurals secundàries a insults prenatals lleus i

crònics estarien essencialment relacionats amb trastorns del patró de desenvolupament i maduració normal dels circuits neuronals.

Finalment, també amb el Projecte 3 descrivim per primera vegada com l'exposició a un ambient enriquit durant el període postnatal primerenc podria millorar l'efecte a llarg termini de RCIU en un model animal en el desenvolupament neurològic, observant recuperacions funcionals que es correlacionen amb milloria estructural. La correspondència clínica de l'evidència aquí descrita es basariaa en estimular als nens d'una manera cognitiva, sensorial i social.

En general, aquesta tesi aporta noves evidències sobre el mecanismes fisiopatològic subjacents a la RCIU, observant com a llarg plaç els canvis estructurals subjacents a les disfuncions funcionals secundàries a la RCIU podrien correspondre a una alteració a nivell de la connectivitat cerebral. A més, aquesta tesi dóna evidències preliminars que suggereixen que una estratègia basada en l'estimulació física, sensorial i cognitiva, així com l'estimulació social aplicada durant la vida postnatal primerenca, on la plasticitat del cervell és més gran, seria una estratègia potencialment útil per tal de mitigar les conseqüències deletèries del RCIU a llarg plaç.

6. CONCLUSIONS

1. La reducció de nutrients i d'oxígen prenatalment ocasiona una disfunció funcional que es correlaciona amb canvis estructurals a nivell de les xarxes cerebrals.

2. La gravetat de trastorns del neurodesenvolupament i la seva associació amb amb els canvis estructural semblen estar relacionats amb el grau de la lesió prenatal, amb efectes més notables en el model d'hipoperfusió placentària.

3. Alteracions neuroconductuals i cognitives que persisteixen fins al període a llarg termini a causa de la restricció del creixement intrauterí es correlacionen amb canvis microestructurals que afecten a àrees específiques del cervell de a nivell de la substància gris i la nivell de les xarxes cerebrals demostrable mitjançant ressonància magnètica de difusió.

4. Histològicament l'alteració a nivell de la connectivitat cerebral secundària a la restricció de creixement intrauterí correspon a canvis a nivell de la densitat de l'espina dendrítica i a nivell de les xarxes perineurals a l'hipocamp.

5. L'estratègia d'enriquiment ambiental aplicada durant períodes crítics del desenvolupament neurològic minora l'efecte de la restricció de creixement intrauterí sobre el desenvolupament neurològic, observant-se una milloria tant funcional com estructural.

APPENDIX II

UNIVERSITAT DE BARCELONA

C.E.E.A
(Comitè Ètic d'Experimentació Animal)

Centres Científics i Tecnològics
Solé i Sabarís, 1-3
08028 Barcelona
Tel. 934037044
ceea@ccit.ub.edu

COMITÈ ÈTIC D'EXPERIMENTACIÓ ANIMAL (CEEA)

Formulari d'acceptació de procediments

DADES PROCEDIMENT

Títol: **Prevenció de la lesió neurològica por hipòxia crònica perinatal en model animal d'hiponutrició en conilla gestant.**

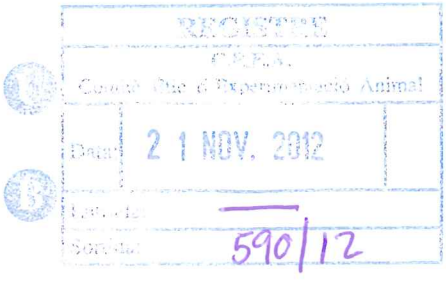
Investigador Responsable: **Miriam Illa Armengol**

Un cop examinada la documentació presentada, en compliment del Decret 214/97 de la Generalitat de Catalunya, el CEEA de la UB ha resolt **ACCEPTAR** el procediment sol·licitat.

Signat pels membres del CEEA que han pres l'acord

Barcelona, 23 d'octubre del 2012

NOTA: El CEEA delega en el/la responsable en benestar animal de la Unitat d'Experimentació Animal on s'allotjaran els animals, el seguiment de la realització del procediment d'acord amb el que està establert a la memòria aprovada per aquest comitè.



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C.E.E.A
(Comitè Ètic d'Experimentació Animal)

Centres Científics i Tecnològics
Solé i Sabarís, 1-3
08028 Barcelona
Tel. 934037044
ceea@ccit.ub.edu

COMITÈ ÈTIC D'EXPERIMENTACIÓ ANIMAL (CEEA)

Formulari d'acceptació de procediments

DADES PROCEDIMENT

Títol: **Avaluació de diferents estratègies neuroprotectores en la prevenció de la lesió neurològica secundària a insuficiència placentària crònica en un model animal de lligadura quirúrgica en conilla gestant**

Investigador Responsable: **Miriam Illa Armengol**

Un cop examinada la documentació presentada, en compliment del Decret 214/97 de la Generalitat de Catalunya, el CEEA de la UB ha resolt **ACCEPTAR** el procediment sol·licitat.

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Barcelona, 3 d'octubre del 2013

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C.E.E.A
(Comitè Ètic d'Experimentació Animal)

Centres Científics i Tecnològics
Edifici Florensa, C/Adolf Florensa nº8, 1ª planta, despatx F14
08028 Barcelona
Telf. 934 034 786
ceea@ccit.ub.edu

COMITÈ ÈTIC D'EXPERIMENTACIÓ ANIMAL (CEEA)

Formulari d'acceptació de procediments

DADES PROCEDIMENT

Títol: **Caracterización estructural a corto y a largo plazo del impacto de estrategias terapéuticas en la prevención de la lesión neurológica secundaria a la insuficiencia placentaria crónica en un modelo animal de conejo**

Investigador Responsable: **Miriam Illa Armengol**

Un cop examinada la documentació presentada, en compliment del Decret 214/97 de la Generalitat de Catalunya, el CEEA de la UB ha resolt **ACCEPTAR** el procediment sol·licitat.

Signat pels membres del CEEA que han pres l'acord

Barcelona, 14 de desembre del 2016

NOTA: El CEEA delega en el/la responsable en benestar animal de la Unitat d'Experimentació Animal on s'allotjaran els animals, el seguiment de la realització del procediment d'acord amb el que està establert a la memòria aprovada per aquest comitè.

APPENDIX III

Original Paper

Fetal Diagnosis and Therapy

Fetal Diagn Ther
DOI: 10.1159/000454859

Received: July 7, 2016
Accepted after revision: November 25, 2016
Published online: January 5, 2017

Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models

Míriam Illa[a] Elisenda Eixarch[a, b] Emma Muñoz-Moreno[a, d] Dafnis Batalle[a, e] Rocío Leal-Campanario[c] Agnès Gruart[c] José María Delgado-García[c] Francesc Figueras[a, b] Eduard Gratacós[a, b]

[a]Fetal i+D Fetal Medicine Research Center, BCNatal – Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Déu), IDIBAPS, University of Barcelona, Barcelona, [b]Centre for Biomedical Research on Rare Diseases (CIBER-ER), Madrid, [c]Division of Neurosciences, Pablo de Olavide University, Seville, and [d]Experimental MRI 7T Unit, Institut d'Investigacions August Pi i Sunyer (IDIBAPS), Barcelona, Spain; [e]Centre for the Developing Brain, Division of Imaging Sciences & Biomedical Engineering, King's College London, London, UK

Keywords
Animal model · Behavior · Brain networks · Fetal growth restriction · Placental insufficiency · Undernutrition

Abstract
Introduction: Chronic reduction of oxygen and nutrient delivery to the fetus has been related to neurodevelopmental problems. Placental underperfusion induces a significant reduction in oxygen and nutrient delivery, whereas maternal undernutrition causes mainly nutrient deficiency. A comparison of the neurodevelopmental effects of both situations in pregnant rabbits was performed. ***Materials and Methods:*** The placental underperfusion model was induced after uteroplacental vessel ligation at 25 days of pregnancy. The undernutrition model was induced after a reduction of 70% of the basal maternal intake at 22 days of pregnancy. Neurobehavioral tests were applied in the derived offspring at the neonatal period and over the long term. Structural brain differences were evaluated by brain networks obtained from diffusion magnetic resonance imaging. ***Results:*** Birth weight was significantly lower in both cases. However, stillbirth was only increased in the placental underperfusion model. Cases from both models presented poorer neurobehavioral performance and network infrastructure, being more pronounced in the placental underperfusion model. ***Discussion:*** Prenatal insults during the last third of gestation resulted in functional and structural disturbances. The degree of neurodevelopmental impairment and its association with structural brain reorganization seemed to be related to the type of the prenatal insult, showing stronger effects in the placental underperfusion model. © 2017 S. Karger AG, Basel

Introduction

Fetal growth restriction (FGR) has been related to neurobehavioral problems during the childhood period [1, 2] that also persist in the long term [3, 4]. Interestingly, long-term follow-up studies have described cognitive impairments and learning difficulties at school age [5] involving short-term memory, and attention and anxiety problems [3, 4].

Although this evidence exists, the exact mechanisms underlying fetal brain programming due to FGR are still unknown. Notwithstanding their obvious limitations, animal models provide an opportunity to advance the investigation of the pathophysiology of this condition. There are two major approaches to reproduce preclinical

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E-Mail karger@karger.com
www.karger.com/fdt

Míriam Illa, MD, Fetal i+D Fetal Medicine Research Center
BCNatal – Barcelona Center for Maternal-Fetal and Neonatal Medicine
(Hospital Clínic and Hospital Sant Joan de Déu), IDIBAPS
University of Barcelona, Sabino de Arana 1, ES–08028 Barcelona (Spain)
E-Mail miriamil@clinic.ub.es

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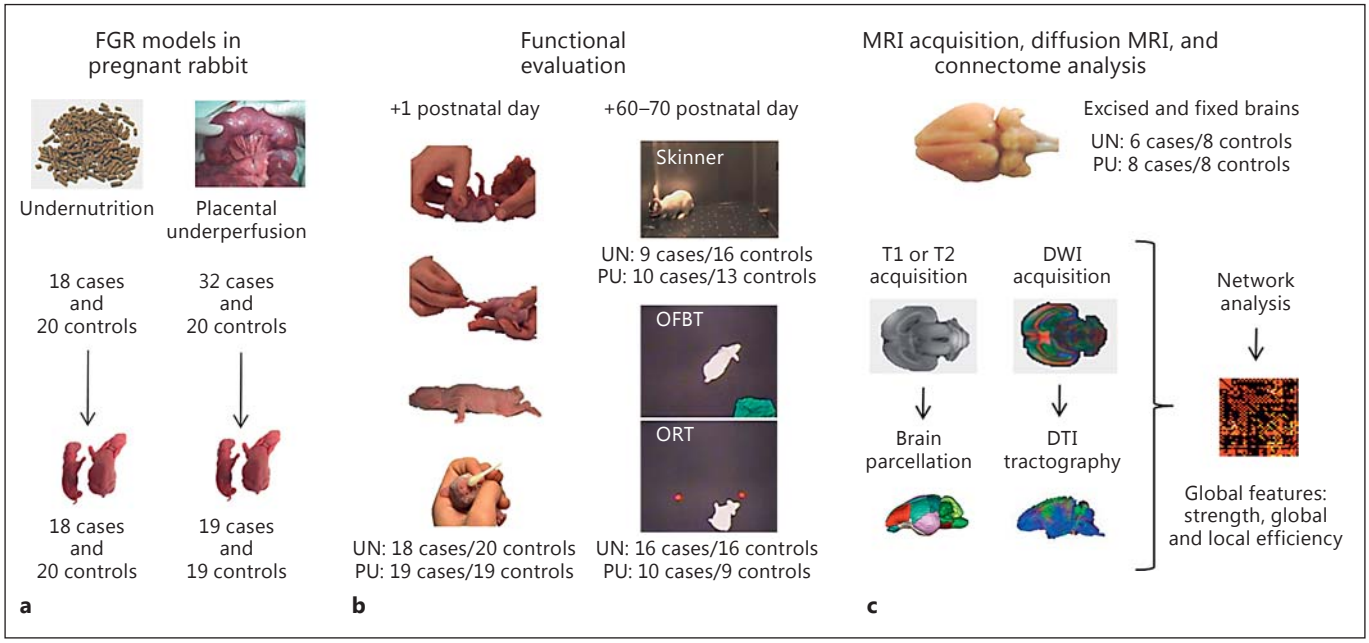

Fig. 1. Study design, methods, and flow chart of the animals included. **a** Illustrative images and scheme of fetal growth restriction (FGR) induction models and number of animals derived from both models. **b** Illustrative images of neurobehavioral tests applied and number of animals evaluated. **c** Illustrative images of MRI acquisition, processing, and connectome analysis. UN, undernutrition; PU, placental underperfusion; OFBT, open field behavioral test; ORT, object recognition task; DWI, diffusion-weighted image; DTI, diffusion tensor image.

FGR models: models based on placental underperfusion (PU) and models based on undernutrition (UN) [6]. PU models are based on the reduction of uteroplacental blood flow, which decreases supply of both oxygen and nutrients, whereas UN models are based on the reduction of maternal intake, which results mainly in a decrease of nutrients. Regarding PU models, different methods have been described. While generalized reduction in the uteroplacental blood supply by means of uteroplacental embolization and bilateral uterine artery ligature resulted in nonpredictable reductions of placental perfusion [7], selective ligature of uteroplacental vessels performed in pregnant rabbits has been demonstrated to develop a gradable model of FGR [8]. Regarding UN models, maternal food deprivation has also demonstrated human FGR characteristics in pregnant rabbits [9, 10].

Although nutrients and oxygen have been described to be essential elements needed to complete normal fetal brain programming [11], we hypothesized that neurodevelopmental effects of oxygen and nutrient restriction during fetal neurodevelopment would have a greater impact on brain programming in comparison to a nutrient deficiency alone. For that purpose, the current study attempts to describe the neurodevelopmental impact of what are probably the two major causes worldwide of adverse intrauterine environment affecting the biologic growth potential (PU model by means of uteroplacental vessel ligation and UN model during pregnancy) induced during the last third of gestation [8]. In the offspring derived from these models, neurobehavioral tests at the neonatal and long-term period and structural brain changes using structural brain networks from ex vivo diffusion magnetic resonance imaging (MRI) at the long-term period were evaluated.

Materials and Methods

The methodology of the study and flow chart are summarized in Figure 1.

Animals, FGR Induction, and Ethics Statement

FGR was induced in pregnant rabbits during the last third of gestation following two different approaches: PU and UN. The PU model was induced in nine pregnant rabbits following the detailed protocol previously described [8]. Briefly, ligation of 40–50% of the uteroplacental vessels of all the gestational sacs of one horn was performed at 25 days of pregnancy, whereas the contralateral horn

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was used as a control. At 30 days of pregnancy, a cesarean section was performed. The UN model was performed in three pregnant rabbits, reducing 70% of the basal food intake, corresponding to approximately 45 g/day of the standard chow specifically designed for the pregnant and lactating rabbit mother (2030 Teklad Global Rabbit Diet). Basal food reduction was started from 22 days of gestation up to 31 days of gestation, when a vaginal delivery was allowed [12]. As a control for the UN model, we included three rabbits that were feed ad libitum. Vaginal delivery was allowed or induced with oxytocin at 31 days of pregnancy. Pups from both models were weighed and identified with a subcutaneous chip. The PU model pups were housed and breastfed by a surrogate, whereas in the UN model, pups were fed by their own mother, with a maximum of eight pups in both models. Pups were housed until the 30th postnatal day, when they were weaned. Thereafter, both groups of rabbits were housed in groups of three with a reversed 12/12-hour light cycle and free access to water and food. The animal experimentation in this study was approved by the Animal Experimental Ethics Committee of the University of Barcelona (permit number: 206/10-5440).

Functional Test Protocol Evaluation

At postnatal day 1, neurobehavioral evaluation was performed following the methodology previously described [13, 14], evaluating general motor skills, reflexes, and olfactory sensitivity.

Between postnatal days 60 and 70, evaluation of learning, anxiety, and memory was performed. Concerning the learning evaluation, a Skinner box was constructed as detailed in Leal-Campanario et al. (box for operant conditioning and instrumental learning for rabbits, 2012. Inscription number in Spain: P2001231369), and the protocol was adapted from the methodology previously described [15] with food reward reinforcement and a continuous reinforcement schedule. One week before starting the evaluation, rabbits were food deprived (~20 g/day of food chow) to increase their motivation to get the food reward. After observing a 10–15% reduction in their basal weight, the first shaping phase was started. This phase lasted 5 days, and any advancement toward the feeder bar was rewarded. After 2 days of rest, the training phase lasting 5 more days was performed, and a reward was given only when the animal specifically pressed the lever. In this phase, the learning criterion was considered to be when the animal pressed the lever and went directly toward the food dispenser to obtain the reward at least three times in one session. All sessions lasted 10 min and were recorded for posterior analysis. After the Skinner test, the animals were allowed to rest for 2 days before continuing with the open field behavioral test (OFBT) and the object recognition task (ORT), respectively. These tests were applied following methodology published previously [16], and examinations were recorded and analyzed using a video tracking software (SMART Software Tracking System from Panlab). OFBT variables included "latency" (time in seconds the animal leaves the familiar starting point and starts exploring the open field) and "number of internal and external areas explored." Variables recorded in the ORT included: time in seconds exploring the two objects presented in the familiarization phase and time in seconds exploring the familiar and the novel objects in the testing phase. The discriminatory index (DI) was then calculated as follows:

$$\mathrm{DI} = \frac{\text{time exploring novel object} - \text{time exploring the familiar one}}{\text{time exploring novel object} + \text{time exploring the familiar one}}.$$

After the neurobehavioral tests, rabbits were anesthetized (ketamine + xylacine) and euthanized with an endovenous overdose of sodium pentobarbital. Brains were then fixed with 10% buffered formalin solution through a cardiac perfusion fixation protocol. After fixation, brains were removed and placed in 10% buffered formalin solution overnight.

MRI Acquisition, Tractography, Brain Parcellation, Network Extraction, and Analysis

A subsample of fixed brains was selected to be scanned (16 animals from the PU and 14 from the UN group). MRI was performed using a 7T animal MRI scanner (BrukerBioSpin MRI GmbH). Due to technical issues, high-resolution three-dimensional T1-weighted images were obtained in the UN group's brain samples by a modified driven equilibrium Fourier transform (MDEFT) sequence, whereas T2-weighted images were obtained in the PU group's brain samples by a rapid acquisition with relaxation enhancement (RARE) sequence. In both models, diffusion-weighted images were acquired using a diffusion sequence covering 30 gradient directions with a b value of 3,000 s/mm^2 together with a baseline (b = 0 s/mm^2) image. Preprocessing, tractography, brain parcellation, and brain network extraction were performed following the methodology previously described [17], obtaining a fractional anisotropy (FA)-weighted network for each subject. The Brain Connectivity Toolbox was used to characterize global functioning of each network by means of graph theory network features [18]. Particularly, we assessed infrastructure (average strength), integration (global efficiency), and segregation (local efficiency) of each FA-weighted network (see online suppl. material for a more detailed methodology explanation; for all online suppl. material, see www.karger.com/doi/10.1159/000454859).

Statistics

Statistical comparisons were performed by general linear models (GLM) and were adjusted by gender in long-term data. Interaction of group (controls and cases) and gender was first included into the model, but as it did not show any significant effect, was excluded of the final model. Significance was declared at $p < 0.05$ (uncorrected). Normality was assessed by Shapiro-Wilk test and homoscedasticity by Levene's test, and when the null hypothesis was rejected, log transformation was performed before GLM analysis. Descriptives of the variables were expressed as mean and standard deviation for normal distributions, whereas median and interquartile range were used for non-normal distributions. To evaluate functional impairments in both models, the mean difference (mean result in controls minus mean result in cases) and its 95% confidence interval (CI) were calculated for each model and for each functional variable. Association of network features with functional results was performed by means of a partial correlation or GLM, as needed. The software package STATA13.0 was used for the statistical analyses.

Results

Perinatal Data

Stillbirth was statistically higher in cases coming from the PU model compared to their respective controls (44 vs. 5%, $p < 0.001$). No stillbirth was observed in the UN

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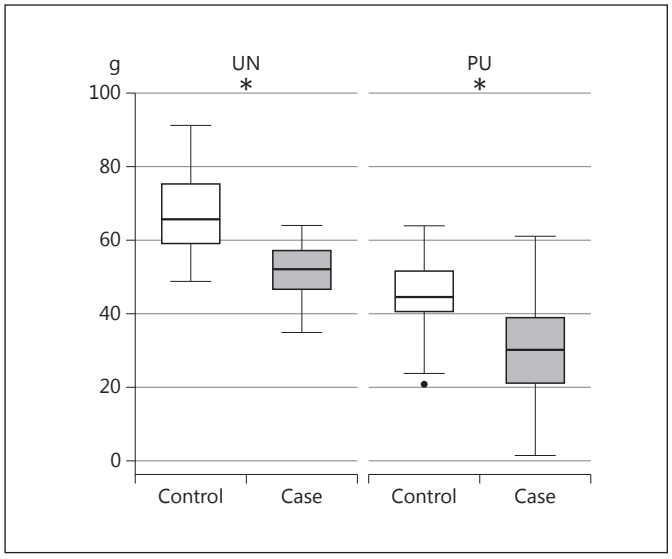

Fig. 2. Birth weight differences in the study groups. Birth weight (g) in controls and cases for both models. UN, undernutrition; PU, placental underperfusion. * $p < 0.05$, statistical significance.

model. Postnatal mortality rate did not differ between cases and controls in both models (42 vs. 32%, $p = 0.55$ in PU; 6 vs. 5%, $p = 0.94$ in UN, cases vs. controls, respectively). Regarding birth weight, both models had a similar effect, observing a significant birth weight decrease in cases compared to their respective controls (Fig. 2). The degree of growth restriction induced by both models was similar (birth weight in PU: 30.23 g [SD 12.08]; birth weight in UN: 51.92 g [SD 7.57]), as both FGR's birth weights corresponded to their 10th percentile derived from normal birth weight distribution (10th percentile from PU: 33 g; 10th percentile from UN: 53 g).

At the long-term period evaluation, no differences in weight (1,444 g [SD 136] vs. 1,589 g [SD 376], $p = 0.24$ in PU; 1,378 g [SD 101] vs. 1,442 g [SD 111], $p = 0.17$ in UN, cases vs. controls, respectively) and gender distribution (percent of females: 55 vs. 62%, $p = 0.73$ in PU; 33 vs. 31% in UN, cases vs. controls, respectively) were observed in these models.

Functional Results

At the neonatal period, cases from both models showed poorer results in almost all the parameters (online suppl. Table S1). These differences were more pronounced in the PU model, as shown in Figure 3, where mean difference and its 95% CI for each functional variable were higher in the PU model.

At the long-term period, all the animals that reached that period did not present any motor abnormality that could have interfered in the execution of the neurobehavioral tasks. Due to technical problems, Skinner tests and OFBT were not available in 11 animals from PU and 8 animals from UN. ORT was attempted in all animals with a successful OFBT test; however, only 17 animals from the PU and 23 animals from the UN model were suitable to be included in the ORT analyses, since they explored at least one object in the familiarization phase and at least one time both objects in the testing phase [19]. Skinner test results showed a lower proportion of cases from the PU model reaching the learning criteria when compared with their controls (30 vs. 77%, $p = 0.03$, cases vs. controls, respectively), whereas no differences were observed in the UN model (44 vs. 56%, $p = 0.56$, cases vs. controls, respectively). Regarding OFBT results, cases from both models presented a significantly increased latency of leaving the familiar starting point and a reduced number of external and internal boxes explored. When ORT was assessed, a decreased DI was observed in cases compared to their respective controls in both models (online suppl. Table S2). Again, these differences at the long-term period were more pronounced in the PU model, as shown in Figure 4, where mean difference and its 95% CI for each functional variable were higher in the PU model compared with the UN model.

Brain Network Results

Overall, animals with a history of FGR presented a significant decrease in brain network parameters when compared with their respective controls in both models at the long-term period. Regarding global and local efficiencies, cases presented decreased values, although these differences were only statistically significant in the PU model (Fig. 5). In addition, significant correlations were observed between global network features and neurobehavioral results, especially in the OFBT variables (online suppl. Table S3).

Discussion

Our results showed that FGR models induced functional impairments in the neonatal and the long-term periods that correlate with structural changes observed by network analysis. Interestingly, these differences were more pronounced in the PU model, suggesting a link between severity of the prenatal insult and the degree of the neurodevelopmental consequences later in life.

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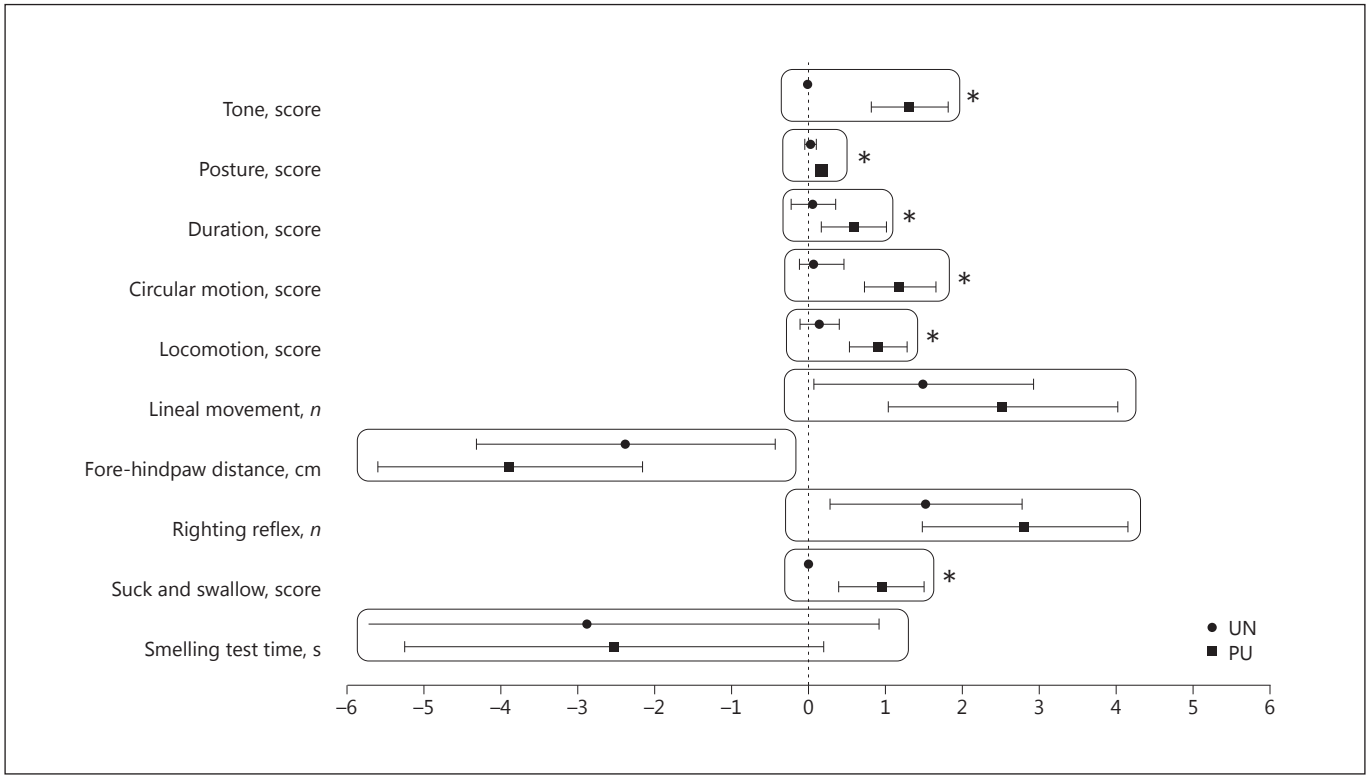

Fig. 3. Mean difference of neonatal functional performance in both models. Mean difference (mean result in controls minus mean result in cases) and its 95% confidence interval between controls and cases for each model and for each functional log-transformed variable at the neonatal period. UN, undernutrition; PU, placental underperfusion. * $p < 0.05$, statistical significance.

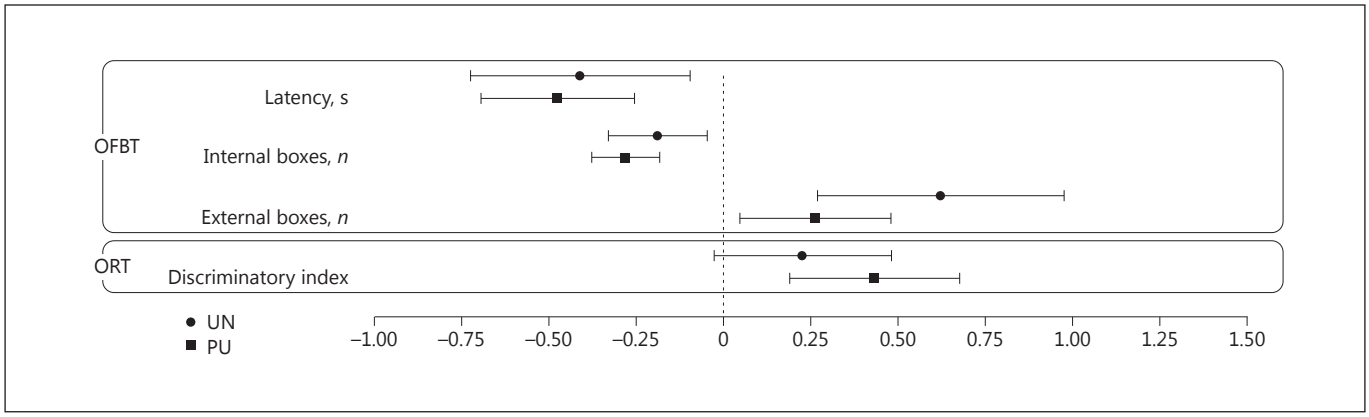

Fig. 4. Mean difference of long-term functional performance in both models. Mean difference (mean result in controls minus mean result in cases) and its 95% confidence interval between controls and cases for each model and for each functional log-transformed variable at the long-term period. OFBT, open field behavioral test; ORT, object recognition task; UN, undernutrition; PU, placental underperfusion. * $p < 0.05$, statistical significance.

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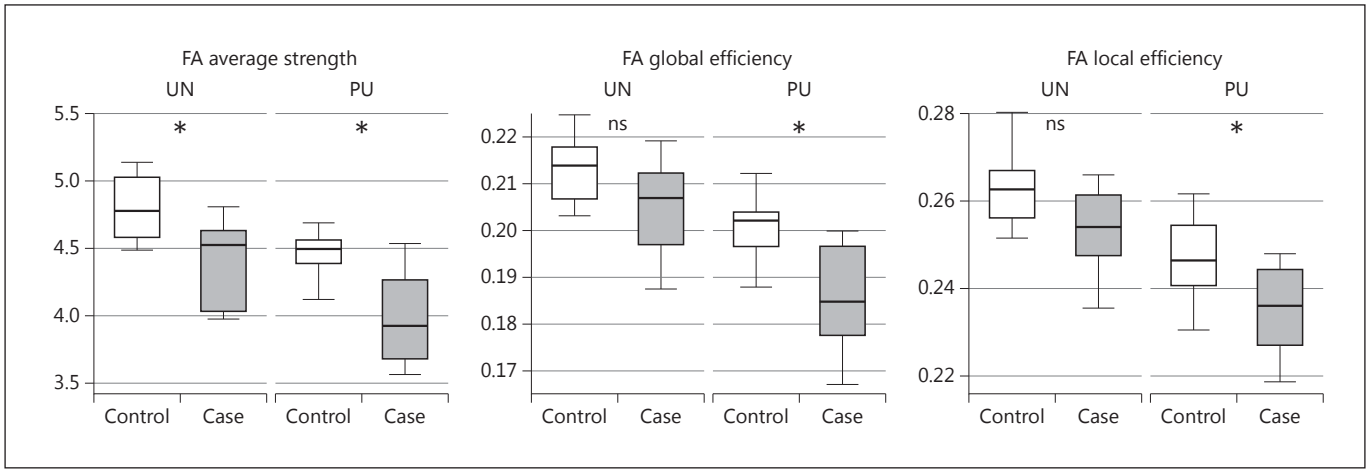

Fig. 5. Fractional anisotropy (FA)-weighted network features. FA-weighted network features in controls and cases for both models, including average strength, and global and local efficiency of weighted FA network. UN, undernutrition; PU, placental underperfusion; ns, not significant. * $p < 0.05$, statistical significance.

Regarding perinatal results, both models induced a reduction of birth weight, but only the PU model was related to an increased fetal and early postnatal mortality, reproducing severe forms of human FGR [20]. The same findings were observed in previous animal studies, in which PU was associated with changes in cardiovascular Doppler parameters, leading to increased fetal mortality [9]. This contrasted with the UN models, based either on global nutrient reduction or low-protein diet, which were associated with birth weight reduction with no significant increase in fetal mortality [6, 9, 21].

Neurobehavioral data confirm previous studies showing that both FGR models correlate with neonatal and long-term neurobehavioral impairments, especially for PU cases. During the neonatal period, clinical studies have described neurobehavioral problems related to FGR, including psychomotor delays and cerebral palsy in the most severe cases [22, 23], or subtler neurocognitive difficulties in less severe forms [24, 25]. Along this line, important motor [26] and olfactory problems [27] were observed in a severe and acute hypoxic-ischemic model in pregnant rabbits, whereas weaker functional disturbances were observed in less severe and chronic PU exposure [28]. At the end of the spectrum, moderate nutrient restriction in pregnant mice has been related to subtle neurobehavioral impairment, such as delayed development of physical and coordinated movements [21, 29]. At the long-term period, reports on infants having suffered from FGR showed neurocognitive difficulties [30] that were even more prevalent in those cases with evident signs of placental insufficiency [31]. In basic research, FGR animal models including UN, a low-protein diet, and PU showed higher degrees of anxiety, reduced social interaction, and depression-related behaviors [16, 29, 32], as well as learning, short-term memory, and attention problems [21, 33–35]. Our results support the notion that severity and type of insult during the prenatal period results in a differential effect on neurobehavior, with more remarkable changes in the PU model.

In addition, this study provides new evidence on brain reorganization underlying neurobehavioral and cognition impairments in both models. Global reduction in FA-weighted average strength in both models supported the idea that FGR has an impaired network infrastructure. These results are in line with previous results in a rabbit model, in which average degree of structural brain networks was also decreased [17]. However, altered regional organization evidenced by means of reduced global and local efficiencies was only found in the PU model, demonstrating a more severe effect at this level. Because FA has been related to axonal packing, neuronal density, and myelination of fiber tracts [36], these results suggested that altered network connectivity could be mainly associated with less mature connections. These results are in line with previous studies in humans [37] and animal models [17], showing significantly reduced FA-weighted network efficiencies in FGR at the long-term period.

Overall, this work provides evidence demonstrating that chronic reduction of nutrients with or without a re-

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duction of oxygen, even when started at later stages of pregnancy, still results in a real impact on brain programming. Data presented in this work strengthen the concept that poor nutrition during prenatal life has an impact on later neurobehavioral and cognitive development [38]. Moreover, this study also proves that chronic hypoxia added to undernutrition during the prenatal period has a more severe effect on functional and structural neurodevelopment, thus making the PU model suitable to study neurodevelopmental consequences of severe forms of FGR. On the contrary, the UN model can be of interest to study effects of less severe forms of FGR.

The main strength of this study is the evaluation of neurodevelopmental consequences in two models of FGR by using the same animal species during the same period. There are a high number of studies describing neurodevelopmental problems in FGR by using different animal species that have provided an undoubted value. However, the rabbit model may have some advantages over the rodent model, as it closely resembles humans in terms of timing of perinatal brain white matter maturation as compared to rats [13]. As in humans, brain maturation begins in the intrauterine period and continues during the postnatal period. Apart from that, the suitability of PU and UN in rabbits to reproduce human features of FGR has been established [8, 10, 26]. Finally, another strength of this study is the fact that both models followed the same evaluation protocol in terms of functional test and brain connectivity assessment, offering the possibility to compare the two models.

Limitations of the study include methodological differences between the designs of both models. First, animals from the PU group were delivered at 30 days of pregnancy (near term) by cesarean section, whereas animals from the UN group were allowed to deliver vaginally at 31 days' gestation. In reality, these differences make our results more transferable to clinics, as severe FGR cases tend to be delivered earlier during pregnancy by means of a cesarean section, whereas less severe cases, which seems to be more accurately reproduced by the UN model, usually are delivered near term by vaginal delivery. Depending on the method of delivery, fetal oxytocin exposure was different. In addition, the difference in the time of birth between the two models has a direct impact on the weight at birth. This difference was evident in the birth weight of the control animals, where controls in the PU model were smaller than the controls in the UN model. Finally, the difference in rearing of the pup could also have an important effect in later neurodevelopment observed in both models. Animals coming from the PU model were fed by a surrogate, whereas animals from the UN model were fed by their mother, who had, however, been undernourished. In order to limit bias due to these design differences, structural and functional differences were assessed, comparing each FGR animal with their matched control, minimizing potential confounders between models. Regarding the brain network extraction, we have applied a tractography method based on diffusion tensor imaging appropriate for the acquisition of the 30 gradient directions. It is known that this technique is less robust in fiber-crossing areas than techniques based on high-angular resolution diffusion imaging, leading to a lower number of recovered fiber trajectories. However, it has been shown that, from the point of view of case-control studies based on brain network analysis, diffusion tensor imaging-based tractography could reduce inter-subject variability, being more sensitive to intergroup variance [39].

Conclusion

This study demonstrates that sustained intrauterine exposure to placental underperfusion or undernutrition results in functional disturbances and correlates with brain network reorganization. The severity of neurodevelopmental impairment and its association with structural brain reorganization seem to be related to the degree of the prenatal insult, with more remarkable effects in the placental underperfusion model.

The present study adds new evidence regarding neurodevelopmental problems of prenatal origin and improves the understanding of brain programming due to prenatal insults associated with neurobehavioral dysfunctions in FGR. Moreover, it demonstrates the feasibility of using brain network features from diffusion MRI as biomarkers to assess and monitor potential treatments using different experimental models.

Acknowledgments

We would like to acknowledge Guadalupe Soria and Xavi Lopez Tudela from Experimental 7T MRI Unit of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) for their help in the performance of MRI acquisitions. We are also indebted to Isabel Salas and the rest of the animal housing staff from Hospital Sant Joan de Déu – Animal Housing for their sensitivity in the care of the animal during the procedures. The Image Registration Toolkit was used under license from Ixico Ltd.

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Disclosure Statement

The authors report no conflict of interest.

Funding Sources

This study was partly supported by grants from Ministerio de Economía y Competitividad (ref. SAF2012-37196) and the Instituto de Salud Carlos III (ref. (PI12/00851, PI13/01018) integrado en el Plan Nacional de I+D+I y cofinanciado por el ISCIII-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER) "Otra manera de hacer Europa"; the research leading to these results has received funding from "la Caixa" Foundation and AGAUR 2014 SGR grant No. 928 (Barcelona, Spain). Funding source(s) had no direct involvement in the design of the study, in the data collection, in the analysis, in in the writing of the report, or in the decision to submit the article for publication.

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Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction

Miriam Illa[1,2], Elisenda Eixarch[1,2,3], Dafnis Batalle[2], Ariadna Arbat-Plana[2], Emma Muñoz-Moreno[2], Francesc Figueras[1,2,3], Eduard Gratacos[1,2,3]*

1 Department of Maternal-Fetal Medicine, Institut Clinic de Ginecologia, Obstetricia i Neonatologia (ICGON), Hospital Clinic, Barcelona, Spain, **2** Fetal and Perinatal Medicine Research Group, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain, **3** Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Barcelona, Spain

Abstract

Background: Intrauterine growth restriction (IUGR) affects 5–10% of all newborns and is associated with increased risk of memory, attention and anxiety problems in late childhood and adolescence. The neurostructural correlates of long-term abnormal neurodevelopment associated with IUGR are unknown. Thus, the aim of this study was to provide a comprehensive description of the long-term functional and neurostructural correlates of abnormal neurodevelopment associated with IUGR in a near-term rabbit model (delivered at 30 days of gestation) and evaluate the development of quantitative imaging biomarkers of abnormal neurodevelopment based on diffusion magnetic resonance imaging (MRI) parameters and connectivity.

Methodology: At +70 postnatal days, 10 cases and 11 controls were functionally evaluated with the Open Field Behavioral Test which evaluates anxiety and attention and the Object Recognition Task that evaluates short-term memory and attention. Subsequently, brains were collected, fixed and a high resolution MRI was performed. Differences in diffusion parameters were analyzed by means of voxel-based and connectivity analysis measuring the number of fibers reconstructed within anxiety, attention and short-term memory networks over the total fibers.

Principal Findings: The results of the neurobehavioral and cognitive assessment showed a significant higher degree of anxiety, attention and memory problems in cases compared to controls in most of the variables explored. Voxel-based analysis (VBA) revealed significant differences between groups in multiple brain regions mainly in grey matter structures, whereas connectivity analysis demonstrated lower ratios of fibers within the networks in cases, reaching the statistical significance only in the left hemisphere for both networks. Finally, VBA and connectivity results were also correlated with functional outcome.

Conclusions: The rabbit model used reproduced long-term functional impairments and their neurostructural correlates of abnormal neurodevelopment associated with IUGR. The description of the pattern of microstructural changes underlying functional defects may help to develop biomarkers based in diffusion MRI and connectivity analysis.

Citation: Illa M, Eixarch E, Batalle D, Arbat-Plana A, Muñoz-Moreno E, et al. (2013) Long-Term Functional Outcomes and Correlation with Regional Brain Connectivity by MRI Diffusion Tractography Metrics in a Near-Term Rabbit Model of Intrauterine Growth Restriction. PLoS ONE 8(10): e76453. doi:10.1371/journal.pone.0076453

Editor: Martin Gerbert Frasch, Université de Montréal, Canada

Received January 23, 2013; **Accepted** August 27, 2013; **Published** October 15, 2013

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Funding: This work was supported by Fondo de Investigación Sanitaria (PI08/0230 and PI12/00851), Rio Hortega grant (CM11/00032 to M.I.) and Sara Borrell grant (CD11/00048 to E.M.) from Instituto de Salud Carlos III; " Obra Social "la Caixa"; and Emili Letang fellowship by Hospital Clinic (to M.I.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: egratacos@clinic.ub.es

Introduction

Intrauterine Growth Restriction (IUGR) due to placental insufficiency occurs in 5–10% of all gestations [1] and it is thought to increase due to the delay in the maternal childbearing in modern societies [2]. Chronic reduction of placental blood supply results in sustained exposure to hypoxemia and undernutrition with the subsequent consequences on the developing brain [3]. The association between IUGR and short- term neurodevelopmental dysfunctions has been extensively described [4–9]. During neonatal period, term IUGR have poorer neurobehavioral and cognitive performance when compared with control term infants [4]. These neurobehavioral impairments seem to be even more pronounced in preterm IUGR when compared with term IUGR newborns, although these differences were not observed at long-term period [5]. Besides this, long-term follow-up studies

have reported that abnormal neurodevelopment after IUGR persists until late childhood and adolescence [3,10–24]. Recent reports have shown that children born with IUGR have long-term cognitive impairment and learning difficulties in school [13,14,17,20,22], being related to a characteristic pattern involving short-term memory, attention and anxiety, and increased risk of attention-deficit hyperactivity disorder (ADHD) [13–15]. These abnormalities have been suggested to reflect changes in specific areas including the anterior hippocampal-prefrontal network, parahippocampal complex, striatum, and thalamus [13,14,25–27]. Magnetic resonance imaging (MRI) studies have consistently demonstrated structural brain changes in IUGR during fetal and neonatal period including changes in brain texture analysis [6] and decreased volume in cortical grey matter (GM) [7], in the hippocampus [8] and differences in cortical development [9]. However, no long-term imaging studies have evaluated the neurostructural substrates underlying functional impairments in IUGR. This knowledge is required to explore the development of imaging biomarkers for early diagnosis and monitoring of abnormal neurodevelopment of prenatal origin [28].

IUGR is associated with disruption of normal brain neurodevelopment rather than gross tissue destruction [29], requiring the use of MRI modalities to identify subtle structural changes. Diffusion MRI is a noninvasive approach based on the measurement of the diffusion of water molecules in tissues [30], which provides indirect information about brain microstructure. Diffusion MRI has been used to assess brain reorganization in response to brain injury in both developing and adult brain [31,32]. Specifically, diffusion MRI has been shown to detect changes occurring in IUGR [33–35] and other fetal conditions also associated with reduced brain oxygen supply such as fetal cardiac defects [36]. Aside from water diffusion parameters, quantitative tractography metrics can be obtained in order to estimate the connectivity of WM pathways among brain regions regulating specific brain functions. This approach has been used to identify changes in diseases of neurodevelopment such as ADHD [37], autism spectrum disorders [38] and periventricular leucomalacia [39,40].

Evaluation of the long-term effects of IUGR on the human brain is limited by the difficulty of conducting prospective studies in sufficiently large sample sizes, and the potential influence of uncontrolled environmental factors [41]. Notwithstanding the obvious limitations, animal models provide the opportunity to conduct comprehensive studies spanning long maturational periods in homogeneous groups. Aside from the reproducibility of experimental conditions, MRI can be performed in isolated whole brain preparations allowing very long acquisition times with high-resolution [42]. The rabbit is a suitable model to reproduce IUGR [43–46] and it presents a human-like timing of perinatal brain WM maturation [44]. We have previously used this model to describe regional changes in fractional anisotropy in newborns which correlated with poorer outcome in neurobehavioral tests [34].

In this study we aimed at providing a comprehensive description of the long-term functional and neurostructural correlates of abnormal neurodevelopment associated with IUGR using a near-term rabbit model. Furthermore, we evaluated the development of quantitative imaging biomarkers of abnormal neurodevelopment based on regional changes in diffusion MRI parameters and connectivity. For all these purposes, we firstly assessed long-term neurodevelopment at a preadolescent equivalent age with functional tests extensively used in rodents. We tested the hypothesis that the rabbit model would display similar changes to humans, involving short-term memory, attention and anxiety problems. Secondly, brain microstructural changes were studied by means of diffusion MRI with high angular resolution schemes. Differences in diffusion parameters were analyzed by voxel-based analysis (VBA), to avoid the need for a priori hypothesis or previous delineation [47]. We also evaluated the presence of differences in the connectivity between brain areas described to be involved in anxiety and attention (including amygdala, hippocampus formation, striatum, thalamus and prefrontal, temporal and cingulate cortices) and short-term memory (including hippocampal formation, hippocampus, thalamus, prefrontal and temporal cortices). Finally, VBA and connectivity results were also correlated with the functional outcomes.

Materials and Methods

The methodology of the study is shown in Figure 1. Each step of the procedure is detailed in this section.

1- Study protocol and procedures

1.1- Ethics Statement. The animal experimentation of this study was approved by the Animal Experimental Ethics Committee of the University of Barcelona (permit number: 206/10–5440). Animal handling and all the procedures were performed following all applicable regulations and guidelines of the Animal Experimental Ethics Committee of the University of Barcelona, and all efforts were made to minimize suffering.

1.2- Animals and study protocol. The study groups were composed of 10 cases with induced IUGR and 11 sham controls obtained from New Zealand pregnant rabbits provided by a certified breeder. Dams were housed for 1 week before surgery in separate cages on a reversed 12/12 h light cycle, with free access to water and standard chow. At 25 days of gestation (term at 31 days), we performed ligation of 40–50% of uteroplacental vessels following a previously described protocol [45] in 10 pregnant rabbits. Cesarean section was performed at 30 days of gestation and living pups were obtained. On the 70th postnatal day, which is considered to be equivalent to preadolescence period in humans in terms of sexual maturity [48], functional tests were applied and the rabbits were sacrificed thereafter. The brains were then collected and fixed with 4% paraformaldehyde phosphate-buffered saline (PBS).

1.3- Surgical model. Briefly, after midline abdominal laparotomy, the gestational sacs of both horns were counted and numbered. Afterwards, only one uterine horn was kept outside the abdomen and the induction of IUGR proper was performed by ligating 40–50% of the uteroplacental vessels of all the gestational sacs from this horn. After the procedure, the abdomen was closed in two layers and postoperative analgesia (meloxicam) was administered for 48 hours. After surgery, the animals were allowed free access to water and standard chow for 5 days until delivery. Cesarean section was performed at 30 days of gestation and living and stillborn fetuses were obtained. All living newborns were weighed and identified by a subcutaneous microchip inserted in their back (Microchip MUSICC, Avid Microchip S.L., Barcelona, Spain). Cases were considered those pups delivered from the ligated horn, whereas controls were those delivered from the contralateral horn (non-ligated). Both cases and controls were housed with a wet nurse rabbit with part of the offspring (total number of rabbit pups in all litters: 8) until the 30th postnatal day when they were weaned. Thereafter both groups of rabbits were housed in groups of three with a reversed 12/12 h light cycle with free access to water and standard chow.

1.4- Neurobehavioral and cognitive evaluation: functional tests. In order to assess functional changes, especially those

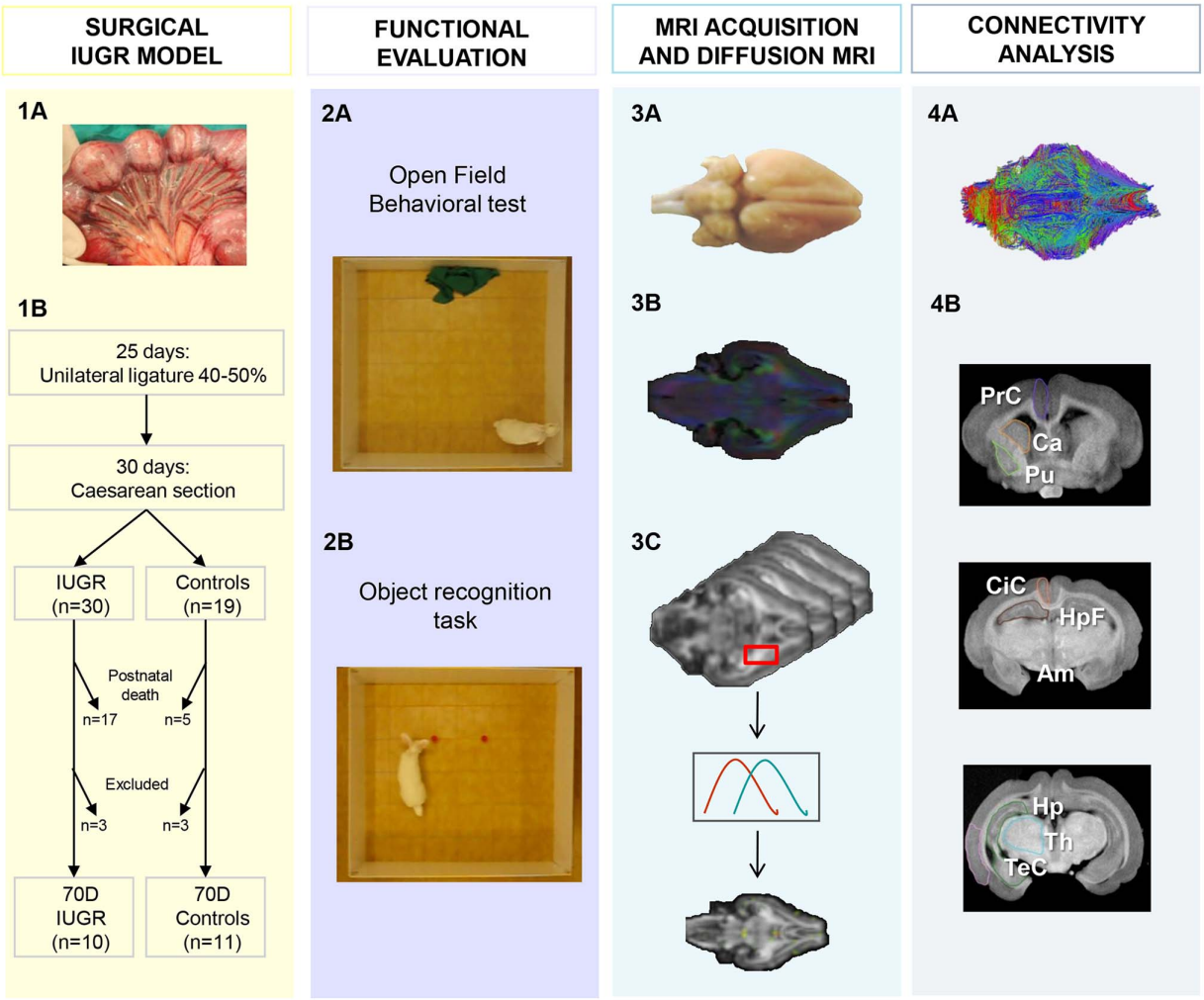


Figure 1. Schematic and graphical representation of the study design and methods. PANEL 1: (A) Illustrative image of unilateral ligation of 40–50% of uteroplacental vessels at 25 days of pregnancy, (B) Scheme of surgical procedures and study groups. **PANEL 2**: Illustrative pictures of neurobehavioral and cognitive evaluation in the Open Field Behavioral Test (A) and Object Recognition Task (B). **PANEL 3**: MRI acquisitions: (A) Fixed brains were scanned to obtain high resolution T1 weighted images and diffusion-weighted images. After masking brain volume, (B) global analysis was performed to obtain average DTI parameters (FA, linearity, planarity and sphericity coefficients). (C) Then voxel-based analysis of diffusion-related parameters was performed by elastic registration to a reference FA map. **PANEL 4**: (A) Illustrative image of tractography used for connectivity analysis. It was performed by measuring the ratio of fibers involved in anxiety and short-term memory networks over the total number of fibers reconstructed. (B) Manual delineation of brain regions involved in anxiety, attention and memory networks in coronal slices including prefrontal cortex (PrC), striatum (Ca + Pu), cingulate cortex (CiC), temporal cortex (TeC), thalamus (Th), amygdala (Am), hippocampus (Hp) and hippocampus formation (HpF).
doi:10.1371/journal.pone.0076453.g001

related to emotion and cognition, two standard tests used extensively in rodents, such as the Open Field Behavioral Test and Object Recognition Task, were adapted for application in rabbits. Specifically, the Open Field Test evaluates locomotion and exploratory activities that compete against fear, anxiety and attention [49–51]. The Object Recognition Task evaluates declarative short-term memory, specifically recognition [52], as well as attention capacity [53] and is based on the tendency of rodents to explore new stimuli for a longer time compared to familiar stimuli [54–56]. Both tests were performed by placing each animal in a squared arena (140 cm ×140 cm) surrounded by opaque plastic walls (height 40 cm). First, we evaluated the Open Field Test with their first contact to the novel environment. As we sought to evaluate any degree of anxiety, we decided not to habituate the animals to the novel area as suggested previously [57]. After the Open Field Test, the animals were removed from the arena and in 30 to 60 minutes were again placed in the arena to evaluate the Object Recognition Task. Both tests were applied between 10 am to 5 pm and after each session the exploring area was cleaned with a 10% ethanol in order to erase any olfactory cue. The room was insulated from sound and with full overhead illumination. To minimize interference due to human contact, each session was video-taped and later evaluated by two blinded observers (MI, AAP).

The Open Field Behavioral Test was designed and used in accordance with the procedure previously described [51]. The testing area was divided into 36 squares of 23×23 cm, the 4 central squares were considered as the internal area and the remaining squares were defined as the peripheral area. For testing, the rabbits were taken out of their cage wrapped with a cloth and placed close to one of the lateral walls (starting point) and behavior was assessed during 10 minutes. Multiple parameters were

recorded including latency of leaving the starting point (seconds), number of squares explored (internal or external), total time spent in internal and peripheral areas (seconds) and other general activities such as number of rearing and grooming.

The Object Recognition Task was performed, being adapted from the original description [58] including some modifications in the stimulus used. Instead of using visual stimulus, odour-based stimulus was used by means of placing pieces of fruit (apple or orange) inside perforated plastic boxes, since olfactory sensitivity is highly developed in rabbits [59]. This is in agreement with the notion that the type of stimulus presented must be one in which the sensory perception of the species chosen is adequate [59]. The test was divided into two consecutives phases. First, two boxes containing the same odour-based stimuli (apple) were presented to the animal during 5 minutes. This constituted the Familiarization phase. The rabbit was then returned to its cage for a 30-minute retention interval. Then, one of the objects was removed and replaced by a novel stimulus (orange) and the animal was again placed in the area with the novel and familiar objects for 5 minutes more in the Testing phase. Exploration of the object was considered when the rabbit showed sniffing, touching and having moving vibrissae while directing the nose towards the object at a distance of less than 1 cm. Cumulative time (seconds) exploring each object in the two sessions was recorded (right and left objects in the Familiarization phase, whereas novel and the familiar objects in the Testing phase). Finally, the discrimination index (DI), which represents the ability to discriminate the novel from the familiar object, was calculated as follows: DI = (Novel Object Exploration Time – Familiar Object Exploration Time)/(Novel Object Exploration Time + Familiar Object Exploration Time). Learning criteria was considered when the DI was above 0. Animals that did not explore the familiar object at least once in the Testing phase or did not explore any of the objects in the Familiarization phase were excluded from the analysis, as previously suggested [60].

1.5- Sample collection. After the functional tests, the rabbits were anaesthetized with ketamine 35 mg/kg and xylazine 5 mg/kg given intramuscularly and were sacrificed with an endovenous overdose of sodium pentobarbital (200 mg/kg). The left and right common carotid arteries were cannulated and the brains were perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde PBS. Finally, the brains were dissected and fixed in 4% paraformaldehyde PBS at 4°C for 48 h.

1.6- Magnetic resonance acquisition. MRI was performed on fixed brains using a 7T animal MRI scanner (BrukerBioSpin MRI GMBH). First, high-resolution three-dimensional T1 weighted images were obtained in the brain samples by a Modified Driven Equilibrium Fourier Transform (MDEFT) 3D sequence with the following parameters: Time of Echo (TE) = 3.5 ms, Time of Repetition (TR) = 4000 ms, 0.7 mm slice thickness with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 184×188 and Field of View (FoV) of 28×28 mm^2, resulting in a voxel dimension of 0.15×0.15×0.7 mm^3. Any potential tissue alteration, mainly significant tissue loss that could alter the results of further image-based analysis, was considered as exclusion criteria. Afterwards, diffusion-weighted images (DWI) were acquired using a standard diffusion sequence covering 126 gradient directions with a b-value of 3000 s/mm^2 together with a reference (b = 0) image. Other experimental parameters were: TE = 26 ms, TR = 250 ms, slice thickness = 0.7 mm with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 40×40 , FoV of 28×28 mm^2, resulting in a voxel dimension of 0.7×0.7×0.7 mm^3. The total scan time for both acquisitions was 13 h56 m40 s.

2- MRI processing and analysis

2.1- Post-processing MRI. As a first step, the brain was segmented from the background by means of customized software implemented in Matlab 2011a (The MathworksInc, Natick, MA, USA) similar to what has been described previously [34].

Tensor model of diffusion MRI was estimated at each voxel inside the brain mask [61] using MedINRIA 1.9 [62] (Inria Sophia Antipolis website, available at www-sop.inria.fr/asclepios/software/MedINRIA/. Accessed 2013 September 1). Based on the tensor model, a set of measures describing the diffusion were computed: fractional anisotropy (FA) and the coefficients of linearity, planarity and sphericity [30,63]. These are all based on the three eigenvalues of each voxel tensor ($\lambda1$, $\lambda2$, $\lambda3$). FA describes the anisotropy of the diffusion, being higher in areas occupied by WM tracts [30]. Linearity, planarity and sphericity coefficients describe the shape of the diffusion. High values of linearity indicate that diffusion occurs mainly in one direction, which mainly involves the presence of fiber tracts; high planarity indicates that diffusion is performed mostly in one plane, which could be related to crossing fibers; and high values of sphericity are related to isotropic diffusion [63]. In addition, the orientation diffusion function (ODF) of each voxel was also reconstructed following a Q-Ball approach [64]. The ODF of each voxel was used to reconstruct fiber tracts by means of the deterministic tractography algorithm implemented in MedINRIA 1.9 [62] (Inria Sophia Antipolis website, available at www-sop.inria.fr/asclepios/software/MedINRIA/. Accessed 2013 September 1).

2.2- Voxel-based analysis. To identify regional changes in diffusion-related parameters, VBA was performed. This analysis consists of the normalization of all the volumes to a reference volume and the comparison of the values at the same voxel of all the normalized volumes, thus identifying statistically significant differences. Registration of the diffusion tensor imaging (DTI) volumes to the reference was performed by means of a block matching algorithm, based on a DTI-specific metric [65]. Moreover, to preserve the coherence between DTI orientation information and the transformed volumes, the Preservation of Principal Direction (PPD) algorithm was applied [66]. In order to compensate for possible misregistrations and reduce noise effects, the registered volumes were smoothed. This smoothing also reduces the effective number of multiple comparisons in the statistical testing, thereby improving statistical power [67]. Van Hecke et al. [68] stated that anisotropic smoothing leads to more accurate VBA results, since it preserves the edges between different kinds of tissues, reducing the partial volume effects. For this reason, we applied an anisotropic Wiener filter [69] to the registered volumes. Once the images are aligned to the reference, it can be assumed that voxels in the same location in all the registered images belong to the same structure, and therefore, they can be compared. Voxel-wise t-test was performed, thereby obtaining voxels with a statistically significant different distribution of diffusion-related parameters including FA and linearity, planarity and sphericity coefficients, between controls and IUGR. The main goal of the use of VBA in this study was to explore and suggest potential relationships on all possible structural changes underlying the functional impairments in our IUGR model. Consequently, we decided to set a threshold of $p<0.01$ and we deliberately decided not to perform multiple comparisons correction. In addition to the analysis of differences on DTI parameters between cases and controls, the Spearman correlation between diffusion parameters and functional outcomes at each voxel was also calculated to identify which regions were related to the changes observed in the neurobehavioral and cognitive evaluation.

Since VBA requires the definition of a reference brain, the results may be biased by this choice. In order to avoid this bias and to increase the reliability of the results obtained, the VBA procedure was repeated taking all the subjects as template, and only the regions where differences were consistently noted in all the templates were considered. In this way, the variability produced by the arbitrary choice of the reference template is discarded.

2.3- Connectivity analysis. Connectivity analysis within specific brain areas involved in anxiety, attention and short-term memory were evaluated. We defined two main brain networks:

- Anxiety and attention network. The selection of areas was based on previous evidence that regulation of attention and emotional reactivity depends on the correct interaction between brainstem, limbic and cortical systems [70,71]. Within the limbic system, the amygdala and hippocampus were included because of their role in fear and anxiety [72–74]. In addition, several cortical areas (frontal, temporal, cingulate cortices) and deep grey nuclei (striatum and thalamus) were selected due to their relation with attention and emotion [27,75–78]. Moreover, some of these brain areas have been identified as components of the Papez circuit which has been proposed to play a major role in emotion [79]. Given these evidence, we arbitrarily defined the "anxiety and attentional network" as all those WM fibers passing through the amygdala and the hippocampus formation, and which additionally passed through at least one of the following structures: striatum, thalamus, prefrontal cortex, temporal cortex or cingulate cortex.
- Short-term memory network. Brain areas proposed to be involved in short-term memory were selected. Although the exact type of memory encoded remains under debate, there is universal agreement that the hippocampus [80,81], and especially the hippocampal formation [82,83], have important roles in declarative memory. In addition, memory based on olfactory recognition depends of the temporal lobe, mainly of the perirhinal cortex [84] and performance of the Object Recognition Task has been proposed to rely on the correct interaction within the perirhinal-hippocampal-medial prefrontal network [85–87]. Finally, recent evidence has suggested the involvement of the thalamus in the regulation of short-term memory [88]. Based on these data, we arbitrarily defined the "short-term memory network" as all those WM fibers passing through the hippocampal formation and which additionally passed through at least one of the following structures: hippocampus, thalamus, prefrontal or temporal cortices.

Manual delineation of GM structures was performed in T1 weighted images including multiple cortical areas (prefrontal, cingulate, temporal), putamen, caudate nucleus, thalamus, amygdala, hippocampus and hippocampal formation (Figure 1, PANEL 4). Combining these regions with previously calculated tractography, WM fiber tracts involved in the two networks of short-term memory and anxiety were extracted. The measurement of connectivity within each network was assessed applying two different quantitative tractography metrics: 1) number of fibers within proposed networks corrected by the total number of fibers in each brain and 2) measurement of mean FA in fibers involved in the proposed networks. For both networks, we analyzed global circuit connectivity considering both right and left hemisphere fibers together (bilateral analysis), and specific right and left circuit connectivity, considering each hemisphere separately (right and left analysis). In addition, correlation between ratio of fibers and mean FA with functional test scores was also analyzed adjusting for gender.

3- Statistical analysis

For quantitative variables, normality was assessed by Shapiro-Wilk [89]. Results were expressed as mean and standard deviation (SD); whereas median and interquartile range (IQR) was used in non-normal variables. Differences between cases and controls were studied after adjusting for gender by means of general lineal model. In non-normal variables such analysis were performed after a log-transformation. For categorical variables, chi-squared test was used. SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for this statistical analysis. In VBA approach, registered and smoothed volumes of FA, linearity, planarity and sphericity coefficients were used to obtain volumetric maps of t-statistics, showing the voxels that presented a significant difference between groups (uncorrected $p<0.01$). In addition, a correlation volume (ρ) was also calculated for each functional item, expressing positive and negative Spearman correlations between FA, linearity, planarity and sphericity coefficients and neurobehavioral and cognitive outcomes. VBA differences between groups and also in functional correlations were analyzed adjusting for gender. Image analysis, processing and regression analysis was performed by means of in-house software implemented in Matlab 2011a (The MathworksInc, Natick, MA, USA).

Results

1- Sample characteristics

There were no surgical or postoperative complications in the 10 dams included. A total of 69 fetuses were included at the time of the induction (23 controls and 47 cases), 49 of which were alive at delivery (19 controls and 30 cases). Postnatally, 5 controls and 17 cases died within the first week of life, thus, 14 controls and 13 cases reached the long-term period. Overall, both the fetal and neonatal mortality rate was higher in cases (stillbirth 17.4% vs. 36.2%, $p = 0.08$ and neonatal mortality 26.3% vs. 56.7%, $p = 0.01$, controls vs. cases respectively). The birth weight was significantly lower in cases compared to controls (49.54 g (SD 5.85) vs. 38.34 g (SD 5.36), $p \leq 0.001$). Nevertheless, these differences were not observed at the 70th postnatal day (2747 g (SD 190) vs. 2626 g (SD 489), $p = 0.41$). Neither were any differences found in the time of postnatal evaluation (71 (IQR 3) vs. 70 (IQR 4) postnatal days, $p = 0.099$) nor in gender distribution (63.6% vs. 50% females, $p = 0.425$).

Of the 27 animals that were functionally evaluated, 6 animals (3 controls and 3 cases) were excluded from the final analysis due to gross tissue abnormalities resulting from sample extraction or manipulation observed in the standard MRI acquisition, with 21 animals in the final sample (11 controls and 10 cases). During the postnatal period, no gross motor abnormalities such as paresia or spasticity were observed in either group.

2- Neurobehavioral and cognitive outcomes: functional tests results

Concordance between the two functional tests from each blinded observer was explored using the interclass correlation coefficient which demonstrated good reliability (mean: 0.941).

In the Open Field Test, IUGR rabbits presented reduced exploratory activities, with a significantly increased latency of leaving the starting point and a trend to present reduced speed while exploring and less rearing. In addition, cases showed a significant reduction in time spent in the internal area as well as a

Table 1. Open field behavioral results in study groups adjusted by gender.

	Controls n = 11	*Cases n = 10*	*p*
Latency of leaving the starting point, seconds †	3.0 (29.0)	59.0 (217.5)	*0.036*
Total squares crossed, number †	113.0 (26.0)	74.5 (54.0)	*0.272*
Total time exploring, seconds	424.5 (106.3)	330.1 (149.5)	*0.139*
Velocity of travelling (total squares/total time)	0.3 (0.1)	0.2 (0.1)	*0.395*
External squares crossed, number	101.2 (37.3)	65.2 (35.4)	*0.034*
Time in external squares, seconds †	578.0 (16.0)	598.0 (8.0)	*0.017*
Internal squares crossed, number	9.2 (4.5)	3.3 (3.2)	*0.004*
Time in internal squares, seconds †	22.0 (16.0)	2.0 (8.0)	*0.083*
Grooming, number †	1.0 (2.0)	0.0 (0.0)	*0.268*
Rearing, number	23.3 (10.3)	15.7 (11.2)	*0.165*

Results are mean and standard deviation (mean (SD)) in normal variables, with median and interquartile range (median (IQR)) in non-normal variables †.
doi:10.1371/journal.pone.0076453.t001

reduction in the number of areas crossed in both the internal and external areas (Table 1).

Regarding the Object Recognition Task, 7 cases and 8 controls fulfilled the previously established criteria. No differences were found in the time exploring right and left objects between groups in the Familiarization phase (right object: 9.50 s (SD 5.31) vs. 7.85 s (SD 0.04), p = 0.585; left object: 6.00 s (IQR 6.75) vs, 2.00 s (IQR 11.00), p = 0.69, controls vs. cases respectively). On the contrary, in the Testing phase controls spent significantly less time exploring the familiar object compared to cases (3.63 s (SD 1.92) vs. 6.71 s (SD 1.80), p = 0.011, controls vs. Cases, respectively). Interestingly, significantly decreased DI was observed in cases as well as a decreased proportion of rabbits achieving learning criteria (Figure 2).

Additionally we explored the relationship between birth weight and the neurobehavioral and cognitive measures, and as expected, we observed a significant correlation in almost all the parameters (Table S1).

3- MRI analysis

3.1- Regional analysis: Voxel-based analysis. When VBA analysis was applied, statistically significant differences were found in FA distribution with a decreased FA in cases compared to controls in multiple structures including cortical regions (insular and temporal) and subventricular WM. The coefficient of linearity was also lower in cases in multiple areas including cortical regions (insular, temporal, prefrontal, and occipital), thalamus, superior colliculus, hippocampal formation and fimbria of hippocampus. The coefficient of planarity showed increased values in the occipital cortex and thalamus in IUGR rabbits, but decreased values in the insular cortex and cerebellar hemispheres. Finally, an

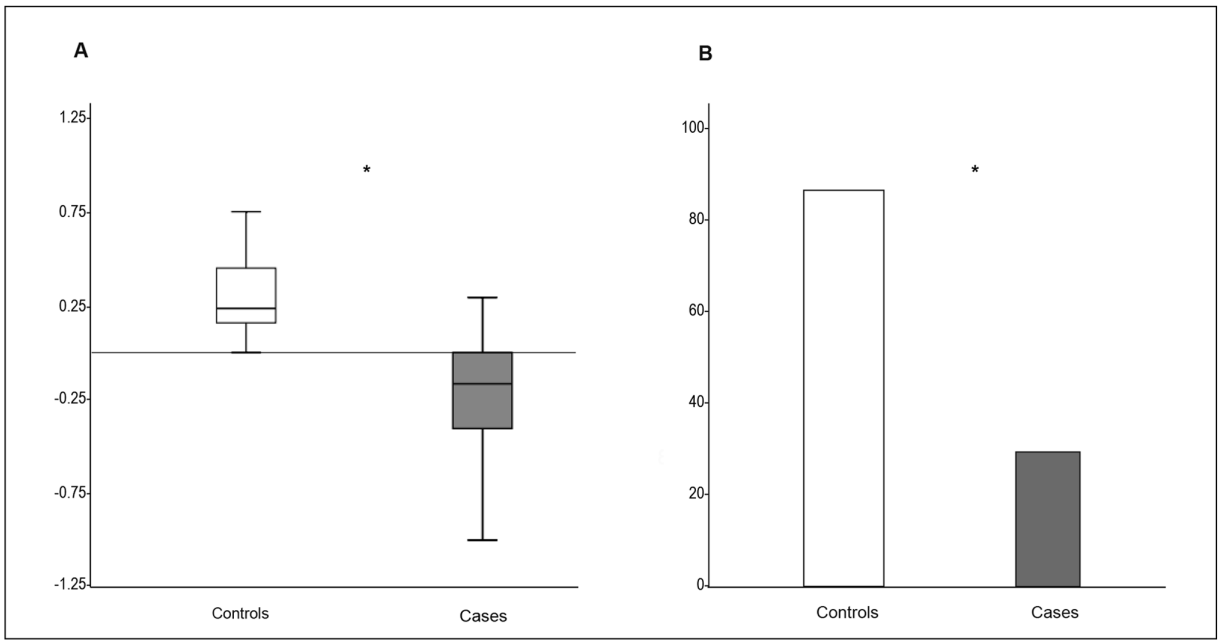


Figure 2. Discriminatory index results and percentage of learning in study groups. (A) Discriminatory index values of the study group (p = 0.013, adjusted for gender); (B) Percentage of controls and cases that reached the learning criteria (p = 0.03, adjusted for gender).
doi:10.1371/journal.pone.0076453.g002

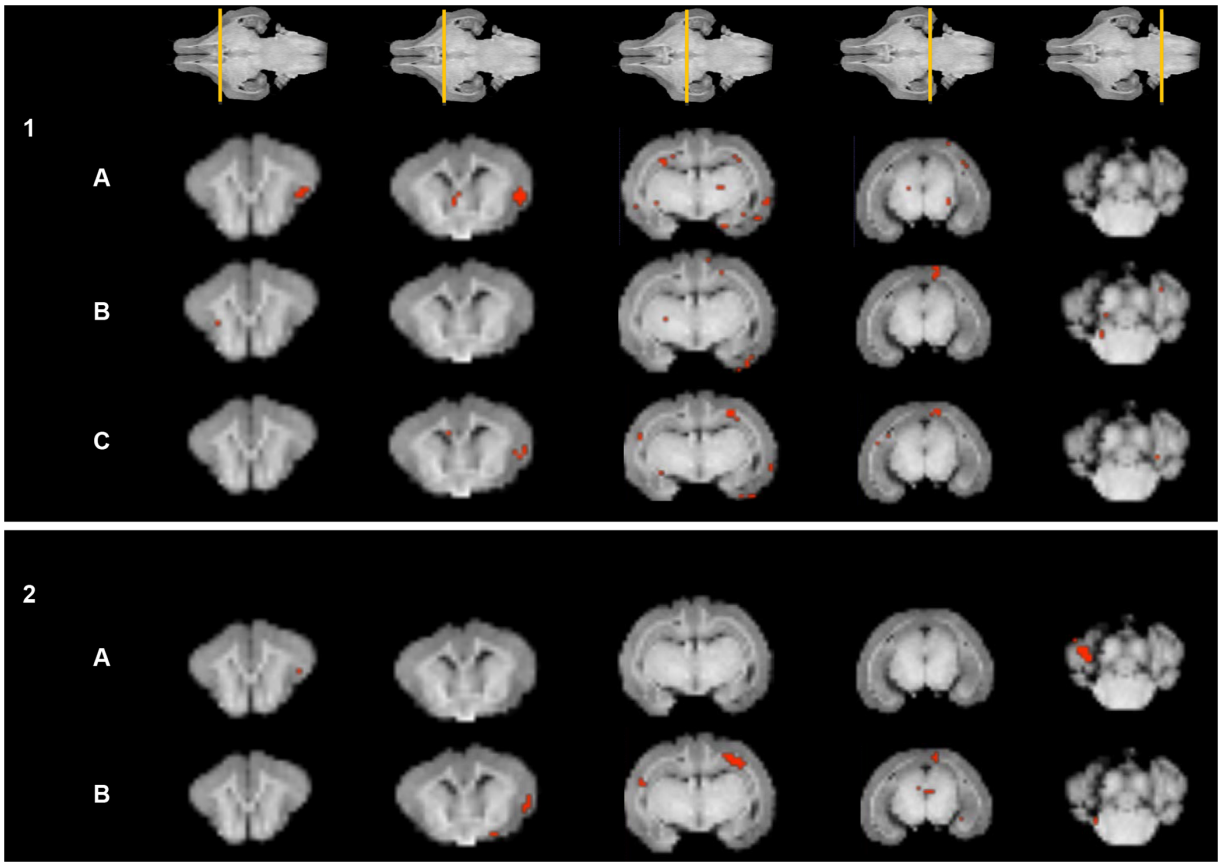


Figure 3. Fractional anisotropy, linearity, planarity and sphericity coefficients: regions showing statistically significant differences ($p<0.01$) between cases and controls. Coronal slices of the 3D reference image displaying representative anatomical structures for specific coefficients. Slice locations are shown in the T1 weighted images at the top. **PANEL 1:** Representative anatomical regions showing a significant decrease in linearity (A) and planarity (B) coefficients and in fractional anisotropy (C) in cases compared to controls. **PANEL 2:** Representative anatomical regions showing a significant increase in planarity (A) and sphericity (B) coefficients in cases compared to controls.
doi:10.1371/journal.pone.0076453.g003

increased coefficient of sphericity was observed in insular cortex and subventricular WM (Figure 3).

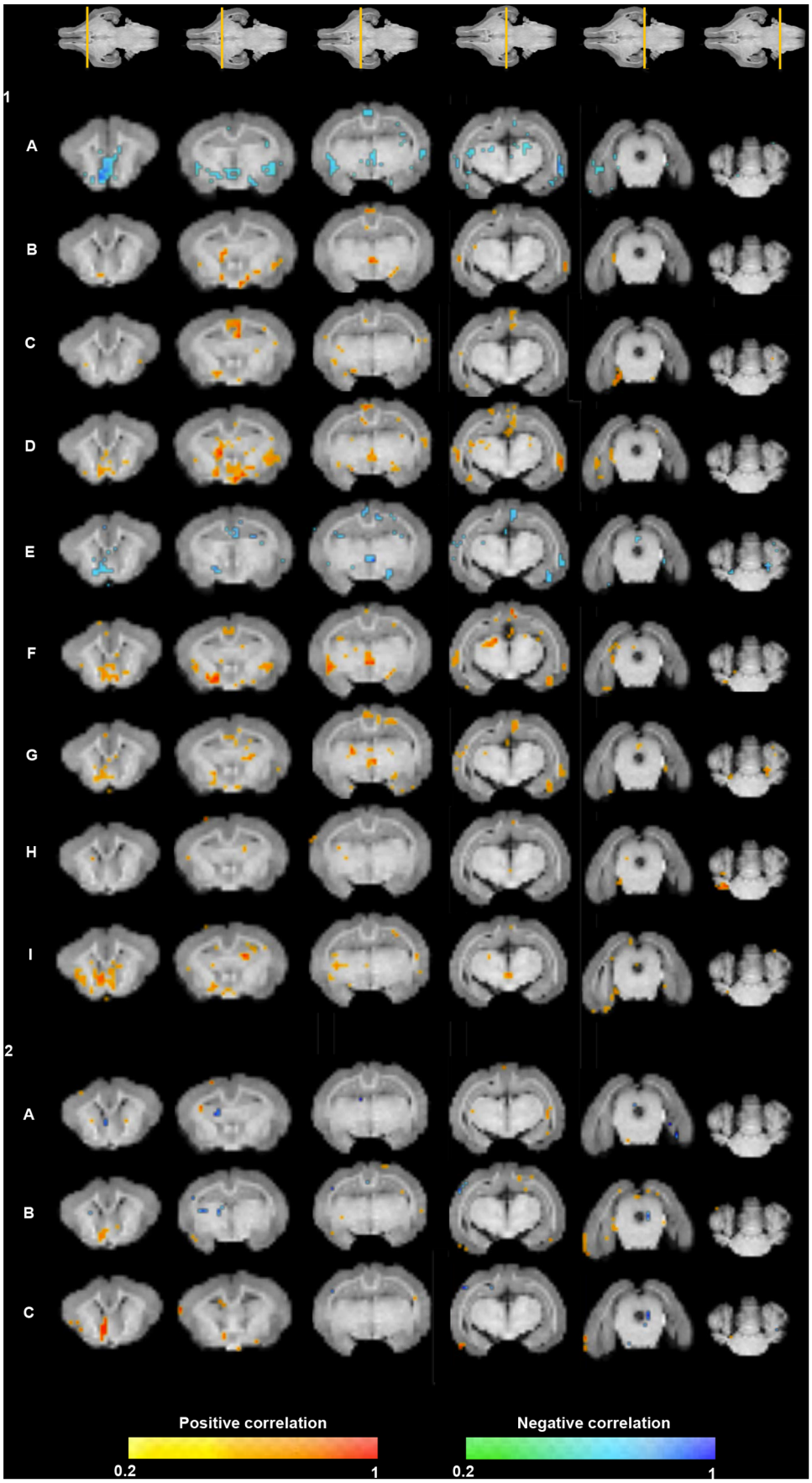
3.2- Correlation between MRI diffusion and neurobehavioral and cognitive outcomes. The FA map shows correlations between functional variables, especially for the Open Field Test, and multiple brain areas (Figure 4 and Table 2). Regarding the GM structures, FA changes in hippocampus and hippocampal formation and in the cingulate and temporal cortex were correlated with more neurobehavioral domains; followed by the prefrontal cortex, thalamus and putamen nucleus. Interestingly, the amygdala presented a significant correlation with two of the variables that are strongly related to anxiety (number of squares crossed and time spent in the internal area). Within the WM structures, the anterior commissure and corona radiata areas showed more correlations with neurobehavioral and cognitive domains. All these findings were supported by similar changes in linearity, sphericity and planarity coefficients (Figure S1, Figure S2 and Figure S3).

3.3- Connectivity analysis. Analysis of the total number of WM fiber tracts reconstructed for the whole brain did not differ between groups (14775 (SD 2332) vs. 13921 (SD 2148), $p = 0.371$, controls vs. cases). Nevertheless, on evaluation of the percentage of fibers involved in a specific network, the cases showed a trend to present a lower ratio of fibers in both networks; being statistically significant in the left hemisphere for both networks (Figure 5 and 6). Table 3 depicts the mean correlation coefficients between the percentage of fibers and functional test results. Regarding the anxiety network, the left hemisphere was significantly correlated with nearly all the variables in the Open Field Test, whereas in the memory network any variable achieved statistical significance. Finally, we did not observe significant differences in the mean FA in the two networks, although there was a trend to presenting a lower FA in cases compared to controls, especially in the anxiety network (Table 4). In addition, we did not observe a correlation between FA results and the functional results (data not shown).

Again, we explored the relationship between birth weight and the ratio of fibers in both networks observing significant correlations in almost all ratios for both networks (Table S2).

Discussion

To the best of our knowledge, this is the first report simultaneously characterizing long-term cognitive neurobehavioral and cognitive dysfunctions and the related neuroanatomical changes in a near-term IUGR rabbit model using advanced imaging techniques. It has been described that neurobehavioral and cognitive impairments associated with IUGR mainly comprise short-term memory, attention and anxiety, but neuroanatomical correlations have not as yet been reported.


1
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Positive correlation
0.2
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Negative correlation
0.2
1

Figure 4. Correlation maps between neurobehavioral and cognitive tests items and fractional anisotropy values. Coronal slices (from anterior to posterior) of the 3D reference image are displayed. Colormap highlights the areas where the correlation coefficient is higher than 0.2. Spearman correlation $p<0.001$. **PLANEL 1:** (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, (I) Rearing. **PLANEL 2:** (A) Time exploring familiar object, (B) Time exploring novel object and (C) Discriminatory index.
doi:10.1371/journal.pone.0076453.g004

1- Long-term neurobehavior and cognitive results

Results from the Open Field Test showed that IUGR rabbits presented a higher degree of anxiety expressed by reduced exploratory activities similar to what has been described in rat models after acute hypoxic-ischemic injury [90]. In addition, there was an increase in the time spent in the periphery and in the latency after leaving the starting point, a characteristic sign of anxiety in animals [91]. Likewise, the IUGR rabbits presented decreased grooming activity. Although the interpretation of grooming behavior in rodents is complex, changes in the incidence of this particular behavior have been also related to altered levels of anxiety [92]. Increased anxiety has been described in rats after perinatal hypoxic insult [93], and in human adolescents and adults with a history of IUGR [11,24]. Data derived from the Object Recognition Task have demonstrated that the IUGR rabbit model demonstrates short-term memory and attentional disorders similar to what has been reported in humans [13]. Our results are comparable to those obtained in rats after prenatal unilateral uterine artery occlusion [94,95].

Overall, with the application of these two tests we have demonstrated that the surgical model of IUGR in pregnant rabbits reproduces some of the cognitive and neuropsychological features described in IUGR children.

2- MRI regional analysis

Long-term structural changes were more remarkable in GM areas and included multiple cortical regions (insular, temporal, prefrontal, occipital cortices and cerebellar hemisphere) and deep GM nuclei (thalamus and hippocampus). Interestingly, our findings of DTI changes in the prefrontal and entorhinal cortices and hippocampus are in line with previous evidence aimed at describing histology changes in the long-term period in the offspring of pregnant rats with IUGR after prenatal occlusion of the unilateral uterine artery. These histological changes include a decreased number of neurons, astrogliosis, an increase in GABAergic neurons and diffuse axonal degeneration [94,95]. Changes in GM detected by DTI have been proposed to reflect changes in the dendritic architecture of pyramidal cells [31,96] which could, in turn, suggest a connectivity impairment of these GM structures. Concerning WM, regional analysis of DTI parameters revealed significant differences with decreased FA and linearity and increased sphericity values in the fimbria of the hippocampus and in the subventricular WM in IUGR group. FA values are closely related to myelination process, increasing its values in WM areas during brain maturation [31]. Decreased values of FA in WM tracts have previously been described after mild hypoxic-isquemic injury and correlated with decreased myelin content, persisting these changes after the recovery period [97]. Consistently with decreased FA, IUGR showed decreased linearity and increased sphericity coefficients that are related with less organized fiber tracts in WM bundles [63]. Therefore, our

Table 2. Significant correlations ($p<0.01$) between neurobehavioral and cognitive tests items and fractional anisotropy in brain regions adjusted by gender.

	Positive correlation	*Negative correlation*
Open Field Behavioral Test		
A		Temporal and cingulate cortices, putamen, thalamus, claustrum, anterior commissure
B	Cingulate cortex, claustrum, hippocampus, corpus callosum, anterior commissure, lateral lemniscus	
C	Cingulate, prefrontal and occipital cortices, hippocampal formation, corona radiata	
D	Prefrontal, temporal, cingulate and insular cortices, putamen, thalamus, claustrum, lateral lemniscus	
E		Cingulate cortex and hippocampus
F	Cingulate cortex, thalamus, amygdala, hippocampus, claustrum, superior colliculus, lateral lemniscus	
G	Cingulate and occipital cortices, amygdala, anterior comissure	
H	Caudate nucleus, cerebellar hemisphere	
I	Temporal cortex, hippocampus, thalamus, claustrum	
Object Recognition Task		
A	Occipital cortex, corona radiata	
B	Occipital cortex, anterior comissure	
C	Cingulate cortex, anterior comissure	

Open Field Behavioral Test items:(A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, (I) Rearing; Object Recognition Task items: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.
doi:10.1371/journal.pone.0076453.t002

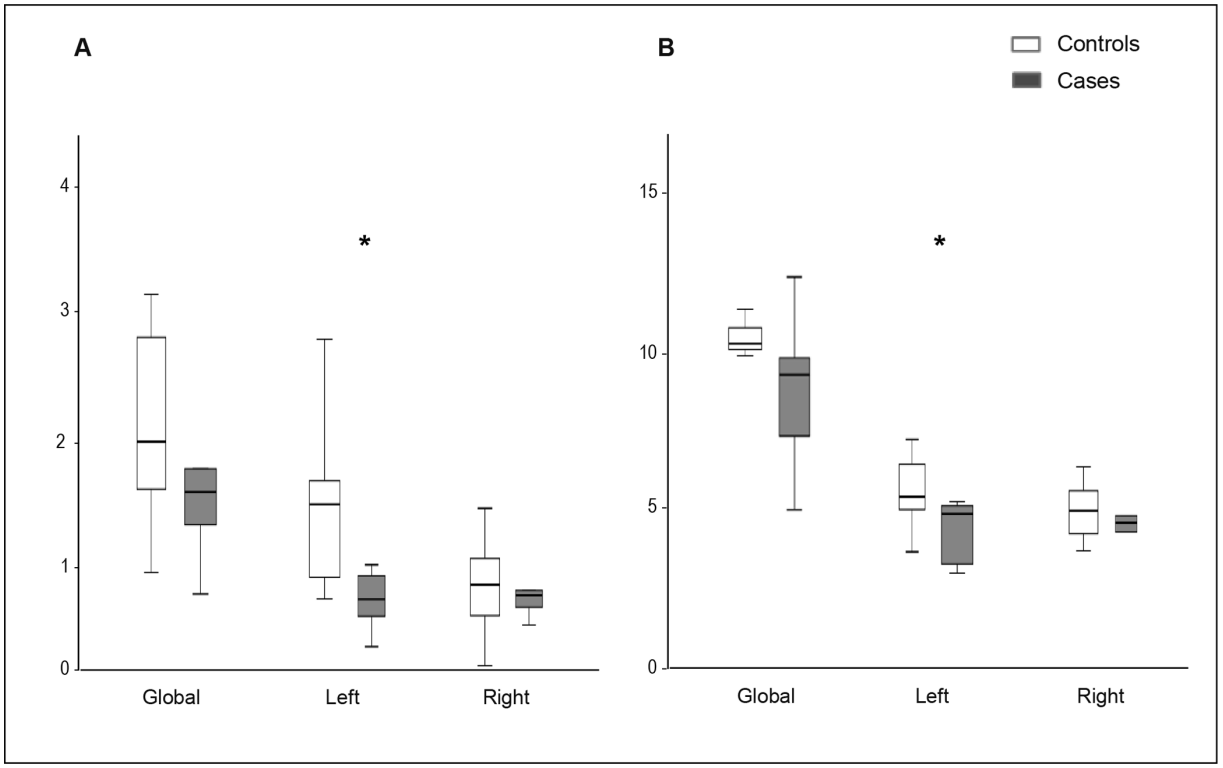

Figure 5. Ratio of fibers involved in the anxiety or memory networks over the total number of fibers. (A) Ratio of fibers adjusted for gender in anxiety network; in global analysis ($p = 0.10$), in left ($p = 0.01$) and right hemisphere ($p = 0.59$ †); (B) Ratio of fibers adjusted for gender in memory network; in global analysis ($p = 0.08$), in left ($p = 0.03$ †) and right hemisphere ($p = 0.92$ †). † Non-normal variables.
doi:10.1371/journal.pone.0076453.g005

results support the hypothesis that IUGR is related with an altered and delayed WM organization and maturation that persists even at long-term period. It should be noted that WM changes seemed to be less pronounced in comparison with our previous findings in which structural brain changes in the neonatal period were assessed using the same animal model [34]. One explanation for the few differences observed in WM structures could be derived for the voxel size used. It should be taken into account that a voxel size of $0.7 \times 0.7 \times 0.7$ mm^3 may produce some partial volume effects which may hinder the presence of differences in some small brain areas, such as thin WM tracts. If these partial volume effects had been present, they would have resulted in a conservative bias, thus attenuating the existing differences and not affecting the validity of the differences observed. Aside from methodological limitations, the assignment of most of the diffusion changes observed to GM compared to WM may indicate that long-term brain plasticity throughout childhood and adolescence [98,99] is more efficient at correcting WM than GM deficits. In line with this notion, myelin content increases from the neonatal period up to young adulthood in an IUGR surgical guinea pig model [100]. The same histological findings, together with a reduction in the magnitude of differences with respect to controls in FA, have been reported in long-term as compared with neonatal measurements in rats [97]. Regional changes in FA showed significant correlations mostly in GM structures with functional results, especially those related to the Open Field Test. With this test, the hippocampal complex, prefrontal and cingulate cortices presented the highest number of correlations. Animal studies have demonstrated the important role of a normal functioning of the hippocampus in the regulation of anxiety [73,101,102]. Concerning prefrontal and cingulate cortices, reduced volumes in children with ADHD [103] and healthy individuals [104] as well as histological changes in rodents [105] in these structures have been associated with attention and anxiety traits. In addition, changes in diffusion MRI parameters of the amygdala were correlated with the number of squares crossed and the time spent in the internal area, two items strongly related to anxiety. These findings are in line with the reported role of the amygdala in the processing of fear and anxiety [72,73].

Concerning correlations with the Object Recognition Task, within GM structures we observed a significant correlation between regional FA changes in the cingulate cortex and the Object Recognition Task results. Several experimental studies in rodents have found that the cingulate cortex plays a key role in novelty detection, attention and memory in fearful situations [106–109], and any disruption in this structure could impair memory consolidation [110]. Taking this into account, although the Object Recognition Task was conducted in the same arena in which the rabbit had previously performed the Open Field Test, persistence of any degree of anxiety while performing the Object Recognition Task could not be ruled out. This could impair memory consolidation in those animals with structural changes in the cingulate, such as our results suggest. This suggestion is in line with clinical studies that have postulated that short-term memory problems observed in IUGR children may be accounted for by a lack of sufficient attention rather than a deficit in processing the information per se [13], impeding short-term memory function. Regarding WM and Object Recognition Task results, the most consistent correlations, as they were observed in all the DTI parameters, were found in the anterior commissure and corona radiata. These tracts connect several brain areas that are engaged in memory and attention [111–113]. Contrary to our original

Table 3. Mean correlation coefficients between ratio of fibers in anxiety and short-term memory networks and neurobehavioral and cognitive tests item results (Spearman's correlation) adjusted by gender.

	Global	*Left Hemisphere*	*Right hemisphere*
Anxiety network			
A	−0.30	**−0.50***	−0.06
B	0.30	**0.56****	−0.05
C	−0.01	0.13	−0.01
D	0.30	**0.55***	−0.05
E	−0.43	**−0.49***	−0.10
F	0.35	**0.52***	0.10
G	0.43	**0.48***	0.10
H	0.05	−0.05	0.24
I	0.18	0.18	0.22
Memory network			
A	−0.51	−0.31	−0.42
B	0.18	0.34	−0.45
C	0.50	0.46	0.11

Open Field Behavioral Test items: (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, (I) Rearing. Object Recognition Task items: (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.
*<p 0.05.
**<p 0.01.
doi:10.1371/journal.pone.0076453.t003

hypotheses, we did not observe any significant correlations between GM and Object Recognition Task results in brain areas classically described to be involved in memory recognition, such as hippocampal formation, temporal lobe and prefrontal cortex [80–82,84,86]. These findings suggest that short-term memory impairment induced by IUGR as reflected in the Object Recognition Task could depend more on the connectivity between relevant regions than on intrinsic changes in their GM. This notion is in line with previous findings supporting strong dependence of memory formation and on the integrity of the perirhinal-hippocampal-medial prefrontal network [85–87].

Table 4. Fractional anisotropy values of fiber tracts within each network adjusted by gender.

	Controls n = 11	*Cases n = 10*	*p*
Anxiety network			
Global	0.2539 (0.0161)	0.2448 (0.0250)	*0.319*
Left	0.2512 (0.0164)	0.2478 (0.0332)	*0.759*
Right	0.2519 (0.0408)	0.2391 (0.0283)	*0.367*
Memory network			
Global †	0.2310 (0.0112)	0.2197 (0.0181)	*0.128*
Left	0.2322 (0.0118)	0.2203 (0.0216)	*0.162*
Right †	0.2233 (0.0273)	0.2219 (0.0128)	*0.128*

Results are mean and standard deviation (mean (SD)) in normal variables, with median and interquartile range (median (IQR)) in non-normal variables †.
doi:10.1371/journal.pone.0076453.t004

In summary, these results partially confirm the hypotheses formulated in clinical studies on children and adolescents with IUGR, but provide new insight as to the specific structural anomalies underlying neurobehavioral and cognitive impairments.

3- Connectivity analysis

IUGR showed a decreased number of fibers in anxiety, attention and memory networks over the total number of fibers reconstructed. These differences were statistically significant in the left hemisphere, with a trend to decreased FA in both networks. Moreover, a significant correlation was observed between the ratio of fiber in the left hemisphere for anxiety network and functional results. Overall, our results are in line with previous MRI diffusion studies in patients with anxiety and attention disorders or memory impairments. Changes in connectivity within the prefrontal and anterior cingulate cortexes and the amygdala have been correlated with anxiety [78,114,115]. In addition, microstructural changes in the connectivity within the fronto-striatal pathway and WM tracts connecting the amygdala and the prefrontal cortex have been described to be strongly related to the ADHD disorder in children and adolescents [37,116–118]. Concerning the memory network, reduced FA has been observed in WM tracts connecting the temporal cortex and the hippocampus in children [119] and in the corona radiata in adults with mild traumatic injury [112]. In these studies, decreased FA was correlated with object recognition results in children, and with attentional and memory impairment in adults. In addition, changes in parahippocampal WM that connects the entorhinal cortex with the hippocampus were correlated with declarative memory problems in elderly patients with mild cognitive impairment [120,121]. Most of the differences observed in our study were restricted to the left hemisphere. Differences observed in the left anxiety network are consistent with previous evidence suggesting left hemisphere lateralization in fear-related anxiety processing [122].

Altogether, the results of this study support the contention that altered connectivity patterns within regions involved in anxiety, attention and memory are involved in the functional impairment associated with IUGR that persists up to the preadolescent period and suggests the importance of completing the normal programming of neuronal connectivity patterns for the achievement of normal neurodevelopment. The data reported demonstrate a decreased number of fibers in combination with more modest changes in FA. These results are different to those observed in the neonatal period [34], and support the idea that, in the long-term, structural changes are essentially related to the distribution rather than with the integrity of fibers. These findings are in line with previous evidence demonstrating that delayed myelination during critical developmental periods can be restored later [100], but will lead to long-term consequences in the patterns of connectivity, as has been consistently demonstrated in human and experimental studies [123,124].

4- Methodological considerations and limitations of the study

The methodology used to perform both VBA and connectivity analysis in this study deserves some discussion. With regard to connectivity analysis, we acknowledge that the networks defined in this study have not been fully validated, although we used consistent evidence from the literature demonstrating the involvement of all the selected regions in the functions of interest. In addition, we acknowledge that there are no standard or widely validated approaches for quantifying tractography metrics in defined networks. Several studies have previously used this approach in human studies to characterize changes in brain

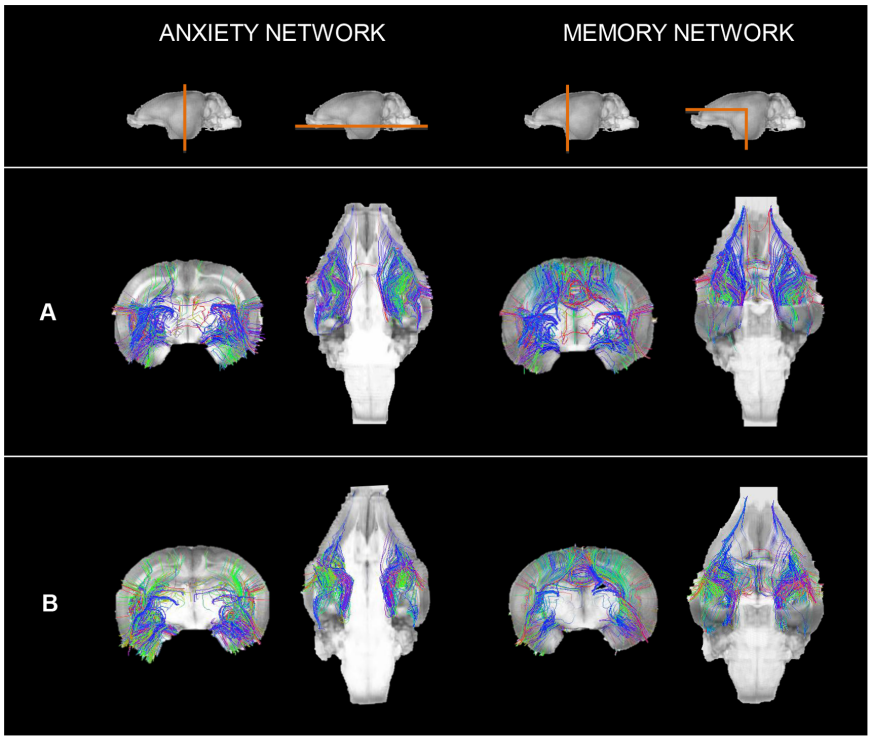

Figure 6. Reconstructed fibers in anxiety and memory networks in the experimental groups. Coronal and axial views of anxiety and memory networks of one control (A) and one case (B). Reconstructed fibers are overlapped to the 3D reconstruction of T1 weighted images.
doi:10.1371/journal.pone.0076453.g006

structure and its neurobehavioral correlates in neurodevelopmental diseases such as ADHD [37], focal perinatal brain injury [125], and periventricular leucomalacia [39,40]. Only a few studies have used fiber count to assess the connectivity within specific brain areas [39,40,126] and these studies did not adjust for brain size or total number of fibers reconstructed. In the present study we introduced this methodological change in order to counter the potential bias of differences in the total number of fibers reconstructed from case to case. Regarding VBA, the use of this approach implies weaker statistical power due to the large number of voxels tested [67], increasing type I error rate. This is partially compensated by the smoothing after registering the DTI volumes to the reference. By smoothing the DTI maps, the effective number of multiple comparisons in the statistical testing was reduced, thereby improving statistical power [67]. We acknowledge that not correcting for multiple comparisons introduces a bias in the interpretation of results. However, as noted above, we intended to use this method in an exploratory fashion which allowed to suggest potential relationships. We would like to stress that confirmation of the relationships here suggested requires further studies with larger sample sizes. Another issue concerning VBA is that the method requires registration of all the subjects in the dataset to a template volume, and therefore the arbitrary choice of this template could bias the result [67]. As described in the methodology section, this issue was addressed by repeating the VBA considering each of the subjects as the reference. Finally, we did not include ADC data in the regional analysis since the fixation process decreases the water content in brain tissue, reducing absolute ADC values in a non-homogeneous and, therefore, non-predictable manner [127], especially in hypoxic tissue [128].

From the point of view of the experimental design, the high mortality rate during the first postnatal week may have selected less severe cases for the long-term follow up, thus attenuating the true impact of the condition. Despite this conservative bias, we were able to demonstrate structural and functional changes after IUGR. Finally, we acknowledge that our sample size may be underpowered to evaluate gender differences in the variables assessed. However, we decided to include gender as a potential confounder in our analysis since adjustment is recommended when biologically confounding is likely, as occurs in many neurobehavioral processes [129].

Conclusions

In conclusion, we have developed a rabbit model reproducing functional and neurostructural consequences of near-term IUGR which persist up to young adulthood. Diffusion MRI demonstrated differences in the specific brain regions involved in the regulation of anxiety, attention and memory and in their related networks which were correlated with long-term functional impairments. The study provides evidence of the type of structural changes involved in long-term neurodevelopmental anomalies associated with IUGR and support the potential value of methods based on diffusion quantitative metrics to assess changes associated with brain reorganization that are not demonstrable by standard imaging techniques. Using the methodology described herein, further multi-scale studies could be performed in order to advance the understanding of the prenatal mechanisms underlying abnormal neurodevelopment to thereby target potential biomarkers based on diffusion MRI and connectivity analysis for early diagnosis and monitoring of the impact of interventional studies.

Supporting Information

Figure S1 Correlation maps between neurobehavioral and cognitive tests items and linearity coefficient. Coronal slices (from anterior to posterior) of the 3D reference image are displayed. Colormap highlights the areas where each correlation coefficient is higher than 0.2. Spearman correlation $p<0.001$. **PLANEL 1:** (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, and (I) Rearing. **PLANEL 2:** (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.
(TIF)

Figure S2 Correlation maps between neurobehavioral and cognitive tests items and sphericity coefficient. Coronal slices (from anterior to posterior) of the 3D reference image are displayed. Colormap highlights the areas where each correlation coefficient is higher than 0.2. Spearman correlation $p<0.001$. **PLANEL 1:** (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, and (I) Rearing. **PLANEL 2:** (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.
(TIF)

Figure S3 Correlation maps between neurobehavioral and cognitive tests items and planarity coefficient. Coronal slices (from anterior to posterior) of the 3D reference image are displayed. Colormap highlights the areas where each correlation coefficient is higher than 0.2. Spearman correlation $p<0.001$. **PLANEL 1:** (A) Latency of leaving the starting point, (B) Total squares crossed, (C) Total time exploring, (D) External squares crossed, (E) Time in external area, (F) Internal squares crossed, (G) Time in internal area, (H) Grooming, and (I) Rearing. **PLANEL 2:** (A) Time exploring familiar object, (B) Time exploring novel object, and (C) Discriminatory index.
(TIF)

Table S1 Mean correlation coefficients between functional results and birth weight (Spearman's correlation).
(DOC)

Table S2 Mean correlation coefficients between ratios of fibers and birth weight (Spearman's correlation).
(DOC)

Acknowledgments

We thank Professor V. D. Chamizo and coworkers (Universitat de Barcelona) for their support in the evaluation of the neurobehavioral tests.

Author Contributions

Conceived and designed the experiments: MI EE DB EMM FF EG. Performed the experiments: MI EE DB AAP EMM. Analyzed the data: MI EE DB AAP EMM FF EG. Contributed reagents/materials/analysis tools: MI EE DB EMM AAP. Wrote the paper: MI EE DB EMM FF EG. Manual segmentation MRI: MI AAP. Evaluation of neurobehavioral test: MI AAP.

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Manuscript Type	Original Paper-Basic Science and Pathophysiology
TITLE	Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction

Running Title	Environmental enrichment enhances brain connectivity in IUGR
Author List	Illa M., Brito V., Pla L., Eixarch E., Arbat-Plana A., Batalle D., Muñoz-Moreno E., Crispi F., Udina E., Figueras F., Ginés S., Gratacós E.
Keywords	Animal model, Intrauterine growth restriction
Author Suggested Keywords	Dendritic spine; Diffusion magnetic resonance; environmental enrichment; object recognition task; open field behavioral test; perineural nets; skinner test; therapy
Preferred Reviewer	

Abstract

Introduction: Structural correspondence of neurodevelopmental impairments related with Intrauterine growth restriction (IUGR) that persists later in life remains elusive. Moreover, early postnatal stimulation strategies have been proposed to mitigate these effects. Brain connectivity abnormalities at long-term period in an IUGR rabbit model and the effects of early postnatal environmental enrichment have been explored. Material and Methods: IUGR was surgically induced in one horn, whereas the contralateral produced the controls. Postnatally, a subgroup of IUGR animals was housed in an enriched environment. Functional assessment was performed at the neonatal and long-term periods. At the long-term period, structural brain connectivity was evaluated by means of diffusion brain resonance imaging and by histological assessment focused in hippocampus. Results: IUGR animals displayed poorer functional results and presented an altered whole-brain networks and decreased median fractional anisotropy in the hippocampus. Reduced density of dendritic spines and perineural nets from hippocampal neurons were also observed. Of note, IUGR animals exposed to enriched environment presented an improvement in terms of both function and structure. Discussion: IUGR is associated with altered brain connectivity at global and cellular level. A strategy based on early environmental enrichment has the potential to restore IUGR neurodevelopmental consequences.

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Early environmental enrichment enhances abnormal brain connectivity in a rabbit model of intrauterine growth restriction

Miriam Illa[a,b], Verónica Brito[b-d], Laura Pla[a,b], Elisenda Eixarch[a,b,e], Ariadna Arbat-Plana[d,f], Dafnis Batalle[g], Emma Muñoz-Moreno[a,h], Fatima Crispi[a,b,e], Esther Udina[d,f], Francesc Figueras[a,b,e], Sílvia Ginés[b-d], Eduard Gratacós[a,b,e]

[a] Fetal i+D Fetal Medicine Research Center, BCNatal - Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Deu), Institut Clínic de Ginecologia, Obstetricia i Neonatologia, Universitat de Barcelona, Barcelona, Spain

[b] Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

[c] Departament de Biomedicina, Facultat de Medicina, Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain

[d] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Bellaterra, Spain

[e] Centre for Biomedical Research on Rare Diseases (CIBER-ER), Barcelona, Spain

[f] Institute of Neurosciences, Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain

[g] Centre for the Developing Brain, Division of Imaging Sciences & Biomedical Engineering, King's College London, London, SE17EH, United Kingdom

[h] Experimental MRI 7T Unit, Institut d'Investigacions August Pi i Sunyer (IDIBAPS), Barcelona, Spain

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*** Corresponding Author:**

Elisenda Eixarch, MD PhD.

Fetal i+D Fetal Medicine Research Center, BCNatal - Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Déu), IDIBAPS, University of Barcelona, Barcelona, Spain.

Sabino de Arana 1, 08028 Barcelona, Spain.

Tel. +34 932 275 400 EXT. 7339.

Email: eixarch@clinic.ub.es

***Short title:** Environmental enrichment enhances brain connectivity in IUGR

*** Conflicts of Interest:**

The authors report no conflict of interest.

*** Source of Funding:**

The research leading to these results has received funding from “la Caixa” Foundation. Additionally, this study was supported by Instituto de Salud Carlos III (PI15/00130; PI12/00851; PI13/01018) integrated in the “Plan Nacional de I+D+I” and copaid by “ISCIII-Subdirección General de Evaluación” and “Fondo Europeo de Desarrollo Regional” (FEDER) “Una manera de hacer Europa”; AGAUR 2014 SGR grant nº 928; Ministerio de Ciencia e Innovación (SAF2015 -67474-R;MINECO/FEDER to S.G); and Centro de Investigaciones Biomédicas en Red sobre Enfermedades Neurodegenerativas (CIBERNED).

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ABSTRACT:

Introduction: Structural correspondence of neurodevelopmental impairments related with Intrauterine growth restriction (IUGR) that persists later in life remains elusive. Moreover, early postnatal stimulation strategies have been proposed to mitigate these effects. Brain connectivity abnormalities at long-term period in an IUGR rabbit model and the effects of early postnatal environmental enrichment have been explored.

Material and Methods: IUGR was surgically induced in one horn, whereas the contralateral produced the controls. Postnatally, a subgroup of IUGR animals was housed in an enriched environment. Functional assessment was performed at the neonatal and long-term periods. At the long-term period, structural brain connectivity was evaluated by means of diffusion brain resonance imaging and by histological assessment focused in hippocampus.

Results: IUGR animals displayed poorer functional results and presented an altered whole-brain networks and decreased median fractional anisotropy in the hippocampus. Reduced density of dendritic spines and perineural nets from hippocampal neurons were also observed. Of note, IUGR animals exposed to enriched environment presented an improvement in terms of both function and structure.

Discussion: IUGR is associated with altered brain connectivity at global and cellular level. A strategy based on early environmental enrichment has the potential to restore IUGR neurodevelopmental consequences.

Key words:

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Animal model, dendritic spine density, diffusion magnetic resonance, environmental enrichment, intrauterine growth restriction, object recognition task, open field behavioral test, perineural nets, Skinner test, therapy.

Abbreviations: DS: dendritic spine; EE: environmental enrichment; FA: fractional anisotropy; IUGR: intrauterine growth restriction; OFBT: open field behavioral test; ORT: object recognition task; PNNs: perineural nets.

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INTRODUCTION

Intrauterine growth restriction (IUGR) due to placenta insufficiency is a well-recognized cause of neurobehavioral and cognitive impairments extending beyond childhood [1, 2] and early adulthood period [3-5]. These neurodevelopmental problems have not been only associated to severe cases, but also milder forms of IUGR are at risk for abnormal neurodevelopment [6]. While severe IUGR affects 3% of pregnancies, mild IUGR affects up to 7% of deliveries, that is about 600,000 cases in Europe [7], representing a huge public health issue. Actually, IUGR is considered, together with prematurity, as the cause of one-quarter of cases for special educational needs [8]. However, the structural ground of these functional impairments is not fully characterized.

The description of the brain changes underlying long-term neurodevelopmental impairments of IUGR is essential for the development of imaging biomarkers for early diagnosis, monitoring [9], and selection of specific therapeutic strategies. With the significant advance of magnetic resonance imaging (MRI) in the recent years, diffusion-weighted imaging (DWI) techniques have demonstrated altered brain network organization could play an important role in this disorder [10-13]. At cellular level, standard histological assessment has not been able to show the structural brain changes underlying brain injury of IUGR that persist up to long-term period. More specific techniques focused in neuronal connectivity could better reflect the structural changes in IUGR, as changes in axonal and dendrite development have been suggested to be the histological basis of brain changes assessed by diffusion tensor imaging (DTI) studies [14,15].

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IUGR might have long lasting consequences, and currently, breastfeeding has been demonstrated to be one of the more effective strategy to partially ameliorate the long-term neurodevelopmental sequelae of IUGR [16]. Recently, other therapies are now arising as promising strategies to overcome brain diseases. Environmental enrichment (EE) strategy has consistently been demonstrated to exert beneficial effects on stress conditions and cognitive impairments by improving complex cognitive functions [17] and animal's emotional and stress reactivity [18-20]. This functional improvement was accompanied by changes at the cellular level, in terms of increased dendritic arborization, number of dendritic spines, synaptic density and postsynaptic thickening, particularly in the hippocampus [17,19,21]. Moreover, it has been demonstrated that neural circuits display a heightened sensitivity from the external environment inputs in specific periods of early postnatal life [22]. However, the neurodevelopmental effect of early EE has never been evaluated in an animal model of IUGR.

Hence, in this study we tested the hypotheses that 1) IUGR alters global and regional neuronal connectivity, and the changes persist up to the long-term period and, 2) a strategy based on EE applied during early postnatal period mitigates the neurodevelopmental impairments related with this condition. For this purpose, we evaluated neurobehaviour and structural changes in a rabbit model of placental insufficiency undergoing early EE [23]. Structural changes were assessed by advanced *ex vivo* DW-MRI and histological markers of neuronal connectivity, including dendritic spine (DS) density and perineuronal network (PNNs) in hippocampus.

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MATERIAL AND METHODS

Animals, IUGR induction and ethics Statement

Animal experimentation was approved by the Animal Experimental Ethics Committee of the University of Barcelona (permit number: 553/13). IUGR was induced in 10 New Zealand pregnant rabbit dams following selective uteroplacental artery ligation procedure in one of the horns at 25 days of pregnancy as previously described [23]. Five days later, a cesarean section was performed obtaining the animals. IUGR animals were obtained from the gestational sacs with arteries ligation, whereas the contralateral gestational sacs produced normally grown subjects (control). All surviving pups were weighed, identified by a subcutaneous microchip inserted in their back (Microchip MUSICC, Avid Microchip S.L., Barcelona, Spain) and were housed with a wet nurse rabbit with part of its offspring (maximum of 8 pups for each wet nurse), until the 30^{th} postnatal day when they were weaned.

Environmental enrichment strategy

After weaning (>30 postnatal days), the animals were housed in groups of three with a reversed 12/12h light cycle with free access to water and standard chow. The animals were housed in standard conditions, except for a subgroup of IUGR animals (n=15) that were housed following an EE strategy (t-IUGR group). The designed EE protocol was based on previous knowledge of behavioral needs and data available from enrichment studies in rabbits [24]. The implemented strategy aimed to increase the animal sensory, physical, cognitive and social stimulation. For that purpose, the animals were housed in larger cages (150x70x40cm) in comparison to the standard ones

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(75x70x40cm). Inside both types of cages, an upper platform allowing the animal to look out was placed, as a basic environmental refinement. However, only inside the t-IUGR animals cage different inanimate objects (wooden bridge, colored balls, bricks) and different flavors of food were placed. Every three days the objects and the food were changed in order to induce novelty and cognitive stimulation. In addition, social stimulation was induced by placing the animals in a big room during one hour twice per week, allowing them to freely explore the environment and to interact with a researcher (M.I., L.P.). This protocol was kept during 30 days up to the sacrifice of the animals. The study design is summarized in Figure 1.

Functional tests protocol and sampling collection

During the neonatal period (at +1 postnatal day), general motor skills, reflexes and sensitivity were evaluated (controls: n= 24; IUGR: n= 14; t-IUGR: n= 15) as previously described by Derrick et al [25,26].

At the long-term period (+60 postnatal days), a set of neurodevelopmental tests was applied in both IUGR and t-IUGR animals (IUGR: n= 14; t-IUGR: n=15). In the control group, due to the high number of animals eligible to be evaluated (n=24), only a subsample of them was included in this analysis (n= 13). In order to assess learning skills, Skinner test was applied following previously described methodology [27]. Learning was considered when the animal pressed the lever and went directly towards the food dispenser at least in three different times in the same session. For anxiety and memory evaluation, Open Field Behavioral Test (OFBT) and Object Recognition Task (ORT) were also applied [28]. ORT was attempted in all animals with a

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successful OFBT test, but only 11 controls, 14 IUGR and 13 t-IUGR fulfilled the ORT's established criteria as previously suggested [29]. The SMART Software Tracking System (from Panlab Harvard Apparatus, UK) was used to record the variables from OFBT (time in seconds exploring the internal area) and ORT (time in seconds exploring familiar and novel objects). The cumulative time exploring both objects from the ORT was recorded and discrimination index (DI) was then calculated as follows:

$$DI = \frac{time\ exploring\ novel\ object - time\ exploring\ the\ familiar\ one}{time\ exploring\ novel\ object + time\ exploring\ the\ familiar\ one}$$

A preserved memory was considered with DI > 0, whereas a DI ≤ 0 indicated problems in short-term memory. All functional tests were evaluated by two blinded observers (M.I, L.P).

Sample collection

After the neurobehavioral tests (at +70 postnatal days), rabbits were anaesthetized with ketamine 35 mg/kg and xylazine 5 mg/kg given intramuscularly, and were sacrificed with an endovenous overdose of sodium pentobarbital (200 mg/kg). Immediately after, 4 animals from each experimental group were randomly assigned to be included in the Dentritic spine (DS) evaluation and were processed according to this, whereas the rest of the animals followed a standard fixative protocol. In DS group, brains were fixed through cardiac perfusion with phosphate-buffered saline (PBS) followed by 2% paraformaldehyde (PFA), whereas the rest of the animals were fixed by 10% buffered 10% formalin. Finally, cranial bone was removed and brains were also fixed by 10 minutes immersion in 2% PFA in the brains included in the DS

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evaluation, whereas the rest were followed by an overnight immersion in 10% buffered formalin.

Magnetic resonance evaluation

After the functional evaluation, brains fixed by the standard protocol were randomly selected to perform MRI, obtaining 8 animals for each group. MRI was performed on fixed brains using a 7T animal MRI scanner (BrukerBioSpin MRI GmbH, Ettlingen, Germany). High-resolution three-dimensional T2-weighted and diffusion-weighted images (DWI) were acquired. Diffusion Tensor model was then fitted and fractional anisotropy (FA) was estimated in each voxel. Automatic parcellation of the subjects' brain was performed based on the New Zealand Rabbit MRI atlas [30]. Brain FA-weighted network for each subject was extracted and infrastructure (average strength), integration (weighted global efficiency) and segregation (weighted local efficiency) were assessed. In addition, median FA from the hippocampal regions was computed for each hemisphere, as well as median FA of the reconstructed streamlines crossing hippocampal regions.

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Histology assessment

a) Dendritic spine evaluation: 15 to 20 basal dendrites from each subject's hemisphere were selected to be evaluated from CA1 of the dorsal hippocampus (see Figure 1) using the Helios Gene Gun System (Bio-Rad) [31]. CA1 was selected for this analysis, as it has been described to be the hippocampal area that receives the major input connections [32,33]. Density of

DS (number of spines/µm) was calculated, including a final sample of 138 dendrites from controls, 155 dendrites from IUGR and 128 t-IUGR.

b) Perineural nets (PNNs): 16 brains fixed by the standard protocol explained previously (controls: n= 4; IUGR: n= 6; t-IUGR: n=6) were randomly selected to evaluate the PNNs. This analysis was done using lectin histochemistry Wisteria Floribunda (WFA) -binding and quantifying the average density of immunolabeling (contact/µm^2) from CA3 of the hippocampus (see Figure 1). Similarly to previous works [34], CA3 area from the hippocampus was preferred to analyze PNNs since a greatest amount of WFA staining was observed in comparison to the CA1 area.

For a more detailed description of MRI processing and histology assessment, see Supplementary Material.

Statistics

For quantitative variables, normality was assessed by Shapiro-Wilk Test and homoscedasticity by Levene's Test. Results were expressed as mean and standard deviation (SD) for normal variables; whereas median and interquartile rates (IQR) were used in non-normal variables. In neonatal data, normal-distributed quantitative variables were analyzed by t-test or with Kruskall-wallis test when needed. For categorical variables, chi-squared test was used. At the long-term period, differences between cases and controls were analyzed using general lineal model (GLM) adjusting by gender. In this case, when the null hypothesis in Shapiro-Wilk or in Levene's Test was rejected, log-transformation was performed prior to the analysis. The software package STATA13.0 was used for the statistical analyses. Significance was declared at $p<0.05$.

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RESULTS

Survival and growth parameters

Stillbirth was higher in non-treated IUGR compared with controls (55% vs. 8%, p<0.001), with no statistical differences when comparing t-IUGR and IUGR (45% vs. 55%, p=0.125). Birth weight was significantly lower in IUGR than in controls (33.6g (SD 1.3) vs. 46.7g (SD 1.3), p<0.001), without any significant difference between IUGR groups (IUGR 34.3g (SD 2.4)) and t-IUGR (33.6g (SD 1.3), p=0.871). At +60P, no differences between groups were observed either in weight or in gender distribution.

Functional results

At neonatal period, non-treated IUGR pups showed poorer results in almost all the neurodevelopmental parameters assessed when compared to controls, whereas no significant differences were observed when comparing IUGR and t-IUGR groups (Table S1).

At the long-term period, non-treated IUGR animals presented functional impairments compared to controls, showing a trend to present reduced learning skills although not being statistically significant, significant memory impairment and higher degree of anxiety (lower DI and less time exploring the internal area). Of note, t-IUGR animals presented an improvement in memory and anxiety trait when compared with IUGR subjects (Figure 2).

MRI results

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Analysis of global network features evidenced a significant decrease in average strength, global and local efficiencies in non-treated IUGR when compared to controls. A significant increase with respect to IUGR was observed in all these variables in the IUGR group in which therapy was applied (Figure 3). Regional analysis revealed no significant differences in the brain volume of the hippocampus within the different groups (Table S2). Analysis of regional FA parameters showed reduced median FA in both the left hippocampus region and the fibers crossing it in non-treated IUGR animals with respect to controls. Interestingly, when compared with IUGR group, t-IUGR animals showed a significant increase in these parameters with similar values to the control group (Figure 4).

Histology results: DS and PNNs

Non-treated IUGR animals presented a significant decrease in DS density when compared to controls, with a significant increase in the t-IUGR animals compared to IUGR (Figure 5a). Similarly, non-treated IUGR animals presented a significantly decrease in PNNs immunoreactivity when compared to controls, with trends to increase and normalize to control levels if the therapy has been applied (Figure 5b).

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DISCUSSION

To our knowledge, this is the first study using connectivity analysis at whole brain and cellular level to show an altered brain connectivity following IUGR that persists beyond adolescence. We hypothesize that these structural brain changes could underpin the neurobehavioral disabilities observed in our animal model. Additionally, we demonstrated that exposure to an enriched environment during early postnatal period ameliorates these effects on brain development after IUGR, partially recovering connectivity and neurobehavioral impairments.

In this study, advanced *ex vivo* MRI combined with histological markers of neuronal connectivity described changes in brain connectivity that persists up to the long-term period after IUGR. MRI results support previous findings in the rabbit model showing impaired global network infrastructure, integration and segregation evidenced by reduction in FA-weighted strength, global and local efficiencies [12,27]. Likewise, alterations in brain networks have been previously described in humans to persist in childhood and early adolescence [10, 11, 13, 35]. Apart from global changes, regional analysis of hippocampus was also explored due to its important role in memory and cognition in animals and humans [36] and for their vulnerability to IUGR [37]. Regional analysis showed decreased FA in the left hippocampus together with a reduction of median FA of fibers passing through the hippocampus. These results suggested the presence of less mature connections, since FA has been related to axonal packing, neuronal density, and myelination of fiber tracts [38]. Predominant changes affecting one of the brain hemispheres is coherent with the idea that some neural functions tend to be more dominant in one hemisphere than in the

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other [39]. In particular, left hippocampus has been described to be related with memory and neurobehavioral impairments in the considered rabbit model [28] as well as in rodents [40,41].

Regarding histological assessment, a significant reduction of DS and PNNs density in CA1 and CA3 hippocampal pyramidal neurons was observed. Both DS and PNNs have been involved in the regulation of synaptic connectivity and plasticity [42-45]. Our results showing decreased levels of DS in IUGR rabbits are in line with previously described studies on guinea pig and sheep model showing changes in DS density and morphology along with changes in synaptic receptors after acute and chronic intrauterine insults [15, 46-48]. On the contrary, although there is growing interest in the description of PNNs alterations related with specific brain diseases such as Alzheimer, schizophrenia and epilepsy [44,49], the pattern of alterations in the PNNs related with IUGR had not been previously evaluated. It has been described that normal completion of PNNs guarantees, in the adult brain, the stability of the established neuronal connections [50]. Therefore, decreased PNNs density in the IUGR animals in CA3 suggests less consolidated connections in the hippocampus, which is coherent with the lower amount of DS found in CA1. Indeed, preliminary evidence suggests that reduction of synapses expressed as reduced DS is associated with reduced PNNs formation [51]. These changes at cellular level were related with MRI findings, especially with regional reduction of FA in the hippocampus and white matter tracts connection.

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Our results demonstrate for the first time that an early postnatal strategy based on EE can improve behavioral performance and brain connectivity after IUGR. This is in agreement with previous basic research where the potential of EE as a non-invasive rehabilitation strategy has been established in rat models of hypoxic-ischemic neonatal injury [52] and prenatal exposition to alcohol [53]. Previous evidences have demonstrated the beneficial effects of EE in animal models as modulator of key sites of brain connectivity [17, 21]. Moreover, our data go in line with clinical evidence showing that NIDCAP program (physical and emotional support to premature infant during neonatal intensive care unit admission) is related with neurobehavioral and structural improvement in severe IUGR preterm infants [54]. Together with positive effects in function, we also observed a recovery in brain connectivity with improved global network feature and increased DS density and PNNs. These changes at cellular level after EE have also been showed in a rat model of neonatal hypoxia–ischemia with preserved DS [52] and in addiction based model with increased PNNs density [55]. The improvement at both behavioral and structural level is crucial to demonstrate the actual effect of EE therapy identifying those functions and regions more sensitive to its effects and to support its implementation in clinical conditions.

This study has some strengths and limitations that merit comment. Despite the limitations of animal research, one of its major strengths is the potential to test therapies and the transferability of these results to humans. On one hand, rabbit brain shows a timing of perinatal brain white matter maturation closer to humans compared to other species [25]. Regarding the IUGR model, either

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perinatal results and the reported neonatal and long-term neurodevelopmental impairments are in good agreement with the literature for this model [12,23,27,28] and also with clinical observations [1-5,7]. Regarding histological assessment, DS are plastic structures and they are constantly subjected to external inputs [56]. However, the experimental setting reduces this variability. Indeed, other features of structural synaptic plasticity, such as dendritic spine morphology and distribution patterns, dendritic branching and length, or analyses of specific perineuronal net component of the extracellular may be of equal interest to be evaluated in the IUGR and may give additional insights in our results. Further studies should be considered to evaluate all this additional features of structural synapsis and also evaluate them in other brain areas different from the hippocampus, as imaging studies of IUGR have revealed reduced volumes and diffusion MRI changes of other grey matter structures [15]. Finally, due to sample size, we acknowledge that we were underpowered for some of the comparisons. In order to quantify the degree of the underpowerment of the reported variables, a supplementary table reporting the mean and risk differences, as appropriate, and its 95% confidence interval was provided (Table S3).

Hence, by combining MRI with histological results we observed that IUGR may disrupt the normal pattern of brain development affecting special key sites for synaptic activity. These connectivity impairments either at global or at cellular level that persist up to the long-term period may explain, at least in part, the basis for the neurodevelopmental disorders associated with IUGR. Environmental enrichment during early postnatal period could ameliorate the

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effect of prenatal insults on neurodevelopment, with functional and structural changes that partially recovers normal conditions. Overall, our results reinforce the notion that environmental factors during critical periods of neurodevelopment could modify development and predispose the individual to lifelong health problems or enhancing it. Further evaluation of EE effects in a clinical setting is needed to explore its real effects and also to determine the exact moment to apply such strategy in IUGR infants.

Acknowledgments

We would like to acknowledge Guadalupe Soria, Xavi Lopez and Raúl Tudela from Experimental 7T MRI Unit of the Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS) for their help in the performance of MRI acquisitions. We are also indebted to Isabel Salas and the rest of the animal housing staff from Hospital Sant Joan de Déu - Animal Housing for their sensitivity in the care of the animal during the procedures. The Image Registration Toolkit was used under Licence from Ixico Ltd.

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Figures legends

Figure 1: Graphical representation of the study design and methods

A) Illustrative images and scheme of IUGR induction. After the breastfeeding period (>30 postnatal day), a cohort of IUGR animals were randomized into the environmental enrichment protocol (t-IUGR) whereas the rest were housed conventionally.

B) Illustrative images of neurobehavioral tests applied. At postnatal day +1, tone, spontaneous locomotion, reflex motor activity, coordination of suck and swallow and motor responses to olfactory stimuli were assessed. At postnatal day +60 and +70P, Skinner test, OFBT and ORT were applied.

C) Magnetic resonance imaging. Fixed brains were scanned, obtaining anatomical and diffusion-weighted images. FA brain networks were extracted and global graph theory features were applied. Regional analysis was done with mean FA of hippocampus and mean FA of fibbers crossing it.

D) Histology. Connectivity characteristics of hippocampal regions were assessed including dendritic spine density and perineural nets evaluation.

Abbreviations: Cont: control; IUGR: intrauterine growth restriction; t-IUGR: Treated intrauterine growth restriction animals; OFBT: open field behavioral test; ORT: object recognition task.

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Figure 2: Functional evaluation at long-term period

A) Percentage of learning in the study groups (controls, IUGR and t-IUGR) obtained from the Skinner test.

B) Discriminatory index in the study groups (controls, IUGR and t-IUGR) from the ORT.

C) Time spent in the internal area in the study groups (controls, IUGR and t-IUGR) from the OFBT.

Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals; OFBT: open field behavioral test; ORT: object recognition task. * p< 0.05 statistical significance.

Figure 3: Global FA network features

Global fractional anisotropy (FA) network features in the study groups (controls, IUGR and t-IUGR). Networks features included average strength, global and local efficiency of weighted FA network.

Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals. * p< 0.05 statistical significance.

Figure 4: MRI regional Hippocampus

Median FA from the hippocampal regions and from the reconstructed fibers crossing hippocampal regions were evaluated in the study groups (controls, IUGR, and t-IUGR).

Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals. * p< 0.05 statistical significance.

Figure 5: Histology

A) Dendritic spine analysis:

A.1 Density of dendritic spine from basal dendrites of CA1 pyramidal neurons from the dorsal hippocampus in the study groups (controls, IUGR and t-IUGR).

A.2 Illustrative images of the dendritic spine density in the study groups

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(controls, IUGR and t-IUGR).

B) Perineural nets analysis:

A.1 Average density of immunolabelling from hippocampus CA3 zone in the study groups (controls, IUGR and t-IUGR).

A.2 Illustrative images of the immunolabelling of perineural nets in the study groups (controls, IUGR and t-IUGR).

Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals. * $p < 0.05$ statistical significance.

Supplementary material

Supplementary Materials and Methods. Brain network analysis and Histology assessment methodology.

Supplementary Table S1: Functional results at neonatal period in study groups.

Supplementary Table S2: Hippocampus volume results.

Supplementary Table S3: Mean or risk difference, as appropriate, and its 95% confidence interval (CI) for reported variables.

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Figure 1

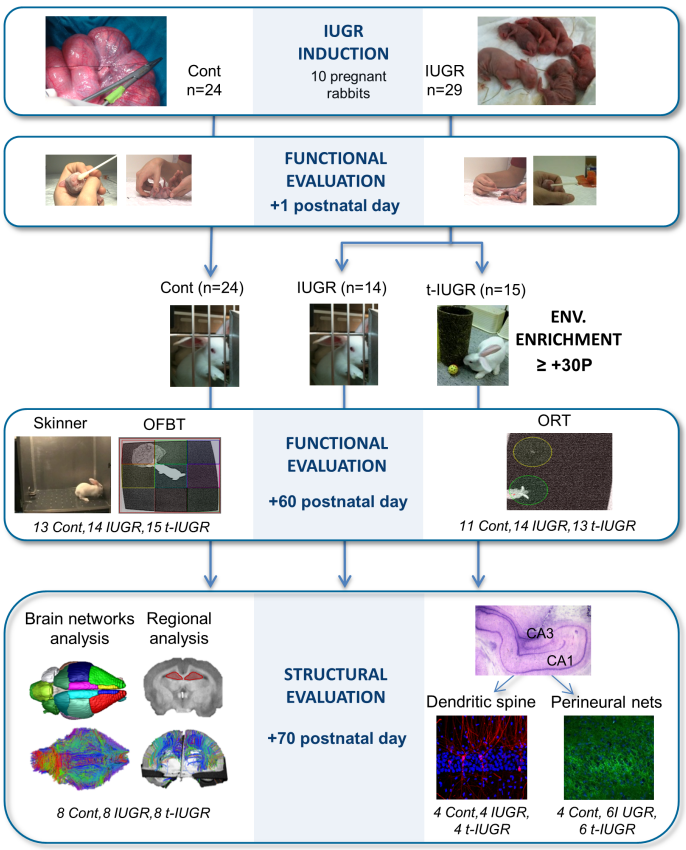

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Figure 2

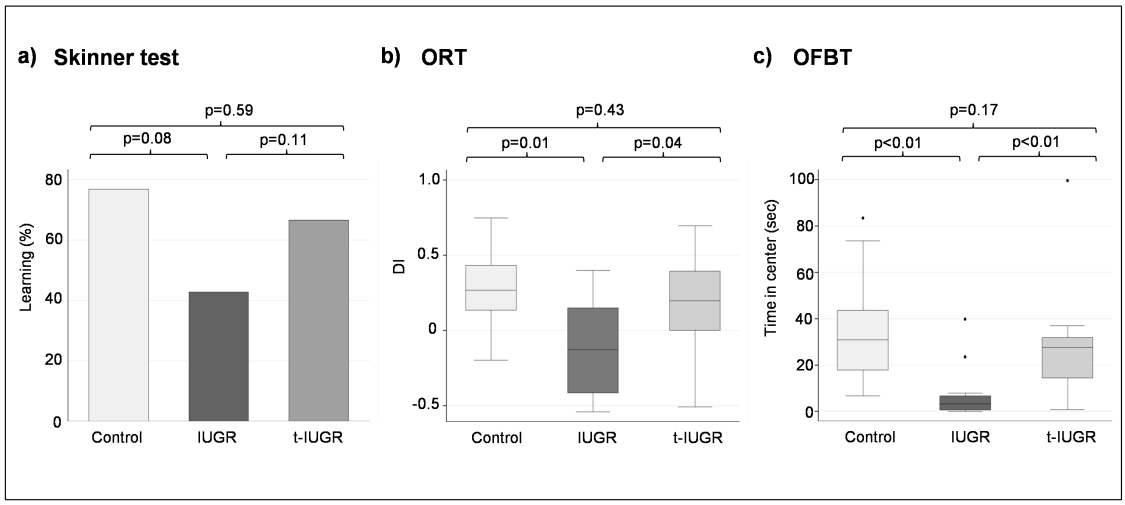

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Figure 3

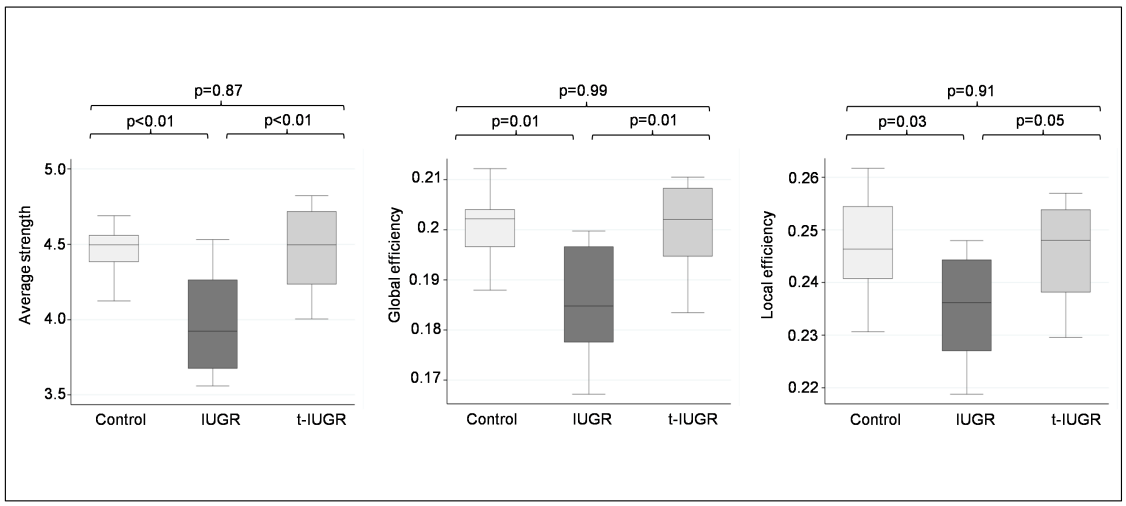

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Figure 4

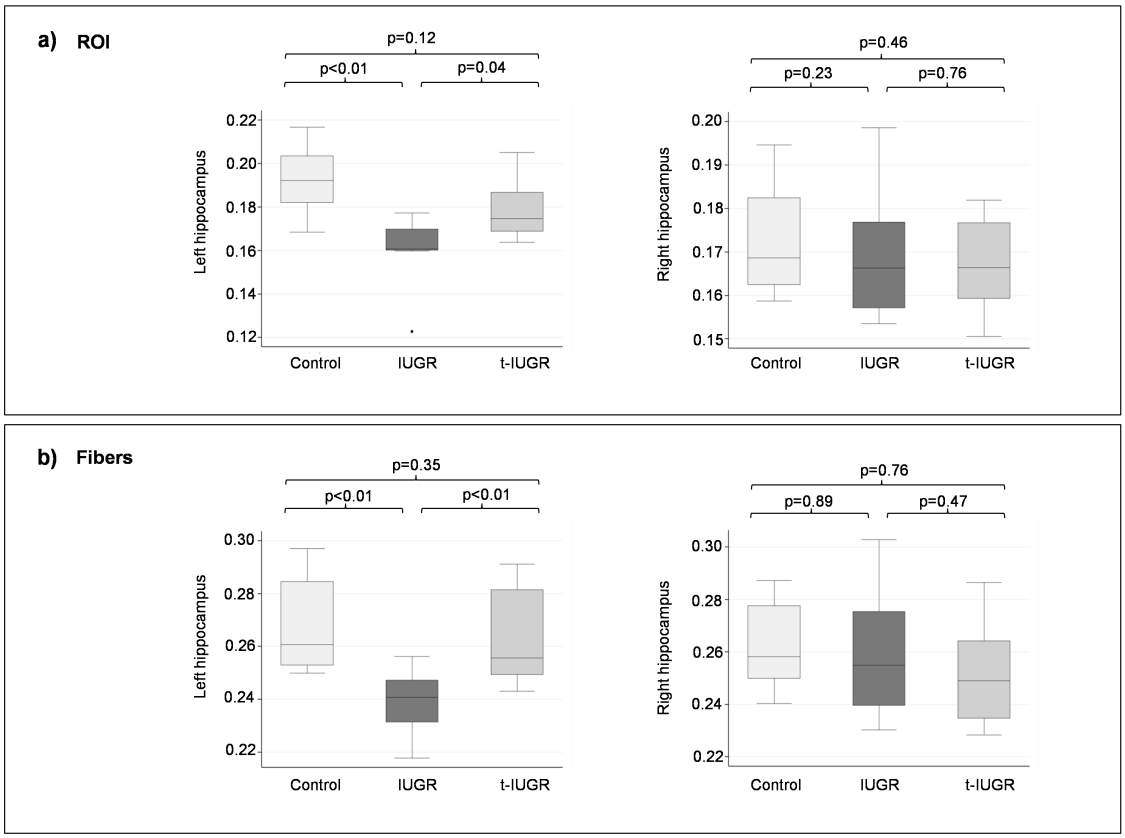

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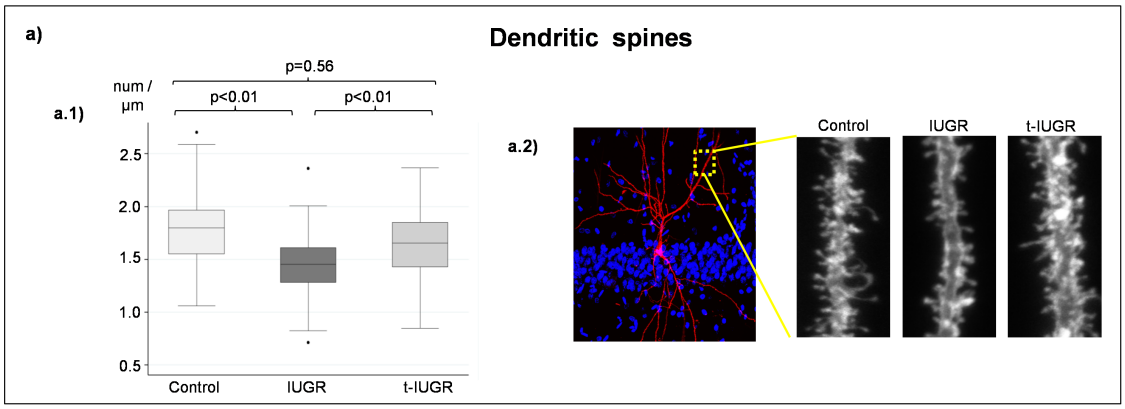
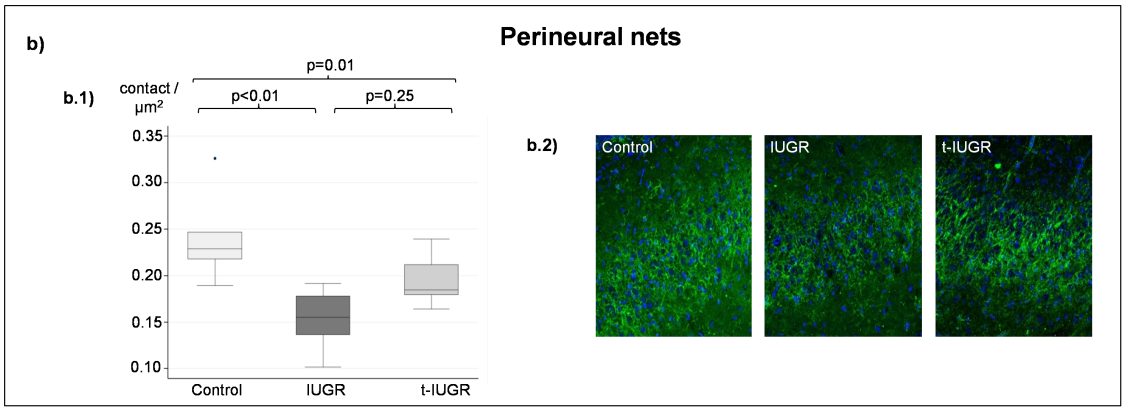
Figure 5





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Supplementary Materials and Methods:

A) MRI methodology

A.1.- MRI acquisition

MRI was performed on fixed brains using a 7T animal MRI scanner (BrukerBioSpin MRI GmbH). High-resolution three-dimensional T2-weighted images were obtained in the brain samples by a rapid acquisition with relaxation enhancement (RARE) sequence with the following parameters: TE=9 ms, TR=4843.7 ms, RARE factor=4, 0.7mm slice thickness with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 256 × 256 and Field of View (FoV) of 32×32 mm^2, resulting in a voxel dimension of 0.125 × 0.125 × 0.7 mm^3. Diffusion-weighted images (DWI) were acquired using a diffusion sequence covering 30 gradient directions with a b-value of 3000 s/mm^2 together with a baseline (b = 0 s/mm^2) image. Other experimental parameters were: TE = 26 ms, TR = 250 ms, 0.7 mm slice thickness with no interslice gap, 70 coronal slices, in-plane acquisition matrix of 40 × 40, FoV of 28 × 28 mm^2, resulting in a voxel dimension of 0.7 × 0.7 × 0.7 mm^3. The total scan time for DWI was 3h6 min and 3min52s for RARE-T2 acquisitions.

A.2.- Pre-processing and tractography

Brain tissue was segmented from the background in the T2 volumes based on the Otsu threshold method.[1] In the case of DWI, brain tissue was segmented from the background by means of an in-house algorithm previously described[2] that takes advantage of the high SNR of the brain tissue on the average diffusion volume. Diffusion Toolkit (http://trackvis.org/dtk/) was used to

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estimate the diffusion tensor model and perform tractography, considering a fractional anisotropy (FA) threshold of 0.1.

A.3.- Brain parcellation

Automatic brain parcellation of the subjects' brain was performed using the New Zealand Rabbit MRI atlas.[3] The atlas was defined considering a T1 template, so a previous step was required by modifying image intensity in order to simulate RARE acquisition contrast. Then, elastic registration was performed between the correspondent atlas template (T1 or RARE-adapted) to each subject's brain using a consistent block matching algorithm.[4] The elastic transformation was applied to the ROI labels, obtaining a parcellation of each brain in 60 ROIs. Coherence between the T1- and RARE-based parcellation had been previously evaluated by scanning one subject using both modalities. Parcellation obtained from both images had been compared, observing similar results in both cases (global Dice Coefficient = 0.97).[5]

In order to align the labels obtained for each subject in T2 volumes to its corresponding DWI, affine registration between T2 and the baseline diffusion image was performed with IRTK (www.doc.ic.ac.uk/~dr/software/).[6] Discrete values of the labels were preserved by nearest neighbor interpolation in both transformations. ROIs comprising only white matter (WM) tissue were discarded, leaving a total of 44 regions for each subject (see Table A at the end of this document), each of them considered as a brain network node.

A.4.- Network extraction

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Brain network of each subject was extracted by means of an in-house algorithm as previously described,[7] defining a network edge e_{ij} between two nodes if there is at least one streamline starting in one node and ending in the other one. In order to assign weights to each edge $e_{ij,}$ we considered the average fractional anisotropy (FA) along all the streamlines connecting each pair of regions *i* and *j*.[7] Hence, FA-weighted (FA-w) were obtained from each subject.

B) Histology assessment

B.1 Dendritic spine

Dendritic spine (DS) density (number of spines / μm) was analyzed in the selected brains using the Helios Gene Gun System (Bio-Rad). A suspension containing 3 mg of DiI (Molecular Probes, Invitrogen) dissolved in 100 μl of methylene chloride (Sigma- Aldrich) and mixed with 50 mg of tungsten particles (1.7 mm diameter; Bio-Rad) was spread on a glass slide and air-dried. The mixture was resuspended in 3.5 ml distilled water and sonicated. Subsequently, the mixture was drawn into Tefzel tubing (Bio-Rad), and then removed to allow tube drying during 5 minutes under a nitrogen flow gas. Then, the tube was cut into 13-mm pieces to be used as gene gun cartridges. Particles were delivered to the hippocampus using a modification of the gun to enhance accuracy by restricting the target are.[8] Daye-coated particles were delivered in the hippocampus shooting over 150-μm coronal sections at 80 psi through a membrane filter of 3 μm pore size and 8 × 10 pores/cm^2 (Millipore). Sections were stored at room temperature in PBS for 3 hours protected from light and then incubated with DAPI, and mounted in Mowiol to be analyzed. DiI-labeled

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pyramidal neurons from CA1 of the dorsal hippocampus were imaged using a Leica Confocal SP5 with a ×63 oil-immersion objective. Conditions such as pinhole size (1 AU) and frame averaging (4 frames per z-step) were held constant throughout the study. Confocal z-stacks were taken with a digital zoom of 5, a z-step of 0.5 µm, and at 1.024 × 1.024 pixel resolution, yielding an image with pixel dimensions of 49.25 × 49.25 µm. 2 or 3 basal dendrites of various neurons were selected for the analysis of spine density according the criteria described in Brito et al 2014:[9] (a) segments with no overlap with other branches that would obscure visualization of spines and (b) segments either "parallel" to or "at acute angles" relative to the coronal surface of the section to avoid ambiguous identification of spines. Only spines arising from the lateral surfaces of the dendrites were included in the study; spines located on the top or bottom of the dendrite surface were ignored. Given that spine density increases as a function of the distance from the soma, reaching a plateau 45 µm away from the soma, we selected dendritic segments of basal dendrites 45 µm away from the cell body.

B.2 Perineuronal nets

For perineuronal nets (PNN) analysis, the frozen block that contains basal ganglia was embedded in Tissue-Tek, serially cut in 20- µm-thick transverse sections with a cryostat, and collected onto gelatin-coated glass slides. All sections were first blocked with 2% normal bovine serum for 1 h, followed by overnight incubation at 4°C with Wisteria Floribunda Lectin (1:20, Sigma). After washes, immunoreactive sites were revealed by using species-specific secondary antibodies conjugated to Streptavidin 488 Alexa Fluor

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(1:200, Invitrogen). After incubation, the sections were thoroughly washed, counterstained with Hoechst 33258 (1:1000, Thermofischer), mounted on slides, and cover-slipped with Fluoromount-G (Sigma). Labeled neurons were localized in CA3 zone, a region of interest (ROI) was manually selected and images were acquired with a scanning confocal microscope (Leica Confocal SP5, 40×/1.3 Oil DIC M27). Image analysis and processing were performed by means of imageJ software. Three representative serial sections from each animal in CA3 area were used and a constant threshold was applied to obtain an estimated average density of immunolabelling (contact/μm^2).

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Table A: Regions of interest used as nodes in the structural brain networks.

ID	Label	Name	ID	Label	Name
1	FCx-L	Frontal cortex L	**23**	Len-L	Lenticular nucleus L
2	FCx-R	Frontal cortex R	**24**	Len-R	Lenticular nucleus R
3	MFCx-L	Medial frontal cortex L	**25**	Th-L	Thalamus L
4	MFCx-R	Medial frontal cortex R	**26**	Th-R	Thalamus R
5	CiCx-L	Cingulate cortex L	**27**	Am-L	Amygdala L
6	CiCx-R	Cingulate cortex R	**28**	Am-R	Amygdala R
7	PiCx-L	Piriform cortex L	**29**	OlB-L	Olfactory bulb L
8	PiCx-R	Piriform cortex R	**30**	OlB-R	Olfactory bulb R
9	ECx-L	Entorhinal cortex L	**31**	Hc-L	Hippocampus L
10	ECx-R	Entorhinal cortex R	**32**	Hc-R	Hippocampus R
11	PaCx-L	Parietal cortex L	**33**	FB-L	Forebrain L
12	PaCx-R	Parietal cortex R	**34**	FB-R	Forebrain R
13	OcCx-L	Occipital cortex L	**35**	CeH-L	Cerebellar hemisphere L
14	OcCx-R	Occipital cortex R	**36**	CeH-R	Cerebellar hemisphere R
15	InCx-L	Insular cortex L	**37**	Ht	Hypothalamus
16	InCx-R	Insular cortex R	**38**	Ve	Vermis
17	TeCx-L	Temporal cortex L	**39**	BF	Basal forebrain
18	TeCx-R	Temporal cortex R	**40**	De	Diencephalon
19	Cl-L	Claustrum L	**41**	Me	Mesencephalon
20	Cl-R	Claustrum R	**42**	Po	Pons
21	Cau-L	Caudate nucleus L	**43**	MO	Medulla oblongata
22	Cau-R	Caudate nucleus R	**44**	Spt	Septal nuclei

Abbreviations: R: right, L: Left

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Table S1:

Functional results at neonatal period in study groups.

variables	*Control* *n=24*	*IUGR* *n=14*	*t-IUGR* *n=15*	*p **	*p ***
Tone (score)†	4 (0)	4 (3)	2 (1)	*<0.001*	*0.234*
Posture (score)†	3 (0)	3 (1)	3 (0)	*<0.001*	*0.530*
Duration (score)†	3 (0)	3 (1)	3 (2)	*0.007*	*0.756*
Circular motion (score)	2.4 (0.6)	1.4 (1.0)	0.9 (0.8)	*0.003*	*0.123*
Locomotion (score)†	3 (0)	2 (1)	3 (2)	*0.001*	*0.697*
Lineal movement (numb)†	3 (0)	0 (2)	0 (1)	*0.004*	*0.792*
Righting reflex (number)†	10 (0)	9.5 (5)	7 (2)	*0.062*	*0.280*
Suck & Swallow (score)†	3 (0)	3 (2)	2 (2)	*0.020*	*0.556*
Smelling test (seconds)†	2 (4)	5 (6)	3 (1)	*0.082*	*0.203*

Results are mean and standard deviation or median and interquartile range (median (IQR))†. Statistical comparisons between groups were performed by Kruskall-Wallis between control and IUGR groups (*) and between IUGR and t-IUGR (**).

Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals

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Table S2:

Hippocampus volume (mm^3).

	Control n=8	***IUGR n=8***	***t-IUGR n=8***	***p ****	***p *****
Left	1238 (12)	1111 (268)	1187 (79)	*0.146*	*0.408*
Right	1201 (116)	1086 (224)	1185 (83)	*0.115*	*0.391*

Results are median and interquartile range (median (IQR)). Statistical comparisons between groups were performed by Kruskall-Wallis between control and IUGR groups (*) and between IUGR and t-IUGR (**).

Abbreviations: IUGR= intrauterine growth restriction; t-IUGR=Treated intrauterine growth restriction animals

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Table S3: Mean or risk difference in the outcome variables.

Mean or risk difference, as appropriate, and its 95% confidence interval (CI) for reported variables

Functional evaluation		
	Control vs. IUGR (n= 13 and 14)	**t-IUGR vs. IUGR (n= 15 and 14)**
Skinner (% learning)	34.2% (-7.59 to 65.15)	24% (-16.01 to 57.09)
OFBT- Internal boxes (time in center, sec)	-27.7 (-42.38 to -13.06)	-24.6 (-34.7 to -14.4)
	Control vs. IUGR (n= 11 and 14)	**t-IUGR vs. IUGR (n= 14 and 13)**
ORT (DI)	-0.18 (-0.41 to 0.06)	-0.08 (-0.08 to 0.19)
Global brain network analysis (MRI)		
	Control vs. IUGR (n= 8 and 8)	**t-IUGR vs. IUGR (n= 8 and 8)**
Average strength	-0.48 (-0.79 to -0.18)	-0.49 (-0.84 to -0.14)
Global efficiency	-0.02 (-0.02 to -0.01)	-0.02 (-0.02 to -0.01)
Local efficiency	-0.01 (-0.02 to -0.00)	-0.01 (-0.02 to 0.00)
Regional MRI analysis (Hippocampus)		
	Control vs. IUGR (n= 8 and 8)	**t-IUGR vs. IUGR (n= 8 and 8)**
Left volume	-27 (-49.11 to -4.89)	-7 (-69.18 to 55.18)
Right volume	-115 (-305.47 to 76.47)	-99 (-279.3 to 82.37)
Left ROI FA	-0.03 (-0.05 to -0.02)	-0.02 (-0.03 to -0.01)
Right ROI FA	-0.01 (-0.02 to 0.01)	-0.00 (-0.17 to 0.02)
Left FA fibers	-0.03 (-0.05 to -0.01)	-0.02 (-0.04 to -0.01)
Right FA fibers	-0.01 (-0.3 to 0.01)	0.00 (-0.02 to 0.21)
Histology assessment		
DS	**Control vs. IUGR (n= 138 and 155)**	**t-IUGR vs. IUGR (n= 128 and 155)**
	-0.35 (-0.42 to -0.29)	-0.2 (-0.26 to -0.13)
PNNs	**Control vs. IUGR (n= 4 and 6)**	**t-IUGR vs. IUGR (n= 6 and 6)**
	-0.09 (-0.15 to -0.03)	-0.04 (-0.08 to 0.00)

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