Facultad de Ciencias Biologicas

Departamento de Bioquímica y Biología Molecular
Centro de Investigación Principe Felipe

Differentiation of human Embryonic Stem Cells (hESC) into neural progenitors as a tool to study both the pathways during early brain development and the neuroteratogenic effects of ethanol

TESIS DOCTORAL

Jelena Kostic, 2012

Facultad de Ciencias Biologicas
Departamento de Bioquímica y Biología Molecular

DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS (hESC) INTO NEURAL PROGENITORS AS A TOOL TO STUDY BOTH THE PATHWAYS DURING EARLY BRAIN DEVELOPMENT AND THE NEUROTERATOGENIC EFFECTS OF ETHANOL

Tesis Doctoral

Presentada por *Jelena Kostic*
Dirigida *por* *Dra. Consuelo Guerri Sirera*
Valencia, 2012

PRINCIPE FELIPE
CENTRO DE INVESTIGACION

CONSUELO GUERRI SIRERA, Dra en Ciencias Biológicas y Jefe del Laboratorio de Patología Celular e del Centro de Investigación Príncipe Felipe

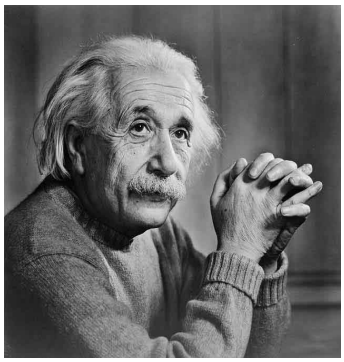
CERTIFICA:

Que la memoria de Tesis Doctoral realizada por Jelena Kostic que lleva por título "***Differentiation of human embryonic Stem Cells (hESC) into neural progenitors as a tool to study both the pathways during early brain development and the neuroteratogenic effects of ethanol"*** ha sido realizado bajo mi dirección y reúne los requisitos necesarios para su juicio y calificación

Valencia a 16 de Julio de 2012

Eduard Punset

"Aprender, soñar y recordar es el poder exclusivo de la mente. A todos los que han descubierto ese poder quisieran fiarse de el y saber lo que pasa por dentro."



"The important thing is not to stop questioning. Curiosity has its own reason for existing."

Albert Einstein

"Los ideales que iluminaron y colmaron mi vida desde siempre son: bondad, belleza y verdad. La vida me habría parecido vacía sin la sensación de participar de las opiniones de muchos, sin contestarme en objetivos siempre inalcanzables tanto en el arte como en la investigación." (Mi visión del mundo)

Agradecimientos

Primero, quiero agradecer a Dra. Consuelo Guerri Sirera una lo primero persona grande y luego una grande científica, por su gran apoyo durante de mi tesis doctoral, sin reservas. Me ha dado siempre consejos importantes, conocimiento impresionante y me ha guiado en el mí camino de formación científica. Gracias Chelo!

Gracias a un laboratorio excelente I-05 y todos mis queridos compañeros (mi segunda familia): Marisa, Maya, Rosa, Maria José, Blanca, Sara, Silvia, Maria y el parte masculino: Jorge y Juan.

Por apoyo profesional agradezco a Dra Raquel Talens Visconti y Irene Sanches Vera.

Durante de la tesis he colaborado con la gente maravillosa y además los expertos grandes en biología de células madre embrionarias en los tres laboratorios, sucesivamente, donde he obtenido una experiencia muy valuable, como trabajar con muchas personas y funcionar como el grupo tan como independiente. Les agradezco a todos, era una gran experiencia. También, conocí las personas que siguen siendo mis amigos.

Al final, gracias a mis padres, a mi hermano y todos mis amigos y compañeros de la facultad por apoyándome 'desde lejos' de varios puntos del ese mundo pequeño.

Esta tesis era un gran esfuerzo y trabajo duro en muchos sentidos, pero con razón y puedo decir, he sacado valores y aprendizajes importantes para toda la vida.

Sin dudas, mí estancia en España cambió mis puntos de vista, mis ángulos de percepción y mí futuro camino profesional, tan como personal.

Abbreviations

7-AAD: 7-aminoactinomycin D

AD: Alzheimer's Disease

AP: alkaline phosphatase

ATTC: American Type Culture Collection

BDNF: Brain Derived Growth Factor

BrDU: Bromodeoxyuridine

bFGF: basic Fibroblast Growth Factor

BMP: bone morphogenic proteins

$CaCl_2$: Calcium Chloride

CB1: cannabinoid receptor type 1

CB2: cannabinoid receptor type 2

CC:corpus callosum

cDNA: complementary Deoxyribonucleic acid

CM: conditioned medium

CNS: central nervous system

CO_2: carbon dioxide

DAMP: damage- (or danger-) associated molecular patterns

DMEM: Dulbecco´s Modified Eagle´s Medium

DMSO: dimetyl sulfoxyde

dl: deciliter

DPBS: Dulbecco's Phosphate Buffered Saline

DTI: diffusion tensor imaging

DTT: dithiothreitol

EBs: embryoid bodies

EG: embryonic germ cells

EC: embryonic carcinoma cells

EDTA: ethylenediaminetetraacetic acid

ERK: extracellular signal-regulated kinases

FAAH: fatty acid amide hydrolase

FAS: foetal alcohol syndrome

FASD: foetal alcohol spectrum disorders

FBS : fetal bovine serum

FITC: fluorescein isothiocyanate

fMRI: functional magnetic resonance imaging

FSK: fibroblast medium

GABA: γ-Aminobutyric acid

GalC: galactocerebroside

GARG16: Glucocorticoid Attenuated Response Gene 16

GFAP: glial fibrillar acidic protein

GFP: green fluorescent protein

GPCRs:7-transmembrane G-protein coupled receptors

gr: gram

hESC: human Embryonic Stem Cell

hNP: human neuroprogenitor

hNPC: numan neural progenitor cell

HFF: human foreskin fibroblast

ICM: inner cell mass

IFN-β: interferon β

IL-6: interleukin 6

IL-12: interleukin 12

IL-27: interleukin 27

IL-1ß: interleukin 1ß

IP-10: interferon gamma-induced protein 10 kDa

iPSC: induced Pluripotent Stem Cell

IRAK: interleukin-1 receptor-associated kinase 1

IRF3: interferon regulatory factor

IRF9: interferon regulatory factor

JNK: c-Jun N-terminal kinases

KCl: Potassium Chloride

LMW-HA: low molecular weight hyaluronic acid

LPS: lipopolysaccharide

MEF: mouse embryonic fibroblast

MAP2: microtubule associated protein 2

$MgCl_2$: Magnesium Chloride

mgr: miligram

mM: milimolar

ml: mililiter

MRI: magnetic resonance imaging

MRS: magnetic resonance spectroscopy

MyD88: myeloid differentiation primary response gene 88

NaCl: Sodium Chloride

NaF: Sodium Fluoride

Na_2HPO_4 : Disodium hydrogen phosphate

N-Cad: N- Cadherin

nm: nanometer

Na3VO4: Sodium Vanadate

NAPE-PLD: N-Acylphosphatidylethanolamine (NAPE)-hydrolyzing phospholipase

NDM: Neural Differentiation Medium

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

NIM: Neural Induction Medium

NPM: Neural Proliferatium Medium

NPC: Neural Progenitor Cell

NP-40: nonyl phenoxypolyethoxylethanol

PAMPs: pathogen-associated molecular patterns

PBS: phosphate buffered saline

pERK: phosphorylated extracellular signal-regulated kinases

PET: positron emission tomography

PMSF: phenylmethylsulfonyl fluoride

PVDF: polyvinylidene fluoride

P/S: Penicillin/Streptomycin

RA: retinoic acid

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

RT-PCR: reverse transcription-polymerase chain reaction

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SPECT: single photon emission computed tomography

SSEA3/4: stage-specific embryonic antigen 3/4

STAT-1/2: Signal Transducer and Activator of Transcription-1/2

TBS-T: Tris-buffered saline+Tween 20

TEMED: Tetramethylethylenediamine

TGF-ß: transforming growth factor

TLRs: Toll-like receptors

TLR2: Toll-like receptor 2

TLR4: Toll-like receptor 4

TNF-α: tumor necrosis factor-α

TRA-1-60: tumour rejection antigen-1-60

TRA-1-81: tumour rejection antigen-1-81

TRIPLE SELECT: trypsyne

TRITC: rodamine

UV: ultraviolet light

VCAM-1: vascular cell adhesion molecule 1

Wnt: wingless signalling

ZO-1: zonula ocludens-1

µg: microgram

µM: micromolar

µl: microliter

µgr: microgram

Contents

----------INTRODUCTION----------

Introduction

1.1. Human embryonic stem cells (hESC)

Embryonic stem cells (ES cells) were firstly derived independently from mouse embryos by two groups in 1981. These groups (Evans and Kaufman, 1981; Martin, 1981) demonstrated a new technique for culturing mouse embryos in the uterus, allowing for the derivation of ES cells from these embryos. Next, 1998 saw a breakthrough when James Thomson and collaborators (Thomson et al, 1988) first developed a technique to isolate human embryonic stem cells (hESC) from the inner cell mass (ICM) of early-stage blastocyst embryos (5-8-day-old blastocysts), obtained from *in vitro* fertilized embryos (Fig.1). Cells were grown on irradiated mouse fibroblast feeder cells (MEF) in the presence of serum after adequate undifferentiated colonies were selected and propagated to new feeder cells in media containing serum replacement and bFGF (basic Fibroblast Growth Factor) (Thomson et al, 1998). Moreover, hESCs are pluripotent, are capable of differentiating into the three germ layers (ectoderm, mesoderm and endoderm) and can given rise to any cell type in the body, which is the most important difference that distinguishes them from endogenous adult stem cells (Thomson et al, 1998).

Human ESC possesses two main characteristics: self-renewing and pluripotency (Fig.2). These cells express transcription factors (OCT4 and NANOG) and specific embryonic antigens 3 and 4

(SSEA3/4), high molecular weight glycoproteins, such as TRA-1-60, TRA-1-81, and typical pluripotent markers, such as high telomerase and alkaline phosphatase activity, which are able to indefinitely divide in culture (Thomson J et al, 2000, Itzkovitz-Eldor J. et al, 2002).

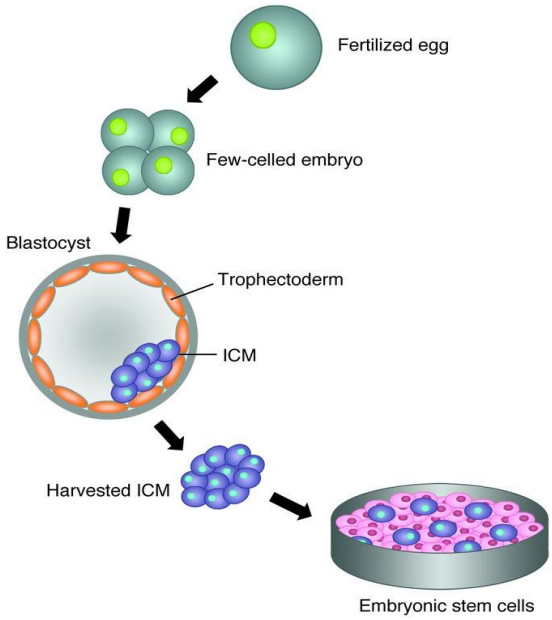


Fig.1. Human embryonic stem cells derivation from the inner cell mass

Another feature of hESC is their ability to maintain karyotype stability with the XX and XY chromosomes. This characteristic is common with karyotypes of other species, but differs from lines of carcinogenic stem cells, which implies their genomic inestability (Thomson et al, 1998). Nevertheless, some reports demonstrated that hESC may have certain level of aneuploidy

(Carpenter et al, 2004), trisomy of chromosome 20 (Rosler et al, 2004) or an aberrant X chromosome (Inzunza et al, 2004).

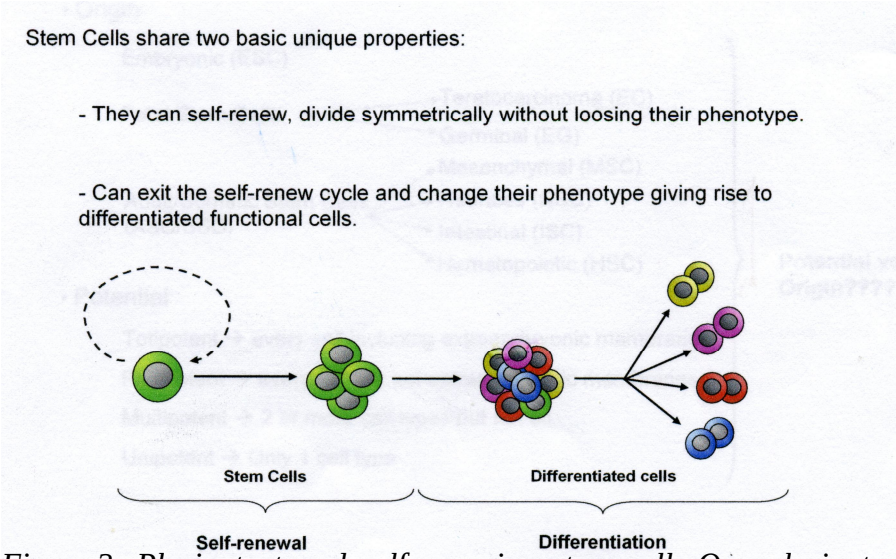


Figure.2. Pluripotent and self-renewing stem cell. One pluripotent stem cell is a potential source of different cell types.

Another feature of hESC is the teratoma formation, which represents a tumor with all three germ layers after their introduction into immune deficiency mice (SCID) (Thomson et al, 1998). Indeed, as comented above, hESCs are pluripotent and capable of differentiating into the three germ layers (ectoderm, mesoderm and endoderm) and can given rise to any cell type in the body, which is the most important difference that distinguishes them from endogenous adult stem cells (Thomson et al, 1998). These properties of ESC are a critical process because they could cause teratoma formation *in vivo*. This is the reason why a well-defined protocol with tissue-specific progenitor cells is very important to avoid *in vivo* side effects.

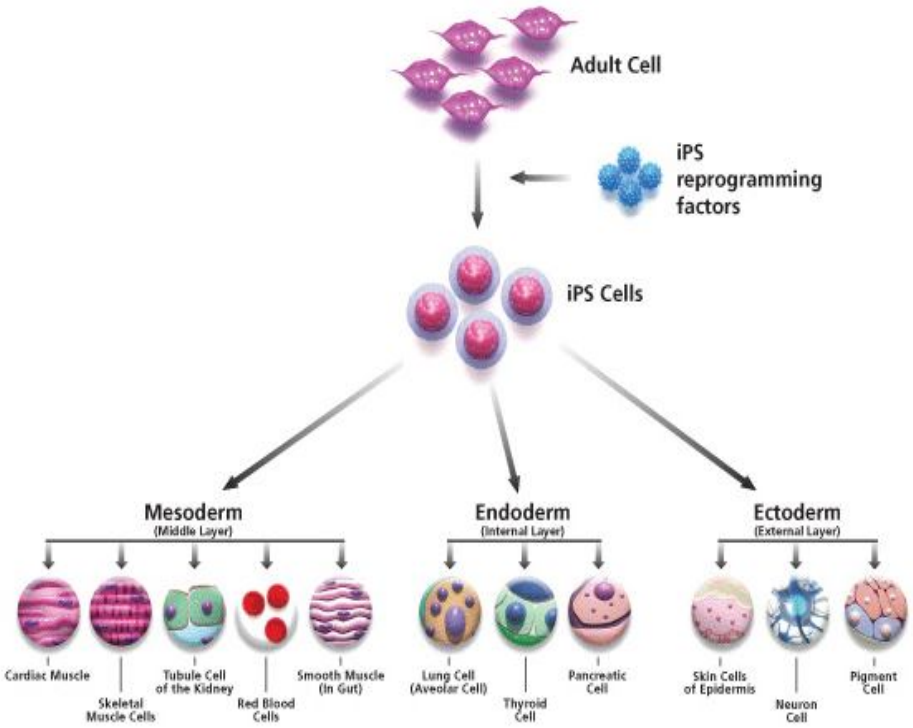


Fig.3. iPSC reprogramming scheme. iPSCs are derived from adult cells by introducing reprogramming factors which are crucial for the induction of pluripotency and the self-renewing property. Cells could be differentiated to the mesodermal, endodermal and ectodermal lines, including all types of characteristic cells for each germ layer.

More recently, induced pluripotent stem cells (iPSC) (Fig.3) were described for the first time by Takashy and Yamanaka (2006). These cells were obtained by introducing four factors inducing the pluripotency state: Oct3 /4,Sox2, c-Myc and Klf4. In addition, iPS

cells have been derived by the introduction of Oct4, Sox2, Nanog and Lin28 (Thomson JA , 2007).

It has been demonstrated that iPSC are very similar to embryonic stem cells and can be generated by the delivery of three genes (*Oct4*, *Sox2*, and *Klf4*) to differentiated cells (Yamanaka et al, 2006) (Fig.3). The delivery of these genes "reprogrammes" differentiated cells into pluripotent stem cells, allowing the generation of pluripotent stem cells without the embryo. Since ethical concerns about embryonic stem cells are typically about their derivation from terminated embryos, it is believed that reprogramming to the iPS may prove less controversial.Human and mouse cells can be reprogrammed by this methodology to generate both human pluripotent stem cells and mouse pluripotent stem cells without an embryo. iPSC express cell surface markers, normal karyotypes, high telomerase activity and the potential to differentiate into all three germ layers (ectoderm, mesoderm and endoderm) showing human embryonic stem cells (hESC). Therefore, iPSCs are challenge in developing new approaches to define various mechanisms in different processes and cell replacement therapy, thus eliminating the controversial and ethical hESC-based issues (Somoza RA, Gallego Rubio FJ, 2012) (Fig.3).

1.2 Early Human Brain development and neural induction. The human brain begins to form very early in prenatal life (just three weeks after conception) (Figs. 4 and 5). The nervous stem derives from the ectoderm, the outermost tissue layer, of the

embryo. derives from the ectoderm, the outermost tissue layer, of the embryo. During the third week of development, the neuroectoderm appears and forms the neural plate along the dorsal side of the embryo. This neural plate is the source of the majority of all neurons and glial cells in the mature human. A groove forms in the neural plate and, by week four of development, the neural plate wraps itself on it to make a hollow neural tube (Fig.5) The most anterior part of the neural tube is called the *telencephalon,* which expands rapidly due to cell proliferation, and eventually gives rise to the brain (Fig. 5). Gradually some cells stop dividing and differentiate into neurons and glial cells, which are the main cellular components of the brain. Newly generated neurons migrate to different parts of the developing brain to self-organise into different brain structures. Once the neurons have reached their regional positions, they extend axons and dendrites, which allow them to communicate with other neurons via synapses. Synaptic communication between neurons leads to the establishment of functional neural circuits that mediate sensory and motor processing, and underlie behavior.The human brain undergoes most of its development in the first 20-23 years of life Complex interactions among genetic, epigenetic, environmental and diffusible factors play critical roles in the developing brain (*Purves, Neuroscience*). For instance, during early embryonic development, the ectoderm becomes specified to give rise to the epidermis (skin) and the neural plate.

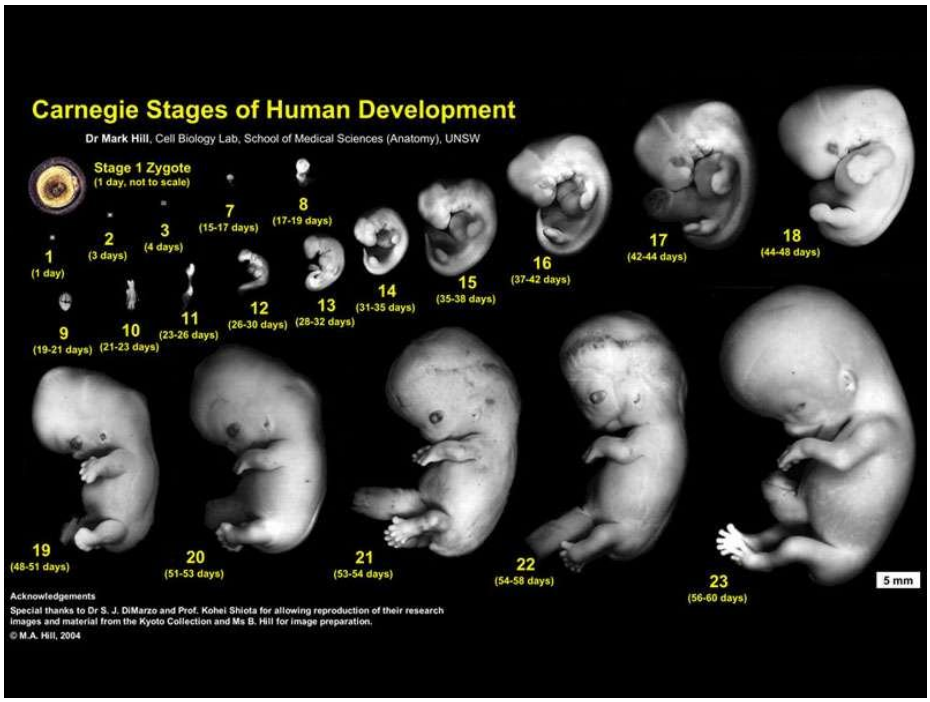


Fig.4. In vivo human development. Dr Mark Hill's Cell Biology lab, School of Medical Sciences (Anatomy)

The conversion of the undifferentiated ectoderm into the neuroectoderm requires signals from the mesoderm. At the onset of gastrulation, mesodermal cells move through the dorsal blastopore lip and form a layer between the endoderm and the ectoderm. These mesodermal cells, which migrate along the dorsal midline, give rise to a structure called the notochord The ectodermal cells overlying the notochord develop into the neural plate in response to a diffusible signal produced by the notochord. The ability of the mesoderm to

convert the overlying ectoderm into neural tissue is called *Neural Induction.*

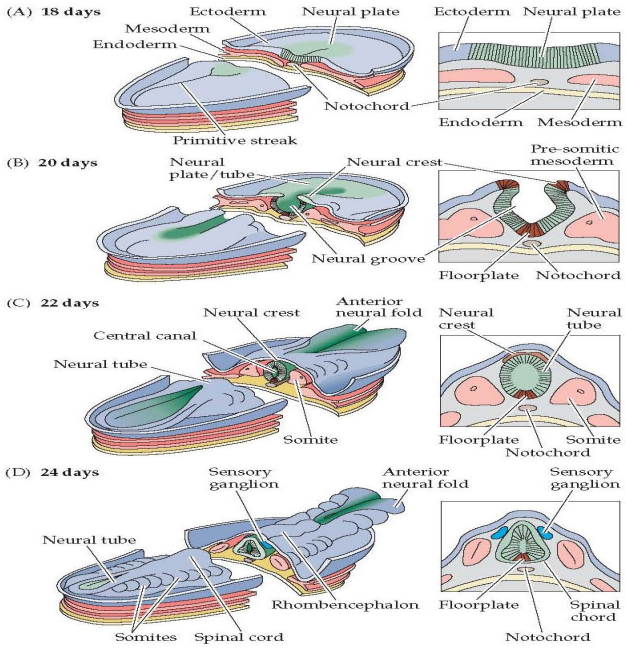
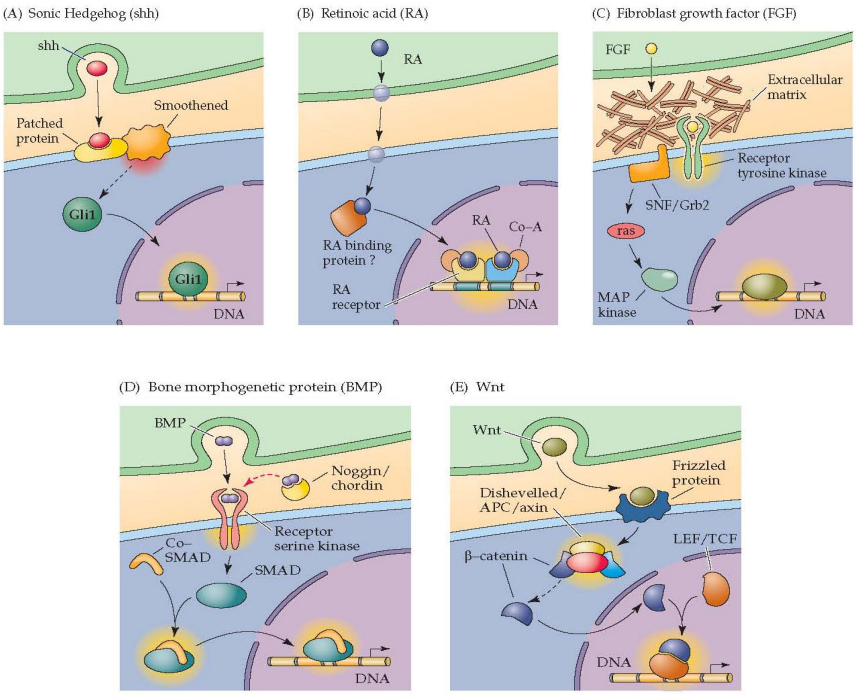


Fig.5. Neurulation in the mammalian embryo. On the left, we can see the dorsal views of the embryo at several different stages of early development (Purves, Neuroscience)

At the molecular level, different signaling pathways, transcription factors and diffusible growth factors (e.g., *sonic hedgehog*, RA, FGF, BMP, Wnt) (Fig.6) participate in many specific processes during neural development. For example, the dorsal neural tube is patterned by BMPs (bone morphogenetic proteins) from the epidermal ectoderm flanking the neural plate. These induce sensory

interneurones by activating Sr/Thr kinases and by altering SMAD transcription factor levels (Wilson and Stice, 2006).



F*ig.6. Different signaling pathways during mammalian development ligands, receptors, downstream molecules; SHH (sonic hedgehog), RA (retinoic acid), FGF (fibroblast growth factor), BMP and Wnt (Purves Neuroscience)*

Likewise, the signals controlling anteroposterior neural development include FGF (fibroblast growth factor) and retinoic acid, which act in the hindbrain and spinal cord.

Another example is Shh, which acts as a morphogen, and induces cell differentiation that is dependent on its concentration. At

low concentrations it forms ventral interneurones, at higher concentrations, it induces motor neuron development, while it induces floor plate differentiation at the highest concentrations. Failure of Shh-modulated differentiation causes holoprosencephaly (Fig.6). These results indicate that the extrinsic (secreted or transmembrane) molecules released by sourounding cells and/or intrinsic signals produced inside the cell are required for the correct patterning of the nervous system during embryogenesis and neurogenesis.

1.3. In vitro differentiation of human neural cells

As mentioned above, during organogenesis, neurulation or neural tube formation is one of the first processes to occur during the initial nervous system configuration (Itskovitz-Eldor et al, 2000). The hESC deriving from the inner cell mass of the blastocyst can be differentiated into embryonic bodies (EBs), which resemble the early post-implantation embryo (a blastocyst containing all three germ layers) (O'Shea, 1999). Indeed, the EBs (Fig.7) formed from hESC express different markers of germ layers, such as ζ-globin, representing mesoderm layers: α-fetoprotein for the endoderm and neurofilament 68Kd for the ectoderm (Itskovitz-Eldor et al, 2000).

hESC can also be differentiated into columnar neuroectodermal cells called *neural rosettes*, and can mimic *in vivo* neuroectodermal development in terms of timing and morphology (Li and Zhang, 2006). *In vitro*, hESC differentiate into primitive neuroectodermal (or neural precursor) cells at around day 10.Then the

neuroectodermal cells exhibit neural *tube-like rosettes* in 14-17 days of differentiation in a chemically defined neural induction media (Gallego et al, 2010; Zhang et al, 2001).

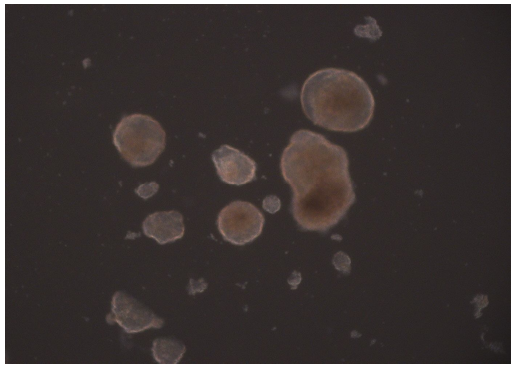


Figure.7. Embryoid bodies (Microscope Zeiss, CIPF)

Neural *rosettes (Fig.8)* contain neural progenitors (NPs) cells, which are a starting point to more restricted progenitors, leading to neuronal and glial cell lines (Elkabetz et al, 2008; Zhang et al, 2001). Proliferating NPs express high levels of Pax6, which is a neuroepithelial marker, as well as nestin, Sox2 and the homogenous expression of Sox1. Sox2 is a transcription factor regulating neural progenitor identity, which inhibits progenitor cells to reach a mature state, but provides proliferation of immature cells and maintenance of the progenitor pool of pro-neural cells. Inhibition of Sox2 restricts the proliferation of progenitor cells and the induction of neuronal and glial differentiation (Graham V, 2003).

In addition, NPs' proliferating cells have been confirmed to express high levels of telomerase as their potential for stem cell maintenance and markers of NPs are Nestin, SOX3, MUSASHI-1 (Stice S et al, 2008). Gene expression analyses have demonstrated induction of the expression of hESC markers during neural progenitor identity, such as a decrease of Nanog, and that the genes characterizing the early neural stage are in progression.

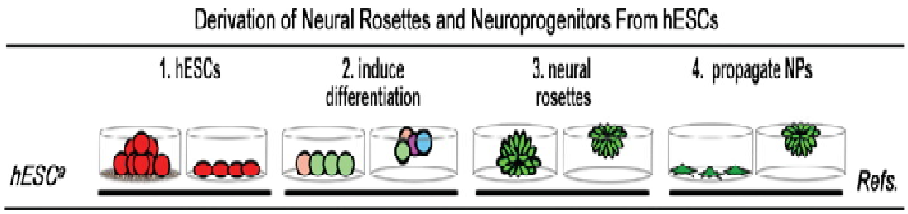


Fig.8. The scheme demonstrates the neural induction process of human embryonic stem cells (hESC) passing through the inductive phase, with tri-dimensional structures called rosettes (agglomerates of neural progenitors-NPs) to propagated NPs (Wilson and Stice, 2006).

Moreover, one of the most important proteins for the tight junction of neuroepithelial cells is ZO-1 (which could be expressed in hESC), while the specific distribution of ZO-1 is a crucial point for neural induction. Besides, N cadherin (N-cad) and CD133, together with the aforementioned ZO-1, contribute to the neural induction process (Elkabetz, 2008). It is important to emphasize that the differentiation potential of rosette cells diminishes with proliferation;

this also occurs *in vivo* during neural development and with neural precursors in neural tube formation (Jessell, 2000).

In short, the results of the derivation of neuroprogenitors from hESC provide an appropriate *in vitro* model to study early stages of the developing brain.

1.4. Ethanol as a teratogen agent: Foetal Alcohol Syndrome (FAS)

1.4.1. FAS and FASD in human studies

Ethanol is a well-recognised teratogen, and defects found in the children of mothers who consumed alcohol during pregnancy are one important brain developmental disorder. Indeed, ethanol is considered to be one of the most common substances that impact the developing brain, and prenatal alcohol exposure is a leading preventable cause of birth defects, mental retardation and neurodevelopmental disorders (American Academy of Pediatrics, 2000).

Among the most recognised consequences of prenatal alcohol exposure is foetal alcohol syndrome (FAS; Jones and Smith, 1973), characterised by pre- and postnatal growth deficiencies, craniofacial anomalies (Fig. 8) and evidence of CNS dysfunction. FAS is now

recognised as an important cause of intellectual disabilities and behaviour problems in many countries (Streissguth *et al*, 1991; Alati *et al*, 2006; Autti-Rämö *et al*, 2006; Ceccanti *et al*, 2007; Spohr *et al*, 2007). However, over the last 30 years, it has become clear that FAS is not the only outcome resulting from prenatal alcohol exposure. Indeed, the effects of prenatal alcohol exposure lie in a continuum of physical anomalies and behavioural and cognitive deficits. The term foetal alcohol spectrum disorders (FASD) has been adopted (Sokol *et al*, 2003) as a nondiagnostic umbrella term to describe this range of effects. Prevalence of FASD, which can be physical, mental or behavioural, is estimated to be ~1% of all births (May and Gossage, 2001).

However, not until recently has it been possible to evaluate the effects of ethanol on human brain structure and function *in vivo*. Novel neuroimaging techniques such as magnetic resonance imaging (MRI), functional MRI (fMRI), diffusion tensor imaging (DTI), magnetic resonance spectroscopy (MRS), positron emission tomography (PET) and single photon emission computed tomography **(**SPECT) have been employed on individuals with FASD to provide insights into the structural and functional alterations caused by prenatal ethanol exposure These studies have demonstrated that prenatal ethanol exposure reduces the overall volume in the cranial, cerebral and cerebellar vaults in FASD (Swayze II et al**,** 1997; Archibald et al, 2001; Sowell et al, 2002a; Autti-Rämö et al**,** 2002

Fig.9. Discriminating associated facial features of the FAS syndrome. (P.D. Sampson et al, 1997)

Human neuroimaging studies have also demonstrated that reduction on different brain areas is not uniform and that the parietal lobe (Archibald et al**,** 2001; Sowell et al, 2002a, 2002b), portions of the frontal lobe (Sowell et al**,** 2002a) and specific areas of the cerebellum (Sowell et al**,** 1996; Autti-Rämö et al**,** 2002; O'Hare et al**,** 2005) appear to be especially sensitive to alcohol insult. Basic animal studies have also evidenced the vulnerability of the CNS to the effects of ethanol, revealing that effects are not uniform and that some brain areas or cell populations are more vulnerable than others (Guerri, 1998, 2002).

1.4.2. Factors influencing the effects of prenatal ethanol exposure on the developing brain

Clinical data indicate considerable variability in the range and magnitude of prenatal ethanol-induced effects on brain abnormalities and behavioural outcomes. Several biological and environmental factors are known to influence the effects of ethanol on the developing brain, including alcohol dose and exposure pattern, developmental timing of exposure, genetic background of the mother and foetus, maternal nutrition, maternal age, socio-economic status and synergistic reactions with other drugs. Among these factors, the levels of alcohol reaching the foetal brain and exposure duration markedly influence the type and extent of damage (Maier *et al*, 1996). In addition, mother and foetus' genetic background such as variations in ethanol metabolism, also influence the risk of ethanol-induced malformations in the foetus (Thomas *et al*, 1998).

1.4.3. Critical periods of brain development and effects of ethanol

Clinical and experimental data demonstrate that the specific brain structure affected and the magnitude of damage are strongly influenced by the developmental timing of ethanol exposure. For example, facial dysmorphology, a salient feature of FAS (Jones and Smith, 1973), appears to arise only when high-peak blood alcohol levels occur during the embryonic stage of gastrulation (Sulik, 2005).

Mice exposed to ethanol on embryonic day 7 (E7) or E8 (Sulik, 2005) and macaques exposed to ethanol on E19 or E20 (Astley *et al*, 1999) exhibit FAS-associated facial dysmorphia. Exposure to alcohol during gastrulation also has a negative impact on the developing brain by reducing the neural progenitors pool (Rubert *et al*, 2006) and causing long-term effects not only on the forebrain (Ashwell and Zhang, 1996; Miller, 2007) but also on mature brainstem nuclei structures (Mooney and Miller, 2007). Human studies demonstrate that both binge drinking and chronic alcohol abuse during early stage human embryogenesis (corresponding to the third week of human gestation) are associated with a greater incidence of craniofacial defects and mental disabilities (Ernhart *et al*, 1987). Although embryogenesis is the main critical period for alcohol teratogenesis, alcohol can also affect other ontogenetic stages of brain development. For example, a second critical period is the stage of neuroepithelial cell proliferation and migration, which occurs in humans from 7 to 20 weeks of gestation (Suzuki, 2007) and from gestation day 12 (G12) to G20–21 in rats. At this stage, most nervous system areas (except the cerebellum) begin to differentiate.

Experimental studies report that ethanol exposure at this stage alters neuronal migration and affects the timing and pattern of cell generation by reducing the number of neurons and glial cells in the neocortex, the hippocampus and the sensory nucleus (Gressens *et al*, 1992; Miller, 1995b; Valles *et al*, 1997; Rubert *et al*, 2006).

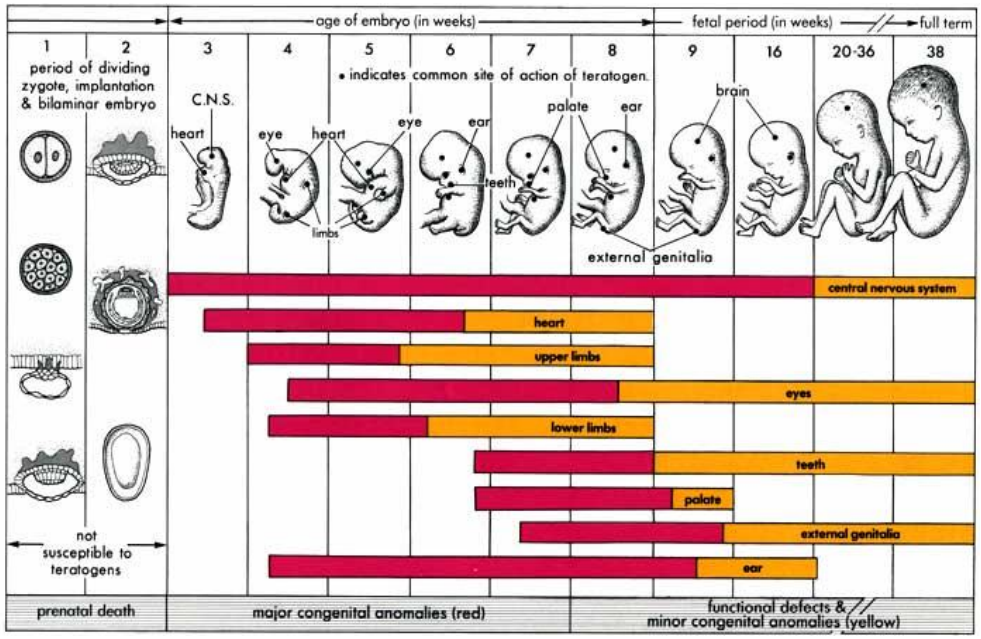


Fig.10. *Sensitive periods for alcohol toxicity during fetal development. Major congenital malformations are represented in red. Functional defects and minor congenital anomalies are depicted in yellow.*

As with other neurodevelopmental disorders (Suzuki, 2007), perturbation of neuroglial proliferation and migration by ethanol could cause long-term abnormalities in the cerebral cortex and in brain size, as noted in individuals with FAS and FASD (e.g, Archibald *et al*, 2001; Sowell *et al*, 2001a, 2002a), and likely contribute to the cognitive defects observed in adults with FASD. Additionally, since formation of the corpus callosum (CC) and the glial midline starts at around 7 weeks in humans (Paul *et al*, 2007; Richards *et al*, 2004), exposure to alcohol at this stage might disrupt early events in callosal formation, and may later dysregulate axonal pruning, leading to

agenesis, hypoplasia or abnormalities in the CC (e.g., Riley *et al*, 1995; Swayze II *et al*, 1997; Bhatara *et al*, 2002). In fact, administration of a single high dose of ethanol to neonatal rats markedly reduced the thickness of the CC (Olney, 2004), possibly resulting from loss of cerebro-cortical neurons whose axons projections comprise much of the CC mass.

Finally, alcohol also interferes with the 'brain growth spurt' neonatal stage in the rat (roughly the equivalent of the third trimester of human gestation), a period characterised by glial development (astroglial and oligodendroglia), synaptogenesis and development of the cerebellum. Ethanol exposure induces microcephaly, causes neuronal cell loss in both the hippocampus and the cerebellum (Goodlett and Lundahl, 1996; Dikranian *et al*, 2005), alters synaptogenesis and glial development (Guerri and Renau-Piqueras, 1997) and can cause learning/memory deficits, as well as long-term and neurobehavioural dysfunctions (Wozniak *et al*, 2004; Popovic *et al*, 2006). In addition, administration of a single high or moderate dose of ethanol to neonatal mice throughout the early synaptogenesis period triggers a significant neuroapoptosis in several brain regions (e.g., forebrain, midbrain, brainstem, cerebellum, spinal cord and retina) (Ikonomidou *et al*, 2000; Dikranian *et al*, 2005; Tenkova *et al*, 2003), with the caudate nucleus and the frontal and parietal cortices being the most vulnerable regions to the neuroapoptotic effects of ethanol (Young and Olney, 2005). In line with these data, alterations

in the caudate nucleus and in the frontal cortices have also been noted in individuals with FASD (Cortese *et al*, 2006; Fagerlund *et al*, 2006).

Collectively, experimental data indicate that although ethanol can interfere with important ontogenetic stages of the mammalian brain, the levels of ethanol reaching the foetal brain and the developmental timing of ethanol exposure, particularly early embryogenesis, are important determinants of the specific brain structures affected and the resulting degree of damage.

1.4.4. Potential mechanisms involved in ethanol-induced brain damage during development

Several molecular mechanisms have been identified as potential candidates responsible for the range of FASD phenotypes (Guerri 1998, 2002; Goodlett *et al*, 2005). These mechanisms are likely to participate at different stages of development and/or with different doses of ethanol. They include (1) alterations in the regulation of gene expression (e.g. reduced retinoic acid signalling, homeobox gene expression, altered DNA methylation; Deltour *et al*, 1996; Rifas *et al*, 1997): (2) interference with the mitogenic and growth factor responses involved in neural stem cell proliferation, migration and differentiation (see revs., Mooney *et al*, 2006; Siegenthaler and Miller, 2006): (3) disturbances in molecules that mediate cell–cell interactions (L1, NCAM, loss of trophic support; e.g. Wilkemeyer *et al*., 2002; Miñana *et al*, 2000): (4) activation of

molecular signalling that controls cell survival or death (growth factors deprivation, oxidative stress, apoptotic signalling and caspase-3 activation, suppression of NMDA glutamate and GABAA receptors, withdrawal-induced glutamatergic excitotoxicity, (e.g. Guerri *et al*, 1994; Pascual *et al*, 2003; Olney, 2004; Young *et al*, 2005) and (5) derangements in glial proliferation, differentiation and functioning (Guerri *et al*, 2001, 2006). This last mechanism is supported by neuroimaging studies which reveal global white matter reductions and abnormalities in FASD (Archibald *et al*, 2001; Sowell *et al*, 2002a).
Although several mechanisms have been postulated, we still do not know why exposure to alcohol during early embryogenesis results in the facial anomalies associated with FAS and causes mental retardation.

1.5. TLR receptors

Toll-like receptors (TLRs) are transmembrane pattern-recognition receptors that initiate signals in response to diverse pathogen-associated molecular patterns (PAMPs). The first Toll protein was discovered in **Drosophila melanogaster**, where it controls dorso-ventral patterning. A mammalian homologue for Toll, TLR4, was later found to recognise bacterial lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria. Subsequently, many additional homologues have been identified across diverse species.

The TLRs family involves 13 members and they participate in the innate immune response. In addition to the pathogen-derived ligands that activate the different TLRs, endogenous TLR ligands referred to as damage- (or danger-) associated molecular patterns (DAMPs) have been identified. Numerous endogenous ligands have been described and include low molecular weight hyaluronic acid (LMW-HA), fibrinogen, fibronectin, β-defensins, heparin sulphate proteoglycans and heat-shock proteins (Kawai and Akira, 2006).

Recent findings suggest that mammalian TLRs play developmental roles during embryogenesis, as well as physiological and metabolic roles in adults. For example, TLR5-deficient mice exhibit hyperphagia and develop hallmark features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance and increased adiposity. Likewise, TLR2 and TLR4 play an important role in the maintenance or proliferation and differentiation of Neural Progenitors Cells (NPC) directing self-renewal or cell fate decision. In vivo experiments also show that mice deficiency of TLR2 has negative impact on adult hippocampal neurogenesis whereas on the contrary, absence of TLR4 increases the proliferation rate and differentiation process (Okun et al, 2011).

Activation of a given TLR engages distinct signalling pathways in different neural cell types. For example, TLR4 activation results in distinct signalling outcomes in astrocytes, microglia, neurons and neural progenitor cells (NPCs), and these pathways differ

from TLR-induced signalling in dendritic cells (Fig 9). TLR4 activation in dendritic cells signals through a myeloid differentiation primary response gene 88 (MyD88)-dependent pathway to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and to produce cytokines such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and IL-12. The MyD88-independent TRIF pathway is also activated and results in the nuclear translocation of Interferon regulatory factor (IRF)-3 and in the synthesis of interferon β (IFN-β), which then activates its receptor coupled with Signal Transducer and Activator of Transcription-1/2 (STAT-1/2) and IRF-9 and with a secondary wave of transcription of cytokines and chemokines such as interferon gamma-induced protein 10 kDa (IP-10) and Glucocorticoid Attenuated Response Gene 16 (GARG16).

In astrocytes, however, TLR4 activates the MyD88- but not the TRIF-dependent pathway. In these cells, MyD88-mediated signaling leads to the NFκB-induced transcription of TNF-α, vascular cell adhesion molecule 1 (VCAM-1) and IL-27, whereas other signalling mediators such as c-Jun N-terminal kinases (JNK) activate STAT-1 to transcribe IP-10 and to suppress cytokine signalling proteins-1 (SOCS-1)

Extracellular-signal-regulated kinases (ERK) are also activated by LPS in these cells independently of MyD88. TLR4 activation in microglia resembles its activation of dendritic cells, with both the MyD88- and TRIF-dependent signalling pathways in an active state.

These in turn induce NFκB activation which promotes the transcription of cytokines such as TNF-α, IL-6 and IL-1β, and IRF-3 activation resulting in the IFN-β-mediated activation of STAT-1 and

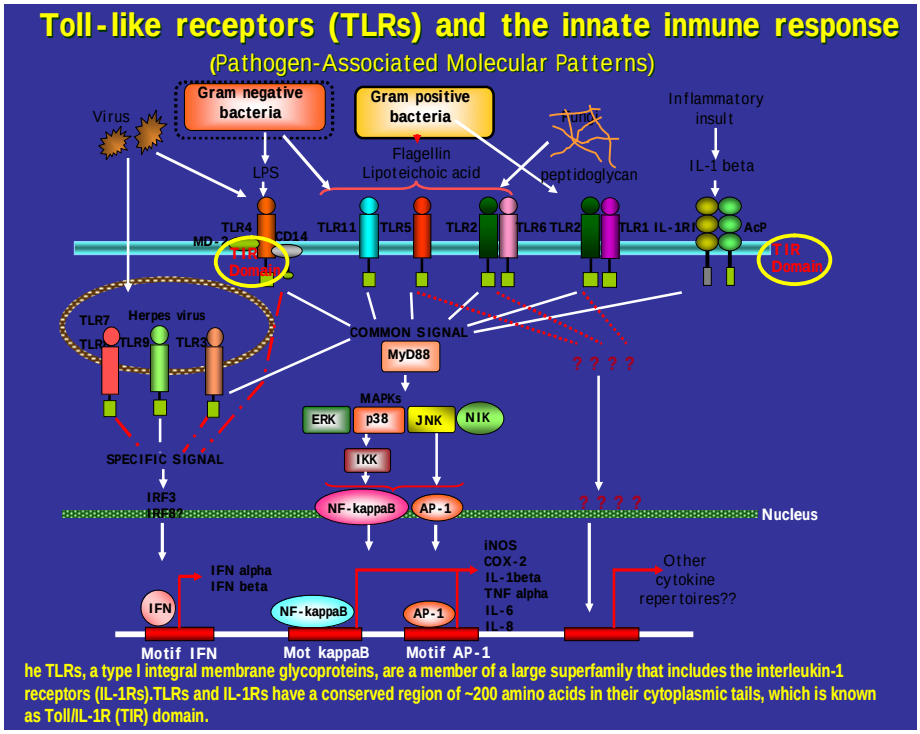


Fig.11. Scheme representing Toll-like receptor signalling. The ligand for TLR4 is LPS (lipopolysaccharide) endotoxin chemically identified and characterised in the most of Gram-negative bacteria and ethanol is also shown ethanol as a potential ligand for this receptor. A downstream pathway is also shown; MAP kinases the activation leads to activation of AP1 and NFkB transcription factors which induce genes that encode cytokines and inflammation- associated molecules.

.

subsequently IRF-1(Kawai and Akira, 2006; Okun et al, 2011)(Fig.10).

Activation of TLRs causes neuroinflammation and TLRs are involved in the pathogenesis of stroke, Alzheimer's disease (AD) and multiple sclerosis (MS) as well as other neurodegenerative diseases (Glass CK et al., Cell 2010). Notably, it has been demonstrated that ethanol induces TLR4 signaling neuroinflammation and brain damage when acting as a TLR4 agonist (Alfonso-Loeches et al, 2010). Recent findings suggest a new role of TLRs as modulators of CNS plasticity. TLRs influence neuroprogenitors proliferation, differentiation, neurite outgrowth and behavioural plasticity.

However, although evidence from mice deficient in TLRs strongly implicates these receptors in neuroplasticity, the distinction between developmental and functional effects of the life-long deficiency of a TLR, as well as the specific roles of TLRs during ontogeny and in neuroplasticity following infection and injury remain unclear.

1.6.Cannabinoid receptors (CBs)

Since the identification of two cannabinoid (CB) receptors for the plants major active constituent Δ9-tetrahydrocannabinol (THC) around 1990, endogenous ligands, endocannabinoids, have been

discovered, and have led to the definition of the 'endocannabinoid-CB receptor (ECBR) system'. Ligands, including anandamide and 2-arachidonylglycerol, are particularly interesting since they are derivatives of arachidonic acid, and they also interact with related lipids such as oleoylethanolamide

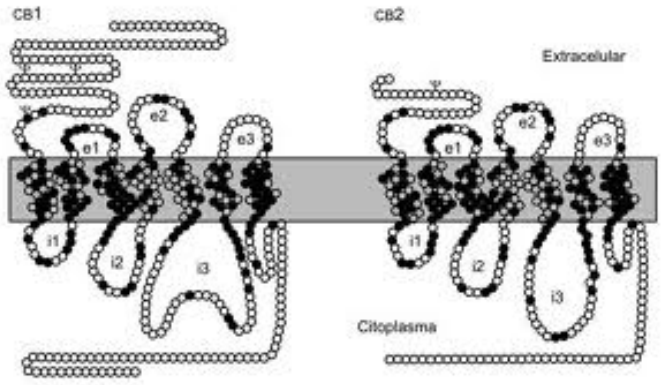


Fig.12. Scheme of cannabinnoid receptors CB1 and CB2

.

There are two main receptor types associated with the endocannabinoid signalling system: cannabinoid receptor 1 (CB_1) and 2 (CB_2). Both receptors are 7-transmembrane G-protein coupled receptors (GPCRs), which inhibit the accumulation of cyclic adenosine monophosphate within cells. CB_1 receptors are present at highest concentration in the brain, but can also be found in the periphery. CB_2 receptors are mostly located in the immune and haematopoietic systems. The enzymes involved in endocannabinoids synthesis and degradation are NAPE-PLD (N-

Acylphosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D) and FAAH (fatty acid amide hydrolase), respectively.

More recent studies have demonstrated that endocannabinoid signalling controls neural progenitor differentiation in the mice adult brain by promoting astroglial differentiation of newly born cells. These results show a novel physiological role of endocannabinoids, which constitute a new family of signalling cues involved in the regulation of the neural progenitor cell function (Aguado T et al., 2005, 2006). However, since all the studies done on the role of endocannabinoids have been preformed in mice, it is presently unknown whether endocannabinoids are expressed in human ESC, and whether they play a role in the proliferation and differentiation of human neuroprogenitors.

----------OBJECTIVES----------

The main objectives of this work are to use neural human neuroprogenitor (NPs) cells from hESC as a tool to study the cellular and molecular events involved in early human neural development under physiological conditions and to study the teratogenic effects of ethanol during the initial formation of the CNS.

Objectives

- To develop an *in vitro* protocol of derivation of human neural progenitors (hNPs) from hESCs, which could mirror early stages of human brain development

- To characterize the *in vitro* culture by evaluating the gene and protein expression of human Neuroprogenitors (hNPs) in culture during their proliferation and differentiation into mature cells (neurons, astrocytes and oligodendrocytes).

- To assess whether the endocannabinoid system, including endocannabinoid receptors (CB1, CB2) and the enzymes involved in their synthesis (NAPE-PLD) and degradation (FAAH) of endocannabinoids (EC), are expressed in human neural progenitors during their differentiation to mature nervous cells.

- To assess the gene and protein expression of TLR4 and TLR2 receptors during neural differentiation from hESC (*in vitro*) and during brain ontogeny in mice.

- Finally, the last objective will investigate the actions of ethanol on the proliferation and differentiation of hNPs. Specifically we will assess the effects of different physiological concentrations of ethanol on: 1) gene and protein expressions during the derivation of hESC to NPs: 2) the proliferation and cells survival of the hNPs, 3) the NPs differentiation processes to mature neural cells, by assessing gene and protein expressions and morphological alterations.

--------MATERIALS AND METHODS-------

Materials and Methods

3.1. Human embryonic stem cells culture (hESC)

Human embryonic stem cells (hESC) (H9 line, WiCell Inc., Madison WI) were grown in human embryonic stem cells medium (hES medium) cultured on commercially available human foreskin fibroblast-HFF (American Type Culture Collection, ATTC, Manassas, VA), MEF (CF-1 MEF, P3) and STO line (CRL-1503™ ATCC) and all of the fibroblast were inactivated with mitomycin C (Sigma ref: I 3390). The hESCs were cultured in appropriate conditions in hoods with termal plates (37°C), floating air, UV protection, incubator at 37°C, 5% CO_2, in humidity. The hESC colonies were replated, according to their size at 6-7 days period, passaging small pieces by mechanical dissection (performed with the insulin needle). The colonies were cleaned mechanically from differentiated cells and medium was changed every second day. For the maintenance of hESC we used also mTeSR™1 Medium kit (StemCell Technologies Ref: #29106) which containes mTeSR™1 Basal Medium and mTeSR™1 5X supplement. In addition, a conditional medium (CM) obtained from hESC cultured on human foreskin fibroblast-HFF feeders were also used. This CM contains grown factors and metabolites which were released by hESC in culture.

3.2. Human foreskin fibroblast cell culture

a)Inactivaton of human foreskin fibroblast (HFF) by mitomycin C

Fibroblasts were inactivated with mitomycin C (Sigma ref: I 3390) 10µgr/ml. Mitomycin C was added to the flask with FSK medium (HFF medium; see bellow) for 2.5-3 hours (a 15 ml flask, we added 150µl of mitomycine C). After three hours, the medium with mitomycin C were aspirated from the flask and inactivated cells were washed 3 times with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.

Then, in order to detach inactivated fibroblasts, a TRIPLE SELECT reagent (Gibco 12563-011) was added to the flask. This reagent is animal origin-free cell dissociation enzyme which can be used instead of porcine trypsin. This step was performed at 37 º C for 5 minutes in the incubator. Cells were then centrifuged at 1200rpm for 3-5 min, the pellet was resuspended with complete fibroblast medium, cells were counted and plated (150.000-200.000 for 6-well plates) on the Gelatin covered plates (Sigma ref: G1890)

b) Fibroblast passaging

Confluent fibroblasts were divided into 3-4 new flasks .For this, the FSK medium was removed, cells were washed three times with DPBS or Dulbecco's Phosphate Buffered Saline not containing calcium, magnesium, and then 3 ml of TRIPLE SELECT (Gibco 12563-011) were added in each 15 ml flask. After 3-5 min, the cells were removed and centrifugated at 12.000 rpm for 5 min. The cells

were the washed 2 times. The last pellet was resuspended in 9.5 ml of FSK medium, fibroblasts were divided into three or four P75 flasks and fresh medium was added to each flask. Flasks were kept in an incubator at 37 ºC, 5% CO2 in humidity conditions. The medium was changed every 3 to 4 days.

FSK (Fibroblast medium)

- ISCOVES Modified Dubelcco's Medium (Sigma: I3390)
- FBS (fetal bovine serum) Ref: 10091-148 Invitrogen (Gibco)
- Glutamax Ref: 35050 Invitrogen (Gibco)
- P-S (Penicillin/Streptomycin) Ref: 15070 Invitrogen (Gibco)

c) Fibroblast freezing and thawing procedure

For fibroblast freezing, the medium of the cells were aspirated and cells were washed once with PBS. Then, the cells were detached, by using Trypsin or TRIPLE SELECT for 3-5 minutes, and centrifuged at 300 rpm for 5 minutes. The supernatant was aspirated and the medium for cell freezing (SBF 90%, DMSO 10% (Sigma D2650) was added to the cells. The tube containing the cells was immersed directly into a freezing container (Mr Frosti) overnight and then the tubes were stored to -80 º C.

For fibroblast thawing, vials containing the cells at -80ºC were thawed by immersion in 37º C water bath. Then, FSK medium was added drop by drop to the cell vial, mixed gently, transferred to the 15 ml tube and centrifuged at 300 rpm for 5 minutes. The supernatant was aspirated and fresh FSK medium (1ml) was added.

3.3. Alkaline phosphatase staining procedure

Alkaline phosphatase is a stem cell membrane marker and elevated expression of this enzyme is associated with undifferentiated pluripotent stem cell. All primate pluripotent stem cells, like Embryonic stem (ES), embryonic germ (EG) and embryonic carcinoma (EC) cells, express alkaline phosphatase activity. For the detection and staining of hESC with of Alkaline phosphatase we used a kit provided by Chemicon(No 001). Briefly,

1. Human embryonic stem cells, H9; were cultured for five days, at low density (*Under our experimental conditions five days of culturing are optimal for good AP stain visualization).*
2. On day five, the medium was aspirated and the hESC were fixed with 4% Paraformaldehyde in PBS for 1-2 minutes.
3. The fixative was aspirated and rinsed with 1X Rinse Buffer, avoiding cell drying.
4. The reagents were prepared for Alkaline Phosphatase staining.
5. The stain solution was added to cover each well and incubated in the dark room temperature for 15 minutes.
6. The staining solution was aspirated and rinsed wells with 1X PBS to prevent drying and then the number of AP expressing colonies were counted (red stem cells colonies), versus the number of different colonies (colourless).

3.4. Neural induction, neuroprogenitors proliferation and differentiation.

Human embryonic stem cells, the H9 line were cultured on commercially available human foreskin fibroblast and cultured in hESC medium (see below) which contains basic Fibroblast Growth Factor, at 37°C in 5% CO2 in a humidified atmosphere.

The hESC colonies were replated, according to their size, after a 6- to 7- day period, by passaging small pieces by mechanical dissection. To obtain embryonic bodies (EB), the hESC colonies were transferred to low attachment plates and maintained in the same medium for 6 days.

For neural induction, after six days in culture, EB were scattered and transferred to adherent conditions on Matrigel-coated plates and cultured with neural induction medium (NIM) . The cells from attached floating formations started to spread radially after 5 days. Rosettes were cut and desegregated to obtain single cells which contain the neuroprogenitors (NPs). Cells were the plated on polyornithine/laminin plates (350,000 cells per 2ml plate) and cultured with neural proliferating medium (NPM). The NPs proliferated in the plates until they became confluent and according to their number, they were passaged into polyornithine-laminin plates with fresh NPM medium (NPM). On day 21 the NPM medium was replaced by a Neural Differentiating Medium (NDM) which contains BDNF. Cells were maintained for an additional 15 days in NDM (Nat et al. 2007).

hESC medium

- DMEM Knockout (Gibco, Cat.No. 10829-018)
- Serum Replacement (Gibco, Cat.No. 10828-028)
- L-Glutamine 200 mM
- P-S (Penicillin/Streptomycin) Ref: 15070 Invitrogen (Gibco)
- MEM (Gibco Cat. No. 11140)
- bFGF (Invitrogen Cat.No. PHG0263)

Neural Induction Medium (NIM),

DMEM/F12 and Glutamax: Neurobasal (1:1), B27 supplement (Invitrogen), N2 supplement (Invitrogen), 2mM Glutamax (Invitrogen), Penicillin/Streptomycin.

Neural proliferation medium

bFGF (20ng/ml), DMEM/F12, Glutamax:Neurobasal (1:1), B27 supplement, N2 supplement, 2mM Glutamax and Penicillin/Streptomycin

Neural differentiation medium

containing Neurobasal medium, B27 (Gibco, Invitrogen), 2mM Glutamax, Penicillin/Streptomicin, 10 ng/ml BDNF (Brain Derived Growth Factor, Invitrogenn Cat. No. 10908-010).

3.5. Cell Proliferation Assay

For cell proliferation assay, BrdU incorporation was assessed using a commercial kit (Roche, cat no. 11810740001) and was performed according to the manufacturer´s instructions. Quantification of the number of cells incorporated BrdU was carrying out by flow cytometry (Cytomics FC500, Beckman Coulter).

3.6. Apoptosis quantification and caspase-3 activity

To analyze apoptosis, cells were labelled with R-phycoerythrin-conjugated annexin-V in combination with the cell-cell-impermeant DNA fluorophore 7-AAD (both Molecular Probes). The assay was performed according to the manufacturer´s protocol. Cells were analyzed by Flow Cytometry (Cytomics FC500, Beckman Coulter).

Caspase-3 activity was measured by a colorimetric assay kit (Sigma-Aldrich Quimica, Spain), using acetyl-DEVD-p-nitroanilide (Ac-DEVD-pNA) as a substrate. Then, the samples were read at 405 nm by the formation of the colorimetric product, p-nitroaniline, and the activity was calculated according to the to the manufacturer´s instructions.

3.7. RNA isolation, Reverse-Transcription PCR and Quantification of mRNA levels

RNA was isolated from undifferentiated, differentiating and differentiated cells at established times, using Tri Reagent (Sigma)

according to manufacturer's instructions. The amount of purified RNA was estimated on nanodrop by measure of absorbance at 260 nm and its purity was assessed by the ratio 260/280 nm. RNA integrity was examined by agarose gel electrophoresis. RNA (1 µg) of each sample was reverse transcribed using Transcriptor First Strand cDNA Synthesis kit (Roche). Diluted cDNA (dilution 1/10) was amplified with a rapid thermal cycler (LightCycler Instrument, Roche Diagnostics) in 20 or 10 µL of LightCycler 480 SYBR Green I Master (Roche) and 0.5 µM of each oligonucleotide. The sequences of both the forward and reverse primers used in this study are shown in Table 1. Each sample was quantified for each gene by triplicates in at least five independent cultures. In parallel, we analyzed the mRNA levels of the human housekeeping GAPDH as an internal control for normalization. To ensure that treatments didn't alter GAPDH mRNA levels, the Ct values were compared between groups. No significant differences were found in GAPDH levels.

Table 1. Sequence of primers used for RT-PCR and length of fragments

Gene	**Forward primer**	**Reverse primer**	**Product (bp)**
αFETOPROTEIN	5'-AGCTTGGTGGTGGATGAAAC-3'	5'-CCCTCTTCAGCAAAGCAGAC -3'	248
GFAP	5'-TCATCGCTCAGGAGGTCCTT-3'	5'-CTGTTGCCAGAGATGGAGGTT-3'	383
GAPDH	5'- AGCCACATCGCTCAGACACC- 3'	5'- GTACTCAGCGCCAGCATCG-3'	301

CB 1	5’-GAACCCCATCATCTATGCTCTG-3’	5’- CAATCTTGACCGTGCTCTTG-3’	194
CB 2	5’- ATGGGCATGTTCTCTGGAAG -3’	5’- GAACCAACAGATGAGGAGCA -3’	152
FAAH	5’- GGAGACCAAACAGAGCCTTG-3’	5’- GGTCCACGAAATCACCTTTG-3’	154
NAPE-PLD	5’- GGTGAGTTAGCTTTGAGGTGTG-3’	5’-GCCAGACGAGTGGAAATAGAAC-3’	246
MAGL	5’- CTTGCGTTGAGTTCCTTCTC-3’	5’- TTTGCCTTCCTCTCACCTTC-3’	151
MAP 2	5’-TTTTCCCTCATGGGAGTCAG-3’	5’-GACATGCAAGGCACAGAAGA-3’	251
NANOG	5’-CAAAGGCAAACAACCCACTT-3’	5’-CTGGATGTTCTGGGTCTGGT-3’	426
NESTIN	5’-AACAGCGACGGAGGTCTCTA-3’	5’-TTCTCTTGTCCCGCAGACTT-3’	220
PAX-6	5’-AACAGACACAGCCCTCACAAACA-3’	5'-CGGGAACTTGAACTGGAACTGAC-3'	275
B-TUBULIN III	5’-ACCTCAACCACCTGGTATCG-3’	5’-GGGATCCACTCCACGAAGTA-3’	449
CASPASE 3	5’-GAACCACTATGAAGCTACCTCAA-3’	5´-CACAGATGCCTAAGTTCTTCCAC-3'	291
SOX 2	5'-GGGAAATGGGAGGGGTGCAAAAGAGG	5'-TTGCGTGAGTGTGGATGGGATTGGTG	150
CD 133	5’-TTGGAGTGCAGCTAACATGAG	5’-GGGAATGCCTACATCTGGAA	300
OLIG 1	5'- TGCCAGTTAAATTCGGCTACTACC	5'-TTGCATCCAGTGTTCCCGATTTAC	390

PCR amplicons were confirmed to be specific by size and melting curve analysis. The real-time monitoring of the PCR reaction, the precise quantification of the products in the exponential phase of the amplification and the melting curve analysis were performed with the

LightCycler 480 quantification software, as recommended by the manufacturer. Table 1 illustrated the sequences of the primers used and the length of the fragments.

3.8. Immunofluorescence

Cells were growing in laminin and poly-L-ornitin-coated glass coverslips (12 mm) in 4- well plates were used for the immunofluorescence studies. Cells were fixed with paraformaldehyde (4% in PBS) for 20 min, permeabilized with 0.25% Triton X-100 for 5 min, as previously described (Rubert et al., 2006). After washing with PBS, the fixed cells were incubated with 5% BSA in PBS for 30 min to block nonspecific antibody binding. Cells were then incubated with the primary antibodies: mouse anti-GFAP (Sigma), rabbit anti-nestin (Chemicon), rabbit anti-MAP2 (Chemicon), mouse anti-TUJ1 (Abcam), rabbit anti-GABA-A (Chemicon. Co), anti-glutamate (Sigma-Aldrich.), anti-PAX6 (Abcam); anti-C2B5 (Sigma-Aldrich, Spain). All antibody incubations were performed in 0.1% Triton-1% BSA in PBS for 1 h at room temperature. Appropriate secondary antibodies (Jackson Immunoresearch, Mississauga, Ontario, Canada) conjugated with fluorophores were used thereafter. For actin filaments staining, cells were incubated for 45 min with **phaloidin**-TRITC (Sigma-Aldrich, Spain), as previously described (Minambres et al., 2006). Nuclei were stained incubating in DNA-binding dye Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) at a dilution 1/40,000 in PBS for 10 minutes at room temperature. Micrographs were digitally recorded with a Leica microscope (Wetzlar, Germany).

To quantify intensity of signal, a minimum of ten fields were taken of at least three different cultures. Immunoreactivity was determined with Metamorph-Offline software (Universal Imaging, Downingtown, TA).

3.9. Stimulation of NPs with LPS

For these experiments neural progenitors at day 15 of their proliferation stage were used. Cells were rinsed and incubated in serum-free medium with for 18 h, and then NPs were stimulated or treated with 50 ng/ml LPS for 10 or 30 min. After the treatment, cells were scraped, centrifuged at 3600 rpm for 5 minutes, in a 0.5 ml Eppendorf tubes. Supernatant was eliminated and the precipitate was resuspended with RIPA Buffer was (80 µl- 100 µl). The cell suspension was incubated in ice for 30 minutes, centrifuged at 13 000 rpm for of 15 minutes. The supernatant was used for protein and kinases determination by Western blotting.

3.10. Nuclear fraction isolation from NPs

Nuclear fraction from NPs was isolated as previously described (Ishida et al., 2002). Briefly, NPs were treated with h LPS (50 ng/ml) for 30 min. Then, cells were washed with PBS, scraped and centrifugated a 350g for 10 min. The pellet was resuspended in Solution 1 + 0.2% NP-40 and left 5 minutes on ice. Then, cell suspension was centrifuged at 1000g for 10 minutes, and supernatant containing the cytoplasmic fraction was removed.

Pellet was the suspended in 1ml of Solution 2 and centrifuged at 1000g for 10 minutes. The pellet was resuspended in Solution 3 and incubated on ice for 20-30 minutes. The next step was centrifugation at 10000g for 10 minutes, supernatant was removed and NUCLEAR fraction was obtained. The nucler fraction was used to assess NFkB.p65 by Western blotting analysis.

SOLUTIONS:

Solution 1:

- Na_2HPO_4 5mM pH 7.4
- NaCl 50mM
- Sucrose 150mM
- KCl 5mM
- DTT 2mM
- $MgCl_2$ 1mM
- $CaCl_2$ 0.5mM
- PMSF 0.1mM

Solution 2:

- 30% (w/v) sucrose
- Tris-HCl 2.5mM pH 7.4
- NaCl 10 mM

Solution3:

- Tris-HCl 2.5mM pH 7.4
- NaCl 300mM
- Triton X-100 0.5%

3.11.Cell lysis

For the cell lysis was used RIPA Buffer with additional components:

RIPA Buffer

Tris-HCl pH 7.5—20mM	0.5M
NaCl—350mM	5M
EDTA—1mM	0.5M
Triton—1%	100%

Na pyrophosphate—2.5mM

Na3VO4—1mM

NaF—1mM

MilliQ Water

Additional components

Roche mini complete (1 tablet for 10 ml) 1 tablet in 1 ml of water

PMSF 0.1M and RIPA Buffer

3.12. Mice and alcohol treatments

Female C57BL/6 wild-type mice (Harlan Ibérica) weighing 18-20 gr were used. All animals were kept under controlled light and dark conditions (12/12h), temperature (23ºC) and humidity (60%). For chronic ethanol treatment, 7-week old female C57BL/6 were housed (3 animals/cage) and maintained with water containing 10% (v/v) ethanol and a solid diet *ad libitum* for 2 months. During this period, the daily food and liquid intake was 3.26±0.91 gr and 3.49±0.5 ml of

10% ethanol in water, respectively. Ethanol concentration in the drinking water was progressively increased for the first two weeks to finally reach 12.8±1.2 gr/kg/bw over 2 months. Blood ethanol levels achieved in ethanol-treated mice were 125±20 mg/dl (range 87-140 mg/dl).

After mating, the control and alcohol-fed dams were placed in separate cages during the gestation and lactation.

All animal experiments were carried out in accordance with the guidelines approved by the European Communities Council Directive (86/609/ECC) and by Spanish R.D. 1201/2005. All the experimental procedures were approved by the Ethical Committee of Animal Experimentation.

3.13. Brain tissue extraction and homogenization

Cerebral cortex of control and ethanol-exposed pups, at different postnatal ages, was used. Brain tissue was homogenized with a Potter in a modified RIPA buffer (see below) (1:50), incubated on ice for 30 min and centrifuged at 13.000 rpm for 15 minutes at 4 degrees. Supernatant was used for protein determination and for Western blotting analysis.

RIPA Buffer (modified)

Chemicals	**Cf**	**Stock**
NP-40	1%	10%
Tris pH8	20mM	1M
NaCl	130mM	5M

NaF	10mM	0.5M
DTT	1mM	1M
Aprotinin	10µgr/ml	1 mg/ml
Leupeptine	40µM	5mg/ml
Na3VO4	1mM	100 mM
PMSF	1 mM	100 mM
Water	up to 1 ml of entire solution	

3.14. Bradford Method for protein quantification

To assess protein determination in the brain and cells extracts, we first performed a standard curve using BSA (bovine serum albumin). Each sample was measured in duplicate with using 2 and 4 µl of the sample + 800 µl of miliQ Water + 200 µl Biorad Reagent, using in parallel BSA standard samples. After 10 min at room temperature, the optical density was measured at 595 nm in a spectrophophotometer.

3.15. Sodium *dodecyl sulfate polyacrylamide gel electrophoresis* (SDS-PAGE) and Western blot analysis

For SDS-PAGE, the different samples were mixed with a buffer containing Tris 350mM pH6.8, glicerol 30% (v/v), β-mercaptoetanol 30% (v/v), SDS 100gr/l and bromophenol blue 200 mg/l. Each sample was boiled at 100º C for 5 minutes. Equal amount of cells or brain lysate of each sample was loaded onto sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). For electrophoresis Mini Protein system (BioRad) was used. The gels

were prepared in two parts: Stacking gel 5% which serves to concentrate the proteins to the same line and pass to the next phase at the same time- to the Resolving gel (7-10% pore) to separate the proteins according to their size and modifications. The electrophoresis was performed in electropforesis buffer containing Trizma base 30gr/l, glycine: 144 gr/l and SDS 10gr/l; in the phase of Stacking gel at 100V and in the other part at 80V. Molecular weight markers were run in parallel (Molecular Weight Rainbow, Amersham).

Gels were then blotted onto polyvinylidene fluoride membrane. The transference was performed in transfer buffer which contained (Tris 25 mM pH 8.3, glycine 192 mM and 20% of methanol). The transference was performed for 1 hour at room temperature at 100 V or at 30V overnight at 4º C (cold room).

Membranes were blocked with 5% dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature on an orbital shaker. The membranes were briefly rinsed for 2 or 3 times with 0.1% TBS-T and then incubated for 2 hour at room temperature or overnight at 4 ºC (see table 2)

Then membranes were incubated with the appropriate secondary antibody, and proteins were visualized with either enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech., Madrid, Spain) or alkaline phosphatase conjugate (Sigma-Aldrich). Band densities were quantified using the AlphaImager 2200 software (Alpha Innotech Corporation, San Leandro, USA).

Membrane stripping

In most cases, membranes were striped and incubated with the GAPDH as a loading control.

Stripping buffer:

4ml Glycine 1M pH 2.5

800µl 10% SDS

15.2 ml MiliQ Water

And after in non-fat dry milk. The next steps were the same.

Table 2: Primary antibodies used

Antibody	***Dilution***	***Made in***	***kDa***	***Location***	***CIA***
TLR4	1:500	*Rabbit*	*80*	*Freeze*	*Abcam*
pERK	*1:250*	*Mouse*	*42-44*	*Fridge*	*Sta.Cruz*
MyD88	*1:300*	*Rabbit*	*35*	*Fridge*	*ProSci*
RhoE	*1:1000*	*Mouse*	*30*	*Freezer*	*Apstate*
NFkB-p65	*1:50*	*Rabbit*	*65*	*Fridge*	*Sta.Cruz*
GAPDH	*1:3000*	*Mouse*	*37*	*Fridge*	*Chemicon*
ERK	*1:250*	*Rabbit*	*42-44*	*Fridge*	*Sta.Cruz*
IRAK	*1:100*	*Rabbit*	*80*	*Fridge*	*Sta.Cruz*
P38	*1:250*	*Rabbit*	*38*	*Fridge*	*Sta.Cruz*
Lamin A/C	*1:1000*	*Rabbit*	*70*	*Freezer*	*Cell Signaling*
TLR2	*1:200*	*Rabbit*	*90*	*Freeze*	*IMGENEX*

Stacking gel (5%)

GEL	1 (4 ml)

H_2O (ml)	2.92
Acril/Bis (ml)	0.5
Tris pH 6,8 (ml)	0.5
SDS 10% (µl)	40
PSA 10% (µl)	40
TEMED (µl)	4

Resolving gel

PERCENTAGE	6%	7,5%	10%	12%
H_2O (ml)	5.8	5.425	4.8	4.3
Acril/Bis (ml)	1.5	1.875	2.5	3
Tris pH 8,8 (ml)	2.5	2.5	2.5	2.5
SDS 10% (µl)	100	100	100	100
PSA 10% (µl)	60	60	60	60
TEMED (µl)	7	7	7	7

3.16. Statistical analyses

Statistical analysis was assessed by one-way ANOVA. Following a significant *F* value, post-hoc analysis (Student – Newman - Keuls) was performed. $P < 0.05$ was considered statistically significant.

----------RESULTS----------

Results

4.1. Experimental approaches to establish the condition for human embryonic stem cells (hESC) growth and maintenance

a) The use of different feeder cells

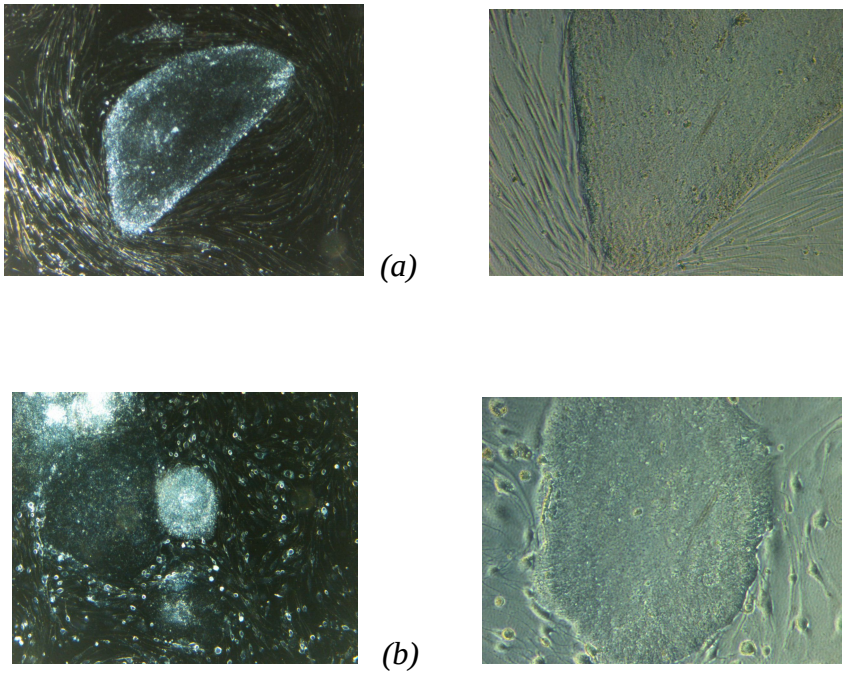


Fig.1. Two different morphologies of the human embryonic stem cells H9 line on HFF (human foreskin feeder)(a) and STO (the mouse embryonic fibroblast cell line)(b)

Previous studies have demonstrated that mouse embryonic fibroblasts (MEF) can be used as a suitable layer to grow human embryonic stem cells (hESC) (Thomson, 1998). Therefore, in order to establish an appropriate feeder layer for growth hESC, H9, we used *two* types of fibroblasts: human foreskin fibroblast (HFF), STO or cells derived from mouse embryos and mouse embryonic fibroblasts

or MEF. As shown in Figure1, the morphology of the H9 colonies was differed depending on the type of feeder layer used. Thus, the cell colonies grown on HFF display a flat morphology (with sharp edges) (Fig1. a), while spherical colonies were found when H9 was grown on STO *(Fig.1,b)*. These results suggest that the shape of the hESC colonies acquired in culture depends on the type of supporting cells where the cells were grown.

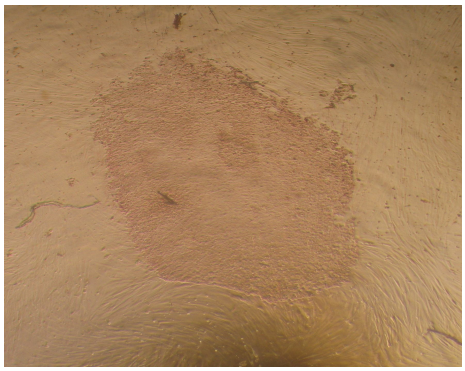


Fig.2. Alkaline phosphatase assay for pluripotency (H9 colony on HFF)

Under all the experimental conditions in which the different feeder layers were used, hESC colonies were cleaned mechanically (every second day) with an insulin needle. Then every 6-7 days, hESC colonies were mechanically cut into small pieces and passed to new gelatin feeder covered plates. An alternative procedure to pass the pieces of colonies was to use collagenase, dispase (enzymatically).

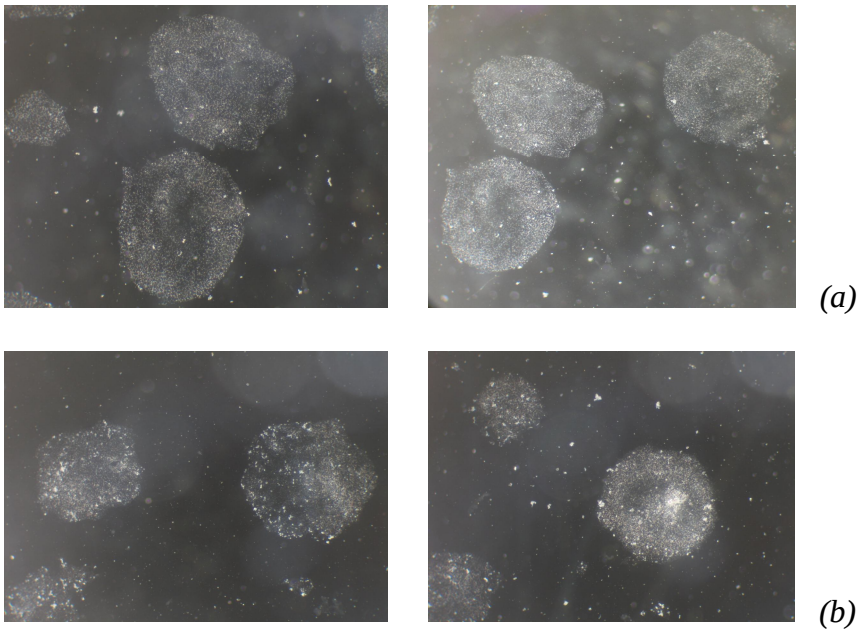
These results suggest that the shape of the hESC colonies acquired in culture depends on the type of supporting cells the cells actually were.

Most undifferentiated embryonic stem cells are characterized by high expression levels of stage-specific embryonic antigens (SSEA-1, 3 and 4), tumour rejection antigens (TRA-1-60 and TRA-1-81), OCT-4 and alkaline phosphatase, a marker of hESC pluripotency. Under our experimental conditions, hESC-H9 expressed alkaline phosphatase, which means that our cells were pluripotent (see Fig. 2).

b) ***The use of different media to culture hESCs under feeder-free conditions.***

We also assessed the appropriate condition for growing hESC under feeder-free conditions and using different media. One of the experimental approaches used was to grow cells in a conditioned medium from H9 growing on MEF since this medium contains all the essential factors needed for self-renewal and cells were also maintained in an undifferentiated state. However, one disadvantage of MEF (mouse embryonic fibroblasts) is that it contains animal-based ingredients, which may increase the risk of cross-transfer pathogens (Fig.3a). Therefore, in order to avoid this risk factor, we grew hESC on Matrigel (BD *Matrigel*^{TM}) under feeder-free conditions and cells were maintained in a serum-free medium containing high levels of bFGF, TGF β and GABA, pipecolic acid, lithium chloride (mTeSR^{TM}1) (Fig.3b). These trophic factors are crucial for the maintenance of human embryonic stem cells, as reported elsewhere

(Ludwig et al, 2006). However, H9 cells proliferate more efficiently when grown in the fibroblast-conditioned medium than with the commercial mTeSR^{TM}1 medium.



(a)

(b)

Fig.3. Feeder-free conditions in two different media. H9 were grown on Matrigel^{TM} in Conditioned Medium (CM) (a) and in the commercially available mTeSR1 medium (b).

Regarding the use of feeder-free conditions, one disadvantage was that the colonies formed were smaller than when H9 were grown on feeder layers. Therefore, in the experiments used in this study, we employed human fibroblast feeders to grow the hESC-H9.

4.2. Characterization of hESC-Derived Neural Progenitors Cultures

To prepare neural stem cells progenitors (hNP) from hESCs and undifferentiated hESCs (the H9 line, Fig.1), we allowed to form embryoid bodies (EBs). Specifically, hESCs-H9 were grown on HFF for 6-7 days, and when cells acquired the appropriate morphology and size, they were transferred to a low-attachment plate.

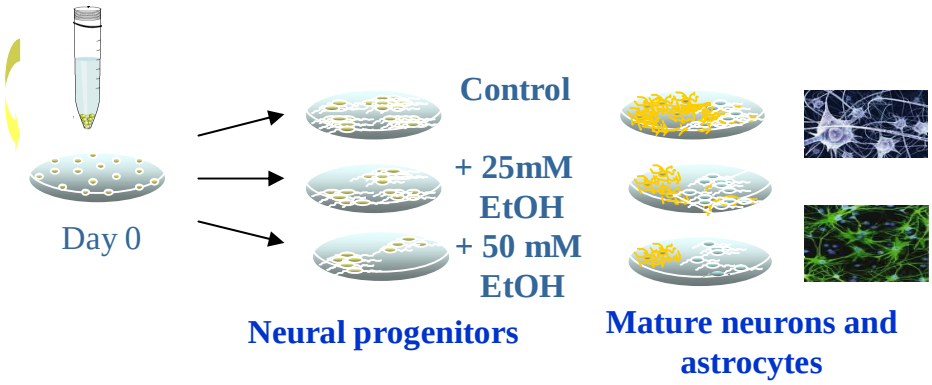


Fig. 4. Scheme of neural differentiation from hESC H9 towards mature neurons and glia. a: The first scheme demonstrates the entire process from hESC to differentiated cells. b: The second scheme demonstrates the phase on polyornithine/laminin-covered plates with three conditions (Ct, 25mM and 50mM).

The colonies formed started to acquire three-dimensional aggregates, called embryoid bodies (EBs), which contain the three embryonic layers (ectoderm, mesoderm and endoderm). The process took approximately six days to achieve mature EBs. For the first two days, floating colonies were grown in ES medium, then two days in

the Neural Induction Medium (NIM) and the ES medium, and finally for two days in NIM (see scheme, Fig. 4).

For neural induction, EBs were scattered and transferred to adherent conditions on Matrigel-coated plates and allowed to differentiate into neurosphere-like structures known as neural rosettes. The morphology of rosettes corresponds to tightly connected, radially spread columnar epithelial cells. Rosettes are neural tube-like structures that contain high levels of neuroprogenitors cells (Zhang et al, 2001; Perrier et al, 2004) (Figs. 5 and 6).

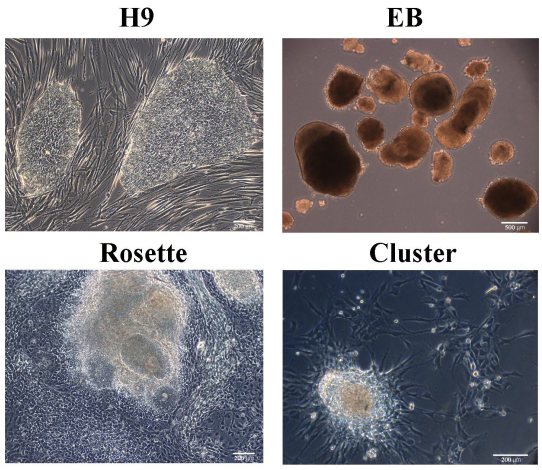


Fig.5. Summary pictures of Neural Differentiation from hESC.

For neural induction, EBs were scattered and transferred to adherent conditions on Matrigel-coated plates and allowed to differentiate into neurosphere-like structures known as neural rosettes. The morphology of rosettes corresponds to radially spreaded columnar epithelial cells tightly connected. After the dissociation of rosettes and

counting by a cell counter using Trypan Blue, cells were plated under adherent conditions and were cultured in a serum-free proliferating medium (PM) containing bFGF (20ng/μl). The number of cells was optimised from 250,000 to 350,000 for 2 ml plates, and approximately to 80,000 cells for the 4-well plates that were used in the experiment.Under these conditions, neural progenitors proliferate and form spherical aggregates or clusters (*Fig. 5*). On days 7-8, aggregates were dissociated; cells were counted again, and were split and re-plated with fresh media. This process was repeated up to 3 times (one/week). For three weeks of hNPs proliferation, cells initially adopted rosette-like structures (*Fig. 5*) and then, as the cell number increased, they formed aggregates or clusters displaying a radial glia array with long filament projections emerging from the central area to the peripheral area (*Fig. 5*). By day 21, clusters were dissociated; cells were cultured in differentiating medium (containing BDNF, 10ng/μl) for 15 days. Under these conditions, cells were allowed to differentiate into mature neurons, astrocytes and oligodendrocytes as a final stage of our differentiation process.

This protocol was simulated (*in vitro*) in the first trimester of human gestation, and proved a very important tool to investigate different genes and protein expression, as well as the neuroteratogenic effect of ethanol, which is one of the most abused neurotoxic agents.

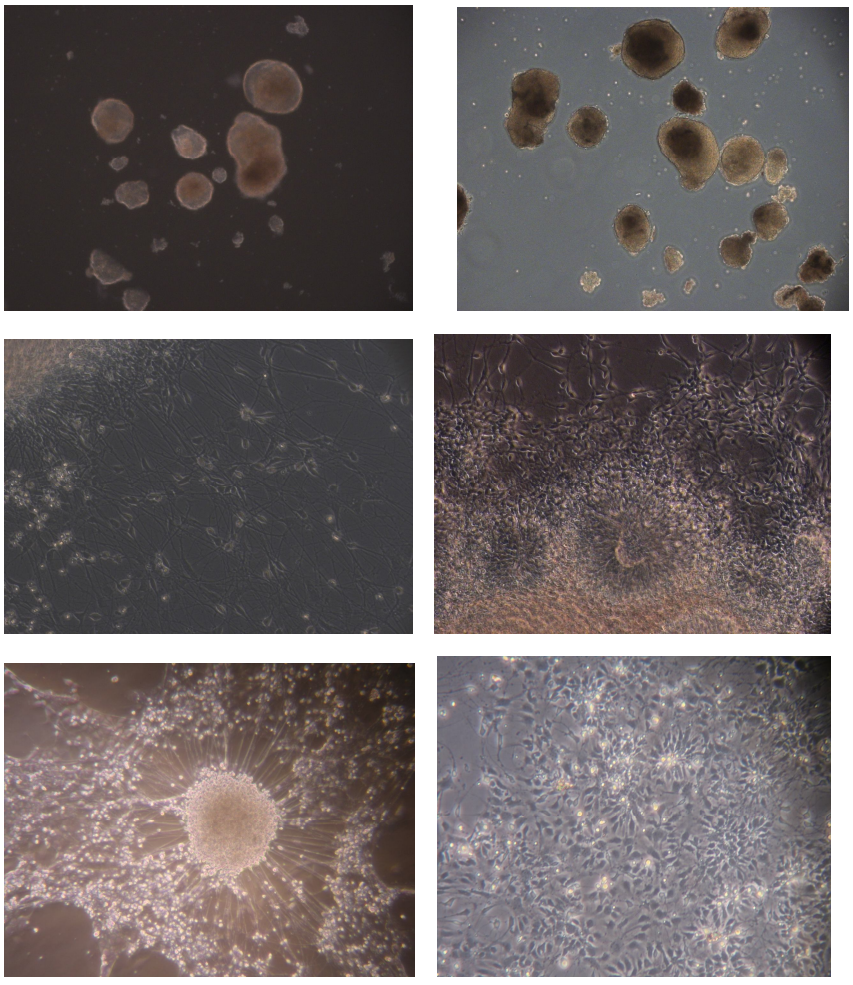


Fig.6. Embryoid bodies (EBs, three-dimensional aggregates representing three germ layers (a). Neural progenitors spreading out of the forming clusters and appearance of neural rosettes in the Matrigel stage, which is not typical for the phase (b). Neural progenitors with the typical cluster shape and with neural extensions, and the cell confluent stage phase (c).

Figure 6 shows different stages of the NPs-expressing GFP during proliferation. The pictures depict the NPs aggregates of immature neuronal cells, as well as the areas where cells spread out.

This figure (a) illustrates the diverse shape and size of EBs. In our experiments, we took EBs of a similar size and shape. This figure also illustrates (b) rosettes and clumps, which are not typical for this stage, although we also detected late rosettes with a lumen, as well as neurons with long extensions separated from attached EBs.

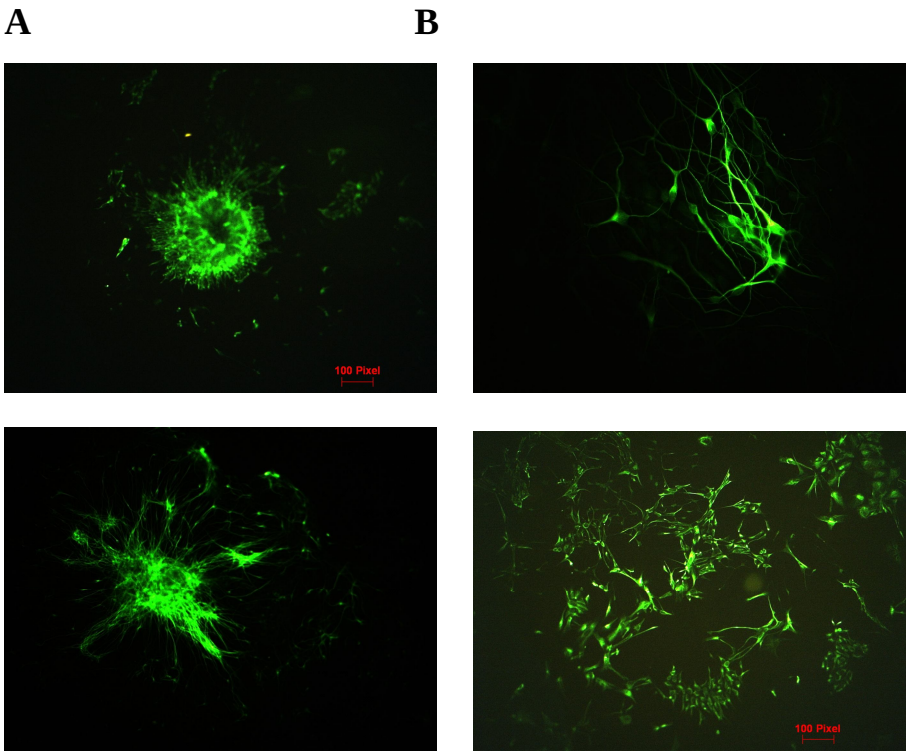


Fig.7. Neural progenitors cells 18 days after growing in NPM medium with bFGF on polyornithine/laminin-coated plates. Cells are with the GFP construct.

Furthermore, Figure 7 also illustrates a typical clump, which appeared during the proliferating period with long extensions around it. This type of cell distribution is typical after NPs platting on the polyornithine/laminin-coated plates. The right picture depicts the

confluent stage of NPs, prepared to be split to other new polyornithine/laminin-coated plates (4-well or 6-well). The passage of NPs usually occurred every 6-7 days after plating. Then cells were prepared to a new exposure to bFGF or in differentiating medium with BDNF (Ahmed S, 1995).

4.3. Differential expression of several genes during NPs proliferation and differentiation.

During the differentiation process from human embryonic stem cells, various genes and signaling pathways were involved. We therefore investigated the gene expression of the different markers of the three embryonic layers (ectoderm, mesoderm and endoderm), as well as the markers of neural progenitors.

An RT-PCR analysis (Figs. 8, 9) indicated that during the first week of culture, cells expressed Nanog (an hESC-specific marker of pluripotency), PAX-6 (a neuroectodermal marker, which is also a marker of neural progenitors) and α-fetoprotein (an endodermic marker). However, the expression of these genes markedly decreased or disappeared (e.g., α-fetoprotein) during the 3 weeks of proliferation, revealing a neuroectodermal linage-like trend towards the most possible pure neural line, leading to mature neuron and glial cells (Figs. 8 and 9). Lack of expression of Brachyury confirmed no mesodermal contamination.

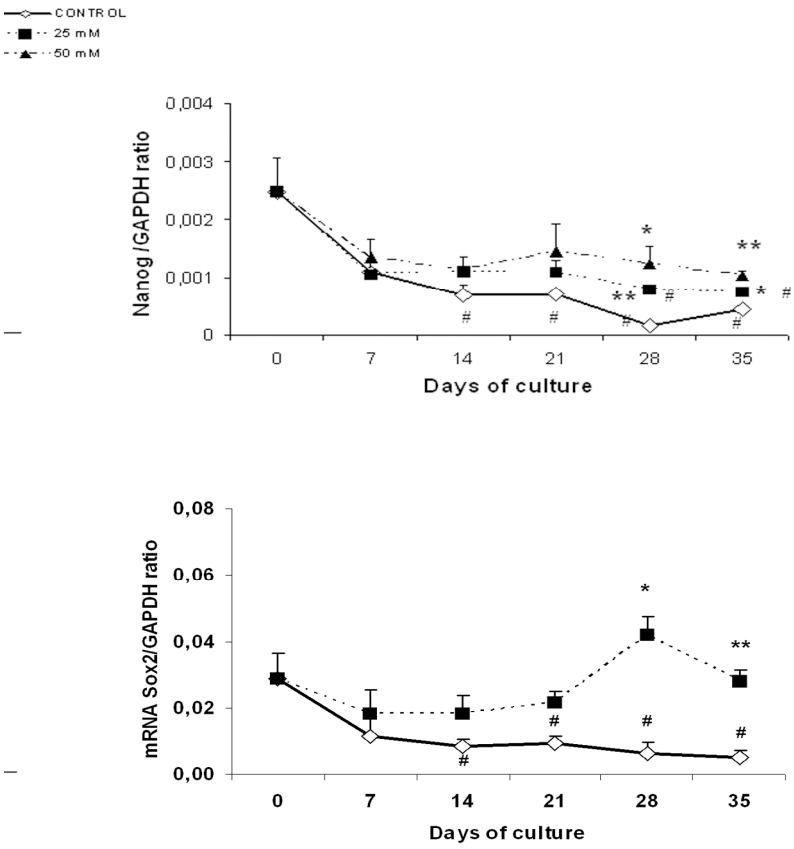


Fig. 8. The RT-PCR analysis of the gene expression NANOG, SOX2 of hNPs during their proliferation and differentiation in the presence or absence of ethanol (25 and 50mM . Data are average ± SD.

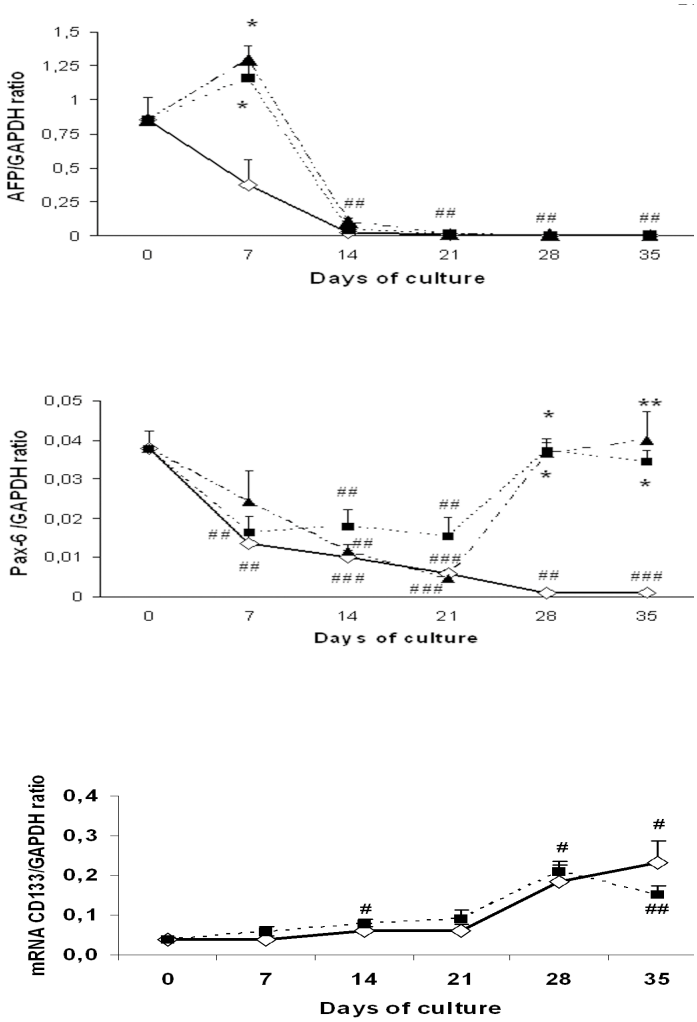


Fig. 9. The RT-PCR analysis of gene expressions of AFP, PAX6 and CD133 of hNPs during their proliferation and differentiation in the presence or absence of ethanol (25 and 50mM). Data are average ± SD.

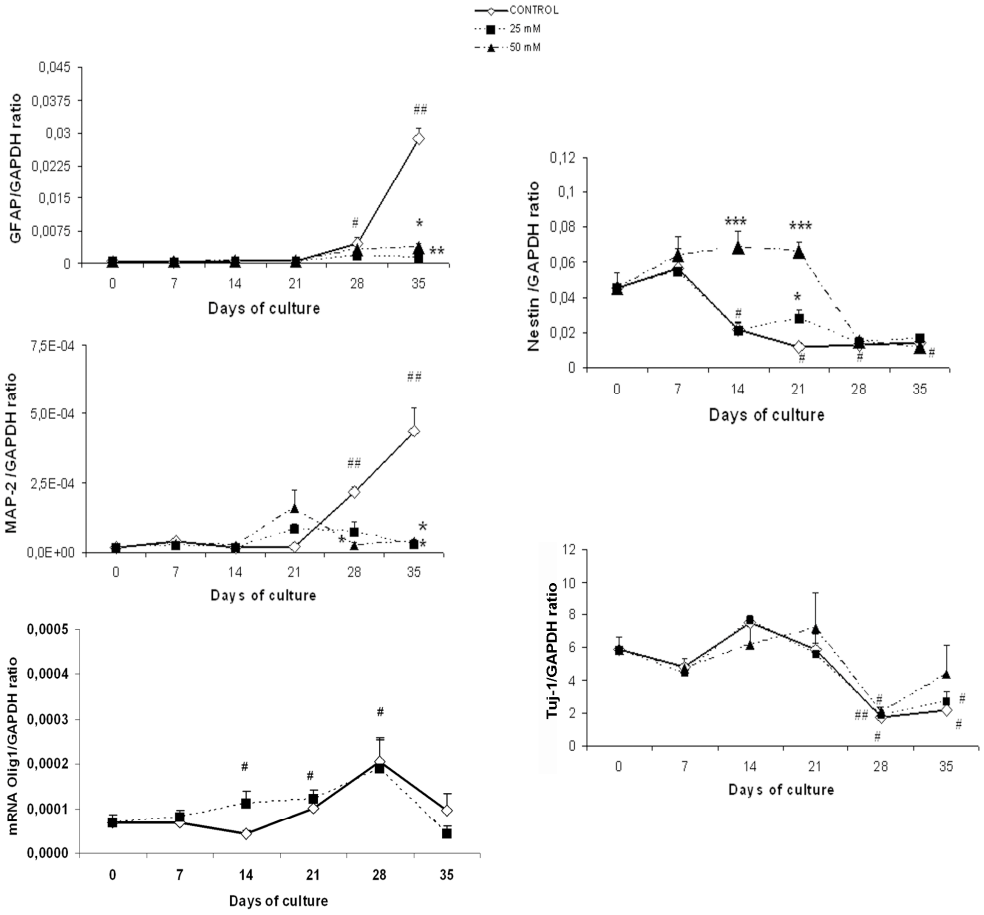


Fig. 10. The RT-PCR analysis of the gene expression of GFAP, MAP2, OLIG1, NESTIN, TUJ1 of hNPs, during the NPs proliferation and differentiation in the presence or absence of 25 and 50mM ethanol. . Data are average ± SD.

Furthermore, the expression of the neural-related genes revealed that, while neural precursors nestin and TUJ-1 were high

during cell proliferation and then decreased during cell differentiation (Fig.10), the levels of MAP-2 and GFAP were undetectable or very low during cell proliferation, but markedly up-regulated into mature cells during cell differentiating (Fig.10).

It is noteworthy that on day 14 of the proliferation stage, the levels of expression of nestin started to decrease, and this event parallels the up-regulation of the TUJ-1 expression, suggesting that neuronal precursors, including radial glia, are already transformed into neuronal precursor TUJ-1. These results indicate that under our experimental conditions, the NPs contained a relatively uniform population of forebrain progenitors, which can be differentiated from hESCs ,and that the temporal pattern of neurogenesis/gliogenesis from *in vitro*-produced human neural progenitors is preserved in our culture system.

4.4. Ethanol affects hNPs proliferation and rosette formation

Our previous studies have demonstrated that *in utero* alcohol exposure impairs the cell proliferation and self-renewal capacity of rat neural stem cells (Rubert et al., 2006). Therefore, to evaluate whether human NPs were sensitive to alcohol effects, we added ethanol (25 or 50 mM) to the medium and we assessed the potential actions of ethanol on hNPs during their proliferation and differentiation. One of the initial effects observed in ethanol-treated cells was a reduction in the size of the aggregates or clusters formed during cell proliferation *(Fig.11*).

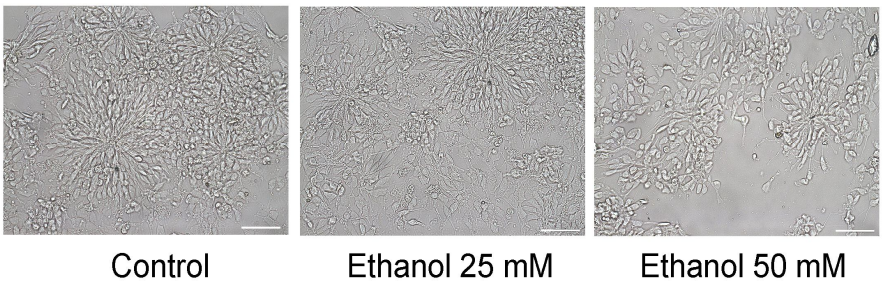


Fig.11. Morphological alterations in NPs exposed to ethanol

Two treatments with 25mM and 50mM ethanol clearly demonstrate a *reduction* in cell number in relation to untreated cells. We observed that ethanol significantly reduces the number of cells during the proliferation phase (Fig.11) and disrupts central aggregates or radially concentrated cells.

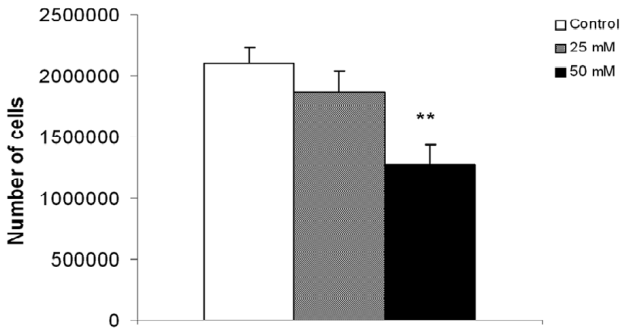


Fig.12. Number of cells in the control and ethanol- (25mM, 50mM) exposed cells. Data are average ± SD.

Quantification of the number of hNPs in clusters demonstrates that ethanol dose-dependently reduces NPs, although the values were only statistical significant in the 50-mM treated cells ($p < 0.01$, *Fig. 12*).

4.5. Ethanol impairs human neuroprecursors survival

To investigate whether the reduction in the number of cells occurs by impairment in cell proliferation or by increased cell death, or by both, we first incorporated BrdU into the control and the ethanol-treated cells.

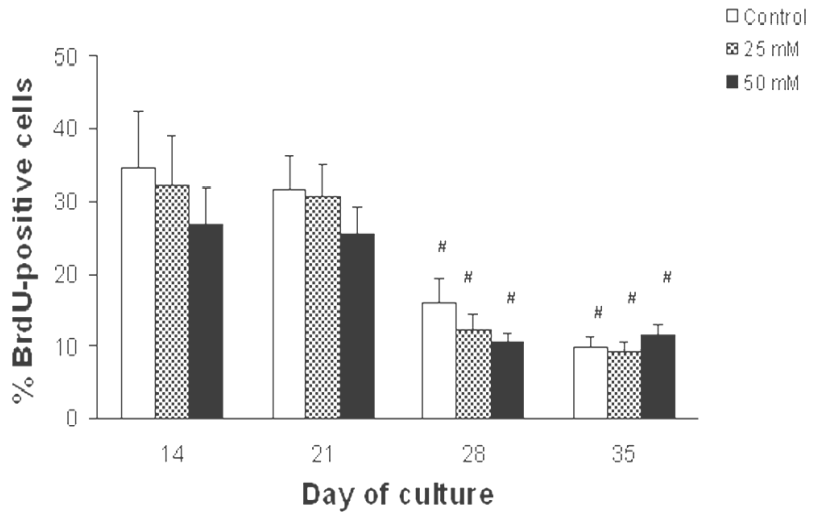


Fig. 13. Incorporation of BrdU and percentage of BrdU-positive cells at various stages of cell proliferation and differentiation in the control and in the alcohol-exposed (25 and 50 mM) cells. Data are average ± SD.

As expected, the percentage of incorporation of BrdU into the control NPs was higher during cell proliferation than during their differentiation (*Fig. 13*). The data in Figure 14 also reveal that ethanol treatment slightly reduced the incorporation of BrdU into hNPs, but this reduction was not statistically significant.

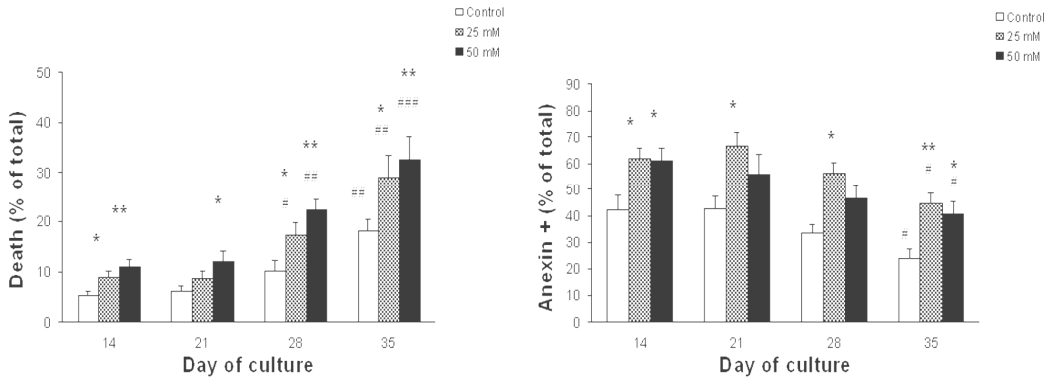


Fig.14. Incorporation of BrdU and the percentage of BrdU-positive cells at various stages of cell proliferation and differentiation in the control and in the alcohol-exposed (25 and 50 mM) cells. Data are average ± SD.

We then analyzed the potential effects of ethanol on cell survival. For this purpose, cells were labeled with annexin V-PE, a marker of early apoptosis stages (Herault, 1999). In addition, and in order to distinguish necrotic cells from apoptotic cells, the annexin V analysis was performed simultaneously with a dye exclusion stain, such as 7-amino-actinomycin D (7-AAD) (Herault, 1999; Lacoeur et al., 2002).

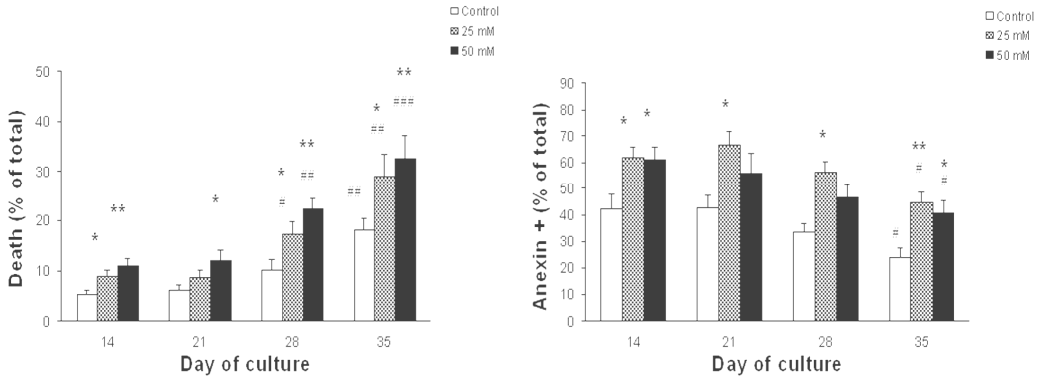


Fig.15. Percentage of death cells measured by the annexin assay (annexin positive cells) using R-phycoerytrin-conjugated annexin-V with cell-cell-impermeant DNA fluorophore 7-AAD.Control and alcohol-exposed (25 and 50mM) cells

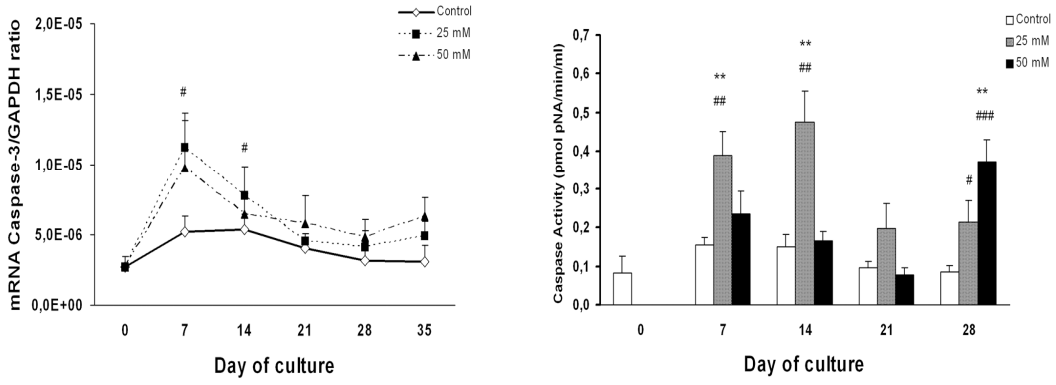


Fig.16.Temporal gene expression of Caspase-3 and Caspase-9 activity. Control and ethanol-exposed (25 and 50mM) cells

The flow cytometry analysis revealed that ethanol increased either the annexin+ population or the number of apoptotic cells (Fig.14).

Moreover, the data in Figure 15 also reveal that while ethanol at 25 mM mainly increased the annexin V+ cells, at higher concentrations, e.g., 50 mM, ethanol also triggered necrotic cell death, as shown by the increasing population of annexin V and AAD + (Fig.15). To confirm these data, an increase in nuclear fragmentation from alcohol-treated cells was also observed with the immunofluorescence studies. According to these data, 25 mM ethanol significantly up-regulated caspase 3 activity, while higher ethanol concentrations, e.g., 50 mM, increased caspase 3 only in the cell differentiation stage (days 28 and 35). Likewise, ethanol also up-regulated the mRNA levels of caspase 3 (Fig.16).

4.6. Ethanol affects the expression of human neurogenic progenitors and impairs their differentiation into mature neural cells

In order to gain additional insight into the potential actions of ethanol on hNPs during their *in vitro* proliferation and differentiation, we determined the expression levels of different gene markers. The data in Figures 8 and 9 illustrate that ethanol, at 25 mM and 50 mM, significantly increased the gene expression of AFP on day 7 of the proliferating stage, suggesting that ethanol affects loss of endodermal markers towards neural precursors. Furthermore, while the levels of transcription factors Nanog and PAX6 were down-regulated during hNPs differentiation, ethanol treatment abolished the down-regulation of Nanog and significantly increased the gene expression of PAX-6 (Fig. 9). An RT-PCR analysis also demonstrates that while ethanol

treatment significantly increased the expression levels of neural precursor Nestin, it reduced the up-regulation of both MAP-2 (a marker of mature neurons) and GFAP (an astrocytes marker), as observed during cell differentiation in the control hNPs. These results suggest that ethanol impairs the transformation of hESC into NPs and mature neural cells, causing an up-regulation of neural precursors (e.g., nestin) and decreasing the expression of genes of mature neurons and astrocytes.

4.7. Morphological characterisation of the human neural precursor culture and its differentiation: effects of ethanol.

An immunocytochemical analysis of hNPs colonies demonstrates that on days 7 to 14 after plating, the majority of neural progenitors form rosette–like pattern structures (Figs. 5 and 6) and then spherical clusters (Figs. 5 and 6), which were mainly composed of a central population of tightly packed small cells forming round structures, along with a flat epithelial-like peripheral population which migrated outwards from the cluster (Figs. 5 and 6). The double immunostaining performed for nestin and TUJ1 reveals that by day 7, about 76% of the epithelial-like peripheral and central cells were positive for nestin (76 ± 15%), while 34 ± 12% of the cells with a unipolar and bipolar morphology on the cell periphery expressed TUJ-1.(Fig.17D).

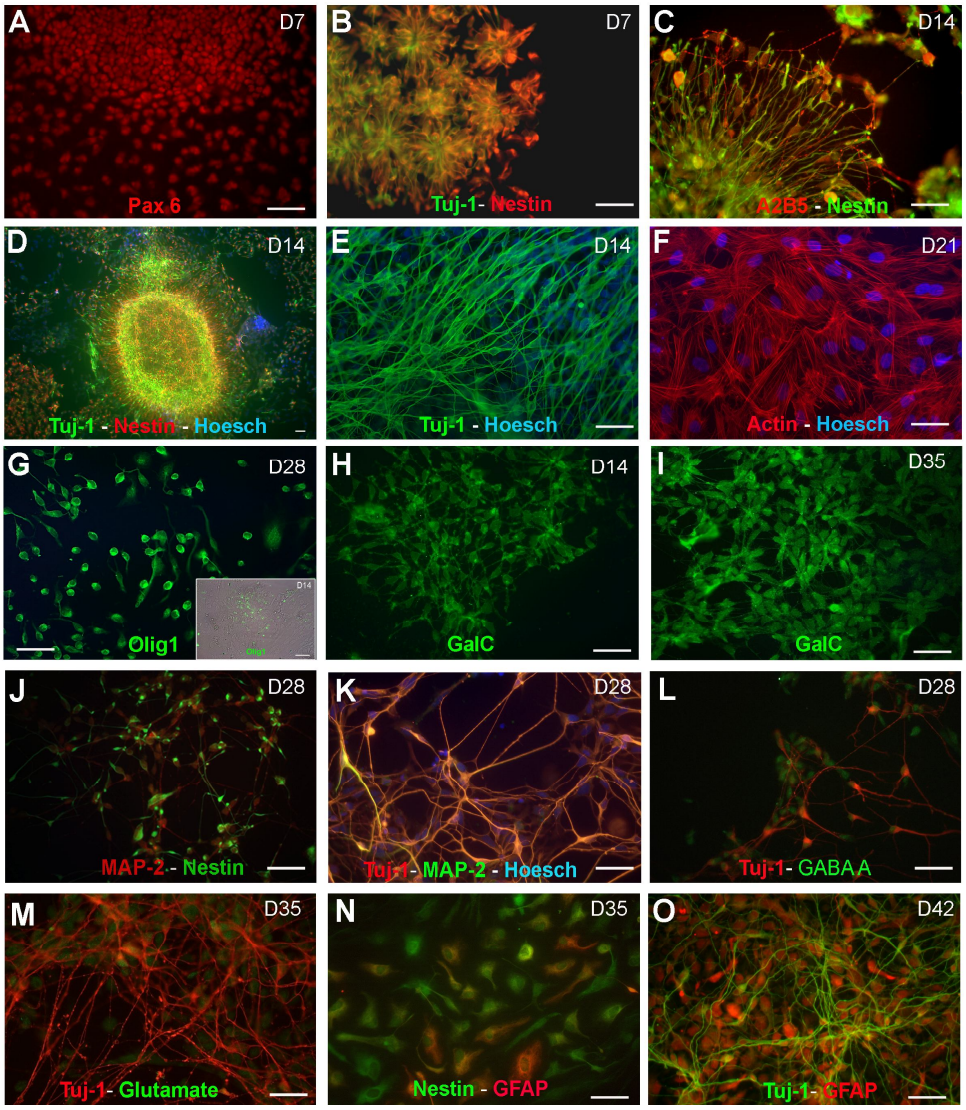


Fig.17. Immunocuytochemical characterisation of hNPs.

At this stage, ~ 80% of cells expressed Pax-6 (Fig. 17A). On day 14, the nestin+ cells showing a RG morphology decreased (43 ±

10%), while the TUJ1+ cells displaying dendritic arborizations and markedly increased (58 ± 13%) (Fig.17E).

On days 14 and 21, 41 ± 17% of cells expressed nestin, while 60 ± 14% were Tuj1+. We were unable to detect glial fibrillary acidic protein (GFAP) and MAP-2 positive cells during cell proliferation. These findings agree with the data obtained by RT-PCT (Fig.10). The immunocytochemical analysis also revealed that during cell differentiation (day 28), the cells which were initially organised as aggregates, displayed more complex dendritic arborizations and that they were stained with nestin and MAP-2, as well as TUJ-1 and MAP-2 (Fig. 17). Some TUJ-1-positive neurons were also immunoreactive for GABA-A^+ (Figure 11-I) and glutamate (Figure 17). During prolonged maintenance, and specifically on day 35, about 15 ± 9% of cells were immunoreactive for the astroglial marker glial fibrillary acidic protein (GFAP) (Figure 5K). On day 42, the percentage of astrocytes (GFAP+) was 20 ± 12% and appeared at the bottom of the MAP-2 positive neurons (Fig. 17).

Ethanol exposure markedly affected the morphology profile of NPs cells. Thus, Figure 11 shows that ethanol altered the morphology of the rosette-like structures (Fig.18) and lowered the number nestin- and TUJ-1-positive cells, forming spherical aggregates during the proliferation stage (days 7-21) (Fig. 18).

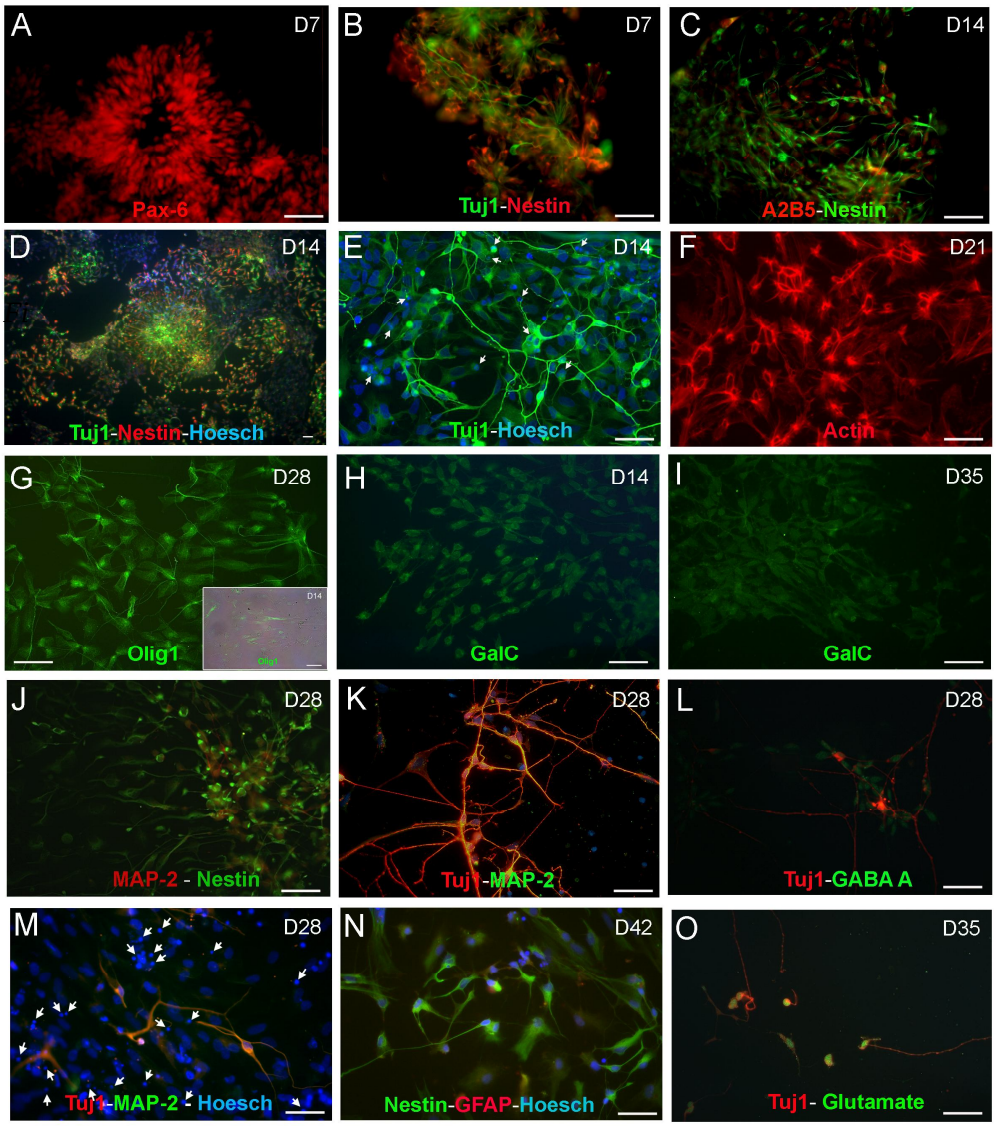


Fig.18. Immunocuytochemical characterisation of hESC – derived neural precursors. Effects of ethanol (25 and 50mM).

However, although ethanol significantly reduced the number of cells, the percentage of the cells expressing nestin and Tuj1 at the different time points of proliferation was similar to that of controls.

Nevertheless, although the number of cells was significantly reduced by ethanol, the percentage of cells expressing nestin and Tuj1 at the different time points of proliferation was similar to that of controls. Furthermore, a striking effect of ethanol was seen, which consisted in an alteration in cell morphology and cytoskeleton changes.

Cells immunoreactive for Nestin (type VI, Intermediate filament) and Tuj1 (Class III, β Tubulin Isotype) displayed shorter processes and abnormal filaments (Fig.18 D, E, H, K). In addition, while actin filaments were organised into stress fibres as bundles running throughout the cytoplasm in the control cells, ethanol exposure markedly disrupted actin organisation by changing stress fibres into actin rings on the cell periphery (Fig.18 F). These effects were more marked in the NPs exposed to 50 mM than those exposed to 25mM. Furthermore, during cell differentiation, ethanol reduced the percentage of cells expressing MAP-2. Thus, while 21 ± 10% of the control cells expressed MAP-2, only 15 ± 7% of the alcohol-exposed (50 mM) cells were MAP-2+ on day 28 of the culture. Similarly, immunoreactive GABA-A and glutamate neurons were also slightly reduced in the alcohol-treated NPs. Likewise, whereas 20 ± 12 % of the control cells were immunoreactive for GFAP on day 42, in the ethanol- (25 and 50mM) treated cells, only 5% were GFAP+.

Taken together, our findings demonstrate that ethanol not only affects the proliferation of NP cells, but markedly reduces their differentiation of NPs into mature neurons and astrocytes. The results also indicate the disruption of several cytoskeletal proteins by ethanol

and suggest that changes in these cytoskeletal proteins might underlie the alterations in cell shape and the functions of the NPs exposed to ethanol (Luo, 2002).

4.8. Expression of new genes in hESCs and human neuroprogenitors.

On the basis that neural human NPs cells derive from hESCs and given an *in vitro* protocol for neural induction, the proliferation and differentiation used in the present study can mirror early forebrain neural development. Thus, we evaluated the expression of new genes, a situation which has only been described in rodents during neural differentiation.

4.8.1: Expression of the endocannabinoid system in hNPs

Cannabinoid receptors are part of the endocannabinoid system, which is comprised of cannabinoid receptors, endogenous cannabinoids (endocannabinoids), and the enzymes that synthesise and degrade endocannabinoids. Endocannabinoids are involved in a wide variety of physiological and pathophysiological processes. Recent studies with rodents have demonstrated the presence of a functional endocannabinoid system in neural progenitor cells that participate in the regulation of cell proliferation and differentiation (Aguado et al., 2005, 2006, 2008). We therefore wondered whether the EC system was also expressed in human NPs.

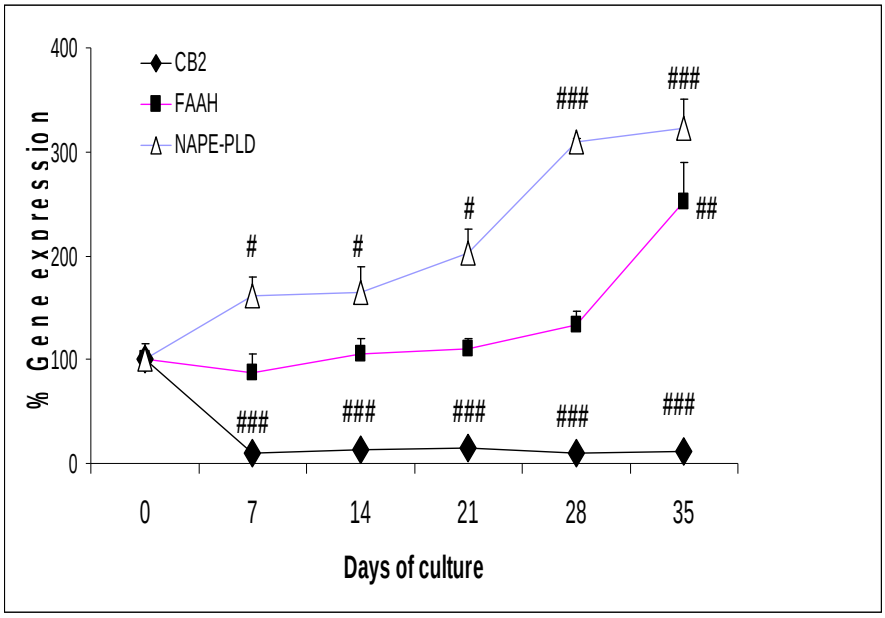


Fig.19.A quantitative PCR (qPCR) analysis of CB2, FAAH and NAPLD receptors during NPs proliferation and differentiation. Data are average ± SD of five different cultures(#p<0.5,##p<0.01,###p<0.001).

For this purpose, the expression of the transcripts of the EC receptors (CB1 and CB2), along with the enzymes involved in EC synthesis (NAPE-PLD) and degradation (FAAH), were assessed. As Figure 19 illustrates, CB2 decreased from day 0 to day 7, and was then maintained with a very low expression during cell proliferation and differentiation. No significant changes were noted in the CB1 expression (data not shown). Conversely, a progressive increase in

NAPE-PLD was observed throughout the culture time, which reached maximal levels during differentiation. The FAAH expression also significantly increased on day 35 when the maximal NAPE-PLD expression was observed. These results suggest that endocannabinoids are present during the early human neural development stage and may play a role during neural differentiation into neurons and astrocytes. Ethanol exposure did not significantly affect endocannbinoids during hNPs proliferation and differentiation (Fig.19)

4.8.2: TLRs expression in human embryonic stem cells and in hNPs during the proliferation and differentiation stages

Toll- like receptors are transmembrane proteins, which were described for the first time in Drosofila melanogaster, which control the developmental process in dorso-ventral patterning (Valanne S., 2001). Until recently, it was thought that TLRs were involved only in immune responses, but new findings suggest that TLRs signalling may influence multiple dynamic processes in the developing and adult central nervous system, including neurogenesis, axonal growth and structural plasticity.

We therefore evaluated whether TLRs, particularly TLR4, were expressed in human ESCs and in hNPs during their differentiation to mature neural cells. The RT-PCR analysis demonstrates (*Fig.20)* a lack of the TLR4 gene expression in hESC-H9. However, the expression of these receptors was detected in EBs, a structure containing the three germ layers (ectoderm, mesoderm and

endoderm); however, the higher gene expression was obtained in hNPs during their proliferation and differentiation.

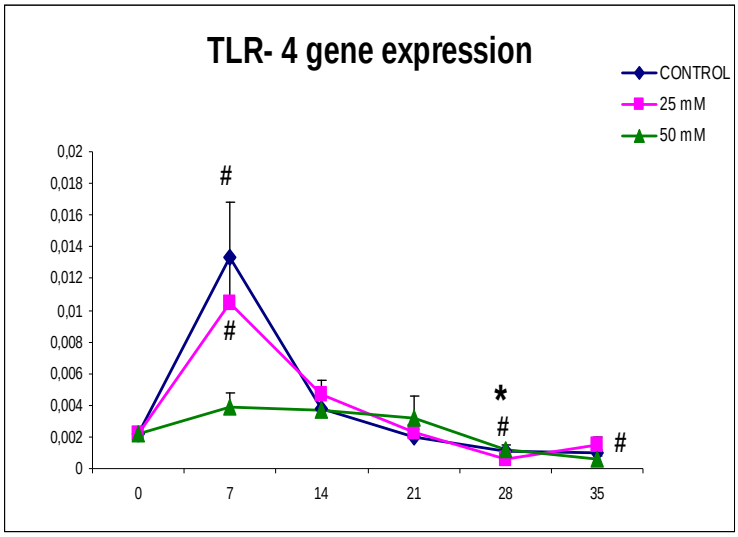


Fig.20. The quantitative PCR (qPCR) analysis of the TLR4 receptor during hNPs proliferation and differentiation control and after 25 and 50mM ethanol exposure. Data are average ± SD of five different cultures ($p<0.5$).

Thus, although we observed that TLR4 is expressed on D0 of hNPs differentiation (Fig. 20), a maximal expression was observed on D7 of NPs proliferation, decreasing thereafter during NPs differentiation (D21-D35).

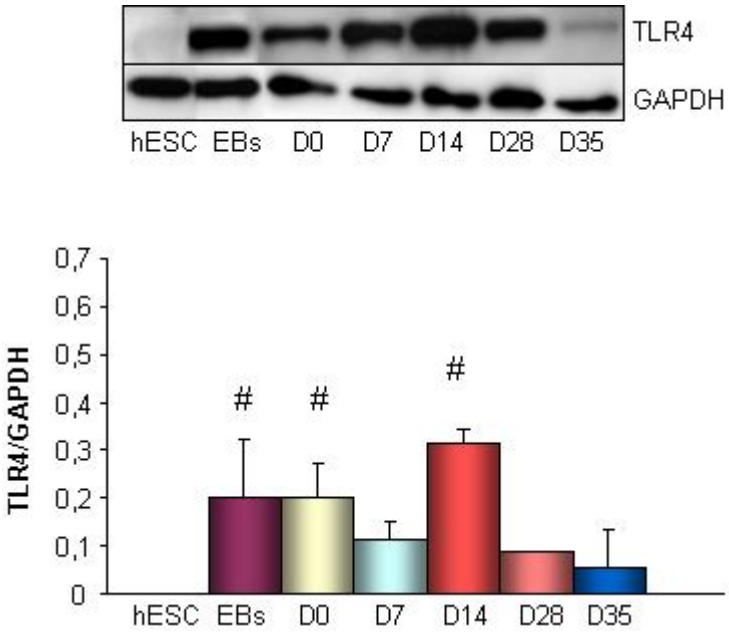


Fig.21. The Western blot analysis of TLR4 in hESC, EBs and NPs during their proliferation (D0-D14) and differentiation (D28-D35). The densitometric analysis of the protein levels of TLR4/GAPDH. Data are average ± SD of three different cultures ($p<0.05$).

We also measured the effects of ethanol on the TLR4 mRNA expression. Although no significantly major changes were noted during NPs differentiation, ethanol significantly reduced the TLR4 mRNA levels on D7 of NPs proliferation and also induced some changes on D20 and D25 of NPs differentiation (Fig. 20).

We next assessed whether the protein expression of TLR4 was also detected in hESC-H9, EBs and hNPs. The Western blot analysis demonstrated that whereas TLR4 was not expressed in H9 cells, the

levels of this protein were markedly expressed in both EBs and NPs (Fig. 21).

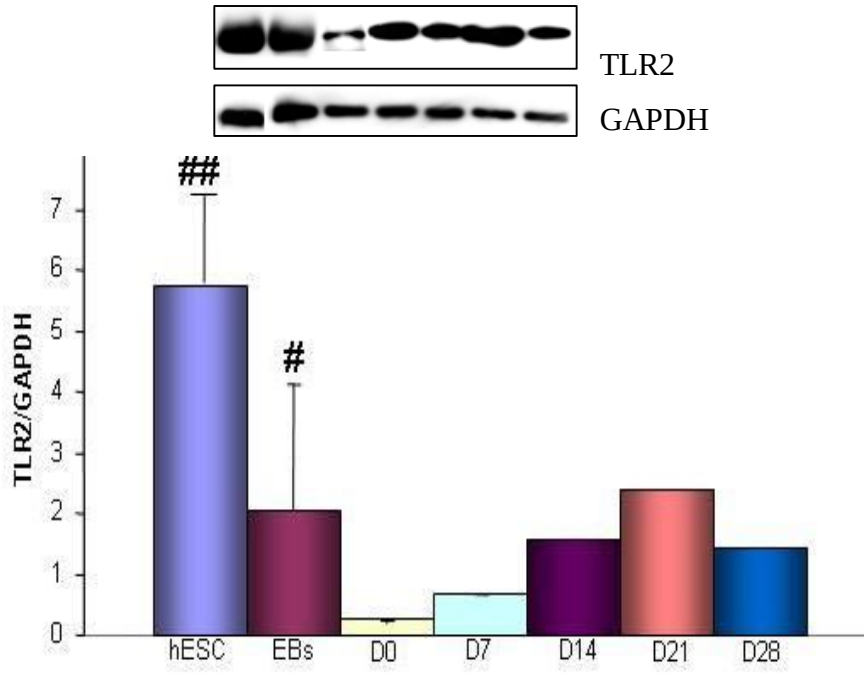


Fig.22. The TLR2 protein expression in hESCs and hNPs during NPs proliferation and differentiation. The blots were densitometrically quantified and the results were expressed as levels of the TLR2/GAPDH ratio. Data are average ± SD of three different cultures (#p<0.05, ##p<0.01).

One notable finding was that the levels of TLR4 increased during NPs proliferation and they peaked on day 14 of NPs proliferation, decreasing thereafter during the differentiation stage (D28-D35) (Figure 21). These results agree with the data obtained by RT-PCR on the gene expression of TLR4 in both hESCs and NPs.

To further evaluate the expression of TLRs in hESCs and NPs, we assessed the protein expression of TLR2, another important receptor in the innate immune system. The Western blot analysis demonstrated a very high expression of TLR2 in hESC-H9 (Fig. 22).

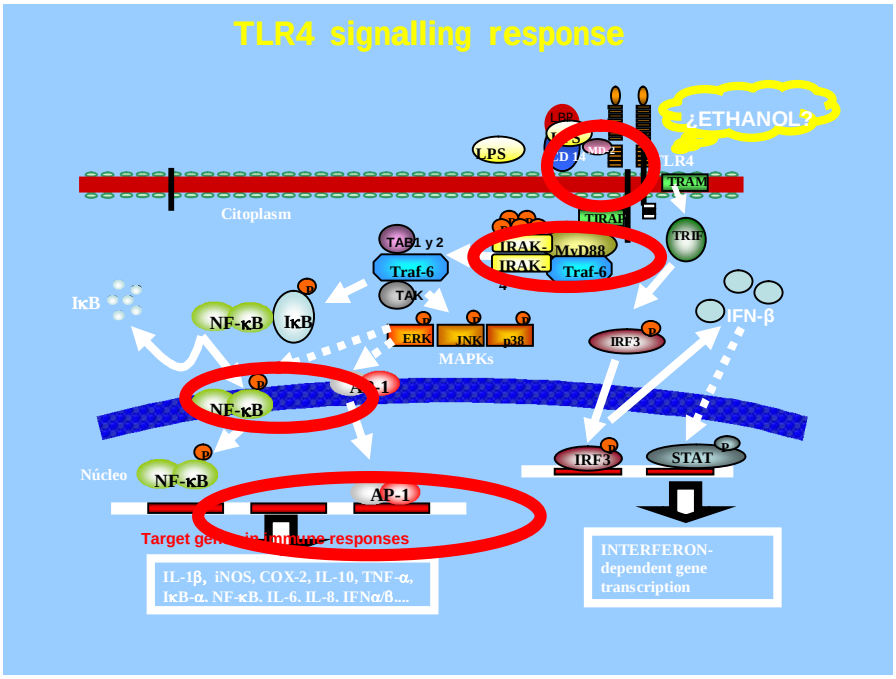


Fig.23. The TLR4 signalling pathway. Activation of TLR4 by its ligands, LPS, leads to downstream signalling molecules (MyD88, Traf-6, IRAKs) to form complex I, which activates complex II formation (Traf-6, IRAKs, TAB-1, -2 and TAK-1) and culminates in the activation of MAPKs and transcriptional factors (NF-kappaB and AP-1) to regulates the expression of the target genes in the immune response. Ethanol can activate TLR4 signalling

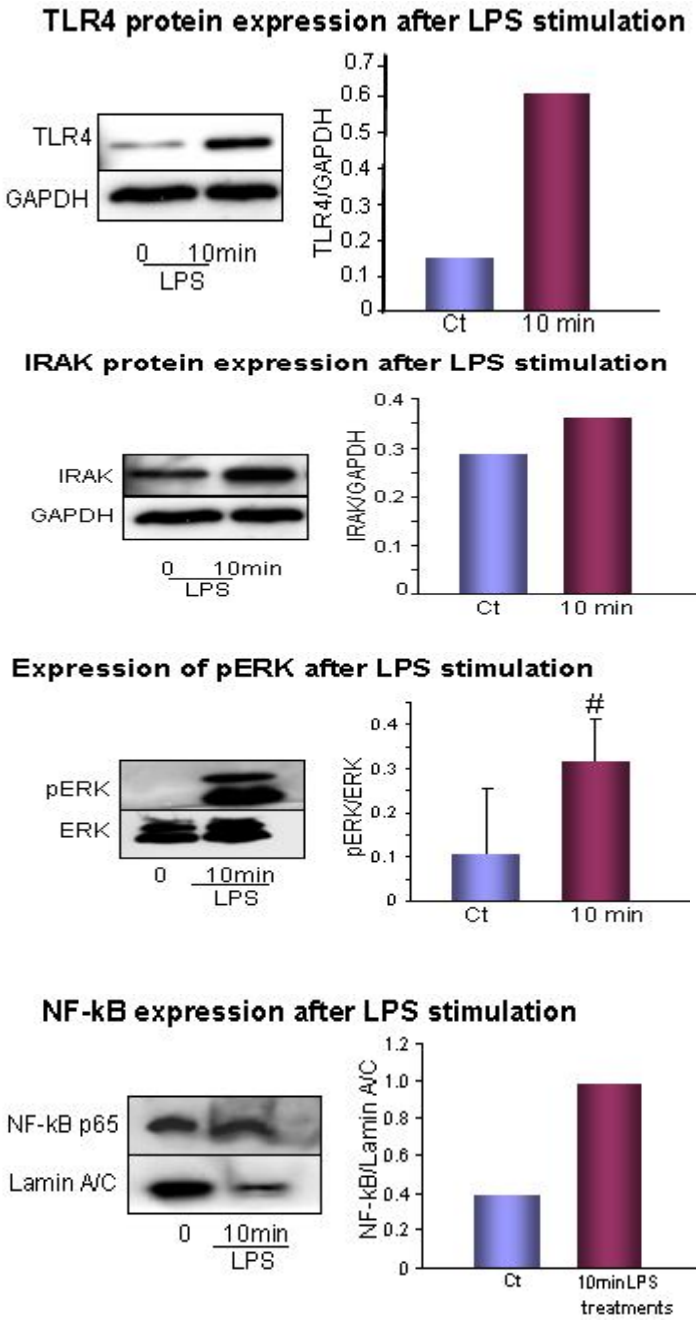


Fig.24. Stimulation of NPs with LPS increases the TLR4 expression and triggers the activation of IRAK and ERK upon 10 min of stimulation, and of NFkB upon 30 min of LPS treatment.

The expression of TLR2 was also detected in both EBs and NPs during their proliferation and differentiation (Fig. 22). Low levels of NPs were observed on D0, then TLR2 increased on days 14 and 21 of NPs proliferation, to then decrease on D28 of NPs differentiation

The expression of the TLRs in NPs raises the question as to whether these receptors are functional. As mentioned above, Toll-like receptors (TLRs) belong to a family of pattern-recognition receptors in the innate immune system. These receptors enable an innate immune recognition of endogenous and exogenous prototypic ligands, and an orchestrated innate and adaptive immune response to infection, inflammation and tissue injury. TLRs response involves the recruitment and activation of complex intracellular signaling cascades, which culminate with the induction of cytokines and other inflammatory mediators. Among these receptors, TLR4 was stimulated by endotoxin LPS, leading to the activation of different kinases, including IRAK (interleukin receptor 1 activated kinase) to trigger a fast downstream stimulation of nuclear factor-κB (NF-κB) and the induction of the genes encoding inflammation-associated molecules and cytokines, as well as the activation of mitogen-activated protein kinases (MAPK) and AP-1 nuclear factor (see Fig 23).

Therefore, in order to assess whether the expression of TLR4 in hNPs was a functional receptor, hNPs on day 15 of proliferation were stimulated with lipopolysaccharide (LPS, 50 ng/μl) for 10 and 30 min. Activation of IRAK, ERK (signal-regulated kinase) and NFkB was assessed. Figure 22 shows that LPS stimulation triggered the

phosphorylation of IRAK and ERK at 10 min, and also activated the nuclear translocation of NFkB at 30 min upon stimulation (Fig. 27). These results suggest the functional role of TLR4 in hNPs.

4.9. Developmental changes of TLR4 and TLR2 protein expressions during mice brain ontogen: effects of ethanol exposure.

In order to gain further insights into TLR4 and TLR2 during brain development and since hNPs provide us with only some information about the expression of TLRs during early embryogenesis we evaluated the expression of TLR2 and TLR4 in

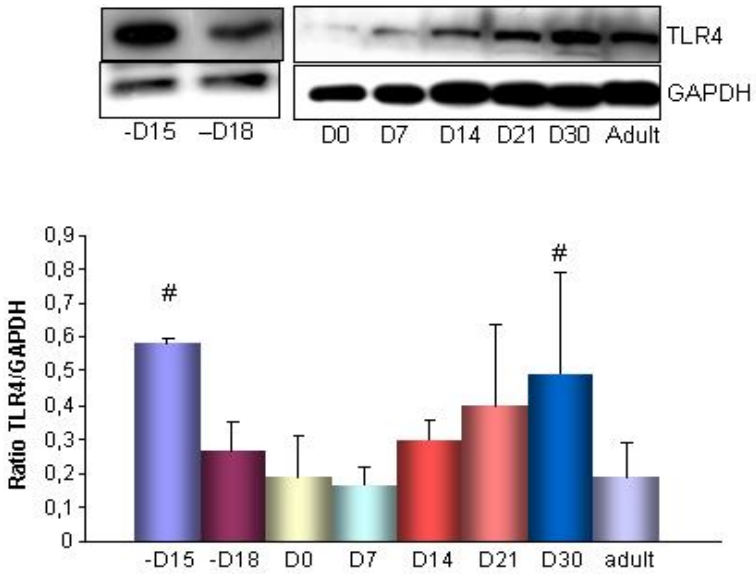


Fig.25. Changes in the TLR4 protein expression in mice cerebral cortices of foetuses and pups during the different stages of brain development. Gestation days 15 and 18 (-D15, -D18), and pups on days 0, 14, 21 and 30 of the postnatal period. The relative expression of TLR4 in the cortices of adult animals is also shown. Data are average ± SD of three or more different animals ($p<0.05$).

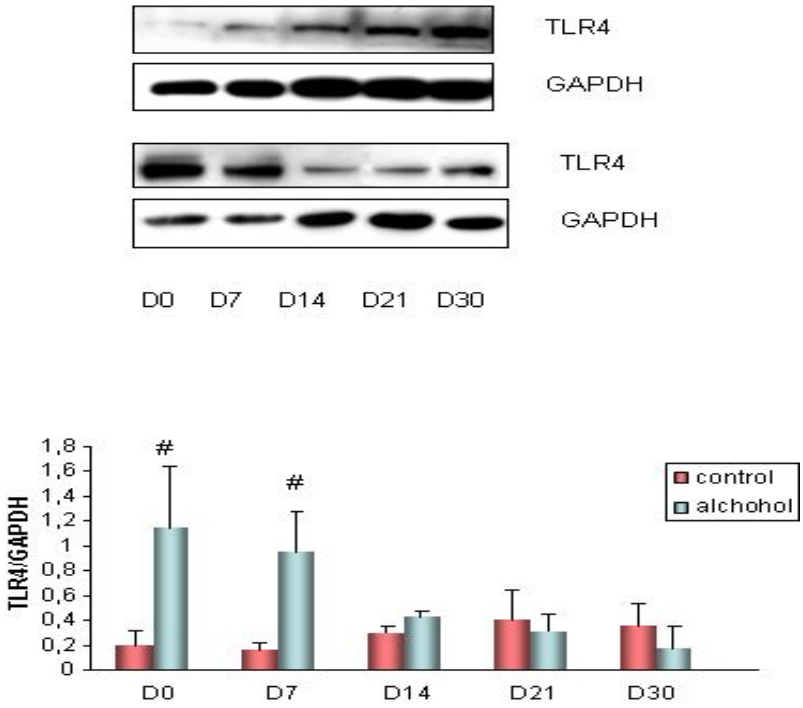


Fig.26. Changes in TLR4 in the cerebral cortices of the control and ethanol-exposed pups during postnatal development. Data are average ± SD of three or more different animals ($p<0.05$).

mice brains during prenatal and postnatal brain development. In addition, to assess the potential action of ethanol on TLRs during development, we also used fetuses and pups of alcohol-fed mice during gestation and lactation.

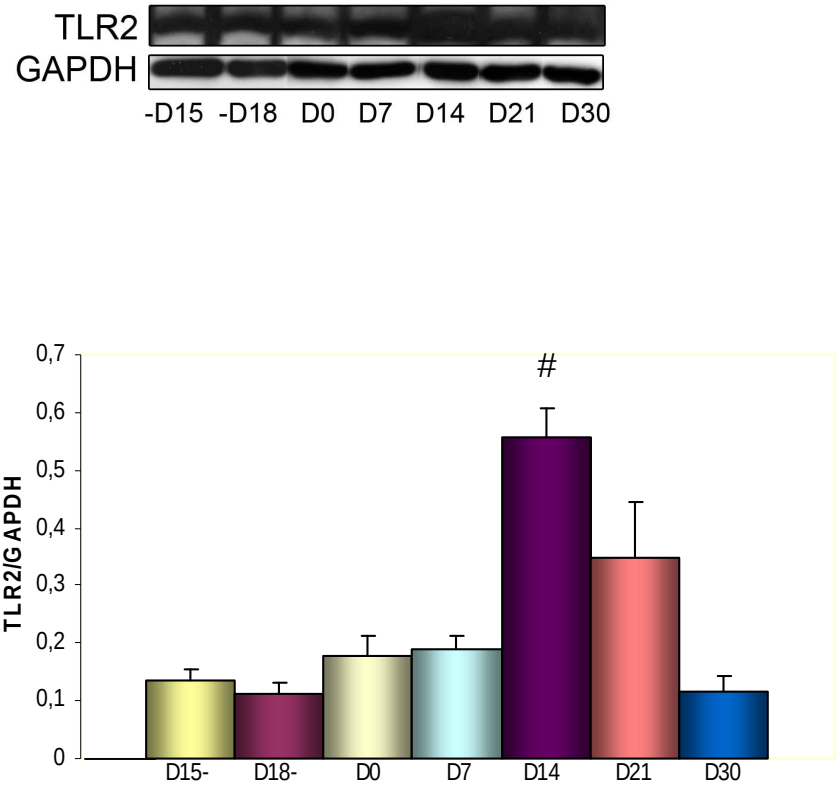


Fig.27. Changes in the TLR2 (TLR2/GAPDH) protein expression in cerebral cortices during mice brain ontogeny. Gestation days 15 and 18 (D15-, D18-) or postnatal period days 0. 7, 21 and 30. Data are average ± SD of three or more different animals ($p<0.05$).

Analysis of the TLR4 expression demonstrates that whereas TLR4 was highly expressed during embryonic development (foetal days 15 and 18), its expression lowered in the cerebral cortices of newborn mice to then increase during the postnatal period (Fig. 25).

Interestingly, alcohol exposure increased TLR4 levels during the early postnatal stages (days 0 and 7) and then slightly lowered the TLR4 levels on postnatal days 21 and 30 (Figure 26). These results

suggest that alcohol either up-regulates TLR4 during the neonatal period or delays the initial maturation of the cerebral cortex.

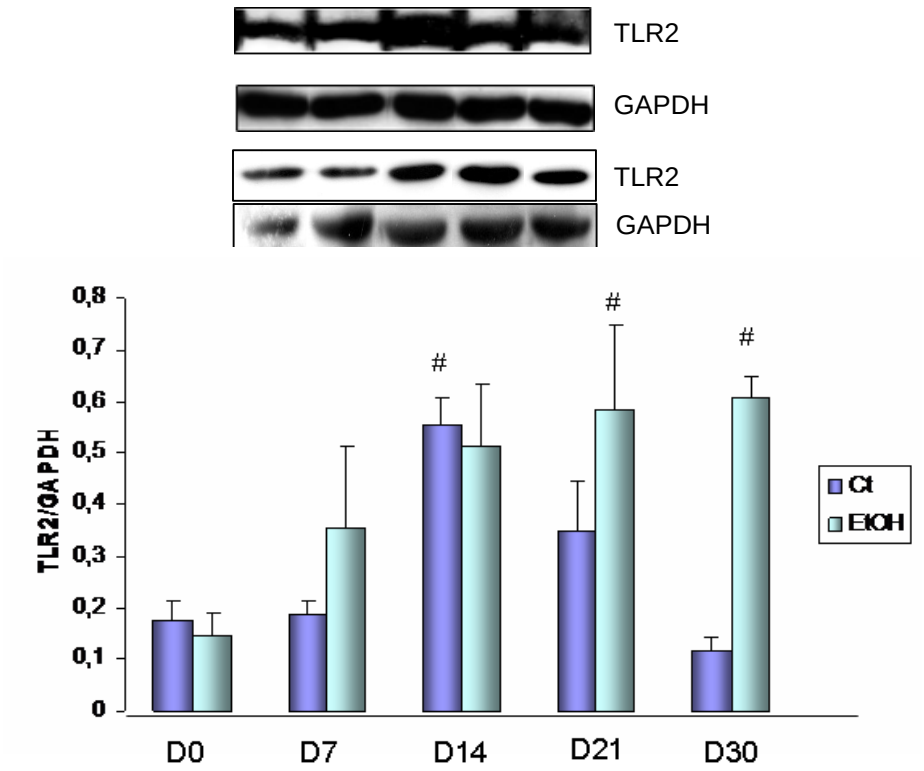


Fig.28. Effects of ethanol exposure on TLR2 in developing mice. Data are average ± SD of three or more different animals ($p<0.05$).

Concerning TLR2, the expression of this receptor was relatively high and remained constant during the prenatal and postnatal periods. Indeed, the levels during the prenatal and postnatal periods did not show statistically significant changes, except for a significantly high level which was noted on day 14 after birth, which

slightly increased on postnatal day 21 (Fig. 27). Unlike the effects of ethanol on increasing the TLR4 expression only during the neonatal period, ethanol exposure up-regulated the TLR2 levels only at the end of the postnatal period, on days 21 and 30 (Figure 28). Different functions of these receptors during postnatal development might explain these differential effects.

TLR2 in female mice treated with 60 % of ethanol shows a different pattern in receptor expression demonstrating increasing level in relative expression of the receptor (Fig.31). The lowest level of the TLR2 we noticed at day of birth and during development was TLR2 reached the highest expression (30 days old mice).

----------DISCUSSION----------

Discussion

5.1.Human Neuroprogenitors (hNPs) derived from human embryonic stem cells (hESC)

Derivation of human neuroprogenitors (hNPs) from human embryonic stem cells (hESC) is of value in the study of early human neurogenesis and in the creation of unlimited sources of donor cells for neural transplantation therapy. In this work, we have characterised and established an *in vitro* culture protocol that allows differentiation of hESC towards human neural precursors (hNPs). The protocol employed has been modified by a previous culture protocol (Nat et al, 2006). In our study, we were able to differentiate hNPs into radial glia (RG), neurons, astrocytes and oligodendrocytes (Carpenter et al, 2001; Kiersted et al, 2005), as demonstrated by the gene expression of different neural cell markers and by immunocytochemistry analysis.

We initially tested different conditions for growing hESC. In particular, hESC were grown in three different feeder conditions: human foreskin fibroblasts (HFF), mouse fibroblasts (STO) and under feeder-free conditions on Matrigel. We demonstrated that the hESC growing on human or STO exhibit typical markers of pluripotency (SSEA-3, SSEA-4, TRA-1-60 and TRA1-81) (Park et al, 2003), normal karyotype, Oct4 and alkaline phosphatise (AP), as demonstrated in previous studies (Park et al, 2003). However, when hESC were grown under feeder-free conditions and maintained on Matrigel, as a protein supporting the cells in mTESR1 (Ludwig et al,

2006; Ludwig et al, 2006), hESC grew less efficiently than in fibroblast feeders. In fact, it is known that fibroblasts support hESC and that they confer them growth factors, adhesion molecules and cellular matrix production. These conditions represent a hESC *in vitro* niche. More recently, hESC culturing moves towards biomaterial supported feeder-free conditions, which are also free of foreign antigens and proteins that could have important applications in clinical and biotechnological studies. For example, they use a polyamide-based 3D nanofibrillar porous matrix, which enhances self-renewal and the proliferation of hESC in comparison with culture dishes. A synthetic polymer matrix has also been described as a cell culture substrate, which could provide a completely defined microenvironment for hESC (Zhang et al., 2011). One disadvantage of feeder-free conditions is that embryonic stem cells give thinner colonies, although this approach offers the advantage of the animal component being absent, which is more suitable and precise for any analysis type and potential clinical use (Ludwig et al, 2006).

Human ESC can be maintained without forming monolayer culture systems since they are propagated as floating aggregates. Under such conditions, hESC form aggregates and embryonic bodies (EBs), they express pluripotency markers and they can differentiate towards the three germ layers *in vivo* and *in vitro* (Steiner et al, 2010; Stojkovic et al, 2004). This procedure is the most widely used method for inducing hESC. Usually, EBs aggregates are exposed to mitogens to be then dissociated and reattached to specific subtracts in order to provoke proliferation and differentiation to specific lineages.

For the neural linage, several methods have been developed to avoid endodermal and ectodermal contamination. For example, Ying and collaborators (2003) identified neural precursor populations and eliminated non-neural populations. However, the most widespread protocol for neural differentiation is the spontaneous formation of three-dimensional aggregates EBs. EBs recapitulate the processes occurring *in vitro* in early embryogenesis, with the appearance of lineage-specific regions similar to formation in early embryos. After 4-6 days in suspension, the EBs from hESC start to differentiate towards epiblast-like cells on the surface of the one EB representing primitive endoderm cells. Culturing EBs for several days results in differentiation into ectoderm, mesoderm end endoderm. This pattern could be directly translated to events occurring in the early human embryo. EBs could be directed to any kind of cell type by using different types of factors and morphogens. For neural cell differentiation, EBs aggregates are treated in early stages with specific morphogens to allow the differentiation of EBs into the ectoderm (Zhong-Wei Do and Su-Chun Zhang, 2004) For example, Shin et al. (2006) used hESC growing on mouse embryonic fibroblasts (MEF) for the first 7 days. Then they incubated hESC with neural induction medium containing a mix of morphogens for 7 days. Finally, they propagated the *rosettes* in proliferation medium and obtained hNPs (Shin et al, 2006).By making full use of previous studies, in our protocol, EBs were cultured on a neural induction medium, then EBs were transferred to adherent conditions on Matrigel-coated plates to

allow differentiation into neurosphere-like structures known as *neural rosettes.*

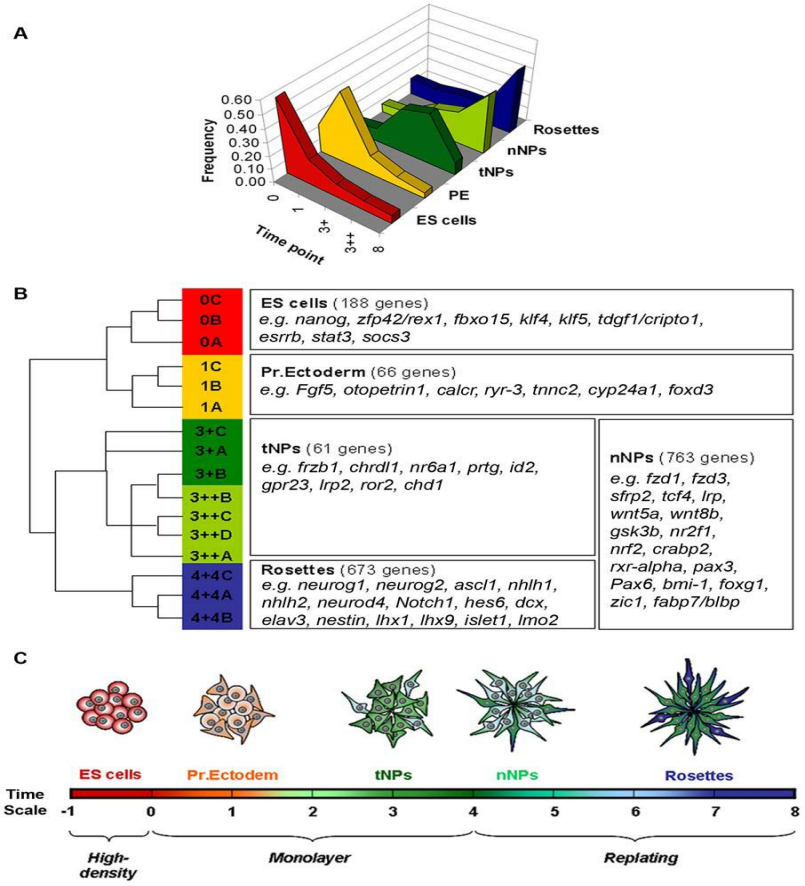


Figure D-1. Clustering analysis of differentially expressed genes. A) Frequency distribution of the expression levels of the genes belonging to the five defined groups. B) Dendogram of the relationship of the expression of the genes belonging to each group (with biological replicates represented by letters A, B, C and D) and an examples of the genes present in the five defined groups. C) Schematic representation of the successive cellular states occurring along the path to neural differentiation (Abranches E, 2009).

Neural rosettes structures are neural tube-like structures that contain high levels of neuroprogenitors cells (Zhang et al, 2001; Perrier et al., 2004) and expressed specific gene types (Fig. D-1). By this procedure, we efficiently obtained relatively pure hNPs in the absence of endodermal and mesodermal contamination, as demonstrated by the expression of α-fetoprotein (an endodermic marker) and Brachyury (a mesodermal marker).

In our cultures of hNPs, in order to control proliferation and differentiation, we used serum-free neural proliferation medium (NPM) containing Fibroblast Growth Factor (bFGF), which represents the main proliferating factor for the neural progenitors pool. This factor directs forebrain specification in the absence of other extrinsic factors (Bartlett et al, 1998; Mason I, 2007). Furthermore for cell maturation, we utilised serum-free neural differentiation medium (NDM) with the key differentiating factor BDNF (Brain Derived Growth Factor) (Ahmed S, 1995). Neuroprogenitors cells were maintained in the Neurobasal medium in which the osmolarity and concentration of several amino acids lowered. In order to maintain NPs, B27, containing RA (retinoic acid), was added to the medium. The other often used supplement that is added to Neurobasal medium is N2, which has a subset of B27 that includes insulin, which could act as a ligand of insulin-like growth factor receptors to induce the differentiation of hESC and the proliferation of the differentiation cells. It has been demonstrated that the survival and proliferation of neural progenitors is more efficient in B27-supplemented medium than in a medium with N2.

5.2.hNPC as a model to study early brain development

One of the major challenges in many studies is to obtain homogenous specific cell population and to renew neural progenitor cell populations capable of differentiating into specific neuronal or glial cell types. A critical point in the differentiation of hESC into NP cells is to maintain a self-renewing pool of NPs. When exposed to the correct environmental signalling cues, NP cells can follow a unique, robust temporal cell differentiation process to form numerous phenotypes (Dhara and Stice, 2008). Another yet unexplored advantage of the derivation of hESCs into NPs is that they can also prove to be a tool to elucidate the mechanisms underlying early human neural and brain development.

In the present work, we used an *in vitro* culture that efficiently directs both the differentiation of hESC towards NPs and the generation of radial glia, neurons and astrocytes in a temporal pattern, which is capable of reproducing early stages of human brain development. The culture protocol maintains progenitors with forebrain specifications in the absence of extrinsic factor, and in the presence of FGF2 for NPs proliferation (Bartlett et al, 1998) and BDNF for their differentiation (Ahmed et al, 1995).

Developing neural systems is a highly complex process that is controlled by complex signalling events which regulate the formation of the neural plate and neural tube. The embryonic neural tube is composed of a pseudostratified layer of neuroepithelial cells with clear apico-basal polarity. Rostro-caudal patterning processes involve WNT (Wingless), FGF (Fibroblast Growth Factor), BMP (Bone

Morphogenic Protein) and RA (Ratinoic Acid) pathways. The final results of the interaction among these pathways are the formation of major segments of the central nervous system (CNS): forebrain, midbrain, hindbrain and spinal cord. Dorso-ventral patterning is controlled by two opposing signals: BMP and SHH (Sonic hedgehog) (Harland R, 2000; Dhara and Stice, 2008; Wilson and Stice, 2006). The cells located in different positions in the dorso-ventral and rostro-caudal directions respond to different and position-specific morphogens.

Although our culture system has its limitations, it mostly reproduces early stages of human brain development. Indeed, neurulation is the first process during organogenesis and it begins from hESCs during early embryonic development events (Schoenwolf and Smith, 2000; Colas and Schoenwolf, 2001, Hornstein and Benvenisty, 2004). Furthermore, neural rosettes represent the *in vitro* analogue of neural tube formation after 2 weeks of hESC neural differentiation (Zhang et al, 2001). This period temporally correlates with the formation of the neural groove by 18 days *in vivo* and its subsequent rostro-caudal delineation by day 20 (O´Rahilly and Muller, 1994). Therefore, this stage is approximately the equivalent to the third week of human gestation if we consider that hESC are derived from 1-week-old embryos.

Regarding the neurogenesis process, recent evidence in developing human embryos has shown that the earliest born neurons are observed within the forebrain by days 31-35 (Bystron, 2006) and that these neurons precede local cortical neurogenesis. During

embryonic development, neural tube cells produce various types of neurons and glial cells, which will populate the CNS. The phenotypically diverse cells will be organised into different structural and functional units to form different specific synaptic contacts. This process generates neuronal circuits which are responsible for different simple and complex behaviours (McConnell, 1995). Our *in vitro* culture of human neural progenitors (hNPs) deriving from hESC coincide with the above findings. We found that rosette formation took place on days 15-17. Then in the presence of FGF2, which is involved in NPs proliferation and maintenance and generally in the stabilisation of neural identity (Carpenter et al 2001, Zhang 2004), epithelial cells, radial glia (RG) and immature neurons (TUI-1+) were observed during the maximal proliferation of hNP (days 18-39 of hESC or days 1-21 of hNPs proliferation) and postmitotic neurons (days 50-53, or day 35 in the differentiating medium including BDNF) which mirrors the early neural development phase of human embryos. In addition, quantitative PCR and immunocytochemical analyses reveal a temporal decrease in the expressions of the mesodermal marker (Brachyury), the endodermal marker (AFP) and the ectodermal marker (PAX6) in parallel with the up-regulation of neural progenitor markers, such as TUJ1 and Nestin, during the hNPs proliferation stage. A drop in the expression of the pluripotency markers Nanog and Sox2, which are crucial for the maintenance of hESC in an undifferentiated state (Thomson et al, 1998), was also noted during hNPs proliferation. The gene expression of Sox2,

together with c-MYc, KLF4 and Oct4, induce pluripotency in iPSC from somatic stem cells (Takashi and Yamanaka, 2006).

Our results further evidence that during their differentiation, hNPs are capable of giving rise to neurons, astrocytes and oligodendrocytes, as revealed by the up-regulation of the protein and gene expression of MAP-2, GABA and glutamatergic neurons, as well as the markers of glial cells GFAP (an astrocytes marker) and A2B5 (an early oligodendrocytes marker). These results indicate that under our experimental conditions, hESCs-derived neural progenitors can reproduce the processes occurring in early nervous system development, and can be used as a tool to study both the molecular and cellular mechanisms during early forebrain development. In many CNS regions, including various layered structures, common progenitors produce both neurons and glial cells. Research into the developing cerebral cortex has suggested that neurons, astrocytes and oligodendrocytes derive from largely separated lineages (McConnell, 1995). However NPs possess both a neurogenic and gliogenic potential, which means that the progenitor cells in rosette cultures could be converted into early neuron-producing or glia-producing cells (Abranches et al, 2009).

The nervous system comprises different regions and derivation-specific neural progenitors. Those cells with a particular regional identity have a given function in the forming brain with a specific identity and phenotype. Forebrain-specific NPs appear first and express homeodomain-specific factors OTX1, OTX2, and BF1 Forse 1, the recently described marker (Elkabetz et al, 2008). The cells

positive to these markers have been maintained in the presence of FGF2 (Zhang et al, 2006). Midbrain cells deriving from NPs are of much interest for therapeutic use in Parkinson's disease. Markers for midbrain-specific neurons include TH, RAX, NURR1, PTX, LMX1a/b, PAX2 and EN. The main growth factors responsible for midbrain differentiation are FGF8 and FGF2 (Perrier et al, 2004). Finally, published data indicate that along the anterior-posterior axis, hindbrain formation is specified by the expression of HOX genes and by the influence of FGF2, SHH and Retinoic Acid (RA). The dorso-ventral region is regulated with SHH (the ventral area) and BMPs (the dorsal area) and retinoic acid (RA) regulates the caudalisation process. By considering all these facts, researchers have successfully developed protocols for specific cell types (Li et al, 2005, 2008; Shin et al, 2005).

5.3. TLRs and endocannabinoids during neural development

TLRs in hNPs and during mice brain ontogeny

Toll-like receptors (TLRs) were described for the first time in the Drosophila, and were seen to play a major role in dorso-ventral polarity, synaptogenesis and in axon guidance during the embryogenesis of the fruit fly embryo (Anderson et al, 1985; Halfon et al., 1995; Rose et al., 1997). However, recent findings suggest that mammalian TLRs also play developmental roles during embryogenesis (Okun et al, 2011), and that they might also perform

physiological functions during development (Okun et al, 2011). TLRs are mainly expressed in the immune system. However, some non-immune cells, such as glial cells and neurons, also express these receptors. Yet the functional role of neuronal TLRs still remains uncertain (Okun et al, 2011). New roles of TLRs involve their participation in neuronal plasticity and in myelin sparing after spinal cord injuries or brain damage (Kigerl KA et al, 2006, Babcock AA et al, 2006, Kim D et al, 2007).

On the bases of the potential role of TLRs during brain development, we explored the possibility that TLR4 and TLR2 can be expressed in both hESC and hNPs. We demonstrate for the first time that although TLR4 is not expressed in hESC H9, the gene and protein expression of TLR4 are detected in EBs, structures containing the three germ layers (ectoderm, mesoderm and endoderm). We also reveal that the levels of TLR4, protein and gene expression increase during hNPs proliferation, with a maximal expression on D7 (mRNA) or D14 (protein), which lower thereafter during NPs differentiation (D21-D35). Conversely, protein expression TLR2 is high in hESC-H9 and decreases in EBs. Furthermore during hNPs proliferation, TLR2 increases on days 14 and 21, but decreases during NPs differentiation. These results indicate that TLR4 and TLR2 are expressed to NPs during not only hESC differentiation, but also during NPs proliferation and differentiation, suggesting a potential physiological role of these receptors during early stages of human CNS development.

Recent studies in mice have demonstrated that TLRs play a role during the proliferation and differentiation of neural progenitor cells (NPCs). For example, deficiency of TLR3 increases the proliferation of the NPCs in the subventricular zone (SVZ) (Lathia et al, 2008; Okun E et al, 2010), while deficiency of TLR4, MyD88 or TRIF enhances the proliferation of retinal progenitor cells (RPCs) in mice neonates (Shechter et al, 2008) and in adult NPCs (Lathia JD et al, 2008). Accordingly, activation of TLR2, TLR3 or TLR4 inhibits the proliferation of SVZ-derived embryonic NPCs (Lathia et al, 2008; Okun et al, 2010; Rolls A et al. 2007) (Fig. 2).

Studies conducted in mice also indicate that TLRs modulate adult hippocampal neurogenesis since TLR2 and TLR4 influence self-renewal and the cell-fate decision in adult NSCs in culture (Rolls A, 2007). TLR2 deficiency changes the level of the differentiation patterns of neural progenitors. This influence diminishes the number of neurons expressing early neuronal markers Tuj1 and increase differentiation into the cells with a glial phenotype, such as GFAP+ cells (Okun et al, 2011). These studies also demonstrate that the activation of TLRs in NPCs triggers MyD88 and NFkB signalling (Fig. 2). Our results with hNPs also indicate that the activation of TLR4 with LPS is capable of inducing downstream signalling as ERK phosphorylation and NFkB activation, suggesting that functional TLR4 signalling also occurs in hNPs.

Using the cerebral cortex of mice during the prenatal and postnatal stages, our results further demonstrate that the expression of

TLR4 is high on day 15 of the foetal (F15) stage, but then lowers at the end of gestation to increase again during postnatal development

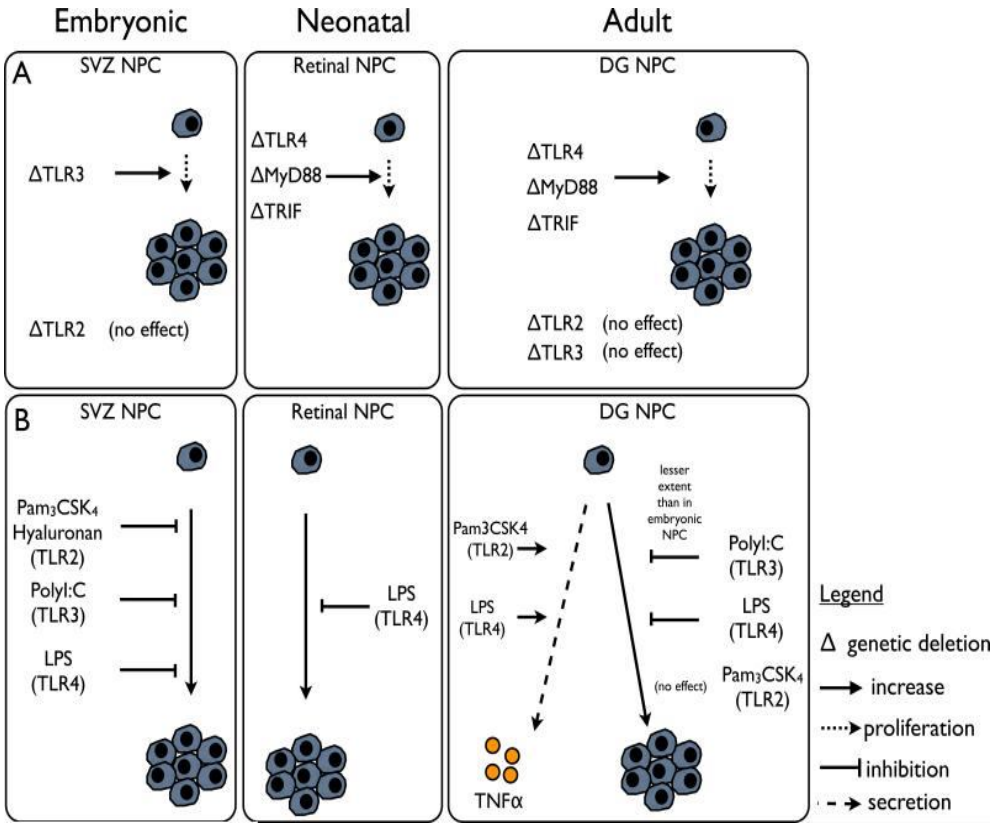


Fig. 2 (A) *Deficiency of TLR3, but not of TLR2, increases the proliferation of SVZ-derived embryonic NPCs. Deficiency of TLR4, MyD88 or TRIF increases the proliferation of RPCs in neonates. Deficiency of TLR4, MyD88 or TRIF, but not of TLR2 or TLR3, increases the proliferation of DG-derived adult NPCs.* ***(B)*** *Activation of TLR2 by Pam3CSK4, TLR3 by PolyI:C or TLR4 by LPS inhibits the proliferation of SVZ-derived embryonic NPCs (middle panel). Activation of TLR4 using LPS inhibits the proliferation of RPCs in neonates (right panel). Activation of TLR4 by LPS, and to a lesser extent activation of TLR3 with PolyI:C, but not of TLR2 using Pam3CSK4, inhibits DG-derived adult NPC proliferation . Activation of TLR2 and TLR4 with the above ligands also induces the release of TNF-α in these cells. This figure describes the work performed in mice (Okun et al., 2011).*

Furthermore, the expression of TLR4 at F15 and on postnatal day 30 is higher than in the adult cerebral cortex, suggesting a potential role of TL4 during both the proliferation and plasticity of the CNS (Okun et al, 2011).

Regarding TLR2, previous studies have shown that TLR2 activation has different effects on embryonic and adult neural progenitor cells. During the embryonic proliferation of neural progenitors, TLR2 exhibits a negative regulation, whereas it has no effect on the self-renewal potential in adult NPC (Okun et al, 2010; Rolls et al, 2007; Covacu R et al, 2009).

We noted that TLR2 is expressed during the early stages of mice cerebral cortex development, with a maximum level on postnatal day 14. In agreement with our results, a new study shows that TLR2 is expressed in the developing telencephalon from early embryonic stages and that it slightly increases during postnatal development (Okun et al., 2010). Infectious agent-related activation of TLR2 inhibits NPC proliferation. Finally, the above findings suggest that if we consider the roles of TLR4 and TLR2 of NPC proliferation, differentiation, plasticity and their role in inflammation, it can be speculated that either the reduction or activation of these receptors during brain development, as in infection, ischaemia and inflammation, and alcohol exposure, can adversely affect brain development (Okun et al., 2010). In fact, our results demonstrate that ethanol exposure during the prenatal and postnatal periods increases the levels of TLR2 and TLR4, which might cause neuroinflammation

and brain damage (Alfonso-Loeches et al., 2010), thus contributing to the neuroteratogenic effects of ethanol.

Endocannabinoids expression in hNPs

Endocannabinoids (EC), the endogenous counterparts of marijuana-derived cannabinoids, act as neuromodulators via presynaptic CB1 receptors, and also control neural cell death and survival. Studies conducted in the last 10 years have demonstrated the role of EC in the regulation of cell proliferation and differentiation of mice neural progenitor cells (Aguado et al., 2005, 2006, 2008). Indeed, mice NPs produce EC, and they express the CB1 receptor and endocannabinoid-inactivating enzyme fatty acid amide hydrolase (FAAH). CB1 receptor activation promotes cell proliferation and neurosphere generation, an action that is abrogated in CB1-deficient NPs. These results demonstrate that EC constitute a new group of signalling cues that regulate NP. At the same time, the CB2 receptor is also expressed, both *in vitro* and *in vivo*, in mice neural progenitors from late embryonic stages to the adult brain, and the pharmacological activation of the CB2 receptor *in vitro* promotes neural progenitor cell proliferation and neurosphere generation (Palazuelos et al., 2006) (Molina-Holgado et al, 2007).

Although, we did not assess the role of EC in the proliferation and differentiation of hNPs, we demonstrate for the first time that hNPs express CB1, CB2 and the enzymes involved in EC synthesis (NAPE-PLD) and degradation (FAAH), suggesting that EC can modulate hNPs proliferation and differentiation. In addition, although

EC play a modulatory role in alcohol dependence (Pava MJ and Woodward JJ, Alcohol 2012), the present study indicates that ethanol exposure during early embryogenesis has no significant effects on the expressions of NAPE- PLD, FAAH, CB1 or CB2 in hNPs.

5.4. Effects of ethanol on human embryogenesis and neuroprogenitors.

A large number of studies have evidenced the sensitivity of the developing brain to the damage caused by environmental chemicals and toxic compounds (Weiss, 2000). Ethanol is one of the most common and most important toxic substances to affect the developing foetal brain. Its consumption during pregnancy can produce a wide range of cognitive, behavioural and physical anomalies, and it is one of the leading preventable causes of birth defects and neurodevelopmental disorders (American Academy of Pediatricts, 2000). In the most severe cases, these anomalies make up a pattern of malformations termed foetal alcohol syndrome (FAS) (Jones et al, 1973). This syndrome is characterised by the following diagnostic criteria: 1) Maternal alcohol dependence or alcohol abuse during pregnancy, 2) Pre- and postnatal growth deficiency, 3) Central nervous system (CNS) dysfunctions, and 4) A particular pattern of facial features. However, CNS dysfunctions are the most distressing and permanent consequence of heavy or moderate alcohol consumption, and are manifested by long-term cognitive and behavioural deficits rather than physical anomalies. The incidence of these neurobehavioral dysfunctions is higher (5-19/1,000 births) than FAS (0.5-3/1,000 births) (Stratton et al., 1996).

Accumulating clinical and experimental evidence also demonstrates the vulnerability of the developing CSN to effects of alcohol. Experimental evidence indicates that ethanol interferes with many ontogenetic phases of brain development. Therefore, the pattern of damage may explain the wide complex pattern of the behavioural and neurological abnormalities observed in human and animals exposed to alcohol at different times during brain development. These studies demonstrate that an important critical period for craniofacial malformations to appear in FAS is early embryogenesis. High levels of alcohol during the embryonic stage of gastrulation (7 days after fertilisation in mice) (Webster et al, 1983; Kotch and Sulik, 1992, Sulik, 2005) and macaques exposed to ethanol on E19 or E20 (Astley et al, 1999) can lead to the craniofacial abnormalities and brain abnormalities associated with FAS. Human studies have also shown that both binge drinking and chronic alcohol abuse during an early stage of human embryogenesis (corresponding to the third week of human gestation) is associated with a greater incidence of craniofacial defects and mental disabilities (Ernhart et al, 1987). The mechanisms of ethanol-induced malformations during human embryogenesis are unknown.

By using hNPs from hESC, we provide evidence that ethanol exposure, at concentrations that can be reached in the blood of alcoholics (Jones, 1992), induces cell death by apoptosis, as revealed by the increment in annexin + cells and caspase 3 activity, or necrosis at higher ethanol concentrations, such as 50 mM. We also observed that ethanol reduces hNP proliferation. In agreement with our human NPs results, some studies have demonstrated that ethanol inhibits mice neural progenitors proliferation by changing the expression of

cyclin/cyclin-dependent kinases, the p53 gene and DNA methyltransferase, these being the key genes involved in cell cycle check points (Hicks et al, 2010).

Our previous studies have also shown that alcohol exposure during early rat embryogenesis *in vivo* (E12) reduces the proliferation of the radial glial progenitor pool, impairs the self-renewal capacity of thelencephalic multipotent progenitor cells and affects neurons and astrocytes generation (Rubert et al, 2006). In agreement with these results, our immunocytochemical and RT-PCR data reveal that *in vitro* ethanol exposure impairs the transformation of immature neuronal (Tuj1) and glial (Nestin) markers into mature neurons (e.g., MAP2) and astrocytes (e.g., GFAP). These results suggest that ethanol impairs the neuroprogenitors maturation process, as demonstrated in previous studies (Tateno et al, 2005, Rubert et al., 2006). Alterations in the methylation process or changes in the DNA demethylation of the genes participating in neural cell maturation (Valles et al., 1997) might involve impairing effects of ethanol on neural cell maturation. Recent studies have confirmed that alcohol delays the cellular DNA methylation programme and also retards embryonic growth. Since the direct inhibition of DNA methylation results in a similar retardation, it has been suggested that alcohol affects embryonic development through epigenetic mechanisms (Zhou FC et al, 2011).

The present findings also reveal that the derivations of NPs from hESCs towards neuroepithelial cells are affected by ethanol. Indeed, ethanol treatment alters the disappearance of AFP (an endodermal marker) during NPs proliferation, and it up-regulates

transcription factors Nanog and Pax-6 during their differentiation. Alterations in Pax6 induce defects in neural stem, while progenitor cells can cause forebrain abnormalities in humans and in experimental animals (see rev. Manuel and Price, 2005). Mice mutants of Pax6 show defects in neural stem and progenitor cell proliferation, resulting in microcephaly and abnormal development in the subventricular zone (Warren et al, 1999; Quinn et al, 2007). Likewise, the overexpression of Pax6 also leads to the depletion of the stem cell pool by driving stem cells to a basal progenitor fate, leading to an overproduction of early-born, deep-layer cortical neurons, causing progenitors apoptosis (Manuel et al, 2007, Sansom et al, 2009) and resulting in forebrain abnormalities (Berger et al, 2007). If we consider the similarities of the later alteration and those induced by ethanol in NPs in culture, it can be postulated that the ethanol-induced up-regulation of PAX6 possibly underlies some of the ethanol-induced defects noted in neuronal differentiation.

Notch signalling is also critical for the maintenance of the progenitor pool as it controls cell renewal, and regulates cell differentiation and cell fate determination. Our preliminary results indicate that the Notch expression decreases in the hNPs exposed to 25mM ethanol at the initiation of astrogliogenesis (on day 28; data not shown). According to these findings, our previous results show that *in utero* ethanol exposure lowers the levels of activated Notch1 and FGFR2, and decreases astrogliogenesis (Rubert et al., 2006). Since one of the prominent roles of Notch is to promote astrocytic fate (Gaiano and Fishell, 2002), an ethanol-induced decrease in Notch

activation at the onset of gliogenesis might account for the reduced GFAP expression during NPs differentiation.

Another important finding of the present study is that our results demonstrate that ethanol affects cell morphology and impairs several cytoskeletal proteins, including nestin, Tuj1 and actin. Interestingly, the actin reorganisation (e.g., loss of stress fibres and actin ring formation) observed in NPs is similar to that described in ethanol-exposed astrocytes (Minambres R et al., 2006, Guasch RM et al., 2003), and that these effects are associated with loss of focal adhesions, activation of the RhoA/ROCK-I/MLC pathway and apoptosis by anoikis in astrocytes (Miñambres et al, 2006). Therefore, ethanol-induced actin cytoskeletal reorganisation may not only underlie disruptions in cell morphology, synaptic formation and plasticity (Luo et al, 2002), but may also participate in ethanol-induced NPs apoptosis. Deregulation of actin dynamics leads to cellular and cognitive anomalies in flies and mice (Sordela and Aelst , 2006). *In vitro* ethanol exposure has a negative impact on endocytosis, exocytosis and nucleocytoplasmic traffic in astrocytes, and alters endocytosis in cultured neurons. In astrocytes, these effects relate to changes in the organisation and/or function of microtubules and the actin cytoskeleton. In primary culture in rats, a difference in actin, tubulin and MAP2 distribution has been reported in control and ethanol-treated animals (Romero et al, 2010).

In summary, by using hESC-derived NPs in culture, we demonstrate that ethanol exposure during early embryogenesis impairs NPs survival, affects the differentiation of NPs into neurons and astrocytes,

disrupts several cytoskeleton components and affects the expression of the different genes associated with neural differentiation.

Summary,

We tested several conditions for human embryonic stem cells (hESC) by growing them on different feeder layers and under feeder-free conditions in order to evaluate the best conditions to establish the derivatisation of hESC into human neuroprogenitors.

By employing the *in vitro* culture that efficiently directs the differentiation of hESC towards NPs, we report the generation of radial glia, neurons and astrocytes in a temporal pattern, which is capable of reproducing early stages of human brain development.

Neuroepithelial progenitors display both the morphological and functional characteristics of their embryonic counterparts and the proper timing of neurons and glia cells generation. Immunocytochemical and real-time (RT) polymerase chain reaction analyses reveal that cells appear as clusters during neuroepithelial cell proliferation and that the genes associated with the neuroectodermal (Pax-6) and the endodermic (α-fetoprotein) lineages decrease in parallel with the up-regulation of NPs genes (nestin and Tuj1), followed by their differentiation into neurons (MAP-2+, GABA+), oligodendrocytes [galactocerebroside (GalC+)] and astrocytes (GFAP+). We further demonstrate for the first time that human NPs express endocannabinoid receptors (CB1 and CB2) and the enzymes involved in endocannabinoids synthesis (NAPE-PLD) and degradation (FAAH). We also show for the first time that TL4 and TLR2 are

expressed in both hESC and hNPs. In parallel, we demonstrate that TLR4 and TLR2 are expressed during mice brain development because both receptors are expressed in both the prenatal and postnatal periods.

Finally by using this *in vitro* culture model, we demonstrate that ethanol impairs NPs survival, affects the differentiation of NPs into neurons and astrocytes, disrupts the actin cytoskeleton, and affects the expression of different genes associated with neural differentiation such as affects the expression of different genes associated with neural differentiation, such as TLR4, TLR2, CB1, MAP2, GFAP. The results provide new insights into the effects of ethanol on human embryogenesis and neuroprogenitors, and offer an opportunity to delineate potential therapeutic strategies to restore early ethanol-induced brain damage.

----------CONCLUSIONS----------

Conclusions

1- We establish an *in vitro* model for the generation of neural progenitors (NPs) from hESCs.

2- Neuroepithelial progenitors display the morphological and functional characteristics of their embryonic counterparts, and the proper timing of neurons and glia cells generation.

3- Immunocytochemical and RT-PCR analyses reveal that hESC appeared as clusters during neuroepithelial cell proliferation, and that the genes associated with the neuroectodermal and endodermal lineages decrease in parallel with the up-regulation of the genes of neural progenitors, followed by their differentiation into neurons oligodendrocytes and astrocytes.

4- We demonstrate for the first time that human NPs express the endocannabinoid receptors (CB1, CB2) and the enzymes involved in endocannabinoids synthesis and degradation, as well as the TLR4 and TLR2 receptors.

5- By using this *in vitro* model, we show that ethanol exposure:

- Impairs the derivation of hESCs towards neural cells, alters the disappearance of the endodermal marker AFP during NPs

proliferation and up-regulates the transcription factors Nanog, Sox2 and Pax-6 during their differentiation.

- Impairs NPs survival, affects the differentiation of NPs into neurons and astrocytes, disrupts the actin cytoskeleton and affects the expression of different genes associated with neural differentiation.

6- The results provide new insights into the effects of ethanol on early human embryogenesis and offer an opportunity to delineate potential therapeutic strategies to restore early brain damage induced by ethanol.

7- Finally, the present study provides not only a new tool to elucidate the mechanisms underlying early human neural and brain development, but also offers the possibility to assess the neuroteratogenic actions of ethanol during early embryogenesis.

----------RESUMEN ----------

Resumen

Diferenciación de células progenitoras neurales a partir de células humanas embrionarias (hESC) como modelo para el estudio de las fases iniciales en el desarrollo del cerebro en humanos y de los efectos neuroteratogénicos del etanol

Las células madre o troncales embrionarias humanas (human Embryonic Stem Cells, hESC) poseen dos características cruciales: capacidad ilimitada de proliferar y potencial de dar lugar a diferentes tipos celulares. Estas dos características las hacen idóneas como herramienta para evaluar su potencial para regenerar el tejido nervioso dañado, como ocurre en diferentes enfermedades neurodegenerativas. Al mismo tiempo, la diferenciación de las hESCs hacia neuroectodermo y células progenitoras neurales puede servir como un modelo *in vitro* para el investigar los procesos celulares y moleculares que ocurren durante las primeras etapas del desarrollo del cerebro así como para analizar la acción de ciertos neurotóxicos y teratógenos (ejem. El alcohol) que afectan el proceso de formación y desarrollo del cerebro durante de la embriogénesis temprana en humanos.

Actualmente existen algunos trabajos que han diferenciado las hESC hacia células del tejido nervioso, pero existen pocos estudios que haya abordado el estudio de las primeras fases del desarrollo del

sistema nervioso central así como los las acciones del alcohol durante la fase temprana de la embriogénesis en humanos

Objetivos

Basados en los anteriores antecedentes, los objetivos de este trabajo han sido:

- Desarrollar un protocolo *in vitro* de derivatización de células humanas neuroprogenitoras a partir de células multipotentes embrionarias humanas (hESC), que pueda reproducir las primeras fases del desarrollo del cerebro en humanos.

- Caracterizar el cultivo *in vitro de células hESC a* neuroprogenitores (hNPs), evaluando la expresión de genes y proteínas durante la transformación de hESC a hNPs y durante la proliferación de NPs y su diferenciación a células maduras neurales (neuronas, astrocitos y oligodendrocitos).

- Evaluar si ciertos sistemas y receptores, como el sistema endocanabinoide, incluyendo los receptores (CB1, CB2) y los enzimas que participan en su síntesis (NAPE-PLD) y degradación (FAAH), se expresan en los

neuroprogenotores humanos durante su proliferación y diferenciación. En roedores el sistema endocanabinoide juega un papel importante en la proliferación de los NPs

- Investigar si los receptores TLR4 and TLR2 se expresan en hESC, hNPs y durante el desarrollo de cerebro en roedores.

- Finalmente, investigar las acciones del etanol durante la diferenciación de hESC a hNPs así como durante la proliferación y diferenciación de los NPs humanos a células maduras neurales. Específicamente se evaluara el efecto del etanol sobre: 1) Expresión de genes y proteínas durante la derivatización de hESC a NPs, 2) la proliferación de hNPs y supervivencia, 3) la expresión de genes y de proteínas así como las alteraciones morfológicas que puedan ocurrir durante la diferenciación de los hNPs a células neurales maduras, 4) expresión del sistema canabinoide y de los TLR4 y TLR2 en hESC, hNPs y desarrollo de cerebro en ratones.

Metodos

Cultivos de hESC: El protocolo que se ha utilizado para establecer los NPs a partir de células embrionaria humanas (hESC) se representa en la Fig.4 de Resultados. Como hESC hemos utilizado la línea H9 (WiCell Inc., Madison WI**)**.. Las células hESC se cultivaron en medio apropiado, en un incubador a 37ºC, 5% CO_2, y en condiciones de humedad. Se realizaron diferentes pases, de acuerdo con el tamaño de las colonias. Para la obtención de cuerpos embrioides, la hESC se transfirieron a condiciones adherentes en una matriz de matrigel. Tras 6 días de cultivo en medio de inducción neural (NIM, que contenía el factor trófico, FGF2) se formaron unas estructuras específicas en forma de "rosetas", ricas en progenitores neurales. Estas estructuras se cortaron, se disgregaron, y se siembraron a una concentración de 300,000 células en placas de 2 ml recubiertas con laminina/poliornitina. Las células se adhirieron y proliferaron en un medio que contenía FGF, que denominamos medio de proliferación. Para la adición del etanol, tras sembrar las placas de cultivo, la células se incubaron toda la noche para permitir su adhesión a la placa, y posteriormente se añadio etanol al medio de cultivo a 2 concentraciones, 25mM y 50 mM..Tras 21 días, el medio de de proliferación se sustituyo por medio de diferenciación que contenía BDNF.

Ensayos para medir proliferación, Viabilidad celular y Caspasa 3

Para evaluar proliferación se incorporo BrdU iy se siguió un protocolo establecido, siguiendo las instrucciones de un kit comercial (Roche, cat no. 11810740001). La cuantificación del número de células que incorporaron BrdU se evaluó mediante citometria de flujo (Cytomics FC500, Beckman Coulter).

Para analizar la viabilidad celular y la apoptosis, las células se incubaron con R-phycoerythrin-conjugatdo annexina-V junto con el fluoroforo 7-AAD (Molecular Probes). Se evaluó la apoptosis y necrosis mediante citometria de flujo (Cytomics FC500, Beckman Coulter).

La actividad Caspase-3 se midió mediante un procedimiento colorimétrico siguiendo las instrucciones de un ensayo comercial (Sigma-Aldrich Quimica, Spain) Este ensayo usa el sustrato acetyl-DEVD-p-nitroanilide (Ac-DEVD-pNA). La intensidad del color y la formación del producto de la reacción, p-nitroaniline, se cuantifico mediante la lectura de las muestras a 405 nm en un espectrofotómetro, usando los controles correspondientes y una curva estándar.

Inmunoelectrotransferencia

La concentración de proteína, tanto en los lisados celulares como en cerebro , se cuantificó mediante el procedimiento de Bradford (Bio-Rad, Hercules, CA, USA).

Para el análisis de proteínas concretas se utilizó la inmunoelectrotransferencia. Para ello se utilizaron geles de 1,5 mm de grosor con porcentajes de acrilamida (en el gel separador) entre el 7 y el 15% según el peso molecular de los fragmentos proteicos a resolver y adicionando dodecil sulfato sódico (SDS). Se mantuvo una relación acrilamida:bis-acrilamida de 30:0,8 en todos los casos. A las muestras obtenidas se les añadió tampón de carga 6x (350mM Tris pH 6.8, 30% glicerol, 30% mercaptoetanol, 100gr/L SDS, 200mg/L azul de bromofenol) y se hirvieron durante 5 min. Para la electroforesis se utilizó un sistema Mini Protean de Bio-Rad en tampón: 6gr/L de Trizma base, 2.88 gr/L de glicina y 20 gr/L de SDS.

Las proteínas separadas por SDS-PAGE se transfirieron a membranas de PVDF (Immobilon Transfer Membrane, Millipore) en tampón 3gr/L de Trizma base, 1.44 gr/L de glicina y un 20% de metanol, durante 1h a 100V.

Las membranas se bloquearon durante 60 minutos en leche desnatada al 5% en TBS-Tween (TBS-T) 0.1% (Tris 20mM y NaCl 500mM pH 7.5) y se incubaron durante toda la noche a 4ºC, en agitación, con los correspondientes anticuerpos primarios (ver Tabla 2, Material y Metodos).

Las membranas se lavaron 3-4 veces con TBS-T 0.1% y se incubaron con los anticuerpos secundarios –anti IgG-conjugado con HRP (anti-ratón peroxidasa, 1:1000, Santa Cruz; anti-conejo peroxidasa, 1:20000,) durante 1 h a temperatura ambiente. Finalmente, las membranas se revelaron mediante quimioluminiscencia utilizando

ECL-Plus (Amersham) y se expusieron a films MXG de Kodak o Hyperfilm de Amersham.

Para la cuantificación de la intensidad de las bandas, estas se capturaron digitalmente con un escáner (EPSON DX 4800) y la intensidad de las mismas se cuantificó por densitometría mediante el uso del programa Alpha-Ease FC, programa de análisis de imagen versión Alpha Imager 2200 (Alpha Innotech Corporation). La intensidad relativa de cada proteína individualmente se expresó como el ratio entre su intensidad y control de carga GAPDH, o su control respectivo

Aislamiento de RNA y RT-PCR

Para aislar ARN se utilizo el Tri-Reagent (Sigma), siguiendo las instrucciones de la casa comercial. La cantidad de ARN purificado se estimó midiendo la absorbancia a 260 nm y 280nm, y la pureza se estableció analizando la relación de la absorbancia 260/280 nm. La integridad del ARN se determinó mediante electroforesis en gel de agarosa. Para el análisis de RT-PCR, 1µg de ARN de cada muestra fue transcrita usando el kit *Transcriptor First Strand cDNA Synthesis* (Roche). El cDNA se diluyó (3µL) y amplificó en un termociclador (LightCycler Instrument; Roche Diagnostics) en 10µL de LightCycler 480 SYBR Green I Master (Roche) y 0,5µM de cada oligonucleótido. La secuencia de cada uno de los cebadores o *primers* de los genes usados viene determinada en la Tabla 1(Material y Metodos).

Los niveles ARNm de los genes SP1 o CypA se utilizó como control interno de normalización de los datos. Para asegurarnos de que

los tratamientos no alteraban los niveles de ARNm del control interno, los valores de Control se compararon entre los grupos experimentales. No se encontraron diferencias en los niveles de ARNm de SP1 o CypA. Para la cuantificación de los productos de la PCR, se analizó la fase exponencial de la amplificación de la curva de meelting y se cuantificó mediante el programa de *LightCycler 480 quantification*, siguiendo las recomendaciones de la casa comercial.

Inmunofluorescencia

Para este procedimiento laa celulas se cultivaron en cubres (12mm) recubiertos de laminina y poli-lisina ien placas de 4 pocillos.. Las celulas se fijaron con paraformaldehido (4% in PBS) durante 20 min, se permeabilizaron con 0.25% Triton X-100 durante 5 min, como se describe en nuestros previos estudios (Rubert et al., 2006). Tras un lavado con PBS, las celulas fijadas se incubaron con 5% BSA en PBS durante 30 min, para bloquear los lugares no especificos. Tras el bloqueo, las celulas se incubaron con los anticuerpos primarios: anti-GFAP (Sigma), anti-nestin (Chemicon), anti-MAP2 (Chemicon), anti-TUJ1 (Abcam), anti-GABA-A (Chemicon. Co), anti-glutamate (Sigma-Aldrich.), anti-PAX6 (Abcam); anti-C2B5 (Sigma-Aldrich, Spain). Todos los anticuerpos se incubaron con 0.1% Triton-1% BSA en PBS durante 1 h a temperature ambiente. Tras lavar las celulas, esta se incubaron con los anticuerpos secundarios apropiados (Jackson Immunoresearch, Mississauga, Ontario, Canada) conjugados con diferentes fluoroforos. Los núcleos se tiñeron con el reactivo que se

une al ADN, Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Las fotos se tomaron con un microscopio Leica (Wetzlar, Germany). Para cuantificar la intensidad de la fuorescencia, se tomaronal menos 10 campos por cultivo, y se usaron 3-4 cultivos. La inmunoreactividad se determino mediante el programa Metamorph-Offline software (Universal Imaging, Downingtown, TA).

Conclusiones

1- Hemos establecido un modelo *in vitro* en donde generamos progenitores neurales (NPs) a partir de células embrionarias humanas (hESC).

2- Los progenitores neuroepiteliales y los NPs humanos muestran características morfológicas y funcionales similares a las que se producen en el desarrollo embrionario, dando lugar a la generación de células gliales y neuronas a tiempos similares a los que se observan durante el desarrollo de cerebro.

3- El análisis mediante inmunocitoquimica y RT-PCR muestra que la hESC forma agregados durante la fase de proliferación, disminuyendo durante esta fase la expresión de genes asociados al neuroendodermo y neuroectodermo e incrementando la expresión de genes asociados a progenitores neurales. Posteriormente, durante la diferenciación de los NPs

se produce la aparición de genes y proteínas marcadoras de neuronas, astrocitos y oligodendrocitos.

4- Por primera vez demostramos que los NPs humanos expresan los receptores de endocanabinoides (CB1 y CB2), los enzimas que participan en la síntesis y en la degradación de los endocanabinoides, así como los receptores TLR4 y TLR2

5- Mediante la utilización del modelo *in vitro* de hESC y de NPs demostramos que la exposición al etanol:

- Altera la derivatización de hESC hacia células neurales, como lo demuestra la elevada expresión del marcador de endodermo *AFP* durante la proliferación y el aumento en los niveles de los factores de transcripción *Nanog, Sox2 y Pax6* durante la diferenciación de los NPs.
- Afecta la supervivencia de los NPs humanos, altera la diferenciación de los NPs hacia células neurales maduras (astrocitos, neuronas) afectando tanto la expresión de genes como de proteínas marcadoras de células neurales maduras y finalmente cambia la morfología celular afectando la estructura del citoesqueleto de actina.

6- Los resultados de los efectos del etanol durante la embriogénesis temprana pueden proporcionar la posibilidad de desarrollar estrategias terapéuticas para restaurar los efectos

del etanol durante el inicio de la formación del sistema nervioso.

7- Finalmente, el presente estudio describe un nuevo procedimiento para evaluar los mecanismos que subyacen al inicio del desarrollo del cerebro humano y también ofrecen la posibilidad de investigar los efectos neuroteratogenicos del etanol durante la embriogénesis temprana.

----------REFERENCES----------

References

Abel EL (ed). *Fetal Alcohol Syndrome: From Mechanism to Prevention*. Boca Raton, FL: CRC Press, 27-50.

Abranches E, Silva M, Pradier L, Shulz H, Hummel O, Henrique D and Beckman E. (2009) Neural Differentiation of Embryonic Stem Cells *In Vitro*: A Road Map to Neurogenesis in the Embryo. *PLoS ONE 4(7): e6286.*

Adachi K, Suemori H, Yasuda S, Naktsui N and Kawase E. Role of SOX2 in maintaining pluripotency of human embryonic stem cells *Genes to Cells* (2010) 15, 455–469

Aguado T, Monory K, Palazuelos J, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzmán M, Galve-Roperh I (2005). The endoccanabinoid system drives neural progenitor proliferation *FASEB* J. (12):1704-6.

Aguado T, Palazuelos J, Monory K, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzmán M, Galve-Roperh I (2006). The endoccanabinoid system promotes astroglial differentiation by acting on neural progenitor cells . *J Neurosci.* 1;26(5):1551-61.

Ahmed S, BA Reynolds and S Weiss. (1995) BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. *J Neurosci* 15: 5765-78.

Amit M and Itskovitz-Eldor (2002). Derivation and spontaneous differentiation of human embryonic stem cells. *J Anat* 200:225-232.

Anderson KV, Bokla L and Nuslein-Volhard C. (1985) Establishment of dorsal ventral polarity in the Drosofila embryo: the induction of polarity by Toll gene product. *Cell*. 42:791-798.

Alati R, Al Mamun A, Williams GM et al. (2006) In utero alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. *Arch Gen Psychiatry* 63: 1009-16.

Alfonso-Loeches S, Pascual-Lucas M, Blanco AM, Sanches-Vera I, Guerri C. (2010) Pivotal role of TLR4 receptors in alchohol-induced neuroinflammation and brain damage. *The Journal of Neuroscience,* 30(24): 8285-8295.

Arnold B. Scheibel. (2011) Embriological Development of the Human Brain.

Ashwell KW, Zhang LI. (1996) Forebrain hypoplasia following prenatal ethanol exposure: quantitative analysis of effects on specific forebrain nuclei. *Pathology* 28:161-6.

Astley SJ, Magnuson SI, Omnel LM et al. (1999) Fetal alcohol syndrome: changes in craniofacial form with age, cognition and timing of ethanol exposure in the macaque. *Teratology* 59: 169-72.

Autti-Rämö I, Autti T, Korkman M et al. (2002) MRI findings in children with school problems who had been exposed prenatally to alcohol. *Dev. Med. Child Neurol*. 44: 98-106.

Autti- Rämö I, Fagerlund A, Ervalahti N et al. (2006) Fetal alcohol spectrum disordersin Finland: clinical delineation of 77 older children and adolescent. *Am J Med Genet A* 140: 137-43.

American Academy of Pediatrics (2000) " Committee on Substance Abuse and Committee on Children and Disabilities. Fetal alcohol syndrome and alcohol-related neurodevelopmental disorders", *Pediatrics* 106, 358-361.

Archibald SL, Fennema-Notestine C, Gamst A et al. (2001) Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Dev. Med. Child Neurol*. 43: 148-54.

Babcock AA, Wirenfeldt M, Holm, T, Nielsen, HH, Dissing-Olesen, L, Toft-Hansen H, Millward JM, Landmann R, Rivest S, Finsen B, and Owens T (2006). Toll-like receptor 2 signaling in response to brain injury: an innate bridge to neuroinflammation. *J Neurosci* 26:12826-12837.

Bartlett PF, Brooker GJ, Faux CH, Dutton R, Murphy M, Turnley A and Kilpatrick TJ (1998). Regulation of neural stem cell differentiation in the forebrain. *Immunol Cell Biol* 76: 414-418.

Berger J, S Berger, TC Tuoc, M D'Amelio, F Cecconi, JA Gorski, KR Jones, P Gruss and A Stoykova. (2007). Conditional activation of Pax6 in the developing cortex of transgenic mice causes progenitor apoptosis. *Development* 134:1311-22.

Bhatara VS, Lovrein F, Kirkeby J (2002). Brain function in fetal alcohol syndrome assessed by single photon emission computed tomography. *S D J Med* 55: 59-62.

Bystron I, Rakic P, Molnar Z, Blakemore C (2006). The first neurons of the human cerebral cortex. *Nat. Neurosci* 9:880-886.

Charry ABC and Daley GQ (2012). Reprogramming Cellular Identity for Regenerative Medicine. *Cell* 148(6):1110-22.

Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS (2001). Enrichement of neurons and neural precursors from human embryonic stem cells. *Exp Neurol* 172:383-397.

Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T, Lucero M and Rao MS (2004). Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. *Developmental Dynamics* 229 243-258.

Ceccanti M, Alessandra Spagnolo P, Tarani L et al. (2007). Clinical delineation of fetal alcohol spectrum disorder (FASD) in Italian children : comparison and contrast with other racial/ethnic groups and implications for diagnostics and prevention. *Neurosci Biobehav Rev* 31: 270-7.

Colas JF, Schoenwolf GC. Towards a cellular and molecular understanding of neurulation (2001). *Dev Dyn*. 221(2):117-45.

Cortese BM, Moore GJ, Bailey BA (2006). Magnetic resonance and spectostropic imaging in prenatal alcohol-exposed children: preliminary findings in the caudate nucleus. *Neurotoxicol Teratol* 28:597-606.

Covacu R et al. (2009). TLR activation induces TNF-alpha production from adult neural stem/progenitor cells. *J. Immunol*. 182, 6889-6895.

Crisostomo P, Wang Y, Markel T, Wang M, Lahm T, Daniel R. Human mesenchymal stem cells stimulated by TNF, LPS or hypoxia produce growth factors by an NFkB but not JNK dependent mechanism. (2008). *Am J Physiol Cell Physiol.* 294:C675-C682.

Dhara, SK. and Stice**,** SL. (2008). Neural differentiation of human embryonic stem cells. *J. Cell. Biochem*., 105: 633–640.

Deltour L, Ang HL, Duester G. (1996) Ethanol inhibition of retinoic acid synthesis as a potential mechanism for fetal alcohol syndrome. *FASEB J* 10:1050-7.

Dikranian K, Qin YQ, Labruyere J (2005). Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. *Brain Res Dev* 155:1-13.

Du ZW and Zhang SC (2004) Neural Differentiation from Embryonic Stem Cells: Which Way? *Stem Cells and Development*. 13(4): 372-381.

Edlund T, and Jessell TM. Progression from extrinsic to instrinsic signaling in cell fate specification: A view from the Nervous System. *Cell*, Vol. 96, 211-224, January 22, 1999.

Elkabetz Y, Panagiotakos G, Al Shamy G, D. Socci N, Tabar V and Lorenz Studer (2008). Human ES cell-derived neural rossetes reveal a functionally distinct early neural stem cell stage. *Genes&Dev*. 22:152-165.

Evans MJ, Kaufman MH (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154-156.

Ernhart CB, Sokol RJ, Martier S et al. (1987) Alcohol teratogenicity in the human: a detailed assessment of specificity, critical period, and treshold. *Am J Obstet Gynecol* 156:33-9.

Fagerlund A, Heikkinen S, Autti-Ramo I (2006) Brain metabolic alterations in adolescents and young adults with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 30:2097-104.

Feng C. Zhou, Yokesh Balaraman, MingXiang Teng, Yunlong Liu, Rabindra P. Singh, Kenneth P. Nephew. Alcohol Alters DNA Methylation Patterns and Inhibits Neural Stem Cell Differentiation. Alchoholism clinical and experimental research (2011). Vol:35 4:735-746.

Fernandez-Lizarbe S, Pascual M, Gascon MS, Blanco A, Guerri C (2008). Lipid rafts regulate ethanol-induced activation of TLR4 signalingin murine macrophage. *Mol Immunol* 45: 2007-2016.

Gallego MJ, Porayette P, Kaltcheva M, Bowen RL, Vadakkadath Meethal S & Atwood CS 2010 The pregnancy hormones hCG and progesterone induce human embryonic stem cell proliferation and differentiation into neuroectodermal rosettes. *Stem Cell Research and Therapy* 1:28.

Galve-Roperh I, Aguado T, Palazuelos J, Guzmán M. Mechanism of control of neural survival by endocannabinoids system. *Curr Pharm Des*. 2008;14(23):2279-88.

Gaiano N and G Fishell. (2002). The role of notch in promoting glial and neural stem cell fates. Annu Rev Neurosci 25:471-90.

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell. 2010 Mar 19;140(6):918-34.

Gomez-Lopez S, Wiskow O, Favaro L, Nikolis S, Price D, Pollard SM, Smith A. Sox2 and Pax6 maintain the proliferative and developmental potential of gliogenic neural stem cells (2011). Glia 59:11 1588-1599.

Goodlett CR, Lundahl KR (1996). Temporal determinants of neonatal alcohol-induced cerebellar damage and motor performance deficits. *Pharmacol Biochem Behav* 55:531-40.

Goodlet CR, Horn KH, Zhou FC (2005). Alcohol teratogenesis: mechanisms of damage and strategies for intervention. Exp Biol Med 230: 394-406.

Gressens P, Lammens M, Picard JJ et al. (1992). Ethanol –induced disturbancec of gliogenesis and neurogenesisn in the developing murine brain: an in vitro and in vivo immunohystochemical and ultrastructural study. *Alcohol Alcohol* 27:219-26.

Graham V, Khudyakov J, Ellis P, Pevny L. (2003). Sox2 function to maintain neural progenitor identity. Neuron. Aug 28;39(5):749-65.

Guasch RM, M Tomas, R Minambres, S Valles, J Renau-Piqueras and C Guerri. (2003). RhoA and lysophosphatidic acid are involved in the actin cytoskeleton reorganization of astrocytes exposed to ethanol. J Neurosci Res 72:487-502

Guerri C, Montoliu C, Renau-Piqueras J. (1994). Involvment of free radical mechanism in the toxic effects of alcohol: implication for fetal alcohol syndrome. *Adv Exp Med Biol* 366:291-305.

Guerri C, Renau- Piqueras J. (1997). Alcohol, astroglia and brain development. *Mol Neurobiol* 15:65-81.

Guerri C. (1998) Neuroanatomical and neurophysiological

mechanisms involved in central nervous system dysfunction induced by prenatal alcohol exposure. *Alcohol Clin Exp Res* 22: 304-12.

Guerri C. (2002) Mechanisms involved in central nervous system dysfunction induced by prenatal exposure. Neurotoxicology Res 4:327-35.

Guerri C, Alissa Bazinet and Edward P. Riley (2002). Foetal Alcohol Spectrum Disorders and alterations in brain and behaviour.*Alcohol and alcoholism* Mar-Apr;44(2):108-14.

Guerri C, Rubert G, Pascual M. (2006) Glial targets of developmental exposure to ethanol. In Miller MW (ed*). Brain Development*. Oxford: Oxford University Press, 295-312.

GR Martin**.** Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *PNAS* Vol. 78, No. 12, pp. 7634-7638, December 1981.

Hao Huang, Rong Xue, Jiangyang Zhang, Tianbo Ren, Linda J.Richards, Paul Yarowsky, Michael I. Miller. (2009). *The journal of Neuroscience*. 29(13): 4263-4273.

Halfon MS, Hashimoto C and Keshishian H. (1995) The Drosofila Toll gene functions zygotically and is necessary for proper motoneuron and muscle development. *Dev. Biol*. 169: 151-167.

Harland R. 2000 Neural induction. *Curr. Opinion in Genetics and Development* 10: 357-362.

Herault O, Colombat P, Domenech J, Deggene M, Bremond JL, Sensebe L, Bernard MC and Binet C (1999). A rapid single laser flow cytometric method for discrimination of early apoptotic cells in a heterogeneous cell population. Br J Haematol 104: 530-537.

Hicks SD, Middleton FA, Miller MW. (2010). Ethanol-induced methylation of cell cycle genes in neural stem cells. *J Neurochem* 114(6):1767-80.

Ikonomidou C, Bittigau P, Ishimaru MJ (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* 287:1056-60.

Inzunza J, Sahlen S, Holmberg K, Stromberg AM, Teerijoki H, Blennow E, Howatta O and Malmgren (2004). Comparative genomic hybridization and of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long term cultivation. *Mol Hum Reprod* 10(6) : 461-466.

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, and Benvenisty N. (2000). Differentiation of

human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol. Med.* Feb;6(2):88-95.

Jessell, TM. (2000). Neuronal specification in the spinal cord: Inductive signals and transcriptional codes. *Nat. Rev. Genet.* 1:20-29.

Jones KL, Smith DW., Ulleland CN and Streissguth AP. (1973) Pattern of malformation in offspring of chronic alcoholic mothers. Lancet 1(7815), 1267-1271.

Jones, AW. (1992) Blood and breath alcohol concentrations.
British Medical Journal 305, 955.

Katherine G Akers, Steven A Kushner, Ana T Leslie, Laura Clarke, Derek van der Kooy, Jason P Lerch, and Paul W Frankland. Fetal alcohol exposure leads to abnormal olfactory bulb development and impaired odor discrimination in adult mice. *Mol Brain* (2011) 4:29.

Kawai T and Akira S (2007) TLR signalling. *Seminars Immunol.* 19:24-32.

Kierstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, Steward O. (2005) Human embryonic stem cell derived oligodendrocytes progenitor cell transplats remyelinate and restore locomotion after spinal cord injury. *J Neurosci* 25:4694-4705.

Kikegarl KA, McGaughy VM, Popovich PG. (2006) A Comparative Analysis of Lesion Development and Intraspinal Inflammation in Four Strains of Mice Following Spinal Contusion Injury. J Comp Neurol. February 1; 494(4): 578–594.

Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, Choi SY, Park K, Kim JS, Akira S, Na HS, Oh SB, Lee SJ. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J Biol Chem. 2007 May 18;282(20):14975-83.

Koch P, Opitz T, Steinbeck JA, Ladewig J, and Brustle O. (2009) A rossete type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. *PNAS* 106(9):3225-30.

Kobel S and Lutolf MP. High-throughpot methods to define complex stem cell niches. (2010) *Focus on Cell Culture Technology Vol 48. No.4*

Kotch LE, and Sulik KK. (1992). Experimental fetal alcohol syndrome: proposed pathogenic basis for a variety of associated craniofacial and brain anomalies. *Am J Med Genet 44*, 168–176.

Maier SE, Chen W, West JR. (1996) The effects of timing and duration of alcohol exposure on development of the fetal brain. In

Abel EL. (ed) *Foetal Alcohol Syndrome*: *From Mechanism to prevention.* Boca Raton, FL:CRC Press, 27-50.

Manuel M and DJ Price. (2005). Role of Pax6 in forebrain regionalization. *Brain Res Bull* 66:387-93.

Manuel M, PA Georgala, CB Carr, S Chanas, DA Kleinjan, B Martynoga, JO Mason, M Molinek, J Pinson, T Pratt, JC Quinn, TI Simpson, DA Tyas, V van Heyningen, JD West and DJ Price. (2007). Controlled overexpression of Pax6 in vivo negatively autoregulates the Pax6 locus, causing cell-autonomous defects of late cortical progenitor proliferation with little effect on cortical arealization. *Development* 134:545-55.

Makriyannis A., Avraham S., Avraham HK. Expression end function of cannabinoid receptors CB1 and CB2 and their cognate cannabinoid ligands in murine embryonic stem cells. *PlosOne* (2007) 2(7):e641.

Martin GR (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Nat. Acad. Sci.* USA 78, 7634-7638.

Mason I (2007). Initiation to end point: the multiple roles of fibroblast growth factors in neural development. *Nature Rev Neurosci* (8):583-96.

May PA, Gossage JP. (2001) Estimating the prevalence of fetal alcohol syndrome: a summary. *Alcohol Res Health* 25:159-67.

McConnell SK. Strategies for the Generation of Neuronal Diversity in the Developing Central Nervous System (1995) *J Neurosci*. (11):6987-98.

Miller MW (1995). Effect of pre- or postnatal exposure to ethanol on the total number of neurons in the trigeminal nerve: cell proliferation and neuronal death. *Alcohol Clin Exp Res* 19:1359-63.

Miller MW (2006). Early exposure to ethanol affects the proliferation of neuronal precursors. *Brain Development. Oxford: Oxford University Press, 182-98.*

Miller MW (2007). Exposure to ethanol during gastrulation alters somatosensory –motor cortices and the underlying white matter in the macaque. *Cereb Cortex* 17:2961-71.

Minambres R, RM Guasch, A Perez-Arago and C Guerri. (2006). The RhoA/ROCK-I/MLC pathway is involved in the ethanol-induced apoptosis by anoikis in astrocytes. *J Cell Sci* 119:271-82.

Miñana R, Climent E, Barettino D (2000). Alchohol exposure alters the expression pattern of neural cell adhesion molecules during brain development. *J Neurochem* 75:954-64.

Molina-Holgado S, et al. (2007). CB2 cannabinoid receptors promote mouse neural stem cell proliferation. *Europian Journal of Neuroscience* 25:629-634.

Mooney SM, Miller MW, Henderson GI (2006) Intracellular events in ethanol- induced neuronal death. *Brain development. Normal Processes and Effects of Alcohol and Nicotine.* Oxford: Oxford University Press, 267-78.

Mooney SM, Miller MW (2007). Time-specific effects of ethanol exposure on cranial nerve nuclei: gastrulation and neurogenesis. *Exp Neurol* 205:56- 63.

Nat R, M Nilbratt, S Narkilahti, B Winblad, O Hovatta and A Nordberg. (2007). Neurogenic neuroepithelial and radial glia cells generated from six human embryonic stem cell lines in serum free suspension and adherent cultures. *Glia* 55:385-99.

Lacoeur H, LM de Oliveira and Gougeon MN (2002). Multiparametric flow cytometric analysis of biochemical and functional events associated with apoptosis and oncosis using the 7-aminoactinomycin D assay. J Immunol Methods 265:81-96.

Larsen P, Holm T, Owens T (2007) Toll-like receptors in brain development and homeostasis. *Science STKE Issue 402:47.*

Lathia JD et al. (2007) The microenvironment of the embryonic neural stem cell: lessons from adult stem cell niches? *Dev. Dyn.* 236, 3267-3282.

Lathia JD., Okun E, Tang SC. et al. (2008) Toll-like receptor 3 is a negative regulator of embryonic neural progenitor cell proliferation. *J. Neurosci*. 28, 13978–13984.

Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC (2005). Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23: 215-221.

Li XJ, Zhang SC. In vitro differentiation of neural precursors from human embryonic stem cells from (2006). *Methods Mol Biol*. 331:169-77.

Li XJ, Hu BY, Jones SA, Zhang YS, Lavaute T, Du ZW, Zhang (2008) Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. *Stem Cells* 26:886-893.

Lujan E, Chanda S, Ahlenius H, Sudhof T and Wernig M. (2012) Direct conversion of mouse fibroblasts to self-renewing, tripotent neural precursor cells. *PNAS* 109 | no. 7 | 2527–2532.

Lindvall O and Kokaia Z (2006). Stem cells for the treatment of neurological disorders. *Nature* 441(7097):1094-6.

Ludwig TE, Bergendahl V, Levenstein ME, Yu J, Probasco MD, Thomson JA. (2006) Feeder-independent culture of human embryonic stem cells. *Nat Methods* 3:637-46.

Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piecarczyk MS, Llanas RA, Thomson JA. Derivation of human embryonic stem cells in defined conditions. *Nat Biotecnol* 24: 185-7, 2006.

Luo L. (2002). Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annu Rev Cell Dev Biol* 18:601-35.

O'Hare ED, Kan E, Yoshii J et al. (2005) Mapping cerebellar vermal morphology and cognitive correlatos in prenatal alcohol exposure. *NeuroReport* 16:1285-90.

Okun E, Griffoen KJ, Lathia JD, Tang SC, Matson MP and Arumugam TV. (2008) Toll-like receptors in neurodegeneration. *Brain Res. Rev.* 59(2): 278-292.

Okun E et al. (2010). TLR2 activation inhibits embryonic neural progenitor cell proliferation. *J. Cell Biol.* 9, 1081-1088.

Okun E, Griffioen KJ and Mattson MP. (2011). Toll-like receptor signaling in neural plasticity and disease. *Trends in Neurosciences,* Vol.34, No5.

Olney JW (2004) Fetal alcohol syndrome at the cellular level. *Addict Biol* 9: 137-49.

O'Rahilly R, Muller F. (1994) Neurulation in the normal human embryo. *Ciba Found Symp* 181: 70-82.

O'Shea KS. Embryonic stem cells model in development. *Anat Rec*. 1999 Feb 15;257(1):32-41.

Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M. Pax6 Transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. *STEM CELLS 2008;26:1663-1672*

Pascual M, Valles SL, Renau-Piqueras J (2003) Ceramide pathways modulate ethanol-induced cell death in astrocytes. J Neurochem 87:1535-45.

Palazuelos J, Aguado T, Egia A, Mechoulam R, Guzman M, Galve-Roperh I. Non-Psychoactive CB2 cannabinoid agonists stimulate neural progenitor proliferation. *FASEB J*. 20, E1773-E1779. (2006).

Park JH, Kim SJ, Oh Ej, Moon SY, Roh SI, Kim CG and Yoon HS 2003. Establishment an maintainance of human embryonic stem cells on STO, a permanently growing cell line. *Biology of reproduction*. 69 200-2014.

Paul LK, Brown WS, Adolphs R (2007). Agenesis of the corpus callosum: genetic, developmental and functional aspects of connectivity. *Nat Rev Neurosci* 8:287-99.

Paul H Lerou, Akiko Yabuuchi, Hongguang Huo, Justine D Miller, Leah F Boyer,Thorsten M Schlaeger and George Q Daley. (2008). Derivation and maintenance of human embryonic stem cells from poor-quality in vitro fertilization embryos. *Nat. Protocols* 3: 923-933.

Pava MJ, Woodward JJ (2012) A review of the interactions between alcohol and the endocannabinoid system: implications for alcohol dependence and future directions for research. *Alcohol* May; 46(3):185-204.

Perrier AL, Tabar V, Barbery T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L. (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA* 101: 12543-12548.

P.M.P. Yean (1989). Fetal Alcohol Syndrome. *Journal of the Hong Kong Medical Association* Vol.41, No. 1, 1989.

Popovic M, Caballero-Bleda M, Guerri C. (2006) Adult's rats offspring of alcoholic mothers are impaired on spatial learning and object recognition in the Can test. *Behav Brain Res* 174:101-11.

Quinn JC, M Molinek, BS Martynoga, PA Zaki, A Faedo, A Bulfone, RF Hevner, JD West and DJ Price. (2007). Pax6 controls cerebral cortical cell number by regulating exit from the cell cycle and specifies cortical cell identity by a cell autonomous mechanism. Dev Biol 302:50-65.

Richards LJ, Plachez C, Ren T. (2004). Mechanisms regulating the development of the corpus callosum and its agenesis in mouse and human. *Clin Genet* 66: 276-89.

Rifas L, Towler DA, Avioli LV (1997). Gestational exposure to etanol suppresses msx2 expression in developing mouse embryos. *Proc Natl Acad Sci* USA 94: 7549-54.

Riley EP, Mattson SN, Sowell ER (1995). Abnormalities of the corpus callosum in children prenatally exposed to alcohol. *Alcohol Clin Exp Res* 19:1198-202.

Romero AM, Esteban-Pretel G, Marin M, Ponsoda HX, Ballestin R, Canales JJ, and Renau-Piqueras J (2010). Chronic Ethanol Exposure Alters the Levels, Assembly, and Cellular Organization of the Actin and Microtubules in Hyppocampal Neurons in Primary Culture *Toxicol Sci.* 118(2):602-12.

Rolls A, Shechter R, London A, Ziv Y, Ayal R, Levy R, Schwartz M. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. *Nat. Cell Biol*. 9, 1081-1088.

Rose D, Zhu X, Kose H, Hoang B, Cho J and Chiba A (1997) Toll, a muscle cell surface molecule, locally inhibits synaptic initiation of the RP3 motoneuron growth cone in Drosofila. *Development*. 124: 1561-1571.

Rosler ES, Fisk GJ, Ares X, Irving J, Miura T, Rao MS, Carpenter MK. (2004) Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn.* 229(2):259-74.

Rubert G, Miñana R, Pascual M et al. (2006) Ethanol exposure during embryogenesis decreases the radial glial progenitorpool and affects the generation of neurons and astrocytes. *J Neurosci Res* 84: 483-96.

Sansom SN, DS Griffiths, A Faedo, DJ Kleinjan, Y Ruan, J Smith, V van Heyningen, JL Rubenstein and FJ Livesey. (2009). The level of the transcription factor Pax6 is essential for controlling the balance

between neural stem cell self-renewal and neurogenesis. PLoS Genet 5:e1000511.

Schoenwolf GC, Smith JL (2000). Mechanisms of neurulation Meth Mol Biol 136: 125-134.

Siegenthaler JA, Miller (2006) Mechanism of ethanol-induced alterations in neuronal migration. *Brain development. Normal Processes and Effects of Alcohol and Nicotine*. Oxford: Oxford University Press, 216-29.

Shechter R, Ronen A, Rolls A, London A, Bakalash S, Young MJ, Schwartz M. Toll-like receptor 4 restricts retinal progenitor cell proliferation. (2008) JCB: Vol.183 No3 393-400.

Shin S, Dalton S, Stice SL (2005) Human motor neuron differentiation from human embryonic stem cells. *Stem Cells Dev* 14:266-269.

Shin S, Mitalipova M, Noggle S, Tibbitts D, Venable A, Rao R, Stice SL. (2006) Long term proliferation of human embryonic stem cell-derived neuroepithelial cells using defined adherent culture conditions. *Stem Cells* 24:125-138.

Sloane JA et al. (2010) Hyaluronan blocks oligodendrocyte progenitors maturation and remyelination through TLR2. *Proc. Natl. Acad. Sci. USA*. 107, 11555-11560.

Sokol RG, Delaney-Black V, Nordstrom B. (2003) Fetal alcohol spectrum disorder. *JAMA* 290:2996-9.

Somoza RA, Gallego Rubio FJ (2012). Cell therapy using pluripotent stem cells. *Curr Stem Cell Res Ther.* 7(3):191-6.

Sordella R and Van Aelst L. Driving Actin Dynamics under theInfluence of Alcohol. *Cell* 127(1):37-9.

Sowell ER, Jerningan TL, Mattson SN et al. (1996) Abnormal development of the cerebellar vermis in children prenatally exposed to alcohol: size reduction in lobules I-V. *Alcohol Clin Exp Res* 20: 31-4.

Sowell ER, Thompson PM, Mattson SN et al. (2002a). Regional brain shape abnormalities persist into adolescente persists into adolescente after heavy prenatal alcohol exposure. *Neuro Image* 17:1807-19.

Sowell ER, Thompson PM, Peterson BS et al. (2002b). Mapping cortical gray matter asymmetry patterns in adolescents with heavy prenatal alcohol exposure. *Neuro Image* 17:1807-19.

Spohr HL, Willms J, Steinhausen HC. (2007) Fetal alcohol spectrum disorders in young adulthood. *J Pediatr* 150: 175:-9.

Stadtfeld M and Hochedlinger (2011) Induced pluripotency: history, mechanisms and applications. *Genes Dev* 24:2239-2263.

Stojkovic M, Lako M, Strahnan T, Murdoch A. Derivation , growth and applications of human embryonic stem cells. *Reproduction* (2004) 128 259-267.

Steiner D, Khaner H, Cohen M, Even-Ram S, Gil Y, Itsykson P, Turetsky T, Idelson M, Aizenman E, Ram R, Berman-Zaken Y, Reubinoff B. Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension. Nat Biotechnol. 2010 Apr;28(4):361-4.

Streissgut AP, Aase JM, Clarren SK et al. (1991) Fetal alcohol syndrome in adolescent and adults. *JAMA* 265: 1961-7.

Stratton K, Howe C, Battaglia F (1996). Foetal alcohol syndrome: diagnosis, epidemiology, prevention and treatment. *Washington DC: National Academy Press*

Sulik KK. (2005) Genesis of alcohol-induced craniofacial dysmorfysm. *Exp Biol Med* 230:366-375.

Suzuki K. (2007) Neuropathology of developmental abnormalities. *Brain Dev* 29: 129-41.

Swayze VW, Johnson VP, Hanson JW et al. (1997). Magnetic resonance imaging of brain anomalies in fetal alcohol syndrome. *Pediatrics* 99:232-40.

Takashi K and Yamanaka. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676.

Tammi Taylor , Young-June Kim , Xuan Ou , Wilbert Derbigny , and Hal E. Broxmeyer. (2010) Toll-Like Receptor 2 Mediates Proliferation, Survival, NF-κB Translocation, and Cytokine mRNA Expression in LIF-Maintained Mouse Embryonic Stem Cells. *Stem Cells and Dev*. Volume 19, Number 9

Tateno M, Ukai W, Yamamoto M, Hashimoto E, Ikeda H, Saito T. The effect of ethanol on cell fate determination of neural stem cells. *Alcohol Clin Exp Res*. 2005 Dec; 29 (12 Suppl): 225S-9S.

Thomas JD, Melcer T, Weinert S et al. (1998) Neonatal alcohol exposure produces hyperactivity in high alcohol sensitive (HAS) but not in low alcohol sensitive (LAS) rats. *Alcohol* 16:237-42.

Tenkova T, Young C, Dikranian K (2003). Ethanol-induced apoptosis in the developing visual system during synaptogenesis. *Invest Ophthalmol Vis Sci* 44: 2809-17.

Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145-1147.

Thomson JA (2007) . Induced pluripotent stem cell lines derived from numan somatic cells. *Science* VOL 318.

Toft-Hansen H, Millward JM, Landman R, Rivest S, Finsen B and Owens T. Toll-Like Receptor 2 Signaling in Response to Brain Injury: An Innate Bridge to Neuroinflammation. The Journal of Neuroscience, December 6, 2006 • 26(49):12826 –12837

Valanne S, et al. (2011). The Drosophila signaling pathway. J. Immunology 186, 649-656.

Vallés S, Pitarch J, Renau-Piqueras J, Guerri C. Ethanol exposure affects glial fibrillary acidic protein gene expression and transcription during rat brain development. J Neurochem. 1997 Dec;69(6):2484-93.

Valles S, Pitarch J, Renau-Piqueras (1997). Ethanol exposure affects glial fibrillary acidic protein gene expression and transcription during rat brain development. *J Neurochem* 69:2484-93.

Warren N, D Caric, T Pratt, JA Clausen, P Asavaritikrai, JO Mason, RE Hill and DJ Price. (1999). The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. Cereb Cortex 9:627-35.

Webster WS, Lipson AH, Sulik KK (1988). Interference with gastrulation during the third week of pregnancy as a cause of some facial abnormalities and CNS defects. *Am J Med Genet*. (3):505-12.

Weiss B (2000). Vulnerability of children and developing brain to neurotoxic hazards. *Environ Health Prospect*.108:(3) 175-81.

Wilkemeyer MF, Menkari CE, Charnes ME. (2002) Novel antagonists of alcohol inhibition of L1- mediated cell adhession: multiple mechanism of action. *Mol Pharmacol* 62:1053-60.

Wilson PG and Stice SS (2006). Development and differentiation of neural rosettes derived from human embryonic stem cells. *Stem Cell Rev*. 2(1):67-77.

Wozniak DF, Hartman RE, Boyle MP (2004) Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. *Neurobiol Dis* 17:403-14.

Ying QL, Smith AG. Defined conditions for neural commitment and differentiation. *Methods Enzymol*. 2003;365:327-41.

Young C, Olney JW (2005). Neuroapoptosis in the infant mouse brain triggered by a transient small increase in blood alcohol concentration. *Neurobiol Dis* 22:548-54.

Zampetaki A, Xiao Q, Zeng L, Y Hu, Q Xu. (2006). TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulating by epigenetic regulations. *Biochemical and Biophisical Research Communication*. Vol. 347: 89-99.

Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol.*19:1129–1133.

Zhang SC (2006) Neural subtype specification from embryonic item cells. *Brain Pathol* 16: 132-142.

Zhang H, Dai S, Bi J and Liu KK (2011) Biomimetic three dimensional microenvironment for controling stem cell fate. *Interface focus* 5:792-803.

Zhang X, Huang TH, Chen J Pankratz MT, Li X, Ayala M, Bondarenko GI, Du ZW, Jin Y, Golos T and Zhang SC (2012). Pax6 Is a Human Neuroectoderm Cell Fate Determinant *Cell Stem Cell* 1: 90-100.

Zhou FC, Chen Y, Love A. (2011). Cellular DNA methylation program during neurulation and its alteration by alcohol exposure. *Birth Defects Res A Clin Mol Teratol*. 91(8):703-15.