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ALGINATE-BASED HYDROGELS FOR CENTRAL NERVOUS SYSTEM TISSUE REGENERATION

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Declaration

I, Angela Bozza, confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signature of the PhD Candidate:

Signature of the Tutor:

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LIST OF ABBREVIATIONS

ALG: Alginate

ALS: Amyotrophic Lateral Sclerosis

BBB: Blood-brain barrier

BDNF: Brain Derived Neurotrophic Factor

BLI: Bioluminescence

CNS: Central Nervous System

DAPI: 4',6-diamidino-2-phenylindole

DG: Dentate Gyrus

ECM: Extracellular Matrix

EGF: Epithelial Growth Factor

FGF: Fibroblast Growth Factor

FN: Fibronectin

GDL: Glucono – delta - lactone

GDNF: Glial derived Neurotrophic Factor

GFAP: Glial fibrillary acidic protein

HA: Hyaluronic Acid

IP: Ischemic penumbra

MAP2: Microtubule-associated protein 2

ESCs: Embryonic Stem Cells

NSCs: Neural Stem Cells

NCAM: Neural cell adhesion molecule

PNS: Peripheral Nervous System

PSD-95: Post-synaptic density 95

RGD: arginine – glycine - aspartic acid

SVZ: Subventricular zone

TIA: Transient ischemic attack

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor- α

VAMP2: Vesicle-associated membrane protein 2

VEGF: Vascular Endothelial Growth Factor

WFA: Wisteria Floribunda Agglutinin

ßIIItub: ß-III Tubulin

ABSTRACT

As the central nervous system shows very little capability for self-repair following injury, regenerative medicine approaches are increasingly interested in the use of stem cells for cell replacement strategies. Biomaterials are an interesting tool to carry out this type of therapies. They allow three-dimensional cultures for stem cells differentiation and are helpful in order to obtain cells at the right developmental stage for transplantation. Moreover, they could help to enhance and control cell survival after transplantation, minimizing cell death.

Stroke is a very severe form of brain injury and one of the leading causes of death worldwide, as no effective cures are available. Several studies show that neural stem cells (NSCs) are able to integrate and improve functional recovery once transplanted in stroke animal models. However, the majority of the grafted NSCs die within weeks after transplantation, limiting treatment efficacy. Tissue engineering approaches aim to restore tissue functions combining principles of cell biology and engineering, using designed and tailored three-dimensional biomaterial scaffolds.

In this study we tested alginate as candidate biomaterial for neural tissue repair. We studied its ability to support mouse embryonic stem cells (mESCs) neural differentiation *in vitro*. We evaluated whether changes in its concentration or modifications with extracellular matrix components could influence cell differentiation, analysing the mechanical and physical properties of the generated scaffolds.

In the first part, we evaluated the suitability of alginate as a scaffold for three-dimensional cultures able to enhance differentiation of mESCs towards neural lineages. We tested whether encapsulation of mESCs within alginate beads could support and/or enhance neural differentiation with respect to two-dimensional cultures. We encapsulated cells in beads of alginate at two different concentrations, with or without modification by fibronectin, RGD peptide or hyaluronic acid. Cells survive and differentiate inside our scaffolds, forming clusters. Gene expression analyses showed that cells grown in alginate scaffolds increase differentiation toward neural lineages with respect to the two dimensional controls. Immunocytochemistry analyses confirmed these results, further showing terminal differentiation of neurons by the expression of synaptic markers. Cells showed also the capability to form networks among themselves and with cells of other clusters. All the scaffolds we prepared resembled brain tissue characteristics, thus we decided to test alginate as potential support for tissue engineering approaches in the injured brain.

In the second part of the work we tested alginate as support for NSCs injection in the brain. We evaluated *in vivo* crosslinking of alginate after injection, and verified inflammation levels due to its presence in mouse brain tissue. Our preliminary studies suggest that alginate polymerizes *in vivo*, forming a hydrogel, and that it does not elicit any inflammatory response following injection.

Our data show that alginate, alone or modified, is a suitable biomaterial to promote *in vitro* differentiation of pluripotent cells toward neural fates. Moreover, it could be used as injectable hydrogel for brain tissue regeneration. We plan to co-inject alginate with NSCs in stroke mouse models in order to enhance viability and integration of the engrafted cells in the damaged tissue. We plan to study alginate permanence in the brain and NSCs viability, integration and capability to stimulate regeneration after ischemic injury.

1. INTRODUCTION

1.1 The central nervous system

1.1.1 Central nervous system (CNS)

The nervous system is the organ system which receives, transmits and elaborates internal and external stimuli through complex networks of specialized cells. It is divided into central (CNS) and peripheral nervous system (PNS). The CNS is composed by the brain, the most complex part, the spinal cord and by the optic, olfactory and auditory systems, and it is responsible for the control and coordination of organs and functions in the body.

Neurons are the functional elements of this system. These electrically excitable cells sense, process and transmit signals to other cells. From the cell body (soma) of a neuron originate several dendrites, that receive signals from afferent neurons and carry them to the cell body, and a single axon that can extend through long distances and carries signals to the axon terminal. In this region, the axon of a pre-synaptic neuron takes contact with other post-synaptic neurons through junctions called synapses, in which chemical signals (neurotransmitters) are released in order to excite, inhibit or modulate the post-synaptic neuron.

The nervous system also contains many supporting cells, the glial cells. They are involved in the homeostasis of the tissue, support and protection of neurons, and myelin production. Glial cells are subdivided in microglia and macroglia. The microglia, present only in the CNS, is composed by resident innate immune cells involved in immune response and inflammation. The macroglial cells present in the CNS are of different types. The astrocytes or astroglia are the most abundant type, important for their trophic and structural support to neurons. They induce synapses formation, function and plasticity (Eroglu and Barres, 2010; Ullian et al., 2004; Ullian et al., 2001), regulate the turnover of neurotransmitter molecules thus modulating synaptic strength and activity (Colangelo et al., 2014; Pellerin et al., 2007; Simard and Nedergaard, 2004). Through interactions with endothelial cells of blood capillaries, they control the blood-brain barrier formation and support, and the related blood flow within the tissue (Attwell et al., 2010; Mulligan and MacVicar, 2004; Zonta et al., 2003). The oligodendrocytes are the cells that produce the *myelin sheat* around the axons, allowing the propagation of electrical signals; whereas the ependymal cells line the ventricular system.

1.1.2 CNS Development

During embryogenesis three germinal layers form: the inner endoderm; the mesoderm in the middle and the outer ectoderm, which gives rise to neural tissue, neural crest cells and epidermis. In the dorsal part of the ectoderm a region acquires neural properties and forms the neural plate, a single-layered pseudo-stratified epithelium. Right after its formation, the neural plate starts to fold on itself into a tubular structure, the neural tube, which eventually will give rise to the brain and the spinal cord (Lawson, 2009). The cell populations in the neural tube undergo patterning and their neural differentiation is promoted by different molecules. During gastrulation, ectodermal cells are allowed to differentiate into neuroectoderm by the action of bone morphogenetic proteins (BMPs) pathway inhibitors, (chordin, noggin and follistatin) secreted by cells of a specialized region called the Organizer, and inhibitors of the Wnt signaling pathway (Dickopf, frzb and Cerberus). These factors, in combination with fibroblast grow factors (FGFs) and Activin/Nodal pathway inhibitors, regulate the processes that lead to neural differentiation (neurogenesis) (Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1997; Hemmati-Brivanlou and Melton, 1994; Levine and Brivanlou, 2007; Stern, 2006).

1.1.3 Embryonic and adult neurogenesis

Embryonic neurogenesis begins with the formation of the neural tube. Here, neuroepithelial cells (or neural stem cells) extend from the luminal part to the outside surface with their nuclei positioned at different heights, mimicking a multilayered structure (pseudo-stratified epithelium). Nuclei movements are related to cell cycle. During the S phase, nuclei are located at the outside edge of the neural tube and they migrate luminally as the cell cycle proceeds (Fig.1) (Paridaen and Huttner, 2014).

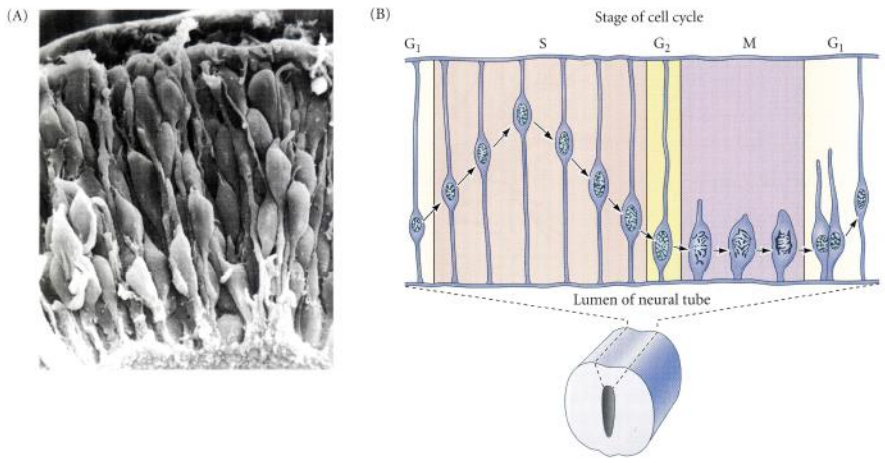

Fig. 1 *Neuroepithelial cells (neural stem cells) in the germinal epithelium*. **A**) SEM image of the chick neural tube; **B**) Scheme of the nuclei of neuroepithelial cells in the neural tube as function of the cell cycle stage, (Gilbert, 2010).

Neuroepithelial stem cells give rise to cells that retain their stemness by remaining connected with the luminal surface (symmetric division), or to daughter cells that migrate and further differentiate (asymmetric division). The timings of the asymmetric divisions are different and specific for the type of neuron or glial cell which will later originate. Neurons are indeed generated during embryonic and early postnatal stages while gliogenesis (generation of glia) starts late in embryonic development and continues in postnatal stages (Guerout et al., 2014).

Sox1 is the earliest marker for neural precursors identified in mouse embryos at neural plate and neural tube stages, whereas in human embryos Pax6 is the first, preceding Sox1 activation (Conti and Cattaneo, 2010). When development proceeds, neuroepithelial cells lose Sox1 expression and acquire Sox2, Nestin and Pax6 expression, becoming radial glia cells. These bipolar-shaped cells share characteristics with both neuroepithelial cells and astrocytes, expressing markers such as radial glial marker-2 (RG-2), glial fibrillary acidic protein (GFAP) and vimentin (Gotz and Huttner, 2005; Solozobova et al., 2012). At the onset of neurogenesis, RGCs switch from symmetric to asymmetric divisions, giving rise to an RGC daughter cell and a differentiating cell which can become either an astrocyte, an oligodendrocyte or a neuron. In addition, they act as scaffold for newly generating neurons, which migrate along their fibres in order to reach their right final destination. In fact, the disruption of radial glia integrity harms the spatiotemporal differentiation pattern, since both neuronal migratory activity and maturation result impaired (Sizonenko et al., 2007). In many lower vertebrates radial glia persists during adulthood, whereas these cells differentiate into astrocytes in most CNS regions of adult mammals (McDermott et al., 2005). However, radial glia cells are present in neurogenic niches where they are involved in neurogenesis processes, acting as stem cells (Barry et al., 2014; Dimou and Gotz, 2014).

As neurons are post-mitotic cells unable to proliferate, it has been believed for a long time that neurogenesis does not take place in the mammalian postnatal CNS. Contrasting findings by Altman and colleagues dated in the 1960 first demonstrated that neurogenesis can also occur in the adult brain, though limited to two forebrain regions: the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus (Altman, 1962). These two neurogenic regions are the niches in which adult neural stem cells reside and where they are activated by many physiological stimuli (maintenance function) and pathological states (reparative function) in order to stimulate regenerative processes (Martino et al., 2011; Urban and Guillemot, 2014). The dentate gyrus (DG) of the hippocampus supports the generation of new granular neurons during life, in several mammalian species (Kempermann and Gage, 2000; Ming and Song, 2005; Shapiro and Ribak, 2005; Zhao et al., 2006), from rodents (Ernst et al., 2014) to primates (Gould et al., 1999; Kornack and Rakic, 1999) and humans (Eriksson et al., 1998)(Eriksson et al., 1998b). Its neurogenic

potential is involved in memory and learning processes (Deng et al., 2010). In rodents, NPCs in the subventricular zone (SVZ) generate neurons which migrate into the olfactory bulb (Carleton et al., 2003; Doetsch et al., 1999; Doetsch et al., 1997; Lois and Alvarez-Buylla, 1994), whereas in humans this migration process seems to be directed to the striatum (Ernst et al., 2014; 2015) . The newly generated neurons are important in rodents for odours discrimination and in humans for pattern separation in memory in order to distinguish and store similar experiences as different memories (Spalding et al., 2013). Alterations in adult neurogenesis have been indeed associated with neurodevelopmental and neurodegenerative diseases (i.e Alzheimer's disease) and with psychiatric conditions in humans (Ernst et al., 2014; Steiner et al., 2006). Adult neural stem cells demonstrated the ability to generate both neurons and glia when isolated and cultured *in vitro*, and the possibility to integrate into pre-existing neural circuits contributing to brain functions (Ming and Song, 2005; Murrell et al., 2013; Taupin, 2006).

Despite the presence of this neurogenic potential during adulthood, the brain is in any case not capable of self-repair following trauma or injury (Kelamangalath and Smith, 2013). After injury the survival of the newly generated neurons is low, due to both intrinsic factors and to the environment, which is not permissive for neurogenesis as it is during embryonic development. In addition, there is an age-related decrease in neurogenic potential, due to the depletion of the self-renewing cell populations which already starts right after brain development is completed (Ahlenius et al., 2009; Sanai et al., 2011). Following an insult, axonal regrowth is impaired by up-regulation of neuronal growth inhibitors and formation of a physical barrier (glial scar) by reactive neuroglia (Pekny and Nilsson, 2005; Pekny et al., 2014).

1.2 Central Nervous System Injuries

1.2.1 Stroke

Injuries to the CNS can be caused by different types of insult such as infections, hypoxia, ischemia (stroke), acute trauma or degenerative diseases (Li et al., 2012).

Stroke is one of the most severe forms of brain injury, the third leading cause of death worldwide and the major cause of disability (Donnan et al., 2008). Thanks to high blood pressure control its incidence is decreasing in developing countries, but globally the number of cases is increasing, mainly because of the ageing population, also leading to a high economic impact on the society.

Stroke is caused by the interruption of blood supply and it is classified on the base of the starting event in ischemic stroke, hemorrhagic stroke or transient ischemic attack (TIA). About 80% of strokes are due to ischemia following the occlusion of a major artery in the brain. This can be caused by a blood clot (thrombotic, 50% of all strokes) or by an embolus

formed somewhere in the body and that travels up to the brain (embolic). The hemorrhagic stroke is caused by the burst of a blood vessel with consequent bleeding in the tissue. In the TIA the interruption of blood supply is temporary and stroke-like symptoms resolve in less than 24 hours, but it is considered a warning sign for a stronger stroke event (Donnan et al., 2008; Hinkle and Guanci, 2007).

1.2.2 Consequences and responses to an ischemic stroke

The outcomes of a stroke depend on which part of the brain is interested, how severely the brain is damaged and other factors (i.e. collateral circulation). Stroke can cause reduced capability in sensory processing, communication, cognition and motor functions, with consequent reduced quality of life in patients. Although in few patients there is a weak recovery of some lost functions, in the majority of the cases the functional circuitry disruption results in long-term functional disability.

The lack of blood supply decreases the oxygen and nutrients available for the cells which suffer and die, impairing the overall tissue functions. Stroke typically leads to tissue infarction with non-selective death of all cell types present in the affected area. After an ischemic insult cells are exposed to an inhospitable environment with growth-inhibitory factors and without growth-supporting molecules. In the core region, where the blood flow is most severely impaired, high cell death rates due to hypoxia create irreversible injuries in the tissue. Between the core region and the healthy tissue there is a less affected region, the ischemic penumbra (IP). Vessels adjacent to the site of the injury contribute with small perfusion, thus IP presents a functionally impaired but structurally intact tissue and a partially preserved metabolism. In this region cells remain viable for several hours but, as time elapses, its extension decreases (Donnan et al., 2008).

The loss of neurons and/or glial cells is due to many mechanisms triggered by reduced oxidative metabolism and consequent changes in energy-related metabolites. The impairment of glucose, oxygen and essential substrates delivery leads to the consumption of all ATP present in the brain without any new production, causing disruption of ionic gradients and the consequent depolarization of the plasma membrane, decrease of intracellular potassium and accumulation of calcium (Ca^{2+}) in the cell. The low levels of oxygen availability lead to anaerobic glycolysis and accumulation of lactate that is not removed by the impaired blood flow, resulting in a decrease in pH (Sims and Muyderman, 2010).

At the pre-synaptic level the depolarization activates voltage-dependent channels that release excitatory amino acids which accumulate in the extracellular space, due to impaired energy-dependent pre-synaptic uptake. This accumulation results in an excitotoxic effect in which the major player is glutamate. In fact, it over-stimulates its α-amino-3-hydroxy-5-

methyl-4-isoxazole propionic acid (AMPA) and N-methyl-d-aspartic acid (NMDA) receptors on other neurons, causing their depolarization (Won et al., 2002). Consequently more Ca^{2+} enters the cell and more glutamate is released, amplifying the initial ischemic insult and leading cells to apoptosis (Fig.2) (Won et al., 2002).

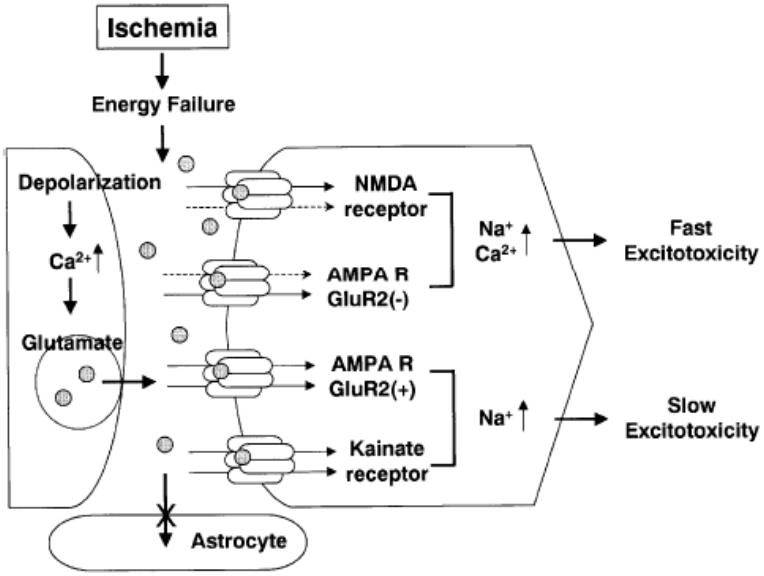

Fig. 2 Scheme of excitotoxic neuronal death in hypoxic-ischemic brain injury, (from Won et al., 2002).

High intracellular Ca^{2+} levels activate many calcium-dependent enzymes such as proteases, endonucleases or enzymes involved in the generation of free radicals (ROS). ROS activate enzymes that degrade macromolecules and bring cells to death (Won et al., 2002). Moreover, as neurons lack endogenous antioxidants, they are highly susceptible to this type of stress (Swanson et al., 2004). Oxidative stress interferes also with blood-brain barrier (BBB) integrity, involved in the protection of neuronal microenvironment, by the activation of metalloproteases (MMPs) which degrade collagen and laminins in the basal lamina. In addition, the recruitment of leucocytes by reactive astrocytes increases BBB disruption and permeability, causing brain edema (Brouns and De Deyn, 2009; Doyle et al., 2008). High calcium concentrations attract water in the cells, causing cytotoxic edema.

Later after stroke, inflammation arises from activated microglia, astrocytes and other cell types of the immune system, which release both pro- and anti-inflammatory modulators, increasing the complexity of events (Brouns and De Deyn, 2009; Lakhan et al., 2009). The first cells to be activated are microglia cells which transform into phagocytes that can get rid of cellular debris and release pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukins (IL-1, IL-6), ROS and nitric oxide (NO). However, they also stimulate neuroprotection by producing molecules such as BDNF and insulin-like growth factor I (IGF-I) (Lakhan et al., 2009).

In the *reactive astrogliosis* process that follows brain injury, activated astrocytes undergo important changes in morphology, gene expression and functions. They become hypertrophic and upregulate the expression of the intermediate filaments GFAP and vimentin, and of markers characteristic of NSCs, NPCs and radial glia such as nestin, brain lipid-binding protein (BLBP), DSD1 proteoglycan, CD15 and tenascin-c (Tn-C). Based on their distance from the lesion and consequent expression of these proteins, they are subdivided in subpopulations, as shown in Fig.3 (Roll and Faissner, 2014). In the presence of severe injuries some of them can re-enter the cell cycle, proliferate and de-differentiate (Robel et al., 2011; Roll and Faissner, 2014), as also demonstrated by their neurosphere-forming potential *in vitro* (Buffo et al., 2008).

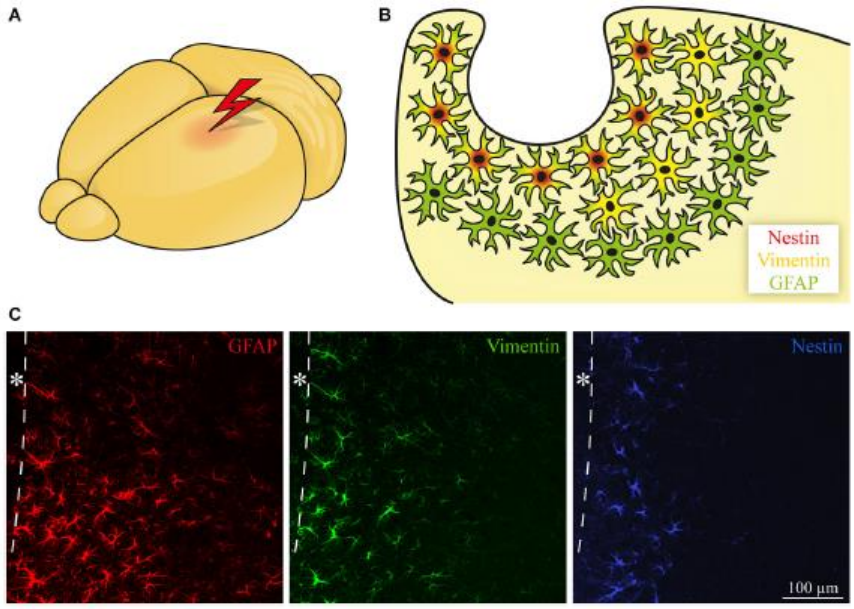

Fig. 3 *Astrocytes activation following brain damage* (**a,b**). The position with respect to the lesion reflects in marker expression, with Nestin expressed near the lesion, Vimentin in a broader area and GFAP with a widespread upregulation, as shown in the scheme (**b**) and by immunohistochemical stainings (**c**). * stroke area. (Roll and Faissner, 2014).

Activated astrocytes form the glial scar, characterized by both beneficial and adverse effects. It is very important in early phases after stroke onset as a barrier for the healthy tissue, preventing extensive bleeding and further tissue loss (Pekny and Nilsson, 2005; Pekny et al., 2014). Following injury, astrocytes are also a support for neurons, protecting them from ROS damage and contributing to glutamate re-uptake through their glutamate transporters GLAST and GLT-1 (Barreto et al., 2011). Evidences of the beneficial role of activated astrocytes and glial scar come from GFAP, vimentin or astrocytes depletion studies in several injury models, including stroke, which report increased tissue damage, lesion size and neuronal loss, but not increased recovery (Nawashiro et al., 2000; Robel et al., 2011) (Li et al., 2008). However, reactive astrogliosis is also considered a physical and

chemical barrier for regenerative processes. Negative outcomes are associated to a prolonged glial scar permanence, astrocytes activation and secretion of molecules (e.i. chondroitin sulphate proteoglycans, CSPGs) which inhibit axonal regrowth (Barreto et al., 2011).

Astrocytes also upregulate the expression of cell adhesion molecules (CAMs) which mediate circulating cell interactions with the vascular endothelium, resulting in blood cell infiltration. Circulating leukocytes adhere to vessel walls and migrate into the brain accumulating and obstructing microcirculation, releasing pro-inflammatory modulators including ROS and proteolytic enzymes (Huang et al., 2006).

Finally, neurogenesis is also activated in response to brain injury, as demonstrated by NSCs increased proliferation and the upregulation of some signalling pathways (epithelial growth factor EGF, vascular endothelial growth factor, VEGF and FGF) and ECM components typical of NSC niches (Yi et al., 2013). In the SVZ new neuroblasts are generated and start migrating along vessels towards the lesion thanks to gradient of molecules produced by glial and inflammatory cells (Young et al., 2011).

1.2.3 Treatments after brain injury

No long-term effective clinical treatments are available for cerebral ischemia. Treatment with tissue-type plasminogen activator (t-Pa) in order to induce thrombolysis to limit the acute effects of stroke is the most effective approach in routine clinical use. However, it is characterized by a very short time window for administration in order to obtain efficient outcomes (within 3 hours from the stroke onset), which limits the number of patients that can benefit from it. Moreover, it can lead to intracranial hemorrhage, a side effect registered in about 6-7% of the cases (Marler, 2006; Murray et al., 2010; Wardlaw et al., 2003). Patients can also undergo physical therapies in order to restore motor functions, but the majority of them do not improve their disability (Li et al., 2012)(Wang et al., 2014). Also the oral administration of aspirin within 48 hours from the stroke onset is associated to reduced damage (Donnan et al., 2008). Several neuroprotective drugs have been tested, but the majority has failed in clinical trials (Lapchak et al., 2011). Recently, glutamate receptors have been tested as therapeutic targets and some studies demonstrate that pharmacological blockade of ionotropic glutamate receptors helps in reducing ischemic damage (Besancon et al., 2008; Doyle et al., 2008; Mehta et al., 2007). Therapeutic approaches for stroke treatment also focus on targeting the glial scar, mostly through its modulation rather than its suppression, considering its important role in the early phases after injury (Shen et al., 2014).

Currently, great importance is given to the development of approaches aimed to stimulate and support endogenous neurogenesis, which occurs after brain injury but is not sufficient for the recovery of neural tissue integrity and functions.

1.3 Neural tissue engineering and regenerative medicine

1.3.1 Regenerative medicine

As just described, the CNS, like many other organs and tissues, lacks the ability to efficiently regenerate following injury and disease. Its neurogenic potential is insufficient for self-repair and in some cases the formation of scar tissue worsens the situation, further impairing the restoration of normal tissue functions. Current clinical treatments following stroke mainly work on minimizing tissue loss and recovering the mobility through rehabilitation, but results are still limited (Pettikiriarachchi et al., 2010).

Regenerative medicine is a multidisciplinary field that addresses tissue and organ functions re-establishment through cell replacement therapies or stimulation of regeneration. This is carried out with two approaches that can be also combined. The *ex vivo* approach is based on the transplant of new cells that should survive, differentiate and integrate in the host tissue replacing lost cells and functions, whereas the *in vivo* approach involves the delivery of drugs, proteins or compounds that should promote endogenous cell stimulation and regeneration (Ikada et al., 2006).

A critical and limiting factor for the application of this type of therapies is the cell source. Transplantation of patient own cells (autologous) could avoid problems of immune response and immunosuppressive treatments. However, this is often limited by the difficulty in harvesting an adequate amount of cells, especially when the patient is old or severely diseased (Ikada, 2006). The discovery of stem cells as a potentially unlimited and renewable cell source opened new possibilities for regenerative medicine.

1.3.2 Stem cells

Stem cells are characterized by two main features: their ability to *self-renew* and their *potency*. These undifferentiated cells proliferate and renew themselves, generating daughter cells with equivalent proliferative and developmental potential through symmetrical cell divisions (self-renewal). They can also differentiate into any cell type of the body (potency) through asymmetrical cell divisions, which give rise to one cell identical to the mother and one committed to differentiation. Based on their potency, stem cells are classified in *totipotent* cells that can give rise to all cell types, both embryonic and extraembryonic (i.e. zygote); *pluripotent* cells that can generate all cell types of the three embryonic germ layers but not extraembryonic cells (i.e. embryonic stem cells); *multipotent* cells that can differentiate into cell types related to a specific lineage; *oligopotent* cells that

can differentiate into few cell types (i.e. lymphoid or myeloid stem cells) and *unipotent* cells that can differentiate into only one type of cells (i.e. some types of skin stem cells).
Stem cells can be also classified based on the source from which they are obtained. For example embryonic stem cells (ESC) are the cells derived from the inner cell mass (ICM) of the blastocyst stage of a mammalian embryo and adult stem cells are derived from specific tissues during the life of an individual (Avasthi et al., 2008).
The great interest in stem cells arises from the possibility to use them as *in vitro* disease models for drug testing and screening or for studying developmental processes and disease mechanisms, as they can be derived directly from patients or be genetically engineered. Moreover, thanks to their unlimited proliferative potential, they are suitable sources for regenerative approaches, in which they can be expanded, differentiated into specific cell types and further transplanted.

Embryonic stem cells (ESCs) are cells derived from the ICM of an embryo and can differentiate into all cell types, except for extraembrionic tissues (i.e. trophoectoderm). They can be kept in culture in an undifferentiated state for long periods, retaining the ability to differentiate into cells of all three germ layers. ESCs were first isolated from mouse embryos and put in culture in 1981 (Evans and Kaufman, 1981; Martin, 1981), while in 1998 they were isolated also from frozen human embryos no longer needed for *in vitro* fertilization (Thomson et al., 1998). Due to their origin, the discovery of human ESCs led to a big and still ongoing debate about the ethical and legal positions concerning their therapeutic use.

In order to be defined as stem cells, the real pluripotency of isolated mouse cells is commonly assessed by the ability to differentiate into all three germinal layers and to integrate and contribute to the development of all tissues, including germinal cell lineages, when re-implanted in a blastocyst. Moreover, when transplanted in adult immunodeficient mice, they should give rise to teratomas, the germline tumours in which components from all three germ layers can be found (Smith, 2001). Initially, mouse embryonic stem cells (mESCs) were cultured on monolayers of inactivated mouse embryonic fibroblasts (mMEFs). Later, the identification of the cytokine leukaemia inhibitory factor (LIF) produced by MEFs enabled feeder-free cultures (Smith et al., 1988; Williams et al., 1988), as LIF can replace MEFs in both derivation and long-term culture of mESCs (Rathjen et al., 1990a; Rathjen et al., 1990b). LIF stimulates mESCs self-renewal but is not sufficient to sustain it (Martello et al., 2013). When applied to serum-free mESCs cultures, cells start to differentiate, mostly into neural precursors (Ying et al., 2003b) whereas presence of serum in the culture provides additional signals able to fully suppress differentiation (Martello and Smith, 2014). Neural differentiation is naturally inhibited by Bone Morphogenetic Proteins

(BMPs) that, when added in culture, can replace serum and sustain self-renewal in coordination with LIF (Ying et al., 2003a).

Undifferentiated mESCs express specific cell surface antigens and membrane-bound receptors used as markers, such as the stage-specific embryonic antigen 1 (SSEA-1) (Tropepe et al., 2001) and gp130 (Nichols et al., 2001). They can be identified also for their enzymatic activities of alkaline phosphatase (ALP) (Wobus et al., 1984) and telomerases (Armstrong et al., 2000). Several transcription factors have been functionally characterized as being necessary for the maintenance of pluripotency, and used as stemness and pluripotency markers as well. The first identified was the POU-domain transcription factor Oct-3/4. Its expression, necessary to maintain mESCs pluripotency (Pesce et al., 1999; Scholer et al., 1989), is found in oocytes and early embryos and it is maintained in the germ cell lineages. Its overexpression does not enhance mESCs self-renewal but leads cells to differentiate into primitive endoderm and mesoderm, while its inactivation causes pluripotency failure in the embryo, with ICM cells located normally but differentiating into trophectoderm (Nichols et al., 1998; Niwa et al., 2000). The homeodomain transcription factor Nanog is another important regulator of pluripotency (Chambers et al., 2003; Mitsui et al., 2003) and its expression levels decrease when mESCs start differentiating. Forced expression of this protein in mESCs confers them the ability to self-renew without the presence of LIF, while its loss destabilizes pluripotency both *in vivo* and *in vitro* (Chambers et al., 2003). Together with Oct-3/4, Nanog is necessary and sufficient to maintain mESCs in an undifferentiated state (Mitsui et al., 2003). Finally, the SRY-box transcription factor Sox2 is also essential for self-renewal. It is expressed in the pre- and post-implantation epiblast but also later in neuroectodermal cells and in some endodermal and epithelial tissues (Martello and Smith, 2014). Sox2 interacts with Oct3/4 and binds together with it to DNA (Masui et al., 2007). Its inactivation in mESCs leads to trophoblast formation, and when overexpressed it induces mESCs differentiation (Kopp et al., 2008).

Human embryonic stem cells (hESCs) can be derived from pre-implantation embryos produced by *in vitro* fertilizations. They are characterized by growth in colonies, groups of cells with a distinct morphology and nuclei of big dimensions. They share many characteristics with mESCs, such as Oct-3/4 expression, telomerase activity, the ability to form teratomas when transplanted in immunodeficient mice and to retain pluripotency after long periods in culture.

The maintenance of ESCs pluripotency involves also several signaling pathways, such as Wnt signaling that, when activated, sustains the expression of Oct-3/4 and Nanog, maintaining both mouse and human ESCs in an undifferentiated state (Sato et al., 2004).

Neural differentiation of ESCs Understanding the mechanisms and differentiation steps involved in neural development *in vivo* helped to recapitulate these processes *in vitro*

(Fehling et al., 2003; Kubo et al., 2004; Yasunaga et al., 2005; Ying et al., 2003b). In fact, even if ESCs induction to ectodermal fate is referred as a “default” pathway (Bain et al., 1995; Tropepe et al., 2001), *in vitro* ESCs differentiation towards neural lineages has been achieved by activating the same signaling pathways involved in neural development during embryogenesis. Notch (Androutsellis-Theotokis et al., 2006; Hitoshi et al., 2002; Lowell et al., 2006), shh (Maye et al., 2004), Wnt (Davidson et al., 2007), BMPs, FGFs (Rao and Zandstra, 2005) and TGF-β (Smith et al., 2008) signaling pathways have been exploited for ESCs neural differentiation (Fig.3). BMPs, Wnt and activin/nodal signaling inhibit neural differentiation *in vitro*, consistent with their inhibition in the early embryo (Aubert et al., 2002; Czyz and Wobus, 2001; Kubo et al., 2004; Ying et al., 2003a). Differentiation of mESCs carrying the green fluorescent protein (GFP) reporter under the control of the neuroectoderm-specific gene Sox1 showed that neural induction depends on endogenous FGF signaling and that obtained neural precursors terminally differentiate into different neuronal subtypes when exposed to combinations of factors known to regulate these processes *in vivo* (Ying et al., 2003b). The Notch pathway has been shown to promote neural differentiation, requiring FGF signaling, and its inhibition makes cells unable to self-renew and to further differentiate (Hitoshi et al., 2002; Lowell et al., 2006).

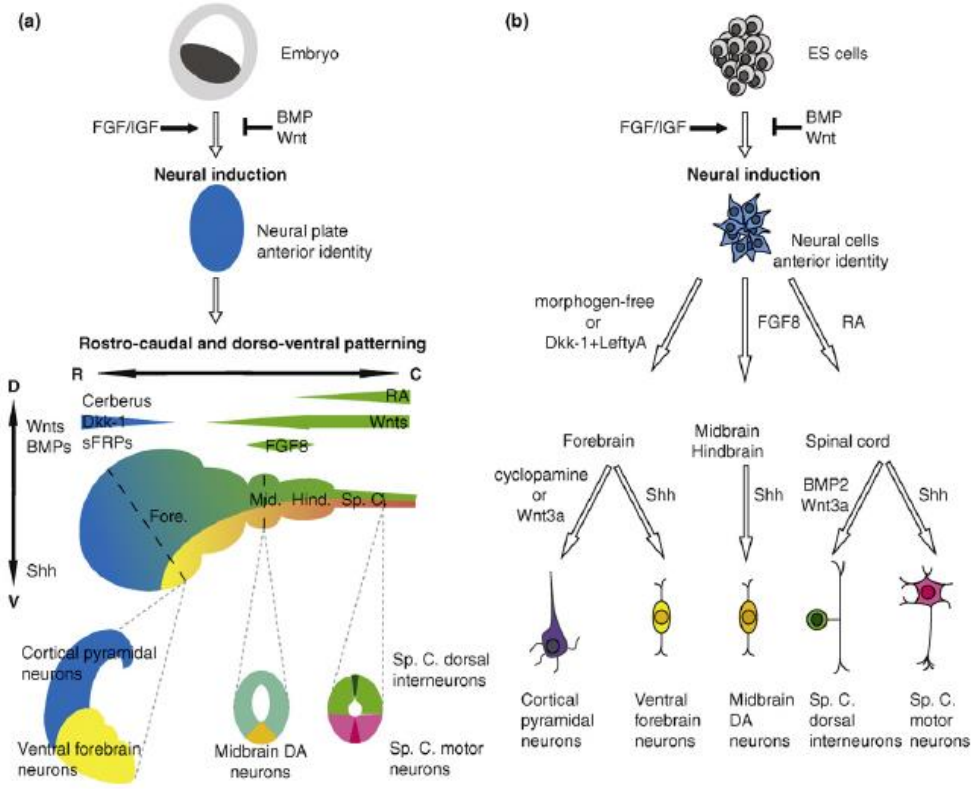

Fig. 4 Scheme of neural induction and patterning *in vivo* (**a**) and *in vitro* (**b**), (Gaspard and Vanderhaeghen, 2010).

ESCs neural differentiation in culture can be achieved by different culture systems. They can involve the initial formation of three-dimensional aggregates (embryoid bodies, EBs) (Hwang et al., 2008; Itskovitz-Eldor et al., 2000; Lee et al., 2000) or develop in two dimensions with cells cultured as monolayers (Chambers et al., 2009; Fico et al., 2008; Ying et al., 2003b). Spontaneous neural differentiation of ESCs was initially increased with retinoic acid (RA) treatment or stromal-conditioned medium, but differentiating neurons presented limited survival and terminal differentiation (Bain et al., 1995; Kawasaki et al., 2000; Soprano et al., 2007). Later-developed protocols involve multi-step differentiation and/or lineage selection. Typically they start with formation of EBs, small aggregates which form from undifferentiated ESCs cultured in suspension in the absence of LIF. They recapitulate early embryonic development *in vivo* and lead to differentiation into all three germ layers. EB-based protocols induce differentiation into neural precursors by treatment with FGF and EGF (Reubinoff et al., 2001; Zhang et al., 2001) or can involve neural selection steps with defined media, such as insulin, transferrin and selenin (ITS) medium (Bibel et al., 2007; Eiraku et al., 2008; Gaspard et al., 2009; Li et al., 2009). Serum-free EB cultures (SFEB) allow direct commitment towards neural fate avoiding variability due to serum presence (Bertacchi et al., 2013; Watanabe et al., 2005; Wataya et al., 2008). However, EBs are characterized by high cell heterogeneity and variability, due to the production of autocrine factors by differentiating cells (Bauwens et al., 2008), leading to the difficult control of ESCs differentiation within these structures.

Culture systems which avoid the aggregation step have been developed for both mouse and human ESCs (Abranches et al., 2009; Baharvand et al., 2007; Chambers et al., 2009; Fico et al., 2008; Ying et al., 2003b). Differentiation has been achieved in low density, adherent, monolayer, feeder-free cultures, using defined serum-free media supplemented with N2, B27 and FGF (Ying et al., 2003b), or with knockout serum replacement (KSR) supplement (Fico et al., 2008). Inhibition of BMPs and SMAD signalling pathways helps in neural commitment, as shown by treatment with Noggin alone (Gerrard et al., 2005) or in combination with another SMAD inhibitor, SB431642 (Chambers et al., 2009; Stover et al., 2013). These protocols often include intermediate steps in which cells are re-plated on ECM components, for selection and further differentiation. Neuronal maturation is achieved by the addition of factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and transforming growth factor β (TGF-β) (Rolletschek et al., 2001). Interestingly, when ESCs are differentiated into neural precursors in adhesion protocols, they acquire the specific conformation of neural rosettes, also present in NSCs cultures. These tube-like structures resemble the neural tube architecture found *in vivo*, characterized by the presence of neural precursors in the centre, whereas differentiating neurons migrate to the periphery (Abranches et al., 2009).

Both human and mouse ESCs have been successfully differentiated into glutamatergic, GABAergic and dopaminergic neurons, astrocytes, oligodendrocytes and photoreceptor progenitors, thanks to the combination of different factors (Carpenter et al., 2001; Erceg et al., 2009; Kawasaki et al., 2000; Lee et al., 2000; Tang et al., 2002; Wichterle et al., 2002). Culture with RA and FGF2 promotes motor neurons differentiation (Wichterle et al., 2002), whereas addition of Wnt and Nodal antagonists promotes production of telencephalic progenitors (Watanabe et al., 2005). Exposure to FGF8 and Shh and subsequently to BNDF and GDNF results in mouse and human ESCs dopaminergic differentiation (Barberi et al., 2003; Cho et al., 2008; Lee et al., 2000; Momcilovic et al., 2012; Park et al., 2005; Perrier et al., 2004; Yan et al., 2005). Neural precursors treated with Shh inhibitors, such as cyclopamine, differentiate into cortical neurons (Gaspard et al., 2009; Gaspard and Vanderhaeghen, 2010).

Astrocytes and oligodendrocytes can also be differentiated from both mouse and human ESC-derived neural progenitors, with FGF and platelet-derived growth factor (PDGF) (Ogawa et al., 2011). Glial cells have been differentiated also from ESCs aggregates with B27 supplement, insulin, FGF-2 and RA and have subsequently shown ability to produce myelin *in vivo*, following transplantation (Kang et al., 2007; Liu et al., 2000; Zhang et al., 2001).

Finally, ESCs have been successfully differentiated into retinal progenitors and pigmented cells of the retinal pigmented epithelium (RPE) (Ikeda et al., 2005; Lamba et al., 2006; Lamba et al., 2010; Levine et al., 1997; Mellough et al., 2012; Osakada et al., 2008). In some cases ESCs-derived photoreceptor progenitors showed ability to integrate in the mouse retina and to terminally differentiate, expressing specific retinal markers (Hambright et al., 2012; Lamba et al., 2009; Lamba et al., 2010).

These protocols can be characterized by poor efficiency and high heterogeneity, since the final populations can be composed also by non-neural cells, neural precursors and still undifferentiated ESCs (Pollard et al., 2006a; Ying et al., 2003b). This indicates that more complex culture conditions could be helpful in order to achieve a more efficient terminal neuronal differentiation. Moreover, these cultures often lack the physiological three-dimensionality of the environment and thus cannot fully recapitulate the *in vivo* development. This has been elegantly shown by Sasai's lab which reported an efficient three-dimensional culture method of self-organizing mESCs that recapitulates the apico-basal polarization of the cortical tissue, generating functional and transplantable neurons (Eiraku and Sasai, 2012; Eiraku et al., 2008). In this protocol cells are cultured as aggregates with a Wnt inhibitor and an inhibitor of Nodal/Activin pathway in a culture medium containing KSR supplement. They showed that pluripotent cells self-organize into three-dimensional structures which resemble the six layered structure found in the cortex,

mimicking both the spatial and temporal *in vivo* development of the cortex. Eiraku and collaborators demonstrated that, under different culture conditions, these self-assembling aggregates of mouse or human ESCs can also give rise to retinal precursors which autonomously fold into an optic cup. These cells are characterized by layered retinal markers expression which resemble the pattern found *in vivo* (Eiraku et al., 2011; Nakano et al., 2012). However, the use of Matrigel, a mixture of ECM components isolated from murine tumours, and the patented serum substitute KSR which composition is unknown limits the possible application of these cultures in therapeutic approaches.

Recently, Dr. Yamanaka was assigned the Nobel prize for discovering the basic cocktail of factors able to reprogram mammalian somatic cells. By screening 24 candidate genes which are known to control embryonic stem cell identity, they found that reprogramming of mouse and human embryonic fibroblasts (MEF) into **induced pluripotent stem cells (iPSCs)** can be achieved by the forced expression of four of them: oct3/4 and sox2, which are involved in pluripotency maintenance, and Klf4 and c-Myc, two oncogenes (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Human fibroblasts were also reprogrammed with similar factors, such as Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007). iPSCs were later obtained from other types of cells, such as liver cells (Aasen et al., 2008), keratinocytes (Aoi et al., 2008), pancreatic β–cells (Stadtfeld et al., 2010), B-cell lymphocytes (Hanna et al., 2008) and neurons (Kim et al., 2011b). iPSCs are morphologically similar to ESCs, express pluripotency markers, lead to teratoma formation and differentiate into all three-germ layer lineages. The fascinating potential of iPSCs is represented by the possibility to derive them from patients, obtaining cells carrying the gene responsible for a specific disease that can be cultured and studied *in vitro* as disease models. Moreover, iPSC-based therapies could bypass the ethical problems concerning the use of ESCs (Takahashi et al., 2007; Yamanaka, 2012). However these approaches are also characterized by some limits. Frequently they need p53 suppression in order to have high reprogramming efficiency and there is a debated possibility that they retain an epigenetic memory of adult cells which can bias their reprogramming and differentiation (Kim et al., 2010). The risk of insertional mutagenesis and oncogenic transformation due to retroviral systems used to obtain these cells has been solved by the development of virus-free reprogramming protocols. Recently the reprogramming of somatic cells without the expression of the oncogene c-Myc has been reported, which reduces tumorigenesis risks (Chen et al., 2014).

Reprogramming of adult somatic cells has also been achieved *in vivo* (Abad et al., 2013) and specialized cells have been instructed to directly turn into another cell type avoiding the pluripotent phase (transdifferentiation) (Pang et al., 2011; Slack, 2007). For example,

human fibroblasts have been transdifferentiated into neurons in different laboratories (Ambasudhan et al., 2011; Pang et al., 2011; Qiang et al., 2014). Vierbuchen and colleagues, using the same approach as Sasai, found that Brn2 (or Pou3f2); Ascl1 and Myt1l expression can convert mESCs and postnatal mouse fibroblasts into neurons which exhibit electrophysiological properties. The addition of NeuroD1 allows the transdifferentiation into neurons also of human dermal fibroblasts (Vierbuchen et al., 2010). Conversion of fibroblasts in neural stem cells was achieved by using various combinations of transcription factors, all having Sox2 as an essential element (Han et al., 2012; Lujan et al., 2012; Ring et al., 2012; Thier et al., 2012).
Mouse and human iPSCs have been successfully differentiated into many cell types, including neural precursors, several neuronal subtypes, glia and retinal precursors (Denham and Dottori, 2011; Hirami et al., 2009; Reddington et al., 2014).

Somatic stem cells are stem cells present in several tissues and organs after development, playing a role in maintenance and repair of the tissue during life. These cells are tissue-specific and exhibit a more restricted potency with respect to ESCs but, as stem cells, they are able to self-renew and to give rise to daughter cells which divide few times before differentiating into terminally mature cells. The different types of somatic stem cells differ for localization, abundance and specialization. The interest in adult stem cells is high because of their endogenous origin and the possibility to bypass ethical problems related to ESCs use (Snippert and Clevers, 2011). In general, adult stem cells are limited in number in the tissue, thus their collection and expansion are often difficult, representing a limit for therapies (Avasthi et al., 2008).

Neural stem cells (NSCs) are multipotent stem cells able to differentiate into neural precursors (NPCs) that can further differentiate into the three neuronal lineages: neurons, oligodendrocytes and astrocytes. They are abundant during development but in the adult brain they are confined in two specialized niches, the dentate gyrus of the hippocampus and the subventricular zone (SVZ) (Batista et al., 2014; Doetsch et al., 1999). NSCs are characterized by the co-expression of Sox2 and Nestin, but they are thought to express also glial markers such as GFAP. In fact, in adult neural niches, radial glia-like cells act as stem cells during neurogenesis processes (Bonfanti and Peretto, 2007; Kriegstein and Alvarez-Buylla, 2009).
NSCs can be isolated both from embryonic and adult nervous tissue (Conover and Notti, 2008; Pollard et al., 2006b) or derived from ESCs, and grown either as spherical aggregates (neurospheres) or as monolayers, in serum-free media supplemented with EGF and FGF. Culture in neurospheres is commonly used for NSCs expansion and later

differentiation, but the population is highly heterogeneous. In fact, some cells undergo spontaneous differentiation and the overall population is composed by NSCs, differentiating precursors and already differentiated cells (Chojnacki et al., 2009; Galli et al., 2003; Pluchino et al., 2005). Dissociation and replating in proliferation medium causes the death of differentiated cells and leads to more homogeneous NSCs populations. However, it has been proven that after several passages as neurospheres, cells decrease telomerase activity and present a more restricted neurogenic potential, more likely toward astrocyte lineage or, when they become neurons, mostly into GABAergic (Conti et al., 2005; Machon et al., 2005). In addition, neuronal differentiation efficiency is low, around 15 - 20% (Tropepe et al., 1999; Vescovi et al., 1993).

Alternative approaches for the culture of NSCs involve adherent conditions, demonstrating that the neurosphere culture is dispensable and that NSCs can be maintained and expanded long-term *in vitro* on laminin-coated supports through exposure to FGF and EGF (Biella et al., 2007; Chambers et al., 2009; Conti et al., 2005). As ESCs-derived neural precursors, NSCs cultured in adhesion organize in neural rosettes. Initially, adhesion protocols where characterized by poor efficiency of neuronal differentiation (5 - 10%) (Biella et al., 2007; Conti et al., 2005). Currently, both ES-derived NSCs and adult SVZ-derived NSCs can be differentiated into GABAergic neurons with high efficiency (65% - 85%), culturing cells in a medium supplemented with N2 and B27, and exposing them to decreasing concentration of FGF and increasing concentration of BDNF (Goffredo et al., 2008; Spiliotopoulos et al., 2009). Differentiated neurons express GABA, GAD67, calbindin and parvalbumin; have functional $GABA_A$ receptors but present variable expression of AMPA, NMDA and kainate receptors, and showed ability of firing action potentials.

1.3.3 Stem cells and their applications in neural tissue repair

Stem cells represent an interesting source for regeneration approaches especially for tissues unable to self-repair such as the CNS. Stem cell-based therapies aim to replace damaged or lost tissue in order to restore its integrity and function. They have been already tested for the treatment of amyotrophic lateral sclerosis (ALS), Parkinson's Disease (PD), Huntington's Disease (HD), spinal cord injury (SCI), stroke and trauma (Becerra et al., 2007; Hoane et al., 2004; Keirstead et al., 2005; Kim et al., 2006; Kimura et al., 2005; Liu et al., 2000; McDonald et al., 1999; Shear et al., 2004; Xu et al., 2006; Yasuhara et al., 2006).

Effective treatments to promote tissue repair and functional recovery after stroke are still lacking and recent studies involving stem cells transplantation in animal models of brain injury, included stroke, report promising results about their neuroprotective effects. When transplanted, NSCs are able to modulate inflammation, stimulate angiogenesis and migrate to the site of the injury. Here they can differentiate into neurons replacing dead cells, or they can exert a "bystander effect" through the release of molecules and factors (nerve

growth factor (NGF), BDNF, GDNF, NT3 and VEGF) that can increase activation, proliferation and differentiation of endogenous NSCs (Doeppner et al., 2014; Fischbach et al., 2013; Hermann et al., 2014; Lindvall and Kokaia, 2011).

In rat and mouse models of intracerebral hemorrhage (ICH), injected hNSCs survive, migrate and help functional recovery. When modified to express Akt1, a serine/threonine kinase with anti-apoptotic effects, they helped the improvement of motor functions, with increased survival and further differentiation into neurons or astrocytes (Jeong et al., 2003; Lee et al., 2009). NSCs overexpressing BDNF or NGF improved functional recovery after injection in a mouse model of stroke (Ding et al., 2013) while NSCs overexpressing GDNF showed ability to stimulate neurogenesis when transplanted in TIA rat models (Yuan et al., 2013). Hippocampal neural progenitors isolated from mice and transplanted in the brain following cerebral ischemia survived for 90 days and differentiated into mature neurons with functional synapses (Tsupykov et al., 2014).

Human iPSCs-derived neuroepithelial-like stem cells (lt-NES) were also tested for stroke treatment in mouse and rat striatum and cortex. After injection they exerted beneficial effects leading to motor function improvements, associated more to enhanced endogenous plasticity due to the secretion of VEGF, rather than to neuronal replacement by grafted cells. lt-NES survived for at least 4 months and differentiated into mature functional neurons of different subtypes (Oki et al., 2012). lt-NES-derived cortical progenitors injected in the stroke-damaged rat cortex differentiated into mature and functional cortical neurons helping the recovery of impaired functions. However, when this improvement is compared to results obtained with injection of non-fated lt-NES, no significant differences can be registered in behavioural improvements between the two treatments (Tornero et al., 2013).

Despite encouraging results, these approaches are still characterized by many issues. Cell integration in the pre-existing circuits and formation of new connections are critical aspects for grafted cells survival, maturation and tissue recovery, thus cells at different stages (non-fated or fate-restricted) are investigated. However, there are still no evidences about which cell type presents better outcomes following transplantation in stroke damaged brains (Pluchino and Peruzzotti-Jametti, 2013). Other critical aspects are the most suitable time point and injection route for cell delivery, in order to obtain the best outcomes. The injection itself could cause enough injury to start glial scarring processes, further limiting survival and integration of grafted cells and newly generated neurons. For this reason both intracerebral and systemic injection are investigated (Doeppner and Hermann, 2014; Lindvall and Kokaia, 2011). Finally, following injection many cells die because of ongoing inflammation, increasing the amount of cells that have to be injected in order to obtain beneficial effects, representing a limiting factor for the application of these therapies in clinical use.

In fact, few clinical trials involving stem cell delivery in stroke patients are ongoing. Many of them involve mesenchymal stem cells transplantation and only few are based on the use of neural stem cells (Aboody et al., 2011; Gage and Temple, 2013; Lindvall and Kokaia, 2011) (ww.clinicaltrials.gov).

In a UK clinical trial, ReNeuron's ReN001 NSCs are used to treat stroke patients. Increasing numbers of immortalized NSCs, isolated from human fetal cortex, were implanted in 12 patients between 6 and 24 months after stroke. These cells were able to differentiate into neurons and oligodendrocytes in rodents, without eliciting tumorigenicity risks and improving sensorimotor functions when transplanted (Cossins, 2013; Lindvall and Kokaia, 2011). However, reports of this study indicate only mild to moderate improvements in five of the nine long term stroke patients. This is mainly due to cell death following transplantation (Aboody et al., 2011).

1.3.4 The neural stem cell niche

During development and patterning of the CNS, the milieu in which NSCs proliferate, migrate and differentiate is permissive, complex and dynamic in order to support these processes. Neural stem cells are indeed known to reside in specialized three-dimensional microenvironments, the neural stem cell niches. Here cells interact with their neighbours and with the ECM and thus are sensitive to stimuli coming from the environment that support and coordinate their long-term self-renewal and differentiation through active interactions (Dellatore et al., 2008). Cellular activities are in this way regulated both by biochemical signals from soluble factors (growth factors or cytokines) and physical stimuli from surrounding cells and ECM associated molecules, involved in signalling transduction events (Dellatore et al., 2008; Doetsch, 2003; Estes et al., 2004). ECM contributes also to the modulation of matrix stiffness and topography in the tissue, known to contribute to cell regulation. In fact, cell attachment to the ECM creates contractile forces which result in stress in the cytoskeleton, influencing processes such as migration or proliferation (Hadjipanayi et al., 2009; Ingber, 2004) (Guo et al. 2006). Consequently, ECM stiffness or elasticity can influence cell shape, which is a regulator of development, cell growth and activity (Guilak et al., 2009).

The brain ECM is mainly composed by proteoglycans, glycoproteins and other components such as collagen, laminins and fibronectin which constitute the basement membrane (Bosman and Stamenkovic, 2003). Cellular adhesion to the ECM is triggered by integrins which interact with many ECM ligands (collagen, laminin, fibronectin, vitronectin), influencing cell behaviour, cell-cell communication and coordinating cell positioning within the niche (Daley et al., 2008; Dellatore et al., 2008; Fuchs et al., 2004; Wade et al., 2014). Proteoglycans (PGs) can bind many extracellular factors, from signaling molecules to

membrane proteins. Different types of PGs are present in the CNS: heparan sulphate (HSPGs), chondroitin sulphate (CSPGs) and dermatan sulphate PGs (DSPGs) (Wade et al., 2014). HSPGs and CSPGs are highly expressed in neurogenic niches and play an important role in cell-ECM adhesion and signaling through their ability to modulate growth factor signaling. They can both inhibit degradation of a ligand or act as co-receptors. HSPGs can stabilize the FGF ligand-receptor complex, promoting its signaling, while HSPGs can bind EGF and VEGF and are involved in BMPs, Shh and Wnt signaling (Doetsch, 2003). BMPs and Notch signaling are important for the balance between quiescent and proliferative NSCs in the hippocampus, whereas Shh, Wnt, FGF and VEGF regulate NSCs proliferation (Lugert et al., 2010; Mira et al., 2010) (Lai et al., 2004; Jin et al., 2003; Lai et al., 2003). Another major constituent of brain ECM is hyarluronic acid (HA) that is released by the cells and interacts with ligands in order to regulate signaling (Preston and Sherman, 2011). Commonly it is involved in neuronal migration, neurite outgrowth and axonal pathfinding (Bandtlow and Zimmermann, 2000). Among glycoproteins, tenascin C (Tn-C) is the most abundant in CNS during development and adulthood, being present also in NSC niche (Wade et al., 2014).

Neurogenic niches are present during development and adulthood and are composed of both neural and non-neural cells, such as astrocytes (Song et al., 2002), endothelial cells (Shen et al., 2004), ependymal cells, microglia (Sierra et al., 2010), blood vessels (Palmer et al., 2000), axon projections and ECM, which together orchestrate signals and modulate stem cells behaviour and new cell production according to needs in the tissue (Faigle and Song, 2013; Fuchs et al., 2004).

The importance of the three-dimensional environment in which cells reside increased the efforts for its better characterization. Currently there is more awareness that recreating the cell-cell and cell-ECM interactions would evoke more physiological responses in stem cells than what soluble factors alone can do in two-dimensional cultures. Natural three-dimensional culture systems, such as EBs and neurospheres, match this idea but they are characterized by heterogeneous populations and consequent poor differentiation efficiency and reproducibility (Bauwens et al., 2008; Conti et al., 2005). The pioneer work of Sasai's group perfectly demonstrates how cells, when put in the right condition within a three dimensional environment, can better recapitulate the processes that occur during development.

1.3.5 Biomaterials in tissue engineering

Regenerative medicine is a very promising strategy to treat damaged CNS but current cell-replacement approaches are still characterized by a small percentage of grafted cells that survive several days after transplantation, limiting their efficacy (Li et al., 2012). When

transplanted in a damaged tissue cells lack the mechanical, chemical and physical support given by the healthy tissue (Ikada, 2006). Tissue engineering, a branch of regenerative medicine, aims to restore tissue functions combining principles of cell biology and engineering, providing an environment in which cells and bioactive molecules can interact synergistically in order to promote tissue repair. This is done with designed and tailored three-dimensional scaffolds, that simulate the *in vivo* microenvironment, giving the biochemical signals and structural features that cells physiologically encounter. Scaffolds can be built according to target tissue characteristics, in order to resemble the specific physiological architecture. They can be composed of ECM constituents or incorporate some of its components and be functionalized with molecules known to stimulate specific stem cell behaviors of interest (i.e. proliferation, differentiation toward specific cell types). They can be used as support for *in vitro* cultures, providing a three-dimensional environment to mimic the physiological microenvironment and guide differentiation of stem cells. Moreover, encapsulated cells can synthesize their own ECM inside scaffolds and thus sense the right stimuli for differentiation to a desired lineage (Dawson et al., 2008; Shakesheff et al., 1998). These cell-seeded scaffolds can be cultured *in vitro* and further implanted. Scaffolds can also be used for the delivery of drugs, proteins and factors that stimulate tissue regeneration or inhibit inflammatory processes, increasing their permanence in the site of injection and allowing different timing of release (Ikada, 2006).

Biomaterials are materials able to interact with biological systems and are commonly used for building scaffolds and matrices for tissue engineering applications. Regardless of the type of tissue they have to be used for, biomaterials should present some common characteristics. They should be biocompatible and allow nutrient and metabolites permeability. Following transplantation in the host tissue they should elicit minimal immune reaction, that can reduce healing and/or provoke rejection. Biodegradability is needed if scaffolds are not used as permanent implants but as support for the viability and initial integration of grafted cells. In this case they should dissolve while cells produce their own ECM, and degradation products should not be toxic.

Biomaterials can be of natural or synthetic origin. Natural biopolymers are polysaccharides, such as agarose, alginate, chitosan; components of the ECM (i.e. hyaluronic acid) or polypeptides, such as collagen, gelatin or silk. Generally natural polymers are biocompatible, enzymatically biodegradable and can contain functional molecules that may help cell attachment, proliferation or differentiation. However their enzymatic degradability could compromise the mechanical integrity, being a limit for their *in vivo* application (Pettikiriarachchi et al., 2010; Yoon and Fisher, 2009). Synthetic polymers are chemically synthesized and can be tailored based on future applications. However, many of them are degradable though hydrolysis and this process can lead to the formation of toxic products,

causing inflammation or fibrous encapsulation responses (Gunatillake and Adhikari, 2003). The most common are polyesters, such as poly(glycolic) acid (PGA), poly(L-lactic) acid (PLA), poly(D,L-lactic-co-glycolic) acid (PLGA) and poly(ε-caprolactone) (PCL); polyanhydrides, polycarbonates and poly(ethylene glycols) (PEGs) (Yoon and Fisher, 2009). Biomaterials polymerization can be achieved through physical or chemical methods, for example ionic crosslinking, photopolymerization, electrospinning, freeze drying or sonication.

In the brain, tissue engineering approaches through scaffold-based cell delivery could help in protecting grafted cells from the inflammation present in the damaged tissue, increasing their viability and time of permanence *in vivo*. Brain is one of the softest tissue and NSCs are able to sense differences in mechanical stiffness, changing their migration, neurite formation or even differentiation (Hynes et al., 2009; Pettikiriarachchi et al., 2010; Seidlits et al., 2010). Biomaterials for brain tissue engineering should thus present characteristics similar to brain, such as elastic modulus values in the range of 0.5-1 kPa (Gefen and Margulies, 2004; Li et al., 2012), should minimize microglia and macrophage activation, avoid neurotoxicity and be easily transplanted. In soft and fragile organs such as the brain, pre-formed scaffold implantation is a difficult and highly invasive procedure. The use of hydrogels, hydrophilic polymers with high water content (typically above 90%) with injectability properties, allows less invasive interventions (Pakulska et al., 2012; Pettikiriarachchi et al., 2010). Hydrogels can be made of natural or synthetic monomers or combinations of the two, and can be neutral, anionic or cationic by charge. Their structure and properties depend on starting monomers, thus can be easily controlled by modulating the manufacturing procedures (Pakulska et al., 2012).

A wide range of scaffolds including hydrogels, nanofibers and self-assembling peptides has been investigated for drug and cell delivery in the CNS (Aurand et al., 2012; Nomura et al., 2008; Prewitz et al., 2012). Chitosan, collagen and fibrin/fibroin scaffolds showed promotion of endogenous cell survival and enhanced grafted cell integration following SCI (Kim et al., 2011a; Lu et al., 2007; McCreedy et al., 2014; Nomura et al., 2008; Zahir et al., 2008). Positive results have been obtained after injection of collagen I and NSCs in rat brain after ischemic insult. Cells co-injected with the biomaterial formed synapses and helped structural and functional recovery (Yu et al., 2010). When encapsulated in hyaluronic acid (HA) hydrogels, human embryonic stem cells proliferate and maintain both their undifferentiated state and pluripotency (Gerecht et al., 2007). Rat neural stem cells adhere and proliferate in HA hydrogels loaded with growth factors (Wang et al., 2011), and murine neural stem cells survive, proliferate and differentiate into mature neurons in scaffolds of HA and Type1-collagen (Brannvall et al., 2007). Mechanical properties of HA-based hydrogels can also influence cell behaviour. Rat neural progenitor cells encapsulated in HA

hydrogels with compressive moduli varying across the range reported for neonatal brain and adult spine (2-8kPa), responded differently to the various mechanical properties. Cells cultured in softer hydrogels with modulus comparable to neonatal brain differentiated into neurons, while those in stiffer hydrogels with properties similar to adult spinal cord showed preference for the astrocytic lineage (Seidlits et al., 2010). Similarly, mesenchymal stem cells (MCS) encapsulated in HA and Type1-collagen scaffolds with elastic moduli ranging from 1-10kPA showed preference for the neural lineage at a modulus of 1kPa and differentiated toward glial cells at a modulus of 10kPa (Her et al., 2013). HA modified with laminin or arginine–glycine-aspartic acid (RGD) peptide showed ability to support cell infiltration, angiogenesis, neurite extension promotion and reduction in glia scar formation when implanted into cortical defects in rats (Cui et al., 2006; Hou et al., 2005). The combination of HA and methylcellulose (HAMC) has been used for the injection of drugs in the stroke injured brain and in SCI models (Cooke et al., 2011; Kang et al., 2009), or as injectable hydrogels for the delivery of retinal stem cells (RSCs) in the sub-retinal space of adult mice (Ballios et al., 2010). Modification of HA hydrogels to better support adhesion and survival of neuronal cells resulted in inhibition of glial scar formation and promotion of axonal growth and angiogenesis once implanted in SCI models (Wei et al., 2010). Despite its animal origins that make Matrigel unsuitable for human applications (Pakulska et al., 2012; Pettikiriarachchi et al., 2010), it has been tested for CNS regeneration approaches. When seeded with rat neurons and astrocytes it supports neurites outgrowth and expression of mature neuronal markers with functional synapses formation (Irons et al., 2008) while, together with collagen, it increases Schwann cells survival *in vitro* and *in vivo* in SCI models (Dewitt et al., 2009; Patel et al., 2010). Matrigel injected together with human NPCs in the post-ischemic rat brain, increased cell survival preventing inflammatory cells infiltration and decreased necrotic infarct cavity with improvements in cognitive and motor functions (Jin et al., 2010).

Among synthetic biomaterials, biodegradable PCL, PLA and PLGA have been tested for CNS tissue engineering. PCL constructs with aligned fibers showed neurite penetration when implanted in adult rat brains (Nisbet et al., 2009). PLGA scaffolds transplanted with NSCs and Schwann cells in hemisected rat spinal cords showed improvements in axon myelination (Xia et al., 2013). PGA scaffolds seeded with mNPCs and implanted in neonatal brains after ischemic stroke facilitated interactions between host and grafted cells, with promotion of neuronal differentiation, helping reconstruction by reducing inflammation and scarring (Park et al., 2002). Poly(*N*-2-(hydroxypropyl) methacrylamide) (pHPMA) showed ability to support cell penetration, angiogenesis, axon growth and ECM deposition after implantation, while poly(hydroxyethylmethacrylate) (pHEMA) allows astrocytes penetration (Lesny et al., 2002). PEG polymers bound to poly(L-lysine) (PLL) support

mNPCs survival and proliferation *in vitro*, allowing their differentiation into mature neurons (Royce Hynes et al., 2007) and PEG-PLA hydrogels promote survival and metabolic activity of neural progenitor cells (NPCs) *in vitro* (Mahoney and Anseth, 2007). PEG-PLA copolymers have been tested for the delivery of NT-3 in the injured spinal cord and for BDNF and GDNF delivery to the brain (Lampe et al., 2011; Piantino et al., 2006), while PEG-PLA nanoparticles, engineered with triiodothyronine (T3), showed ability to decrease tissue infarction and brain edema in mouse stroke model (Mdzinarishvili et al., 2013).

Self-assembling peptide nanofiber scaffolds (SAPNS) have been also tested for CNS regeneration, since they are characterized by high porosity, tissue-like water content and can present bioactive peptide sequences (Collier, 2008; Silva, 2005). They showed promising results *in vitro* (Cheng et al., 2013)(Holmes et al., 2000) and *in vivo* in SCI or TBI animal models (Cheng et al., 2013; Guo et al., 2009; Tysseling-Mattiace et al., 2008). However, SAPNS present a big limit due to their susceptibility to enzymatic degradation *in vivo*, that makes them mechanically weak (Pettikiriarachchi et al., 2010).

Many studies investigate the use of biomaterial for CNS regeneration following SCI or TBI but less efforts have been made regarding treatment of damaged tissue following stroke. They could be a valid tool for enhancing cell transplantation efficiency through isolation of grafted cells from the surrounding inflamed environment present following injury and providing a three-dimensional support for their migration and integration, otherwise inhibited by the damaged tissue and inflammatory response. For this reason new hydrogels and biomaterials should be investigated, in order to obtain efficient and easy support for cell delivery in ischemic neural tissue.

1.3.6 Alginate

Alginate is a natural polymer widespread in nature that can be used as biomaterial in tissue engineering. It is present as a structural component in marine brown algae and as a capsular polysaccharide in soil bacteria and in several species of Pseudomonas. All commercial alginates derive from algae extraction. The interest in alginate as biomaterial for tissue engineering is due to its ability to retain water and to bind cations but also its biocompatibility, non-immunogenicity and hydrophilic nature. Currently this FDA-approved polymer is used in different application such as nutrition supplement or wound dressing (Sun, 2013). It is a linear anionic polysaccharide composed of blocks of (1-4)-linked ß-D-mannuronic acid (M) and α-L-guluronic acid (G) (Frampton et al., 2011; Novikova et al., 2006). The relative amount of each monomer can vary among sources and they can be linked randomly or present in homopolymeric blocks (Fig.5) (Frampton et al., 2011). The carboxylate groups present on the polysaccharide chains provide sites for the covalent attachment of peptides and proteins that can promote cell attachment (Rowley et al., 1999).

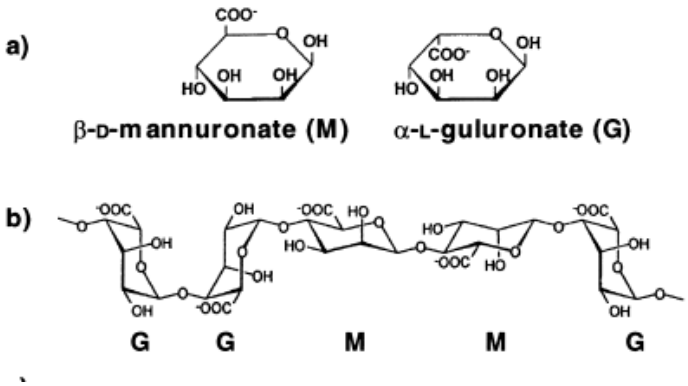

Fig.5 *Structural characteristics of alginates*: **a**) alginate monomers, **b**) chain conformation, (Draget et al., 2005).

Alginate mechanical properties such as viscosity, stiffness and degradability depend on chemical characteristics and can be varied by changing alginate composition, its concentration or the gelling procedure used for crosslinking. Gels containing higher amount of L-guluronic acid are stiffer than ones rich in D-mannuronate and in presence of higher M/G ratio, the pores are characterized by smaller average size (Huang et al., 2012).

Alginate hydrogels can be obtained with different crosslinking procedures, such as phase transition (thermal gelation), cell-crosslinking or free radical polymerization, but the most common is ionic crosslinking, in which unmodified alginate is ionically crosslinked into hydrogels through the exposure to divalent cations, such as Ca^{2+}, Mg^{2+}, Sr^{2+} or Ba^{2+}. In this process, cations bridge the G residues on different chains making scaffold fabrication and cell encapsulation simple, non-toxic to cells, and efficient (Lee and Mooney, 2012; Martinsen et al., 1989; Novikova et al., 2006; Rowley et al., 1999). Typically, $CaCl_2$ is used as Ca^{2+} source bringing to immediate crosslinking. This method allows the easy encapsulation of cells in alginate beads, by dropping alginate-cell mixture into a $CaCl_2$ solution (Fig.6).

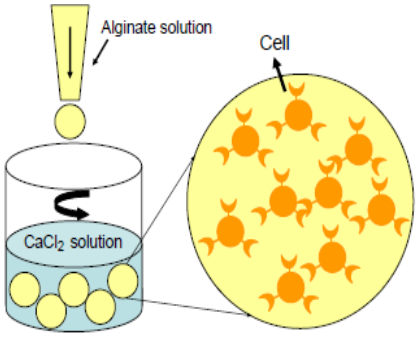

Fig.6 *Ionic crosslinking procedure with Ca^{2+} ions*. With this procedure, cells can be encapsulated in alginate beads, modified from (Sun, 2013).

Cells can also be recovered from the capsules by the addition of Ca^{2+} chelating agents that dissolve the beads. The ionic crosslinking which makes them water-insoluble is slowly disrupted in the body through exchange of Ca^{2+} with Na^{+}, so that alginate becomes again a water-soluble polymer and it is excreted in the urine (Frampton et al., 2011; Ikada, 2006; Novikova et al., 2006). Depolymerization processes can occur also with exposure to acids, alkanes or free radicals and are faster at low and high pH values, or with increasing temperature (Haug et al., 1963). Degradation is influenced also by alginate molecular weight (MW): higher MW decreases the number of reactive positions available for hydrolysis, leading to slower degradation rates (Moya et al., 2012; Sun, 2013).

Alginate properties can also be varied through addition of an external coating, typically of polycations such as poly-L-lysine (PLL), poly-L-ornithine (PLO) and poly-D-lysine (PDL). These coatings should act as a barrier against the host immune system when used for *in vivo* applications, by blocking diffusion of big molecules such as antibodies (De Castro et al., 2005; Wilson et al., 2014).

1.3.7 Alginate applications

Alginate is widely used for drug delivery applications, wound healing, bone and cartilage tissue engineering due to its biocompatibility, biodegradability and non-antigenicity (Sun, 2013). Alginate-supported cultures are routinely used for stem cells growth and differentiation in several lineages, including osteogenic and chondrogenic lineages (Coates and Fisher, 2012); but also cardiac (Bauwens et al., 2008), pancreatic (Wang et al., 2009) and hepatocytic lineages (Lin et al., 2010). Alginate encapsulation of bone marrow-derived stem cells (BMSCs) supports their *in vitro* differentiation into hepatocytes (Lin et al., 2010) and it has successfully been used for type I diabetes treatments through encapsulation of islets of Langerhans (Moya et al., 2012; Soon-Shiong et al., 1993).

Recent studies also demonstrate that neural lineages can be supported and differentiated in alginate hydrogel cultures and that properties such as mechanical stability and elastic modulus strongly influence cell phenotypes (Addae et al., 2012; Candiello et al., 2013; Frampton et al., 2011; Kim et al., 2013; Li et al., 2011; Li et al., 2006; Matyash et al., 2014; Wilson et al., 2014). Murine ESCs encapsulation in alginate microbeads was tested altering alginate concentration (from 1.2% to 2.5% w/v), reporting 2,2% w/v alginate as the optimal concentration for neural commitment. In these gels cells were viable throughout the culture period and expressed an array of neural markers following delivery of soluble differentiation inducers (Li et al., 2011). Addae and colleagues differentiated mESCs into functional GABAergic neurons after encapsulation in 1,1% w/v alginate hydrogels (Addae et al, 2012), while hESCs have been differentiated into dopaminergic neurons after encapsulation in 1,1% w/v alginate microcapsules (Kim et al., 2013). Mouse neural stem cells encapsulated

in 1,5% w/v alginate beads were used to study the optimal initial cell density by quantifying cell expansion after 7-9 days of culture in proliferation medium. Immunocytochemical analyses on neural cell differentiation performed on cells recovered from alginate hydrogels and grown for a couple of days in two dimensions showed that encapsulated cells retain the ability to further differentiate into the three neural lineages (Li et al., 2006). Murine cortical neural stem cells (NSCs) encapsulated in alginate with either high guluronic acid (68%) or high mannuronic acid (58%), with and without a poly-L-lysine (PLL) coating, survived and proliferated in mostly all conditions tested. However the secretion of neuroprotective factors (BDNF, GDNF, NGF) was reported only in non-coated high-G alginates, the hydrogels with the best mechanical stability (Purcell et al., 2009). Encapsulated rat astroglioma cells, astrocytes and hippocampal neurons in 1%w/v alginate hydrogels showed morphologies more similar to that found *in vivo* and extrude processes outgrowth in the scaffold (Frampton et al. 2011). The encapsulation of genetically engineered fibroblasts secreting BDNF or NT-3 in alginate constructs influenced NPCs differentiation into neurons or oligodendrocytes (Shanbhag et al., 2010).

To date, the most promising results have been obtained using soft hydrogels, showing an elastic modulus comparable to brain tissue (Banerjee et al., 2009; Matyash et al., 2012; Purcell et al., 2009). Studies on rat NSCs also demonstrated that mechanical properties can influence the encapsulated cell population. Alginate elastic modulus was varied over two orders of magnitude and NSCs proliferation and neural differentiation were measured. NSCs proliferation increased with decreasing modulus, and the greatest gene expression of neural differentiation markers was observed in the softest gels, which had elastic moduli comparable to brain tissue (180Pa) (Banerjee et al., 2009). Primary rat neurons, neural spheroids, human and rat neural stem cells cultured on ‘soft’ alginate films underwent rapid and abundant neurite outgrowth, and were resistant to oxidative stress injury (Matyash et al., 2012).

Alginate encapsulation also supports *in vivo* proliferation and differentiation of neural linages in rat spinal cord lesions (Kataoka et al., 2004; Prang et al., 2006; Willenberg et al., 2006; Wu et al., 2001) and rat sciatic nerve regeneration (Hashimoto et al., 2002). In one model, rat hippocampus-derived neurosphere cells were transplanted to an alginate-filled lesion of a young rat spinal cord. After four weeks neurosphere cells survived, differentiated, migrated and integrated into the host tissue (Wu et al., 2001). A similar study used alginate-based anisotropic capillary hydrogels seeded with rat neural progenitor cells and reported no inflammatory response and induction of directed axon regeneration after implantation into rat cervical spine lesions (Prang et al., 2006). Schwann cells co-transplanted with alginate hydrogels inhibit cellular apoptosis and promote locomotor function recovery in a rat model of SCI (Wang et al., 2012). Alginate has also been tested

for the delivery and release of growth factors. Injection of alginate loaded with fibrinogen and GDNF in a rat spinal cord injury model supports spinal cord plasticity and regeneration, with increased neurofilaments in the site of the lesion but lower functional recovery with respect to the GDNF bolus injection (Ansorena et al., 2013). Rats with SCI transplanted with Wnt3a-secreting fibroblasts encapsulated in alginate hydrogels showed better recovery and axon regeneration with respect to cells transplanted alone (Park et al., 2013).
Finally, both human (Lu et al., 2012) and mouse (Kuo and Chang, 2013; Kuo and Chung, 2012) induced pluripotent stem cells (iPS) cells have also been encapsulated in alginate-based biomaterials and evaluated for neurogenesis. These studies report induction of neural lineages with benefits by encapsulated growth factors (Kuo et al., 2013) and scaffold-grafted peptide sequences (Kuo et al., 2012).

Some studies report the possibility of obtaining *in situ* crosslinkable alginate hydrogels and few of them use these *in situ*-forming alginates obtained with $CaSO_4$ for CNS repair, studying if they enhance cell transplantation efficiency and neuroregenerative effects in rat SCI models (Chang et al., 2001) (Jin Hook Park et al., 2013). They have been evaluated also for VEGF delivery in the myocardium and in hindlimb ischemia models (Hao et al., 2007; Silva and Mooney, 2007). Injection in rats before inducing cerebral ischemia showed slightly more improvements in motor functions with respect to controls (stroke alone, injection of alginate or VEGF alone) and a more marked reduction in infarct area (Emerich et al., 2010). Two studies report the use of *in situ* crosslinkable alginate hydrogels obtained with calcium carbonate ($CaCO_3$) and glucono-delta-lactone (GDL), testing them as sealant for dural defects (Nunamaker and Kipke, 2010) or modified with RGD for *in vitro* studies as support for endothelial cells delivery (Bidarra et al., 2011).
There are evidences that unmodified alginate does not provide adequate cell adhesion (Lee and Mooney, 2012; Rowley et al., 1999), but it can be modified in order to improve cell attachment and motility with ECM components such as fibrin, fibronectin, collagen or HA. These molecules can also help in recapitulating the native cell environment, providing biochemical and biophysical cues to the cells. Modifications with fibronectin (Fn) can be used to study effects of cell attachment. It is an extracellular glycoprotein that binds both cell integrins and other ECM molecules, and plays a major role in cell adhesion, growth and differentiation (Schwarzbauer and DeSimone, 2011). This glycoprotein is also important for neural development by promoting cell survival, migration, neurite outgrowth and synapse formation with a specific spatial and temporal expression (Perris and Perissinotto, 2000). It is also involved in the remodelling of the tissue after brain injuries, showing promotion of nerve regeneration (Alovskaya et al., 2007). Biomaterials are often associated with the arginine-glycine-aspartic acid (RGD) peptide which is a site of cell attachment via cell surface integrins for ECM binding proteins such as fibronectin, fibrin and laminin (Rouslahti

1987). In addition to promoting adhesion, integrin binding can also stimulate intracellular signaling and gene expression involved in viability (Salinas and Anseth, 2008), migration, and differentiation (Chan and Mooney, 2008; Hwang et al., 2006; Schmidt et al., 2011). It has been shown that RGD modifications increase neural cell attachment and spreading. Adult rat neural stem cells cultured on a lipid bilayer with various RGD-containing peptides underwent increased aggregates formation in the presence of specific peptide sequences and retained their ability to differentiate into both neurons and astrocytes (Ananthanarayanan et al., 2010). Presence of RGD peptide can also help neurite outgrowth and increase neurite length in synthetic hydrogel cultures (Shepard et al., 2012).

The glycosaminoglycan hyaluronan (HA) is one of the major components of the developing CNS extracellular matrix (Margolis et al., 1975) and it is a critical component of the neural stem cell niche (Preston and Sherman, 2011). HA is a linear polysaccharide of (1-*β*-4)D-glucuronic acid and (1-*β*-3)*N*-acetyl-D-glucosamine which binds cell surface receptors CD44 and CD168. CD44 controls HA-induced cell proliferation and survival (Toole, 2004) while CD168 plays a role in HA-induced cell locomotion (Yang et al., 1994). In culture, human embryonic stem cells express high levels of both CD44 (Campbell et al., 1995) and CD168 (Stojkovic et al., 2003). HA is involved in neuronal migration, neurite outgrowth and axonal pathfinding (Bandtlow and Zimmermann, 2000). Many studies sustain the idea that HA efficiently supports differentiation of embryonic and neural stem cells, and that mechanical properties of HA-based hydrogels can also influence cell behaviour (Brannvall et al., 2007; Gerecht et al., 2007; Her et al., 2013; Seidlits et al., 2010; Wang et al., 2011). The use of an HA-rich environment could thus favour neural differentiation.

So far, there is only one report of alginate use for CNS regeneration in patients. In 2008, the Biocompatibles International Company reported the recovery of a stroke patient, in which they transplanted a polypropylene bag, filled with alginate beads (CellBead^{TM}) with encapsulated MSCs, engineered in order to produce Glucagon-like peptide 1 (GLP-1), a protein naturally produced in humans, which has anti-apoptotic effects, preventing cell death. The bag was removed after 2 weeks from the implant and they reported the recovery of speech and use of the arms in the patient (Aboody et al., 2011).

1.4 *In vivo* imaging

1.4.1 Toll-like receptors (TLRs) role in brain injury

The innate immune response is the defence against pathogens and is based on a variety of receptors, including the transmembrane Toll-like receptors (TLRs). They are located on antigen presenting cells such as dendritic cells (DCs), B and T cells, macrophages and microglia, and are part of the class of pattern-recognition receptors (PRRs), which activate the innate immune system and modulate the adaptive immune response. They were first

described for their ability to recognize conserved exogenous pathogen-associated molecular patterns (PAMPs), found in lipopolysaccharide (LPS) in gram negative bacteria, lipoproteins, flagellins and other pathogen components. Subsequently, their role as sentinels of tissue damage and consequent inflammatory response has been discovered. This role is due to some endogenous ligands known as “damage-associated molecular patterns” (DAMPs). Typically they are confined in the intracellular space but are released following injury, damage, stress or death, activating TLRs (Heiman et al., 2014). The effect of endogenous TLRs stimulation can vary, depending on which tissue or cell types are involved, leading to both detrimental and beneficial effects. When activated by their pathogen- or host-derived ligands, TLRs induce signals which result in the release of pro-inflammatory cytokines and chemokines, such as TNF-a, IL-1 and IL-6, known to be responsible of stroke brain damage (Okun et al., 2009; Takeda et al., 2003).

It has been demonstrated that in both human and rodents, TLRs are also expressed in the CNS in cerebral endothelial cells, astrocytes, oligodendrocytes and neurons, with constitutive expression mainly confined to the regions with direct access to the circulation (Heiman et al., 2014; Marsh and Stenzel-Poore, 2008). Through endogenous ligand recognition they are involved in some non-immune physiological processes in the CNS such as neurogenesis and brain development (Okun et al., 2009), but also in neuroinflammation associated with neurological and neurodegenerative conditions, such as Alzheimer’s disease, multiple sclerosis and stroke (Tang et al., 2007).

After cerebral ischemia many endogenous ligands have been identified, together with the upregulation of the expression of TLR2, TLR4 and TLR9. Already one hour from stroke onset, neurons express high levels of TLR2 and TLR4 (Tang et al., 2007; Ziegler et al., 2007) while high TLR2 expression is found in microglia after 24 hours (Lehnardt et al., 2007). Mouse models deficient for TLR2 and TLR4 show better outcomes after cerebral ischemia with respect to WT, presenting reduced infarct volume and edema, and decreased production of inflammatory cytokines such as IL-6 and TNF-α, thus confirming the involvement of TLRs in the response to stroke (Cao et al., 2007; Lehnardt et al., 2007) (Ziegler et al., 2007).

The elimination of downstream elements of TLRs response does not correspond to ischemia protection nor amelioration (Famakin et al., 2011), confirming that the inflammatory response after stroke is important for dead cells clearance and for setting the conditions for tissue repair. In fact, negative effects are linked to its prolonged permanence that limits regeneration. TLRs have been shown to produce the anti-inflammatory cytokine IL-10, which elicits its neuroprotective function through inhibition of the neurotoxic effects of TNF-α and IFNɣ (Nathan and Ding, 2010). IL-10 expression however starts from 12-24 hours after TLRs stimulation. This delay in activation of neuroprotective cascades could be

essential for inflammation to start in order to protect healthy tissue (Samarasinghe et al., 2006). TLRs activation has been proven to be crucial also for hematopoietic stem cells recruitment to the ischemic area, in order to reduce infarct volume (Ziegler et al., 2011). Moreover, TLRs act as mediators of necrotic neurons to microglia but are also responsible of microglia sensitivity to apoptosis, through the production of IFNβ (Jung et al., 2005). This is an important mechanism of prevention of an excessive inflammatory response, which could be deleterious for tissue recovery. These findings highlight the dual role of TLRs in stroke response, which first trigger inflammation and further actively participate in its resolution.

1.4.2 TLR2-luc/GFP mouse strain and *in vivo* bioluminescence assay

Before entering a clinical trial, safety and efficacy of tissue engineering approaches must be evaluated. During preclinical testing in animal models, cell-scaffold constructs are often implanted in nude mice or immunosuppressed animals, that cannot present immunorejection events (Ikada et al., 2006). This easily allows the analysis of efficacy of the treatments, but data on biomaterial biocompatibility in the tissue are distorted. Therefore there is the need of new approaches to study the effects of grafted biomaterials in a tissue.

Recently a transgenic mouse model that carries a dual reporter system with luciferase (luc) and green fluorescent protein (GFP) under the transcriptional control of a murine TLR2 promoter has been developed. This model can be used for more reliable biocompatibility studies. TLR2-luc/GFP mice allow the *in vivo* imaging of TLR2 transcriptional activation, as indication of inflammation levels, by using a biophotonic/bioluminescence imaging and a high resolution charged coupled device camera (CCD) (Lalancette-Hebert et al., 2009). The dual reporter system allows microscopic resolution with the GFP fluorescence signal, while emission of luciferase above 620nm is used for live bioluminescence imaging (BLI). The great advantage of this model is the possibility to visualize inflammation in living animals, which are anesthetized without interfering with the recordings or the treatment under evaluation. In order to detect BLI signals, animals should be injected with the substrate of luciferase, D-luciferin. If luciferase is expressed, in presence of ATP and magnesium ions, the D-luciferin is transformed in its adenilated form, with the release of photons that are detected by the instrument. Basal TLR2 expression in the brain is very low or even undetectable, thus it does not interfere with analyses. However baseline signals should be recorded before starting the experiments, since there could be the presence of basal photon emission from tissues. These measurements are used to normalized values obtained in later time points, allowing the comparison among different conditions and animals.

This model has been tested with systemic or intracerebral LPS injection. Its administration is a well-established model associated with a strong induction of inflammation through TLRs activation, included TLR2, both at the mRNA and protein level in the central nervous system (Laflamme et al., 2001). Results showed the possibility to detect the induced inflammation in the brain and spinal cord of animals after LPS injection (Lalancette-Hebert et al., 2009). Since TLR2 activation is present in microglia cells in response to cerebral ischemia or LPS stimuli, inflammation profile after LPS injection was recorded in mouse brain, demonstrating that TLR2 and GFP co-localize in these cells, recapitulating the induction and expression profile of the endogenous TLR2. In addition, the authors successfully used this mouse strain in order to study spatial and temporal microglia activation after ischemic injury in the brain (Lalancette-Hebert et al., 2009). These studies confirm that TLR2-luc/GFP mouse strain is a reliable tool for study inflammation profiles in the brain, since it is present in this tissue and its expression is induced following injury.

This system has been used in the presented work, in order to analyze biomaterial compatibility once injected in the mouse brain.

2. AIM OF THE THESIS

Cell replacement therapies are currently among the most promising strategies to cure an injured brain and embryonic stem cells (ESCs) represent an important and unlimited cell source for transplantation therapies (Polak and Bishop, 2006). Even if many published neural differentiation protocols for ESCs are based on monolayer cultures, it is also known that stimulation from the surrounding environment is crucial for the differentiation of cells towards the desired lineage. Natural three-dimensional culture systems, such as embryoid bodies or neurospheres, are characterized by highly heterogeneous populations and lack rigorous control of differentiation. Biomaterials can help recapitulating the three-dimensional environment present *in vivo*, allowing to better mimic the physiological interactions and stimuli that cells encounter *in vivo*.

The first aim of this study was to evaluate three-dimensional alginate-based scaffolds for the differentiation of mESCs. We tested whether specific alginate concentrations and modifications could enhance the production of terminally differentiated neurons with respect to two dimensional control cultures. We analyzed cell viability and neural differentiation within our scaffolds, by RT-qPCR and immunocytochemical analyses. In literature it is reported that alginate does not favour cell adhesion (Lee et al., 2012; Rowley et al., 1999), we thus tested its modification with fibronectin, a protein involved in cell adhesion and its adhesion peptide, the RGD peptide (Scwarzbauer et al., 2011). Alginate modification with hyaluronic acid, an ECM component present during neural development and in the adult neural stem cell niche (Margolis et al., 1975; Preston et al., 2011), was tested as well. As stem cells neural differentiation is influenced by scaffold properties (Amit et al., 2000, Pfieger et al., 1997; Teixeira et al., 2009), the mechanical and physical properties of the alginate scaffolds we produced were analyzed testing their water content and stiffness. Furthermore, we tested whether bead dimension could influence stem cell differentiation.

Stroke is one of the major causes of long-term and permanent disability (Donnan et al., 2008). NSCs are shown to integrate and improve functional recovery once transplanted in stroke animal models (Doeppner et al., 2014; Ding et al., 2013; Oki et al., 2012; Lee et al., 2009;Jeong et al., 2003; Yuan et al., 2012). However, the majority of the grafted NSCs die within weeks after transplantation, resulting in a limited efficacy of the treatment (Li et al., 2012). In the second part of this work we evaluated the possibility to use alginate as support for stem cell transplantation in the brain. We tested an alternative crosslinking method in order to obtain injectable alginate hydrogels, which can allow minimal invasive surgery. We tested mNSCs viability and initial differentiation after encapsulation in alginate hydrogels obtained with different crosslinking methods. Biocompatibility and suitability of alginate injection in the mouse brain tissue were then evaluated. Histological analyses were

performed on injected brains in order to confirm alginate crosslinking *in situ* and presence of grafted cells in the site of injection. We took advantage of a mouse strain that carries the luciferase under the control of the TLR2 promoter in order to test alginate biocompatibility in the brain tissue. TLR2 is known to be involved in inflammation after brain injury (Tang et al., 2007), thus this mouse model allows to visualize *in vivo* the activation of TLR2 and consequently to monitor the inflammatory response elicited by alginate injection and presence in the mouse brain.

During my PhD I was involved in other projects ongoing in our laboratory. Reports of the results can be found attached at the end of the thesis.

3. MATERIALS AND METHODS

3.1 *In vitro* murine stem cell culture and differentiation in three-dimensional alginate-based hydrogels

3.1.1 Mouse embryonic stem cell (mESCs) and mouse neural stem cell (mNSCs) cultures

The feeder-independent mouse embryonic stem cell line E14TG2a.4 (obtained from MMRRC, University of California, Davis) and a mESC-derived neural stem cell line (Conti et al., 2005) were used in this study. mESCs were maintained in an undifferentiated state in gelatin-coated dishes in self renewal ES medium (Glasgow Minimal Essential Medium (GMEM, Sigma)), 10% Fetal Calf Serum (FCS, Millipore), 1 mM Sodium Pyruvate (Gibco), 0.1 mM Non Essential Amino Acids (NEAA, Gibco), 2 mM L-Glutamine (Lonza), 100 U/mL Penicillin/Streptomicin (Lonza), 0.05 mM β-mercaptoethanol (Sigma), 1000 U/mL Leukaemia Inhibitory Factor (LIF, Sigma).

mNSCs were maintained in proliferation on uncoated plastic in Self Renewal medium, consisting of Euromed-N medium (Euroclone), 1% N2 supplement (Life Techonogies), 20 ng/ml FGF (Peprotech), 20ng/ml EGF (Peprotech), 2 mM L-Glutamine (Lonza), 100 U/mL Penicillin/Streptomicin (Lonza).

3.1.2 Alginate solution

Alginate solutions (1.0% and 2.0% w/v) were prepared by mixing alginic acid sodium salt (Sigma), 0.15 M NaCl and 0.025 M HEPES in deionized water. The solution was stirred and heated to dissolve the alginate, autoclaved and then filtered with 0,22 μm filters. Fibronectin (Fn, Sigma) was added to the alginate at 0.1 mg/mL final concentration, RGD (Novamatrix) and hyaluronic acid (HA, Sigma) at the final concentration of 5 mg/mL.

3.1.3 Alginate gel characterization

The water content for each alginate formulation was determined by investigating the swollen weight (Ws) and dry weight (Wd). Four 2.0% w/v or 1.0% w/v alginate discs of each formulation (unmodified, HA- or Fn- modified) were crosslinked in 0.3 M $CaCl_2$ overnight using customized molds. Following crosslinking gels were punched out of the molds and equilibrated for 1 hr in PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. After 1 hr, the alginate discs were weighed and the swollen weight (Ws) was recorded. Then the alginate discs were placed into an oven at 100 °C for 1 week and weighed again (Wd). The water content percentage was calculated using the formula: (WsWd)/Ws*100%. Mean water contents and associated standard deviations (n = 4) are reported. The bulk

mechanical properties of all 3 formulations at both 1 and 2% alginate were calculated using the Q-800 Dynamic Mechanical Analyzer (DMA; TA Instruments, New Castle, DE) and Q Series Explorer software. 6 mm thick samples were cyclically compressed at 1 Hz to a strain of 10%. The Young's Modulus was determined from the linear region of the generated stress-strain curve. Four samples of each alginate formulation were tested.

3.1.4 Cell encapsulation and differentiation in alginate beads

For encapsulation cells were washed with phosphate buffered saline (PBS), detached using trypsin-EDTA, and counted. After pelleting the desired quantity, cells were mixed with alginate and dropped into a 0.1 M $CaCl_2$ solution with a syringe with a 19G or 27G needle (day -1). After 10-15 min of incubation alginate beads were rinsed once with medium or PBS buffer and then placed into a 6-well plate with 5-6 large or 10-12 small beads for each well.

mESCs culture Two-dimensional control culture was performed following published protocols (Fico et al.,2008) with minor modifications. Briefly, cells were seeded on gelatin-coated 12-well plates at an initial density of 1000 cells/cm^2 in ES medium. 1 day after plating the medium was changed to serum-free neural differentiation medium (Knock-out DMEM, Life-Technologies) supplemented with 15% Knock Out Serum Replacement (KSR, Life-Technologies). The medium was changed every other day until day 18. For three-dimensional cultures, cells were seeded at an initial density of 2 x 10^6 cells/mL alginate solution, cultured one day in ES medium then transferred in neural differentiation medium until day 18. Medium was changed every other day.

mNSCs culture Two-dimensional control culture was performed following published protocols (Spiliotopoulos et al., 2009) with minor modifications. Cells were seeded on 12-well plates at an initial density of 1,5 x 10^5 cells/cm^2 in D1 medium consisting of Euromed-N (Euroclone) supplemented with 1% N2 supplement (Life Technologies), 1% B27 supplement (Life Technologies), 20ng/ml FGF (Peprotech), 2 mM L-Glutamine (Lonza) and 100 U/mL Penicillin/Streptomicin (Lonza) (Day0). At day3 cells were detached with Accutase and replated at the density of 5 x 10^4 cells/cm^2 on 24-well plate coated with laminin (3ug/ml) in medium A, consisting of DMEM/F12 (Life Technologies) and Neurobasal medium (Life Technologies) at 1:3 ratio, supplemented with 0,5% N2 supplement (Life Technologies), 1% B27 supplement (Life Technologies), 10ng/ml FGF (Peprotech), 20 ng/ml BDNF (Peprotech). At day 6 medium was changed to medium B (DMEM/F12:Neurobasal medium (1:3), 0,5% N2 supplement, 1% B27 supplement, 6,7ng/ml FGF, 30 ng/ml BDNF). At day 9, FGF concentration is lowered to 5 ng/ml and medium is changed every two days until day 12. For three-dimensional cultures cells were encapsulated at a density of 2 x 10^6 cells/mL alginate solution.

3.1.5 Cell encapsulation in alginate *in situ* gelling hydrogels

1.25% w/v alginate solution was prepared as previously described. A 0.2M $CaCO_3$ solution and a 0.8M GDL solution were prepared in sterile water and filtered. 125µl of $CaCO_3$ solution were added to 1mL of alginate and mixed. Prior cell seeding and/or injection, 125µl of GDL solution were added to alg:$CaCO_3$ mixture and mixed.

For cell encapsulation in alginate *in situ* gelling hydrogels, mNSCs were washed with phosphate buffered saline (PBS), detached using Accutase, and counted. After pelleting the desired quantity, cells were mixed with alginate:$CaCO_3$ solution until resuspension. GDL solution was added to the cell suspension and mixed. 400µl of alginate-cell mixture were put in each well of a 24-well plate and incubated at 37°C, 5% CO2 for 10-15 minutes, until hydrogel crosslinking. Fresh medium was added and changed after 30 minutes. mNSCs were differentiated as described above (from Spiliotopoulos et al., 2009).

3.1.6 Cell recovery from alginate beads

Cells were isolated from beads by the addition of 0.05 M EDTA for 20-30 min at 37ºC, which disrupts the polymer by Ca^{2+} chelation. Cells were pelleted by centrifugation at 500x g for 5 min and used for subsequent analyses.

3.1.7 Cell viability assay and flow cytometry

Live/Dead cell viability assay (Live/Dead cell viability assay kit, Life Technologies) was performed at day 7 and day 18. For the assay beads were collected and incubated in PBS for 30 min at 37°C. Beads were then placed in a 24 well-plate and incubated for 30 min in dark conditions with Ethidium Homodimer-1 (EH-1) and Calcein (AM) dissolved in sterile PBS. Results were analyzed using a Zeiss Axio Observer.Z1 microscope. For flow cytometry counts beads were stained as just described and subsequently dissolved. Cells were recovered and analyzed with FACS Canto using the Facs Diva software. Unstained cells were taken as control.

3.1.8 Fixation of encapsulated cells

Beads were collected and placed in 4% paraformaldehyde (PFA) solution in PBS for 24 hrs at 4ºC or 24 hrs at room temperature (RT). Samples were then immersed in 30% sucrose solution for 24 hrs at RT, embedded in OCT (Tissue-Tek) and stored at -80ºC until cryostat sectioning (20 µm).

3.1.9 Immunocytochemistry analyses

Slides were washed for 5 min in PBS, and incubated with blocking solution for 1 hr at room temperature. Cells were incubated for 1.5 hrs with primary antibody in blocking solution and

then washed three times for 5 min with PBS containing Triton 0.1%. Cells were then incubated for 1 hr with secondary antibodies, washed again, incubated for 5 min with Hoechst or DAPI and mounted with Mowiol. The primary antibodies used were: βIII-tubulin (1:1000; Covance), GFAP (1.500, Dako), NCAM (1:500; Millipore), Nestin (1:200; Millipore), MAP2 (1:200; BD Bioscience), PSD95 (1:400; NeuroMab), Sox2 (1:500; Abcam), VAMP2 (1:600; Synaptic System). The secondary antibodies used were: Alexa Fluor 594 Goat anti-rabbit (1:1000; Life Technologies), Alexa Flour 488 Goat anti-mouse (1:1000; Life Technologies). Images were taken using Zeiss Axio Observer.Z1 microscope, Leica TCS SP5 or Zeiss LSM 501Meta confocal microscopes.

3.1.10 Wisteria floribunda agglutinin (WFA) staining

Slides were washed two times for 10 min in PBS and incubated with blocking solution for 1 hr at RT. They were then incubated O/N at 4°C with biotinylated wisteria floribunda lectin (Vector Laboratories) (10µg/mL) and then washed three times for 15 min with PBS. Cells were incubated for 1hr at room temperature with the secondary antibody conjugated with streptavidin and washed three times for 15 min with PBS. After 5 min incubation with Hoechst and a 10 min wash in PBS, they were mounted with Mowiol.

3.1.11 RNA isolation and RT-qPCR analyses

Cells were recovered from beads at day 7 and day 18 and total RNA was isolated using a Nucleospin RNAII Kit (Macherey Nagel). RNA was reverse-transcribed using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative polymerase chain reactions were performed on a real-time PCR machine (Bio-rad CFX96) with KAPA Sybr® Fast qPCR kit (Resnova) for 40 cycles with the following profile: 95 ºC for 15 s, 60 ºC for 20 s, 72 ºC for 40 s. For the melting curve 0.5 ºC was increased every 5 s from 65 ºC to 95 ºC. All reactions were run in triplicate and β-Actin was used as reference gene. Relative gene expression was calculated using the DDCT method. A list of primers used can be found in Table 1. Each experiment was performed at least three times. Values are expressed as mean ± SEM.

3.1.12 Statistical analyses

Data were analyzed using a Student's t-test or a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test as appropriate to determine statistical significance of differences between hydrogel conditions and control cultures. Levels of significance were set at $p < 0.05$ (*), and $p < 0.01$ (**). Significance was calculated with respect to control cultures, unless otherwise stated.

Oligonucleotides	Sequences
β-Actin	Forward: 5'-AATCGTGCGTGACATCAAAG-3'; Reverse: 5'- AAGGAAGGCTGGAAAAGAGC-3'
Brachyury	Forward: 5'-GAACCTCGGATTCACATCGTGAGA-3'; Reverse: 5'-ATCAAGGAAGGCTTTAGCAAATGGG-3'
Gad67	Forward: 5'-TCCAAGAACCTGCTTTCCTG-3'; Reverse: 5'-GAGTATGTCTACCACTTCGAG-3'
GFAP	Forward: 5'-GGAGAGGGACAACTTTGCAC-3'; Reverse: 5'-CCAGCGATTCAACCTTTCTC-3'
HB9	Forward: 5'-GTGCCAGCACCTTCCAAC-3'; Reverse: 5'- CTTCGGCACTTCCCCAAG-3'
Nestin	Forward: 5'-GATCGCTCAGATCCTGGAAG-3'; Reverse: 5'-AGAGAAGGATGTTGGGCTGA-3'
NCAM	Forward: 5'-AGGAGAAATCAGCGTTGGAG-3'; Reverse: 5'-CGATGTTGGCGTTGTAGATG-3'
Oct 3/4	Forward: 5'-CTGAGGGCCAGGCAGGAGCACGAG-3'; Reverse: 5'- CTGTAGGGAGGGCTTCGGGCACTT-3'
Pax6	Forward: 5'- CCTCCTTCACATCAGGTTCC-3'; Reverse: 5'- CATAACTCCGCCCATTCACT-3'
TH	Forward: 5'-TCAGAGCAGGATACCAAGCA-3'; Reverse: 5'-CGAATACCACAGCCTCCAA-3'
Tph2	Forward: 5'-CAGCGGTAGTGTTCTCCTTG-3'; Reverse: 5'- ATTCCGTTTTGCCACATTC-3'
βIII-Tubulin	Forward: 5'-TTCTGGTGGACTTGGAACCT-3'; Reverse: 5'-ACTCTTTCCGCACGACATCT-3'
VGlut2	Forward: 5'- GACAAAGAATAAGTCCCGTGAAG-3'; Reverse: 5'- TCTCTCCTGAGGCAAATAGTG-3'
Sox17	Forward: 5'-GCCAAAGACGAACGCAAGCGGT-3' Reverse: 5'-TCATGCGCTTCACCTGCTTG-3'

Table 1 *List of primers used for the RT-qPCR analyses*.

3.2 *In vivo* injection of alginate hydrogels: crosslinking and biocompatibility analyses

3.2.1 Animals

Three to six months old CD1 and C57/BL6 TLR2-luc/GFP male mice were used for this study. The protocols have been approved by the Ethical Committee at the University of Zagreb School of Medicine.

3.2.2 Mouse NSCs isolation and culture

Embryonic neural stem cells were harvested from E14.5 mouse embryos. Briefly, both hemispheres were dissected from embryos and collected tissue pieces were mechanically triturated. Further enzymatical dissociation was performed by adding few mL of Accutase and incubating 30 min at room temperature. The enzyme was diluted with fresh medium and the suspension was centrifuged 6 min at 300g at room temperature. The obtained cell pellet was resuspended in fresh growth medium, cells were counted, seeded at the density of 2-3 x 10^6 in a T75 flask and cultured in DMEM/F12 with Glutamax (Life Technologies), supplemented with 1% N2 supplement, 1% B27 supplement, 20ng/mL EGF (Peprotech), 10 ng/mL FGF (Peprotech) and 100 U/mL Penicillin/Streptomicin (Lonza). Neurospheres begin to form and they were regularly propagated through enzymatic dissociation with Accutase. The enzyme was diluted with DMEM/F12 and the suspension was centrifuged 6 min at 300g at RT. The pellet was resuspended in growth medium and cells were seeded at a density of 2x10^6 cells/25 mL in a T75 flask. Half of the medium was changed every 3-4 days.

3.2.3 Cell staining

NSCs were stained with a fluorescent dye with long aliphatic tails (PKH26) which is stably incorporated into lipid regions of the cell membrane (PKH26 Cell Linker Kit, Sigma). Neurospheres were disaggregated with Accutase and the cell suspension was washed once with serum free medium and centrifuged at 400 x g for 5 minutes. 1mL of Diluent C, important for cell viability and staining efficiency, was added for resuspending the cell pellet. Immediately prior to staining, 2x Dye Solution (4 x10^{-6} M) was prepared by adding 4 mL of the PKH26 dye solution to 1 mL of Diluent C. The 1 mL of cell suspension is rapidly added to the 1 mL of 2x Dye Solution and immediately mixed by pipetting. The cell/dye suspension was then incubated for 5 minutes with periodic mixing by flicking the tube. 2mL of serum were added to the solution that was incubated for 1 minute in order to stop the staining by binding the dye in excess. Cells were centrifuged at 400 x g for 10 minutes and the cell pellet was resuspended in 10 mL of complete medium and centrifuged at 400 x g

for 5 minutes for washing. Another 2 washes with complete medium were performed in order to remove the unbound dye. After the last wash, the cell pellet was resuspended in complete medium and the cells were counted. The desired quantity was centrifuged at 200 x g for 5 minutes and resuspended at the appropriate concentration for injections.

3.2.4 Transient Middle Cerebral Artery Occlusion (MCAO) procedure

Transient middle cerebral artery occlusion (MCAO) was performed on a 12-week old male TLR2-luc/GFP mouse. Anaesthesia was induced with 3% isoflurane and maintained with a vaporizer. A ventral midline neck incision was made and the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed. The ECA was carefully dissected and the CCA was temporarily sutured. A permanent suture was placed around the left ECA, whereas a temporary suture was tied proximally to the bifurcation of the CCA. The left ICA was isolated and clipped using a vascular clip. A monofilament suture was inserted through the ECA into the CCA. The suture was firmly tied around the monofilament to prevent bleeding. The remaining portion of the ECA was cut to free the stump and insert the monofilament suture into the ICA. The clipped ICA was opened and the filament was advanced into the circle of Willis. The suture in the ECA was tightened to fix the monofilament suture in position. The wound was closed by applying a temporary wound clip.

After 60 minutes middle cerebral artery blood flow was restored. The mouse was re-anesthetize and the incision site was reopened by removing the clips. The suture on the ECA was opened and the filament slowly withdrawn until reaching the bifurcation of the CCA. ICA was clipped with a vascular clip above the end of the intraluminal suture. The monofilament suture was completely removed from the ECA and the suture was retightened firmly. The ICA was opened and the suture was removed from the CCA to allow reperfusion. The wound was closed with a suture.

3.2.5 Stereotactic injection into the mouse brain

Intracerebral transplantations into the mouse striatum were performed with the KOPF stereotactic apparatus (900LS) and Hamilton syringe needle (5µl). Mice were injected intraperitoneally with 0.5g/kg of Avertin for anaesthesia and positioned in the stereotaxic apparatus. A midline incision in the scalp was done to disclose the bregma suture. The striatum coordinates were calculated according to a brain atlas (Comparative cytoarchitectonic atlas of the C57BL/6 and 129/Sv mouse brains, Hof PR et al., Elsevier, 2000), and a hole was drilled in the skull in correspondence to the site of injection. The Hamilton syringe was loaded, lowered in the tissue and 1µl of solution was injected. According to experimental groups, mice received injection of PBS, LPS (2,5 ng/µl), mNSCs

in medium (150 000 cells/µl), 1% w/v alginate with or without $CaCO_3$ (20mM) and GDL (80mM), 1% alginate with encapsulated NSCs (5000 or 50 000 cells/µl). At the end, the needle was left in position 3 minutes in order to avoid backflow of the solution. The wound was then closed with a suture.

3.2.6 Brain fixing, collection and sectioning

Brains were fixed by transcardial perfusion with 4% paraformaldehyde (PFA) and a post-fixation overnight at 4°C. Brains were cut with vibratome (30µm) or cryostat (20µm) following overnight incubation at 4°C in 30% sucrose and embedded in OCT (Tissue-Tek).

3.2.7 Immunocytochemistry analyses

Brain sections were washed for 5 min in PBS, and incubated with blocking solution for 1r h at RT. Sections were incubated for 1.5 hrs with primary antibody in blocking solution and then washed three times for 5 min with PBS containing Triton 0.1%. Cells were then incubated for 1 hr with secondary antibodies, washed again, incubated for 5 min with Hoechst and mounted with Mowiol. The primary antibodies used were: GFAP (1.500, Dako), nestin (1:200; Millipore). The secondary antibody used was Alexa Fluor 488 Goat anti-rabbit (1:1000; Life Technologies). Images were taken using Zeiss Axio Observer.Z1 microscope.

3.2.8 Histological analyses

Brain sections were stained with cresyl violet 0,1% for 2-5 min. Sections were then rinsed in distilled water and dehydrated in a series of increasing concentrated alcohols. Following a brief incubation in xylene (2-5 min), sections were mounted with DPX mountant (Sigma). Images were taken using Zeiss Imager.M2 microscope.

3.2.9 Bioluminescence (BLI) *in vivo* imaging

Three months-old C57BL/6 TLR2-luc/GFP male mice were used in these experiments. One day before the baseline imaging, mice were shaved in order to avoid signal masking from the fur. 20 min before the imaging mice received intraperitoneal injections of the luciferase substrate D-luciferine in 0,9% saline (150mg/kg) (Caliper Life Science). Mice were then put in an anaesthesia box with 2% isofluorane (IVIS - XGI-8). Imaging was performed with the Xenogen IVIS Spectrum (Caliper Life Technology). Before and during acquisition of images, the mouse was positioned in the imaging chamber and kept under anaesthesia with a nose cone attached apparatus. Images were acquired with the Living Image Program. At the end of the measurement, the animal was put back in the cage. Baseline

measurements were performed before the surgery/injection (baseline), and 1 day, 3 days, 7 days and 14 days after the injection.

Data were analyzed with the Living Image In Vivo Analyses Software (Caliper LS-Xenogen). Photon emission values, expressed as photon/second, were exported after drawing the ROI for each time point in each animal. Measurements at each time point were normalized with the corresponding baseline value of the mouse.

4. RESULTS

4.1 Neural differentiation of mouse embryonic stem cells (mESCs) in three-dimensional alginate beads

Part of this work is based on the publication: **Bozza A**, Coates EE, Incitti T, Ferlin KM, Messina A, Menna E, Bozzi Y, Fisher JP and Casarosa S, Neural differentiation of pluripotent cells in 3D alginate-based cultures. Biomaterials 35 (2014) 4636-4645.

4.1.1 Introduction

The central nervous system (CNS) is limited in its capacity for self-repair after damage. Thus, cell replacement therapies or stimulation of endogenous stem cells are currently the most promising strategies to cure an injured brain. Evidences that Embryonic Stem Cells (ESCs) can potentially differentiate into most neuronal subtypes (Lee et al. 2000; Carpenter et al., 2001; Tropepe et al., 2001; Pachernik et al. 2002; Wichterle et al., 2002; Ying et al., 2003; Watanabe et al., 2005; Fico et al., 2008), make them a suitable and unlimited cell source for neural tissue regeneration (Polak et al., 2006). Their differentiation *in vivo* is influenced by mechanical, physical and chemical signals coming from soluble factors and contact with surrounding cells and ECM, (Estes et al., 2004). For this reason it is becoming increasingly evident that a three-dimensional culture system could be more efficient than two-dimensional cultures or Embryoid Bodies (EBs) formation for generating neurons *in vitro* (Bauwens et al., 2009).

Biomaterials can provide a three-dimensional culture environment to mimic the physiological microenvironment and guide differentiation of a stem cell population (Shakesheff et al., 1998; Dawson et al., 2008). It has been recently shown that alginate supports neural lineages differentiation and culture. Its modification with fibronectin or with its adhesion motif, the RGD peptide, can be used to study effects of cell attachment, while the addition of hyaluronan (HA), one of the major components of the developing CNS ECM (Margolis et al., 1975) and of the neural stem cell niche (Preston et al., 2011), can be tested for neural differentiation efficiency and enhancement.

In this part of the study, we developed an approach to drive differentiation of mESCs toward neuronal lineages using cell encapsulation in alginate beads and culture in a simple neural differentiation medium (Fico et al., 2008). We tested two different alginate concentrations and beads dimensions, and different modifications, fibronectin, RGD peptide and hyaluronic acid, characterizing their physical properties such as water content and Young's modulus. Cell survival was quantified and neural differentiation was analyzed by RT-qPCR and immunocytochemistry.

4.1.2 Experimental design

Mouse embryonic stem cells (mESCs) were encapsulated in hydrogel spheres of diameters in the range of 3.5 - 4.5 mm (Table 2). Alginate concentration influences biomaterial properties such as mechanical stability, elastic modulus, and nutrient diffusion within the hydrogel (Wang et al., 2009); while elastic modulus influences cell differentiation (Saha et al., 2008). We thus tested two different alginate concentrations, 1% w/v and 2% w/v, based on the results of previous work (Li et al., 2011; Wang et al., 2009). We also tested alginate beads modified by the addition of Fibronectin (Fn), RGD peptide (RGD) or Hyaluronic acid (HA) in order to test whether these molecules, known to play an important role in brain development and axonal migration, could enhance *in vitro* neural differentiation of mESCs. As control, we used a two-dimensional culture system where cells are grown in monolayer on gelatin coated plates according to a published protocol (Fico et al., 2008). In this protocol, general neural differentiation is achieved with a serum-free differentiation medium without the addition of any growth factor. This allowed us to better evaluate the influence of alginate hydrogels on mESCs neural differentiation without restricting cell differentiation towards specific neuronal subtypes.

Cells were encapsulated at an initial cell density of 2 x 10^6 cells/mL of alginate and were cultured for 18 days.

Condition (day18)	Diameter (mm)	+/- (mm)
Alg 1%	3,59	0,19
HA 1%	4,14	0,05
Fn 1%	3,43	0,48
RGD 1%	3,52	0,09
Alg 2%	4,05	0,07
HA 2%	4,58	0,07
Fn 2%	3,86	0,11
RGD 2%	3,83	0,06

Table 2 *Average beads size* . ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide.

4.1.3 Cell viability analyses of encapsulated cells

Alginate capsules remained transparent throughout the length of the differentiation culture, allowing for cell examination by brightfield microscopy. Beads remained spherically shaped in most of the cases and showed swelling once inside the culture medium but no degradation was evident. Fig. 1 shows how cells aggregate in small clusters following formation of the beads. Clusters do not increase their size throughout the protocol, and remain smaller than canonical EBs formed with the hanging-drop method (Kurosawa et al., 2007). Cell growth in small aggregates has been reported by other groups (Huang et al., 2012; Lu et al., 2012). In some cases, especially in the alginate-Fn experimental group, some cells escaped from the beads and attached to the bottom of the well (Fig. 1 b, d, h, arrows).

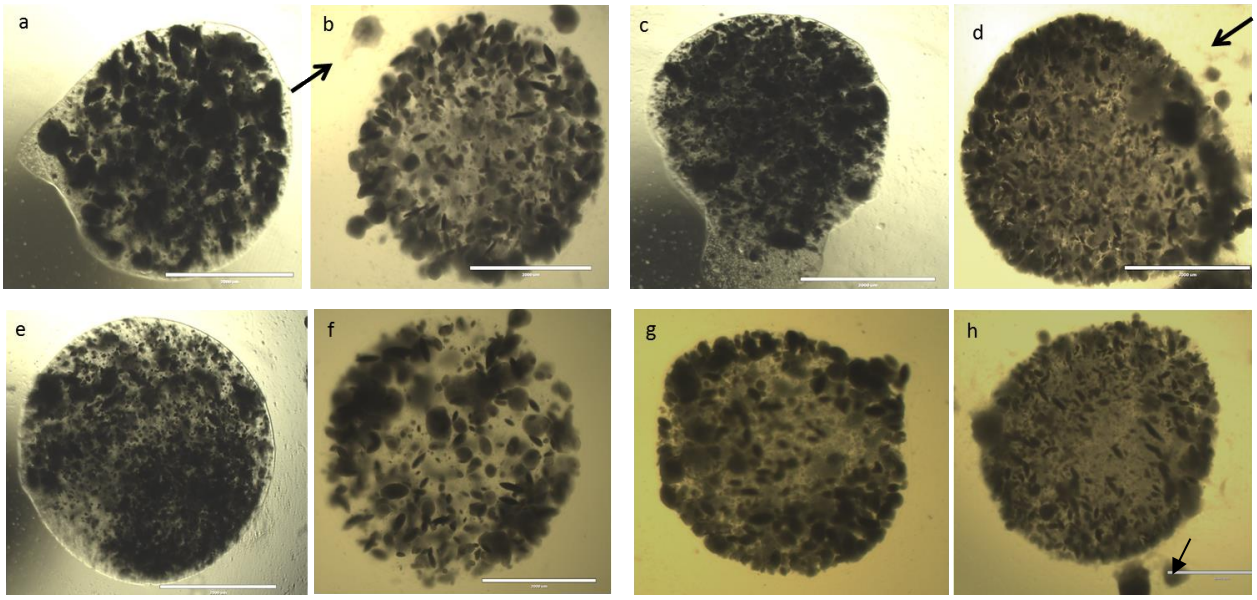

Fig.1 *mESCs encapsulated in alginate beads*. Cells inside alginate hydrogels proliferate and form clusters. 1% w/v alginate (**a**), 2% w/v alginate (**b**), 1% alginate – Fn (**c**), 2% w/v alginate – Fn (**d**), 1% w/v alginate – HA (**e**), 2% w/v alginate – HA (**f**), 1% w/v alginate – RGD (**g**), 2% w/v alginate – RGD (**h**). Magnification 2x (scale bar 2000 μm)

Viability of cells was assessed at three time points: immediately after encapsulation (D0), after 7 days of differentiation (D7) and at the end of the culture period (D18). D7 was chosen since it was shown to be the peak of neural precursor generation in two-dimensional cultures (Fico et al., 2008 and our own unpublished data). A Live/Dead two-color assay was performed on the intact beads; in parallel cells were recovered from the hydrogels and analyzed by flow cytometry for quantification. Fig. 2A shows that the majority of the cells remain viable throughout the culture period in almost all experimental conditions tested, as shown in green by the Calcein-AM staining for live cells at D18 (Fig. 2A). The Ethidium Homodimer-1 (EH1) red staining for dead cells is weak in all groups, with a slightly higher intensity in alginate-Fn group. Modification of alginate with RGD was not

included in the cytometric analyses, due to its variability in terms of stability in culture and homogeneity of cell differentiation, but Live/Dead assays on intact beads at D18 resulted in quite high cell viability (Fig.2A). Fig. 2B reports the results of cytofluorimetric analyses and shows the survival rate of the encapsulated cells, which is satisfactorily high in all tested conditions. The lower cell viability registered at D0 could be explained by cell death due to the encapsulation procedure, while the increase in viability observed from D0 to D18 can be attributed to the fact that some of the cells submitted to a differentiation stimulus can undergo cell death during the first days in culture. At D7 cells cultured in 2% alginate, both unmodified and modified, present lower viability with respect to 1% experimental conditions. This time point is associated with a peak of neural precursors generation in two-dimensional cultures and we can speculate that 1% alginate hydrogels better support mESCs viability during early neural differentiation.

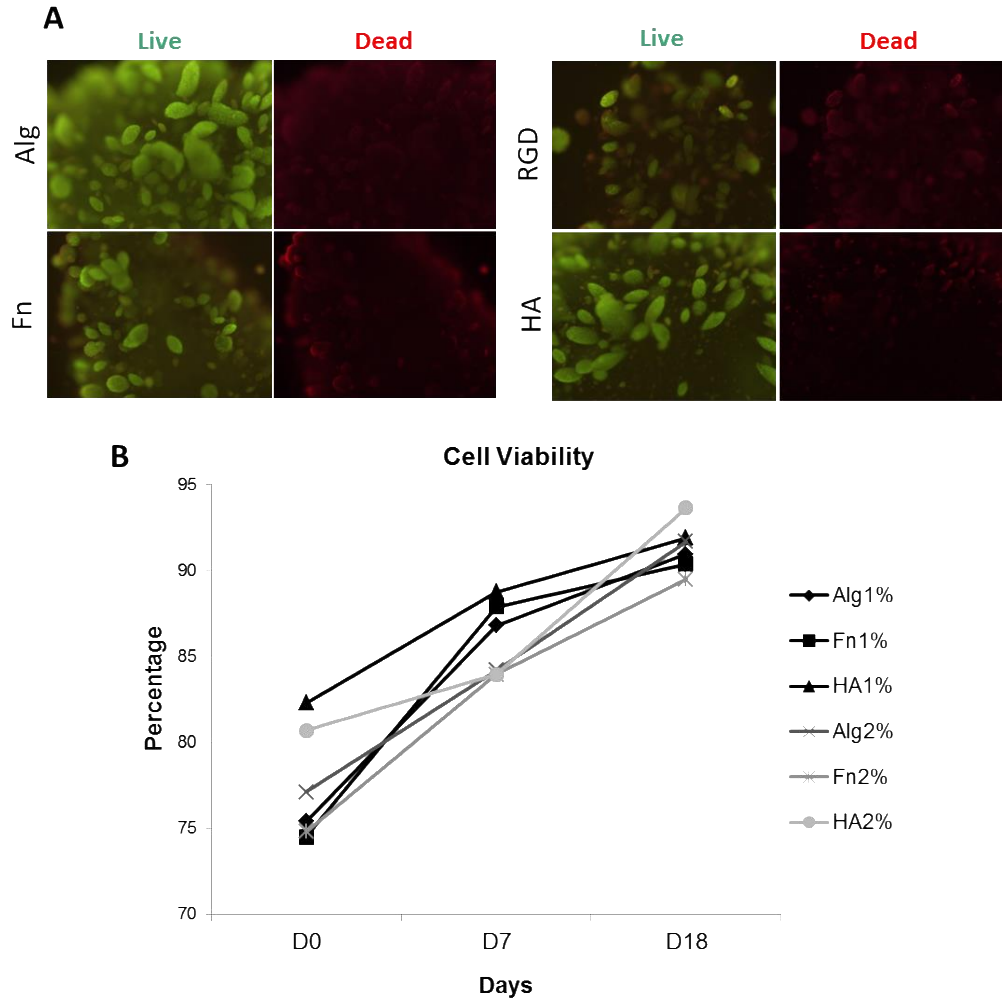

Fig.2 *Cells viability in alginate beads*. Cell viability was analyzed with LIVE/DEAD assay and cytofluorimetric analyses at D0, D7 and D18. **A**: LIVE/DEAD assay on intact beads at D18, **B**: cytofluorimetric analyses at D0, D7 and D18, merge of 2 experiments. ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide.

Cells encapsulated in both 1 and 2% alginate modified with HA present higher percentages of live cells at D0 with respect to cells encapsulated in the other experimental conditions, suggesting that this modification could better support cell viability after the encapsulation procedure. In fact, except for 2% alginate–HA at D7, this modification is associated with the highest cell viability percentages in all time points analyzed.

4.1.4 Molecular analyses of neural differentiation

In order to analyze neural differentiation we performed molecular analyses at D7 and D18. Cells were recovered from the beads and RNA was collected for RT-qPCR analyses. Additionally, some beads were fixed for cryosectioning and immunocytochemistry analyses. At D7 cells still express the pluripotency markers Oct3/4 and Nanog (Fig. 3) with variable levels in both alginate concentrations, indicating that not all cells at this time point have already started to differentiate. However, neural differentiation is occurring as shown by the expression of the pan-neural marker Pax6, which shows significantly higher expression levels with respect to controls in cells grown in all experimental conditions except for RGD–alginate, and with the highest levels in 1% alginate-HA (Fig. 3). At this time point, in two dimensional cultures there is the peak of generation of neural precursors, expressing high levels of Nestin. In the three-dimensional cultures, Nestin expression is variable among the different conditions, with lower levels with respect to control cultures, except for cells grown in alginate–HA at both concentrations. However, even if the differences in expression levels do not present statistical significance, we find higher expression of the neural differentiation marker βIII-tubulin in most experimental groups. Alginate 1% modified with Fn and RGD peptide present the lowest expression levels of βIII-tubulin (Fig. 3).

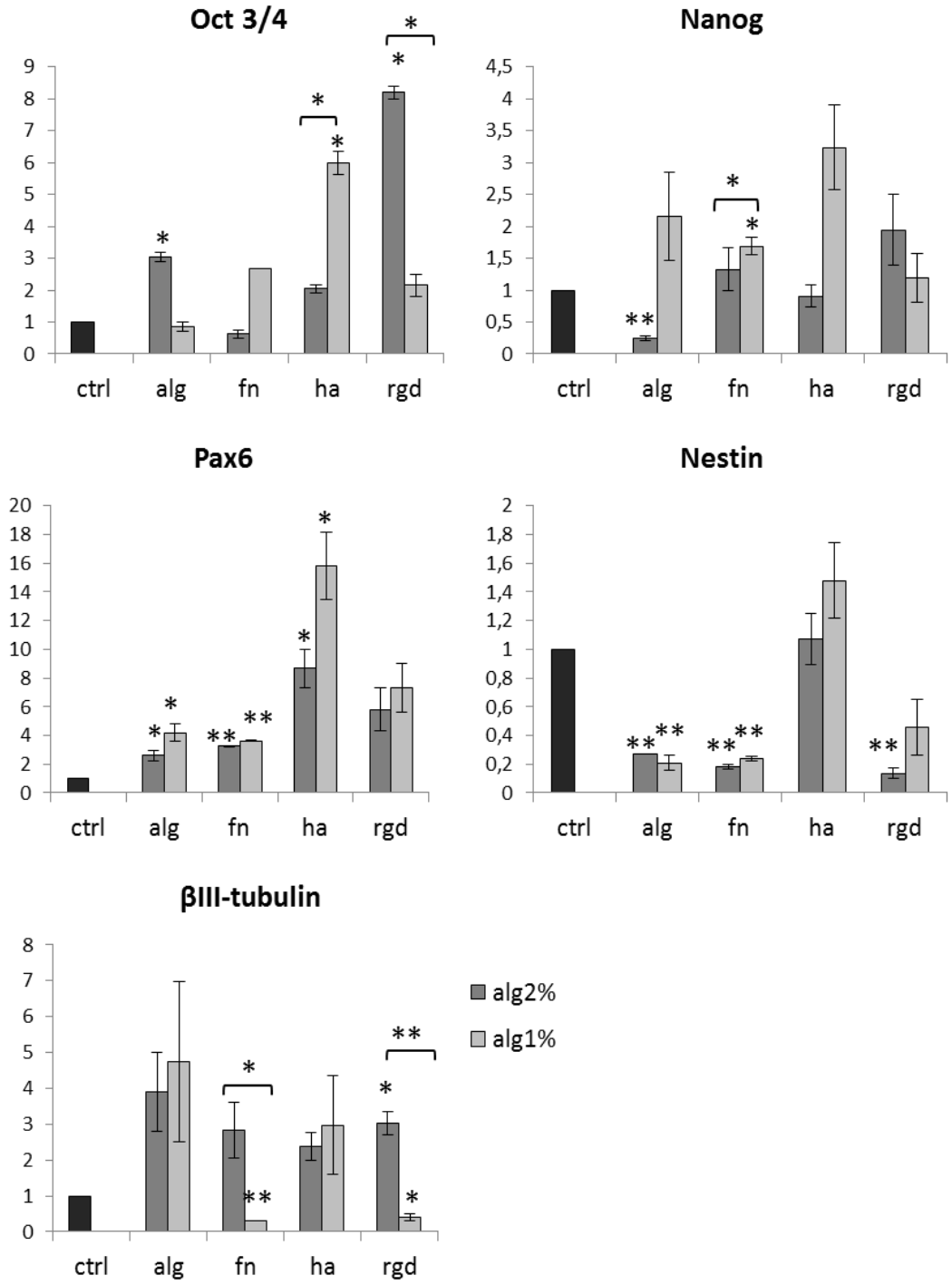

Fig.3 *Early neural differentiation of mESCs encapsulated in alginate beads*. RT-qPCR analyses on pluripotency markers (Oct3/4 and Nanog) and neural differentiation markers (Pax6, Nestin and βIII-tubulin) at D7. Dark gray bars: 2% w/v alginate, light gray bars: 1% w/v alginate. ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide. Statistical significance with respect to the control culture: * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

These data show that after 7 days, neural differentiation is enhanced in three-dimensional cultures with respect to monolayer controls, with lower levels of Nestin expression and higher Pax6 and βIII-tubulin, but it is still not possible to discriminate which alginate

concentration is the best for mESCs neural differentiation. However, differences were evident at the end of the culture period.

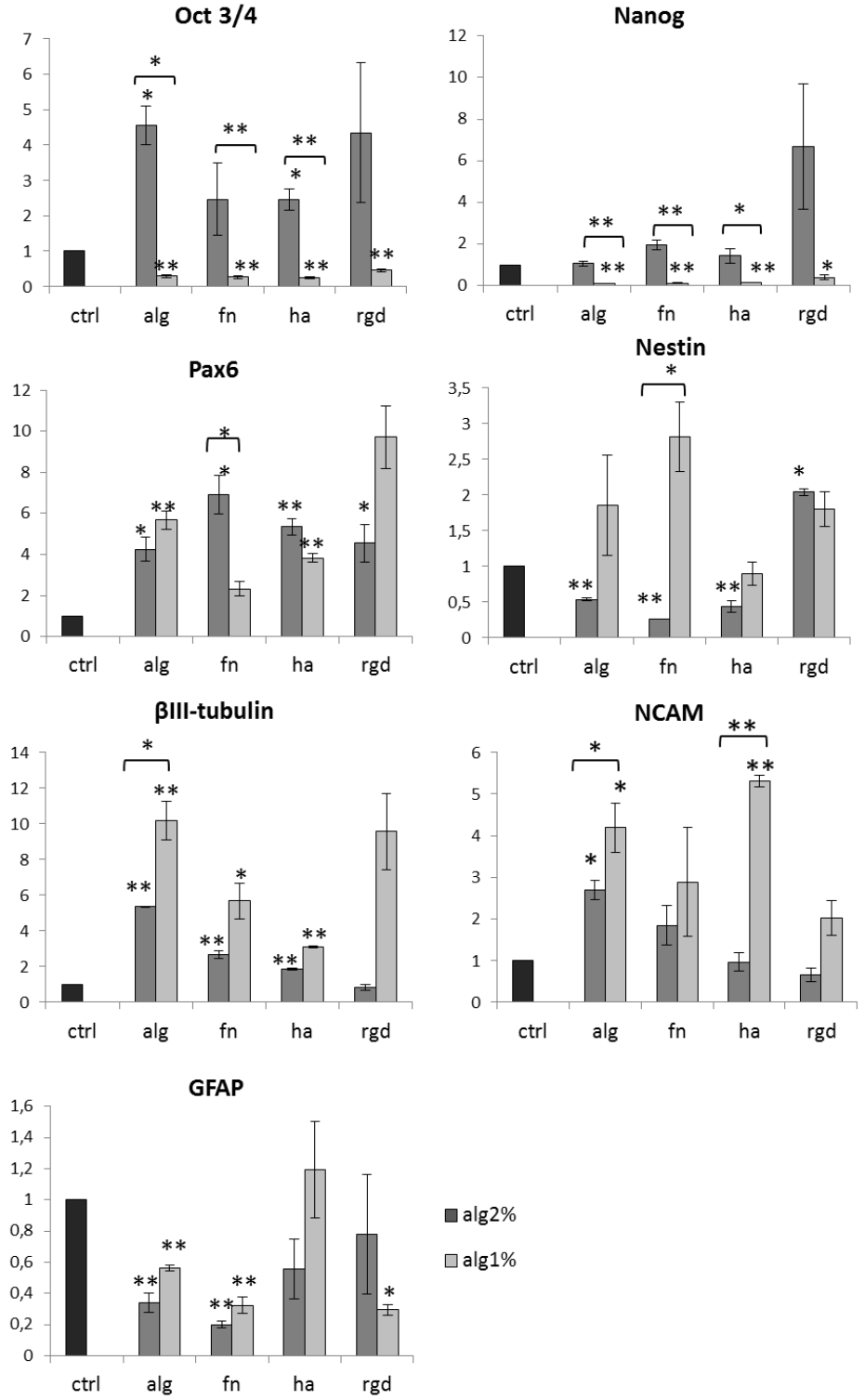

Fig.4 *Terminal neural differentiation of mESCs encapsulated in alginate beads*. RT-qPCR analyses on pluripotency markers (Oct3/4 and Nanog), neural differentiation markers (Pax6, Nestin, βIII-tubulin and NCAM) and glial marker (GFAP) at D18. Dark gray bars: 2% w/v alginate, light gray bars: 1% w/v alginate. ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide. Statistical significance with respect to the control culture: * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

At D18 cells grown in 1% alginate showed a very homogeneous differentiation. Pluripotency markers Oct3/4 and Nanog are strongly reduced with respect to the controls and to 2% alginate (Fig. 4), indicating that in these culture conditions the majority of the cells differentiate. Although Pax6 is present in all conditions with significantly higher levels with respect to controls, neural differentiation is most efficient in alginate and alginate-HA at both concentrations. Nestin expression is significantly decreased in cells grown in 2% alginate, unmodified or modified, with the exception of RGD-alginate group in which cells still present high levels of this marker. Cells grown in 1% alginate hydrogels present a more variable expression of Nestin with higher or comparable levels with respect to two-dimensional controls, however no significant values were found. βIII-tubulin expression is higher in cells grown both in 1% and 2% alginate, alone or modified with HA or Fn, with respect to controls, confirming that neural differentiation is occurring. RGD groups present more variable expression levels of this marker. Cells grown in 2% alginate-RGD are characterized by expression levels comparable to controls, while 1% alginate-RGD has higher expression but without showing any statistical significance with respect to two-dimensional controls. At the end of the protocol NCAM, a neuronal terminal differentiation marker, is highly expressed in cells grown in 1% alginate, unmodified or modified with HA, presenting higher levels with respect to two-dimensional controls and their counterparts at 2% alginate conditions. Furthermore, the glial marker GFAP presented significantly lower expression levels in alginate and alginate-Fn at both concentration and in 1% RGD–alginate with respect to the controls. More variable expression levels are present in alginate-HA at both concentrations and in 2% RGD-alginate, comparable to control ones (Fig. 4).

In order to test the homogeneity of our neural population we also checked differentiation towards lineages of other germ layers. At both D7 and D18 the endodermal marker Sox17 is not present or present at very low and variable levels with respect to the controls. Higher expression levels with respect to the controls are found in few cases, such as in 1% alginate-HA at D7, in 1% alginate-Fn and 1% alginate-HA at D18 (Fig. 5). However, these data refer to the single experiment in which expression of this marker was detectable, as indicated by the absence of error bars in the graph. The mesodermal marker Brachyury is significantly less expressed in all conditions with respect to the controls at D7. At D18 its expression is not detectable in 1% alginate, unmodified and modified, and variable in 2% alginate gels, comparable to controls (Fig. 5). These data indicate that cell differentiation towards endoderm and mesoderm lineages is highly impaired in our culture conditions.

These data allowed us to establish that 1% alginate, alone or modified with HA, supports neural differentiation more efficiently than 2% alginate, showing higher expression levels of late neuronal differentiation markers and the generation of a more homogenous neural cell

population at the end of the culture period. This is shown by the expression of pluripotency markers, which are almost absent in all conditions tested, and by the absence of meso-endodermal markers expression in most of the experiments performed.

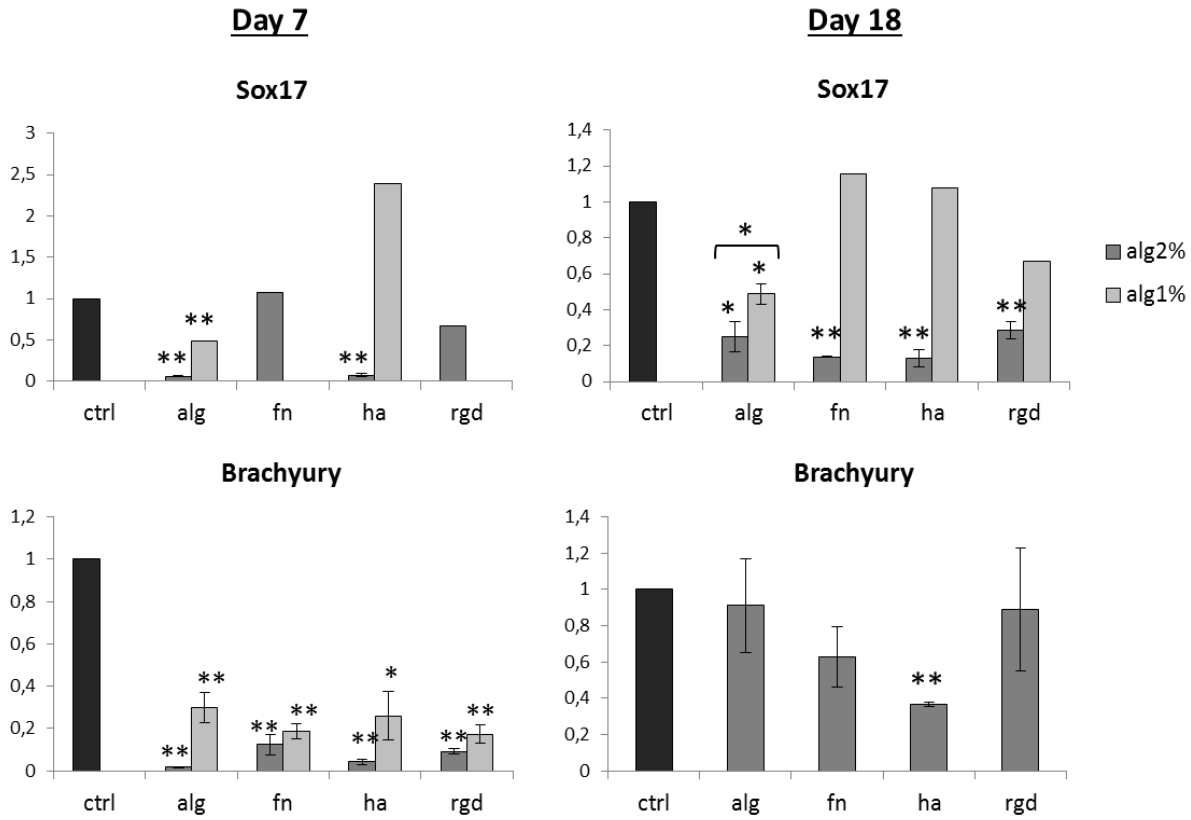

Fig.5 *Differentiation of mESCs encapsulated in alginate beads towards non-neural lineages*. RT-qPCR analyses on endodermal marker (Sox17) and mesodermal marker (Brachyury) at D7 and D18. Dark gray bars: 2% w/v alginate, light gray bars: 1% w/v alginate. ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide. Statistical significance with respect to the control culture: * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

4.1.5 Neural specific and synaptic proteins expression

The RT-qPCR data indicate that the best conditions for neural differentiation are 1% alginate unmodified or, even better, modified with HA. In order to confirm that cells undergo neural differentiation and to understand how cells organize inside clusters, we performed immunocytochemistry analyses on all experimental groups.

At D7 cells express both Nestin and βIII-tubulin in all conditions (Fig. 6). Nestin is less expressed in cells grown in 1% alginate alone or modified than in 2%, while βIII-tubulin is more expressed in the periphery of the clusters in all cases. This could indicate that differentiation proceeds with a periphery-to-centre gradient, possibly due to higher accessibility to nutrients in the periphery of the clusters.

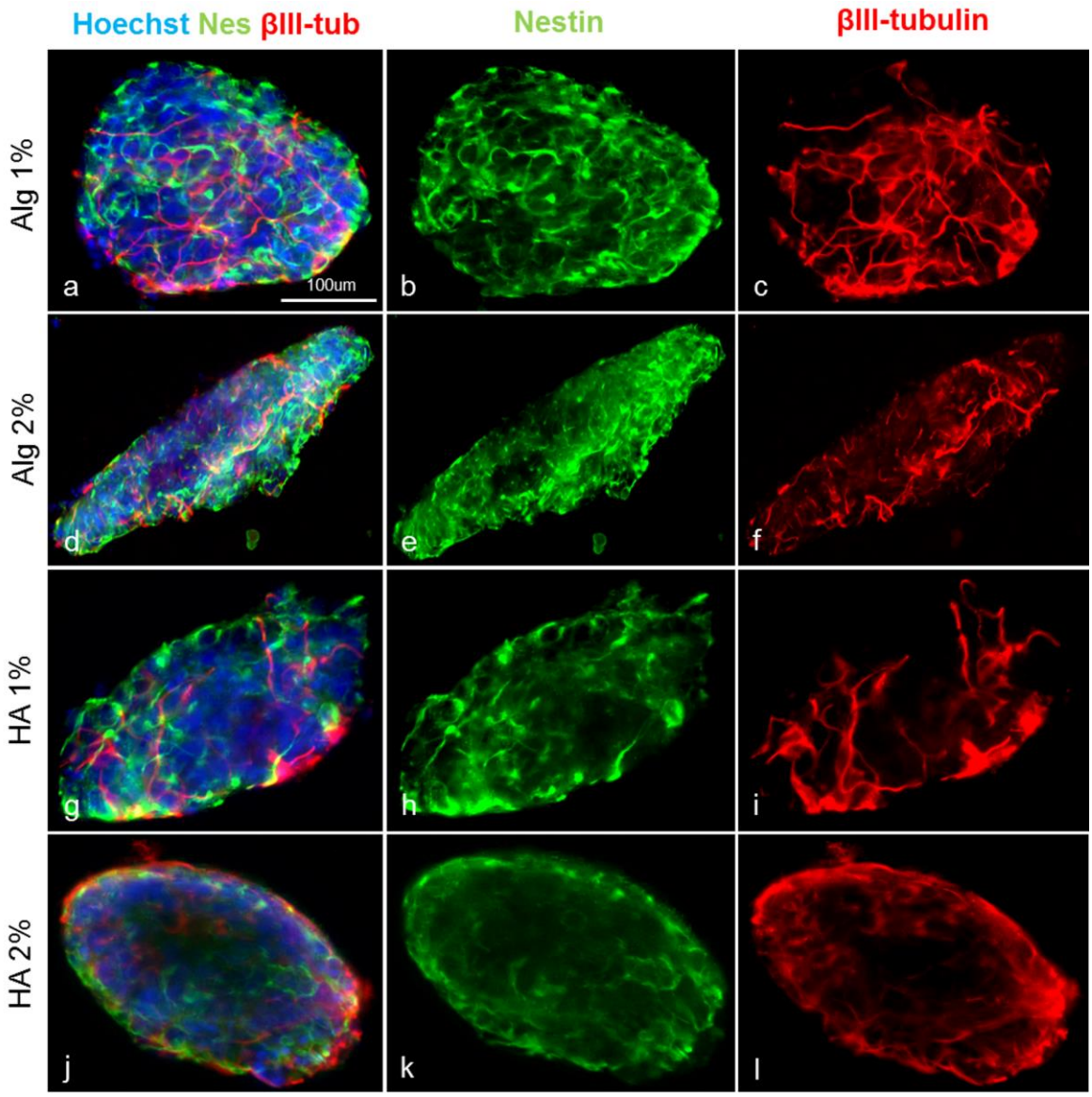

Fig.6 *Early neural differentiation of mESCs encapsulated in alginate and alginate-HA gels*. Immunocytochemistry analyses at D7 on single clusters for early neuronal differentiation markers Nestin, in green (**b, e, h, k**) and βIII-tubulin, in red (**c, f, i, l**). Blue, Hoechst staining (**a, d, g, j**). ALG: alginate, HA: alginate - hyaluronic acid.

At D18 Nestin is still highly expressed in cells grown in 2% alginate alone or modified. In cells grown in 1% alginate and 1% alginate-HA the presence of neural rosettes (Fig. 7 a-c, g-i, white arrows) is a strong indication that neural differentiation is proceeding efficiently. In all panels of Fig. 7, βIII-tubulin expression is localized not only in the cell body but also in neurites, and shows that cells make an intricate network of processes inside clusters.

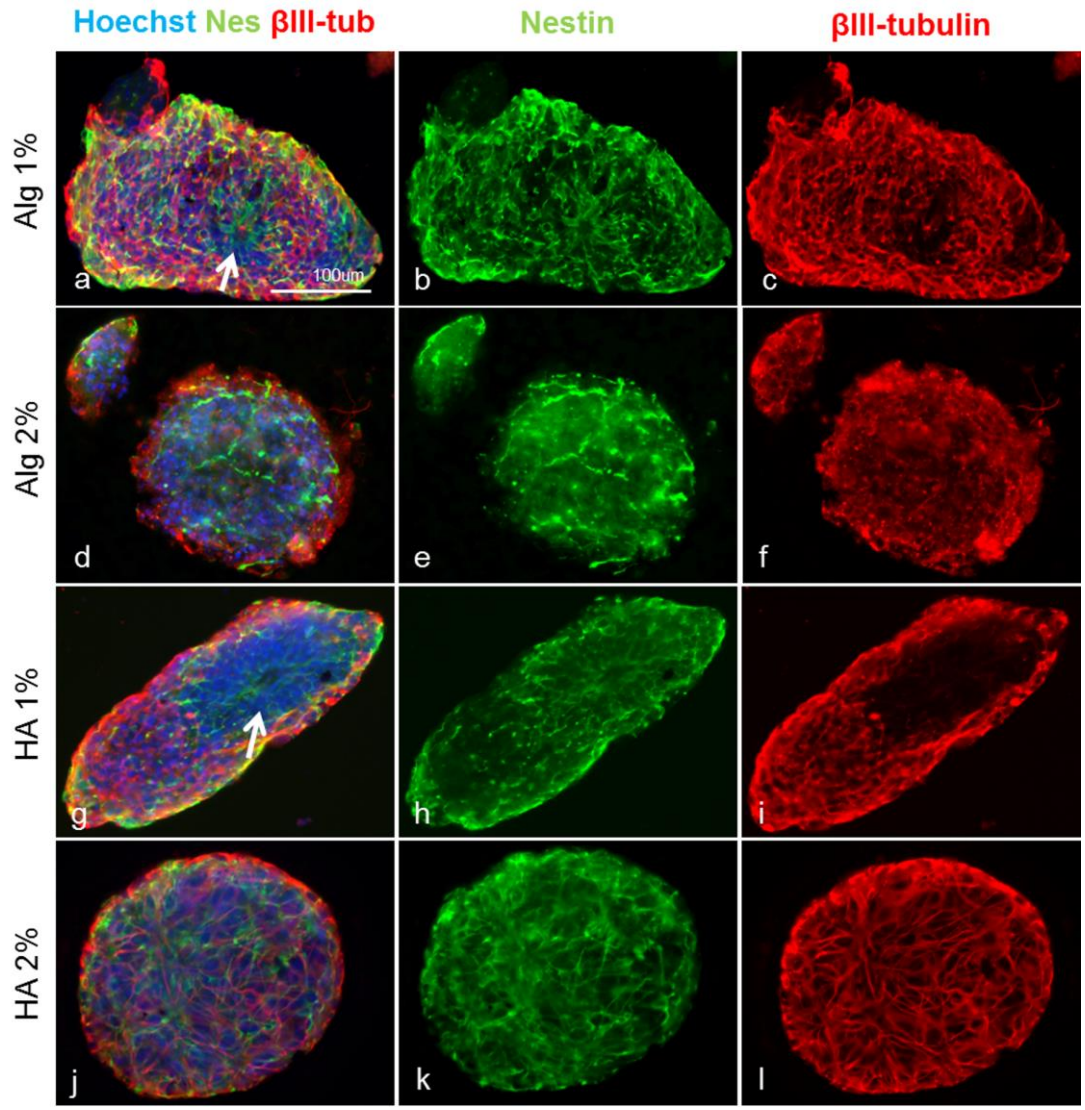

Fig.7 *Neural differentiation of mESCs encapsulated in alginate and alginate-HA gels*. Immunocytochemistry analyses at D18 on single clusters for early neuronal differentiation markers Nestin, in green (**b, e, h, k**) and βIII-tubulin, in red (**c, f, i, l**). Blue, Hoechst nuclear staining (**a, d, g, j**). ALG: alginate, HA: alginate - hyaluronic acid.

We did not include alginate modified with RGD in our analyses, due to its poor reproducibility in terms of differentiation among different experiments, as shown in RT-qPCR analyses. We checked for the expression of Nestin and βIII-tubulin in alginate-Fn groups at D18, confirming that cells grown in this condition undergo neural differentiation (Fig. 8 a-f). Interestingly, we found enclaves of proliferating cells expressing Sox2 but not Nestin in 1% alginate-Fn (Fig. 8 g-i) suggesting that these cells are pluripotent and that, as also shown by the RT-qPCR data, these conditions drive neural differentiation less efficiently.

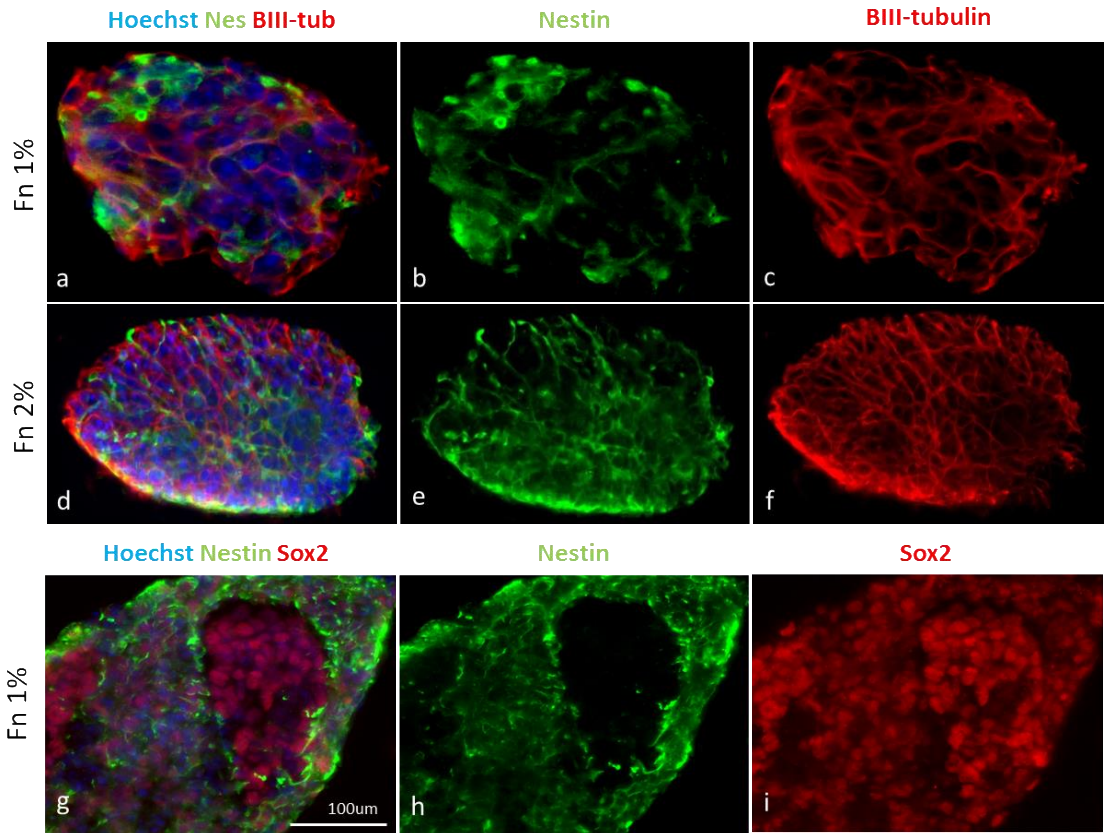

Fig.8 *Neural differentiation of mESCs encapsulated in alginate-Fn gels.* Immunocytochemistry analyses at D18 on single clusters for early neural differentiation markers Nestin, in green (**b, e, h**), βIII-tubulin, in red (**c, f**), Sox2 in red (**i**). Blue, Hoechst nuclear staining (**a, d, g**). Fn: alginate – fibronectin.

To further characterize the extent of differentiation and the morphology of the differentiated cells, we analyzed the expression of later differentiation markers, such as neural cell adhesion molecule (NCAM) and microtubule associated protein-2 (MAP-2). At D18 we obtain terminally differentiated neurons as shown by the expression of both proteins in all conditions (Fig. 9 a-r).

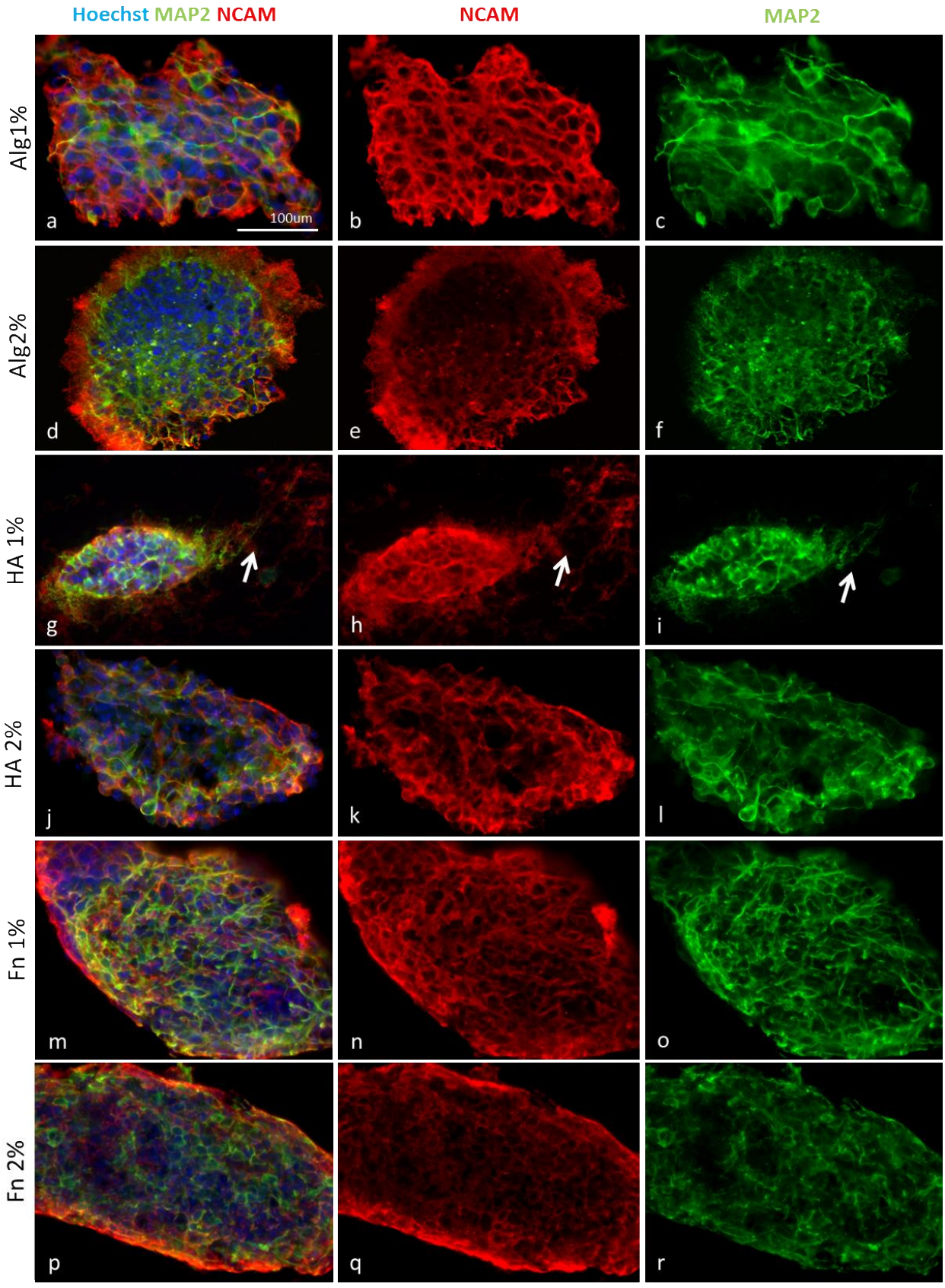

Fig.9 *Terminal neural differentiation of mESCs encapsulated in alginate and alginate-HA gels*. Immunocytochemistry analyses at D18 on single clusters for terminal neuronal differentiation markers NCAM, in red (**b, e, h, k, n, q**) and MAP2, in green (**c, f, i, l, o, r**). Blue, Hoechst nuclear staining (**a, d, g, j, m, p**). ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate - fibronectin.

MAP-2 was more abundantly expressed in cells grown in both 1% alginate conditions than in 2% alginate. We also found that cells were able to make connections among clusters and this is more evident in the cells grown in 1% alginate-HA (Fig. 9 g-i, white arrows).

We also investigated the capability of the neurons obtained in 1% alginate with or without HA to express synaptic proteins such as the presynaptic marker Synaptobrevin/VAMP2 (VAMP2) and the postsynaptic marker post-synaptic-density 95 (PSD-95) (Fig. 10a-c). VAMP2 staining is localized in small puncta (Fig. 10 a, b) and is present in cells inside the cluster and also in projections outside the cluster (Fig. 10a, white arrow). Cells grown in alginate-HA also show expression of PDS-95, with a somato-dendritic pattern (Fig. 10c).

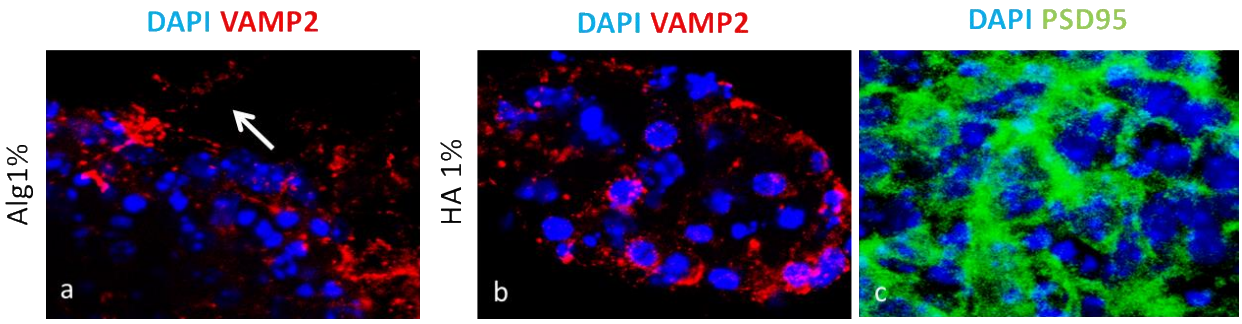

Fig.10 *Synaptic markers expression.* Immunocytochemistry analyses at D18 on single clusters for pre-synaptic marker VAMP2, in red (**a, b**) and post-synaptic marker PSD95 (**c**). Blue, DAPI nuclear staining (**a, b, c**). ALG: alginate, HA: alginate-hyaluronic acid.

We also analyzed GFAP expression. Glial differentiation occurred in few clusters in all culture conditions (Fig. 11). GFAP expression is lowest in cells grown in 1% alginate and 1% alginate-HA. Our data show that in the three-dimensional culture conditions neuronal differentiation is enhanced with respect to glial differentiation.

These data show that in our three dimensional culture system, neurons are able to terminally differentiate, to form a network of neuronal processes, and to express synaptic markers.

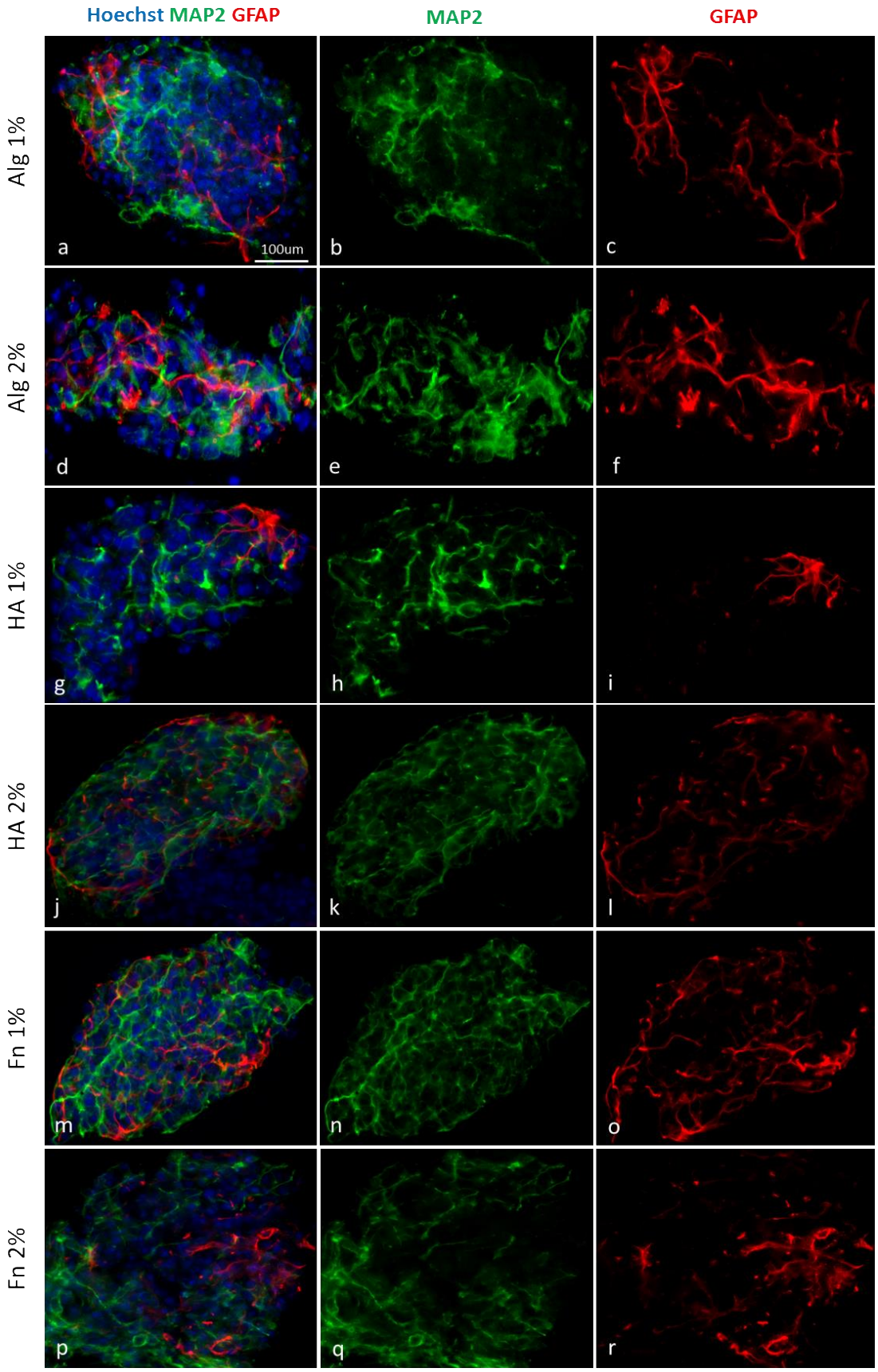

Fig.11 *Glial differentiation of mESCs encapsulated in alginate, alginate-HA and alginate-Fn gels.* Immunocytochemistry analyses at D18 for neuronal marker MAP2 in green (**b, e, h, k, n, q**) and glial marker GFAP in red (**c, f, i, l, o, r**). Blue, Hoechst nuclear staining (**a, d, g, j, m, p**). ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin.

4.1.6 Generation of different neuronal subtypes

To complete our analysis on terminal differentiation, we checked which neuronal subtypes were obtained in the cultures. Fig. 12 reports RT-qPCR analyses showing that cells differentiated in all hydrogels express markers for different neuronal subtypes: the vesicular glutamate transporter 2 (VGLUT2, glutamatergic neurons), the glutamic acid decarboxylase 67 (GAD67, GABAergic neurons) and the tyrosine hydroxylase (TH, dopaminergic neurons). In all experimental conditions the markers for these three neuronal subtypes are significantly higher expressed with respect to the controls. Hyaluronic acid modification, at both alginate concentrations, presents the highest expression of VGLUT-2 and GAD67, whereas TH expression levels are more homogenous among experimental groups. Except for alginate-Fn at both concentrations, no significant differences in marker expression was found between 1% and 2% experimental groups. 2% alginate, unmodified and modified with RGD, resulted to be the most variable condition. Expression of Tph2 (serotonergic neurons) and HB9 (motoneurons) was not found in any of the experimental conditions (data not shown). These data show that under these culture conditions, different subtypes of neurons can be generated without the addition of exogenous factors.

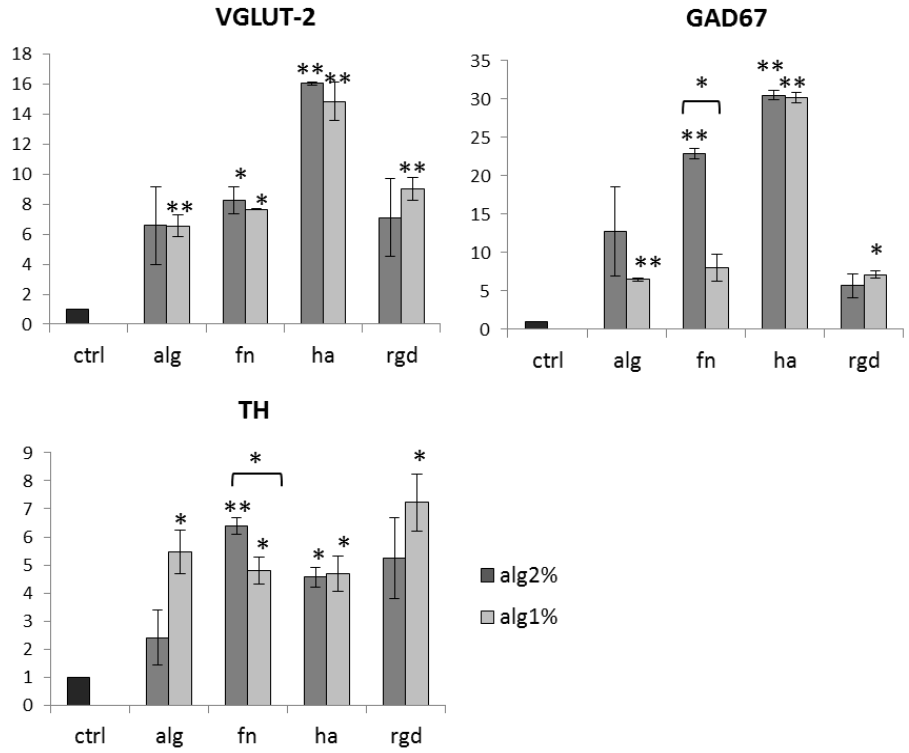

Fig.12 *Generation of different neuronal subtypes in three-dimensional alginate cultures of mESCs*. RT-qPCR analyses at D18 for neuronal subtypes markers: vesicular glutamate transporter-2 (VGLUT-2), tyrosine hydroxylase (TH) and glutamic acid decarboxylase (GAD67). Dark gray bars: 2% w/v alginate, light gray bars: 1% w/v alginate. ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide. Statistical significance with respect to the control culture: * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

4.1.7 Extracellular matrix deposition by encapsulated cells

We checked whether cells are able to produce their own extracellular matrix (ECM) once encapsulated in alginate beads. We stained them with Wisteria floribunda lectin (WFA), which marks chondroitin sulfate proteoglycans (CSPGs). Preliminary results demonstrate that during differentiation cells produced their own ECM rich in CSPGs, as shown by the red staining, both when cultured in 1% unmodified alginate (Fig 13 a, b) and in 1% alginate-HA (Fig. 13 c, d). The majority of the cells inside clusters is WFA positive, but few clusters present some cells in the periphery that are negative for the staining (Fig. 13 a, c, white arrows). This suggests that cells in the internal part of the cluster need to produce their own ECM, while for cells in the periphery interactions with the surrounding alginate might be sufficient to support their growth and differentiation, without the need of endogenous ECM production.

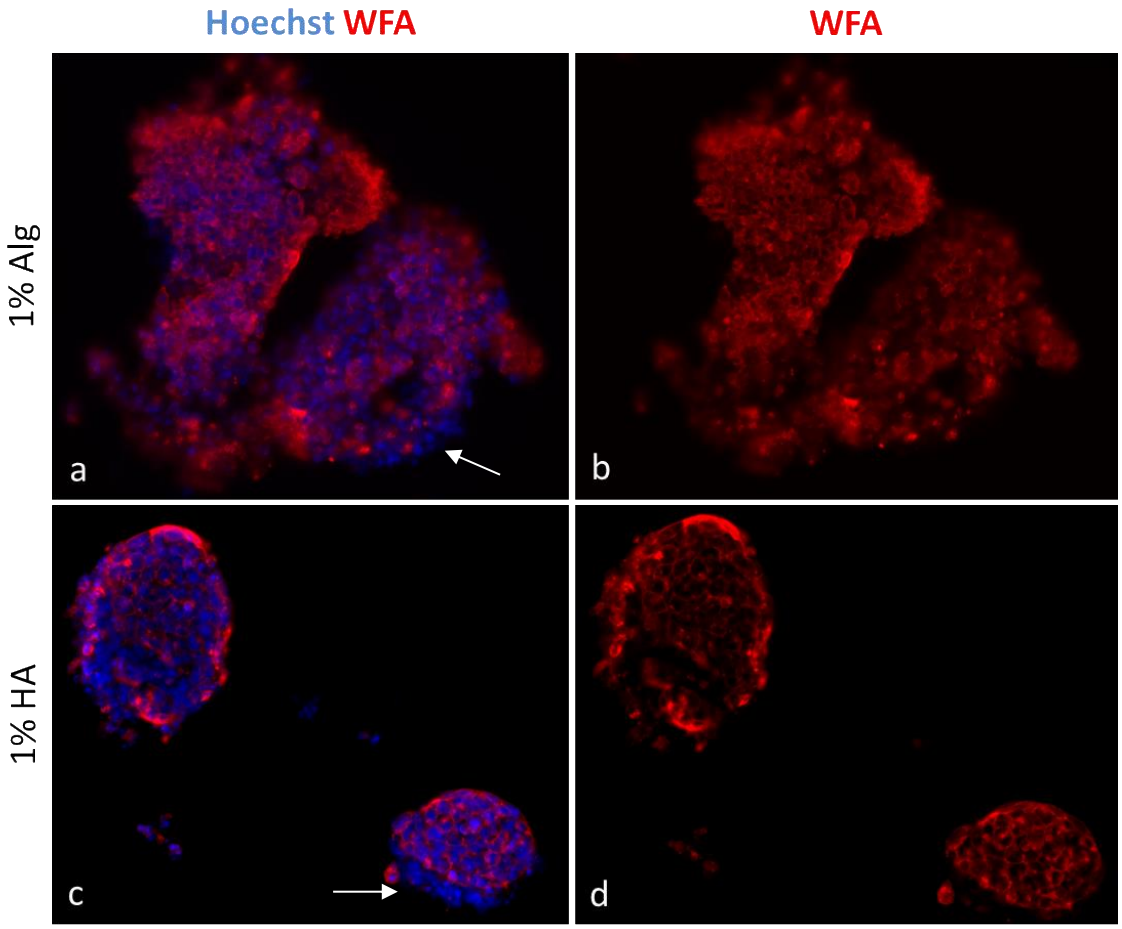

Fig.13 *ECM production by cells encapsulated in alginate hydrogels*. WFA staining for CSPGs (red) on cells encapsulated in 1% unmodified alginate (**a, b**) and in 1% alginate – HA (**c, d**) at D18. Blue, Hoechst nuclear staining (**a, c**). ALG: alginate, HA: alginate - hyaluronic acid.

4.1.8 Alginate gel characterization

Cells grown in 1% alginate-HA undergo better neural differentiation, suggesting that scaffold chemical properties influence cell differentiation. This is also supported from evidences that HA is present during neural development and it is important for migration and axonal growth (Preston et al., 2011). However since there are differences in neural differentiation markers and protein expression between cells cultured in the two alginate concentrations, we tested if physical and mechanical properties of hydrogels could have a role in cell differentiation. We performed water content analyses and the different alginate formulations show no statistical differences among groups (Fig. 14A). Hydrogel stiffness was evaluated as well, showing that elastic moduli (or Young's moduli) of all alginate concentrations and modifications we used fall in the range that is considered to resemble the brain ECM (0.1-1 kPa) (Banerjee et al., 2009; Matyash et al., 2012). For 2% alginate gels, the stiffest hydrogels with the largest Young's Moduli are the unmodified and the RGD-modified alginate, while alginate-Fn and alginate-HA have significantly lower Young's Moduli and exhibit no statistical differences between them (Fig. 14B). 1% alginate hydrogels show no statistical difference among them (Fig. 14C). Comparing the two alginate concentrations, we see that 2% unmodified alginate and alginate-RGD show a statistically greater modulus over all other hydrogels. Regarding alginate modifications, Fn-modified hydrogels show no statistical differences between them, whereas in unmodified alginate, RGD- and HA-modified gels the 2% alginate group has a statistically greater modulus (Fig. 14D). These data indicate that the contrasting differentiation properties that we see in the various conditions can be ascribed to both mechanical and chemical differences in the hydrogels. The more efficient neural differentiation found in cells grown in the lower alginate concentration could be due to differences in elastic moduli between 1% and 2% hydrogels (mechanical properties). Considering 1% alginate experimental groups, we registered no differences in elastic moduli but increased neural differentiation among cells grown in 1% alginate modified with HA, suggesting that the modification of the hydrogel with this macromolecule enhances neural differentiation (chemical properties).

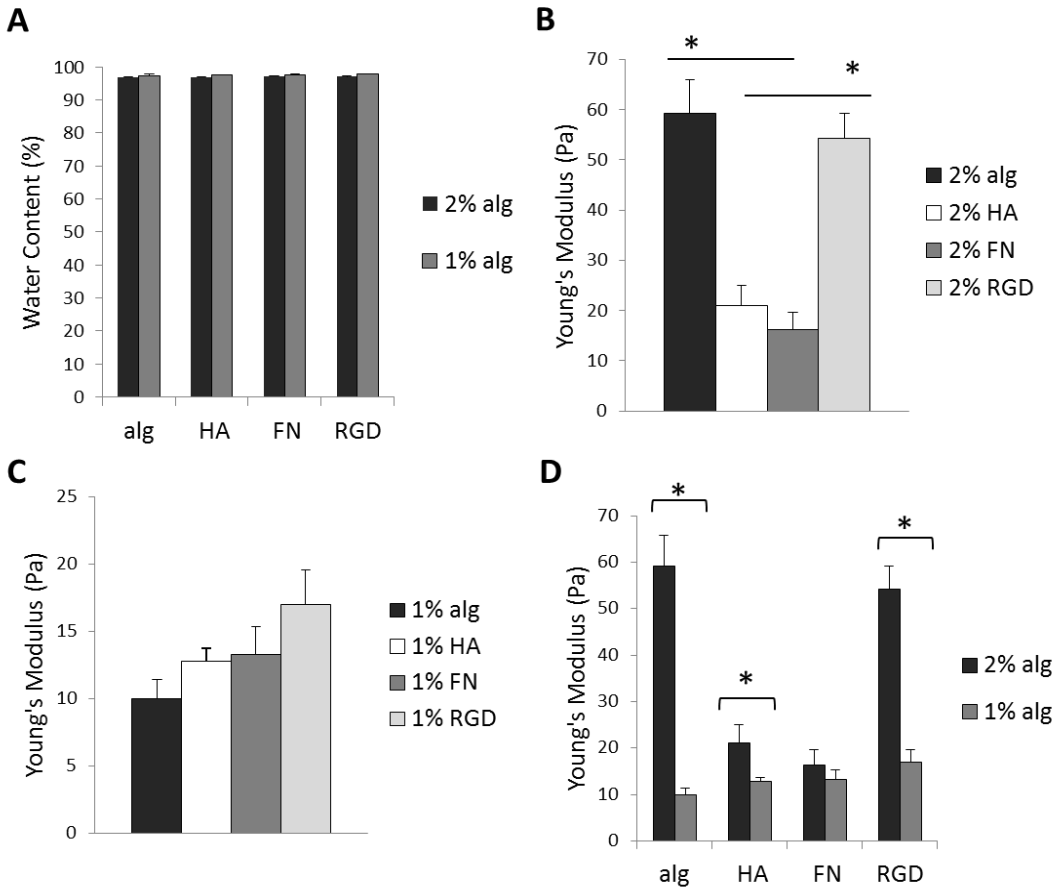

Fig.14 *Mechanical and physical properties of alginate hydrogels*. **A**: water content analyses on 1% and 2% alginate hydrogels, **B**: elastic modulus of 2% alginate hydrogels, **C**: elastic modulus of 1% alginate hydrogels, **D**: comparison of elastic moduli among 1% and 2% alginate hydrogels. ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate - fibronectin, RGD: alginate – RGD peptide. Statistical significance: * p< 0.05, ** p< 0.01.

4.1.9 Beads dimension influence on neural differentiation

Besides alginate concentration and hydrogel stiffness, it has been demonstrated that beads dimension influences stem cells differentiation as well (Wang et al., 2009; Huang et al., 2012). Bead dimension can be modulated using different needle sizes during the encapsulation procedure (see section 3, Materials and Methods). We tested if cell culture in smaller beads, obtained using 27G needles, could enhance nutrients diffusion in the scaffolds and consequently mESCs neural differentiation with respect to both two-dimensional cultures and larger beads (19G needle) used in our previous studies. We showed that culture in 1% alginate allows for a better neural differentiation, therefore we decided to test only this alginate concentration. Alginate modification and cell encapsulation were performed following the same procedures previously used and cells were cultured following the same differentiation protocol (Fico et al., 2008).

mESCs were encapsulated in 1% alginate beads of average 2 mm diameter, which show events of swelling once put in the culture medium and remain transparent throughout the protocol. Cells inside the beads proliferate and form clusters, as previously seen in larger

beads (Fig. 15 and Fig.1). HA is the most stable condition, with bigger beads that remain rounded in shape and do not degrade during the protocol. Alginate-Fn group turned out to give the weakest hydrogel, with smaller and oval shaped beads. In this experimental condition there is a high rate of biomaterial degradation, with cells that escape from the beads, attach to the plate and continue to grow in two dimensions (Fig. 15, black arrow).

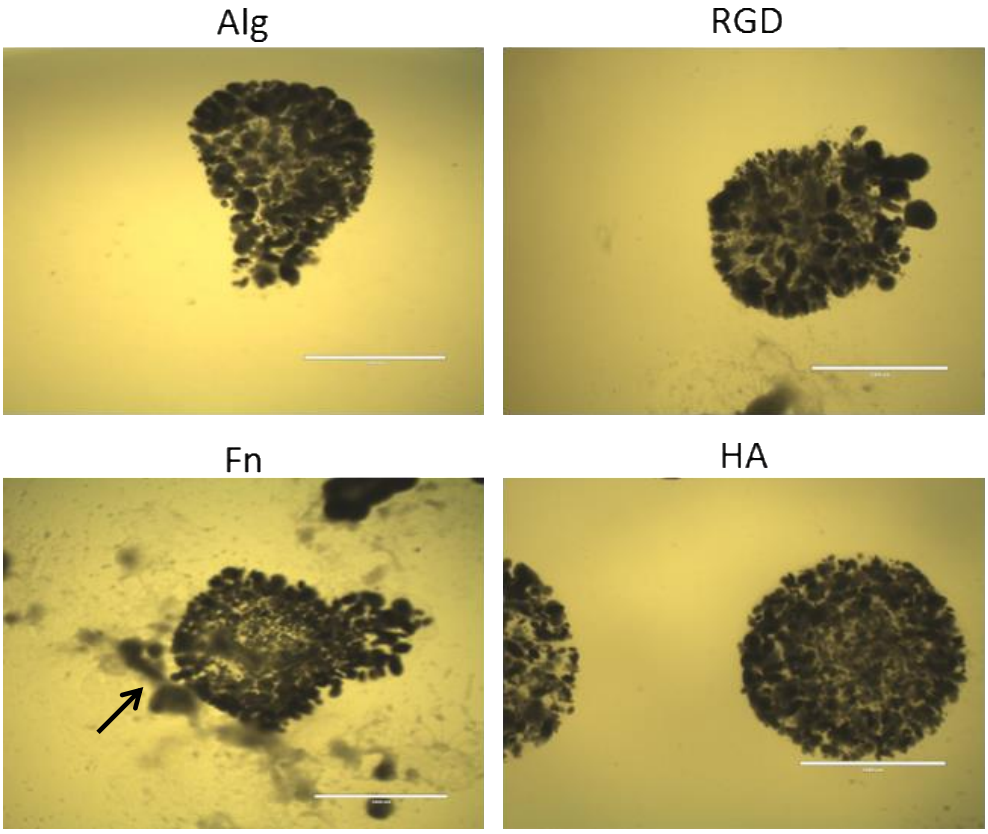

Fig.15 *mESCs encapsulated in small alginate beads*. Cells inside alginate hydrogels proliferate and form clusters. ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide. Scale bar: 2000µm.

Cell viability was analyzed with Live/Dead assay on intact beads. The majority of the cells remain viable throughout the culture period, as shown in green by the Calcein-AM staining for live cells at D18 (Fig. 16). The Ethidium Homodimer-1 (EH1) red staining for dead cells shows slightly higher intensity in alginate-Fn group with respect to the other experimental conditions (Fig. 16), confirming that the poor stability of this hydrogel is not appropriate to support cell viability and culture.

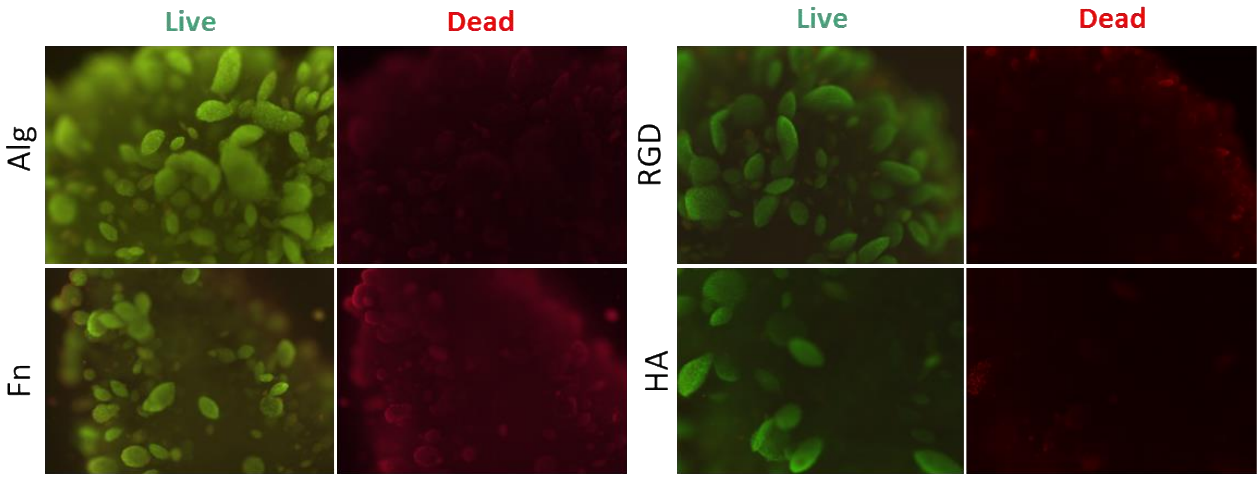

Fig.16 *Cell viability of mESCs encapsulated in small alginate beads*. Cell viability was analyzed with LIVE/DEAD assay on intact beads at D18. ALG: alginate, HA: alginate-hyaluronic acid, Fn: alginate–fibronectin, RGD: alginate–RGD peptide.

We then analyzed the extent of neural differentiation of mESCs encapsulated and cultured in small beads by RT-qPCR and immunocytochemistry analyses at D7 and D18, as previously done with cells grown in the larger beads.

At D7 the expression of pluripotency markers is higher in small beads with respect to controls and larger beads in all tested conditions, but the variability among single experiments is high and no statistical significance can be observed (Fig. 17). Neural differentiation occurs in small beads as shown by the expression of the neural markers Pax6 and Nestin. However their expression is lower than two-dimensional controls and larger beads in all experimental conditions. The later neural differentiation marker βIII-tubulin is also poorly expressed in cells grown in small beads.

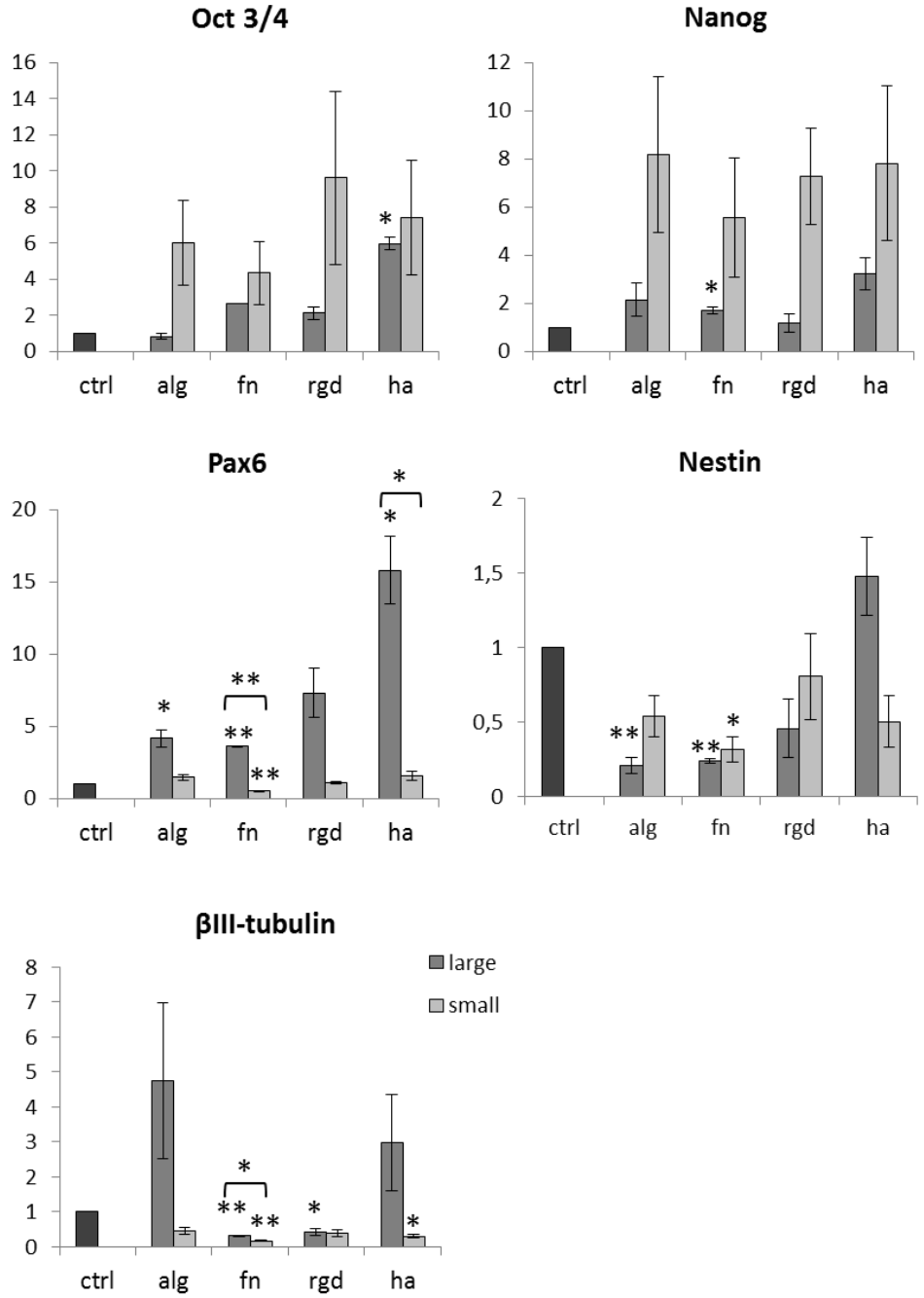

Fig.17 *Early neural differentiation of mESCs encapsulated in small alginate beads, comparison with large beads*. RT-qPCR analyses on pluripotency markers (Oct3/4 and Nanog) and neural differentiation markers (Pax6, Nestin and βIII-tubulin) at D7. Dark gray bars: large beads (19G), light gray bars: small beads (27G). ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate - fibronectin, RGD: alginate - RGD peptide. Statistical significance with respect to the control culture: * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

The high expression of pluripotency markers together with the very low expression of neural differentiation markers suggest that at D7 cells cultured in small beads present a delayed neural differentiation with respect to two-dimensional cultures and three-dimensional cultures in larger beads. In fact, at D18 we find that cells underwent neural differentiation also in these culture conditions. Pluripotency markers expression presents

lower or comparable levels with respect to two-dimensional culture controls, indicating that cells differentiate in these hydrogels (Fig. 18). Pax6 and Nestin expression levels are comparable with control ones, whereas βIII-tubulin and NCAM expression is increased. Furthermore, GFAP expression is quite absent in all experimental conditions tested, indicating that the culture of mESCs in small alginate beads impairs their differentiation towards glial lineages. These data indicate that small beads are also good for neural differentiation of mESCs. However, in almost all the experimental conditions tested, cells cultured in larger beads present lower expression levels of pluripotency markers and higher expression levels of neural differentiation markers, indicating that neural differentiation is not enhanced in small beads with respect to larger beads. Moreover alginate and alginate-HA, which resulted to be the most permissive culture conditions for mESCs neural differentiation in previous experiments, present significantly higher expression levels of βIII-tubulin and NCAM in large beads, confirming a better neural differentiation in these three-dimensional culture systems.

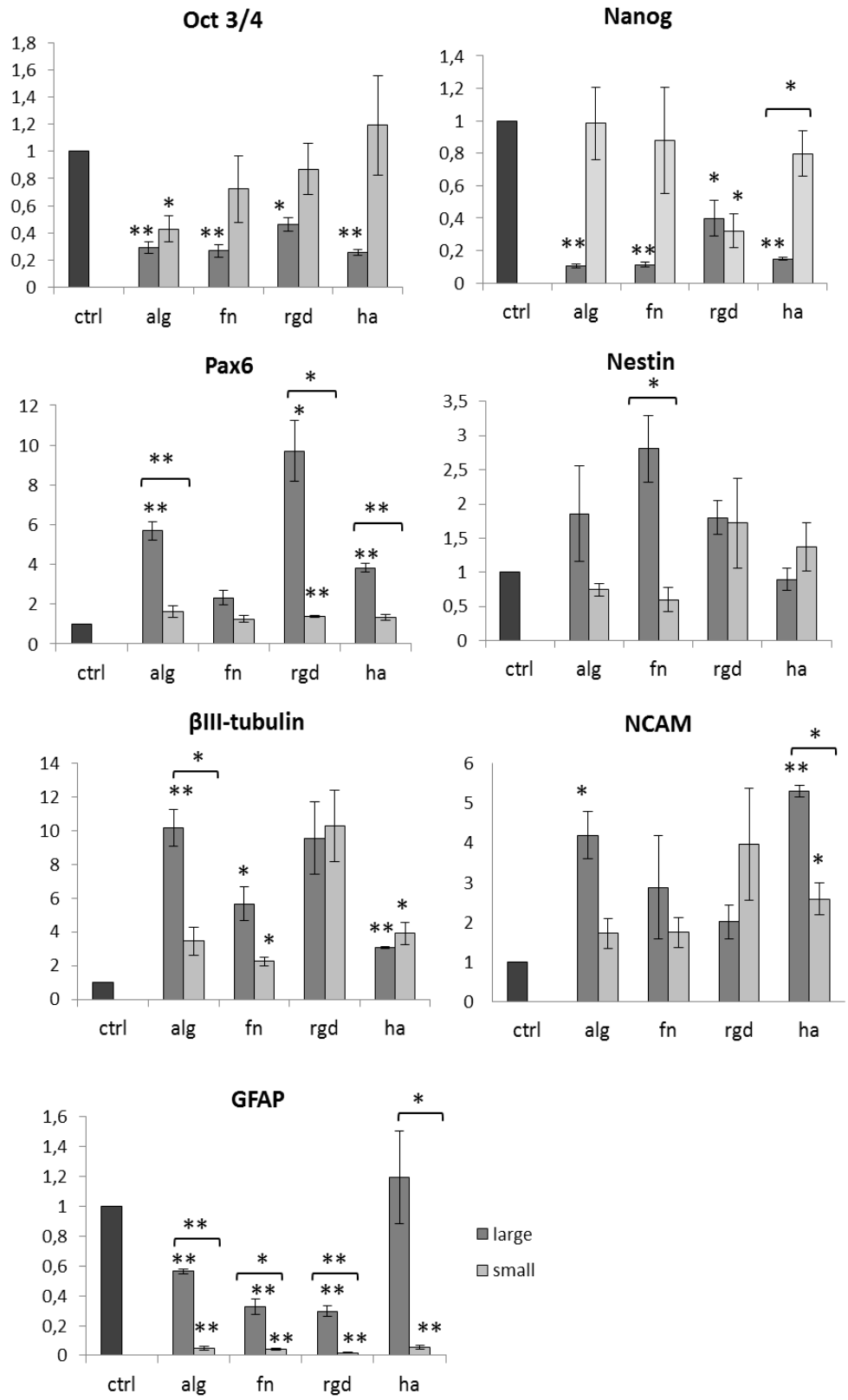

Fig.18 *Terminal neural differentiation of mESCs encapsulated in small alginate beads, comparison with large beads*. RT-qPCR analyses on pluripotency markers (Oct3/4 and Nanog), neural differentiation markers (Pax6, Nestin, βIII-tubulin and NCAM) and glial marker (GFAP) at D18. Dark gray bars: large beads (19G), light gray bars: small beads (27G). ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate - fibronectin, RGD: alginate – RGD peptide. Statistical significance with respect to the control culture: * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

We also checked cell differentiation into non-neural lineages. Expression of the mesodermal marker Brachyury has been found in only one experiment at D7, presenting higher values with respect to larger beads but comparable or lower with respect to two-dimensional controls. At D18 its expression is variable but higher than two-dimensional cultures (Fig. 19). The endodermal marker Sox17 is expressed at D7 in small beads with lower or comparable levels with respect to two-dimensional cultures and it was found at D18 with high expression in alginate-HA and no expression in alginate-RGD in only one experiment. These data indicate that some extent of meso-endodermal differentiation is present in cells grown in small beads. At D18 we could not find Brachyury expression in larger beads while is highly present in small beads, indicating a more heterogeneous differentiation of mESCs when bead dimension is deceased.

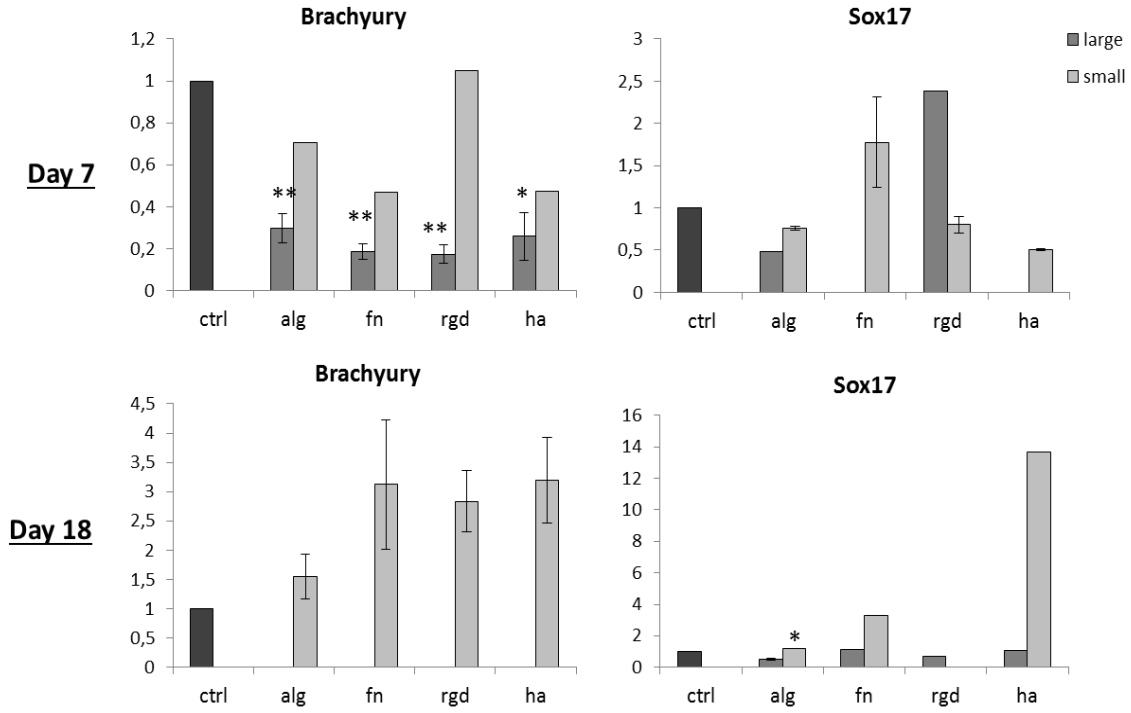

Fig.19 *Differentiation of mESCs encapsulated in small alginate beads towards non-neural lineages, comparison with large beads*. RT-qPCR analyses on endodermal marker (Sox17) and mesodermal marker (Brachyury) at D7 and D18. Dark gray bars: large beads (19G), light gray bars: small beads (27G). ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate – fibronectin, RGD: alginate – RGD peptide. Statistical significance with respect to the control culture: * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

Neural differentiation of mESCs encapsulated in small beads is not enhanced with respect to culture in larger beads and in two-dimensions. Moreover the final population is more heterogeneous, with cells that do not differentiate and cells that differentiate towards non-neural lineages. However, we performed immunocytochemistry analyses on cells grown in small beads in order to confirm that they undergo neural differentiation. At D7 cells still express the pluripotency marker Oct3/4, however in few clusters we find the presence of Nestin and βIII-tubulin positive cells (data not shown). At D18 immunocytochemistry

analyses confirmed that neural differentiation occurs in small beads. In fact neural precursors are present, as indicated by the co-localization of Sox2 and Nestin neural precursor makers in all conditions tested (Fig. 20 a-l). Another indication of neural differentiation is the presence in alginate-HA of neural rosettes, tube-like structures in which neural stem cells arrange, recapitulating the spatial organisation of the neural tube *in vivo* (Fig. 20 g-i).

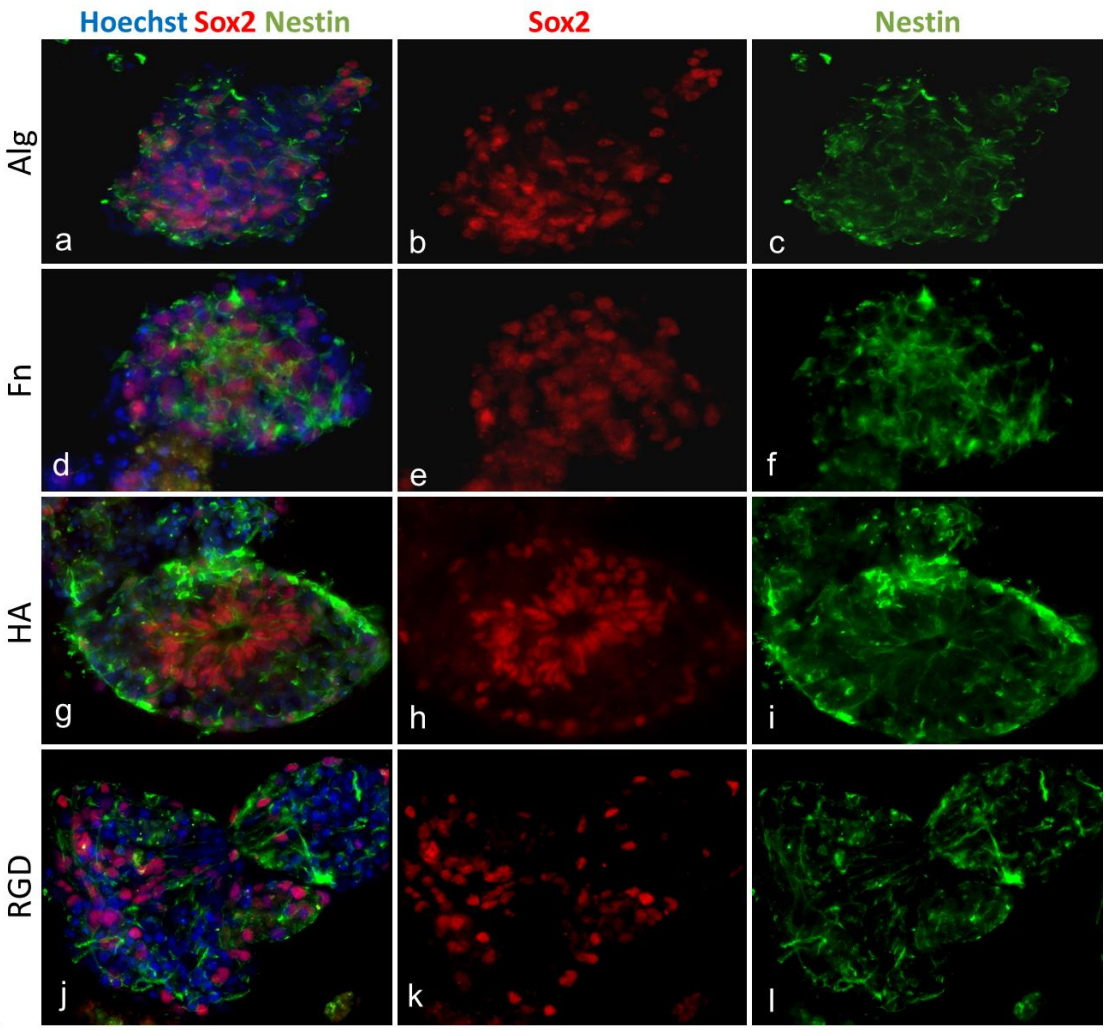

Fig.20 *Early neural differentiation of mESCs encapsulated in small alginate beads*. Immunocytochemistry analyses on single clusters at D18 for neural precursors markers Sox2, in red (**b, e, h, k**) and Nestin, in green (**c, f, i, l**). Blue, Hoechst nuclear staining (**a, d, g, j**). ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate - fibronectin, RGD: alginate - RGD peptide.

The later neuronal marker βIII-tubulin is present as well in cells grown in all experimental conditions (Fig. 21 a-l), indicating that neural differentiation is ongoing. The staining shows that cells are forming networks inside clusters while differentiating. In alginate-HA we detect events of neurite sprouting in the hydrogel, as indicated by the white arrows (Fig. 21 g-i).

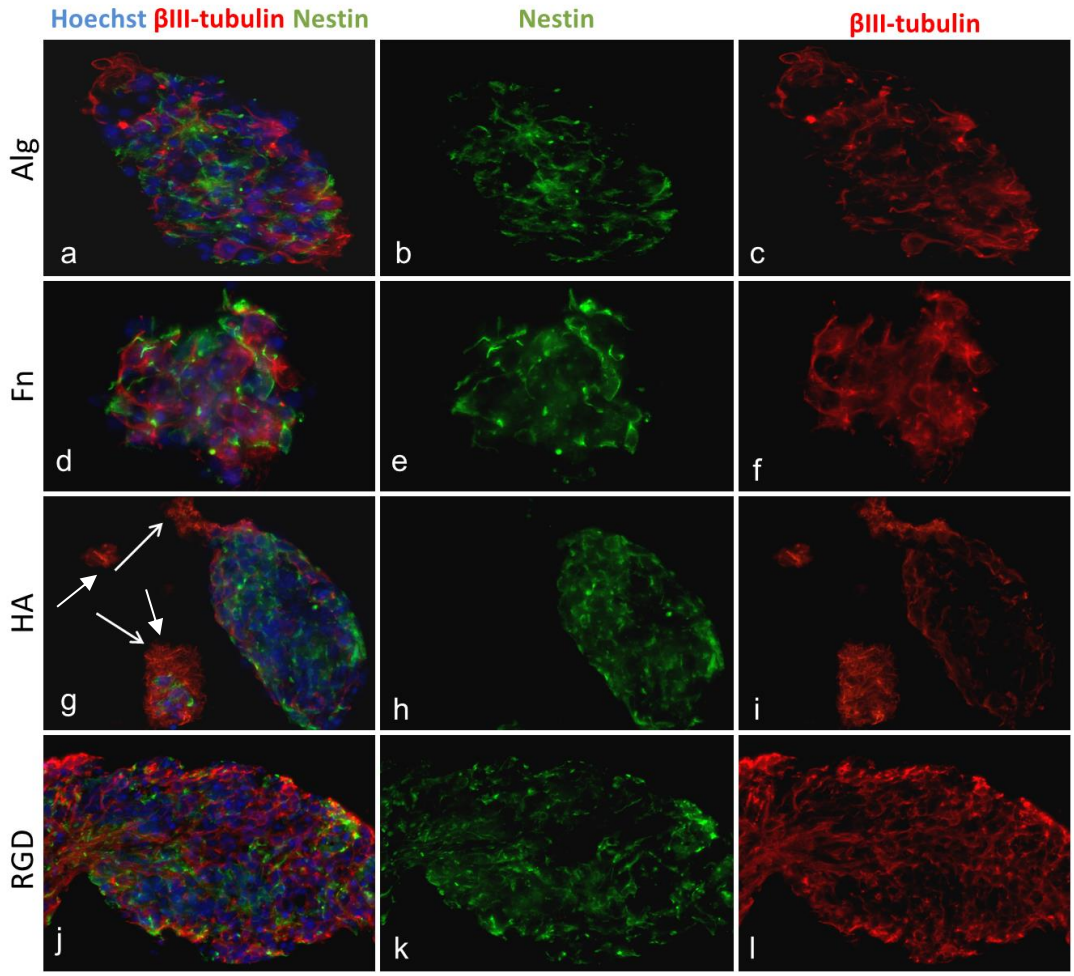

Fig.21 *Neural differentiation of mESCs encapsulated in small alginate beads*. Immunocytochemistry analyses at D18 on single clusters for early neuronal differentiation markers Nestin, in green (b, e, h, k) and βIII-tubulin, in red (c, f, i, l). Blue, Hoechst nuclear staining (a, d, g, j). ALG: alginate, HA: alginate - hyaluronic acid, Fn: alginate - fibronectin, RGD: alginate - RGD peptide.

These data confirm that culture in small alginate beads supports neural differentiation as indicated by the presence of Sox2/Nestin positive neural precursors in all experimental conditions. Some cells proceed with neural differentiation showing positivity for the neural differentiation marker βIII-tubulin and start to form networks with other cells inside clusters. Cells grown in alginate-HA form neural rosettes and show neurite spreading in the hydrogels, supporting previous results in larger alginate beads that indicate 1% alginate-HA as the best culture conditions for mESCs neural differentiation. Cells cultured in alginate-RGD hydrogels show also in this study high variability in differentiation, while modification of alginate with Fn resulted to be the weakest experimental conditions, characterized by high degradation rates and lower cell viability.

The comparison between data obtained by culturing mESCs in large and small beads indicates that decreasing beads dimension does not result in an increased mESCs neural differentiation within our culture system. Results indicate also that nutrient diffusion is not

limited in large beads, where the majority of the cells homogenously differentiate towards neural lineages, further confirming that our three-dimensional culture system is a valuable tool for the efficient neural differentiation of pluripotent cells.

4.2 Neural stem cells and alginate co-injection for CNS regeneration following cerebral ischemia

Part of this work has been performed in collaboration with Prof. Srecko Gajovic Lab at the Croatian Institute for Brain Research in Zagreb (Croatia).

4.2.1 Introduction

In the second part of this work we present preliminary studies about the use of injectable alginate hydrogels for *in vivo* applications in the brain tissue.

The work described in the previous part shows that our alginate-based hydrogels support neural differentiation of mESCs and that scaffolds present physical and mechanical characteristics comparable to brain tissue, thus they could be of interest for tissue engineering approaches in the damaged brain.

We showed that our alginate three-dimensional culture system supports efficient pluripotent cells neural differentiation and we hypothesized that it could support neural stem cells viability and differentiation as well, allowing the possibility to explore its applications as hydrogel for brain tissue regeneration. The culture of neural stem cells in alginate hydrogels is reported in few studies, showing that their differentiation is supported after encapsulation in 1,5% w/v alginate beads (Li et al., 2006), 1% w/v alginate hydrogels (Purcell et al., 2009) or in alginate hydrogels with elastic moduli comparable to brain tissue ones (Banerjee et al., 2009).

In the first part of the following study we tested mNSCs viability and early neural differentiation in three-dimensional alginate beads. According to our previous findings, cells were encapsulated in 1% alginate, unmodified and modified with HA. We initially evaluated the optimal starting cell density for encapsulation by analyzing cell viability. Cells were then cultured and differentiated following an established protocol (Spiliotopoulos et al., 2009) and evaluated for their neuronal differentiation. We further hypothesize that NSCs encapsulation in alginate hydrogels could help in controlling cell delivery and in enhancing cell survival by protecting NSCs from the inflammatory environment present in the tissue.

The crosslinking method with $CaCl_2$ used in previous studies leads to immediate alginate polymerization which is difficult to control due to the high solubility of calcium chloride in the solution. This limits its application for *in vivo* approaches, since the short time in which crosslinking takes place does not allow the injection of the solution. For this reason we investigated an alternative *in situ* gelling method, in order to slow down and control alginate crosslinking, so to obtain injectable hydrogels for applications in the brain. Few studies report the slow polymerization of alginate, which can be obtained by mixing alginate solution with $CaCO_3$. This mixture does not crosslink until the addition of glucono-δ-lactone (GDL), which slowly acidifies the pH allowing alginate crosslinking. These *in situ* crosslinkable alginate hydrogels have been tested as sealant for dural defects (Nunamaker

et al., 2010) or modified with RGD for *in vitro* studies as a scaffold for delivery of endothelial cells (Bidarra et al., 2011).

In this study we evaluated the use of *in situ* gelling alginate hydrogels as support for NSCs injection in mouse brain tissue. First we encapsulated mNSCs in alginate hydrogels, crosslinked with the $CaCO_3$ - GDL method, testing mNSCs viability. We then evaluated occurrence of alginate crosslinking *in vivo* in the brain tissue, by staining alginate with a dye and studying its localisation profile following injection and with histological stainings.

In order to evaluate alginate biocompatibility in the brain tissue we took advantage of a recently developed mouse strain which carries a dual reporter system with luciferase (Luc) and green fluorescent protein (GFP) under the transcriptional control of a murine toll-like receptor-2 (TLR2) promoter (Lalancette-Hebert et al., 2009). This mouse model allows *in vivo* imaging of TLR2 transcriptional activation, which can be used as an indicator of inflammation. We monitored TLR2 expression following alginate injection into the brain up to 2 weeks in this mouse model.

4.2.2 Experimental design

We encapsulated mNSCs in alginate hydrogels, testing cell viability and neural differentiation. According to our previous studies, 1% alginate, unmodified and modified with HA, resulted to be the best culture conditions for neural differentiation of mESCs. We tested mNSCs encapsulation in these two culture conditions.

As control we used a published two dimensional neuronal differentiation protocol where cells are grown in monolayer and differentiated prevalently into GABAergic neurons by culture in medium supplemented with N2 and B27 and exposure to decreasing FGF and increasing BDNF concentrations (Spiliotopoulos et al., 2009). The protocol last 21 days but since we were interested in testing whether alginate support mNSCs survival and initial differentiation and not full differentiation into specific neuronal subtypes, we cultured encapsulated cells with this protocol for only 12 days. We first tested different initial cell encapsulation densities. 50 000 (Li et al., 2006), 100 000 (Banerjee et al., 2009), 500 000 (Purcell et al., 2009) or 2 x 10^6 (from our previous data) cells/mL alginate were encapsulated in alginate beads and cell viability was assessed by Live/Dead assay on the intact beads. Analyses show that in our culture system the highest cell density results in the highest cell viability after some days in culture (data not shown). Consequently, in further studies cells were encapsulated at an initial cell density of 2 x 10^6 cells/mL of alginate, cultured for 12 days and evaluated for their differentiation by RT-qPCR and immunocytochemistry analyses.

4.2.3 Encapsulated mNSCs viability in alginate beads

Following encapsulation a Live/Dead assay was performed at different time points on the intact beads. Fig.22 shows high viability of cells encapsulated in alginate hydrogels during the first days in culture. These results confirm that mNSCs survive the gelling procedure and the conditions found inside alginate hydrogels. Dead cells are present in all experimental groups but the positivity for Ethidium Homodimer-1 (EH-1) is very low in all time points analyzed. The presence of some dead cells due to the encapsulation procedure and the stress caused by the differentiation stimuli that cells receive can be expected. mNSCs encapsulated in alginate hydrogels form small clusters.

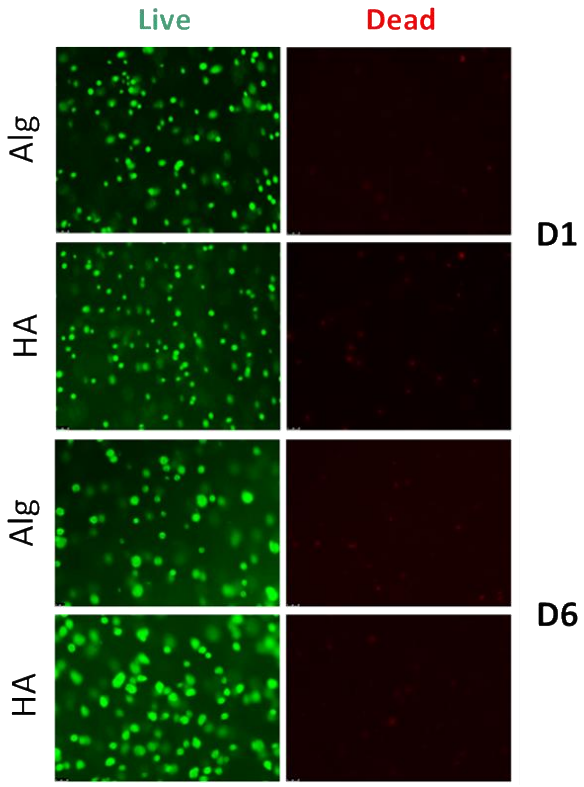

Fig.22 *Live/dead assay on mNSCs encapsulated in alginate gels*. Live cells are stained in green (Calcein-AM), dead cells are stained in red (Ethidium Homodimer-1). ALG: alginate, HA: alginate – hyaluronic acid.

4.2.4 Neural differentiation of mNSCs encapsulated in alginate beads

In order to verify if our three-dimensional alginate system supports neural differentiation of mNSCs, cells were recovered from the beads and RNA was collected for RT-qPCR analyses at D0, D3, D7 and D12 (Fig. 23). Two dimensional cultures were used as controls. Neural differentiation occurs in alginate beads, but with lower extent with respect to two-dimensional culture controls. In fact, the expression of the neural precursor marker Sox2, which should disappear as neural differentiation proceeds, significantly decreases during the culture period in both alginate and alginate-HA. Cells cultured in three-dimensional scaffolds present a peak of Nestin expression at D3 that decreases during the following days in culture, with variable expression levels but always comparable to control ones. We hypothesized that mNSCs encapsulation could initially slow cell differentiation with respect to three-dimensional cultures because of delayed nutrients and factors diffusion that leads to prolonged proliferation. However neural differentiation occurs also in three-dimensional cultures as demonstrated by the increasing expression of the marker for differentiating neurons βIII-tubulin. Three- and two- dimensional cultures follow the same trend but cells encapsulated in alginate and alginate-HA present lower levels of βIII-tubulin expression at all time points analyzed. The terminal neural differentiation marker NCAM is still not highly expressed after 12 days in three-dimensional cultures with respect to two-dimensional controls, but its levels seem to increase during the culture period. The glial marker GFAP is

expressed in encapsulated mNSCs with variable levels in all conditions and time points tested. The two-dimensional protocol does not favour glial cell differentiation (Spiliotopoulos et al., 2009) and we can thus speculate that neither does our culture system, as encapsulated cells present lower GFAP expression levels during all culture period.

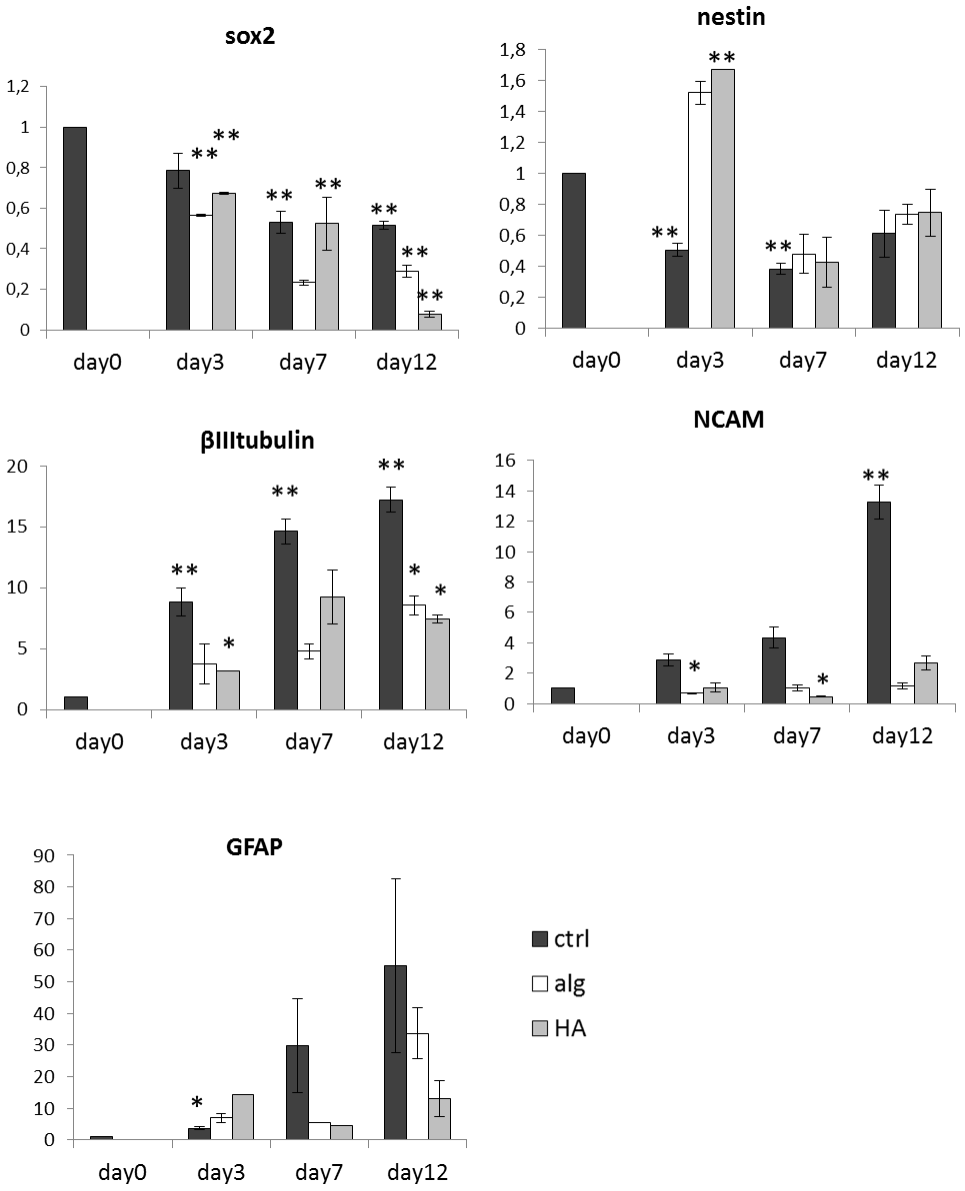

Fig.23 N*eural differentiation of mNSCs encapsulated in alginate beads*. RT-qPCR analyses on neural precursors markers (Sox2 and Nestin) and later neural differentiation markers (βIII-tubulin and NCAM) and glial marker (GFAP) at D0, D3, D7 and D12. ALG: alginate, HA: alginate - hyaluronic acid. Statistical significance with respect to the control (D0): * $p < 0.05$, ** $p < 0.01$. Merge of 3 experiments.

We checked differentiation of mNSCs encapsulated in alginate beads by immunocytochemistry analyses for the terminal differentiation markers NCAM and MAP2. NCAM expression was found already at D7 in cells grown both in alginate and alginate-HA (Fig.24 a-f). NCAM and MAP2 expression, shown in Fig. 24 g-l, are present at D12 in both

conditions tested, confirming that neural differentiation occurred in our three-dimensional culture systems. Results show how cells form connections and networks among themselves and that they start spreading neurites outside the clusters into the hydrogel.

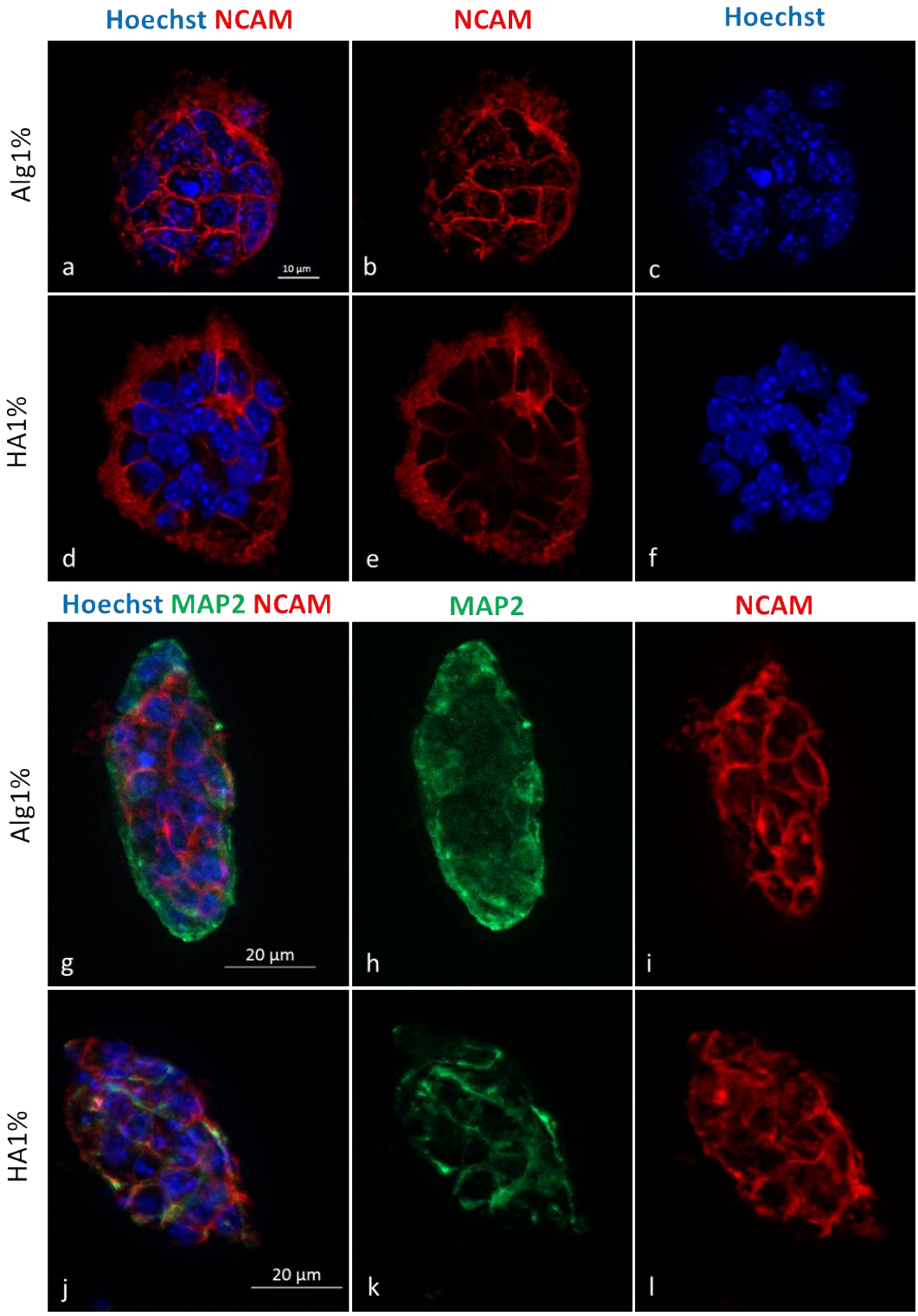

Fig.24 *Differentiation of mNSCs encapsulated in alginate and alginate–HA beads*. Immunocytochemistry analyses on single clusters at D7 for terminal neuronal differentiation marker NCAM in red (**a, b, d, e**). Immunocytochemistry analyses on single clusters at D12 for terminal neuronal differentiation markers MAP2 in green (**g, h, j, k**) and NCAM in red (**g, i, j, l**). Blue, Hoechst nuclear staining (**a, c, d, f, g, j**). ALG: alginate, HA: alginate - hyaluronic acid.

These results confirm that mNSCs survive and proliferate inside alginate hydrogels, forming small clusters in the first days of culture. mNSCs differentiate inside alginate scaffolds, even if with a lower extent with respect to two-dimensional cultures. βIII-tubulin expression increases during the culture period, following the trend observed in two-dimensional cultures, whereas NCAM expression increases more slowly with respect to controls. This can be explained by different nutrients availability which is immediate in two-dimensional cultures, whereas it could be slower in three-dimensional cultures. No significant differences are found among cells differentiated in unmodified alginate or modified with HA. Cells present similar expression levels of neuronal markers both in RT-qPCR and immunocytochemistry analyses. Alginate-HA is highly characterized by neurites which extend out from the clusters into the hydrogels and that can be visualized also by brightfield microscopy (data not shown).
These results demonstrate that our three-dimensional alginate-based culture system can be also used for the *in vitro* culture and differentiation of mNSCs.

4.2.5 mNSCs encapsulation in injectable alginate hydrogels

In order to obtain injectable alginate hydrogels for cell delivery in brain tissue, we investigated a gelling procedure that allows alginate crosslinking after injection, *in situ*. The alginate solution, when mixed with $CaCO_3$, does not crosslink until GDL is added. As the pH lowers during time after GDL addition, Ca^{2+} are slowly released by $CaCO_3$. In this way, calcium ions are not all immediately disposable to alginate G residues, thus allowing enough time for the injection of the alginate solution before it completely polymerized. Crosslinking time is influenced by $CaCO_3$ and GDL concentration and by the temperature at which the reaction occurs.
Even if calcium concentrations used during *in situ* alginate crosslinking are much lower than the ones used with $CaCl_2$, they could still be harmful for cells. For this reason, we initially tested viability of mNSCs after encapsulation in *in situ* gelling alginate hydrogels. $2x10^6$ cells/mL alginate were encapsulated in 1% w/v alginate, unmodified or modified with HA. Cell encapsulation does not interfere with alginate crosslinking, which occurs in 10 minutes at 37°C. A Live/Dead assay was performed on alginate gels the day after encapsulation (D1) and after 3 days of culture (D3). Fig. 25 shows that cells survive the gelling procedure and that they remain viable during the first days in culture. Few dead cells can be seen in some experimental conditions, probably due to the encapsulation procedure and induction of differentiation.

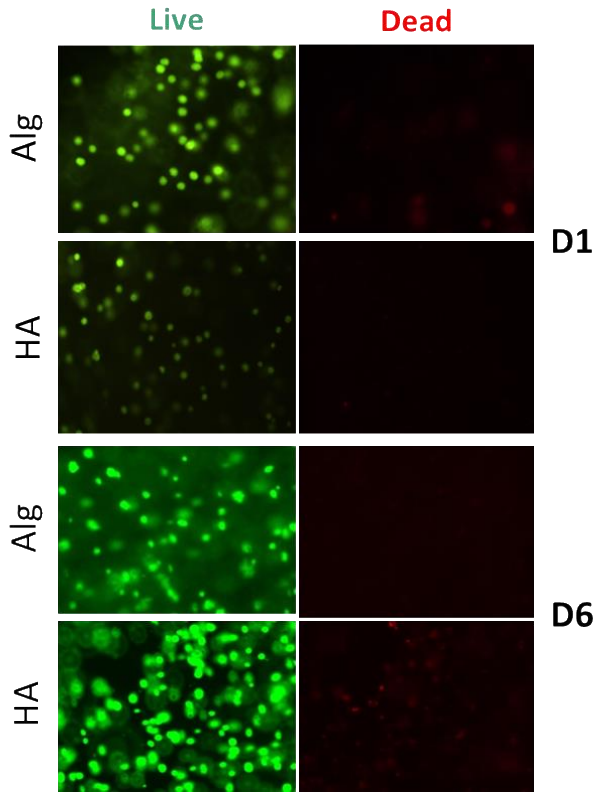

Fig.25 *Live/dead assay on alginate hydrogels crosslinked with the in situ gelling method*. Staining of cells encapsulated in alginate beads: live cells are stained in green (Calcein-AM), dead cells are stained in red (Ethidium Homodimer-1). ALG: alginate, HA: alginate – hyaluronic acid.

4.2.6 Alginate *in vivo* crosslinking

We initially tested if the *in situ* gelling procedure efficiently leads to alginate polymerization following injection in the brain. In order to trace alginate injection, we stained alginate solution with a blue dye, Astra blue. Stained alginate was injected with and without the crosslinking agent $CaCO_3$, in order to analyze the differences in its distribution and polymerization. A group of animals was injected with the dye alone. Three to six months old CD1 male mice were stereotactically injected with 1µl of solution into the striatum, one of the regions where stroke is commonly induced in animal models. Brains were collected after 24 hours or 5 days post injection (p. i.), cut with an adult brain slicer matrix for coronal sectioning and analyzed for blue staining presence and distribution. All mice injected with alginate survived the procedure and did not present any side effects due to alginate presence or calcium release in the brain. Results suggest that alginate polymerization *in vivo* occurs (Fig. 26). In fact, injection of alginate together with $CaCO_3$ and GDL results in a confined blue staining in the site of injection even after 5 days (Fig. 26 a, b), whereas injection of alginate without the crosslinking agent results in a more diffuse distribution, with the presence of some blue precipitates (Fig. 26 c, d, black arrows). Coordinates for the injection were calculated based on an atlas for C57/BL6 adult mouse brain, which is slightly smaller with respect to CD1 adult brains. This explains why some of the injected solution

went into the ventricle (Fig. 26 d). However, in the image it is clearly visible how the blue diffuses more in the tissue with respect to alginate injection with $CaCO_3$. Furthermore, injection of the dye alone results in a less intense and more widely spread blue staining (Fig. 26 e, f).

These preliminary data suggest that alginate polymerization occurs *in vivo* in the mouse brain and that the *in situ* gelling procedure is a good method for obtaining injectable hydrogels, without harming the animals with highly invasive surgery.

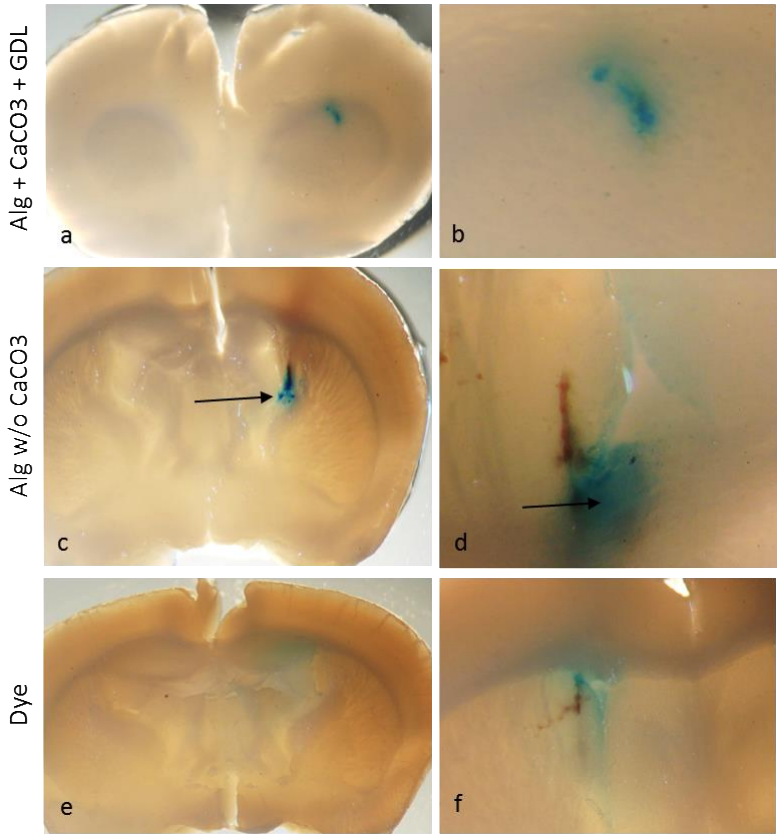

Fig.26 *In situ polymerization of alginate in the brain tissue*. Brains collected after 24 hours or 5 days from the injection. Alginate + $CaCO_3$ + GDL 5 days p.i. (**a, b**), alginate without $CaCO_3$ 24 hours p.i (**c, d**), injection of dye 24 hours p.i. (**e, f**).

In order to confirm these data, brains of C57/BL6 male mice stereotactically injected into the striatum with stained alginate were collected, fixed and cut at the vibratome or cryostat for histological analyses. A group of animals was injected with alginate and mNSCs, in order to test if the presence of cells could impair alginate polymerization. We tested two different cell concentrations, 5000 cells/µl, close to cell density used in our previous *in vitro* studies and 50.000 cells/µl which is higher than cell density used *in vitro* but lower than the cell density commonly used for NSCs injection in the brain (Oki et al., 2012; Tornero et al., 2013). Alginate was not stained in order to avoid any cell death, whereas cells were labelled with a fluorescent membrane dye (PKH26) prior to injection, allowing their localization in the tissue. Brains of mice injected with alginate were collected after 24 hours

post injection, brains injected with alginate and NSCs after 24 hours, 7 days and 14 days from injection.

We initially processed the injected brains by vibratome sectioning. Apparently no crosslinked alginate was present in our sections. However, we noticed that all sections show a hole in the site of injection, in which dark residues are often visible (Fig. 27a, white arrows). Moreover, following Hoechst nuclear staining, some cells are visible inside these residues (Fig. 27b, white arrows), suggesting that what we see are remaining pieces of crosslinked alginate. We concluded that we were losing information about the possible alginate crosslinking because of the methodology used for the analyses. In fact the sections obtained by vibratome are collected floating in PBS and, since the volume of the injected solution is very small, we cannot exclude that the tiny amount of gel present in each section, even if it is crosslinked, is lost once the slice is cut and starts floating. We thus decided to cut remaining brains at the cryostat. Following this protocol, brain sections do not display any hole in the site of the injection and it is possible to observe the presence of crosslinked alginate (Fig. 27c). Following nuclear staining, the presence of cells is visible inside the site of injection and the alginate (Fig. 27d, white arrow).

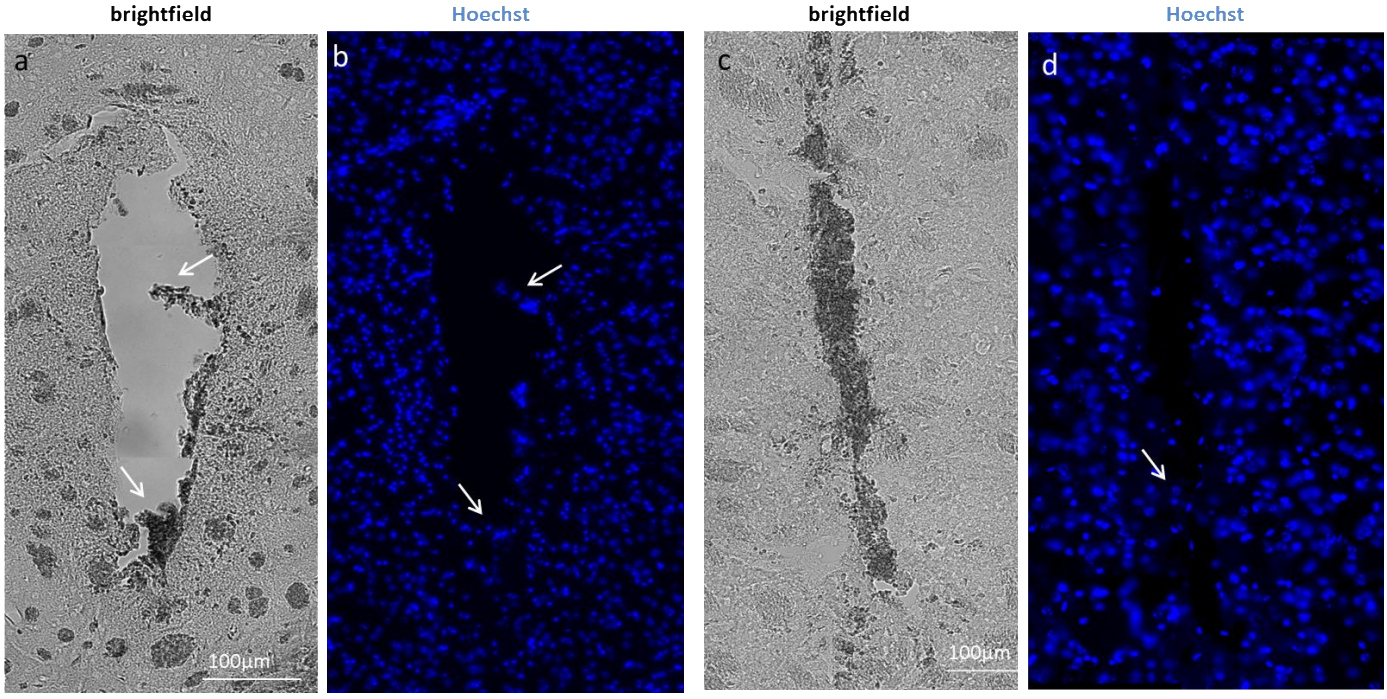

Fig.27 *Alginate crosslinking in the brain tissue.* Brain sections collected 24 h p.i. and cut at the vibratome (**a, b**), brain sections cut at the cryostat (**c, d**). Blue, Hoechst nuclear staining (**b, d**).

Injected cells are present in the site of injection, as shown by the red fluorescence of PKH26 (Fig. 28 b, c). Cells were visible only in the brains injected with the higher cell density, whereas in the brains injected with the lower amount of cells no red fluorescence

was visible. This could be due to poor cell resuspension in the alginate solution at the moment of the transplantation which prevented cell injection in the brain, but could also be a limit of the dye used for cell labelling. PKH26 intercalates in the cell membrane and it can be lost together with the lipidic cell components during the washes of the immunocytochemistry procedure. The injection of GFP-NSCs could help in identifying the origin of the problem.

In order to confirm that cells present in the injection site are grafted NSCs, we performed immunocytochemical analyses for the neural marker Nestin. Cells characterized by red fluorescence seems to co-express Nestin (Fig. 29d-f), suggesting that they are the mNSCs we injected. However the staining protocol needs to be improved in order to avoid the high background around the site of injection. Staining for GFAP, which marks astrocytes that are activated following injury and overexpress this protein, also showed a high background and non-specific fluorescence signal around the site of the injection (Fig. 29b).

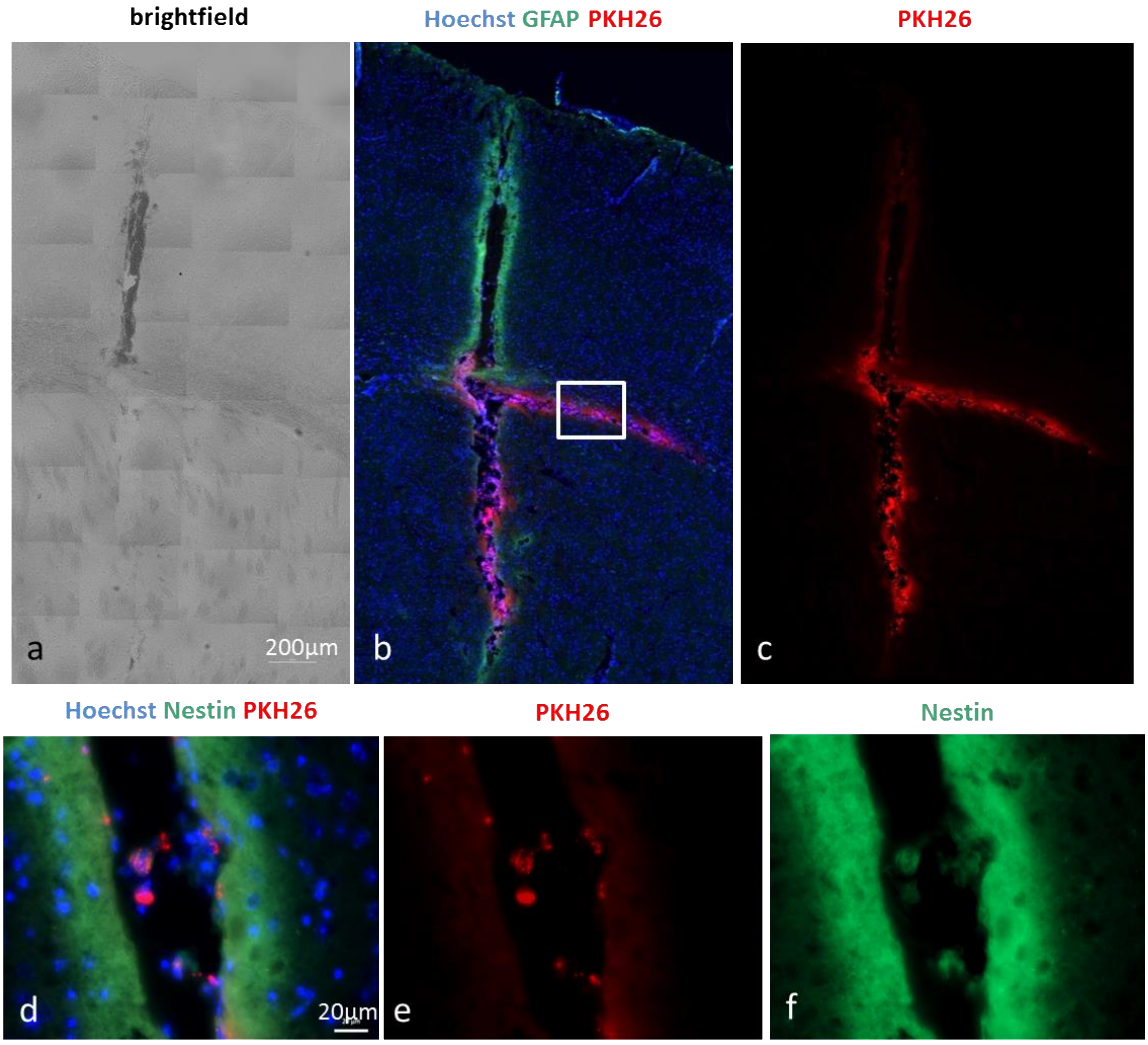

Fig.28 *Localization of cells co-injected with alginate in the brain.* Immunocytochemistry analyses on brains collected 24 hours after injection of alginate and NSCs for activate astrocyte marker GFAP (**b**) and neural precursor marker Nestin (**d, f**) in green. Red, fluorescence membrane dye (**b, c, e**), blue, Hoechst nuclear staining (**b, d**).

Histological analyses with the Cresyl Violet staining were performed on sections of brain collected after 24 hours from injection and confirmed the presence of crosslinked alginate and injected mNSCs in the site of the lesion, as shown in Fig. 29 (a, b). Injected cells present deep violet staining (Fig. 29a) whereas alginate is characterized by a light pink staining (Fig. 29b, black arrow). Crosslinked alginate presence can be found in the entire hole left by the needle. In order to confirm these preliminary results, polymerized alginate presence should also be investigated with other histological stainings specific for polysaccharides, such as Alcian Blue or Astra Blue.

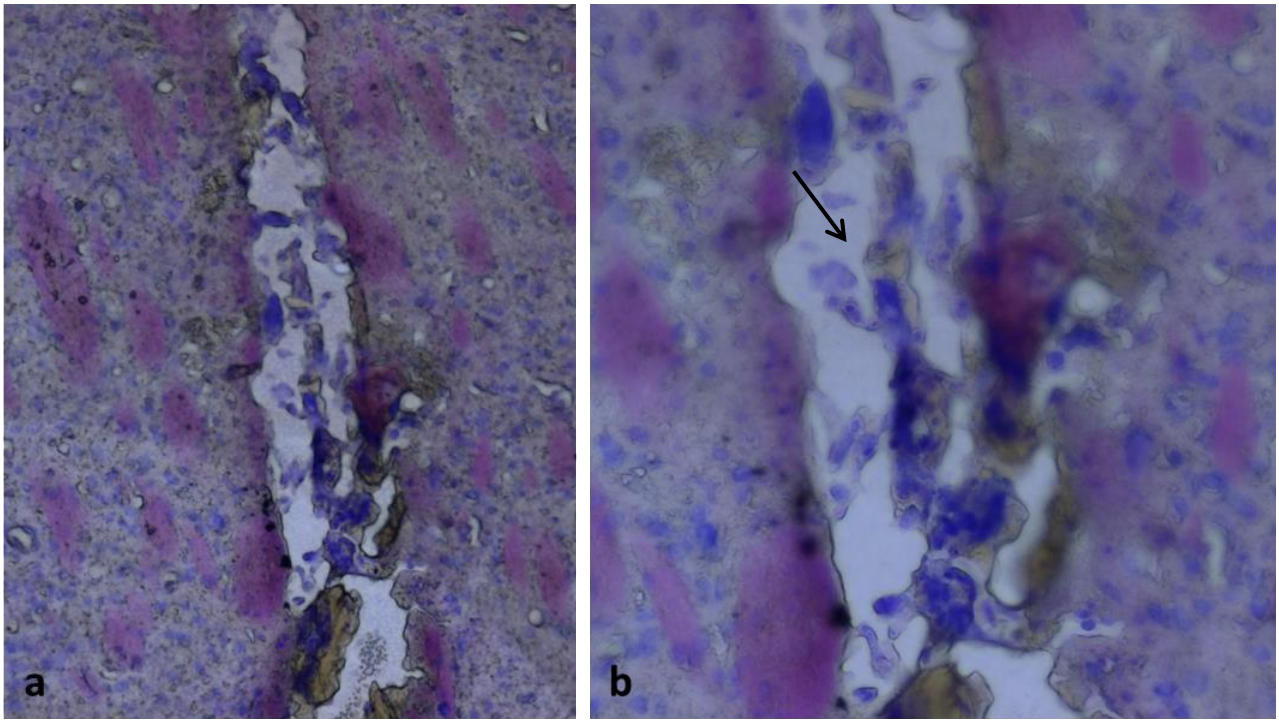

Fig.29 *Presence of crosslinked alginate and injected cells in the site of injection*. Cresyl violet staining on sections of brains injected with alginate and cells and collected 24hours after injection (**a, b**).

These results show that alginate crosslinking occurs *in vivo* in brain tissue. Cell encapsulation does not interfere with alginate crosslinking, allowing efficient cell delivery in the brain, with cells mainly localizing in the injection site.

4.2.7 Alginate biocompatibility in the brain tissue

In situ-forming alginate hydrogels support mNSCs viability and differentiation, indicating that crosslinking procedure is not harmful for the cells. Moreover, our preliminary data demonstrated the feasibility of the injection of these hydrogels in mouse brain tissue and their efficient *in vivo* polymerization. We further investigated if alginate crosslinking and presence in the tissue is causing inflammatory response. We thus evaluated its

biocompatibility in the brain tissue taking advantage of the mouse strain carrying the dual reporter system (luciferase and GFP) under the control of the TRL2 promoter. This mouse models allows to monitor the *in vivo* activation of TLR2, involved in inflammation processes following brain injury.

Three months old TLR2-luc/GFP male mice were used to test inflammation profile after alginate injection in the mouse brain. Baseline values were recorded before stereotactic injection into the striatum. A group of mice (n=2) received lipopolysaccharide (LPS) injection as positive control for inflammation. Its administration is a well-established model associated with a strong induction of inflammation through TLRs activation, included TLR2 (Laflamme et al., 2001). Since we are interested in the use of alginate as hydrogel for brain regeneration following stroke, one mouse underwent MCAO procedure and was also considered as a positive control for inflammation. Another group of mice (n=3) received injection of 1% alginate, a second group (n=6) was co-injected with 1% alginate and NSCs, while a third one (n=2) received administration of NSCs alone. Since the injection procedure causes some levels of inflammation itself, a group of mice (n=5) was injected with PBS for "basal" inflammation values and was considered as a negative control for inflammation. Following injection, mice were imaged 1 day, 3 days, 7 days and 2 weeks after surgery. Each measurement at each time point is normalized on the baseline value.

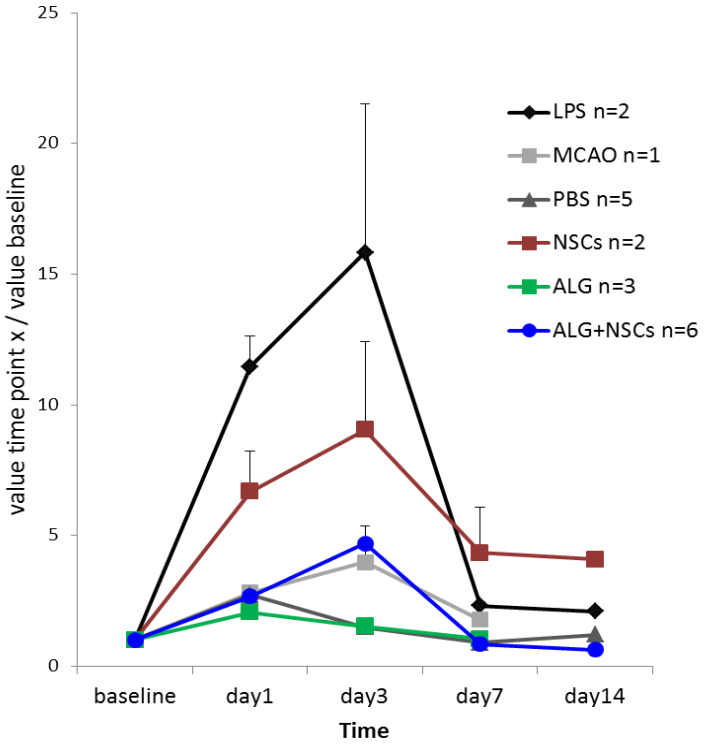

Fig.30 *Biocompatibility of alginate in the brain tissue*. *In vivo* bioluminescence analyses. LPS: lipopolysaccharide, MCAO: middle cerebral artery occlusion, PBS: phosphate buffered saline, ALG: alginate.

Mice injected with alginate survived up to 4 months, indicating that alginate injection is not harmful for the animals. Moreover bioluminescence results show that alginate injection in the brain tissue does not elicit inflammation (Fig. 30). During the first three days following injection, LPS (black line) highly stimulates an inflammatory response that spontaneously regresses in the following days, but does not return to baseline values. Alginate injection (green line) presents an inflammation profile comparable to PBS (dark gray line), indicating that the presence of the hydrogel in the brain does not increase the inflammation caused by the injection procedure itself. These two experimental groups follow the same trend, with a peak of inflammation at day1 after which they start to recover, reaching again baseline values at day7. Alginate (and PBS) values are much lower than LPS values, indicating that its injection is safe and not detrimental for the tissue. Injection of NSCs (red line) results in an inflammation profile similar to LPS injection, but with lower values, thus suggesting that the presence of exogenous cells in the brain could stimulate inflammation. Moreover, when NSCs are co-injected with alginate, the inflammation profile follows the same trend of NSCs alone, with a peak at day3 and a later decrease, but values are much lower that injection of NSCs alone. This suggests that not only alginate does not cause inflammation in the brain tissue, but it prevents inflammation when injected together with NSCs, probably avoiding interactions between the grafted cells and host tissue. The inflammation profile following MCAO procedure (light gray line) is characterized by a peak at day3 that resolves in the following days. This mouse presents lower values with respect to LPS but also to NSCs and alginate-NSCs injections. Probably this result is not truly representative of the inflammation caused by stroke in mouse brain. In fact, mice that undergo MCAO procedure can exhibit very different outcomes in terms of inflammation profile (data not shown, from Prof. Gajovic Lab), therefore we need to increase the number of animals in this group in order to have a more representative profile of inflammation after ischemic insult in the brain. We need to increase the number of animals in our experimental groups, however these preliminary results suggest that alginate injection in the brain is not harmful for the animals and does not elicit inflammation. In fact, alginate inflammation profile presents much lower values with respect to LPS injection and already after 1 week, injection of alginate both alone and with NSCs, presents values comparable to baseline. Interestingly, co-injection of alginate and NSCs decreases the inflammation caused by the presence of grafted NSCs in brain tissue.

In this part of the study we demonstrated that our three-dimensional culture system also supports mNSCs survival and differentiation. We investigated the use of injectable alginate hydrogels for the delivery of NSCs in brain tissue, showing that the crosslinking procedure is not harmful for encapsulated cells and calcium concentrations are not toxic. Moreover,

our preliminary data demonstrated the feasibility of *in situ* gelling alginate hydrogels injection in the brain, showing its efficient crosslinking *in vivo* and suggesting also that its presence in the brain tissue does not elicit any inflammatory response.

Injectable alginate hydrogels could be used in tissue engineering approaches for the efficient transplantation of stem cells in the injured brain, allowing minimal invasive surgery

5. DISCUSSION

5.1 Neural differentiation of mouse embryonic stem cells (mESCs) in three-dimensional alginate beads

Pluripotent cells can differentiate into many different cell types, including neurons (Lee et al., 2000; Carpenter et al., 2001; Tropepe et al., 2001; Wichterle et al., 2002; Ying et al., 2003; Watanabe et al., 2005; Fico et al., 2008), making them potentially suitable for cell-replacement therapies for injured brains, that are unable to self-repair. Many neural differentiation protocols develop in two dimensions or start with EBs formation (Lee et al., 2000; Hwang et al., 2008; Itskovitz-Eldor et al., 2000). However these culture systems present low homogeneity or do not represent the physiological environment in which cells grow and differentiate. Given the importance of cell-cell and cell-ECM interactions, biomaterials are good candidates for recapitulating the *in vivo* conditions *in vitro*.

Alginate is widely used in bone and cartilage tissue engineering (Sun et al., 2013), and recently its ability to support the differentiation of stem cells towards neural lineages has also been reported (Frampton et al., Li et al., 2011; Addae et al., 2012; Candiello et al., 2013; Kim et al., 2013). In our study we evaluated the possibility to differentiate mouse embryonic stem cells encapsulated in alginate beads at 1% and 2% w/v alginate concentration towards neural lineages. The advantage of this method is the ease of crosslinking when alginate is exposed to bivalent cations and the possibility to recover cells from the hydrogel. We tested the hypothesis that this three-dimensional culture system would enhance neural differentiation with respect to traditional two-dimensional cultures. Alginate was modified with fibronectin, RGD peptide and hyaluronic acid in order to assess whether these modifications could favor cell attachment (Schwarzbauer et al., 2011) and neural differentiation (Perris et al., 2000; Margolis et al., 1975; Preston et al., 2011; Bandtlow et al., 2000).

We showed that alginate allows encapsulated cells to survive and grow, showing a viability of around 90% at the end of the culture period, for all experimental conditions tested. Alginate polymerization is obtained with ionic crosslinking through exposure to a Ca^{2+} ions concentration which is 100-fold higher than physiological concentration. Abnormal Ca^{2+} ions concentrations can damage cells and, after injury, increased intracellular calcium uptake is associated with neuronal apoptosis (Wingrave et al., 2003). These data reporting high cell viability during the culture period indicate that the high calcium concentration used is not toxic for mESCs and that the crosslinking procedure does not harm cell viability. The day after encapsulation, cells embedded in alginate-HA at both concentrations present slightly higher viability compared to other conditions, suggesting that this modification is

able to support a better initial cell viability. At D18 cells cultured in all experimental conditions show same levels of viability whereas at D7 2% alginate conditions present lower viability levels with respect to 1% gels. A differentiation stimulus can cause some cells to die but from RT-qPCR analyses in the two alginate concentrations at this time point it is not possible to observe a difference in terms of neural differentiation. This indicates that lower cell survival rates cannot be related to more efficient differentiation and we can thus speculate that a lower alginate concentration better supports cell viability.

Cells form clusters that increase in size the first days of culture but are smaller than canonical EBs, allowing for a more homogeneous differentiation. In fact, the simple culture medium that we used enables a limited amount of proliferation during the first days of the differentiation culture, a phenomenon that has previously been shown (Amit et al., 2000). Alginate beads swell but do not degrade throughout the culture period and at the end of the experiment we have evidence of successful neural differentiation, at different levels, in all experimental conditions. We confirmed the presence of neural precursors after 7 days in culture by the expression of early neural markers Nestin and βIII-tubulin, especially among cells grown in alginate and alginate-HA at both concentrations. βIII-tubulin expression was confined to cells in the periphery of clusters suggesting that these are the first cells to differentiate. It has been reported (Wang et al., 2009) that stiffness and dimension of alginate beads influences nutrients diffusion, thus this periphery-to-center gradient of differentiation timings could be due to the diffusion of nutrients. Diffusion studies with molecules of different molecular weight (MW) within alginate beads showed that this process is influenced both by MW of diffusing molecules and alginate concentration, with decreased rates as alginate concentration increases. However, only high MW molecules (500 kDa) presented impaired diffusion rate, remaining confined in the periphery of the bead. Other factors with MW similar to growth factors used during *in vitro* cultures are free to diffuse to the centre of the bead within 24 hours of incubation (Wang et al., 2009). In addition, no differences in differentiation were found among clusters in the periphery with respect to the centre of the beads at D18 in our three-dimensional cultures (data not shown). These data prompted us to hypothesize that the more precocious differentiation observed in the periphery of each cluster may depend from interactions between the cells and the surrounding biomaterial, rather than from molecule diffusion. The differentiation signals could then spread toward the center of each cluster, as at D18 the gradient observed at D7 has disappeared.

After 18 days we demonstrated that cells underwent neural differentiation in all experimental groups, and that differentiation was enhanced with respect to two dimensional cultures. Cells cultured in the lower alginate concentration presented the most homogeneous differentiation, with nearly no expression of pluripotency markers, whereas

these markers were more present in cells grown in 2% alginate. The most efficient neural differentiation occurred in cells grown in 1% alginate and 1% alginate-HA. These two groups, especially 1% alginate-HA, had higher expression levels of the markers for terminally differentiated neurons MAP2 and NCAM, as shown both by RT-qPCR and immunocytochemistry analyses. In these two experimental conditions we observed the presence of neural rosettes, a neural hallmark (Abranches et al., 2009), confirming that three-dimensional culture under these conditions is optimal for neural differentiation.

Modification of alginate with the adhesion protein fibronectin or the RGD peptide did not enhance neural differentiation. Cells encapsulated in alginate-RGD presented variable expression of neural differentiation markers among different experiments. Moreover, cells encapsulated in 2% alginate with these modifications showed high expression levels of pluripotency markers at D7 and still at D18, both by RT-qPCR and immunocytochemical analyses (data not shown), indicating a more heterogeneous differentiation within these experimental groups. Cells differentiated in alginate-Fn presented at D18 enclaves of cells that are still undifferentiated, Sox2 positive but Nestin negative, and no significant expression of markers for terminally differentiated neurons, shown by RT-qPCR analyses. This confirms the heterogeneity and the poor reproducibility of differentiation performed with this culture condition. We thus decided to not further characterize cells differentiated in these hydrogels.

Immunocytochemistry analyses revealed the presence of GFAP positive cells in all experimental conditions, confined only to very few clusters, and with lower expression levels than in two-dimensional cultures. Among the different hydrogel conditions, GFAP expression was highest in 2% alginate. Increasing hydrogel stiffness is in fact reported to increase glial differentiation (Franze et al., 2013). The presence of glial cells in the cultures could be helpful or even necessary for the maturation and sustenance of newly generated neurons. Evidences for the trophic role of glia for neuronal maturation and synapse formation come from both *in vivo* and *in vitro* studies (Freeman et al., 2006). βIII-tubulin and MAP2 staining clearly demonstrated that cells are able to extend neurites connecting them inside clusters. The capability of creating three-dimensional networks inside alginate hydrogels, poorly described in previous studies (Li et al., 2011; Kim et al., 2013), shows that this system is able to properly recapitulate the cell-cell and cell-ECM interactions occurring *in vivo*. This allows a more homogenous differentiation than that occurring in EBs. Connections among cells are more abundantly present in 1% alginate and 1% alginate-HA, suggesting that scaffold stiffness plays a role in differentiation. Both conditions show projections outside clusters, found especially in the 1% alginate-HA group, confirming that three-dimensional alginate scaffolds support neurite growth and expansion. Specifically, our data suggest that 1% alginate-HA is the best culture condition for neural differentiation.

Furthermore, differentiated neurons in this experimental group express both pre- and post-synaptic markers, suggesting that they can form synapses inside our three-dimensional cultures. We thus hypothesize that the chemical modification of alginate by HA is able to influence neural differentiation, as expected, likely due to the fact that HA is present during brain development and in the neural stem cell niche (Margolis et al., 1975; Preston et al., 2011), and it is known to be important for migration and axonal growth (Bandtlow et al., 2000). Moreover it is reported that HA hydrogels promote neural differentiation of murine neural stem cells (Brannvall et al., 2007) and mesenchymal stem cells (Her et al., 2013) when combined with type 1 collagen.

mESCs culture in our alginate-based scaffolds indicates that the optimal alginate concentration for stem cells neural commitment is at 1% w/v. Previous studies involving mESCs encapsulation in alginate microbeads with different alginate concentrations (from 1.2% to 2.5% w/v) reported 2.2% w/v alginate as the optimal concentration for neural commitment (Li et al., 2011). Our data are in contrast with these findings but support later studies reporting the differentiation of mESCs into GABAergic neurons in 1.1% w/v alginate hydrogels (Addae et al, 2012) and of hESCs into dopaminergic neurons in 1.1% w/v alginate microcapsules (Kim et al., 2013).

We investigated ECM production by encapsulated cells and the staining for chondroitin sulfate proteoglycans shows that cells inside clusters are surrounded by their own ECM, which could favors their differentiation, resembling the physiological environment encountered *in vivo*. Preliminary results also show that some cells located at the periphery of the clusters do not produce ECM components, suggesting that interactions with the surrounding alginate could be enough for their support, sustenance and differentiation. We should investigate whether in longer period of culture, cells will substitute alginate with their own ECM.

In order to confirm that nutrient diffusion is not limiting cell differentiation, we cultured mESCs encapsulated in small beads. We show that, in our three-dimensional culture system, a reduction in bead dimension does not result in enhanced neural differentiation, which occurs although to a lower extent with respect to two-dimensional controls and to the larger alginate beads. Decreasing beads size enhances hydrogel instability in all experimental conditions we tested, especially in alginate-Fn, and with the exception of alginate-HA. Beads of this experimental group were bigger in dimension and more stable during the protocol, without presenting events of degradation or cell escape from the hydrogel. Moreover, cells cultured in alginate-HA underwent more efficient neural differentiation as indicated by the presence of neural rosettes, terminal differentiation markers expression and ability of differentiating neurons to extend projections into the scaffold. All these characteristics resemble three-dimensional cell culture in larger beads of

alginate-HA, as further demonstration that alginate modification with HA better supports mESCs neural differentiation.

There are evidences that mechanical properties of biomaterials influence cell differentiation, and that neuronal maturation is promoted by soft substrates with elastic moduli in the same range of the brain tissue (100-1000 Pa, Gefen et al., 2004; Li et al., 2012) (Amit et al., 2000, Pfieger et al., 1997; Teixeira et al., 2009). We demonstrate that cell behaviour is influenced both by chemical and mechanical properties of alginate hydrogels. All of our experimental conditions present elastic moduli in the range of those found in brain (Banerjee et al., 2009; Matyash et al., 2012) and can thus support neural differentiation. The more efficient neural differentiation obtained of cells grown in 1% alginate with respect to 2% alginate hydrogels may be due to physical properties, despite the small differences in Young's modulus values. In fact it has been shown that cells can recognize and respond to small changes in material properties, such as elastic modulus (Yoon et al., 2007). Among the experimental conditions using 1% alginate, HA modification supports the most efficient and homogenous neural differentiation. No differences in mechanical properties were found among 1% alginate groups, suggesting that differentiation is influenced by the modification and therefore by the hydrogel chemical properties. It has previously been demonstrated that hyaluronan hydrogels support and enhance neural differentiation of embryonic and neural stem cells (Brannvall et al., 2007; Wang et al., 2009; Her et al., 2013).

The expression of terminal differentiation markers for different neuronal subtypes, the presence of networks among cells and projections outside clusters, and the expression of synaptic proteins, confirmed that this three-dimensional culture system is able to elicit an efficient terminal neuronal differentiation, and generate different neuronal subtypes without the addition of exogenous factors. This system could be a very useful tool to obtain highly pure neuronal subtypes populations by the addition of specific soluble factors. Moreover, alginate hydrogels could be easily modified with other ECM components present in the stem cell niches and which are known to play important roles in stem cells differentiation.

5.2 Neural stem cells and alginate co-injection for CNS regeneration

Stroke is one of the most severe forms of brain injuries and one of the leading causes of death worldwide (Donnan et al., 2008). Neural stem cells (NSCs) are good candidates for cell replacement therapies in the nervous tissue, since they have been shown to stimulate neuroprotection, have the ability to migrate, especially to the site of the injury, and to integrate in the endogenous circuitry (Fischbach et al., 2013, Doeppner et al., 2014). Furthermore, there are reports of improved functional recovery following NSCs

transplantation in stroke animal models (Jeong et al., 2003; Lee et al., 2009; Oki et al., 2012; Yuan et al., 2012; Ding et al., 2013).

ESCs-based approaches for neural tissue regeneration following ischemia have been investigated as well, reporting evidences of beneficial effects and functional recovery (Takagi et al., 2005; Buhnemann et al., 2006; Kim et al., 2014). However ESCs are difficult to control, thus they can lead to the risk of teratoma formation after injection *in vivo* (Fong et al., 2007; Seminatore et al., 2011; Guan et al., 2014). Moreover, also *in vitro* is difficult to obtain pure cell-fated populations, unless some steps of purification or selection are performed.

Despite the promising results of these approaches, the majority of the grafted cells die within weeks after transplantation, resulting in a limited efficacy of the treatments. Very large amounts of cells need to be injected in order to obtain beneficial effects, and this is a strong limit for the translability of these approaches into the clinic (Li et al., 2012). Regenerative medicine and tissue engineering combine the use of stem cells with biomaterials in order to better differentiate them and control their transplantation. They can allow three-dimensional cultures that better recapitulate the three-dimensional physiological environment in which cell grow and differentiate and could also help to enhance and control cell survival after transplantation, minimizing cell death.

The scaffolds we generated are soft enough to resemble brain tissue, as indicated by their elastic modulus values, therefore we turned our attention toward the possibility of using alginate as biomaterial for brain regeneration following injury. Various biomaterials have been tested for brain tissue regeneration following stroke, such as collagen, Matrigel, PGA scaffolds and PEG-PLA nanoparticles (Yu et al., 2010; Jin et al., 2010; Zhong et al., 2010; Park et al., 2002; Mdzinarishvili et al., 2013). Cell encapsulation in alginate hydrogels already showed to support *in vivo* proliferation and differentiation of neural linages after transplantation in spinal cord lesion models (Kataoka et al., 2004; Prang et al., 2006; Willenberg et al., 2006; Wang et al., 2012) but its use for brain tissue regeneration has not been evaluated yet. Few studies report mNSCs encapsulation and culture in alginate hydrogels, showing their viability inside scaffolds and early differentiation (Li et al., 2006; Banerjee et al., 2009; Purcell et al., 2009). In one study alginate was tested as carrier for VEGF administration before inducing stroke in rat brain (Emerich et al., 2010). Finally, injectable alginate hydrogels have been tested as sealants for dural defects (Nunamaker et al., 2010).

Injectable hydrogels are preferable for brain tissue engineering, since they allow a less invasive surgery. *In vivo* injections of alginate crosslinked with the $CaCl_2$ method is not feasible, due to the instant polymerization of the hydrogel. In addition, high concentration of calcium in the tissue could be detrimental for cells and increase inflammation cascades that

follow ischemic injury. We evaluated an alternative *in situ* crosslinking method with the use of $CaCO_3$ and glucono-delta-lactone (GDL), which also involves lower calcium ion concentrations.

We avoided the use of mESCs during *in vivo* studies, thus we first tested mNSCs viability in our system and showed that they survive in alginate hydrogels obtained both by $CaCl_2$ and *in situ* gelling procedures. We reported their differentiation following encapsulation in alginate beads, confirming the possibility to use three-dimensional alginate-based scaffold for NSCs cultures, as reported in other studies (Li et al., 2006; Banerjee et al., 2009; Purcell et al., 2009).

We then tested injectable alginate biocompatibility and suitability for cell replacement therapies in the damaged central nervous system with the final goal to co-inject NSCs and alginate hydrogels in stroke mouse models. We hypothesize that alginate could enhance survival and integration of the engrafted cells in the damaged neural tissue, by protecting them from the host inflammatory response. Moreover, continuous activation of glutamate receptor and consequent excessive intracellular influx of Ca^{2+} has been associated to neuronal death (excitotoxicity) following ischemic insult. *In vitro* studies report that inhibition of Ca^{2+} influx or removal of extracellular calcium result in a decreased neuronal excitotoxicity (Limbrick et al., 2003). The use of alginate hydrogels that rely on ionic crosslinking with Ca^{2+} could potentially reduce this type of damage following injury. In fact, if some calcium-binding sites are still free following injection in the tissue, they can bind extracellular calcium ions, reducing the intracellular uptake.

We report evidences of alginate *in vivo* crosslinking in the brain, indicating that the use of $CaCO_3$ and GDL is an efficient method for obtaining injectable hydrogels. Injection of alginate stained with a dye results in a more localized distribution with respect to its injection without Ca^{2+} ions or injection of the dye alone, suggesting that its crosslinking occurs in the brain tissue. The presence of polymerized alginate was also confirmed by histological analyses, however, biomaterial staining is weak. We need to confirm these data with histological staining specific for polysaccharides (i.e Alcian Blue or Astra Blue), that could better allow the visualization of alginate presence in the brain tissue. We need to investigate the presence of crosslinked alginate also in brains collected at 7 days and 14 days post injection.

After co-injection of alginate and mNSCs, we found cells localized in the site of injection one day after transplantation, though we need to investigate cell viability in more details. The red fluorescence for the PKH-26, the dye used to stain cells before injection, co-localizes with nestin expression, confirming that the cells that we observe are the NSCs we injected, and not cells dislocated from the tissue during needle withdrawal. Histological stainings further confirmed the presence of injected mNSCs in the site of the lesion. With

these preliminary studies we confirm feasibility of co-injection of *in situ* gelling alginate hydrogels and NSCs in brain tissue.

Mice injected with alginate survived up to 4 months, indicating that its presence is not harmful for brain tissue. We confirmed this by evaluating the inflammatory response in the brain tissue after alginate injection. Toll-like receptors (TLRs) are involved in inflammation as defence to pathogens present in the body. They have been shown to be present in the CNS and have been associated with inflammatory responses following injury (Marsh et al., 2007; Heiman et al., 2014 ;Keller 1997, Marte 2008 Waldner 2009 ; Tang et al., 2007). Based on TLRs important role and early involvement in inflammation, a mouse model for the *in vivo* monitoring of inflammation has been recently developed (Lalancette-Hebert et al., 2009). C57/BL6 TLR2-luc/GFP mice have been used in this study to test levels of inflammation due to alginate injection and presence in mouse brain tissue. Alginate injection does not elicit inflammation as its profile is overlapping with that of PBS that causes minimal responses, likely due to the injection procedure itself. Alginate, both injected alone and with NSCs, presents very low values with respect to lipopolysaccharide (LPS) injection. LPS administration strongly induces inflammation through activation of TLRs, including TLR2, representing a useful positive control for inflammation (Laflamme et al., 2001). This data further demonstrates that alginate presence in the brain is not causing inflammatory response in the tissue. NSCs injection elicits quite high inflammatory responses if compared to LPS and PBS, which could be expected in allogenic transplants. Interestingly, when NSCs are injected together with alginate, the resulting inflammation profile is higher than alginate alone but lower than NSCs alone. These data suggest that alginate prevents grafted-host cells interactions and consequent inflammation response. This confirms that alginate can help in increasing cell viability and survival following injection in a damaged brain, by protecting transplanted cells from the host environment. However, this confinement could impair NSCs beneficial role in the damaged tissue. These cells are known to stimulate neuroregeneration by release of neurotrophic factors and to migrate, differentiate and integrate in the host tissue (Lindvall et al., 2011; Fischbach et al., 2013; Hermann et al., 2014; Doeppner et al., 2014). We demonstrated that 1% alginate hydrogels, especially when modified with HA, allow neurites extension into the scaffolds in *in vitro* cultures. We hypothesize that after injection encapsulated cells could be able to extend their projections, sense the environment and form connections with the host cells, therefore having beneficial effects for the tissue without being in direct contact with it.

Middle Cerebral Artery Occlusion (MCAO) is commonly used for inducing cerebral ischaemia in animal models (Gerriets et al., 2003; Bacigaluppi et al., 2010; Rosell et al., 2013). It mimics the blood flow arrest that occurs during human stroke and elicits a similar consequent inflammatory response. Since we are interested in the treatment of brain injury

following stroke, we included an animal which underwent MCAO procedure as a positive control. Its inflammation profile overlaps with that of alginate-NSCs injection. However data about one single animal are not reliable and we need to increase the number of animals in this group in order to obtain a significant and representative trend of the inflammation that occurs in the brain after stroke. In fact, the procedure outcomes can vary among animals and depend also on operator handling.

These preliminary studies suggest that a hydrogel forms in brain tissue following injection of *in situ* gelling alginate and that this does not elicit an inflammatory response in the tissue. Alginate can be a good candidate biomaterial to generate injectable hydrogels for brain tissue regeneration approaches, allowing minimal invasive surgery and ensuring protection to the grafted cells from the host environment, likely increasing cell viability, survival and integration.

6. CONCLUSIONS

In my PhD thesis work we set up an alginate-based culture system able to efficiently support and enhance the neural differentiation of pluripotent cells. We showed that the culture of mouse embryonic stem cells encapsulated in alginate beads allows for increased differentiation with respect to traditional two-dimensional cultures, especially among cells grown in 1% alginate, alone or modified with hyaluronic acid. Cells cultured in these conditions present the highest and most homogeneous expression of neural markers. We demonstrated that generated neurons are able to form networks within clusters and outside clusters, confirming that our hydrogels promote neurite growth and extension. We also showed that without the addition of any exogenous factor we obtain a final neuronal population composed by different neuronal subtypes. In addition, analyses of mechanical and physical properties of the scaffolds we generated show their potentiality for soft tissue regeneration, such as brain. We investigated alginate hydrogels potentiality as support for NSCs injection in the brain. We reported *in vitro* mNSCs viability and initial differentiation in alginate hydrogels. Our preliminary *in vivo* studies demonstrate the possibility to obtain injectable alginate hydrogels that crosslink once injected in the brain tissue. Inflammation profiles obtained after alginate injection suggest that alginate presence is not harmful for the tissue. Taken together these findings suggest that alginate could be an efficient support for mNSCs transplantation in the nervous system, able to increase cell survival and integration in an injury-affected brain.

7. FUTURE PERSPECTIVES

In this study we report the set-up of a three-dimensional alginate-based culture system for the efficient differentiation of pluripotent cells towards neural linages. Traditional two-dimensional culture systems for the derivation of specific neuronal subtypes are often characterized by low efficiency. These systems are based on complex combinations of soluble factors known to stimulate cell differentiation but lack the three-dimensionality of the physiological environment in which cells reside, not providing the adequate physical stimuli, also important for cells growth and fate-commitment. In our cultures instead we used a general differentiation protocol without the addition of growth factors, obtaining different neuronal subtypes in the final cell population and demonstrating that a three-dimensional environment can influence and stimulate as well their differentiation. This system thus can be improved by the addition of growth factors, helping in recapitulating both the biochemical and mechanical stimuli that influence stem cell differentiation *in vivo*, in order to obtain highly enriched population of the desired neuronal subtype. Moreover, three-dimensional culture methods could allow to use less soluble factors, that are expensive and increase the complexity of the system.

A possible application could be the differentiation of ESCs towards dopaminergic neuronal lineages of great interest for Parkinson's disease. Another interesting study could be the differentiation of stem cell towards retinal cells, as it is known that a three-dimensional environment obtained by cell aggregation or with biomaterials enhances differentiation towards this type of lineage (Eiraku et al., 2011; McUsic et al., 2012; Nakano et al., 2012).

We tested the modification of alginate with fibronectin and hyaluronic acid, but alginate could be modified with other specific ECM components, known to be important for differentiation or involved in pathological conditions. Preliminary results in the lab indicate that alginate allows cells to produce their own extracellular matrix inside the scaffolds. Selective enzymatic removal of ECM components could allow analyses of their influence on function, differentiation and behavior of the encapsulated cells.

In the second part of the project, we explored the suitability and feasibility of using injectable *in situ*-forming alginate hydrogels for brain tissue regeneration, in order to enhance viability and integration of the engrafted cells in damaged neural tissue. Our goal is to co-inject NSCs and alginate hydrogels in a mouse model of focal cerebral ischemia obtained by middle cerebral artery occlusion (MCAO). In this MCAO model, stroke is induced by temporary ligation of this artery and the procedure causes a brain damage due to the stop of blood flow that resembles human stroke. As first step forward we need to confirm preliminary data about alginate crosslinking and biocompatibility in the brain tissue, by performing histological analyses with staining specific for polysaccharides, by

Transmission Electron Microscopy (TEM) analyses on the injected alginate and by increasing number of animals monitored for inflammation profile. Viability of cells co-injected with alginate in the brain should be evaluated, as well as alginate permanence and clearance in the tissue. Subsequently, we will approach the MCAO mouse model, treating animals with injections of NSCs, alone or encapsulated in alginate gels, in the site of the lesion. We believe cells will survive, differentiate, integrate, form connections and stimulate neurogenesis in the injured brain, supported by alginate encapsulation.

Our preliminary studies indicate that alginate decreases inflammation caused by the single injection of NSCs, suggesting its role in preventing grafted cells interaction with the host tissue. This could be important when cells are transplanted in the injured brain, where the environment is characterized by inflammation processes and does not support cell viability and integration. Moreover, since cells formed connections in our *in vitro* cultures, we will analyze whether encapsulation supports the formation of connections and synapses from the grafted cells. Immunohistochemical analyses at different time points will be performed in order to evaluate transplanted cells differentiation and integration within host lesioned area. Functional tests will assess improvements in behavioural and neurological function after encapsulated cells transplant.

As glial scar is considered one of the main obstacles for CNS repair as it inhibits cell integration, axonal regrowth and restoration of physiological functions in damaged brain tissue (Buffo et al., 2008; Robel et al., 2011; Roll et al., 2014), we should consider astrocytes infiltration and interaction with the grafted alginate hydrogels. It should be checked whether alginate stimulates the transition of astrocytes to a reactive state, which is known to be detrimental for regeneration if it is prolonged in time.

Finally, once this challenging approach will be set up, it could be improved in different ways. We will test whether the addition of hyaluronic acid to the alginate hydrogel contributes to create an environment for cells able to help cell viability and stimulate regeneration following engraftment. In addition, it could be coupled with pharmaceutical scar-modulating treatments which are now under evaluation (Shen et al., 2014).

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APPENDIX

Published Paper containing results present in the thesis

Neural differentiation of pluripotent cells in 3D alginate-based cultures

Bozza A, Coates EE, Incitti T, Ferlin KM, Messina A, Menna E, Bozzi Y, Fisher JP and Casarosa S.

Biomaterials 35 (2014) 4636-4645.

Doi: 10.1016/j.biomaterials.2014.02.039

In this paper, my contribution was in the set-up and execution of all the experiments, with exception of mechanical and physical analyses on alginate scaffolds. I contributed by analysing and interpreting data obtained from the experiments and by putting them in the right context and current status of the research in the field. I wrote the manuscript and performed the revision requested from the referees.

Results present in this article were included in the thesis.

Published Papers containing results not present in the thesis

1. Noggin expression in the adult retina suggests a conserved role during vertebrate evolution

Messina A, Incitti T, Bozza A, Bozzi Y and Casarosa S.

Journal of Histochemistry and Cytochemistry, 2014; 62(7):532-540.

Doi: 10.1369/0022155414534691

In this study we investigated the expression of Noggin, a BMP inhibitor, in the adult retina of three vertebrate species: fish, frog and mouse.

In this paper, I contributed to the analyses on adult mouse retinae. I processed cryostat samples and performed immunohistochemical analyses on the sections, analyzing the expression of Noggin, of the photoreceptors markers Rhodopsin and Synaptophysin, of the marker for Golgi TNG46 and of Pax6. Results are reported in Fig. 3 (c, f, i, l) and in Fig. 4 (c, f).

2. Noggin-mediated Retinal Induction Reveals a Novel Interplay between BMP Inhibition, TGFβ and SHH Signaling

Messina A, Lan L, Incitti T, Bozza A, Andreazzoli M, Vignali R, Cremisi F, Bozzi Y, Casarosa S.

Stem Cell, *under revision*.

In this study is reported the involvement of Noggin in the regionalization of anterior neural structures.

I participated to this study performing RNA extraction and RT-qPCR analyses on the treated Animal Cap Embryonic Stem Cells (ACES) of *Xenopus Laevis* embryos. I was involved also in the analyses of the data.

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