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Functional neural differentiation of adult hippocampus derived stem cells

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Abstract

Stem cells are defined by their capacity to differentiate into different phenotypes while maintaining their ability to replicate indefinitely. These characteristics depend on their intrinsic capabilities and their interaction with the cellular niche. However, stem cells isolated later in development show a restricted differentiation capability limited to the cell lineage specifically present in the tissue of origin.

Using an adult, hippocampal-derived stem cell line (designated the CHIP stem cell line), I started looking at their integration once they had been implanted into hippocampal organotypic slices. This would have given me an idea of the sequential steps they follow during their integration and how this relates to what is known to happen *in vivo*. Knowing that in the hippocampus, they originate from the sub granular zone and develop into granule cells, I carried out an electrophysiological characterization of dentate gyrus granule cells both in organotypic and in acute slice. It became clear that the survival of stem cells after the implantation was affected by the culture medium used for the organotypic slices. Switching to a different culture medium improved the survival of the stem cells but at the same time they did not show signs of integration (e.g. dendritic-like protrusions).

I therefore started looking at stem cell functional differentiation *in vitro* without the slice. In the CHIP stem cell line plus, in addition, in another hippocampal stem cell line (HCN95) I compared the response to two differentiation protocols. I employed several standard neuronal markers (including NeuN) in order to define their developmental status. Unlike most other studies I also used a marker for the alpha subunit of the voltage-activated sodium channel ($Na_v1.2$) which is responsible for the generation of action potentials in mature neurons. Checking stem cell ability to fire action potentials at various time intervals, in parallel to the expression of neuronal markers, I found that $Na_v1.2$ expression better correlates with the functional maturation of our neuronal stem cell lines when compared to NeuN. Therefore, $Na_v1.2$ expression can be employed as reliable marker of mature neuronal phenotype.

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ACSF	Artificial cerebro-spinal fluid
AIS	Axon initial segment
AP	Action potential
BDNF	Brain derived neurotrophic factor
BrdU	Bromodeoxyurindine (5-bromo-2-deoxyuridine)
CA	Cornu ammonis
CNS	Central nervous system
DAPI	4',6-diamidino-2-hPenylindole
DG	Dentate gyrus
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
EC	Entorhinal cortex
GABA	γ-Aminobutyric acid
GAT	GABA transporter
GC	Granule cell
GCL	Granule cell layer
GFP	Green fluorescent protein
GFAP	Glial fibrillary acidic protein
GFs	Growth factors
HS	Horse serum
IPSC	Inhibitory postsynaptic current

LTP	Long term potentiation
ML	Molecular layer
mIPSC	Miniature inhibitory postsynaptic current
$Na_v1.2$	Sodium channel subunit α 1.2
NeuN	Neuronal nuclei
NGF	Nerve growth factor
NT-3	Neurotrophin-3
p_r	Release probability
SGZ	Sub granular zone
TTX	Tetrodotoxin
VGCCs	Voltage gated calcium channels

1 Introduction

The work I carried out in this thesis is oriented towards developing a better understanding of two aspects of adult neural stem cells. The first is the eventual ability of adult hippocampal stem cells to integrate once implanted onto hippocampal organotypic slices. The second is the neuronal differentiation of two adult hippocampal derived stem cells lines.

Human multipotent neural stem cells can integrate into the developing brain if injected into the ventricles in a xenogenic host brain (Brustle et al., 1998) and we already know that stem cells implants done in clinical trial are promising although still far for being a reliable solution (Lindvall and Kokaia, 2010). To understand better the factors that influence stem cells integration we chose the rat hippocampal organotypic slices as an *in vitro* platform which allows testing the outcome of stem cell implants at different stages. The hippocampus, together with the olfactive bulbs, is one region of the brain where neurogenesis occurs even in adulthood and the availability of stem cells lines derived from the adult rat hippocampus make it possible to recreate the relatively normal condition for their integration.

So far several stem cell markers have been employed in order to define the state of a stem cell population (Kempermann et al., 2004). Different markers define different sequential steps along the differentiation process that leading to a mature neuronal phenotype. However, in regards of neural stem cells functionality, it is implicit to assume that neural maturation have been reached once cells develop the ability to fire action potentials. Therefore, a marker that is structurally essential for this function, like the α-subunit of the voltage-activated sodium channel, can be used to identify functional and thus mature, neural stem cells.

1.1 Stem cell definition

The definition of Stem cell includes both the ability to replicate while remaining undifferentiated and the ability to develop different phenotypes upon differentiation (Morrison et al., 1997; Watt and Hogan, 2000). While the first factor is common to all stem cell lines the second varies enormously depending on the source from which these cells are derived. Stem cells derived from a blastocyst are pluripotent, being able to originate all the cells types present in an adult organism while stem cells isolated late during development are multipotent because the number of cells types that can be obtained is limited. Ultimately stem cells isolated from adult tissues are called progenitor cells because they can only differentiate into lineage-specific phenotypes.

Stem cells were originally discovered thanks to a series of studies carried out by Till and McCulloch in the 1960s. They found that a single population of cells taken from mouse bone marrow is able to differentiate along the erythrocytic, granulocytic and megakaryocytic lines and lead to a recovery of the hematopoietic system once implanted in heavily irradiated mice (Till and McCulloch, 1961). That discovery led a few years later to human bone marrow transplants (Gatti et al., 1968).

Meanwhile, several others tissues from which stem cells can be harvested have been found and technical improvements opened a new series of possibilities. In 1997 the cloning of the sheep Dolly using nuclear transfer technique brought the entire stem cells field to world attention (Wilmut et al., 1997). In 1998 the creation of a human stem cell line from an early embryo (Thomson et al., 1998) contributed to the picture of stem cell therapy as a solution for a vast number of diseases and medical conditions through transplant therapy. Very recently a more advanced technique has been developed allowing, through injection of defined genes and transcription factors, induction of pluripotency in adult cells consequently called induced pluripotency cells or iPS cells (Takahashi and Yamanaka, 2006).

As we observe from these recent developments in the stem cell field, the initial definitions are not absolute and reflect our level of knowledge of cellular commitment to a specific phenotype. In the future these definitions are very like to change following new discoveries.

1.2 Embryonic Stem cell differentiation

Embryonic stem cells form a particular group of cells in the mammalian embryo. After egg fertilization the zygote starts the cleavage process during which it undergoes several mitotic divisions without increasing its dimensions. It then goes through three main step steps: compaction, cavitation and the degeneration of the *zona pellucida* which transform the zygote into a blastocyst. This stage is reached after 4 to 6 days in human and lasts until the embryo implants into the uterus. Stem cells isolated from the inner cell mass of the blastocyst (fig.1) can differentiate into all cell types of the embryo but they are not able to derive the trophoblast again (Hadjantonakis and Papaioannou, 2001).

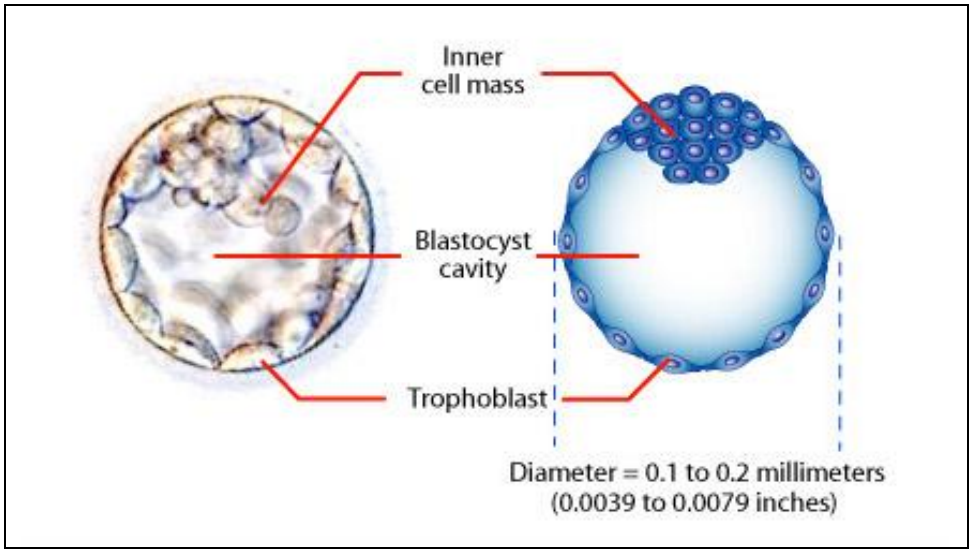

Fig.1. Illustration of a blastocyst with the real photographic image on the left side and a schematic representation on the right side. Red lines indicate the correspondent structures. (Image from Dr. Joseph S. Tash, University of Kansas, Medical Center; http://www.kumc.edu/stemcell/images.html)

The commitment of stem cells to differentiate to a specific phenotype could depend on an internal program or there could be extracellular signals which direct the stem cells' differentiation. *In vivo*, with embryonic development, stem cells progressively lose their multipotency up to a point where they became lineage-restricted stem cells. *In vitro*, where the specific extracellular clues are missing, stem cells behave like lineage restricted cells but some of their characteristics change leading to the idea that in optimal conditions they may be able to exhibit extraordinary plasticity (D'Amour and Gage, 2002).

Another important characteristic is the modality of division. Stem cells can divide symmetrically generating two identical daughter cells or asymmetrically with a daughter cell which retains parent cell' properties and the other daughter cell having different developmental potential. During development and more pronounced in the adult, stem cells or progenitor cells undergo an asymmetric division resulting respectively in a multipotent stem cell and a cell which is committed to differentiate (Mione et al., 1997).

1.3 Neuronal lineage

The central nervous system (CNS) is primarily composed of neurons and glial cells. The neurons are thought to be the main computational units on which rely the functions of transmission, elaboration and storage of the information coming from the sensory system. The glial cells are the non-neuronal cell which functionally "support" the neurons and that can be divided in two main categories: microglia and macroglia. The microglia form the only immune defence of the brain which is isolated from the circulatory system by the blood-brain barrier. The macroglia are mainly astrocytes and oligodendrocytes. Oligodendrocytes form the myelin sheaths which surround the axons allowing fast and efficient nerve conduction. Astrocytes seem to have more complex functions not only supporting (Simard and Nedergaard, 2004) but also modulating synaptic transmission (Ullian et al., 2001).

1.4 Adult Neurogenesis

The central nervous system was long known for its cellular stability as it was thought that in order to maintain its functionality the circuitry should be almost static in time and no new neurons could be added (Geuna et al., 2001; Gross, 2000). However, with the introduction of ^{3}H-Thymidine to label the DNA of mitotic cells during the S-phase, several areas of the brain were found to be mitotically active (Altman, 1962) and within a few decades neurogenesis was demonstrated in canaries (Paton and Nottebohm, 1984) and in a variety of mammals (Kuhn et al., 1996).

The introduction of 5-bromo-2'deoxiuridine (BrdU) gave new emphasis to the neurogenesis field. BrdU is a thymidine analogue, incorporated into DNA during replication like ^{3}H-Thymidine but it can be visualized with immunocytochemistry. These techniques together make it possible to carry out

double labelling in order to investigate the phenotype of the newly formed cells and clearly demonstrate the existence of neurogenesis in specific brain areas (Gould et al., 1999). Two areas of the brain in which we can find an on-going production and integration of newborn neurons are the hippocampus and the olfactory system (van Praag et al., 2002). Although most studies are carried out on rodents it has also been demonstrated that there are newly generated neurons in the adult, human hippocampus (Eriksson et al., 1998). In the olfactory system newly-generated cells migrate from the sub ventricular zone (SVZ) (Chiaramello et al., 2007) along the Rostral Migratory Stream (RMS) to the olfactory bulbs where they can develop into granule cells and periglomerular cells (Whitman and Greer, 2007).

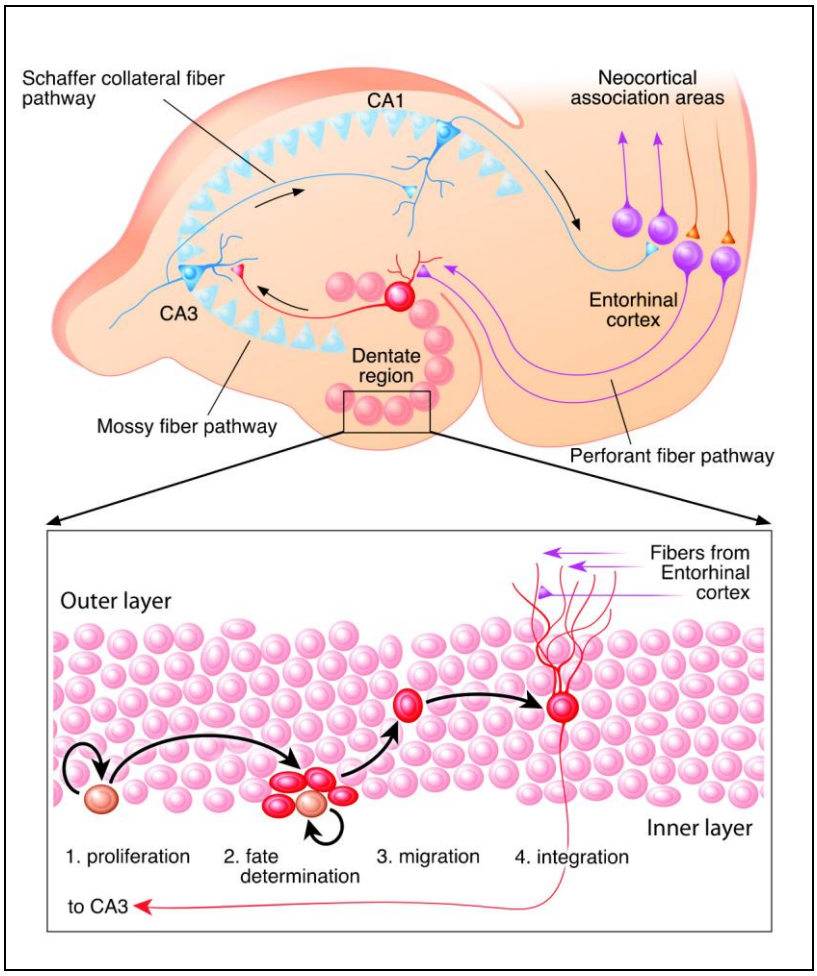

Fig.2. Adult hippocampal stem cells proposed steps of integration. Cells proliferate by symmetrical division then once they undergo asymmetrical division they start determining their fate, migrate and eventually integrate into the molecular layer of the dentate gyrus. (from Lie et al., Annu. Rev. Pharmacol. Toxicol. 2004)

In the hippocampus the neural precursor cells arise from the subgranular zone (SGZ) and then migrate to the granular cell layer of the dentate gyrus (fig.2). Here they undergo a selection where the vast majority are eliminated by apoptosis (Kuhn et al., 2005) and the survivors initiate their maturation process. It is thought that these cells do not replace older granule cells but they integrate into the pre-existing circuitry. This process therefore parallels and counteracts the effect of apoptotic cell death that naturally happens in the brain with aging but also increase the dimension of the dentate gyrus formation (Crespo et al., 1986).

1.5 Characterization of granule cells neuronal maturation

Nestin positive neural precursor cells located in the SGZ have been shown to develop into proper granule cells by transfecting them with a retrovirus containing green fluorescent protein (GFP) (Ide et al., 2008). Nestin positive new-born neurons in the adult hippocampus initially express glial fibrillary acidic protein (GFAP) a well-known astrocytic marker (Seri et al., 2001) but not S100β which is a marker for the mature astrocyte.

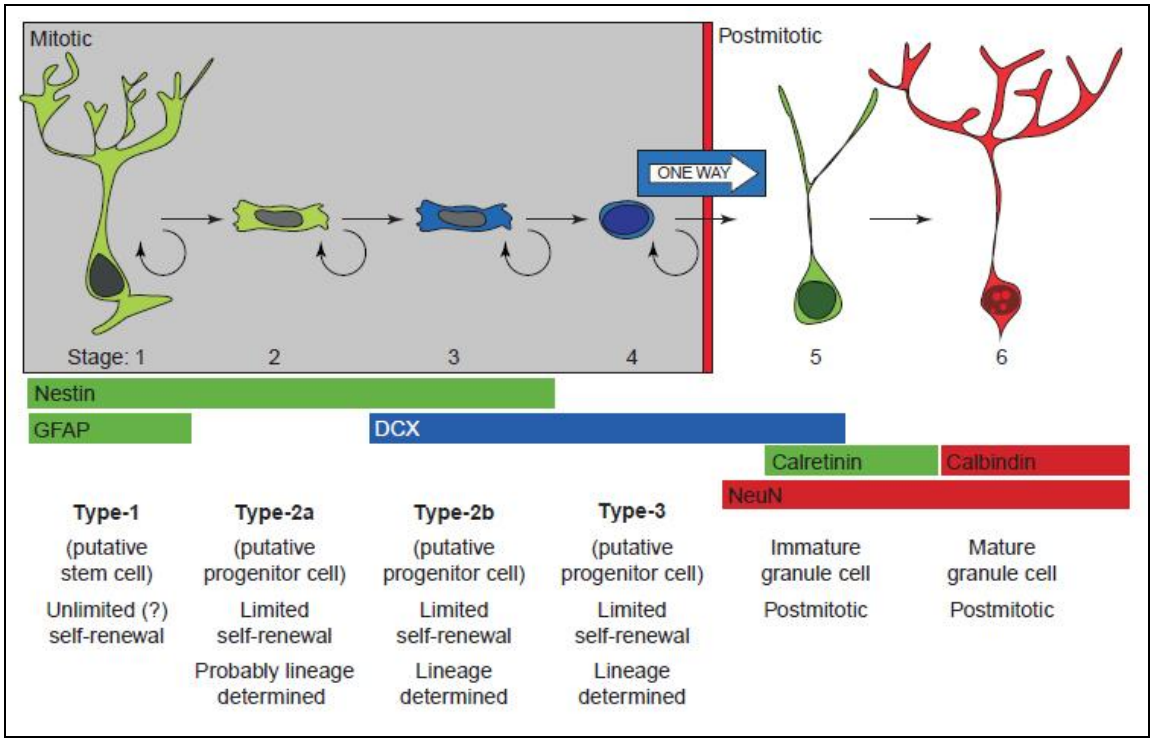

Fig.3. The scheme represent the six steps of neuronal differentiation on the basis of morphology, proliferative capabilities and the presence of specific markers (Figure from Kempermann et al., 2004).

Starting from cells with an immature phenotype expressing GFAP and nestin (fig.3), Kempermann defined six sequential maturation steps during which adult progenitor cells differentiate into a neuronal phenotype (Kempermann et al., 2004). The first step in the neuronal path is the expression of Doublecortin (DCX). Progenitor cells that express this marker have entered the neuronal lineage and although they can still undergo mitosis they cannot generate glial cells anymore, as no co-labelling with GFAP has been found yet. The next step is the expression of NeuN which is associated with the so called post-mitotic neuronal state (Mullen et al., 1992). Once cells express NeuN, they do not undergo mitosis anymore and start expressing first calretinin (Brandt et al., 2003) and then, after 2-3 weeks, calbindin. These two markers, both calcium binding proteins, are associated with early and mature granule cell phenotype, respectively.

1.6 Voltage-sensitive sodium channels

Voltage-activated sodium channels are a particular group of integral membrane proteins that open upon a change of membrane potential (V_m). They have a role in controlling the voltage gradient and are present in cells that require a rapid change in the membrane potential like muscle, secreting cells and particularly in neurons, where they make possible the transmission of a wave of depolarization along the axon that is called the action potentials (Hodgkin and Huxley, 1952). In an action potential (AP) the depolarizing phase is caused by the fast opening of the voltage-activated sodium channels which allows Na^+ ions to flow into the cell. The fast closing and inactivation of the sodium channels plus the opening of the voltage-activated K^+ channels is then responsible for the repolarising phase. Sodium channel inactivation persists until the Vm returns close to the cell resting potential. The potassium current persists much longer than the sodium current, due to the longer time constant related to the opening/closing of the K^+ channels. Together with the voltage-activated potassium channels, specific Ca^{2+}-activated potassium channels are responsible for the after hyperpolarization that occur during the repolarising phase (Lancaster and Nicoll, 1987; Faber and Sah, 2007).

The voltage-sensitive Na^+ channel is a glycoprotein with a transmembrane complex formed by a α-subunit of 260Kd and smaller β subunits: β1 (36kd), β2 (33kd) and β3 (Catterall, 1984; Morgan, 2000). While the α-subunit forms the ionic pore of the channel, the β subunits participate in the channel's transport to the cell membrane and in the regulation of its inactivation. Tetrodotoxin (TTX) is a neurotoxin, originally extracted from the puffer fish of the family Tetraodontidae, which is selective for sodium channels in nerves and muscles. It binds to the extracellular domain of the $Na_v1.2$

physically blocking the flow of ions and therefore blocking the propagation of action potential. The EC_{50} of TTX for the voltage-activated sodium channels varies from 6 to 60nm depending on the channel α-subunit isoform and TTX has the important feature of being reversible, as the channel regains functionality once it has been washed out from the extracellular solution.

Nine α-subunits have been characterized so far and four of these isoforms have been found in the CNS: $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$. Of these, $Na_v1.2$ and $Na_v1.6$, are predominately located in the axon initial segment while both $Na_v1.1$ and $Na_v1.3$ are located in the node of Ranvier (Yu and Catterall, 2003). Recent discoveries have shown that the asymmetric distribution and threshold of activation of $Na_v1.2$ and $Na_v1.6$ can explain some of the properties of the axon in relation to the generation and transmission of action potentials. The $Na_v1.2$ channels have a high threshold of activation and are located preferentially in the first 25μm of the axon initial segment. $Na_v1.6$ in comparison has a lower threshold and is found 25-50 μm from the soma down the axon and up the dendrites. These characteristics suggest $Na_v1.6$ sets the AP initiation site and $Na_v1.2$ sets a high threshold for APs, preventing back propagation to the somatodendritic region of the neuron (Hu et al., 2009; Dulla and Huguenard, 2009). Moreover, as they confer the ability to initiate and propagate APs, $Na_v1.2$ and $Na_v1.6$ can eventually be used as markers of mature neurons.

1.7 Hippocampal formation: circuitry and functions

The hippocampal formation is a neuronal structure of the mammalian brain but it is also present, with a simpler structure, in bird and reptile brain (Ulinski, 1990). It has been extensively studied in rodents, monkeys and human. The rat is the preferred species for testing hippocampus-related function, because of several advantages, like a bigger brain compared to mice and their suitability for behavioural tests. The rat hippocampal formation is a banana shaped structure located on the wall of the lateral ventricle with a long axis bending along the septal-temporal axis in a C-shaped manner (fig. 4).

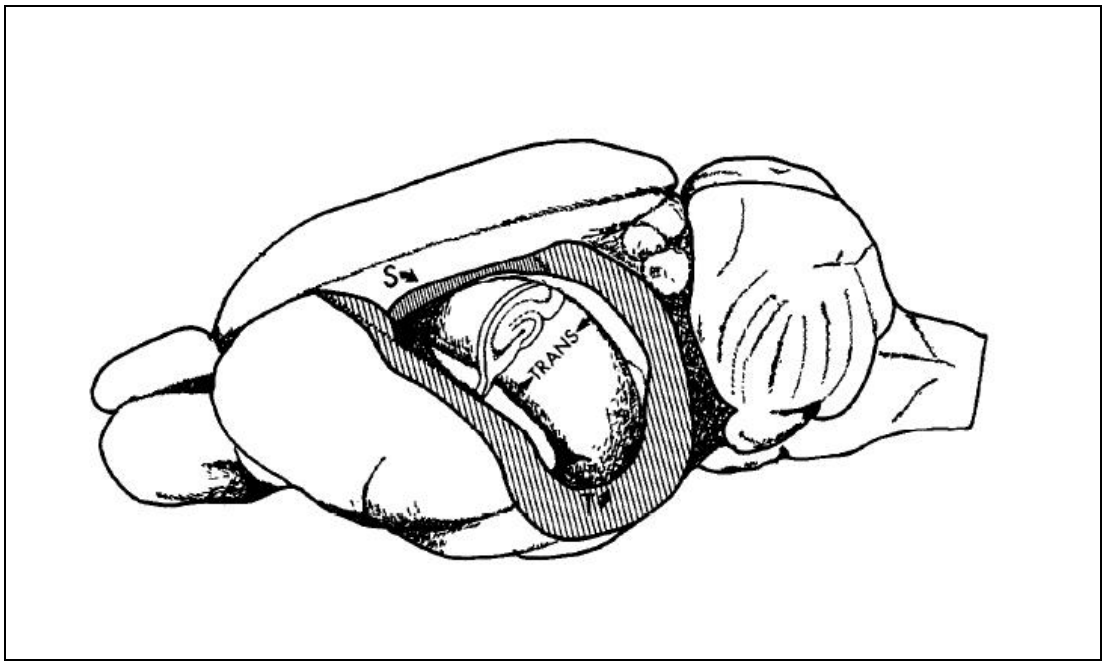

Fig.4. Line drawing of the rat brain showing the hippocampal structure (Amaral and Witter, 1989).

The hippocampus "proper" consist of CA3, CA2 and CA1 areas whereas the term "hippocampal formation" refer to a large region including dentate gyrus (DG), subiculum, presubiculum, parasubiculum and entorhinal cortex (EC). However, the term hippocampus is often used to indicate the whole hippocampal formation, as I do in this manuscript.

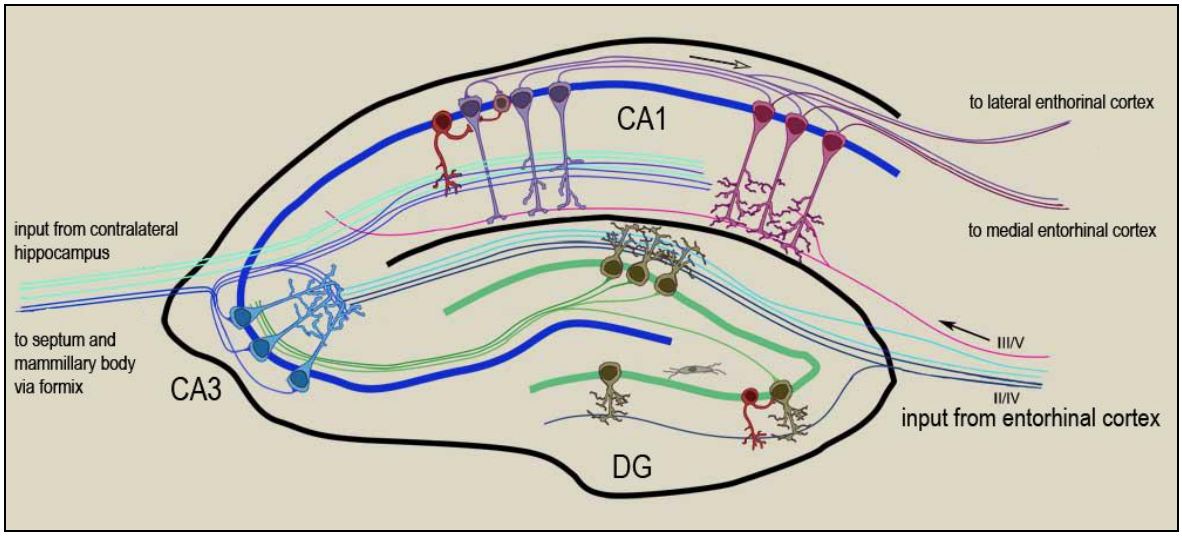

Fig.5. Schematics of the hippocampal formation. Specific areas are labelled as Dentate Gyrus (DG), *Cornu Ammonis* area 3 (CA3) and *Cornu Ammonis* area 1 (CA1) (Figure adapted from Dr. Alexander Straiker; http://www.indiana.edu/~cnnbdlab/Alexhippocampus1.jpg).

The various regions of the hippocampus are connected by mainly unidirectional projections and form the so-called trisynaptic circuit shown in fig.5. In this circuit the entorhinal cortex is the main input-output station of the hippocampus, receiving and sending back information from/to the cortex. EC makes synapses with the dentate gyrus and its projection to the granule cells is called the perforant path. The DG sends its projection through the hilus to the CA3 pyramidal cells by mean of the mossy fibers. The output of the CA3 pyramidal neurons, the Schaffer collateral, synapses with CA1 pyramidal neurons. This is a basic description of the classic trisynaptic circuit. In more detail there are other connections which are worth considering. The CA3 pyramidal neurons receive inputs from the same layer II pyramidal neurons of the entorhinal cortex that contact the dentate gyrus. They are interconnected receiving recurrent connections from axon collateral of other CA3 cells. CA3 pyramidal neurons are also important as their projection through the fornix establishes commissural connection between the two hippocampi (Amaral et al., 1990). CA1 pyramidal neurons receive direct connections from layer III entorhinal cortex which are thought to modulate the output back to the EC (Steward and Scoville, 1976).

1.8 Dentate gyrus structure and functions

In the dentate gyrus it is possible to distinguish 3 layers. The layer closest to the external fissures is cell free and is called the molecular layer (ML). The middle layer which contains the granule cell bodies is called the granular cell layer (GCL). The cellular region enclosed between the v-shape of the GCL is referred to as the polymorphic cell layer.

Granule cell morphology differs from that of pyramidal neurons of CA3-CA1 areas as all the dendritic branches are directed to the ML, conferring the GCs characteristic cone-shaped aspect. Cells in the GCL are closely apposed and their number, about $1.2x10^6$ units in rat, does not vary in adulthood (Rapp and Gallagher, 1996). Granule cells are the only cells of the dentate gyrus which project to the CA3 region. Mossy fibers terminals are distinctive. They form a large expansion of about 3-5 μm in diameter which irregularly interdigitate with the so called thorny excrescences at CA3 proximal dendrite and a single termination can have up to 37 synaptic contacts with a CA3 pyramidal cell dendrite (Rollenhagen et al., 2007).

The others cells in the DG act locally and are therefore called interneurons. The majority of interneurons use γ-aminobutyric acid and are therefore inhibitory (Ribak et al., 1978). The basket cells are the most studied. They derive this name from the peculiar way their axons make synaptic contacts, surrounding the GCs bodies in a way which resembles a basket. Other interneurons are less studied but their role is indeed important for the functionality of the hippocampus. An extensive description has been written by Freund and Buzsaki (1996).

The perforant path is the major glutamatergic input to the DG and derives from layer II of the entorhinal cortex. Observed from the septal to the temporal pole the afferent fibers are not homogeneously distributed with the lateral EC innervating the septal part and the medial EC sending its afferents to the temporal pole of the dentate gyrus (Hjorth-Simonsen and Jeune, 1972). The inner part of the molecular layer of the DG receives commissural afferents from the other hemisphere as well as associational fibers (Deller, 1998).

A different input to the DG derives from the serotoninergic neurons in the medial-raphe which innervate the hippocampus via the supracallosal pathway through the fimbria-formix and via the infracallosal pathway through the cingulate bundle (Azmitia and Segal, 1978).

One less specific but nevertheless important source of afferents come from the medial septum (MS) and is referred as the septohippocampal pathway. It enters the hippocampus through the fimbria-

formix and reaches several hippocampal layers. This connection comprises 2 cell types: cholinergic and GABAergic neurons. The cholinergic fibers afferents to the DG have been demonstrated by electrophysiological studies to excite granule cells (Wheal and Miller, 1980). The GABAergic fibers instead control inhibitory interneurons (Freund and Gulyas, 1997). This connection is supposed to carry autonomic state, emotional and motivational information about the animal. In comparison, the perforant path is thought to carry cortical sensory information relating to the environment (Gulyas et al., 1999).

1.8.1 Synaptic transmission in the hippocampus

The trisynaptic circuit (i.e. EC>DG>CA3>CA1) is the most studied in the hippocampal formation, although the entorhinal cortex is also known to send direct connections to both CA3 and CA1 regions.

All the synapses in this circuit are glutamatergic. These glutamatergic fibers also form synapses with GABAergic interneurons resulting in feed-forward and feed-back inhibition.

Communications between neurons is facilitated by morphological specializations called synapses. These structures comprise pre- and postsynaptic zones specialized for converting electrical signals (e.g. APs) into chemical signals (e.g. release of neurotransmitters) and vice-versa therefore, allowing a fast "point to point" transmission. A slower way of communication that does not require symmetrical membrane specialization is the synaptic spillover or extrasynaptic release of neurotransmitters (Sem'yanov, 2005) which can also involve glial cells (Overstreet, 2005; Matsui and Jahr, 2003).

In "point to point" synaptic transmission, when an action potential reaches the presynaptic terminal it induces the opening of voltage gated calcium channels (VGCCs), located in close proximity to the active zone. The local increase in $[Ca^{2+}]$ causes the fusion of the synaptic vesicles with the plasma membrane and a consequent release of neurotransmitter in the synaptic cleft. The synaptic cleft is a thin space of about 20nm which separates the presynaptic terminal from the postsynaptic terminal where receptors are densely clustered.

1.8.2 Glutamatergic transmission

Glutamate is the main excitatory neurotransmitter in the hippocampus and it relies on ionotrophic and metabotrophic glutamatergic receptors (Kew and Kemp, 2005).

Ionotrophic receptors, once activated, open a channel in the membrane in the form of a selective aqueous pore. Different groups of glutamate ionotrophic receptors show different pharmacokinetics and are named after their competitive ligand, respectively N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isoprsopenylpyrrolidine (kainate).

AMPA receptors are composed of a combination of 4 subunits (GluR1-4) and are present in almost all the excitatory synapses in the hippocampus. The two prominent AMPA receptors tetramers in the hippocampus are GluR1-R2 and GluR2-R3 (Wenthold et al., 1996). They are permeable to monovalent cations (Na^+ and K^+ mostly) but can have a low permeability to Ca^{2+} (Liu and Zukin, 2007).

Kainate receptors have common features with AMPA receptors. They are tetrameric and formed by a combination of different subunits: GLuR5-7 and KA1-2. They are located mostly in the dentate gyrus and CA3 subfields of the hippocampus both pre and post-synaptically (Frerking and Nicoll, 2000).

NMDA receptors are heteromultimers of NR1 and NR2A-D subunits and show two important features. They are highly permeable to Ca^{2+} and are not able to pass current at normal resting potential. Whereas AMPA receptors only need glutamate to be able to open the pore, NMDA receptors need glycine to be bound to a co-agonist binding site and Mg^{2+} blockade to be removed. Glycine is normally bound at its physiological concentration whereas Mg^{2+} needs a depolarization of the membrane by up to -50mV in order to be removed (Rebola et al., 2010). This important property allows the NMDA receptors to function as coincidence detectors of sustained synaptic activity, leading to changes in the synaptic strength but with low contribution to the baseline synaptic transmission (Bliss and Collingridge, 1993).

Metabotropic receptors act with a different mechanism. Once activated their intracellular domain binds G-proteins activating a cascade of second messengers. Metabotropic glutamate receptors are divided into group I (mGluR1 and 5), group II (mGluR2 and 3) and group III (mGluR4, 6, 7, 8) depending on the second messengers they interact with. The group I mGluRs predominantly couple via $G\alpha_q/G\alpha_{11}$ to phospholipase C, whereas the group II and III couple to the inhibition of adenylyl-cyclase activity, via $G\alpha_i/G\alpha_0$ (Anwyl, 1999). For their slower *modus operandi* mGluRs receptors are thought to modulate synaptic transmission and plasticity.

1.8.3 GABAergic transmission

GABA is the principal inhibitory neurotransmitter in the whole brain. In the hippocampus it is released by interneurons although in developing granule cells both markers for glutamatergic and GABAergic transmission coexist (Gutierrez, 2005). The synthesis of GABA from glutamate is mediated by two different isoforms of glutamic acid decarboxylase, GAD67 uniformly present in the cytoplasm and GAD 65 which is found specifically at the presynaptic terminals.

GABA receptors are divided into ionotrophic ($GABA_A$) and metabotropic ($GABA_B$) types. $GABA_A$ receptors are permeable to the monovalent anions Cl^- and HCO^{3-} and their opening induces inhibitory postsynaptic currents (IPSCs) in the adult hippocampus.

$GABA_A$ receptors are heteropentameric and can be composed of several subunits : $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ϵ, π, θ but only the α_1, α_2, α_4, β_3, γ_2 and δ_1 subunits are expressed in the dentate gyrus of the hippocampus (Sperk et al., 1997). The common composition of hippocampal $GABA_A$ receptors is $2\alpha + 2\beta$ subunits plus one γ or δ subunit (Whiting et al., 1999).

The different subunit composition of the $GABA_A$ receptors is responsible for the different channel kinetics and sensitivity to drugs found in different brain areas at sequential developmental stages. In particular α_1, γ_2 and $_{\delta 1}$ subunits in the dentate gyrus are going through developmental changes in postnatal rats (Killisch et al., 1991; Laurie et al., 1992) and influence characteristics like activation and desensitization rates (Gingrich et al., 1995; Angelotti and Macdonald, 1993; Saxena and Macdonald, 1994).

Once GABA has been released in the synaptic cleft, diffusion and high affinity GABA transporters (GATs) play an important role in the depletion of the neurotransmitter. So far four different GATs have been identified by differential amino acid sequences and interaction with specific drugs. While synaptic GATs plays an important role in the definition of magnitude and duration of IPSCs (Dingledine and Korn, 1985; Iversen and Kelly, 1975) extra synaptic GATs regulate the diffusion of GABA that mediate cross-talk between different synapses (Isaacson et al., 1993).

1.9 Dentate Gyrus granule cells in acute hippocampal slice

The characteristics of dentate gyrus granule cells have been investigated by Edwards et al. (1989, 1990) in hippocampal acute slices using whole cell patch-clamp technique. Granule cells have a high input resistance of about 1GΩ. Spontaneously occurring inhibitory post-synaptic currents (IPSCs) had a fast rise time of less than 1ms and a slower decays, respectively < 3ms and > 30ms. The amplitude of the currents varied from few pA up to 100 pA at -50mV resting potential. The amplitude of these IPSCs was reversing at 0 mV in symmetrical Cl^- concentration and was completely blocked by application of bicuculline to the bath solution. The application of GABA also obscured the detection of synaptic currents confirming their origin as $GABA_A$ mediated Cl^- currents.

After TTX application only currents lower than 40 pA were recorded. TTX is a neurotoxin which blocks the activation of the voltage-activated sodium channels, preventing the formation of action potentials. Therefore all the postsynaptic current recorded in TTX derive from spontaneous release of single vesicles from the presynaptic terminals which are referred as miniature inhibitory postsynaptic potentials (mIPSCs).

1.10 Organotypic Hippocampal slices

The inaccessibility of, and difficulty in manipulating the central nervous system over a prolonged period of time (e.g. several weeks) has led the experimenter to develop *in vitro* approaches. Organotypic cultures are one of the most widely used methodologies for maintaining explants from nervous tissue. The roller tube technique was initially introduced by Hogue (1947) and then modified through the years until extensively characterized by Gahwiler (1981).

A different implementation has been developed by Stoppini et al. (1991) in which hippocampal slices are placed onto a porous membrane at the interface between the air (95% O_2, 5% CO_2) and the culture medium. Compared with the roller tube technique this method allows a better preservation of the tissue structure. Long term organotypic hippocampal cultures retain a certain level of three-dimensional structure with a thickness of 5-10 cells bodies and a dense network of cellular processes (Buchs et al., 1993). Also their synapses are very similar to those in acute slices in regard to density and maturity, both structural and functional (De Simoni et al., 2003). Field potentials increase during the first 6-12 days in culture then they become stable. Many forms of synaptic plasticity have been

demonstrated, including paired-pulse facilitation, post-tetanic potentiation and LTP (Muller et al., 1993). Such a system satisfies the needs required to investigate the long-term effects of drugs, trophic factors and also different mechanisms of synaptic plasticity. It is well known that new granule cells are produced *in vivo* throughout life in rodent hippocampus (Altman and Das, 1965) but recently even organotypic slices has been demonstrated to be able to generate new neurons (Raineteau et al., 2004; Raineteau et al., 2006). Therefore this model is well suited to test adult hippocampal stem cell integration and differentiation after implantation.

1.11 Hypothesis for adult hippocampal neurogenesis

The brain retains a high level of plasticity even in adulthood being able to learn and adapt to environmental changes that happen in the surrounding environment. Changes in the morphology, connectivity and synaptic strength of single neurons account for this ability. In the dentate gyrus of the hippocampus, new neurons are generated throughout life adding an additional source of plasticity (Gould and Gross, 2002). These new neurons become functionally integrated into the existing circuitry in a period ranging from 2 to 4 weeks (Jessberger and Kempermann, 2003) and show some interesting characteristics which lead to an enhanced cellular plasticity. They have enhanced long-term potentiation (LTP) due to a low threshold of induction and cannot be inhibited by GABA (Snyder et al., 2001). These factors hypothetically allow them to be much more prone to form synaptic connections and therefore rapidly integrate into the existing circuitry. Fast integration is a prerequisite for their survival and a high level of synaptic stimulation of the dentate gyrus is closely related to a high rate of survival of the newborn neurons. While neurogenesis has been associated with better performance in spatial discrimination (Clelland et al., 2009), amongst aged rats, the group with lower levels of neurogenesis is also the better performer in spatial memory tasks (Bizon et al., 2004; Bizon and Gallagher, 2005). This implies that the role of neurogenesis may have different effects on different spatial memory tasks or change between adult and old age.

2 Materials and Methods

2.1 Preparation of acute slices

Acute rat brain slices from male P14 Sprague-Dawley rats were prepared following the protocol from Edwards et al., (1989). The rat head was removed by decapitation, the brain hemisected and quickly immersed in ice-cold artificial cerebro-spinal fluid (ACSF) within 60s. A segment was cut away from each hemisphere by hand with an angle of approximately 105 degree from the midline surface and that surface was used subsequently to stick the hemisection onto a vibrating tissue slicer (Camden Instruments, Loughborought, UK). The slices were 350 μm thick and during slicing the well was filled with the same ice-cold ACSF. Once removed the slices were transferred into an incubating chamber, continuously bubbled with 95% O_2 5% CO_2, held at 35° C for 30 min, before allow them to return to room temperature (25°C). The recording started 30 min after the slice reached room temperature and last up to 8 hours. All work was carried out under UK Home Office regulations in conformity with national ethics committee guidelines.

2.2 Preparation of organotypic slices

We use the method of Stoppini et al. (1991) well-described by De Simoni and Yu (2006). All the operations were carried out in sterile conditions under a flow cabinet. Culture inserts were placed into a 6 well plate. Each well was filled with 1 ml culture medium and 3 hydrophilic culture membranes (Millipore) were placed in each insert. The six well plates were kept in an incubator at 37°C in 95% O_2 5% CO_2 until the slices were ready for plating. The two brain hemisphere from P5 Sprague-Dawley rat were stuck onto a vibrating manual tissue slicer and immersed in cold (4°C) slicing medium. Parasagittal hippocampal slices 300 μm thick were cut and then transferred onto hydrophilic culture membrane. Each culture insert holds up to 3 slices. The culture plates were kept in the incubator and the culture medium was changed 3 times per week. The enthorinal cortex was not removed from the hippocampal slices as it is thought to help to maintain a more physiological connectivity, avoiding excessive axonal sprouting by the granule cells (Coltman et al., 1995). Each group *in vitro* is representative of a time interval defined as days *in vitro* (DIV): DIV 7 (7-8 DIV), DIV14 (12-14 DIV), DIV21 (19-21 DIV) and DIV28 (27-29 DIV).

Culture medium:

25% horse serum, 50% minimal essential medium, 23% Earle's balanced salt solution (all from GIBCO BRL), Penicillin (50u.i./ml), Nystatin (12u.i./ml) (from Sigma-Aldrich).

Slicing medium: 2.978 g. HEPES, 487.5 ml EBSS. (Hepes is first dissolved in a small quantity of EBSS and then filtered (0.22 syringe filter) into aliquots.

2.3 Cell culture

Two adult hippocampal stem cell lines were used in this study. One was provided by Dr. Victor Nurcombe (Chipperfield et al., 2005) and will be referred to as CHIP; the other one was kindly shipped to us by Dr. Michaela Thallmair (Brain Research institute - Zurich), isolated by Prof. F.H.Gage (La Jolla – California) and will be referred to as HCN95. In both cases, the aliquots containing the cells were stored in the vapour phase of liquid nitrogen upon arrival. Aliquots were then thawed in a 37° C water bath until the ice just start melting and re-suspended in 5 ml of cell culture medium. The cells were cultured in a 25 cm^3 flask or in a 10cm Petri dish and the culture medium was changed every 2-3 days. The flasks/dishes were previously coated using 2 ml of 50 µg/ml poly-D-lysine (30.000-70.000 average molecular weight, Sigma-Aldrich) in H_2O for at least 1 hour and then rinsed twice with PBS and once with culture medium prior to use. The cultures were visually checked and the cells split in a new flask before they reached confluence. CHIP cells were not treated with any proteolytic enzymes during the isolation process and in the subsequent phases in order to avoid any alteration of their extracellular matrix, using the same protocol as during their isolation. HCN95 cells were instead treated with Trypsin or TrypLE™ Select (Invitrogen) in order to dissociate them from the culture plate.

Media compositions were as follows:

CHIP culture medium:

Neurobasal-A minus glutamine medium supplemented with:

B27 (2%), bhFGF2 (20ng/ml), penicillin (100u.i./ml), streptomycin (100µg/ml), glutamine (20mM).

HCN95 culture medium:

DMEM/F12 medium supplemented with:

N2 (1%), bhFGF2 (20ng/ml), penicillin (100u.i./ml), streptomycin (100µg/ml).

2.3.1 Cell differentiation protocol

Differentiations media were obtained adding the following components to the previous media:

Growth-factors media: BDNF (10 ng/ml), NT-3 (10 ng/ml), NGF(3S) (10 ng/ml), Laminin (10 µg/ml).

Serum media: Horse serum 10%

Each stem cell line was cultured separately in both differentiation media over a period of 4 weeks and immunohistochemistry and electrophysiology experiments were carried out at DIV7, DIV14, DIV21 and DIV 28.

2.4 Electrophysiology

Whole cell patch-clamp recordings were made with an Axopatch1D (Axon Instruments), in artificial cerebrospinal fluid (ACSF) at room temperature (24-25 °C), using 4-6 MΩ electrodes pulled from borosilicate glass (World Precision Instruments) with a PP-830 microelectrode puller (Narishige, Tokyo, Japan). During the recording the preparation was immersed in ACSF solution: (in mM) NaCl 125, KCl 2.4, $NaHCO_3$ 26, NaH_2PO_4 1.5, glucose 19.4, $CaCl_2$ 2, $MgCl_2$ 1; and bubbled with 95% O_2 / 5% CO_2.

2.4.1 IPSC recordings

The internal solution used to clamp the cells' voltage contained (in mM): CsCl 140, Hepes 5, EGTA 10, $CaCl_2$ 2 and MgATP 2; with pH adjusted to 7.4 with CsOH. The currents measured were very small ($<1*10^{-10}$ pA) therefore the series resistance was not compensated obtaining a minimal baseline noise. Moreover, series resistance was monitored at the beginning and at the end of the recording by injecting +5 mV pulse. The recordings were rejected if there was more than 20% difference in the

series resistance at the beginning and end. TTX 1μM (Tocris Cookson Ltd) was added to the solution to block the voltage-gated Na^+ channels and record miniature synaptic events. SR93351 6μm (Tocris Cookson Ltd) was added to the bath to block the GABAergic activity and record the current due to glutamatergic receptor activation.

Part (n= 5) of the electrophysiological data recorded from acute slice at P14 and organotypic slice at DIV7 has been collected while supervising and in collaboration with Xiao Wei-Wang (MSc student).

2.4.2 Action potential recordings

K-Gluconate intracellular solution used to record action potential contained (in mM): KGluconate130, NaCl 10, HEPES 10, EGTA 1, MgATP 2, and $MgCl_2$ 1. pH was adjusted to 7.4 with NaOH and osmolarity to 298 mOsm with glucose. This solution has about -15 mV junction potential which has been compensated before touching the cell. Whole cell configuration was reached in voltage clamp configuration and then the amplifier was switched to current clump configuration in order to be able to record the voltage changes.

2.4.3 Signals Recordings & Analysis

The currents and voltage signals were filtered at 2 KHz (8 Bessel 10dB low-pass filter) and sampled at 10 kHz with A-D interface (CED 1401plus). WinEDR and WinWCP signal analysis software (kindly supplied by Dr John Dempster) were used to acquire and analyse the data.

When recording IPSCs, for each cell/condition 320s of data recording were sampled (each including 5 second of +5mV pulse signals at the beginning and at the end of the recording) using the following parameters: threshold spike detection, amplitude -3 pA, time over threshold 5ms, dead time 30ms, running mean period 8 ms. Background noise was measured in each recording and all the events detected were then inspected by eye in order to avoid event detection errors. We accepted only the events in which the rise time was < 3ms and faster than the decay time. (Edwards et al., 1990)

Once the events were selected the following parameters were measured: amplitude, rise time, decay time (t_{50}), frequency. Rise time was measured from 20 to 80% of the maximum amplitude.

When recording action potentials a series of current steps of increasing amplitude was used to elicit the action potentials. Resulting voltage changes were classified as action potential on the basis of parameters including onset voltage, maximum depolarization and after hyperpolarization.

2.5 Immunohistochemistry

Stem cells were plated onto 12mm glass coverslips coated with poly-D-lysine 1 to 3 days before analysis. The plates were taken from the incubator and the coverslips rinsed and fixed in paraformaldyde 4%/glucose 4% solution for 10 minutes. In order to permeabilize the cell's membrane, coverslips were kept for 5 minutes in 0.5% Triton in PBS. A "blocking" solution containing 10% fetal calf serum and 2.5% bovine serum albumin was used to saturate the unspecific binding sites for 20minutes. Primary and secondary antibodies were applied in 10% NGS and 2.5% BSA in PBS solution or 10% NGS in PBS solution, respectively. Coverslips were washed 3 times with PBS between each treatment.

We used antibodies against the following markers to test the phenotype of our cells:

GFAP: Glial Fibrillary Acidic Protein is a type III intermediate filament expressed in astrocytes of the central nervous system. However, it is not a marker of mature astrocytes, as is β-100.

Nestin: Nestin is a Type-VI intermediate filament, transiently expressed during cellular division and development especially in the subventricular zone of the hippocampus. For these characteristics it is used as a marker of neural precursor cells.

MAP-2: Microtubule Associated Protein 2 is expressed in dendrites where it stabilizes the microtubule structure.

NeuN: Neural Nuclear antigen is expressed in vertebrate, including human, neurons (Mullen et al., 1992; Sarnat et al., 1998).

Calretinin: Calretinin is an intracellular calcium binding protein belonging to the troponin-C superfamily. It is expressed in an early post-mitotic stage of neuronal differentiation in hippocampal granule cells (Brandt et al., 2003).

Calbindin-D28k: Calbindin-D is an intracellular calcium binding protein used as dentate gyrus granule cell marker although it does not label immature granule cell (Goodman et al., 1993).

Na_v1.2: splice variant 1.2 of the 260kD α-subunit forming the pore of the voltage-activated sodium channels (Jarnot and Corbett, 2006)

Primary antibodies:

Millipore Rabbit anti-brain type II voltage gated sodium channel ($Na_v1.2$) Cat: AB5206 Dilution factor 1:400	Sigma-Aldrich Mouse Monoclonal anti-MAP2 Clone: HM-2 Cat: M4403 Dilution factor 1:500
Sigma-Aldrich Mouse Monoclonal Anti-Glial Fibrillary Acidic Protein (GFAP) Clone: G-A-5 Cat: G3893 Dilution factor 1:500	Swant Mouse Monoclonal anti-Calbindin D-28k Lot: 07(F) Cat: 300 Dilution factor 1:5000
Millipore Mouse anti-nestin monoclonal Clone: Rat-401 Cat: MAB353 Dilution factor 1:200	Millipore Mouse Monoclonal anti-Calretinin Cat: MAB 1568 Dilution factor 1:2000
Millipore Mouse anti-neuronal nuclei (NeuN) monoclonal Clone: A60 Cat: MAB377 Dilution factor 1:200	

Secondary antibodies:

Cy ™ 5 – conjugated AffiniPure Goat Anti Rabbit IgG (H+L) Jackson Immunoresearch Code: 111-175-003 Lot: 85419	Cy ™ 5 – conjugated AffiniPure Goat Anti Mouse IgG (H+L) Jackson Immunoresearch, Code: 115-175-003 Lot:74552

Before proceeding we tested antibodies against GFAP, Nestin and NeuN for their efficacy on differentiated hippocampal cultures containing both neurons and glial cells. We did not test MAP-2,

Calretinin and Calbindin as we already knew their optimal concentration. Antibodies against GFAP and Nestin showed a good specificity when used in concentration of 1:500 and 1:200 respectively. NeuN Antibody optimal concentration was 1:200 (fig. 6). The labelling of the stem cell line was carried out strictly following the protocol, using the antibodies at their optimal concentration.

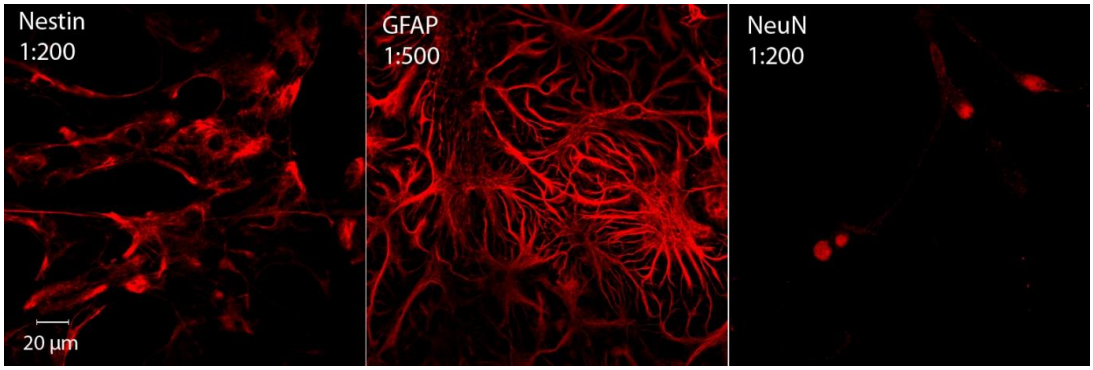

Fig.6. Three different cultures containing differentiated hippocampal cells stained with GFAP (middle), nestin (left) and NeuN (right). Each image shows the labelling obtained using those antibodies at their optimal concentration. Antibody names and concentrations are shown in each image at the top left corner.

CHIP stem cells (Chipperfield et al., 2002) were able to renew themselves when grown in the presence of basic fibroblast growth factor 2 (bFGF2). This stem cell line has been transfected with a replication-deficient adenovirus expressing green fluorescent protein (GFP) from which GFP positive clones have been manually selected and expanded. Unexpectedly, I found that in 2 cases over >100 images, not all the cells were GFP positive (fig.7, white arrow). This lack of expression does not introduce any significative problems because of its small number and therefore does not affect the validity of the results.

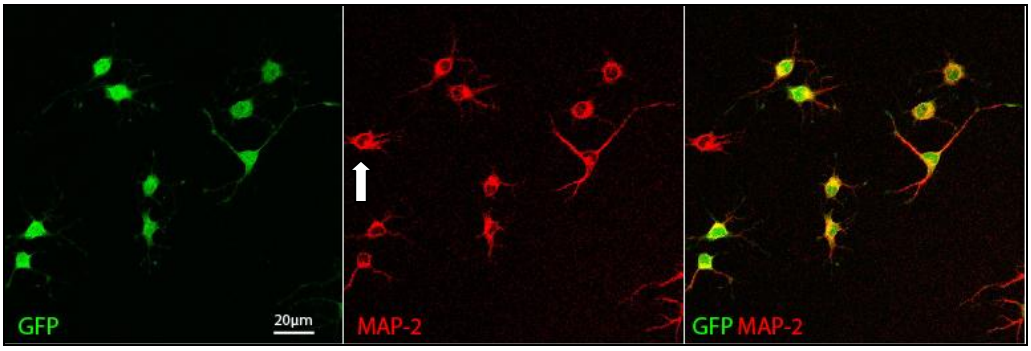

Fig.7. Adult hippocampal progenitor cells stained with antibodies against MAP-2. The white arrow shows the position of the non GFP labelled cell.

As the cells were GFP positive (exc.488 nm, emiss. 509 nm), the secondary antibodies chosen for the detection of the staining were conjugated with CY5 (exc.650nm, em.670nm), in order to avoid bleed through caused by cross-excitation of the signals. Once stained the slice were fixed on glass slides. The pro-long antifade (Molecular Probes) was used as mounting medium to lower the photo-bleaching. The fluorescence cells images were taken using a Zeiss LSM-510 Meta confocal microscope with Argon-Krypton (488nm), Helium-Neon (633nm) and UV (405nm) lasers to excite respectively GFP, Cy5 and DAPI

2.6 Slice imaging

Alexa dyes which do not induce any electrophysiological change in the cells were alternatively included in the intracellular solution at 0.2 mg/ml (all from Molecular Probes Europe BV, Leiden, The Netherlands):

Alexa Fluor 488 product code A10436; absorption 495 nm, emission 519 nm.

Alexa Fluor 594, product code A10438; absorption 590 nm, emission 617nm.

The imaging was performed on an Olympus Fluoview confocal microscope (generously supplied by Olympus, London, UK) on an upright Olympus BX50WI, using the following Olympus objectives: 40x water immersion, NA 0.8 or 60x water immersion, NA 0.9. The photomultiplier and gain were set in order to have the best resolution without reaching saturation. Imaging was usually performed immediately in live tissue continuously perfused with ACSF.

2.7 Statistical Analysis

Electrophysiological data were analysed using WinWCP, WinEDR. The statistical analysis was carried out using Microsoft Excel, GraphPad Prism 5 and SigmaPlot 11. All the data groups were tested for normality. In some cases it was not possible to test a data group for normality because of the small n. Previous studies have however shown that such data are normal distributed. (Note that where raw data distributions are skewed the medians are taken from individual cells. As the medians are normally distributed between cells the means of the medians are then used for analysis (Edwards et al., 1990; Parsley et al., 2007). One-way ANOVA or two-way ANOVA were used to compare the

differences between different sets of data. The differences were considered significant at $p \leq 0.05$. Post-test were applied as appropriate (see results). Data are showed as mean ± SEM if not indicated differently. In cases where the raw data were not normally distributed the means refer to the average of the medians. The p-value is shown using the symbol "*" to indicate a p-value < 0.05, the symbol "**" to indicate a p-value < 0.01 and the symbol "***" to indicate p-value <0.001.

2.8 Limitation of the methods

2.8.1 Cell culture

Neuronal cultures provide a valid alternative to *in vivo* models for studying basic cell properties and cell-cell interactions because of their defined conditions and the high number of manipulations they allow. Culture media used initially must contain serum to provide factors which are critical for the survival of the neurons. Because of the way it is sourced serum composition varies from one lot to another affecting the reliability and reproducibility of experiments. N2 supplement (Bottenstein and Sato, 1979) and B27 supplement (Brewer et al., 1993) were developed in an attempt to define the essential components present in the culture medium and improve the reproducibility of the experiments. This approach solves several problems and drastically reduces the variability of the neuronal culture. However, several laboratories still experience differences using commercially available supplements (Tsui and Malenka, 2006; Schluter et al., 2006). The reason is that several components, like bovine serum albumin and transferrin, are isolated from biological sources and the isolation procedures differ from different vendors. Therefore it is critical to use the same supplier for each product to obtain reproducible results.

3 Results

3.1 Characterization of IPSCs from granule cells in organotypic slices

The aim of the study was to implant hippocampal stem cells onto hippocampal organotypic slices in order to analyse the process of differentiation and integration into a pre-existing network. I also studied the characteristics of the granule cells occurring *in situ* in such slices.

Several works demonstrate that soon after birth the functionality of the dentate gyrus in acute slices is mainly dominated by GABAergic synaptic transmission (Markwardt et al., 2009). Glutamatergic activity increases with postnatal development until it reaches a plateau at the end of the fourth week (Liu et al., 2000), when the vast majority of the granule cells have reached maturity. Moreover, it has been shown that the majority of synaptic currents were blocked in the presence of bicuculline (Edwards et al., 1990) and spontaneous activity was largely GABAergic. I thus studied the spontaneous inhibitory synaptic currents (IPSCs) which occurred in granule cells of organotypic slices.

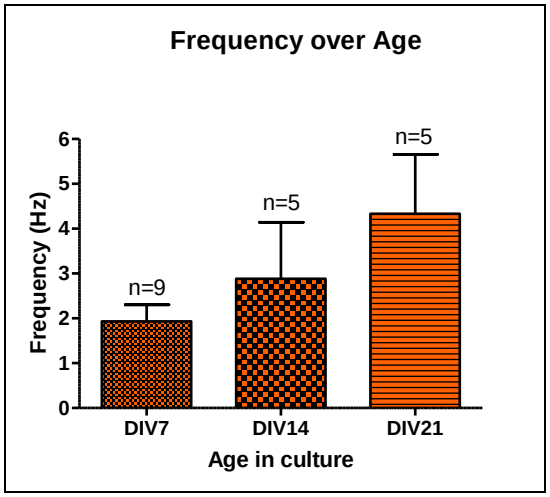

Fig.8. Frequency of IPSCs plotted against age in culture (one-way ANOVA p=0.178, post-tests Bonferroni are not significant).

We know that the general population of granule cells in the dentate gyrus completes its maturation in 4^{th} – 5^{th} postnatal weeks (Piatti et al., 2006). In my recordings of the activity in the granule cells up to 3 weeks of age, I found that the frequency of IPSCs did not significantly increase over time (fig. 8).

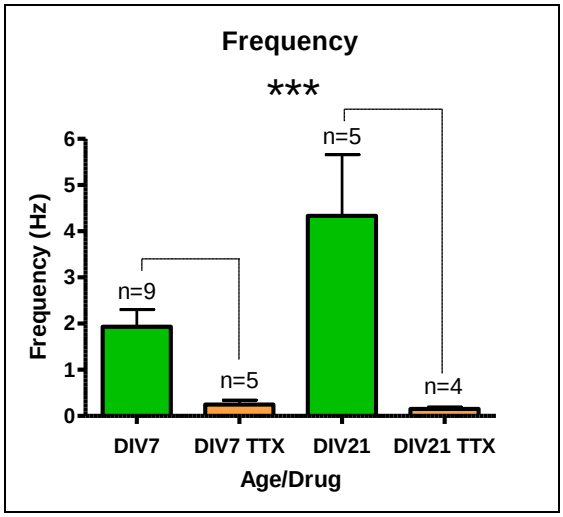

Fig.9. Frequency of IPSCs in granule cells at DIV7 and DIV21 recorded without and with TTX. (Two-way ANOVA: control vs TTX p=0.0003, Age effect and interaction are not significant).

IPSCs frequency comprises both spontaneous and miniature postsynaptic currents (mIPSCs). mIPSCs are due to a single vesicle spontaneously released from the presynaptic terminal. Once TTX was applied to isolate the mIPSCs, the frequency radically decreases at both ages (fig.9).

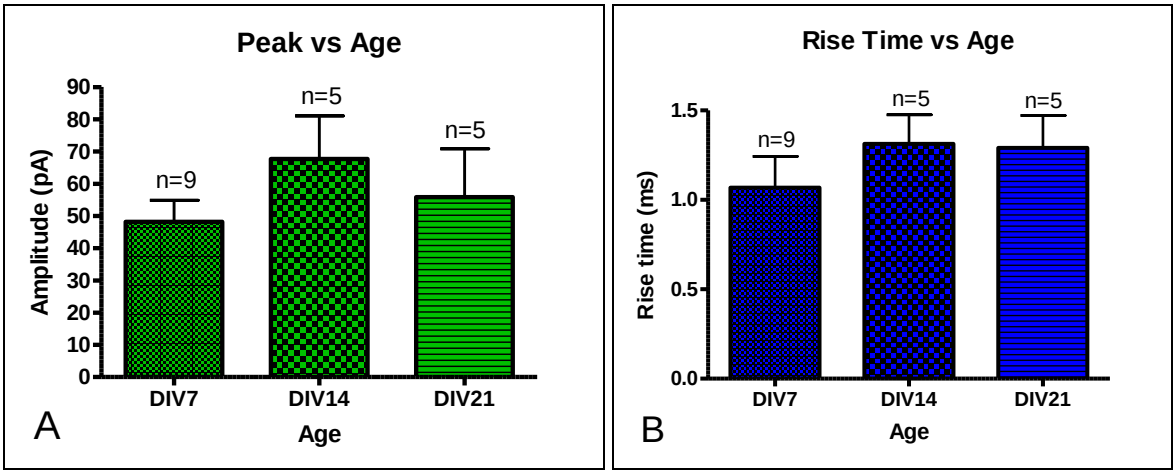

Fig.10. A: Peak of IPSCs over 3 weeks in culture (One way ANOVA p=0.437, post-tests Bonferroni not significant). B: Rise time of IPSCs over 3 weeks in culture (one way ANOVA p=0.552, post-tests Bonferroni not significant).

The peak amplitude of the IPSCs (fig.10A), measured at DIV7, DIV14 and DIV21 show no significant differences. The IPSCs rise time (fig. 10B) is fast and stable throughout the 3 weeks. It ranges from 1.07±0.17ms at DIV7 to 1.31±0.16ms at DIV14.

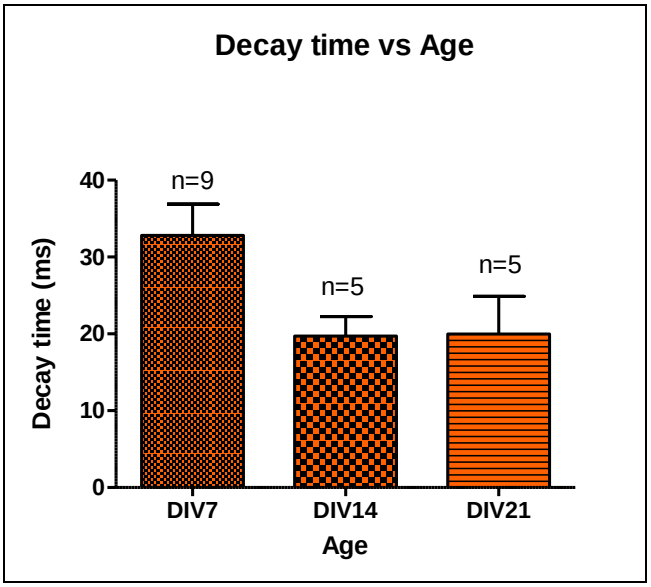

Fig.11. Decay time to half amplitude (t_{50}) across 3 weeks in culture (One way ANOVA p=0.053, post-test Bonferroni DIV7 vs DIV21 $p<0.05$, others post-tests are not significant).

The decay time (t_{50}) is determined by how long the channels remain open or the mean open-time. The decay time constant (t_{50}) showed a clear trend toward decreasing over 2 weeks in culture (fig.11) from DIV7 (32.81±4.06ms) to DIV21 (19.98±4.9ms).

3.2 Comparison of IPSC activity in DIV7 organotypic and P14 acute slice

Postsynaptic potentials in CA1 pyramidal neurons have already been analysed both in organotypic and acute slices (De Simoni et al., 2003). The same is less true for the granule cells of the dentate gyrus. Therefore I did a comparison between DIV7 organotypic slice and P14 acute slice to test whether the results were comparable between the two preparations.

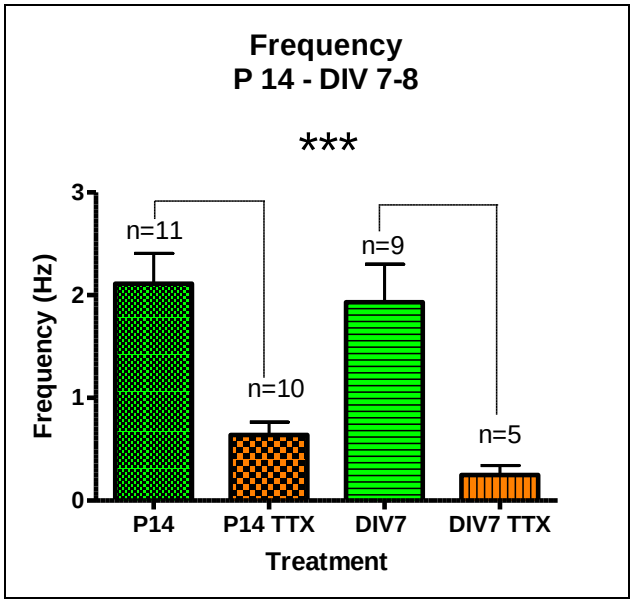

Fig.12. Comparison of IPSCs frequency of P14 acute slices and DIV7 organotypic slices in control condition or TTX (two-way ANOVA control vs TTX $p<0.001$, P14 vs DIV7 $p=0.3378$, interaction $p=0.7247$).

The frequency of IPSCs of DG granule cells from P14 acute (2.11±0.3 Hz) and DIV7 organotypic slices (1.93±0.37 Hz) are similar in control condition (fig. 12). After TTX infusion both slice preparations show a marked decrease in the frequency of IPSCs. There is no difference between mIPSCs frequency at P14 and DIV7.

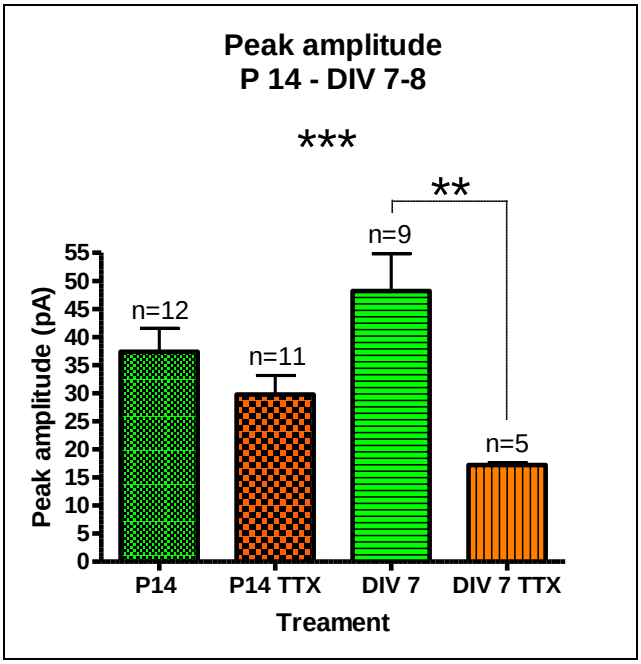

Fig.13 IPSCs peak amplitude in P14 acute slices and DIV7 organotypic slices (two-way ANOVA control vs TTX p=0.0005, P14 vs DIV7 p=0.8665, interaction p=0.0256; post-tests Bonferroni DIV7 vs DIV7 TTX p=0.003, others post-tests are not significant).

There is a significant difference in the peak amplitude between IPSCs and mIPSCs at DIV7 (fig.13). The ratio between the amplitude of mIPSCs and IPSCs, which can be used to estimate the number of release sites activated during an action potential, is close to 3 (2.78) in the organotypic slices.

From the ratio between mIPSCs and IPSCs frequencies (fig.12) it appears that 30% of P14 IPSCs and 13% of DIV7 IPSCs may be mIPSCs. As mIPSCs have significantly lower amplitude, the mean amplitude of AP-mediated events alone at DIV7 will be higher.

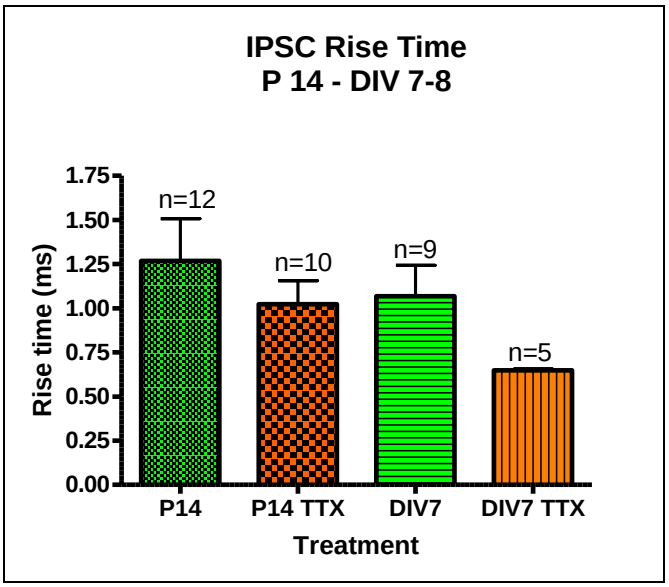

Fig.14 IPSC rise time in P14 Acute slices and DIV7 organotypic slices (Two-way ANOVA control vs TTX p=0.1172, P14 vs DIV7 p=0.1727, interaction p=0.6741).

IPSC rise time (fig.14) is not significantly different between P14 and DIV7. In TTX condition, the rise time detected in DIV7 organotypic slices is not significantly different from in P14 acute slice.

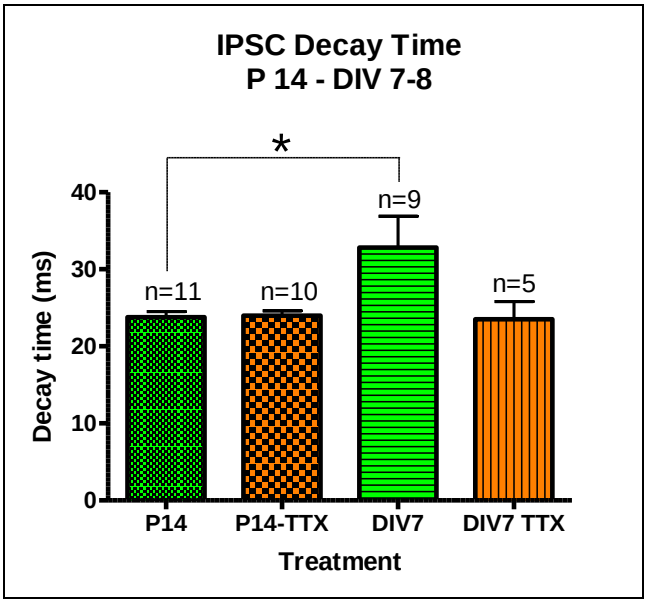

Fig.15 IPSC decay time in P14 acute slices and DIV7 organotypic slices (two-way ANOVA Control vs TTX p=0.0643, P14 vs DIV7 p=0.0818, interaction p=0.0548; post-test Bonferroni P14 vs DIV7 p=0.032, others post-tests are not significant).

Decay time (t_{50}) differs significantly between P14 and DIV7. The effect of TTX does not have a statistically significant effect on the decay time in comparison with the control condition in either acute or organotypic slices.

3.3 Implantation of stem cells into organotypic slices

Stem cells from the line obtained from Chipperfield and expressing GFP were cultured in Neurobasal medium and then implanted into organotypic slices cultured in minimum essential medium (MEM/EBSS based medium). In such a preparation, stem cells do not survive more than two weeks. To check if the organotypic slice medium alone was able to sustain stem cells, I cultured CHIP stem cell in this medium. I found the same results as in the implant, with stem cells that do not survive more than 2 weeks in culture showing a decreased density at DIV14 (fig. 16) and no cells remaining at DIV21 (data not shown).

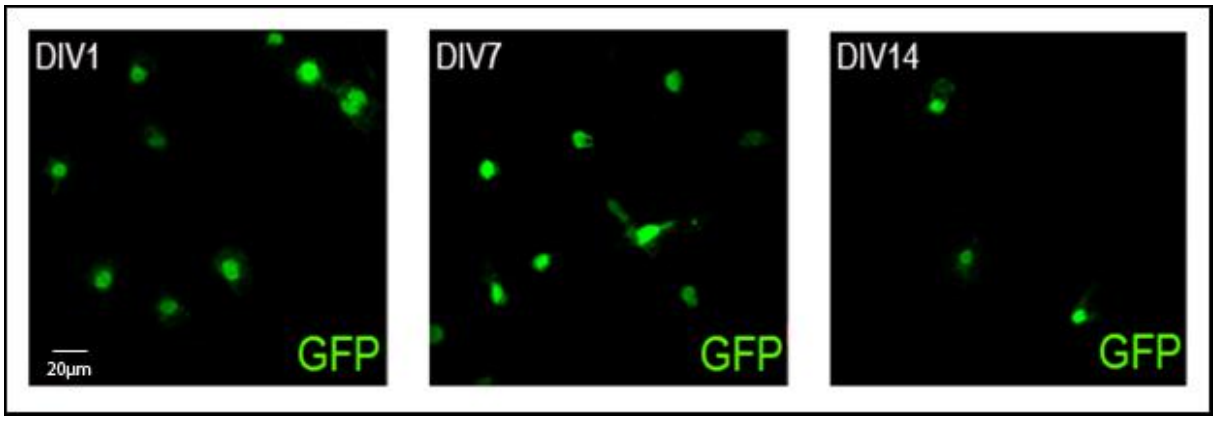

Fig.16. CHIP stem cells cultured in organotypic slice medium. Note the lack of arborisations at DIV7 and DIV14.

I thus investigated whether organotypic slices would survive in the DMEM/F12 based medium. The DMEM/F12 based medium composition does not use horse serum and it includes the F12 nutrient mixture which is specifically formulated to better support neuronal cell culture.

I found that DMEM/F12 based medium is compatible with organotypic slices (fig.17A, B) and stem cell survival (fig. 18). Neurons from organotypic slices cultured in this medium remain healthy until the 6th week showing a well-developed dendritic tree with a high number of spines (fig.17B).

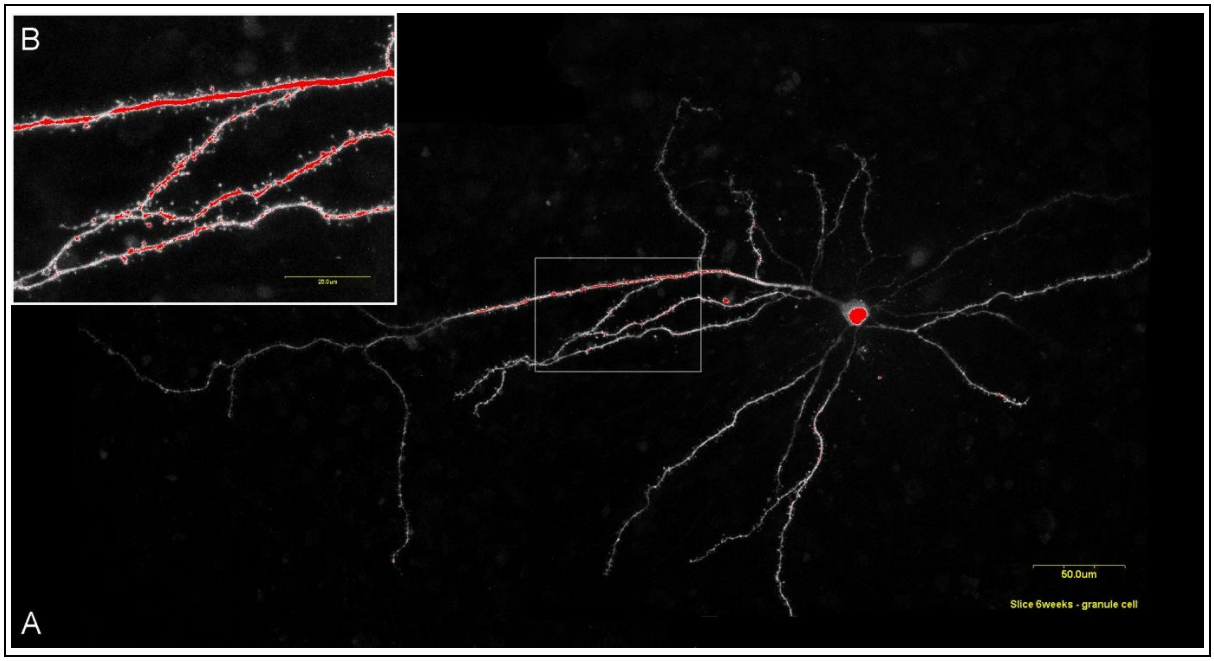

Fig.17. Neuron from 6 weeks hippocampal organotypic slice filled with Alexa Red dye. A: projection of 48 images composing the stack (scale bar 50µm). B: magnification of a group of dendrites with plenty of spines (scale bar 20µm).

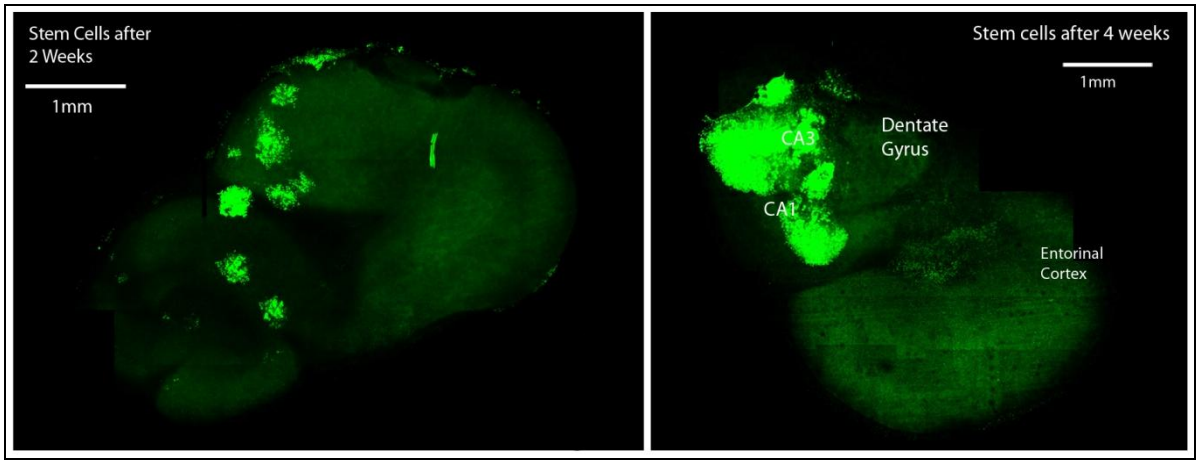

Fig.18 Stem cell implanted onto organotypic slices at 2 and 4 weeks in culture.

The method I used to implant the cells does not allow a precise quantification of the number of cells that remain on the slice but in all the cases I found that they tend to aggregate forming groups of cells of variable dimensions. The stem cells that were washed away from the slice do not survive and die during the first 2 weeks (data not shown). Unfortunately I saw no evidence of glial/neuronal differentiation under these conditions. Cells have no protrusions and hence the project changed to characterizing the differentiation of 2 different stem cell lines in culture.

3.4 Immunohistochemichal characterization of stem cells in culture

The adult hippocampus stem cell line I used for the implantation experiment was found to be stable in culture, maintaining its stem cell features for over 6 months (Chipperfield et al., 2002). As described in the literature (Kempermann et al., 2004) the use of several antibodies is necessary in order to define the several markers that are expressed in a sequential timeline during the differentiation process. I therefore assessed their phenotype with different antibodies: GFAP, Nestin, MAP2, NeuN, Calretinin and Calbindin (fig. 19A-B).

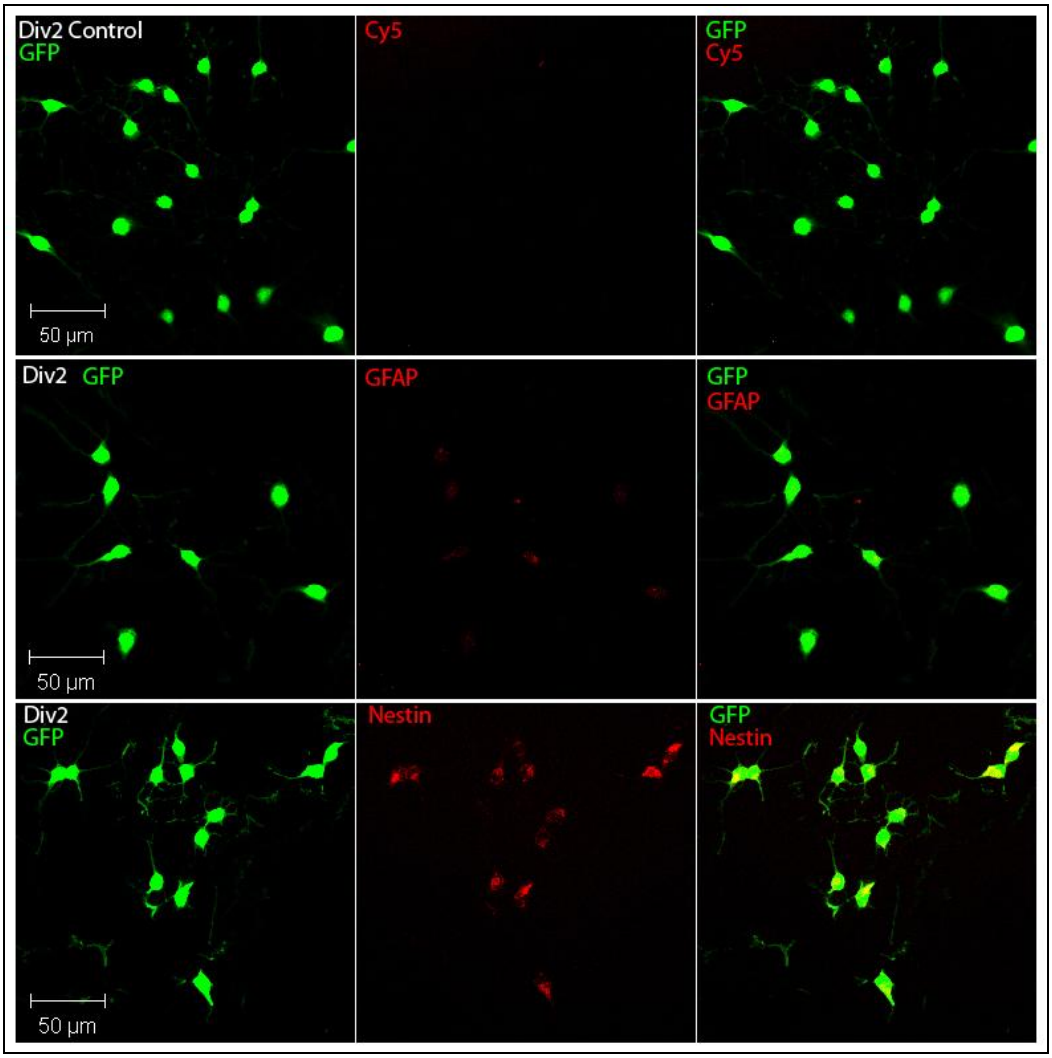

Fig.19A. Immohistochemical characterization of CHIP stem cell line with GFAP and nestin antibodies after 2 days in culture. In green CHIP stem cell line constitutively expressing green fluorescent protein , in red from top to bottom , the control labelling using only the secondary antibody (CY5), the labelling for glial fibrillary acid protein (GFAP) and the labelling for nestin protein (Nestin).

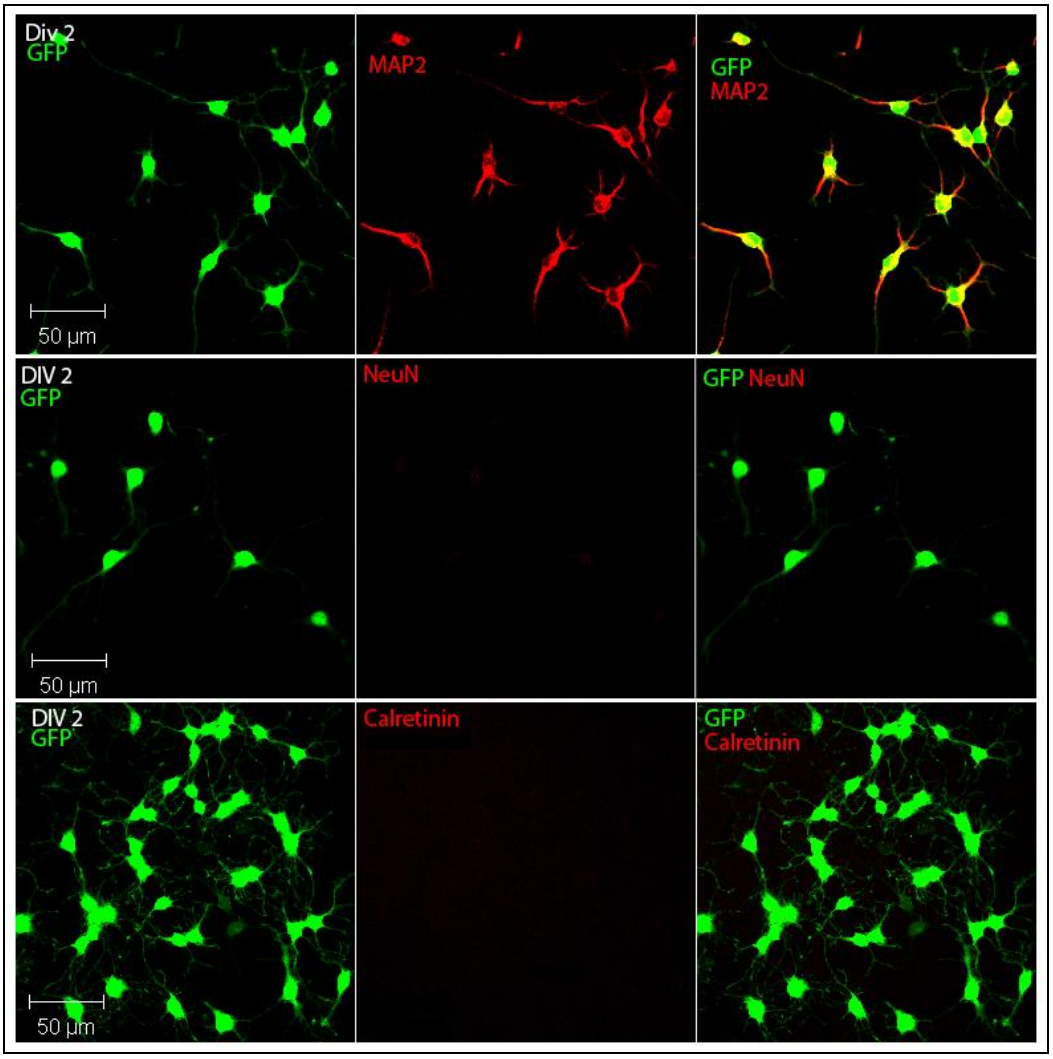

Fig.19B. Immohistochemical characterization of the CHIP stem cell line with MAP2, NeuN and calretinin antibodies after 2 days in culture. In green CHIP stem cells constitutively expressing green fluorescent protein, in red from top to bottom, the labelling for microtubule associated protein 2 (MAP2), the labelling for neural nuclei protein (NeuN) and the labelling for calretinin protein (Calretinin).

The CHIP stem cell line had a very low expression of GFAP but was positive for Nestin and MAP2. They do not express NeuN and Calretinin and/or Calbindin (Calbindin results not shown). So, in agreement with the literature these stem cell lines have a neuronal phenotype as they express MAP2 (Peng et al., 1986) but they are not yet totally differentiated into mature neurons.

3.5 Study of the functional differentiation of two adult hippocampus derived stem cell lines.

In order to correlate the functional differentiation of two stem cell lines, CHIP and HCN95, with the presence of the neuronal marker NeuN, I studied both the cells' abilities to fire action potentials and the presence of sodium channels. The shape of the action potentials recorded from both these cells line were still immature but they correlate well with the presence of sodium channels and were TTX sensitive (fig. 20).

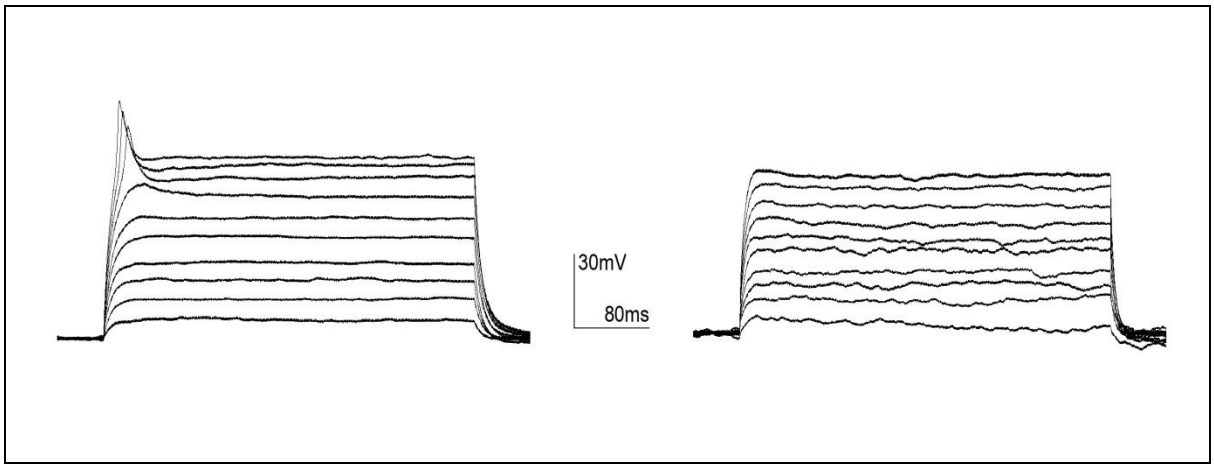

Fig.20. Membrane potentials elicited after current steps injection in HCN95 stem cell. On the right the cells perfused with 10μM TTX Krebs solution to show that the current we were recording was effectively due to voltage gated sodium channel opening.

Both CHIP and HCN95 stem cells were induced to differentiate using two different protocols. In the first protocol 10% horse serum was added to the culture media whereas in the second protocol I used a defined mix of growth factors (BDNF, NT-3, NGF) + Laminin, in order to induce neuronal differentiation.

Chipperfield Stem cells cultured in horse serum differentiation medium for 4 weeks (fig.21) show no markers for sodium channels or NeuN. They also have a low percentage (20%) of neurons able to fire action potentials both at DIV7 and DIV28.

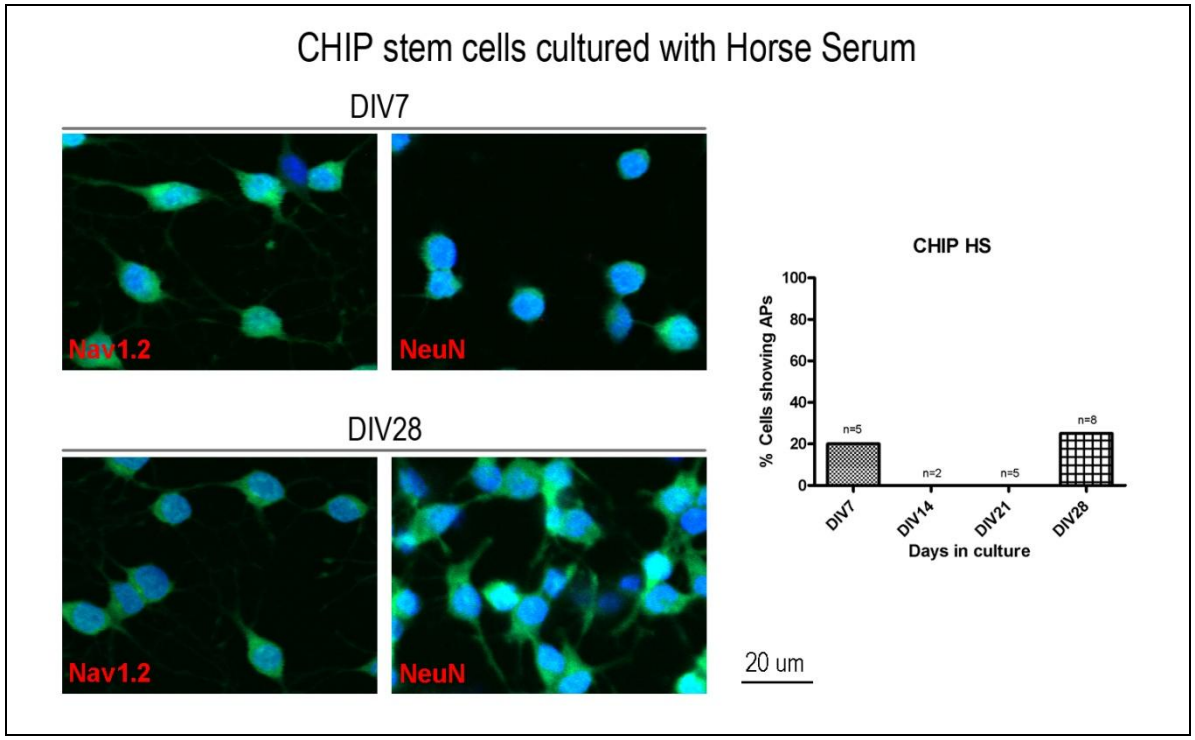

Fig.21. Chipperfield Stem cells cultured in HS differentiating medium and labelled for Nav 1.2 and NeuN. In blue the DAPI staining of the nucleus; in green the GFP for the cell body and in red the staining for Nav1.2 and NeuN which are absent both at DIV7 and DIV28. TOP: Stem cells at DIV7. BOTTOM: Stem cells at DIV 28. RIGHT: Graph of the stem cells with the % of action potential recorded at each age.

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HCN95 stem cells cultured in Horse serum differentiation medium (fig. 22) show no marker for NeuN at DIV7 and DIV 28 but were positive for $Na_v1.2$ at DIV 28. Also the totality of the neurons is able to fire APs starting from DIV21.

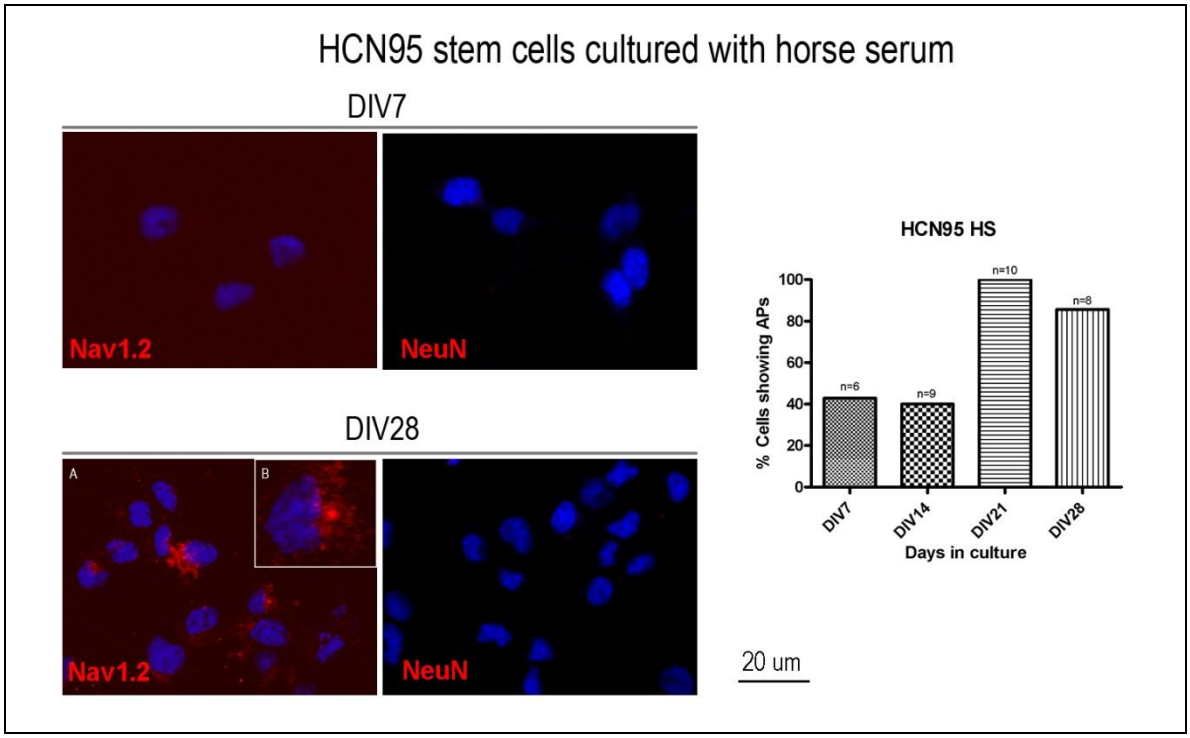

Fig.22. HCN95 Stem cells cultured in HS differentiating medium and labelled for $Na_v1.2$ and NeuN. In blue the DAPI staining of the nucleus and in red the staining for Nav1.2 or NeuN. TOP: Stem cells at DIV7. BOTTOM: Stem cells at DIV 28 with magnification of cells expressing Nav 1.2. RIGHT: Graph of the stem cells with the % of action potential recorded at each age.

CHIP stem cells cultured in growth factor differentiation medium (fig. 23) show no marker for NeuN at DIV7 and DIV 28 but are positive for markers for $Na_v1.2$ at DIV 28. Also the totality of the neurons is able to fire APs once they reach DIV28.

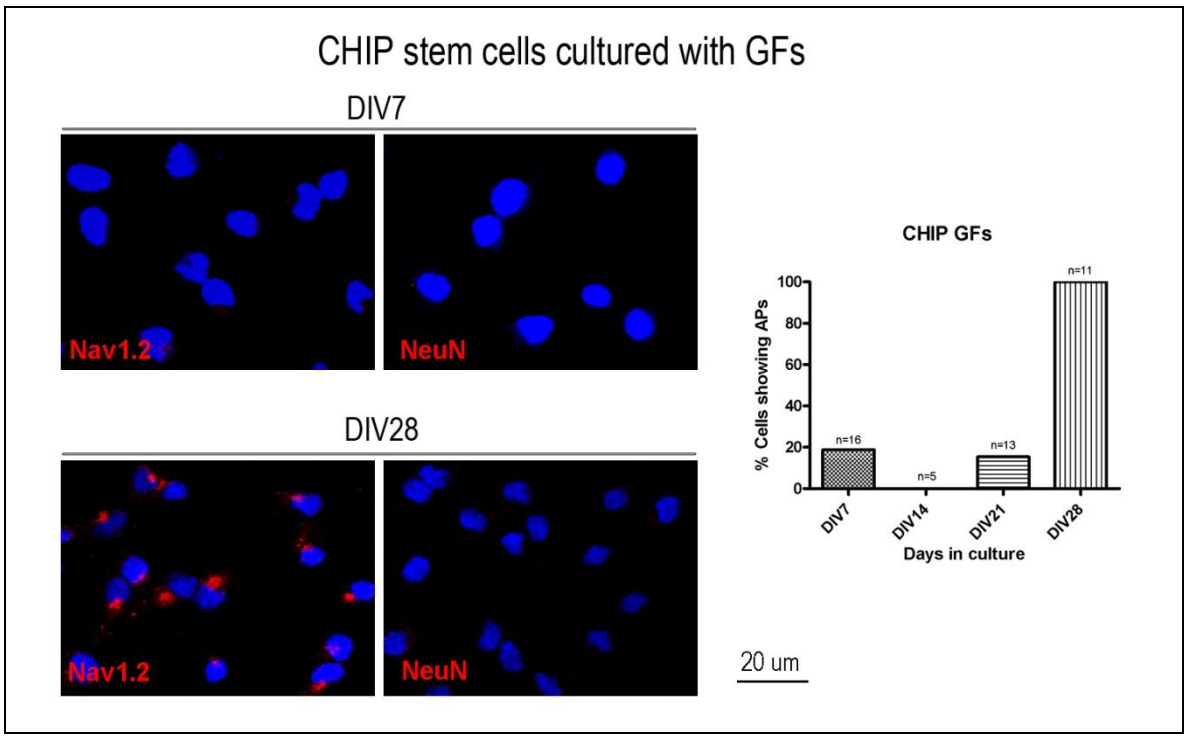

Fig.23. CHIP stem cells cultured in Growth factors differentiating medium and labelled for Na_v 1.2 and NeuN. In blue the DAPI staining of the nucleus. TOP: Stem cells at DIV7. BOTTOM: Stem cells at DIV 28. RIGHT: Graph of the stem cells with the % of action potential recorded at each age.

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HCN95 stem cells cultured in growth factor differentiation medium (fig. 24) show no marker for NeuN at DIV7 and DIV 28 but they do show markers for $Na_v1.2$ at DIV 28. Also the totality of the neurons is able to fire APs at DIV28

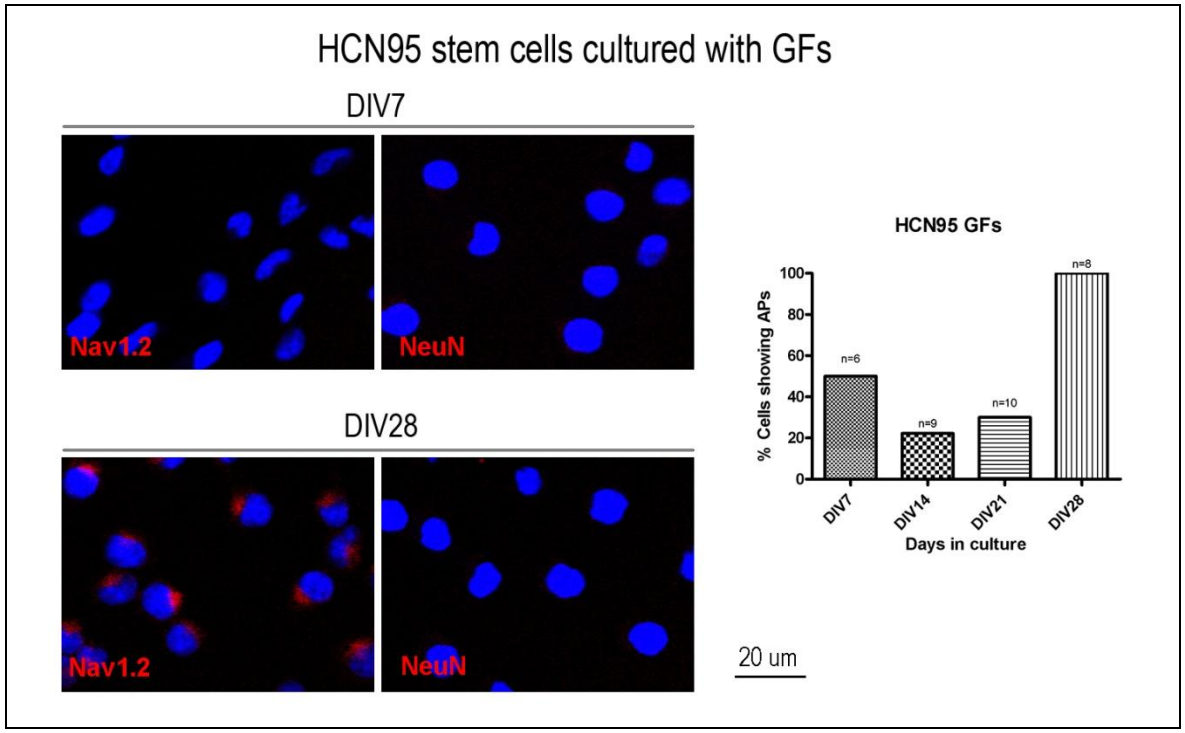

Fig.24. HCN95 Stem cells cultured in growth factors differentiating medium and labelled for Na_v 1.2 and NeuN. In blue the DAPI staining of the nucleus and in red Nan1.2 or NeuN. TOP: Stem cell at DIV7. BOTTOM: Stem cell at DIV 28. RIGHT: Graph of stem cell with the % of action potential recorded at each age.

4 Discussion

Using organotypic slices has allowed me to study which elements influence the survival and the integration of stem cells once they have been implanted. I was able to characterize postsynaptic currents in granule cells over a period of 4 weeks. However, stem cell integration was not achieved and I switched my aim to the study of stem cell differentiation in culture, using immunohistochemical markers and electrophysiology to assess their properties.

4.1 Changes in dentate gyrus granule cell IPSCs properties

Knowing the characteristics of granule cell postsynaptic currents in the dentate gyrus of the hippocampus is an essential step to picture their behaviour. Therefore I started characterizing granule cell postsynaptic currents in organotypic hippocampal slices from 1 to 3 weeks.

Glutamate and GABA are the principal neurotransmitters in the mammalian brain (Freund and Buzsaki, 1996) but the vast majority of postsynaptic currents recorded from granule cells have been shown to be GABAergic in acute hippocampal slice (Edwards et al., 1990) as well as in organotypic hippocampal slices (unpublished data from X.W. Wang MSc Thesis). Therefore, in my recordings I focused on GABAergic IPSCs.

We observed a significant decrease in the frequency of IPSCs in the presence of TTX confirming that a significant proportion of spontaneous events are mediated by action potentials in both preparations. After TTX application to block spontaneous action potentials, we observed that mIPSCs frequency (fig. 9) does not decrease significantly, between the first and the third week in culture. This is in contrast with a previous report where it has been found that mIPSCs frequency increase with age in DG granule cells from P0 to P21 acute slices (Hollrigel and Soltesz, 1997).

When I look at the peak and rise time of the IPSCs there is no significant difference between them at different ages. The IPSCs mean median peak amplitude grouping DIV14-21 (62±9.6) is similar to IPSCs amplitude recorded in mature granule cells (62±6.7 pA, (Markwardt et al., 2009). Considering that dendritic synaptic events are characterized by a slow rise time, most of these events, rising in approximately 1 ms are probably caused by perisomatic synapses (Soltesz et al., 1995)

4.2 Different properties of organotypic and acute hippocampal slices.

The IPSCs recordings of dentate gyrus granule cells from postnatal day 14 (P14) acute slices allow a comparison between corresponding ages of acute slices and organotypic slices.

There was no significant difference in the frequency of spontaneous IPSCs between the two preparations both in control condition and with application of TTX. In both preparations the dentate gyrus is still undergoing a major development but in the organotypic slices the reduction of the number of synapses, induced during the slice preparation by the loss of afferent and efferent connections, may have been recovered at DIV7. Moreover, there is no significant difference in mIPSCs frequency between DIV7 and DIV21 in organotypic slices (fig.9). Excluding changes in p_r, these data lead to the conclusion that the number of functional synapses remain similar in both acute and organotypic slices.

The peak IPSC amplitude (fig. 13) is also similar but once I applied TTX, it significantly decreases in the organotypic slice preparation, down to 35% of the normal amplitude. IPSCs are generated by synchronous release of presynaptic vesicles from one or more release sites present at the activated synapse. In contrast, mIPSCs generated after TTX application are due to a single vesicle released from one release site. This result suggests that one action potential activates nearly 3 releases sites on average in organotypic slices compared with the ratio of approximately 1:1 in acute slices.

The rise time is not significantly different between the two preparations (fig. 14). This may mean that in both preparations the currents derive from synapses which are equally distant from the soma.

Looking at t_{50} there is a significant difference between P14 acute slices and DIV7 organotypic slices (fig. 15). Among several factors, t_{50} is determined by channel subunit composition (Garrett et al., 1990), temperature and membrane potential (Pytel et al., 2006).

We recorded at room temperature while holding the cells at -70mV therefore a switch in subunit composition remains the most feasible explanation. Changes in subunit compositions during development have been shown in terms of changes in sensitivity of GABAergic synaptic currents to neurosteroids (Cooper et al., 1999) and also zolpidem and zinc (Hollrigel and Soltesz, 1997). The α_4, β_1 subunits which start declining at about day 12 *in vivo* and δ subunits that instead become detectable (Laurie et al., 1992). Considering we are studying preparations in this age range, the differences may reflect that development in organotypic slice occurs at a different rate. Withdrawing progesterone

from rats in particular, has shown to increase α_4 in association with a decrease in decay time (Smith et al., 1998).

Another factor which could possibly contribute to change in t_{50} is how fast GABA is cleared out of the synaptic cleft once it has been released from the presynaptic terminal. The depletion of the GABA neurotransmitter from the synaptic cleft relies on diffusion and the GAT-1 transporter.

The GAT-1 transporter seems to develop together with the GABAergic transmission (Yan et al., 1997). After applying the GAT-1 GABA transporter blocker NO711, there was a significant difference in the decay time of evoked IPSCs but no effect on the decay of spontaneous IPSCs recorded from mouse hippocampal slice between P30 and P60 (Wei et al., 2003). Therefore, it probably has no influence on shaping the decay time.

With the maturation of the brain the extracellular volume undergoes a marked reduction between P10 and P20 in rats (Lehmenkuhler et al., 1993). Artificially reducing the extracellular space in acute slice with a hypo-osmotic ACSF solution during the first postnatal week resulted in a prolongation of $t_{50.}$ However, at P13-16, t_{50} results unaffected by the same procedure (Draguhn and Heinemann, 1996).

It is also possible that the differences in decay time reflect an increased extracellular space in organotypic slices.

4.3 Importance of a permissive environment for stem cell integration.

Several studies in which stem cells have been transplanted into the brain *in vivo* reveal that they use local cues to migrate to specific regions and then differentiate (Renfranz et al., 1991; Fishell, 1995; Vicario-Abejon et al., 1995). The initial aim of this project was to understand the process through which adult derived hippocampal stem cells differentiate and integrate once implanted into the hippocampus and in particular into the dentate gyrus.

I used hippocampal organotypic slices as a host tissue and implanted stem cells at DIV8. In this first attempt, stem cell survival, once they have been implanted onto organotypic slice, was limited to 2 weeks. In such a preparation not only the brain slices but also the culture medium plays an important role in defining the environmental conditions that can allow the stem cells to survive and integrate. The presence of serum in the slice culture medium gives the necessary nutrients which allow survival of the organotypic slices but apparently does not allow survival of the stem cells. Culturing CHIP

stem cell in a medium with such composition has been proven to inhibit their survival (fig.16). This result is in line with what was found in other experiments in which neurogenesis was found to be inhibited by serum in the culture medium, even in presence of epidermal growth factor (Raineteau et al., 2004).

The difficulties experienced with the survival of stem cells once they were applied onto the organotypic slices has led me to use a different medium for culturing organotypic slices. The new formulation is based on DMEM-F12 and does not require horse serum. In this condition stem cells survive for up to 6 weeks in culture but again, no signs of differentiation like dendrites or axons protruding from the cell body were detected. The main difference in the new medium formulation is the absence of horse serum and the presence of N2 supplement to sustain cell survival. N2 supplement is chemically defined and has been shown to support and be selective for neuronal cells (Bottenstein and Sato, 1979). The composition of the serum is unknown and while it certainly contains factors which are necessary for the survival of neurons it may also contain factors that are strongly inhibiting the survival of cells in a developing state.

The inhibiting effect of horse serum on stem cell survival is very interesting, we know that *in vivo* stem cell survive and proliferate only in two particular zones, the SVZ and the SGZ, while the other zones may be non-permissive. Thus the horse serum medium appears to behave like a non-permissive zone. Moreover, it suggests that the characteristics of the cellular niche are a major factor in determining the survival of the stem cells.

We implanted the cells by suspension and subsequent deposition on the whole surface of the slice. Once they have been put on the slice, the cells tend to aggregate but no preferential areas on the organotypic slices have been detected. In a similar experiment using the J1ES cell line (Li et al., 1992) stem cells survive and integrate when they are deposited within the hilus of the Dentate Gyrus (Benninger et al., 2003). Therefore another important factor in determining the integration seems to be related to the intrinsic characteristic of the stem cell line used for the implantation. A further experiment could employ different stem cell lines in order to test the importance of particular components of the culture medium in allowing stem cell survival.

4.4 Immunohistochemichal characterization of stem cells in culture

Stem cell development and differentiation have been screened usually by looking at the expression of several markers: each one associates with a particular maturation state. An accepted model for hippocampal neurogenesis has been proposed by Kempermann (2004) who defined six milestones of neuronal differentiation. In this model each stage is associated with the presence or absence of a specific marker and the crucial step is the expression of NeuN which marks the acquisition of a mature granule cell phenotype.

In my work I did an immunohistochemical characterization of the CHIP stem cell line which was positive for GFAP, Nestin and MAP2. Based on the Kempermann classification, cells which express GFAP and Nestin are called "Type-1" cells and are the putative radial-glia stem cells which are found in the SGZ of the adult dentate gyrus. MAP2 in contrast is a protein found on the dendritic microtubules and supposedly only in mature neuron. This stem cell line has the characteristics of "type-1" cells which include the ability to proliferate while the expression of MAP2 collocate this cells a step forward in the path of neuronal differentiation although they do not express NeuN, a commonly used marker of mature neurons.

In previous work, stem cells express a voltage-dependent sodium current which is also a neuronal marker, while they are not yet expressing NeuN (Fukuda et al., 2003). The presence of a voltage-activated sodium current can indeed be interpreted as an earlier sign of neuronal differentiation and furthermore the ability to fire action potentials. In the next section I will discuss the presence of sodium-activated voltage channels, detected using an antibody against $Na_v1.2$, and the presence of NeuN, both in correlation with the acquisition of a mature neuronal phenotype.

4.5 Functional neuronal differentiation of two adult hippocampal stem cell lines

Adult hippocampal progenitor stem cells are expected to develop into granule cells or glial cells on the basis of their internal program and the stimuli which they receive from the environment. Neuronal differentiation is achieved when the cells develop the ability to fire action potentials once they receive the proper stimulation.

Both CHIP and HCN95 stem cell lines were able to remain undifferentiated even after several months in culture. After I introduced the differentiating factors these cells were induced to mature into a neural phenotype but with different outcomes, depending on the specific stem cell line and the differentiation media which was employed.

CHIP stem cells cultured in horse serum do not show sodium channels or NeuN for up to 4 weeks in culture. This stem cell line also shows a very low percentage of cells able to fire action potentials (fig. 21). In the same conditions HCN95 stem cells show the presence of sodium channels after 4 weeks. They also show a much higher percentage of active cells. NeuN is not expressed in either cell line. Excluding a non-efficacy of the antibody against NeuN which has been tested using a neuronal cell culture explanted from rat hippocampus (fig.6) the absence of NeuN could be interpreted as an indicator of a non-neuronal phenotype. However, the presence of sodium channel $Na_v1.2$ subunits and a high percentage of cells able to fire action potentials definitely define these cells as neurons. Different stem cell lines have therefore a different internal program which leads them to a different development even in the presence of the same differentiating factor (HS).

Using a mix of growth factors as differentiating agent, yields a high percentage of neuronal differentiation in both CHIP and HCN95 stem cell lines. Sodium channels were never detected at DIV7 whereas they were present at DIV28 in both stem cell lines. The distribution of the cluster of sodium channels suggest a localization on the plasma membrane but does not rule out the possibility of intracellular labelled Nav1.2 subunits, as found in a similar work (Biella et al., 2007). The channels are localised with high density only in one site for each cell, probably the axon initial segment (fig. 23 and 24) where the higher density of sodium channels is necessary to generate action potential. Indicatively, at DIV28 all the CHIP and HCN95 cells recorded are able to fire action potentials.

NeuN is not expressed in any culture up to 28 days. This led me to think that $Na_v1.2$ is better indicator of neuronal phenotype when applied to young neurons. It is difficult to find a single marker that could define a cell as a neuron, especially when they are immature. Choosing a marker that strictly correlates with a neuronal characteristic, like the ability to fire action potential, has been demonstrated in this study to be more reliable.

Furthermore, as it has been proposed also in other studies (Goffredo et al., 2008), the possibility of generating such a high percentage of electrophysiologically mature neurons *in vitro* will yield a new source of neurons not only for transplant purposes but also for pharmacologic studies.

5 Appendix

5.1 Western blot - result

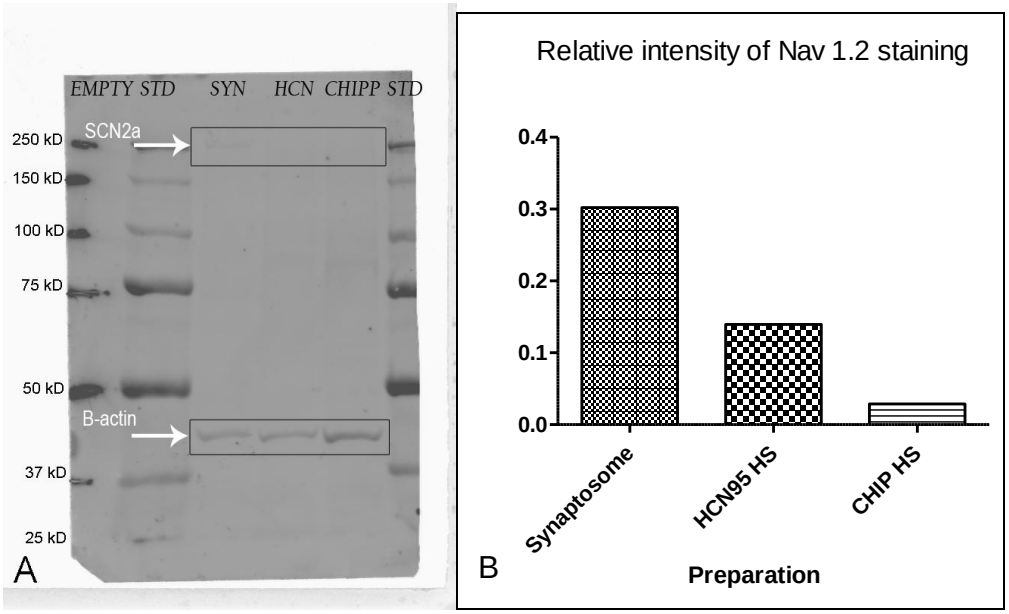

Fig.25. Western blots of HCN95 and CHIP stem cells cultured in Horse serum differentiating media. A: from left to right the column contains: the protein standards, the synaptosome, HCN95 HS and CHIP HS. B: graph of the value of the signals as detected by ImageJ analysis software.

CHIP and HCN95 stem cell were cultured in horse serum differentiating medium for 4 weeks. The western blot in fig. 25 shows the results obtained after running the different samples. Each line had been loaded with 80µg of protein sample. β-actin staining shows the quantity of proteins loaded in each lane. The sodium α-subunit SCN2a is present as a faint signal only in the synaptosome preparation and no signal is visually detectable in the HCN95 and CHIP lanes.

5.2 Western Blot - Material and Methods

The western blot technique allows the detection of specific proteins in tissue extracts or homogenates using gel electrophoresis to separate proteins by the length of the polypeptide in the denatured condition. Once the proteins are separated they are transferred onto a membrane on which they can be

detected using specific antibodies. This technique has been employed to test the increased expression of voltage-activated sodium channels by mean of detecting the SCN2a peptide which is the main channel subunit. Samples from the different preparations were taken at the appropriate times: DIV0, DIV7, DIV14, DIV21 and DIV28 using the following protocol.

The medium was removed and the culture plates washed once with PBS. Cells were harvested by mechanical dislocation and centrifuged once for 3 min at 1000 rpm. The supranatant was removed and the cells were resuspended in 1-1.5 ml PBS into a 1.5 ml vial (Eppendorf). Each vial was centrifuged at about 2000 RPM and the pellet was frozen at -80 ° after removing the supranatant.

The lysis buffer (1x PBS, 1% Nonidet, 0.05% Sodium deoxycholate, 0.1% SDS, Complete Protease Inhibitor Cocktail Tablets) is added to the cell sample as soon as possible after removing the sample vial from the freezer (-80°C). In some cases medium (~100µl) is left into the vial due to low number of cells harvested therefore the buffer composition is adapted in order to maintain the final concentration.

In order to load the same amount of protein in each well it is necessary to measure the protein concentration in each sample. To do that, we use Bovine Serum Albumin, of different known concentrations, to build the calibration curve of the spectrophotometer and then we measure protein concentration in the samples with BCA protein assay kit (Pierce). We quantify the results obtained from our sample by comparison with the curve created with BSA. The right amount of protein from each sample is then mixed 1:1 to 2x sample buffer (Laemmli buffer, Biorad) and heated at 37°C for 30 minutes. The samples are loaded into the gel wells as soon as they are removed from the heater.

The precast gels used are 7.5% Tris-HCL (Ready Gel, Biorad) with 10 wells and 4% stacking gel and 30 µl capacity per well. The first and the last lane of the gel are loaded with protein standards (precision plus protein Standards, Biorad) in order to recognize the molecular weight of the protein of interest and to have a visual control during the electrophoresis.

The gel is immersed into running buffer (25 mM Tris pH 8.3, 192 mM Glycine, 0.1% SDS) and the proteins are allowed to migrate for 120 minutes at 80 volts. The proteins are then transferred from the gel to the membrane filter (Immuno-Blot PVDF Membrane, Biorad) while submerged into a transfer buffer which is the same as the running buffer but with 20% methanol added. SDS is normally withdrawn from the transfer buffer only when the protein of interest is smaller than 80 KD.

After the transfer process is completed the membrane is washed 2 times in TBST 1x (2.43 g. Trizma HCl, 8.0 g. NaCl, pH to 7.6 with pure HCl, 1ml Tween 20, and top up to 1 L dH_2O) and then

incubated in Red Ponceau solution (Sigma) for 5' . The Red Ponceau solution binds to the proteins making it possible to check the outcome of the transfer by eye. The filter is then washed extensively in TBST until the Red Ponceau staining disappears.

5.2.1 Immunostaining for SCN2a

The filter is then placed in 5% milk blocking solution under constant agitation for 1 hour at 4°C. After incubation, the membrane is washed in TBST for 5 seconds. The primary antibody, rabbit anti mouse $Na_v1.2$ (Millipore) is diluted 1:200 in 1-3% milk TBST solution and incubated overnight at 4 ° C in an agitating chamber. Then the membrane is washed several times in TBST to remove the entire residual antibody. To be sure that the difference in the staining is not due to different initial loading quantities, the membrane filter is double stained for β-actin using an antibody made in rabbit (ABCam, Cod. AB8227) with the same procedure as that for SCN2a protein but with a dilution factor 1:5000.

The secondary antibody, goat anti-rabbit CY5 (Jackson Immunoresearch), is diluted 1:800 in blocking buffer and left on the filter for 2 hours at RT while agitating. The filter membrane is then washed several times to remove the residual secondary antibody.

The staining had been detected using a laser scanner (Typhoon 9410) with a laser line of 633nm. The acquisition parameters were: high sensitivity (8 times image average), pixel size 100µm, PMT 700.

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