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presented by
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**Role of Growth/Differentiation Factor (GDF) 15 in the regulation
of embryonic neural precursors.**

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A mis padres y hermano.

To Francesca, with all my gratitude.

Index:

Summary.	1
Zusammenfassung.	2
Articles from this PhD thesis:	3
Chapter 1: Introduction.	4
1.1- Definition of neural stem cells.	4
1.2- Neural precursor cells during development.	6
1.3- FGF-2/EGF responsiveness during embryonic development.	9
1.4- Transforming growth factor-beta (TFG-β) superfamily.	13
1.4.1- Bone morphogenetic proteins (BMPs).	15
1.4.2- Growth/differentiation factor 15 (GDF15).	16
1.5- Aims of the study.	19
Chapter 2: Materials and Methods.	21
2.1- Materials.	21
2.1.1- General reagents, buffers and solutions.	21
2.1.2- Cell Culture Reagents and Media.	21
2.1.3- Reagents for immunostaining.	22
2.1.4- RNA isolation.	23
2.1.5- cDNA synthesis reagents and Reverse Transcriptase-PCR.	23
2.1.6- qPCR reagents.	23
2.1.7- Primary Antibodies.	24
2.1.8- Secondary Antibodies.	24
2.1.9- Software.	24
2.1.10- Genotyping primers.	25
2.2- Methods.	26
2.2.1- Dissection of the tissue.	26
2.2.2- Primary neural precursor cell cultures.	27
2.2.3- Fluorescence activated cell sorting (FACS).	28
2.2.4- Differentiation of neurosphere-derived precursors.	28
2.2.5- BrdU incorporation on differentiating neurosphere-derived precursors.	29
2.2.6- Immunocytochemistry.	29

2.2.7- Immunohistochemistry.	30
2.2.8- Quantitative analysis of immunolabelled cells in vivo.	31
2.2.9- Fluorescence microscopy.	31
2.2.10- RNA isolation.	31
2.2.11- cDNA synthesis by RT PCR (Reverse transcription polymerase chain reaction).	32
2.2.12- Quantitative PCR.	32
2.2.13- BrdU cumulative labelling.	33
Chapter 3: Results.	35
3.1- Analysis of GDF15 expression in the embryonic and adult GE/SVZ and hippocampus.	35
3.2- GDF15 is not a mitogen and its addition at different concentrations to neurosphere cultures does not affect NPC proliferation.	38
3.3- Effect of GDF15 on proliferation and differentiation of NPC derived from the embryonic GE.	39
3.3.1- NPC derived from GDF15 ^{-/-} embryonic GE in vitro give rise to less progeny than their WT counterpart.	39
3.3.2- Absence of GDF15 leads to a decrease in EGFR expression in GE NPC.	41
3.3.3- Decrease on EGFR expression is not mediated by a change in FGF-2 signalling.	48
3.3.4- GDF15 controls cell cycle exit of NPC differentiating in vitro.	49
3.3.5- Role of GDF15 in the regulation of GE NPC in vivo.	53
3.3.6- Absence of GDF15 leads to an increase in Mash1 expression.	58
3.4- Effect of GDF15 on NPC of the hippocampus.	60
3.4.1- NPC derived from GDF15 ^{-/-} embryonic hippocampus give rise to less progeny in vitro than their WT counterpart.	60
3.4.2- Absence of GDF15 leads to a decrease in EGFR expression in hippocampal NPC.	61
3.4.3- Decrease on EGFR expression is not mediated by a change in FGF-2 signaling.	66
3.4.4- Analysis of EGFR expression in vivo.	68
3.4.5- Role of GDF15 in the regulation of hippocampal NPC in vivo.	70
3.4.7- Comparison of EGFR and PHH3 expression in vivo.	74

Chapter 4: Discussion.	76
4.1- GDF15 is expressed by NPC from embryonic and adult GE/SVZ and hippocampus.	76
4.2- GDF15 does not directly affect NPCs proliferation in vitro.	77
4.3- GDF15 directly regulates EGFR expression in NPC and not by modulation of FGF-2 signalling.	79
4.4- GDF15 promotes cell cycle exit of GE derived progenitors in vitro.	80
4.5- GDF15 provides a feed forward signal regulating the cell cycle of proliferating progenitors in the developing GE.	80
4.6- Impaired EGFR expression in GDF15^{-/-} hippocampal NPC leads to a decrease in proliferation in the hippocampal subependyma in vivo.	83
References.	87
Abbreviations.	93
Acknowledgements.	95

Summary.

TGF- β superfamily members play important roles in the regulation of multiple aspects of neural stem cell behaviour. Growth/differentiation factor 15 (GDF15), a new member of this superfamily recently cloned and characterized by our lab and others, has been shown to be expressed at low levels in the rodent brain (Böttner(a) et al., 1999) and to be particularly localised in neurogenic areas as the sub-ventricular zone (SVZ) of the lateral ventricles (Schober et al., 2001). As a follow up of this observation, in this study I investigated the possibility that GDF15 may play a role in the regulation of neural precursor behaviour during brain development.

In this work, I first demonstrated that GDF15 is expressed in neurogenic areas of the mouse brain during development and that neural precursor cells (NPCs) represent the main source of GDF15.

I next analysed a GDF15 KO / lacZ KI mouse line developed in our lab to investigate the effect of lack of GDF15 expression on NPCs. Comparative analysis between NPCs isolated from WT and GDF15^{-/-} mice revealed that absence of GDF15 leads to a decrease in the expression of EGFR in NPCs without affecting the total number of primary clone forming precursors neither in the ganglionic eminence (GE) nor in the Hippocampus. However, in the GE absence of GDF15 alters the timing of cell cycle exit of secondary progenitors differentiating from primary NPCs.

These observations in vitro were also confirmed in vivo. Analysis of brain neurogenic areas by immunohistochemistry showed that lack of GDF15 induces a downregulation of EGFR expression in neural precursor cells in both hippocampus and GE, leading to a decrease in neural precursor proliferation in the hippocampus but not affecting the proliferation of primary precursors in the GE. Instead, I found that in this region in vivo as in vitro, in the absence of GDF15 expression, secondary precursors are going extra round of proliferation leading to an increase in mash1 immunopositive cells in the SVZ and in the lateral GE.

Thus, this is the first study which describes GDF15 as a new regulatory molecule of the neuronal lineage in the developing mouse telencephalon.

Zusammenfassung.

Die Mitglieder der TGF- β Superfamilie spielen eine wichtige Rolle in der Regulierung des Verhaltens neuraler Stammzellen. Ein neues Mitglied dieser Superfamilie, der Wachstums- und Differenzierungsfaktor 15 (Growth/differentiation factor 15; GDF15), wurde kürzlich kloniert und in unserem sowie weiteren Laboren charakterisiert. Es wurde gezeigt, dass GDF15 im Gehirn von Nagetieren schwach exprimiert wird (Böttner(a) et al., 1999) und im Besonderen in neurogenen Regionen, wie der subventrikulären Zone (SVZ) der lateralen Ventrikel zu finden ist (Schober et al., 2001). Aufbauend auf diesen Ergebnissen habe ich in der vorliegenden Arbeit untersucht, ob GDF15 in der Regulierung neuraler Stamm- und Vorläuferzellen während der Entwicklung involviert ist. Dabei konnte erstmals gezeigt werden, dass GDF15 während der Entwicklung in neurogenen Regionen des Mäusegehirns exprimiert wird und dass GDF15 hauptsächlich von neuralen Stamm- und Vorläuferzellen (neural precursor cells; NPCs) gebildet wird. Des Weiteren habe ich an Hand einer in unserem Labor generierten GDF15 KO / lacZ KI Mauslinie den Effekt von GDF15 in seiner Abwesenheit untersucht. Vergleichende Analysen von isolierten NPCs aus Wildtyp- und GDF15^{-/-}-Mäusen zeigen, dass das Fehlen von GDF15 zu einer verringerten Expression von EGFR (epidermal growth factor receptor) führt. Dabei wird die Anzahl an NPCs, die primäre Klone bilden können, weder in der Ganglionic Eminenz (GE) noch im Hippocampus beeinträchtigt. Allerdings führt die Abwesenheit von GDF15 in der GE zu einem zeitlich veränderten Austritt differenzierender sekundärer NPCs aus dem Zellzyklus. Diese *in vitro* Beobachtungen konnten auch *in vivo* bestätigt werden. An Hand immunohistochemischer Untersuchungen neurogener Regionen konnte gezeigt werden, dass der Verlust von GDF15 zu einer verringerten Expression des EGF-Rezeptors in NPCs des Hippocampus als auch der GE führt. Die Herunterregulierung des EGFRs wiederum hat eine verminderte Proliferation von NPCs im Hippocampus, nicht jedoch in der GE zur Folge. Stattdessen verbleiben in dieser Region sekundäre NPCs eine zusätzliche Runde im Zellzyklus und führen dadurch zu einem Anstieg mash1-immunopositiver Zellen in der SVZ und der lateralen GE. Bei der vorliegenden Arbeit handelt es sich somit um die erste Studie, die GDF15 als einen neuen Regulationsfaktor der neuronalen Abstammungslinie im sich entwickelnden Maus-Telencephalon beschreibt.

Articles from this PhD thesis:

Growth/differentiation factor (GDF)-15 regulates cell cycle exit of secondary progenitors in the developing ganglionic eminence.

Carmen Carrillo-García, Jens Strelau, Gabi Hölzl-Wenig, Kerstin Horsch, Volker Eckstein, Klaus Unsicker and Francesca Ciccolini. Manuscript in preparation.

Growth/differentiation factor (GDF)-15 promotes proliferation of hippocampal precursors by regulating EGFR expression.

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Chapter 1: Introduction.

1.1- Definition of neural stem cells.

Stem cells (SCs) are defined by their capacity to self-renew and to differentiate into specialized cell types. During development, the differentiation capacity and self-renewal ability of SC progressively decrease, and in the adult SC are found in specialised tissue niches producing only tissue cells. For example, in the central nervous system (CNS), SC are restricted to a neural potential, giving rise only to neurons and macroglia.

The zygote and the cells derived by the first few division, up to the 8 cell stage, are totipotent SC having the capacity to give rise to any cell type, embryonic and extra-embryonic. After this developmental stage, cells within the blastocyst have a more restricted capacity as they can give rise to every cell of the organism but not to extra-embryonic structures; thus those are pluripotent SC. Multipotent SC are derived from pluripotent cells and can produce only cells of a closely related family of cells (e.g. neural stem cells). Those give rise to unipotent cells which can produce only one cell type but have the property of self-renewal, which distinguishes them from non-stem cells (e.g. neuroblasts). Therefore, the loss of totipotency is related to the development of the organism: embryonic SCs give rise to all the specialized embryonic tissues whereas in adult organisms, SC and progenitors act as a repair system for the body but also maintain the normal turnover of regenerating organs, such as blood or skin.

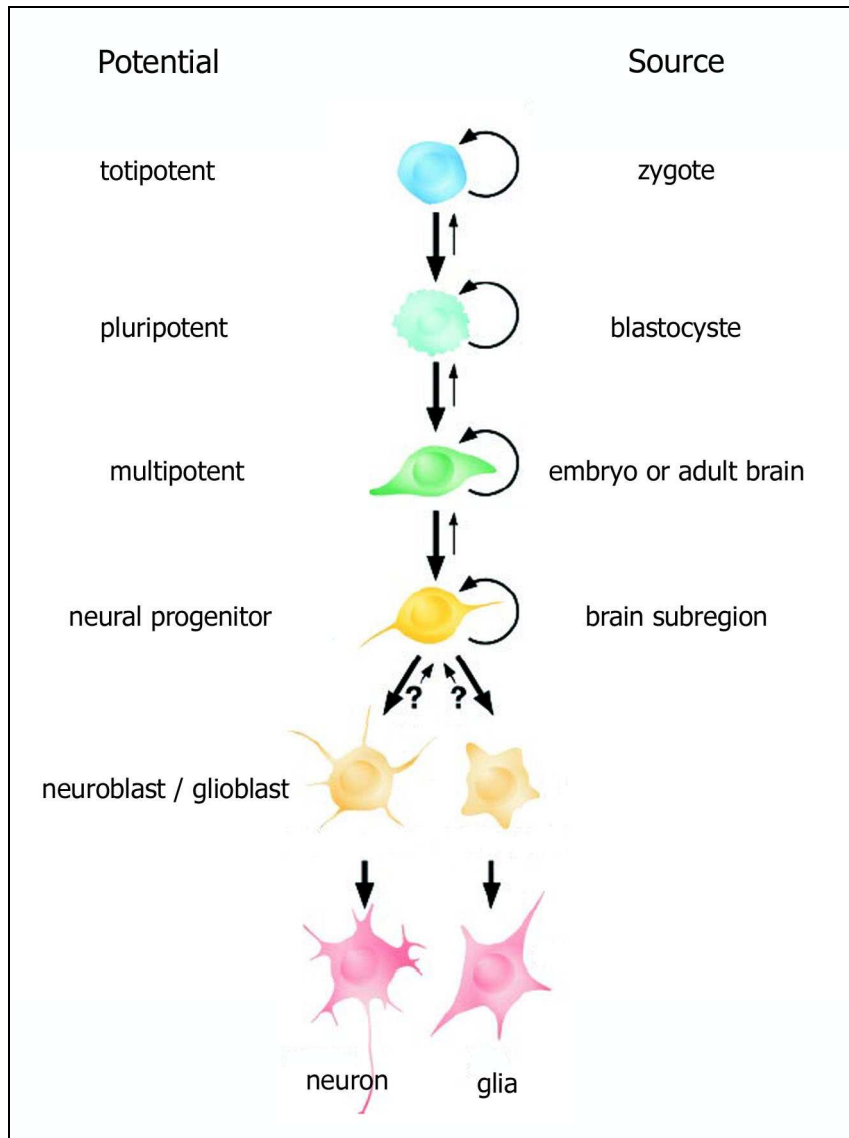


Fig 1.1- Potential of the different cell types in CNS development. During development, the differentiation capacity and self-renewal ability of SC decrease from the totipotency of the embryo to the adult, in which SC are restricted to specific niches with limited potential. Cartoon adapted from (Gage, 2000).

Neural stem cells (NSCs) are multipotent SC that can give rise to the three main cell types within the CNS: neurons, astrocytes, and oligodendrocytes. Progenitor cells are the progeny of stem cells with limited self-renewal and lineage restriction. The term “precursor cell” is used to encompass stem and progenitor cells as well as cells with undetermined but assumed degrees of stemness (Babu et al., 2007). In the embryo, NSC derive from ectodermal cells giving rise to the neural tube and upon its closure they are localised in the germinal epithelium surrounding the central cavity of the neural tube.

Instead, in the adult brain, neural precursor cells (NPC) are mainly found in two neurogenic regions, the subventricular zone of the lateral ventricles (SVZ) and the subgranular layer (SGL) of the dentate gyrus (DG) in the hippocampus. Some NSCs have been found in non-neurogenic regions as the spinal cord (Temple, 2001). Adult NPC in vivo can give rise to only certain neuronal types (as for example, granule neurons in the DG, and olfactory interneurons in the olfactory bulb) and in the case of SVZ precursors, to glial cells (Menn et al., 2006). It has been suggested a lineage-relationship between embryonic and adult NSC by which neuroepithelial (NE) cells give rise to radial glia (RG) cells during embryonic development that in turn will generate adult NSCs.

NSCs are usually identified retrospectively on the basis of their behaviour after isolation. In adherent cultures, NSC produce cell clones containing neurons, glia and more SC; but they can also be cultured as floating, multicellular neurospheres. In vivo, identification of NSC is a difficult issue as specific markers that define NSC remain elusive; so nowadays, a combination of positive and negative markers is generally used to define those cells in vivo.

1.2- Neural precursor cells during development.

The mammalian central nervous system originates from the neural plate, which is a specialised area of the ectoderm, the most external layer of the embryo. The neural plate folds during embryonic development to form the neural tube; its internal cavity will give rise to the ventricular system. The neural tube will differentiate progressively to give rise to the complete CNS. During the early phases of brain development, the anterior portion of the neural tube closes to form the vesicles that will give rise to the telencephalon, the most anterior portions of which form the lateral ventricles in the cerebral hemispheres. At the end of neurulation, the primordium of the CNS is composed of NE cells that maintain contact with both the ventricular and pial surfaces, which span the entire thickness of the neural tube. NE cells undergo a characteristic alternate movement of the nucleus between the basal and the apical surface (interkinetic nuclear migration). Mitosis occurs at the apical surface of the neuroepithelium, thereby generating a pseudostratified appearance. At early stages of embryonic development, neuroepithelial cells undergo symmetric division giving rise two identical NE cells, thereby expanding the NE population. As development proceeds, the NE thickens and

NE cells acquire some of the characteristics of astroglial cells, such as expression of the Brain Lipid Binding Protein (BLBP), the intermediate filament protein Vimentin, and the astrocytic glutamate transporter (Glast), and thereby transform into RG cells. NE and RG cells share many characteristics, as the maintenance of apical-basal polarity and the expression of the intermediate filament protein nestin and interkinetic nuclear migration (Noctor et al., 2002).

RG cells present a characteristic morphology with a short process with an end-foot contacting the ventricular surface and a long basal process in contact with the pial surface. RG as well as NE cells undergo interkinetic nuclear migration during the cell cycle, with the nucleus moving at the abventricular border of the VZ at the beginning of DNA synthesis and then during the G2 phase returning at the apical border. There, mitosis takes place. RG cells express the neural precursor marker nestin and the related marker RC2, in non rodent mammals they express the glial marker GFAP (Malatesta et al., 2008). RG cells show electrophysiological properties associated with precursor cells such as low input resistances and no voltage-dependent conductances (Kriegstein et al., 2006). Originally, it was assumed that the role of RG was to serve as scaffold for neuronal migration, guiding newborn neurons to their final destination. Recently, evidence has been provided that they are also neuronal progenitors, which give rise to new neurons. RG in the VZ generate new progenitors by undergoing symmetric cell divisions that generate new RG cells, and asymmetric divisions, which give rise to neurons that will migrate to more superficial layer along the radial glial fibre process (Fig. 1.2.1). This generates a gradient of neurons at different stages of maturation along the radial fibre. This ensemble of cells, which contains at least one proliferative RG cell and one or more neurons derived from it migrating along its process, is called a “radial clone” (Noctor et al., 2004). This system is well described in the developing cortex; whether it can be applied to neural precursors of the ganglionic eminence (GE) is still unclear. RG cells can also undergo a different type of asymmetric division, by which they give rise to neurons or to basal progenitors (BP) also called intermediate progenitors. BP, which at earlier stages of neurodevelopment are generated by apically dividing NE cells, divide symmetrically at the basal border of the VZ generating two cells which will exit the germinal epithelium and differentiate into neurons. Throughout embryonic neurogenesis BP are significant sources of neurons. Quantitative analysis suggests that the majority (between 50-95%) of early born cortical neurons are generated by BP, whereas only about 20% of upper cortical layer neurons derive from

BP (Pontious et al., 2008; Haubensak et al., 2004; Noctor et al., 2004; Miyata et al., 2004). BP are considered a sort of committed transit amplifying population deriving from NE and from RG at later stages. BP retain the apical-basal polarity, although after mitosis they retract their apical extension, but do not express astroglial markers, thereby differing from RG cells (Malatesta et al., 2008).

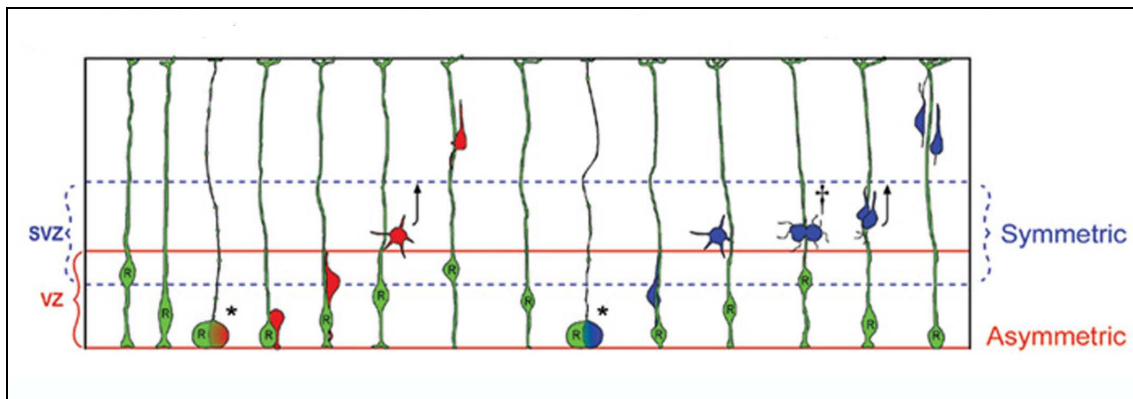


Fig 1.2.1- RG symmetric and asymmetric divisions occur in separated niches. RG (green) divide asymmetrically in the VZ to self-renew and generate a neuron (red) or an intermediate precursor (blue), which divide in the SVZ to give rise to two neurons (blue). Cartoon adapted from (Noctor et al., 2004).

By birth, the vast majority of neuronal production is complete, and RG cells in most regions of the mammalian brain disappear or transform into astrocytes (Merkle et al., 2004; Aguirre et al., 2005; Morshead and van der Kooy, 2004; Noctor et al., 2004). However, in some regions of the adult CNS such as the SGL of the DG in the hippocampus, and the SVZ in the lateral ventricles RG maintain their function as precursor cells. After birth, during early postnatal stages, RG within the GE retract their processes and transform into astrocytes that will persist as part of the neurogenic SVZ through postnatal development and into adulthood (Merkle et al., 2004; Ventura and Goldman, 2007). In hippocampal development, RG from the embryonic ventricular wall detach and move into the SGZ, where they transform into elongated stellar glial like cells and generate neurons of the granule cell layer. Production of new neurons is continuously supported throughout adulthood by the resident stem/progenitor cells of the SGZ. The granule cell layer and subgranular layer of the DG of the hippocampus are not fully established until postnatal developmental stages.

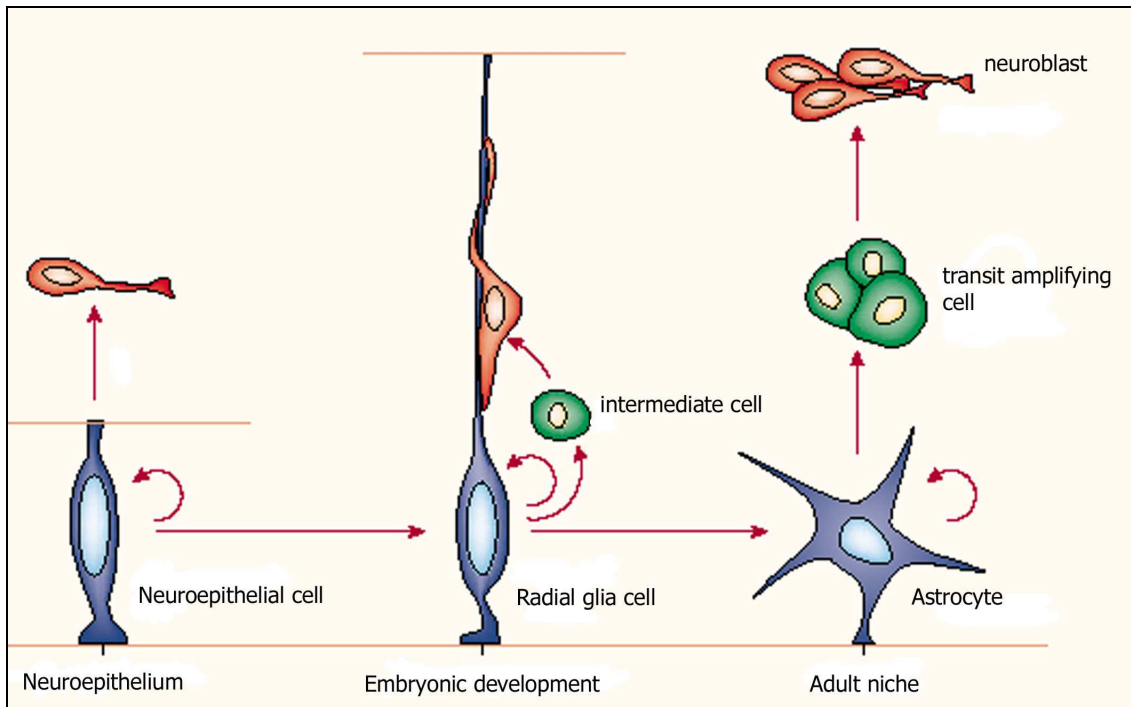


Fig 1.2.2- Neural stem cells at different developmental time points. The cells in blue represent a NSC: left, a NE cell; middle, an RG cell; and right astrocytic adult NSC. The NSC are lineage connected, NE cells give rise to RG cells which will develop into adult astrocytic NSC. Cartoon modified from (Ihrie and Alvarez-Buylla, 2008).

1.3- FGF-2/EGF responsiveness during embryonic development.

During development NPC change their ability to give rise to neuronal and glia progeny. At earlier embryonic stages, when neurogenesis is predominant, progenitor cells that are restricted to a neuronal fate are more abundant, whereas at later embryonic stages, when gliogenesis begins, glia-restricted progenitors are more abundant (see Fig 1.3.1) (Lillien and Raphael, 2000). NPCs also change their responsiveness to extrinsic signals during development. This determines whether cells respond to specific signals in their environment at distinct times and influences their response. One mechanism by which responsiveness to growth factors is regulated during development is control of growth factor receptor expression.

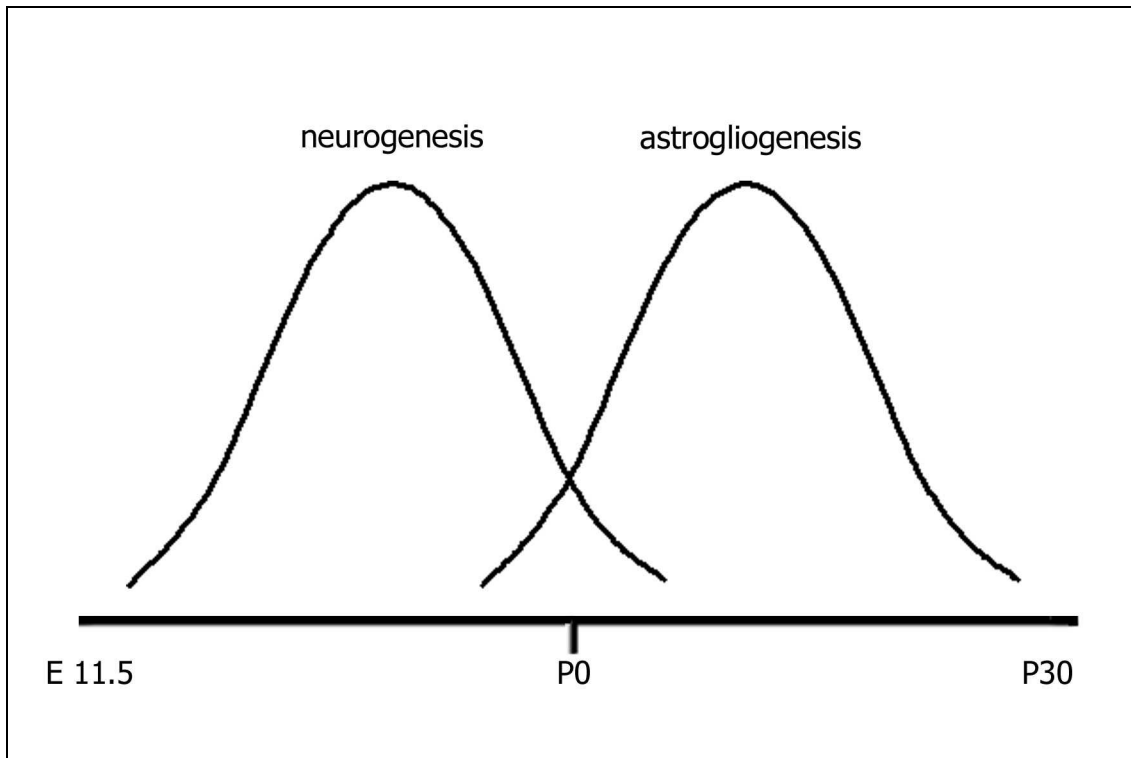


Fig 1.3.1- Scheme representing the time points, at which neurogenesis and astrogliogenesis are predominant in mouse brain development. Neurogenesis starts at early stages of embryonic development, showing a peak at middle embryonic stages, whereas astrogliogenesis starts at late embryonic stages reaching the peak shortly after birth.

Such a control has been involved in the regulation of responsiveness to epidermal growth factor (EGF) in developing NPC. Early NPC proliferate in response to fibroblast growth factor-2 (FGF-2) but not to EGF. In contrast, at later stages of development and in adult, NPC are mitotically responsive to EGF as well (Ciccolini and Svendsen, 1998; Lillien and Raphael, 2000). This change of responsiveness *in vivo* is developmentally programmed. The acquisition of EGF responsiveness is associated with the expression of high levels of EGF receptor (EGFR) protein (Lillien and Raphael, 2000; Ciccolini, 2001) and mRNA (Santa-Olalla and Covarrubias, 1999) by a subpopulation of precursors (Burrows 97). Differences in the levels of EFGR expression determine how progenitor cells interpret an extrinsic signal at specific stages of development (Lillien and Raphael, 2000).

Although these differences exist, both cell types are lineage related and are sequentially generated during embryonic development (Ciccolini, 2001). The generation of EGF responsive NPC normally begins at mid-embryonic stages of development and, at least

in vitro, it is promoted by FGF-2 (Ciccolini and Svendsen, 1998; Lillien and Raphael, 2000). As a consequence of this increase, NPC acquire the competence to respond to EGF family ligands in several ways, including proliferation and astrocytic differentiation (Burrows et al., 1997).

During embryonic forebrain development also the length of the cell cycle and mode of division of NPC change over time. Lengthening of the cell cycle increases the opportunities for NPC to respond to changes in their environment. During early development FGF-responsive cells exhibit an increase in cell cycle with embryonic age (from 17,6 hours to 26,5 hours), and their mode of division switches from being primarily symmetric at early stages to primarily asymmetric at mid-embryonic stages. Asymmetric divisions of FGF-responsive NPC take place in the VZ (Martens et al., 2000). It is not clear whether all FGF-2 responsive cells acquire EGF-responsiveness. It has been suggested that from mid-development onwards two distinct populations are present in the GE: FGF-responsive cells residing in the VZ and generating EGF-responsive cells by undergoing asymmetric cell division. It has been proposed that EGF-responsive cells migrate through the VZ to reside in the SVZ where they divide symmetrically (Martens et al., 2000). However, analysis of the pattern of EGFR expression in situ shows that EGFR is expressed in both VZ and SVZ cells, and that EGFR expressing cells in the VZ mostly represent nestin immunopositive precursors, whereas most EGFR expressing cells in the SVZ are TuJ1 immunopositive (Ciccolini et al., 2005). Furthermore, although late development precursors can be separated into two populations, i.e. EGFR^{low} and EGFR^{high}, on the basis of levels of EGFR expression, both precursor populations proliferate in response to EGF (Ciccolini et al., 2005).

Differences in EGFR expression levels influence several aspects of NPC behaviour. The ability to divide in response to EGFR activation requires the expression of high levels of the receptor. EGFR^{high} cells are multipotent and self-renew (Reynolds et al., 1992; Burrows et al., 1997) suggesting that they are NSC. Expression of high levels of EGFR also promotes NPC migration in vitro and in vivo (Caric et al., 2001; Ciccolini et al., 2005; Aguirre et al., 2005). Concentration of EGF family ligands also determine whether EGFR^{high} progenitors remain multipotent (low concentrations) or generate astrocytes at the expense of neurons and give rise to less neurospheres in culture (high concentrations). Thus, proliferation and astrocytic differentiation are a threshold response to EGFR activation (Lillien and Gulacsi, 2006).

The temporal pattern of changes in the acquisition of EGFR is the consequence of a balance between positive and negative extrinsic signals, in which bone morphogenetic protein 4 (BMP4) and FGF-2 have been implicated (Lillien and Raphael, 2000). FGF-2 is produced by progenitor cells and choroid plexus (Raballo et al., 2000), and its levels increase during mid-late stages of embryonic development (Powell et al., 1991). FGF-2 is believed to act primarily through FGF receptor 1 (FGFR1), which is expressed at early stages of embryonic development (Tropepe et al., 1999). It has been shown that in the absence of FGFR1 the expansion of FGF-2 responsive cells and the generation of EGF responsive cells are severely diminished at E14.5 (Deng et al., 1996; Tropepe et al., 1999). BMPs are produced by RG cells (Schluesener and Meyermann, 1994) and by the choroid plexus (Furuta et al., 1997), and its receptor levels decrease during development in the brain (Zhang et al., 1998). It has been suggested that a reduction in BMP4 signalling triggers the increase in FGF-2 expression in the CNS (Lillien and Raphael, 2000).

In contrast to BMP4, Wnt, Sonic Hedgehog (Shh) and FGFs promote the increase in EGFR expression (Lillien and Raphael, 2000; Viti et al., 2003). These molecules have an additional effect on NPCs in culture, decreasing (BMPs) or increasing (Wnt, Shh and FGF-2) proliferation. This effect on proliferation is concentration dependent in such a way that higher concentrations of FGF-2 or Shh are required to promote EGFR expression than to stimulate proliferation (Lillien and Raphael, 2000; Viti et al., 2003). Vice versa, higher concentrations of BMP4 are required to inhibit the ability of FGF-2 to stimulate proliferation than to block expression of EGFR (Lillien and Raphael, 2000). This shows that acquisition of EGFR responsiveness in NPC is regulated by the integration of multiple modulatory signalling molecules (Lillien and Gulacsi, 2006).

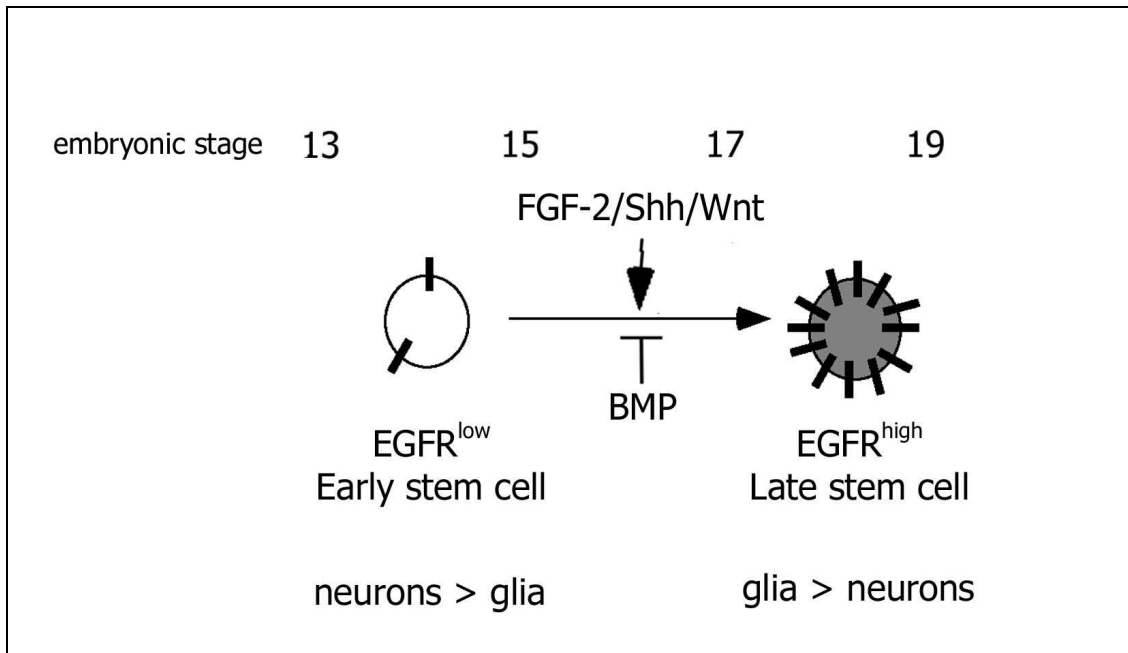


Fig 1.3.2- Model illustrating regulation of EGFR expression in embryonic NPC. At early stages of embryonic development NPCs are not responsive to EGF. During mid-embryonic stages cells acquire high levels of EGFR and thus the capacity to respond to EGF family ligands. The acquisition of EGFR is negatively regulated by BMPs and positively regulated by FGF-2, Shh, and Wnt. Early SC generate mainly neurons whereas late stem cells tend to generate more glia.

1.4- Transforming growth factor-beta (TGF- β) superfamily.

The transforming growth factor beta (TGF- β) superfamily members exert a wide range of activities regulating cell growth, differentiation, matrix formation, and apoptosis (Baek et al., 2001; Munoz-Sanjuan and Brivanlou, 2002; Mishra et al., 2005; Golestaneh and Mishra, 2005; Falk et al., 2008).

Members of this superfamily share a number of important structural characteristics. TGF β s are dimeric secreted proteins with a long propeptide separated from the mature protein by a protease acting on a conserved RXXR sequence. There are several cleavage motifs present; however cleavage usually occurs at the most downstream available site near the N-terminus. Processed TGF- β superfamily proteins all contain a highly conserved seven-cysteine domain spanning about 80 aa that encompasses most of the mature protein and forms the cysteine knot, a structural hallmark of this superfamily. There is complete conservation of the cysteine residues and their spacing in all family members. Sequence alignments based on the seven-cysteine domain are used to classify

proteins within the superfamily into individual families. The major ones are: TGF- β subfamily sensu stricto; the decapentaplegic-Vg-related (DVR) subfamily, which includes bone morphogenetic proteins (BMPs) as well as growth/differentiation factors (GDFs); the activin / inhibin subfamily; and the glial cell line-derived neurotrophic factor (GDNF) subfamily (Böttner(b) et al., 1999). GDF15 shows 15–29% sequence similarity to the other TGF- β superfamily members indicating that it is a divergent member (Bootcov et al., 1997).

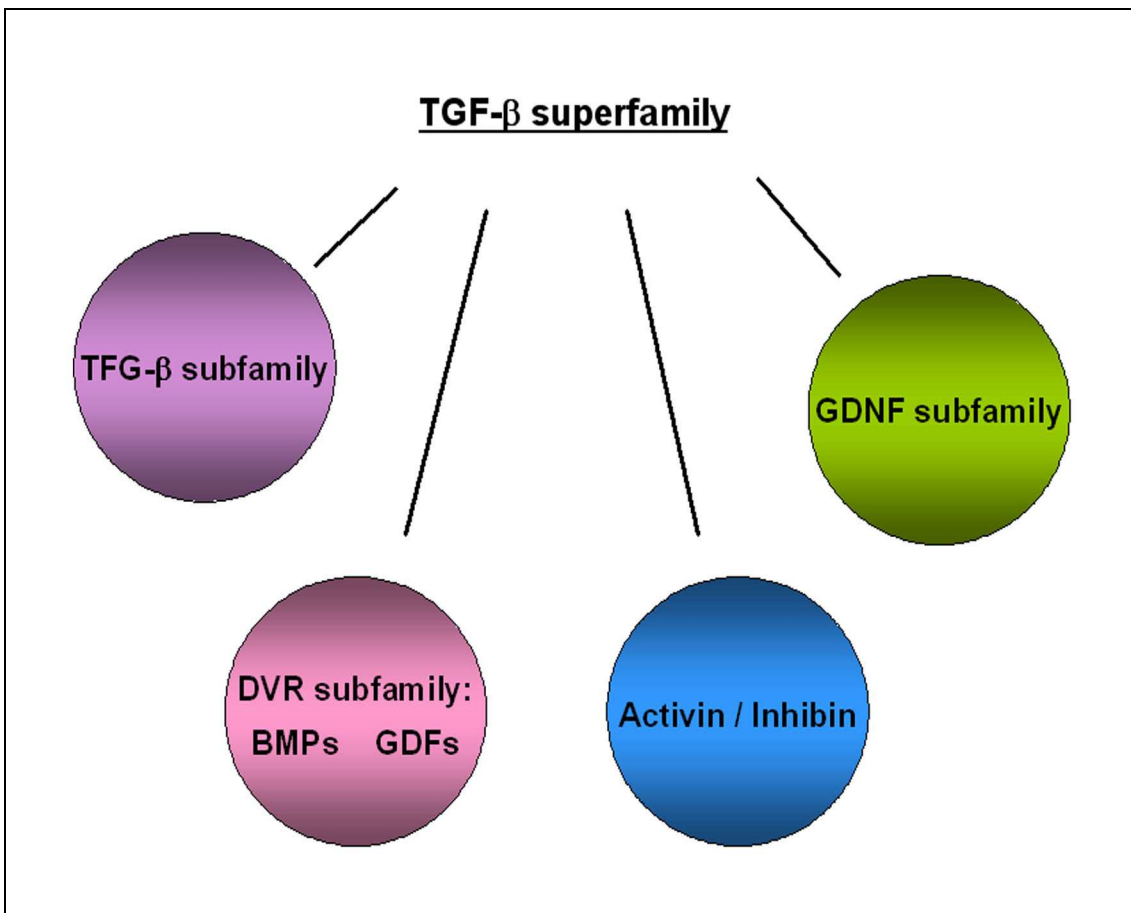


Fig 1.4.1- Cartoon illustrating the four major subfamilies within the TGF- β superfamily.

TGF- β superfamily proteins play important roles in the regulation of multiple aspects of NSC behaviour, as promoting self-renewal, preventing of stem cell differentiation, or biasing stem cell differentiation potential. The effects of TGF- β proteins depend not only on the identities of the target cell and the ligand, but also on the dosage, the differentiation state and environment of the cell (Mishra et al., 2005).

1.4.1- Bone morphogenetic proteins (BMPs).

The bone morphogenetic proteins (BMPs) are members of the TGF- β superfamily which are critical regulators of CNS development. The effect of BMPs on NPC is dependent on the stage of embryonic development. At early embryonic stages, BMP proteins inhibit cell proliferation and increase apoptosis (Furuta et al., 1997). At later embryonic stages, BMPs show a concentration-dependent activity, enhancing neurogenesis at low concentrations and promoting apoptosis at higher concentrations (Mehler et al., 2000). Finally, in perinatal and adult brain BMP signalling promotes astroglial lineage commitment (Gross et al., 1996; Mehler et al., 2000). At all developmental stages, BMPs inhibit oligodendroglial differentiation (Mehler et al., 2000). It is believed that this variability of the effect of BMP is not due to changes in the expression of BMP receptors, as the different BMP receptor subunits are widely expressed by progenitor cells in the embryonic, postnatal, and adult germinal zones (Mehler et al., 2000).

Noggin is a naturally expressed inhibitor that binds to BMPs with high affinity, preventing them from binding to cell surface receptors thereby inhibiting BMP signal transduction (Li and LoTurco, 2000). It has been proposed that a crosstalk between noggin and BMP regulates the differentiation of NPC at different stages prior and after birth (Chen and Panchision, 2007). Noggin inhibits neurogenesis and gliogenesis in early and late NPC, respectively (Li and LoTurco, 2000; Lim et al., 2000), and enhances the formation of oligodendrocytes at every stage of development, suggesting a direct inhibition of BMPs in oligodendrogenesis (Mehler et al., 2000; Colak et al., 2008).

As discussed above, during embryonic brain development BMP4 regulates responsiveness to EGF in embryonic NPC (Lillien and Raphael, 2000). Also the effect of BMPs on NPC is age dependent. Early embryonic NPCs (FGF-responsive) undergo apoptosis or neuronal differentiation depending on the developmental stage and BMP concentration (Mabie et al., 1999); whereas in late and perinatal NPCs (EGF-responsive), BMPs promotes the commitment to the astroglial lineage (Gross et al., 1996).

1.4.2- Growth/differentiation factor 15 (GDF15).

GDF15 is a newly identified member of the TGF- β superfamily, which shares only about 25% sequence identity with other family members. However, it does contain the characteristic consensus cleavage signal for processing the immature pro-form to the active secreted protein (Baek et al., 2001).

In the literature, GDF15 is also known by other names including: macrophage inhibitory cytokine 1 (MIC-1) (Bootcov et al., 1997), nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) (Baek et al., 2001), prostate-derived factor (PDF) (Paralkar et al., 1998), placental transforming growth factor-b (PTGFB) (Lawton et al., 1997), and placental bone morphogenetic protein (PLAB) (Hromas et al., 1997).

The genes of rat, mouse and human GDF15 have been isolated by screening genomic libraries and its comparison revealed a conserved structure consisting of two exons separated by one intron of approximately 3.0 kb, which interrupts the coding sequences within the prepro-domain of the proteins at identical positions. Close to the putative translation start codon there is a conserved TATA-like motif. However, the orthologous GDF15 molecules show the lowest (70%) similarities between rodent and human of all members of the TGF- β superfamily (Massague, 1990). Moreover, GDF15 represents a divergent member of the TGF- β superfamily, which shares only 25% of sequence homology with other family members. This is the lowest degree of sequence conservation within this superfamily, revealing that GDF15 is a distant member, which has been located phylogenetically and structurally next to GDF9 (Böttner(b) et al., 1999).

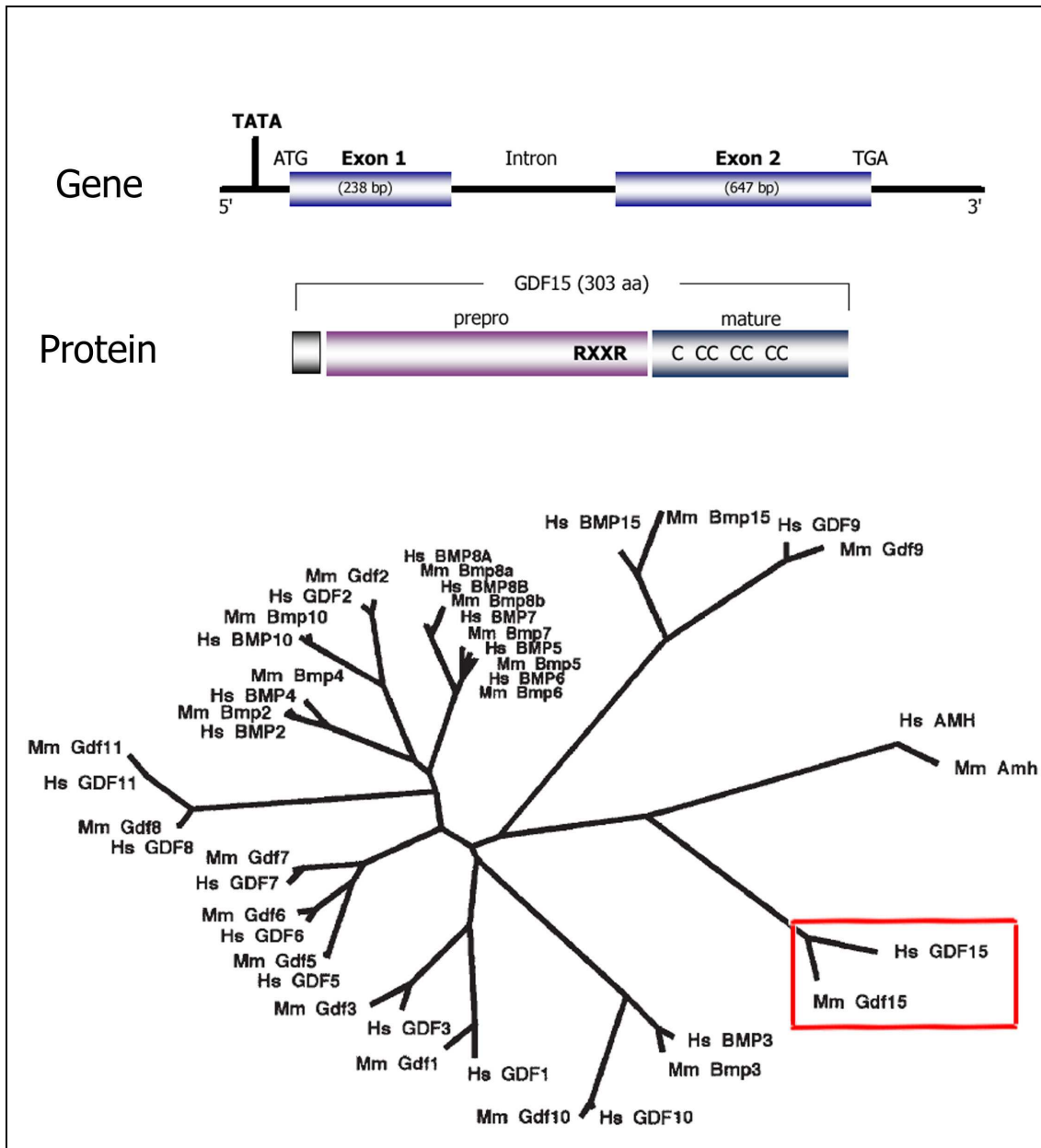


Fig 1.4.2.1- Top: Schematic representation of GDF15 gene and protein structure. Bottom: Phylogenetic tree representing the BMP and GDF families. GDF15 is a divergent member, whose closest related TGF β superfamily member is GDF9.

The GDF15 protein is produced in mouse as a 303-amino acid (aa) polypeptide that includes a 29-aa signal peptide, a 167-aa propeptide (amino terminal) and a 115-aa mature region (carboxy terminal). The protein is generated as a 40-kDa propeptide from which the N-terminus is cleaved and a 30-kDa disulfide-linked homodimer is secreted as the active form (Fairlie et al., 2001). In the carboxy-terminal region, the protein contains nine cysteine residues, seven of which form the characteristic cystine knot

conserved among the superfamily members. The cystine residues of the cystine knot form disulfide bonds (Daopin et al., 1992). The mature protein undergoes disulfide-linked dimerization in the endoplasmic reticulum. Interestingly, in GDF15 the propeptide is not required for the correct folding and secretion of the mature peptide, which has been seen to be essential in other TGF- β superfamily members as is the case of TGF- β 1 or BMP2 (Fairlie et al., 2001). Nevertheless, the propeptide is required as a quality control as only correctly folded and dimerized proteins leave the endoplasmic reticulum for the Golgi apparatus, where it is thought that the proteolytic cleavage at the conserved cleavage site, separating the propeptide from the mature domain, probably occurs.

Like many TGF- β superfamily cytokines, GDF15 is widely expressed, but under normal conditions, placenta and liver are the only tissues expressing large amounts of the protein (Fairlie et al., 2001). Epithelial cells, in a wide variety of other organs such as prostate and colon, express lower amounts of GDF15 mRNA. GDF15 expression is, however, dramatically increased in inflammation and injury. Increased GDF15 expression is a feature of malignant cell transformation, suggesting that it may play a role in controlling cell proliferation. In particular, high levels of GDF15 have been observed in many cancers including breast, colon, pancreas, and prostate tumours. In rodent brain, GDF15 is expressed at low levels in the choroid plexus, subventricular areas and in a cell population between the hippocampal fimbria and the dorsal thalamic area within the lamina affixia (Böttner(a) et al., 1999; Schober et al., 2001). This localization within neurogenic areas suggests a possible role in NPC development.

Many studies have revealed the different roles of GDF15 in various pathologies and cancers: it has been described to be a biomarker for p53 pathway activation (Yang et al., 2003); predictor of miscarriage (Tong et al., 2004); a cardioprotective cytokine (Kempf et al., 2006) and to have a pro-apoptotic role in different cancer cell types as prostate or epithelial tumour cell lines (Liu et al., 2003; Bauskin et al., 2005). The very high level of GDF15 mRNA in placenta and in serum during pregnancy in general suggests a generalized role not only in embryo implantation and placental function but also in other aspects of this process (Fairlie et al., 2001). In the lesioned rat brain, the protein levels are up-regulated in neurons following brain lesion, and GDF15 has been suggested to have a protective effect (Schober et al., 2001). It is also a very effective neurotrophic factor for embryonic dopaminergic neurons isolated from the midbrain

floor and a neuroprotective factor for substantia nigra dopaminergic neurons in a model of Parkinson's disease (6-OHDA) (Strelau et al., 2000). GDF15 also promotes survival of cerebellar granule neurons in culture (Subramaniam et al., 2003) and in sensory neurons of dorsal root ganglia (Strelau et al., 2000).

To study further possible roles of GDF15 *in vivo*, our lab generated a GDF15^{-/-} mouse/lacZ KI (Strelau and Unsicker, unpublished). The mice are viable and fertile with apparently no distinguishing phenotype except the fact that, compared to their WT littermates, the body weight of mutant mice has a greater fat component. This observation is consistent with the fact that the GDF15 overexpressing mice show a smaller body weight and a reduced body fat than their wild type (WT) littermates. It is not clear whether this effect of GDF15 is a consequence of its regulation of the appetite (Baek et al., 2006; Johnen et al., 2007).

In the present study, I have taken advantage of the availability of GDF15 mutant mice to investigate the potential role of GDF15 in regulating the behaviour of embryonic NPC. I have focussed on neural precursors derived from the GE and the hippocampus.

1.5- Aims of the study.

Previous studies have shown low levels of GDF15 expression in the rodent brain (Böttner(a) et al., 1999). GDF15 expression is particularly localised in neurogenic areas as the SVZ of the lateral ventricles (Schober et al., 2001), suggesting that GDF15 may influence NPC behaviour. The overall aim of this study is to investigate the role of GDF15 in regulating precursor behaviour in neurogenic areas of the embryonic mouse brain. To this end, I have investigated the following specific issues:

- 1- Is GDF15 expressed in NPCs from the mouse brain?
- 2- Does GDF15 have a mitogenic effect on NPCs?
- 3- Does GDF15 affect *in vitro* proliferation and/or differentiation of GE and hippocampal derived NPC?
- 4- Does GDF15 affect NPC behaviour in the GE and hippocampus *in vivo*?

I first studied the pattern of expression of GDF15 in mouse brain at different developmental stages and in isolated NPC using real time-PCR. Next, I investigated whether GDF15 affects proliferation, differentiation and survival of putative NPCs

using bulk and clonal cultures from GE and hippocampus derived cells. For this analysis I have also taken advantage of the availability of a mouse line lacking GDF15 expression to compare NPCs cells derived from mutant and WT animals. Purification of NPCs by FACS (Ciccolini et al., 2005) allowed me to directly study the properties of NPC. This analysis revealed that in both regions, GE and hippocampus, NPCs represent the main source of GDF15, and that absence of GDF15 leads to a decrease in the expression of EGFR in NPC but does not affect the total number of primary clone forming precursors. I have also found that in the absence of GDF15 GE derived differentiating progenitors undergo an extra round of proliferation causing a delay in neuronal differentiation. In the last part of this study, I have designed experiments to investigate the role of GDF15 in vivo in both GE and hippocampal formation, in light of my in vitro findings. My results suggest that in vivo as in vitro absence of GDF15 downregulates EGFR expression in NPC leading to a decrease in NPCs proliferation in the hippocampus. In the GE I found that despite EGFR downregulation, the proliferation of primary precursors in the VZ is not affected. Instead I found that in this region in vivo as in vitro secondary precursors are undergoing an extra round of proliferation leading to an increase in mash1 expressing cells in the SVZ and in the lateral GE.

Chapter 2: Materials and Methods.

2.1- *Materials.*

2.1.1- General reagents, buffers and solutions.

Aqua Brawn	Brawn
Ethanol	J.T. Baker
Glucose	Merck
MilliQ Purificated Water	Millipore
PBS (Phosphate buffered saline)	
10 mM Phosphate	AppliChem
137 mM Sodium chloride	Prolabo
2,7 mM Potassium chloride	J.T. Baker
pH 7.4	

For the preparation of aqueous solutions desalted water from a “MilliQ Water Purification System” (Millipore) was used.

2.1.2- Cell Culture Reagents and Media.

B-27	Gibco
BrdU	Roche
EGF*Alexa 488	Molecular probes
Euromed-N medium	Euroclone
FCS	BioWhittaker
GDF15 human	R&D
Glucose	SIGMA
Human recombinant EGF	Peptotech
Human recombinant FGF-2	Peptotech
Leibovitz medium	Gibco
L-Glutamine	Gibco
Penicillin/Streptomycin	Gibco
PI (propidium iodide)	SIGMA
PL-Ornithin	SIGMA

Trypan Blue SIGMA

Culture medium:

Euromed-N medium
100 U/ml penicillin/streptomycin
2 mM Glutamine
2% B27
20 ng/ml EGF
10 ng/ml FGF-2

Sorting medium:

1:1 Euromed-N/Leibovitz
2% B27
1% FCS
10 ng/ml FGF-2
30% Glucose

Differentiation medium:

Euromed-N medium
100 U/ml penicillin/streptomycin
2 mM Glutamine
2% B27
1% FCS
10 ng/ml FGF-2

2.1.3- Reagents for immunostaining.

Ammonium chloride	Merck
DAPI (4', 6-diamidino-2-phenylindole)	Boehringer
Glycine	Sigma
HCl	J.T. Baker
Horse serum	Gibco
Mowiol®	Calbiotech
NP-40	CN Biomedicals Inc.
PFA (Paraformaldehyde 4% w/v)	Fluka

Roti [®] -liquid barrier marker	Roth
Sodium tetraborat	Merck
Sucrose	Riedel-deHaën
TissueTec [®]	Sakura
Triton-x-100	Merck

Blocking solution:

- 0,1% Triton-x-100
- 1% BSA
- 1,5% Horse serum
- 5% FCS
- PBS

2.1.4- RNA isolation.

Chloroform	J.T. Baker
Isopropanol	Merck
TriFast [®]	Peqlab

2.1.5- cDNA synthesis reagents and Reverse Transcriptase-PCR.

5X Buffer Transcription Buffer	Promega
dNTPs	Promega
First strand buffer	Promega
M-MLV Reverse Transcriptase	Promega
Random Hexamer Primers	Promega
RNase inhibitor	Promega
RQ1 DNase (RNase free)	Promega
Taq DNA polymerase	Promega

2.1.6- qPCR reagents.

Assay Mix	Applied Biosystems
Assays-on-Demand (AOD)	Applied Biosystems
β-Actin	Applied Biosystems
18s	Applied Biosystems

FGF receptor 1	Applied Biosystems
FGF receptor 2	Applied Biosystems
FGF-2	Applied Biosystems
GAPDH	Applied Biosystems
GDF15	Applied Biosystems
TaqMan [®] Universal PCR Master Mix	Applied Biosystems

2.1.7- Primary Antibodies.

Mouse anti-BrdU	Roche	1:10
Mouse anti-O4 (a gift of Dr. Jackelin Trotter, University Mainz, Germany)	selfmade	1:100
Mouse anti-Tuj1	SIGMA	1:400
Rabbit anti-PHH3	Upstate	1:500
Sheep anti-EGFR	Upstate	1:50

2.1.8- Secondary Antibodies.

Donkey α sheep IgG*Cy3 against sheep α EGFR	Dianova	1:500
Goat α rabbit IgG*Alexa 488 against rabbit α PHH3	Molecular Probes	1:1000
Goat α mouse IgG*Alexa 488 against mouse α Tuj1 mouse α BrdU	Molecular Probes	1:1000
Goat α mouse IgM*PE α mouse anti-O4	Jackson ImmunoRes. Lab.	1:200

2.1.9- Software.

ABi Prism [®] 7000 SDS v1.1 with RQ Study Application v1.1	Applied Biosystems
Acrobat Reader v5.1.0	Adobe
Adobe Photoshop v6.0.1	Adobe
AxioVs40 V 4.5.0.00	Carl Zeiss Imaging
EndNote v8.0	Thomson ISI Researchsoft

FACSVantage and FACS Aria sorter	Becton Dickinson
GraphPad PRISM v.3.03	GraphPad Software
Image Tool v3.00	UTHSCSA
ImageJ 1.36b	National Institutes of Health, USA.
Microsoft® Windows XP Home Edition:	Microsoft
(Excel, Word, Power point)	

2.1.10- Genotyping primers.

Sequences of the genotyping primers for the GDF15^{-/-} mice:

5INTEX2

Gene	Primers	Expected band
GDF15	forward primer F30: 5'-TTG GGA AAA GGT TGG AGA GA-3' reverse primer R18: 5'-GAT ACA GGT GGG GAC ACT CG-3'	806bp
LacZ	forward primer F21: 5'-GCA GAG AGG CTG AGG AAC TT-3' reverse primer LacZR4: 5'-GTT CTT GTT GGT CAA AGT AAA CGA C-3'	1810bp

NEOEX2

Gene	Primers	Expected band
GDF15	forward primer F26: 5'-ATG CGC ACC CAA GAG ACT-3' reverse primer R21: 5'-GGC CAC CAG GTC ATC ATA AG-3'	320bp
LacZ	forward primer NeoF1: 5'-TCG CCT TCT TGA CGA GTT CT-3' reverse primer R20: 5'-CCC AGT CTT GTA GAC AGA GCA A-3'	690bp

2.2- Methods.

2.2.1- Dissection of the tissue.

Embryos: Time-mated pregnant (plug day =1.0) C57/Bl6 mice (Charles River) were killed by increasing CO₂ concentration followed by neck dislocation. Brain dissection was done in ice cold Euromed-N basal medium. NPC were obtained from dissecting GE and hippocampus (and/or cortex where specified) of embryonic day 14, 16 and 18 (E14, E16 and E18)

Young and adult: Adult (8 weeks) C57/Bl6 (Charles River) and GDF15 mutant mice were killed by increasing CO₂ concentration followed by neck dislocation, whereas postnatal (1 week) mice were killed by decapitation. Brains were removed from the skull and the SVZ region and the hippocampus (and cortex where specified) were dissected out, placed in cold Euromed-N basal and used for further experiments.

Transgenic animals and Genotyping.

The GDF15 knock-out/lacZ knock-in mouse (GDF15^{-/-}) was generated in the lab of Prof. K. Unsicker by Dr. J. Strelau. GDF15^{+/-} males were mated with GDF15^{+/-} females to obtain littermate embryos of the three genotypes (WT and GDF15^{-/-} homozygous and heterozygous). Tail biopsies were used for genotyping.

Pure genomic DNA was isolated using the GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manufacturer's protocol. A piece of tail from each mouse was cut and frozen at -20°C. Before DNA extraction, the sample was allowed to thaw slightly on ice to protect against degradation. Then, 180 µl of Lysis Solution T and 20 µl of Proteinase K were added to each sample and incubated at 55°C for 6 hours to digest the tissue. After digestion, the samples were incubated with 20 µl of RNase A Solution during 2 minutes at room temperature to obtain an RNA-free genomic DNA. Cells were then lysed by mixing the samples with 200 µl of Lysis Solution C. The lysate was transferred into a GeneElute Miniprep Binding Column (previously pre-assembled and prepared with Column Preparation Solution) and centrifuged at 6500 g for 1 minute. The column was washed 3 times with 500 µl of Wash Solution and 1 minute centrifugation at 6500 g each time. Then DNA was eluted by addition of 200 µl of Elution Solution and 1 minute centrifugation at 6500 g after 5 minutes of incubation at room temperature to increase the elution efficiency. Two µl of the eluted DNA were used for PCR amplification purposes. Mice were genotyped by

two different PCR reactions using primers specific for the WT and the GDF15^{-/-} allele. 15 µl of each sample were loaded on 1% (5IntEx2) and 1,5% (NeoEx2) agarose gels prepared according to the standard procedures and run at 120 V until the gel front reached the bottom of the gel. Then gels were stained in a 1% ethidium bromide bath for 10 minutes and destained for further ten minutes in a water bath. Bands were then visualized under a UV detection system. PCR mixture with water was used as a negative control, and samples with a known genotype were used as a positive control.

2.2.2- Primary neural precursor cell cultures.

Serum-free CNS stem cell cultures represent a selective system in which most primary differentiated neural cells are eliminated at early stages of culturing, whereas the undifferentiated precursors including SC enter into an active proliferation state (Gritti et al., 2001). Many parameters may influence growth efficiency, but at least some conditions should be satisfied for the NPC to become the main cell type in these cultures: the absence of serum; the addition of the appropriate mitogens, i. e, EGF (20 ng/ml) and FGF-2 (10 ng/ml); and the absence of an adherent substrate.

Tissue was transferred into culture medium consisting of ice cold Euromed-N basal serum free culture medium consisting of, Penicillin-Streptomycin (100 U/ml), glutamine (2 mM) and 2% B27 supplement and gently triturated with a fire-polished Pasteur pipette. To induce NPC proliferation human recombinant EGF and FGF-2 were added to the culture medium at a concentration of 20 ng/ml and 10 ng/ml, respectively.

2.2.2.1- Neurosphere cultures.

Following dissociation, cells were plated at a density of 10⁵ cells/ml in 24 well plates in culture medium in the absence (as control) and presence of GDF15 (10 ng/ml). Cells were fed with 1/2 the volume of fresh culture medium every four days. NPCs were allowed to proliferate in suspension culture for a week. During this time they formed floating cell clusters, termed neurospheres, consisting of aggregated proliferating NPCs and more differentiated cells. Total cell number was counted after one week in culture.

2.2.2.2- Primary clonal cultures.

After dissociation cells were plated at a density of one cell per well in 96 well/plates containing 50 µl culture medium supplemented with EGF and FGF-2 in presence or absence of GDF15 (10 ng/ml). Sorted cells were plated using a FACS automated cell

deposition unit under the conditions mentioned above. Cultures were kept in the incubator for a week during which a subset of plated NPCs (putative neural stem cells) proliferate and give rise to clones. After 7 days the clones per plate were counted to estimate the frequency of original NPCs.

2.2.2.3- Secondary clonal cultures.

To determine the self renewal ability of NPCs single spheres from clonal cultures were transferred to eppendorf tubes, mechanically dissociated by trituration, and re-plated in 96 well plates in EGF and FGF-2 supplemented culture medium at a density of 10^3 cells per well. Secondary neurospheres were scored after 7 days in vitro.

2.2.3- Fluorescence activated cell sorting (FACS).

Freshly dissected tissue was mechanically dissociated and cells were resuspended in sorting medium consisting of Euromed/Leibovitz medium (1:1), 2% B27, 1% FCS, 10 ng/ml human recombinant FGF-2 and 30% glucose immediately after dissociation or 24 hours after incubation in FGF-2 culture medium. The cell suspension was filtered using polypropylene round-bottom tube with cell strainer cap and samples were stained by adding an equal volume of sorting medium containing EGF (40 ng/ml) conjugated to a fluorophor (Alexa 488). Sorting gates were set using unstained cells and cells that had been incubated in culture medium with unlabelled EGF for at least 20 minutes previous to the staining with EGF Alexa. Viable cells were revealed by propidium iodide exclusion (PI 1 μ g/ml). Sorting gates were set to collect cells displaying the strongest (EGFR^{high}) and the lowest (EGFR^{low}) EGF alexa fluorescent signal. Cells were sorted using a FACSVantage and a FACSAria sorter (Becton Dickinson).

2.2.4- Differentiation of neurosphere-derived precursors.

Following one week in culture, neurospheres were collected by centrifugation at 800 rpm for 3 minutes. The medium was discarded and the cell pellet was re-suspended in 3 ml of ice-cold PBS containing 0,6% of glucose and kept on ice for 5 minutes. During this step, cell-cell adhesion weakens in the absence of divalent ions and a single cell suspension can be obtained with a milder trituration procedure. Neurospheres were then centrifuged at 800 rpm for 3 minutes and the pellet was re-suspended in 200 μ l of culture medium and mechanically triturated through a fire-polished Pasteur pipette. Differentiation was induced by plating 5×10^4 neurosphere-derived cells in

differentiation medium, consisting of culture medium supplemented with 1% foetal calf serum and 2 ng/ml FGF-2, onto polylysine coated chamber slides. Cells were left in differentiating conditions for up to 14 days. The phenotype of differentiated cells was determined by immunocytochemistry at day after plating (DAP) 7, 10 and 14 using antibodies against type III tubulin (TuJ1), O4 and 5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for nuclear counterstain.

2.2.5- BrdU incorporation on differentiating neurosphere-derived precursors.

Differentiating cultures were exposed to BrdU for 16 hours at various time-points (DAP2, 4 and 7). BrdU was directly added into the media to a final concentration of 6,7 µg/ml to avoid cytotoxicity. After 16 hours, cells were processed for immunocytochemistry as described below.

2.2.6- Immunocytochemistry.

Cells were fixed with 3% paraformaldehyde (PFA) in PBS containing 4% sucrose for 10 minutes and rinsed several times in PBS at room temperature. Cells were permeabilised with NP-40 (0,5% in PBS) for 5 minutes. For BrdU stainings, cells were incubated with HCl 2 N for 30 minutes in order to denature the DNA followed by neutralization washes with sodium tetraborate 0,1 M pH 8,5 for 30 minutes more. After permeabilisation, cells were incubated with primary antibodies overnight at 4°C. Next day, cells were washed with PBS, 2X5 min, to washout extra primary antibody and then incubated with fluorescently labelled secondary antibodies for 1 hour. Cells were washed 2X5 minutes with PBS and rinsed with water to remove PBS. Excess water was removed by gently tilting and tapping the chamber-slide or coverslip on a tissue paper. 5 µl of Mowiol was placed on each well of a chamber-slide and a glass coverslip was gently placed on top of it. Chamber- slides or coverslips were stored at 4°C in darkness to preserve fluorescence.

For O4 detection, cells were incubated for one hour with anti O4 antibody added to the media. After the incubation time, cells were fixed, permeabilised and incubated directly with the secondary antibody as described before.

Immunopositive cells were counted by taking pictures of 5 to 10 culture fields for each animal. DAPI and immunopositive cells were scored using Image Tool v3.00

(UTHSCSA) software to estimate the number of positive cells as percentage of total cells.

2.2.7- Immunohistochemistry.

Whole brains were removed and fixed by immersion in 4% PFA overnight. Tissue was cryoprotected by transferring it into 30% sucrose o/n and then freezed in Tissue-Tek[®] and kept at -80°C. Brains were cryosectioned by sequential coronal sections of 16 µm thick using a Leica CM3050S cryostat, placed on Super-Frost[®] Plus Microscope Slides (Menzel-Glaser[®]) coated slides and freezed and kept at -20°C until immunohistochemistry was performed. Immunohistochemistry was developed in wet chambers to avoid exsiccation of the samples. Sections were defrosted at room temperature for 15 minutes and then rehydrated with PBS for another 15 minutes. To avoid leaks of the solutions, slices were encircled with a barrier marker creating a water repellent barrier around the tissue. Slices were permeabilised by a 10 minutes wash with NP-40 (0,5% in PBS). For BrdU stainings, the DNA was denatured by a 30 minutes incubation with HCl 2 N followed by neutralization with sodium tetraborate 0,1 M pH 8,5 for at least 30 minutes. Afterwards slices were incubated with glycine for 30 minutes (0,1M in PBS) followed by 30 minutes incubation with ammonium chloride (50 mM in PBS) to quench the autofluorescence. Afterwards, the tissue was incubated for 60 to 90 minutes in a blocking solution consisting of: 5% FCS in PBS or 1,5% horse serum, 1% BSA and 0,1% Triton-x-100 in PBS. After the blocking step, sections were processed for immunostaining by incubation with primary antibodies diluted in blocking solution overnight at 4°C.

Next day, sections were washed, 3X10 minutes, with PBS and then incubated with fluorescently labelled secondary antibodies for 1 hour at room temperature. Samples were then washed 3X 40 minutes with PBS. DAPI was added to stain cell nuclei and then samples were washed 10 minutes with PBS and rinsed with water to remove PBS. Excess water was removed by gently tilting and tapping the slide on a tissue paper. Mowiol was placed on each slide and a glass coverslip was gently placed on top of it. Then, slides were stored at 4°C in darkness to preserve fluorescence.

2.2.8- Quantitative analysis of immunolabelled cells in vivo.

Series of coronal sections were collected for immunostaining. For each mouse immunostained cells were counted in 3 16 μm sections separated from each other by five intervening sections (80 μm in total). Care was taken to select sections at comparable rostrocaudal levels of the aSVZ and hSVZ. After, immunostaining fluorescent micrographs from the appropriate rostrocaudal levels were taken to perform a quantitative analysis. The micrographs were taken using a 20x or 40x objectives and the number of positive cells was counted. For the quantification in the CA3 and the hilar field of DG the entire regions were counted. In the CA1 and in the aSVZ, cells were counted within a square region of interest with an area of 50 μm^2 . The aSVZ was divided into three subregions: the “apical border”, consisting of the first two layers of cells lining the ventricle, the VZ representing the adjacent area included into a 50 μm^2 square, and the SVZ in which cells were also quantified using a 50 μm^2 square. In the striatum, cells were quantified within a region of interest of about 40000 μm^2 . For each section cell counts in the striatum were obtained by pooling the number of cells scored within three areas of interest.

For each group at least three mice were analysed in order to obtain statistical significance. Immunostaining with only secondary antibodies were carried out as negative controls.

2.2.9- Fluorescence microscopy.

Samples immunostained with fluorescently labelled antibodies were analysed using Zeiss Axioplan 2 imaging microscope (Zeiss, Germany) equipped with a AxioCam digital camera (AxioCam HRc Zeiss, Germany) under control of AxioVision software (AxioVs40 V 4.5.0.00, Carl Zeiss Imaging, Germany). Photomicrographs from different fields were captured under different filters according to the dye used. Cell counting was performed using Image Tool v3.00 (UTHSCSA) software or manually.

2.2.10- RNA isolation.

RNA isolation was performed using the TriFast[®] reagent from peqlab following the manufacturer’s instructions. TriFast[®] reagent was added directly to the tissue/cell pellet or to the cell culture dish when sample cells were cultured in the presence of a substrate. Samples were homogenised by repeated pipetting and trituration with a “Penökel”

homogenizer. The homogenate was then incubated for 5 minutes at room temperature to allow dissociation of the nucleoprotein complexes. 200 µl of chloroform per 1 ml of TriFast[®] were added to the homogenate and samples were shaken vigorously to mix the reagents and then incubated for 10 minutes at room temperature. The mixture was separated into two phases by centrifugation at 4°C with 12.000 g for 15 minutes. The RNA contained in the upper aqueous phase was then transferred into a new tube and precipitated with 500 µl isopropanol per 1 ml of TriFast[®] for 15 minutes at room temperature or overnight at -20°C. The RNA was pelleted by centrifugation at 4°C and 12.000g for 10 minutes, rinsed with 70% ethanol, dried and resuspended in water (Aqua Braun). RNA concentration was measured by optic densitometry using an eppendorf BioPhotometer[®]. RNA samples were stored at -80°C.

2.2.11- cDNA synthesis by RT PCR (Reverse transcription polymerase chain reaction).

For each reaction 2 µg of total RNA were incubated with 0,5 µl of RNAase inhibitor (40 u/µl, Promega) and 0,5 µl of RQ1 DNase (1 u/µl, Promega) for 15 minutes at 37°C in a thermocycler (eppendorf Mastercycler gradient). The mixture was heated to 70°C for 5 minutes and then slowly cooled down to room temperature. Reverse transcription was performed by adding to the samples the following reaction mixture:

8 µl first strand buffer (5x)
4 µl dNTP (40 mM, each nucleotide 10 mM)
2 µl random hexamer primers (500 ng/µl)
2 µl M-MLV (reverse transcriptase, 200 u/µl)
3 µl water (Aqua Braun)
Total volume 19 µl

Samples were incubated for 2 hours at 37°C, heated to 90°C for 5 minutes and stored at -20°C for further use.

2.2.12- Quantitative PCR.

qPCR analysis based on the TaqMan[®] methodology was performed using an ABI Prism[®] 7000 Sequence Detection System (Applied Biosystems) under control of ABI Prism[®] 7000 SDS Software v1.1 with RQ Study Application v1.1 (Applied Biosystems).

Genes of interest GDF15, FGF-2, FGF receptors 1 and 2, and housekeeping genes 18s, GAPDH and β -Actin gene-specific TaqMan[®] probes and primer sets were obtained from Applied Biosystems as Assays-on-Demand (AOD) gene expression products. The AOD ID's were GDF15, Mm00442228-m1; FGF-2 Mm00433287-m1; FGFR1 Mm00438923-m1; FGFR2 Mm00438941-m1; 18s, Hs99999901-s1; GAPDH, Mm00000015-s1; β -Actin, Mm00607939-s1.

Two μ g of total RNA isolated using the TriFast[®] reagent (peqlab) were used for synthesis of complementary DNA (cDNA) by Reverse transcription polymerase chain reaction as previously described.

The qPCR reaction mixture contained 3 μ l cDNA (diluted 1:10 for housekeeping genes), 15 μ l 2X TaqMan Universal PCR Master Mix, 1,5 μ l 20X Assay Mix and 10,5 μ l Aqua Braun in a total reaction volume of 30 μ l. The cDNA was pipetted first into 96 well plates and afterwards the reaction mixture was added to each well. All assays were performed in triplicate. Reaction wells without template cDNA and with water served as negative controls. After addition of all the reagents, the plate was sealed with a plastic foil and centrifuged for a minute to bring all solutions to the bottom of the well and to eliminate air bubbles. Then the RT-PCR was run for 45 cycles using standard programme. Results were finally expressed as $2^{-\Delta\text{CT}}$ which is an index of the relative amount of mRNA present in each sample.

2.2.13- BrdU cumulative labelling.

Cumulative BrdU labelling was used to investigate eventual differences in the number of proliferating precursors between WT and GDF15^{-/-} animals. Time mated (E18) pregnant GDF15^{+/-} females from heterozygous matings were injected once or twice intraperitoneally with BrdU (100 μ l/g body weight) and sacrificed 2 and 6 hours after the first injection respectively.

Embryos were taken out from the mother and brains were removed from the skull. One hemisphere of each brain was processed for immunohistochemistry as described before. From the other hemisphere, striata and hippocampus were dissected out, mechanically dissociated by pipetting up and down and then plated onto PLO-coated chamberslides and left to adhere to the substrate for two hours. Then cells were processed for

immunocytochemistry as described before. A piece of tail of each embryo was kept for the analysis of the genotype. Immunopositive cells were counted as described above.

Chapter 3: Results.

3.1- Analysis of GDF15 expression in the embryonic and adult GE/SVZ and hippocampus.

Immunohistochemistry with specific antibodies and in situ hybridization had previously revealed that GDF15 is expressed in the germinal epithelium surrounding the lateral ventricle of the neonatal rat brain and mainly in the epithelial cells of the choroid plexus in adult rats (Schober et al., 2001).

To investigate in more detail GDF15 expression in the murine periventricular epithelium I used quantitative RT-PCR. The germinal epithelium of the GE and the hippocampus at days 14, 16 and 18 (E14, E16 and E18) of embryonic development and in the SVZ of postnatal mice were dissected, total RNA was extracted and processed for RT-PCR. Analysis of three independent samples revealed GDF15 mRNA in all samples examined, independently of the region of origin and the age. I found that levels of GDF15 mRNA significantly increase between mid (E14) to late development (E16/E18) in the GE and remained high in the postnatal SVZ (Fig. 3.1, A). In contrast I found that GDF15 expression in the hippocampus is higher (around three-fold difference) in embryonic and early postnatal hippocampus, than in adult mice (Fig. 3.1, B). These observations suggested that the differential pattern of GDF15 expression observed may be due to differences in the composition of the dissected tissue, rather than to an age-dependent regulation of GDF15. In particular the observation that GDF15 is highly expressed in the postnatal SVZ indicates that NPC express GDF15.

Results

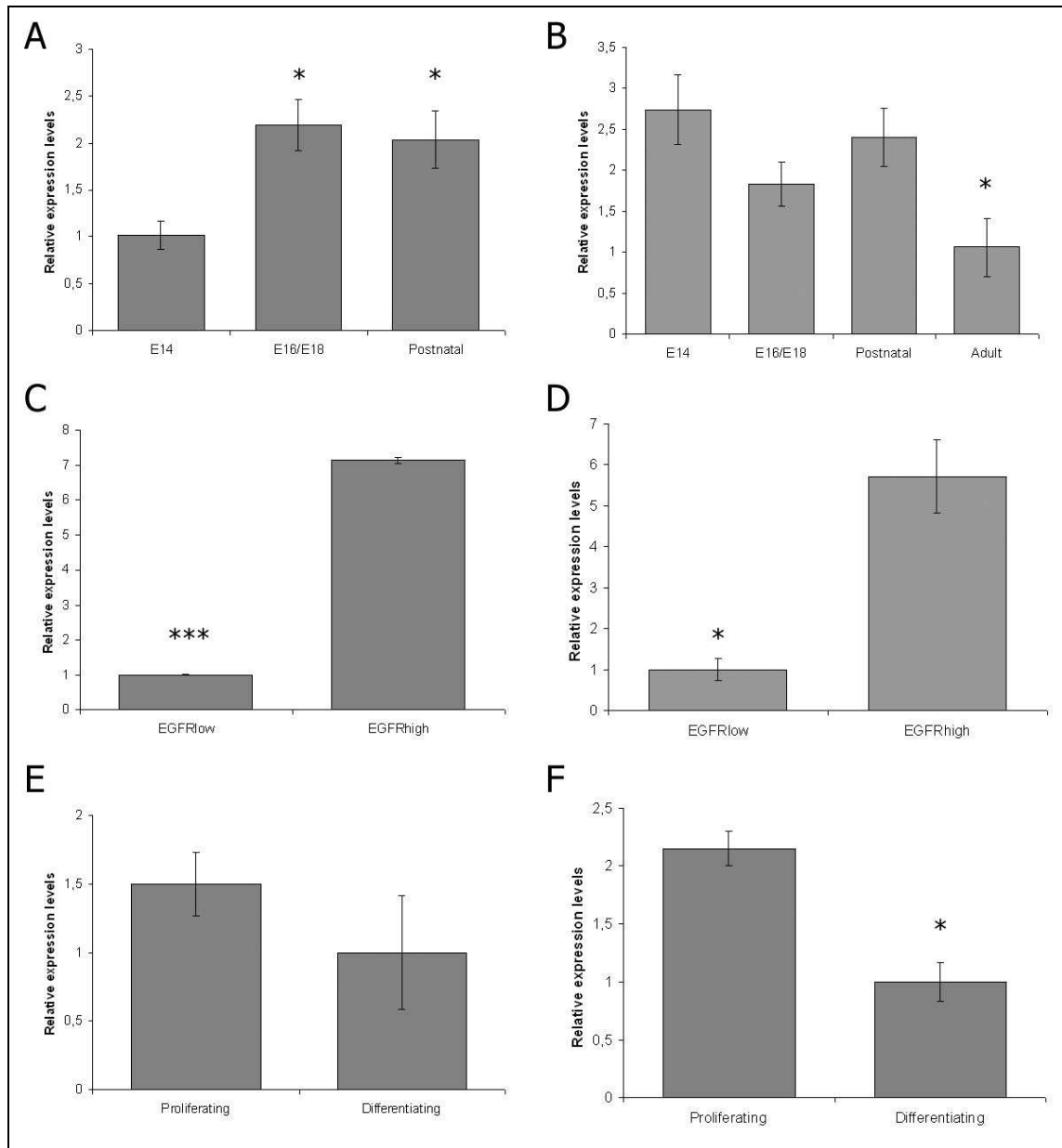


Fig. 3.1- Levels of GDF15 expression correlate with degree of composition in NPC. Analysis of GDF15 expression in the mouse GE/SVZ and hippocampus by quantitative RT-PCR. (A, B) Comparison of GDF15 expression, relative to 18s ribosomal RNA, of tissue dissected from the GE/SVZ (A) and hippocampus (B) at the indicated ages. (C, D) Comparison of GDF15 expression, relative to 18s ribosomal RNA, of sorted E18 GE (C) and hippocampus (D) derived EGFR^{low} and EGFR^{high} cells. (E, F) Comparison of GDF15 expression, relative to GAPDH and β -Actin, of proliferating and differentiating neurosphere precursors derived from E18 GE and hippocampus. Data are given as means \pm SEM of at least three independent animals and experiments.

To further investigate this issue I followed two approaches. Firstly, I expanded the precursor pool by culturing the dissociated tissue to induce neurosphere formation.

Under these conditions most differentiated cells die and only the precursors proliferate and aggregate to form cell clusters called neurospheres. After an 8 day expansion, neurospheres were collected and single cells were induced to differentiate for further 7 days. Cells derived from both proliferating and differentiating cultures were collected and the expression of GDF15 was analysed by RT-PCR. This analysis revealed that both proliferating and differentiating GE-derived precursors express GDF15 since no difference was observed in GDF15 expression between the two groups (Fig. 3.1, E). In contrast, in hippocampal cultures, I found a higher expression (two-fold) in proliferating neurosphere precursors compared to differentiating cells (Fig. 3.1, F). I next analysed the number of cells that are still proliferating in cultures that had been differentiating for one week. To do this I exposed the cultures to BrdU overnight. Next, I performed immunocytochemistry and scored the number of cells that incorporated BrdU as a percentage of total cells in the culture determined by DAPI counterstaining of the nuclei. I observed that in GE derived cultures 8.38 ± 1.09 % of the cells are still proliferating NPC that produce high levels of GDF15; while in hippocampal derived cultures only 2.43 ± 0.53 % were proliferating precursors. This shows that GE-derived cultures even after a week in differentiating conditions contain significantly more NPC than differentiating hippocampal cultures, and that this may account for the fact that GE but not hippocampal differentiating cultures continue to produce high levels of GDF15.

Secondly, I took advantage of a FACS based procedure to isolate NSCs to directly investigate their ability to express GDF15 (Ciccolini et al., 2005). Using this approach, it was previously shown that in the telencephalic germinal epithelium a high proportion (around 1 in 4) of cells expressing high levels of epidermal growth factor receptor ($EGFR^{\text{high}}$ cells) display *in vitro* properties of NSC such as long term self renewal and multipotency. I therefore used flow cytometry to purify cells expressing high levels of EGFR ($EGFR^{\text{high}}$) and cells expressing low levels of EGFR ($EGFR^{\text{low}}$) from the E18 GE and hippocampus and measured the expression of GDF15 in these two cell populations by quantitative RT-PCR. I found that in both regions GDF15 mRNA levels were higher (GE seven-fold; hippocampus five-fold) in $EGFR^{\text{high}}$ cells than in the $EGFR^{\text{low}}$ population (Fig. 3.1, C, D).

Taken together, these data indicate that in the hippocampus GDF15 is mainly expressed by proliferating progenitors, whereas in the GE GDF15 is expressed by proliferating NPC of the periventricular area and by their progeny.

3.2- GDF15 is not a mitogen and its addition at different concentrations to neurosphere cultures does not affect NPC proliferation.

To analyse the potential role of GDF15 in the regulation of NPC behaviour I investigated first the possibility that GDF15 may act as a mitogen. To this end, I established bulk neurosphere cultures from the GE and the hippocampus of E18 WT animals. Freshly dissected cells were left to proliferate in growth medium and total cell number was scored after a week. Cells were cultured in the presence and absence of known mitogens, such as EGF and FGF-2, with or without exogenous GDF15. Since, *in vivo* levels of GDF15 in the mouse brain are very low (Böttner et al., 1999), I used for these experiments a concentration of 10 ng/ml of GDF15 that I expected to be saturating. This analysis showed that GDF15 alone did not promote neurosphere proliferation (Fig. 3.2 A and B). Moreover, GDF15 did not act synergistically with exogenous EGF and FGF-2 to promote proliferation (Fig. 3.2 A and B). Since the biological activity of the protein (acquired from R&D) is routinely tested in our lab using cultured midbrain dopaminergic neurons, it is unlikely that these results are due to a defective batch of protein. Similar observations were made using various concentrations of GDF15 ranging from 1 to 100 ng/ml (Fig. 3.2 C-F). Taken together, these data suggest that GDF15 does not act as a mitogen *in vitro* for precursors derived from either the GE or the hippocampus.

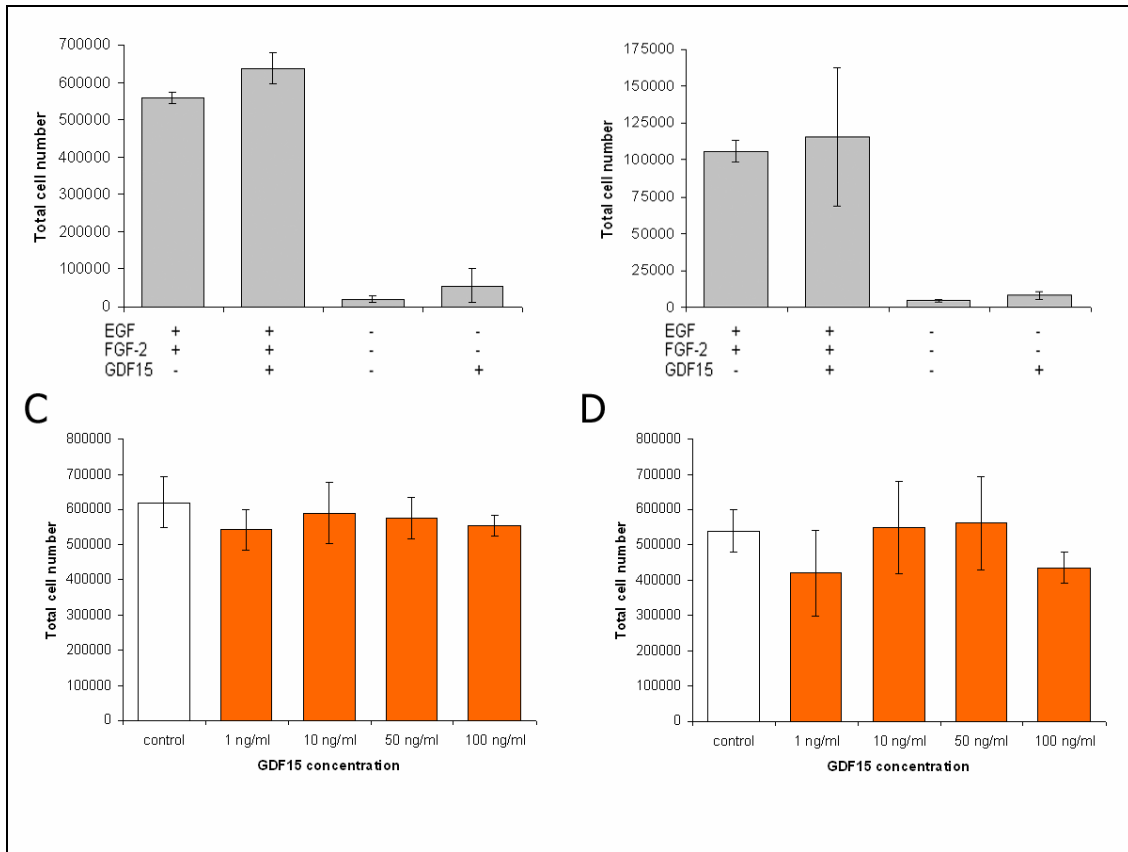


Fig. 3.2- GDF15 is not a mitogen for GE or hippocampal NPC. (A, B) Quantitative analysis of the effect of GDF15 on total cell number in GE (A) and hippocampal (B) neurosphere cultures that had been grown for a week in the presence or absence of EGF, FGF-2 and GDF15 as indicated. (C-D) Quantitative analysis of total cell number in neurosphere cultures established from E18 WT GE (C) and hippocampus (D) grown in the presence of both EGF and FGF-2 and the indicated concentrations of GDF15. Data are given as means \pm SEM of at least three independent experiments.

3.3- Effect of GDF15 on proliferation and differentiation of NPC derived from the embryonic GE.

3.3.1- NPC derived from GDF15^{-/-} embryonic GE in vitro give rise to less progeny than their WT counterpart.

The analysis of GDF15 expression had shown that it is highly expressed in the periventricular germinal zone and that NPC represent the main source of GDF15 in this area. Therefore I investigated whether GDF15 affects neural precursor behaviour. I first analysed whether GDF15 modulates neural precursor proliferation in bulk neurosphere cultures derived from the GE of E14 and E18 WT and GDF15^{-/-} embryos.

After dissection, tissue was triturated and plated at a density of 10^5 cells/ml in 24 well plates in culture medium in presence and absence of exogenous GDF15. Cells were allowed to proliferate 8 days before being collected and counted.

This analysis showed that at both ages cultures derived from GDF15^{-/-} animals contained significantly less cells than the WT counterparts (Fig. 3.3.1, A as E14 and B as E18). Furthermore, consistent with my previous findings addition of exogenous GDF15 did not alter the proliferation of either WT or GDF15^{-/-} GE precursors.

To investigate whether the above decrease in NPC proliferation reflected a decrease in the neural precursor pool I next used clonal analysis to determine the frequency of clone forming cells. Dissected GE of E14 and E18 WT and GDF15^{-/-} embryos were mechanically dissociated and cells plated at a density of one cell per well by limiting dilution. The number of clones, reflecting the amount of NPC present in the original tissue, was counted after one week in culture (Fig. 3.3.1 C as E14 and D as E18). No significant difference was found in the number of clone forming cells between WT and GDF15^{-/-} mice, although at E18, GDF15^{-/-} animals displayed a trend in terms of reduction in the number of clone forming cells (Fig. 3.3.1. D). Furthermore, addition of exogenous GDF15 did not alter clone formation of either WT or GDF15^{-/-} GE derived precursors (Fig. 3.3.1.C and D, WT+G and KO+G).

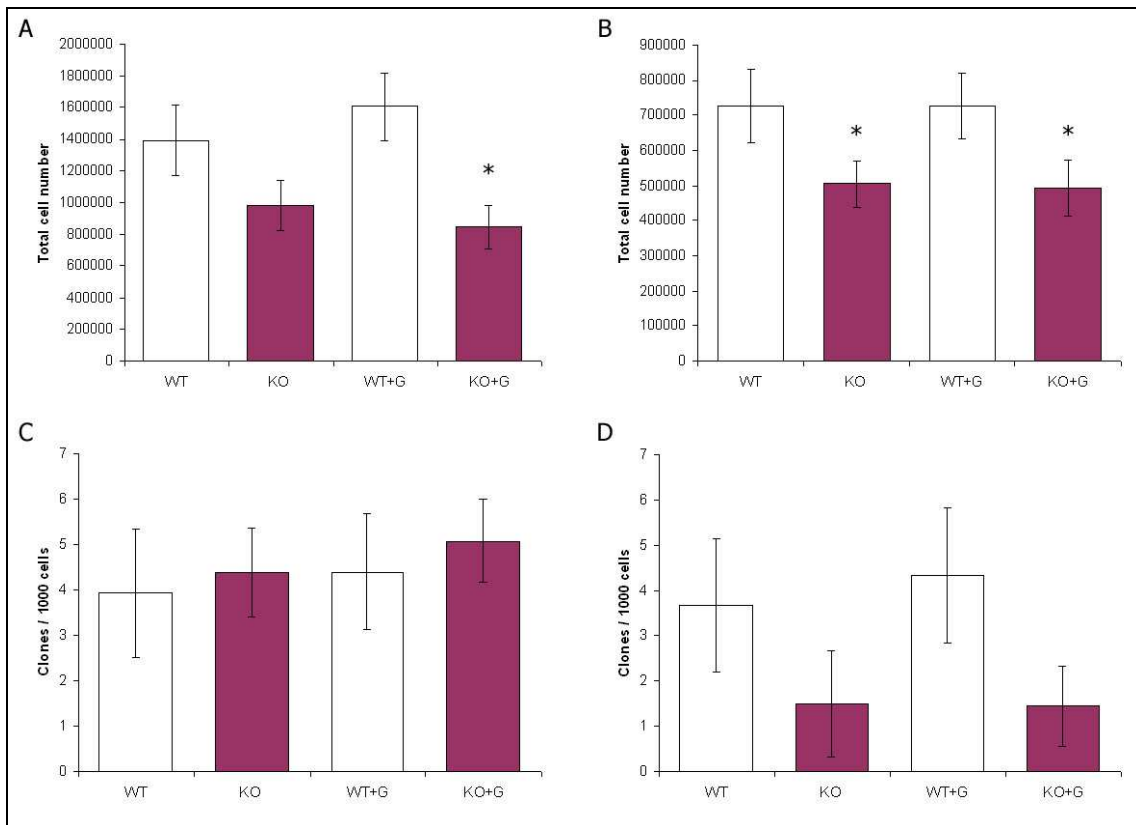


Fig. 3.3.1- GDF15 affects NPC proliferation *in vitro*. (A, B) Total cell number generated after a week in GE bulk neurosphere cultures from E14 (A) and E18 (B) WT and $GDF15^{-/-}$ grown in the presence or absence of exogenous GDF15 (WT+G and KO+G). (C, D) Quantitative analysis of the number of primary clones generated after 8 days in clonal GE derived cultures from E14 (C) and E18 (D) WT and $GDF15^{-/-}$, grown in growth media in the presence or absence of exogenous GDF15 (WT+G and KO+G). Data represent the means \pm SEM of at least three independent experiments.

Taken together, these data indicate that the decrease in the number of cells derived from $GDF15^{-/-}$ NPC is not a consequence of a reduction in the NPC pool in $GDF15^{-/-}$ animals.

3.3.2- Absence of GDF15 leads to a decrease in EGFR expression in GE NPC.

Since both types of assays (bulk neurosphere and clonal cultures) are associated with a high experimental variability, they may not be suitable for the detection of subtle differences. To reduce intrinsic experimental variability and to directly investigate the properties of clone forming cells, I isolated them on the basis of EGFR expression as reported above. Using this approach clone forming cells were previously found both in the $EGFR^{low}$ and in the $EGFR^{high}$ populations, however at a different frequency.

One in four EGFR^{high} cells generated clones, whereas in EGFR^{low} only one out of about 200 shows properties of NPC (Ciccolini et al., 2005). Furthermore, it was shown that at E18 NPC of the GE and hippocampus (see chapter 3.4) already express high levels of EGFR and therefore can be directly analysed after dissection. In contrast, at E14 NPC still express low levels of EGFR. Hence they can be sorted using the same approach only after they have been grown *in vitro* in the presence of FGF-2. At this age 24 hours of exposure to exogenous FGF-2 promotes EGFR expression in a subset of NPC, whereas after a prolonged (48 hours) exposure to FGF-2 most stem cells become EGFR^{high} cells (Ciccolini et al., 2005). Therefore, in these experiments I sorted E18 samples directly after dissection (DIV 0) and after cells had been exposed to FGF-2 for 24 hours (DIV 1) whereas E14 cells were analysed at DIV 1 and 2.

I found that, compared to WT mice, E18 GDF15^{-/-} GE contained significantly less EGFR^{high} cells. In addition, this difference was not detected anymore at DIV1 after cells had been exposed for 24 hours to FGF-2 (Fig. 3.3.2, D and E). At E14, both at DIV 1 and 2, I observed a similar trend however it was not significant. The observation that exposure to exogenous FGF-2 restores the number of EGFR^{high} cells in samples derived from GDF15^{-/-} embryos shows that absence of GDF15 affects levels of EGFR expression in NPC but not their absolute number.

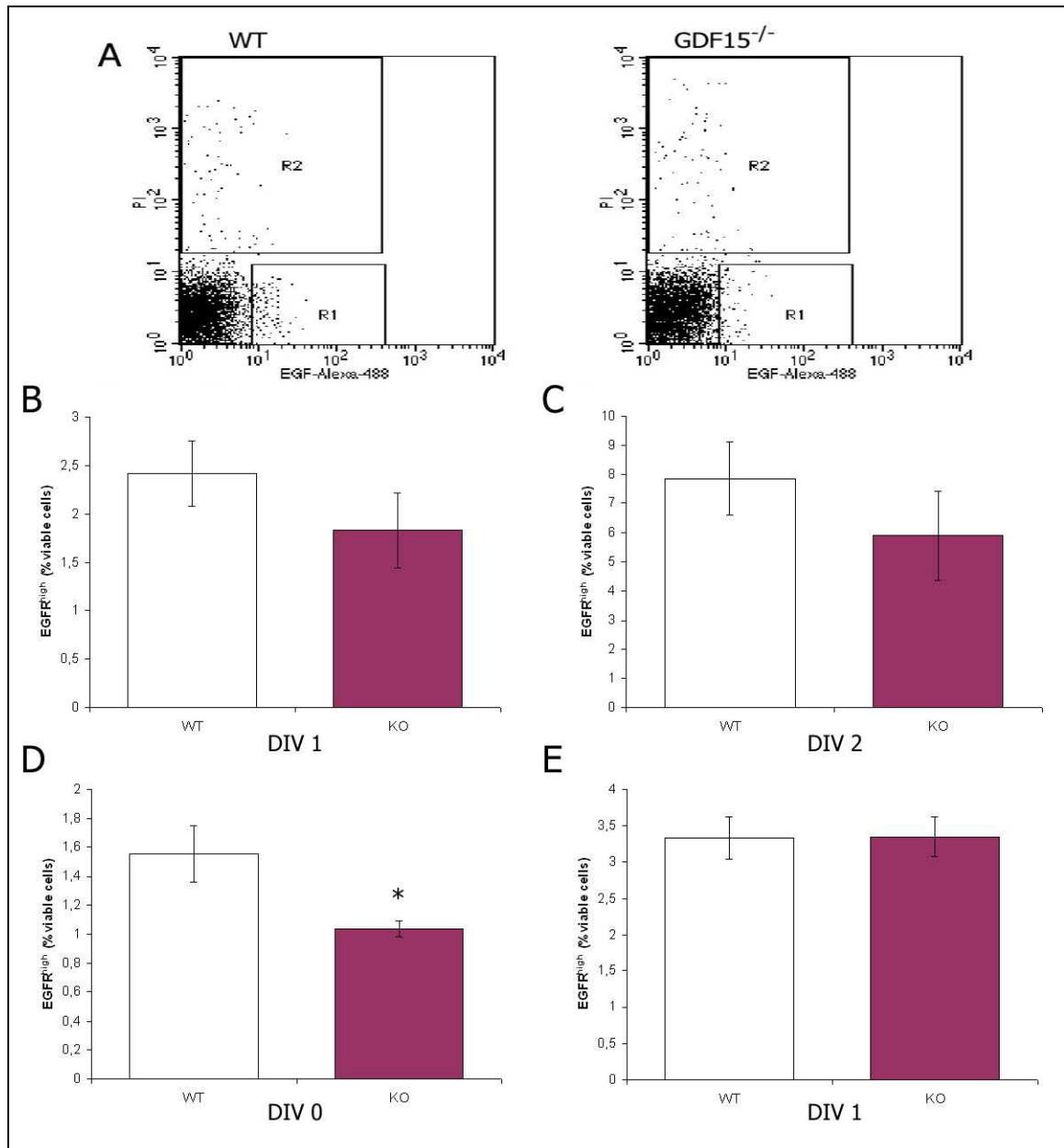


Fig. 3.3.2- Absence of GDF15 affects expression of EGFR in GE NPC but not their absolute number. (A) Representative dot plots of dissociated E18 GE derived cells from WT and GDF15^{-/-} animals after staining with EGF-Alexa 488 and PI to reveal EGFR^{high} cells (R1) and dead cells (R2). (B-E). Quantitative analysis of numbers of EGFR^{high} cells isolated from the GE of WT and GDF15^{-/-} embryos. (B, C). Analysis of cells derived from E14 animals, one and two days after dissection and exposure to FGF-2 (day *in vitro* = DIV 1 and 2). (D, E) Analysis of cells derived from E18 animals, immediately after dissection (DIV 0) (D) or at DIV 1 after overnight treatment with FGF2 (E). Data are given as means ± SEM of at least three independent experiments.

I next investigated the clone forming capacity of EGFR^{high} and EGFR^{low} cells sorted from WT and GDF15^{-/-} animals. Sorted cells were plated at a clonal density of one cell

per well on 96 well/plates in the case of EGFR^{high} cells, and 10 cells per well the EGFR^{low} cells. After two weeks in culture, I counted the number of clones derived from EGFR^{high} and EGFR^{low} cells in WT and GDF15^{-/-} derived samples. I found that, independently of age and day of analysis, EGFR^{low} cells isolated from WT and GDF15^{-/-} embryos gave rise to clones with similar efficiency. Furthermore, no differences were found between the clone formation capacity of EGFR^{high} cells isolated from E14 WT and GDF15^{-/-} embryos either at DIV 1 or 2. In contrast, EGFR^{high} cells isolated from E18 GDF15^{-/-} embryos displayed at DIV 0 a significantly lower clone forming capacity than their WT counterparts. Such a decrease was not observed, however, when cells were analysed at DIV1 (Fig. 3.3.3 A and C respectively).

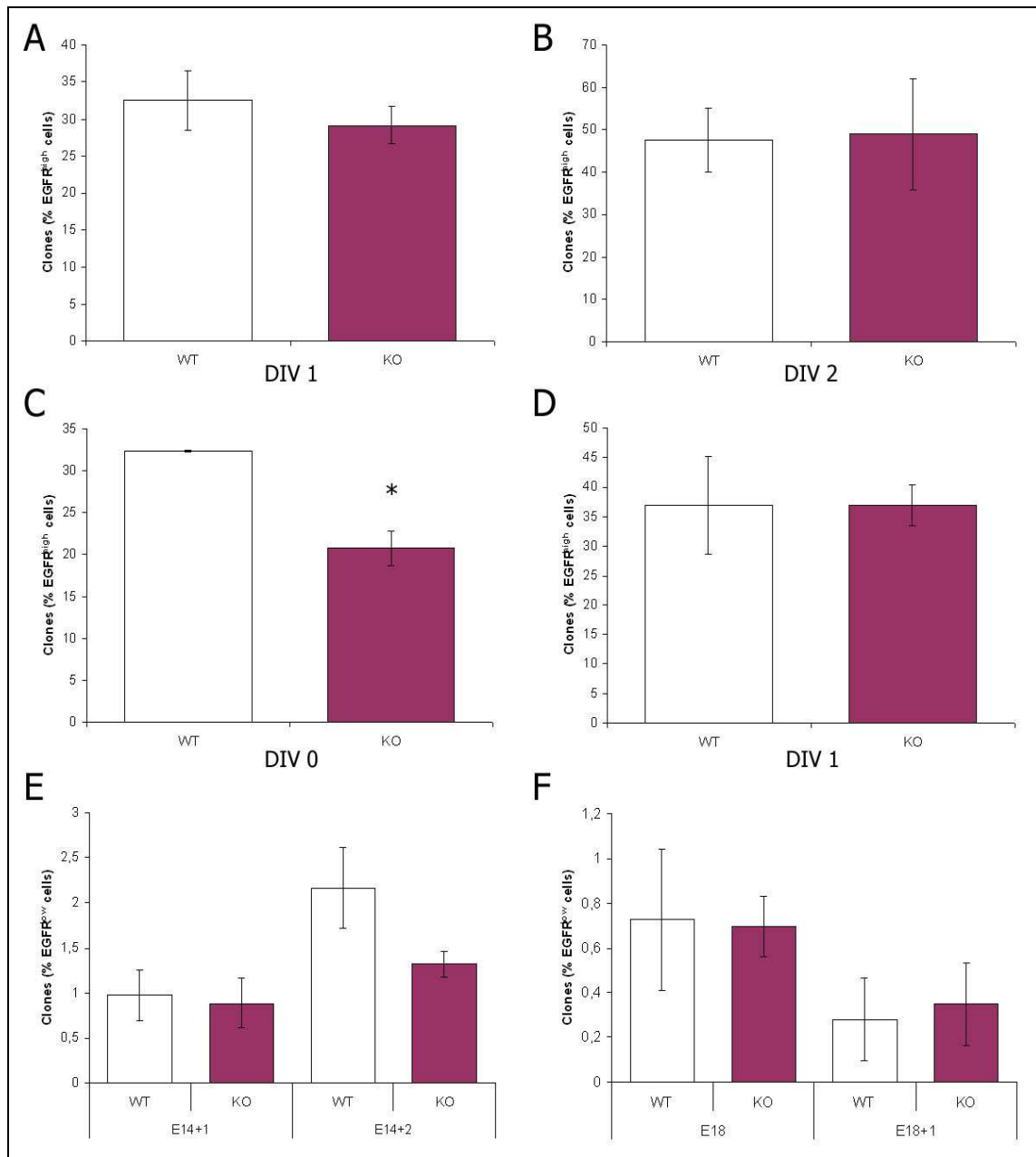


Fig. 3.3.3- EGFR^{high} cells isolated from E18 GDF15^{-/-} embryos show reduced clone forming capacity as compared to WT cells. (A-D) Quantitative analysis of the clone forming capacity of EGFR^{high} cells isolated from the GE of WT and GDF15^{-/-} animals analysed by FACS. (A, B) E14 derived cells were analysed one and two days after dissection (DIV 1 and DIV2) following exposure to FGF-2. (C, D) E18 derived cells were analysed immediately after dissection (DIV 0) (C) or at DIV 1 after overnight treatment with FGF2 (D). (E, F) Quantitative analysis of clones derived from EGFR^{low} cells isolated from WT and GDF15^{-/-} mice, from E14 (E) and E18 (F) animals. Data are given as means \pm SEM of at least three independent experiments.

To investigate whether the absence of GDF15 may affect the proliferation mode rather than number of NPC, I next analysed the proliferative and self-renewal capacity of

EGFR^{high} clone forming cells sorted from E14 and E18 WT and GDF15^{-/-} embryos. To analyse the proliferative potential, individual clones were dissociated and the number of cells per clone counted, considering clone size (number of cells per clone) as a representative score of cell proliferation. Self-renewal was scored as the number of secondary clones generated from 1000 cells plated from dissociated single clones. This analysis revealed no significant difference with respect to either parameter between WT and GDF15^{-/-} derived EGFR^{high} cells at any age tested (E18 DIV 0 and E14 DIV 1) (Fig. 3.3.4 C as E14 and D as E18). To investigate whether the reduction in the number of EGFR^{high} cells in GDF15^{-/-} animals was a consequence of cell death I used propidium iodide (PI) exclusion and FACS analysis to investigate cell viability in cell preparations obtained from WT and GDF15^{-/-} dissociated tissue. PI is a nucleic acid dye, membrane impermeable, generally excluded by viable cells and incorporated by cells with a damaged membrane. It may therefore be used to identify dying cells in a given cell population. This analysis revealed no significant difference between WT and GDF15^{-/-} GE derived cells in the number of PI⁺ cells (Fig. 3.3.4 A as E14 and B as E18), indicating that changes in cell viability were not the reason for the decrease in the number of EGFR^{high} cells observed in the GE of GDF15^{-/-} animals.

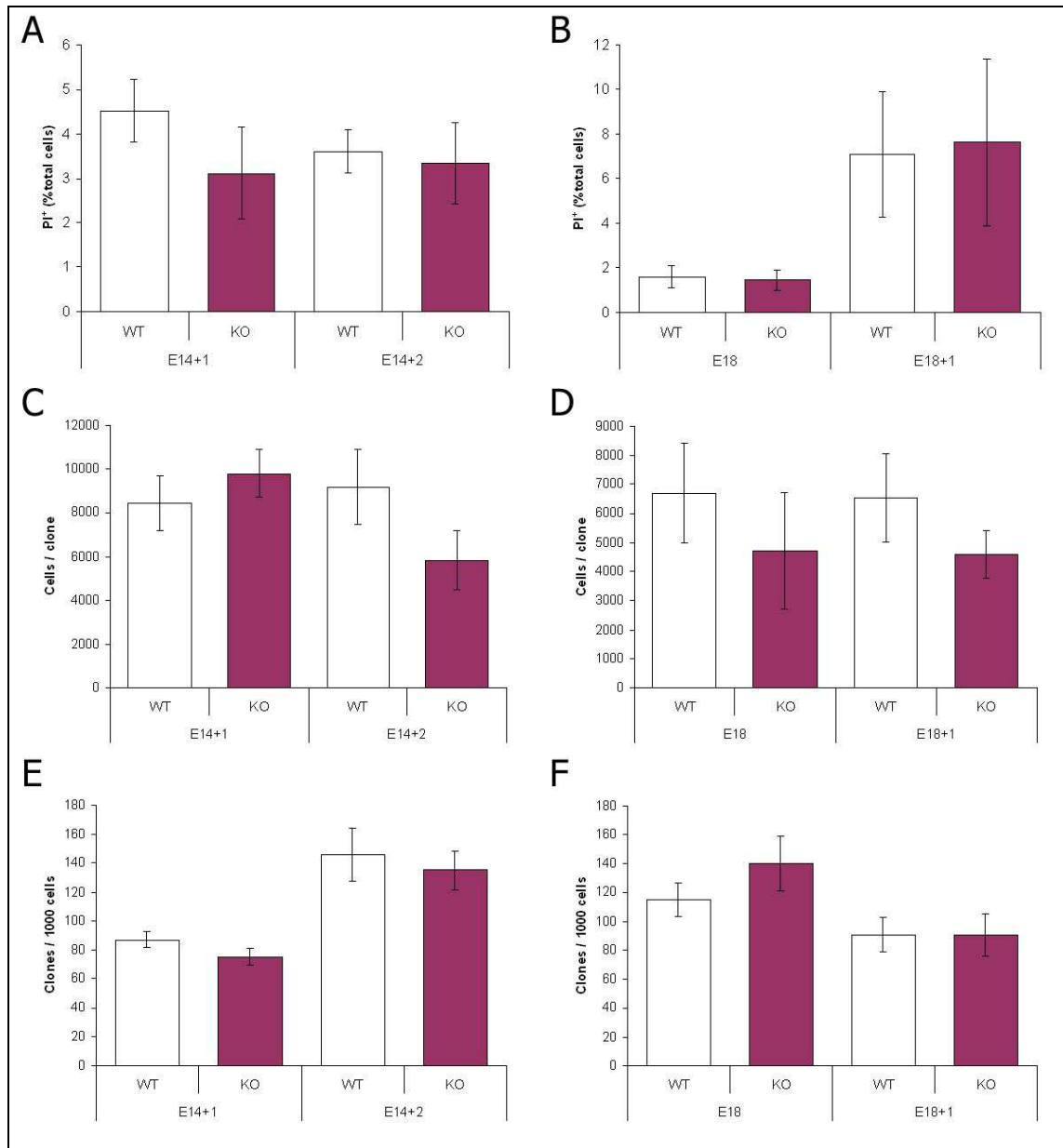


Fig. 3.3.4- Absence of GDF15 does not affect cell viability, proliferation and self renewal of EGFR^{high} cells. (A, B) Quantitative analysis of the number of PI positive cells found in dissociated cells of E14 (A) and E18 (B) GE dissected from WT and GDF15^{-/-} mice. (C, D) Quantitative analysis of the number of cells generated per clone, from clones derived from EGFR^{high} E14 (C) and E18 (D) derived cells. (E, F) Quantitative analysis of the number of secondary clones obtained per 1000 primary clone cells plated from E14 (E) and E18 (F) derived neurospheres. Data are given as means \pm SEM of at least three independent experiments.

Taken together, consistent with our previous observations, these data show that absence of GDF15 does not directly affect the number of clone forming cells or their ability to proliferate *in vitro*. They also show that in the absence of GDF15 the number of NPC

that have acquired high levels of EGFR is decreased. Such a decrease in EGFR expression likely explains the lower clone formation efficiency of E18 GE cells at DIV0. The observation that both the number and the clone formation capacity of EGFR^{high} cells is rescued by exposure to exogenous FGF-2 further underscores that absence of GDF15 prevents expression of high levels of EGFR but does not decrease the size of the stem cell pool.

I next investigated whether exogenous GDF15 could rescue the decrease in EGFR expression. For these experiments dissociated tissue derived from WT and GDF15^{-/-} embryos was incubated overnight in NSA-B27 media in the presence of varying concentrations of FGF-2 (1, 5 and 10 ng/ml) with or without GDF15. Afterwards, cells were stained with EGF Alexa and FACS analysed as described above. These experiments revealed no differences in the number of EGFR^{high} cells at any FGF-2 concentration tested, neither in the presence nor in the absence of GDF15 (Fig. 3.3.5 A, B).

Since it was conceivable that a prolonged exposure to FGF-2, even at low concentrations, would mask the effect of GDF15 on EGFR expression, I incubated cells obtained from dissociated WT and GDF15^{-/-} GE with or without FGF-2 and GDF15 for only 6 hours prior to FACS analysis. These experiments revealed that in WT cultures all treatments led to a significant increase of the number of EGFR^{high} cells compared to the untreated control. Furthermore, such an increase was greatest when both GDF15 and FGF-2 were added to the culture, suggesting a synergistic effect of the two growth factors. A similar reduction was observed in parallel experiments with GDF15^{-/-} derived cells (Fig. 3.3.5 C and D show WT and GDF15^{-/-} respectively). Interestingly, in this case the increase of EGFR expression was lower than the one previously observed with WT derived cells, suggesting that in the absence of GDF15 there is a general downregulation of the molecular machinery regulating expression of EGFR in NPC.

3.3.3- Decrease on EGFR expression is not mediated by a change in FGF-2 signalling.

Since it is well known that EGFR expression levels in GE NPC are regulated by FGF-2 signalling (Santa-Olalla and Covarrubias, 1999; Lillien and Raphael, 2000; Ciccolini, 2001), I next investigated expression of FGF-2 and its principal receptors in the CNS, FGFR1 and 2, in the SVZ of WT and GDF15^{-/-} perinatal mice by quantitative RT-PCR (Fig. 3.3.5 E-G respectively). This analysis revealed no apparent differences between

WT and GDF15^{-/-} animals, indicating that the effect of GDF15 on EGFR expression is unlikely to be mediated by an alteration in FGF receptor expression.

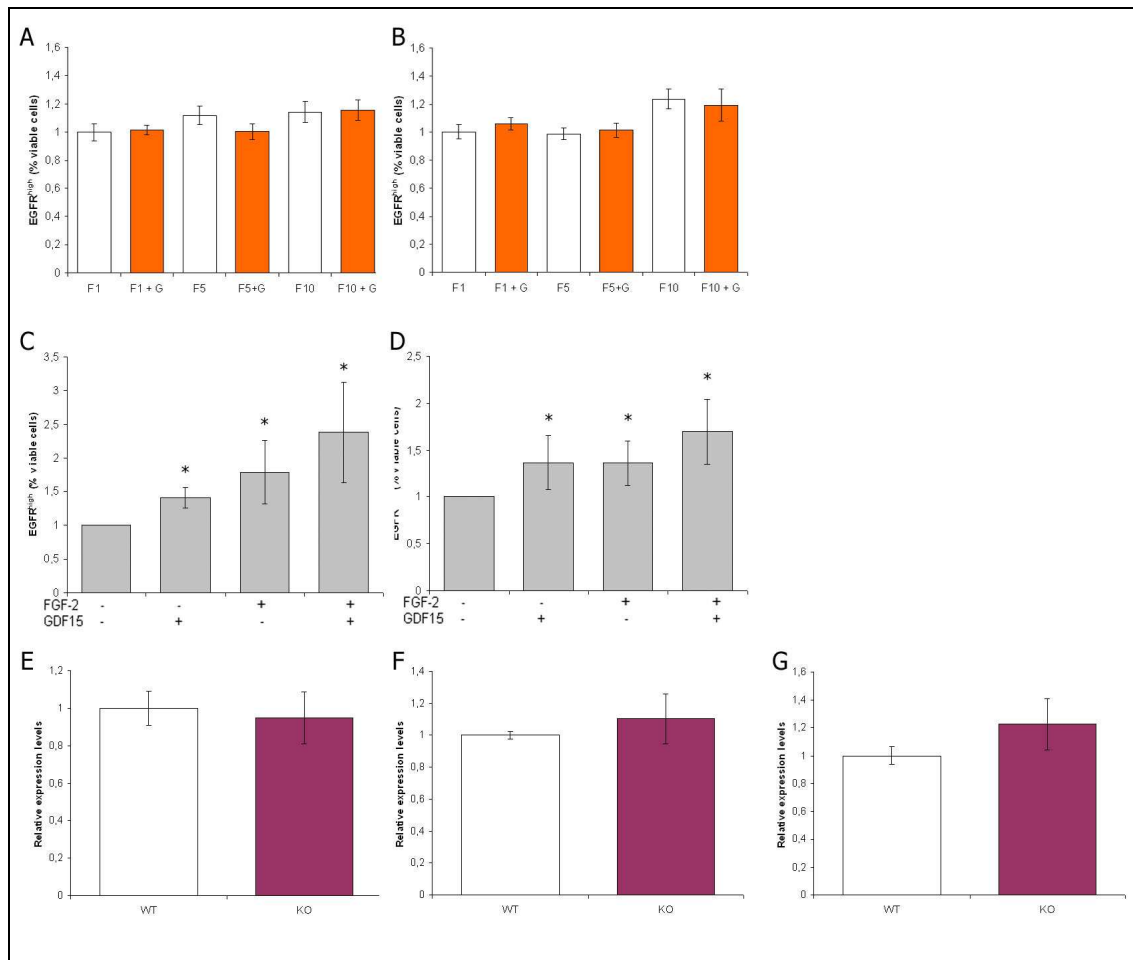


Fig. 3.3.5- GDF15 modulates EGFR expression in NPC independently of FGF signalling. (A, B) Quantitative analysis of the number EGFR^{high} cells isolated from E18 GE of WT (A) and GDF15^{-/-} (B) animals FACS analysed after overnight exposure to different concentrations of FGF-2 in the presence or absence of GDF15. (C, D) Quantitative analysis of the number of EGFR^{high} cells isolated from E18 GE of WT (C) and GDF15^{-/-} (D) after 6 hours of incubation in the absence or presence of FGF-2 and GDF15. Data were analysed by one-way ANOVA. (E to G respectively) Analysis of FGF-2, FGFR1 and 2 expression in the perinatal SVZ by RT-PCR. Comparison of FGF-2, FGFR1 and 2 mRNA levels, relative to GAPDH and β -Actin mRNAs, of SVZ dissected from WT and GDF15^{-/-}. Data are given as means \pm SEM of at least three animals.

3.3.4- GDF15 controls cell cycle exit of NPC differentiating in vitro.

Next I analysed whether GDF15 affects NPC differentiation. Neurospheres obtained from E18 GE precursors were differentiated by dissociation and plating onto a substrate in the absence of EGF and at low concentrations of FGF-2. Generation of neurons and

oligodendrocytes was monitored one week after induction of differentiation by immunostaining with TuJ1 and O4 antibodies, respectively.

Exogenous GDF15 was added to the medium either during the period of neurosphere generation (proliferation phase) or during differentiation. As control I set up parallel cultures not exposed to exogenous GDF15.

No difference was observed in the number of oligodendrocytes between WT and GDF15^{-/-} in any tested condition (Fig. 3.3.6 C). In contrast, I found that, seven days after induction of differentiation, cultures derived from GDF15^{-/-} animals contained significantly less neurons than neurosphere cultures obtained from WT animals. Addition of exogenous GDF15, either during the proliferation or during the differentiation period, did not affect the number of neurons in cultures derived from WT animals. In contrast, exogenous GDF15 rescued neurogenesis in cultures established from GDF15^{-/-} animals. However, this rescue was observed only in cultures exposed to GDF15 during the differentiation phase and not in cultures treated during the proliferation period (Fig. 3.3.6 A). Analysis of the number of pycnotic nuclei revealed no effect of the different treatments on cell death in WT or GDF15^{-/-} cultures (Fig. 3.3.6 B). Furthermore, I found no differences between GDF15^{-/-} and WT cultures when neurogenesis was monitored after a longer differentiation period (DAP 10 and 14) (Fig. 3.3.6 D and F).

Taken together, these data indicate that GDF15 does not affect the survival of NPC, but accelerates the differentiation of neurons.

Results

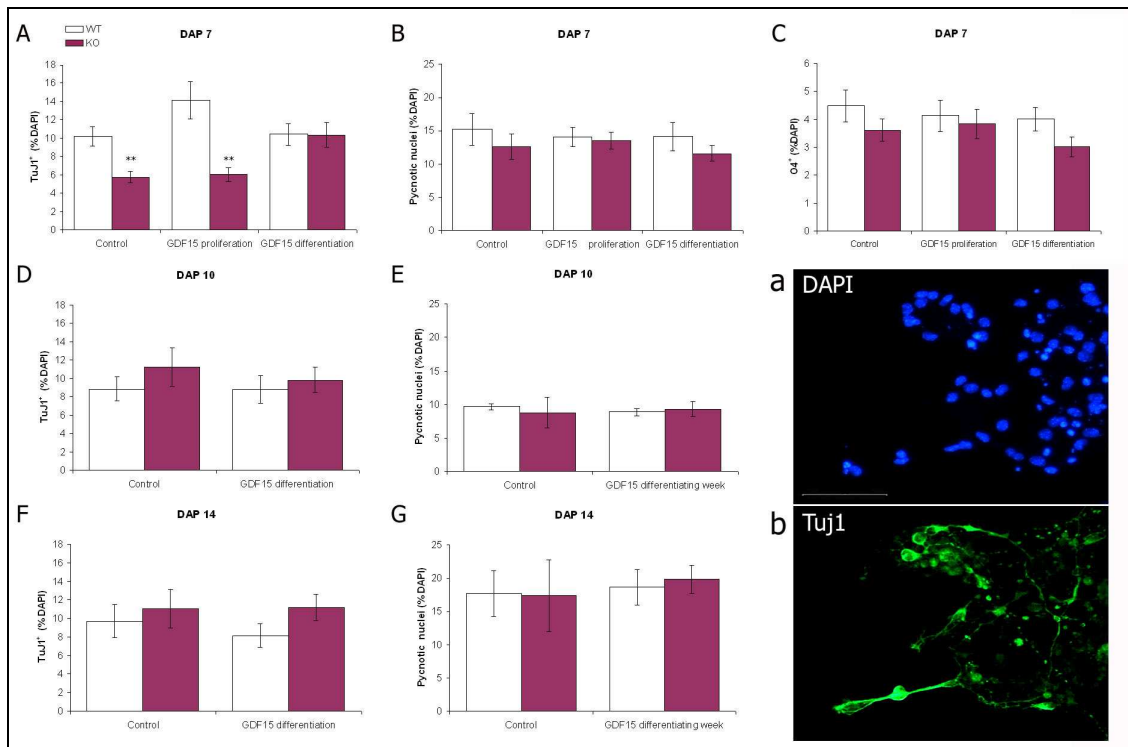


Fig. 3.3.6- Effect of GDF15 on differentiation of neurosphere derived precursors. (A, D and F) Quantitative analysis of the number of neurons (TuJ-1⁺) found in WT and GDF15^{-/-} neurosphere cultures at 7, 10 and 14 days after plating (DAP) (respectively A, D and F) . WT and GDF15^{-/-} cultures were expanded and differentiated in the absence (control) or presence of GDF15 as indicated. (B, E, and G) Quantitative analysis of pycnotic nuclei found in WT and GDF15^{-/-} neurosphere cultures differentiating in the indicated condition at DAP 7, 10 and 14 (respectively B, E and G). (C) Quantitative analysis of oligodendrocytes (O4⁺) found in WT and GDF15^{-/-} neurosphere cultures at DAP 7 in the presence or absence (control) of exogenous GDF15 as indicated. (a, b) Representative examples of TuJ1-immunoreactive neurons of differentiated GE neurosphere-derived precursors. Scale bar: 50 μ m. Data are given as means \pm SEM of at least three independent animals.

I next investigated whether such an effect of GDF15 on neural precursor differentiation is associated to a change in precursor proliferation. To do this, I analysed BrdU incorporation in differentiating cultures at different time points: DAP 2, 4 and 7. This analysis revealed a greater number of proliferating cells in GDF15^{-/-} derived cultures than in their WT counterpart. The increase in BrdU incorporation was transient and observed only at DAP 2 (Fig. 3.3.7 B) but not at later stages of differentiation (Fig. 3.3.7 D and E). Furthermore, addition of exogenous GDF15 caused BrdU incorporation in GDF15^{-/-} cultures to revert to WT control levels, while it did not affect cell proliferation in WT cultures (Fig. 3.3.7 B). Taken together, these results indicate

that in NPC differentiating in the absence of GDF15 the timing of cell cycle exit is delayed.

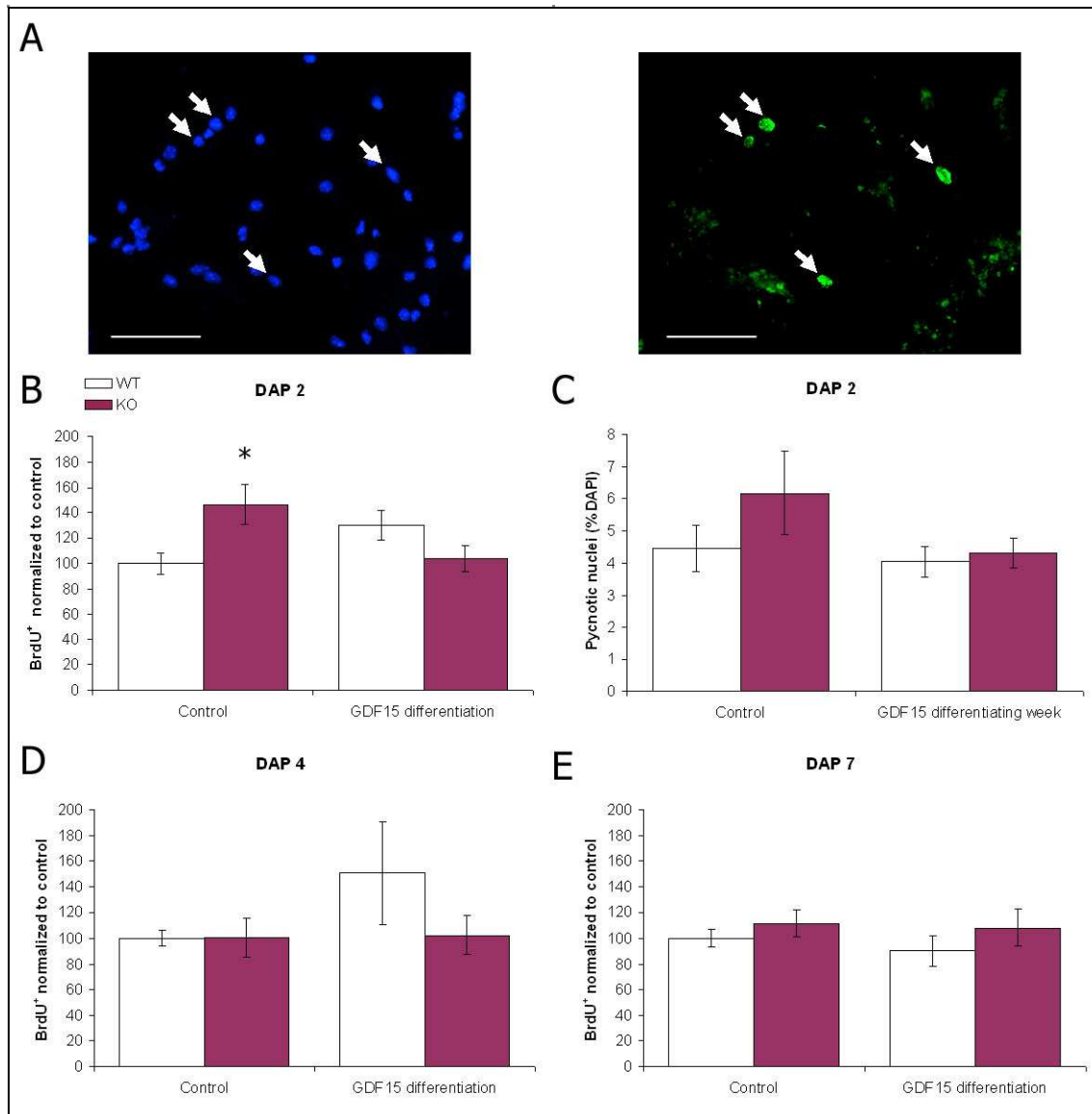


Fig. 3.3.7- Effect of GDF15 on cell cycle exit during neurosphere differentiation. (A) Representative examples of BrdU-immunoreactive cells of differentiated GE neurosphere-derived precursors. Scale bar: 50 μ m. (B, D and E) Quantitative analysis of the number of dividing cells in neurosphere cells differentiating in the presence or absence of GDF15. Cultures were incubated O/N with BrdU and analysed on DAP2 (B), 4 (D) and 7 (E). (C) Quantitative analysis of the number of pycnotic nuclei analysed in B. Data represent the means \pm SEM of at least three independent experiments.

3.3.5- Role of GDF15 in the regulation of GE NPC *in vivo*.

Data presented so far suggest that absence of GDF15 does not directly affect the maintenance of primitive precursors in the GE. Moreover, *in vitro* GDF15 is directly involved in the regulation of cell cycle exit of more differentiated neuronal progenitors, while it indirectly affects proliferation of more primitive precursors by regulating expression of EGFR in NPC. I therefore investigated next whether absence of GDF15 has a similar effect *in vivo*. I first analysed EGFR expression in the GE by immunohistochemistry on coronal telencephalic sections. EGFR^{high} cells were localised in the VZ and SVZ of the periventricular germinal epithelium (Fig. 3.3.8). Positive stained cells were also found in the striatum. Most immunopositive cells showed radial orientation although I also found tangentially oriented EGFR^{high} cells, as previously shown (Ciccolini et al., 2005; Lillien and Gulacsi, 2006).

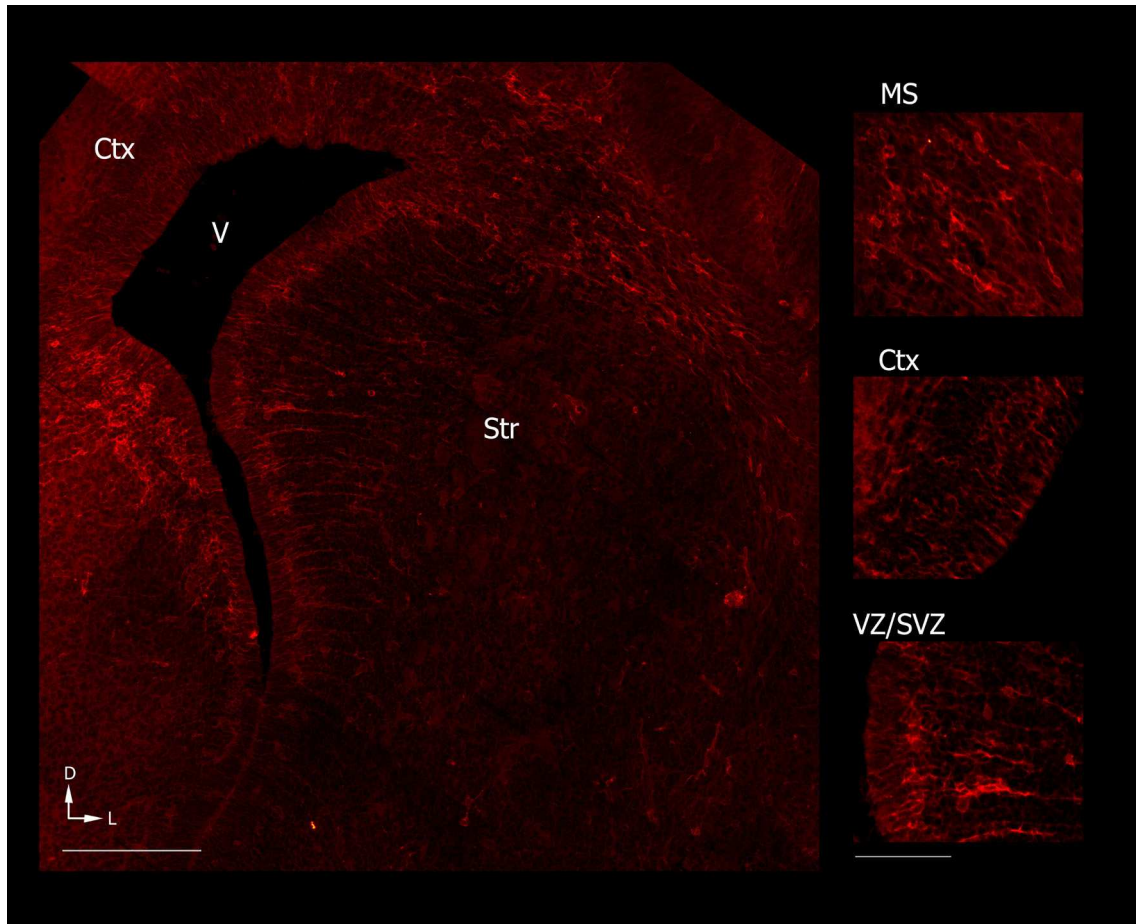


Fig. 3.3.8– EGFR expression in the E18 mouse telencephalon. Left picture: Immunohistochemistry of a coronal section of one E18 hemisphere. On the right: higher magnification image of the migratory stream (MS), cortex (Ctx) and GE ventricular and subventricular zones (VZ/SVZ) showing EGFR^{high} cells in all three areas. V=ventricle; Str= striatum. Scale bar: 200 μ m left picture; 100 μ m right panels.

Comparison between WT and GDF15^{-/-} VZ showed a clear difference in the organisation of the EGFR immunolabelled cells. In WT animals, EGFR^{high} cells are organised in columns of radially oriented cells (white arrows in Fig. 3.3.9) which cannot be found in the GDF15^{-/-} where instead, cells are distributed in clusters.

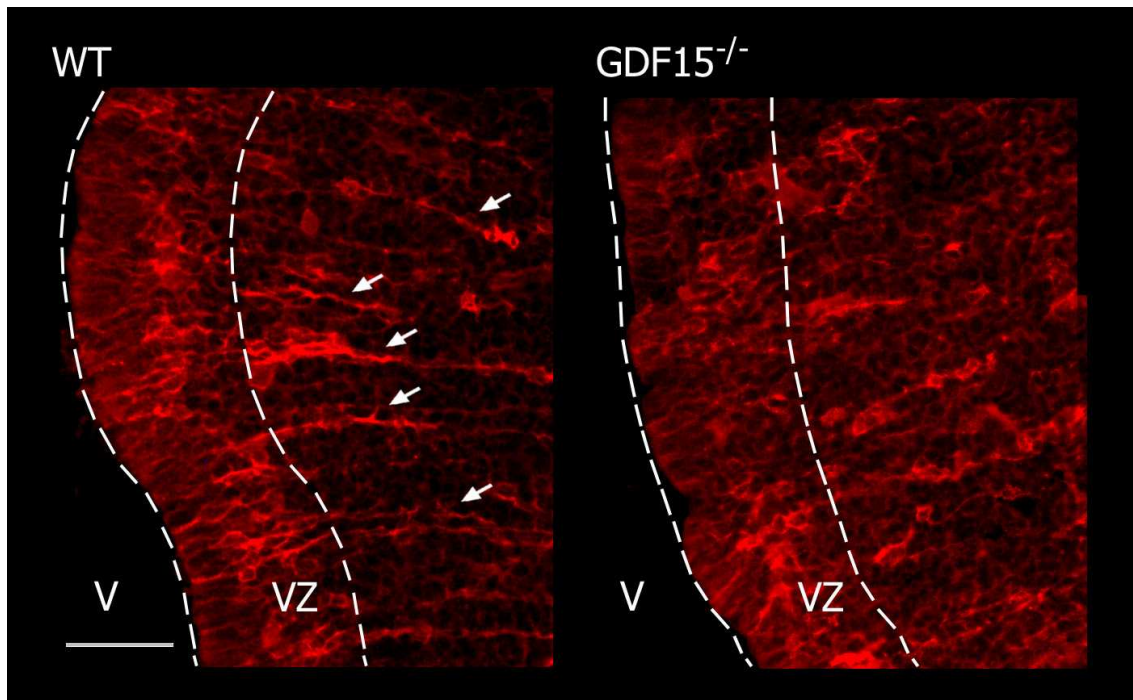


Fig. 3.3.9 – EGFR^{high} cells do not form columns of radially oriented cells in the GDF15^{-/-} in vivo. Fluorescent micrographs showing representative examples of coronal sections from WT and GDF15^{-/-} E18 mice immunostained for EGFR. V indicates the relative position of the ventricle; VZ of the ventricular zone; white arrows show the columns of radially oriented EGFR labelled cells. Scale bar is 50 μ m.

Next, I studied the number of cells undergoing mitosis in the VZ and SVZ of E18 WT and GDF15^{-/-} GE by immunostaining with antibodies recognising phospho-histone H3 (PHH3). Mitotic cells were grouped into three categories: cells undergoing mitosis at the apical border of the VZ: more primitive precursors, basal mitotic progenitors in the SVZ, and cells undergoing mitosis between these two regions. This analysis revealed no significant difference between WT and GDF15^{-/-} animals in the number of mitotic cells at the apical border of the VZ (Fig. 3.3.10 A). Interestingly, the numbers of cells dividing in the SVZ and in the remaining VZ (i.e. the region intermediate between the apical border and the SVZ), where secondary neuronal and glia progenitors divide, were both significantly increased in GDF15^{-/-} animals compared to WT mice (Fig. 3.3.10 B and C).

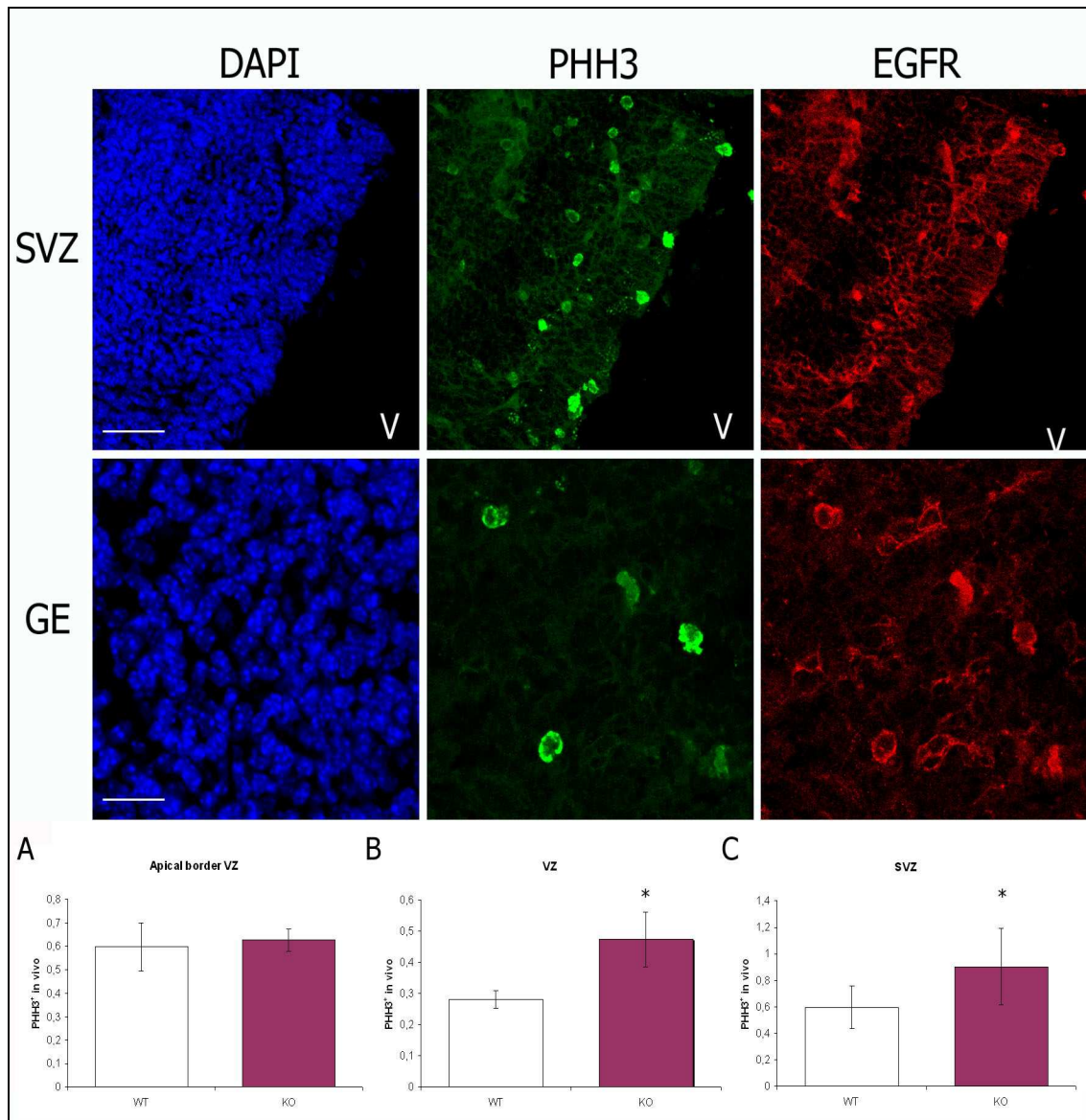


Fig. 3.3.10- Analysis of cell proliferation *in vivo* in WT and $GDF15^{-/-}$ animals. Confocal images showing coronal sections of the GE germinal region of E18 WT mice immunostained with PHH3 antibodies (central panels) and EGFR (right panels). Left panels show DAPI staining of nuclei. V indicates the relative position of the ventricle. Scale bar: SVZ 75 μm , Str 37,5 μm . (A, B and C) Quantitative analysis of the number of PHH3 positive cells undergoing mitosis at the apical border (A) within the VZ (B) or the SVZ (C). Data represent the means \pm SEM of at least three independent experiments.

To further characterise the nature of the extra proliferating cells I investigated if they represented precursors residing in the germinal epithelium, or more mature progenitors undergoing a last round of cell division before migrating towards more basal regions of the GE. To this end I set up heterozygous matings. Pregnant animals were given one or

two intraperitoneal BrdU injections and sacrificed 2 and 6 hours after the first injection. Striata of one hemisphere from WT and GDF15^{-/-} embryos were fixed in PFA and processed for immunohistochemistry, whereas striata of the other hemisphere were dissected and dissociated cells plated onto polyornithine coated coverslips and analysed by BrdU immunocytochemistry. After a two hour BrdU pulse more proliferating cells were found in the VZ of GDF15^{-/-} animals than in WT embryos, whereas no difference was found in the number of proliferating cells in the striatum. Similarly, GDF15^{-/-} embryos displayed an increased number of dividing cells as compared to WT after a 6 hour BrdU pulse. Analysis of the dissociated tissue showed that GDF15^{-/-} GE contained 47,3% more BrdU immunopositive cells than the WT counterpart (Fig. 3.3.11 B). A similar increase (around 41,3%) was observed by immunohistochemistry (Fig. 3.3.11 C). However, the extra proliferating cells were not in the germinal epithelium as observed after the 2 hour BrdU pulse where at this time point I found less proliferating cells, but rather localized in the more internal region of the GE (Fig. 3.3.11 D). Taken together, these data suggest that supernumerary proliferating precursors found in GDF15^{-/-} embryos do not represent primitive NPC resident in the VZ and SVZ but differentiating progenitors that migrate away from the germinal epithelium.

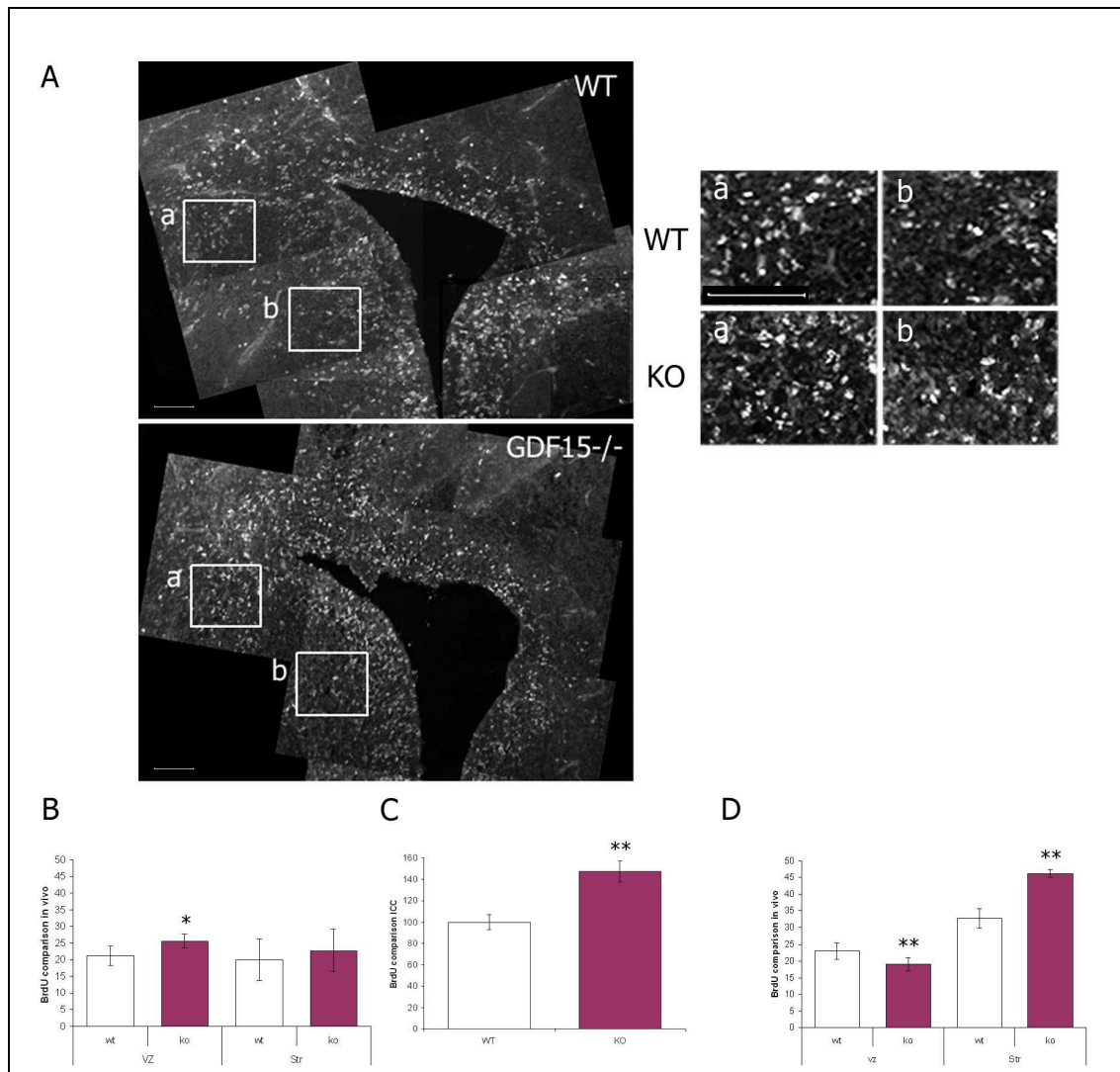


Fig. 3.3.11- Effect of GDF15 on NPC proliferation *in vivo*. (A) Coronal sections showing BrdU immunolabelling in the periventricular region of WT and GDF15^{-/-} mice. (B) Quantitative analysis of the number of BrdU positive cells after a 2 hour pulse found in the VZ and striatum of WT and GDF15^{-/-}. (C) Quantitative analysis of the number of BrdU positive cells found in WT and GDF15^{-/-} dissociated GE after a 6 hour BrdU pulse (n=8) expressed as a percentage of the WT. (D) Quantitative analysis of the number of BrdU positive cells found in VZ and striatum in WT and GDF15^{-/-} (n=5) after a 6 hour BrdU pulse. Scale bars, 100 μm. Data represent the means ± SEM of at least three independent experiments.

3.3.6- Absence of GDF15 leads to an increase in Mash1 expression.

Taken together, the above analysis of proliferation both *in vitro* and *in vivo* had indicated that GDF15 regulates proliferation of NPC. In particular, both *in vitro* and *in vivo*, absence of GDF15 leads to extra proliferation in the compartment of secondary progenitors, whereas it does not cause major changes in the proliferation rate of more

primitive precursors. To further analyse this issue and investigate the nature of the extra-proliferating cells, I next used immunohistochemistry to monitor expression of Mash1, a transcription factor expressed at initial steps of neurogenesis in relatively undifferentiated secondary precursors but not in primary stem cells (Torii et al., 1999), in the GE of E18 WT and GDF15^{-/-} littermate embryos. In addition, as previously described for BrdU, I analysed both dissociated cells of the dissected GE and coronal telencephalic sections. Using this approach I found that dissociated cells of GDF15^{-/-} GE contained more Mash1 immunopositive cells than the corresponding WT tissue (see graph in Fig.3.3.12). This increase was also confirmed by immunohistochemistry which clearly showed a higher amount of Mash1 positive cells in the subependymal layer of the GDF15^{-/-} (Fig. 3.3.12).

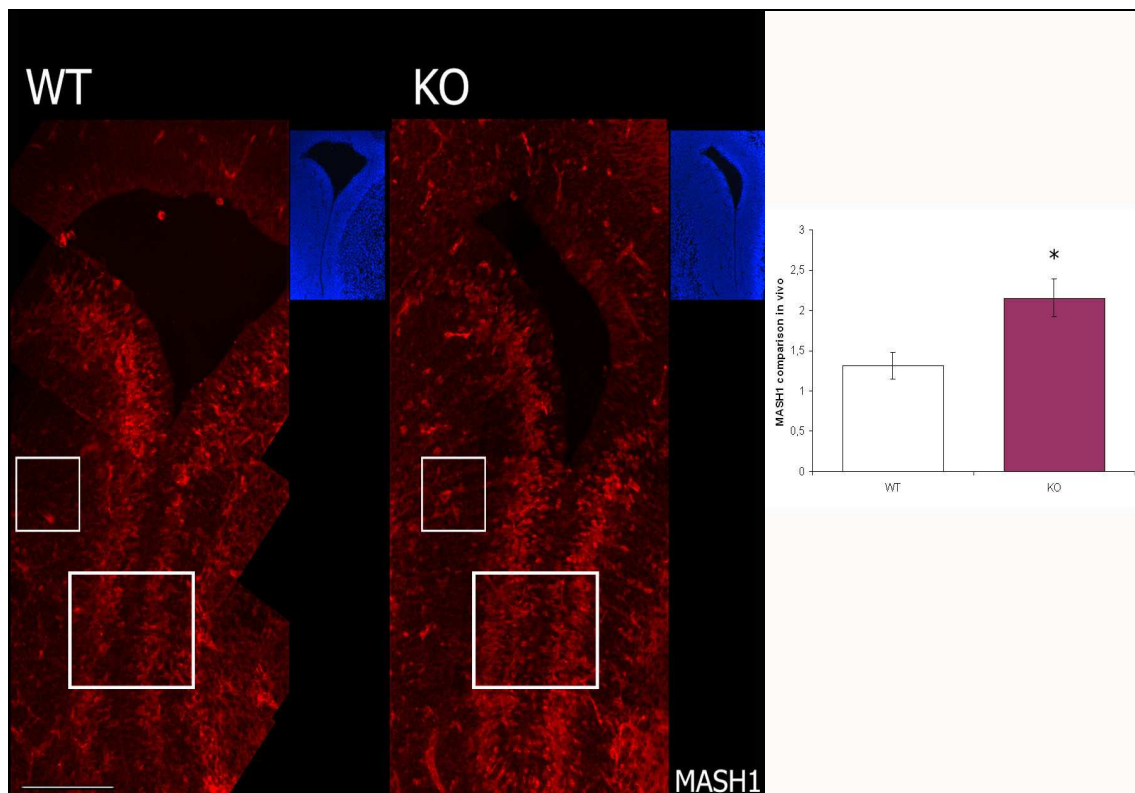


Fig. 3.3.12- Increased number of Mash1 immunopositive cells in the subependymal layer of GDF15^{-/-} embryos. Fluorescent micrographs showing representative examples of coronal sections of the subependymal region of the lateral ventricle of E18 WT and GDF15^{-/-} embryos, immunostained with Mash1 antibodies and counterstained with DAPI (small insets). The graph shows a quantitative analysis of the percentage of Mash1 positive cells found in WT and GDF15^{-/-} dissociated GE (n=6). Data represent the means \pm SEM of at least three independent experiments. Scale bar is 50 μ m.

3.4- Effect of GDF15 on NPC of the hippocampus.

3.4.1- NPC derived from GDF15^{-/-} embryonic hippocampus give rise to less progeny in vitro than their WT counterpart.

As shown above GDF15 is highly expressed in the hippocampus during development and its expression decreases in adulthood (Fig. 3.1 B). I have also shown that expression of GDF15 mRNA in NPC cultures derived from the hippocampus is downregulated during differentiation, indicating that GDF15 in this region is highly expressed by proliferating precursors (Fig. 3.1.F). To further study the possible effect of GDF15 on NPC proliferation I first generated neurosphere cultures from dissociated E18 and E14 hippocampal cells of WT and GDF15^{-/-} mice. Dissected tissue was triturated and plated in culture medium in the presence or absence of exogenous GDF15 as described. This analysis revealed a significant reduction in the number of cells obtained after 8 days of proliferation in GDF15^{-/-} derived cultures compared to WT cells at both E14 and E18 (Fig. 3.4.1 A and B). Consistent with the data obtained from the GE (see Fig. 3.3.1 A and B for a comparison), this reduction was not rescued by addition of exogenous GDF15, showing that also in the hippocampus GDF15 does not directly affect NPC proliferation.

To investigate whether such a decrease reflected a depletion of the NPC pool I used clonal analysis from E14 and E18 WT and GDF15^{-/-} animals. Hippocampal derived cells were plated at a density of one cell per well in 96 well plates and clones were scored after 8 days in culture. As shown in figure 3.4.1, panels C and D, this analysis revealed no significant difference in the number of clone forming cells between WT (5.7 %) and GDF15^{-/-} animals (6.8 %).

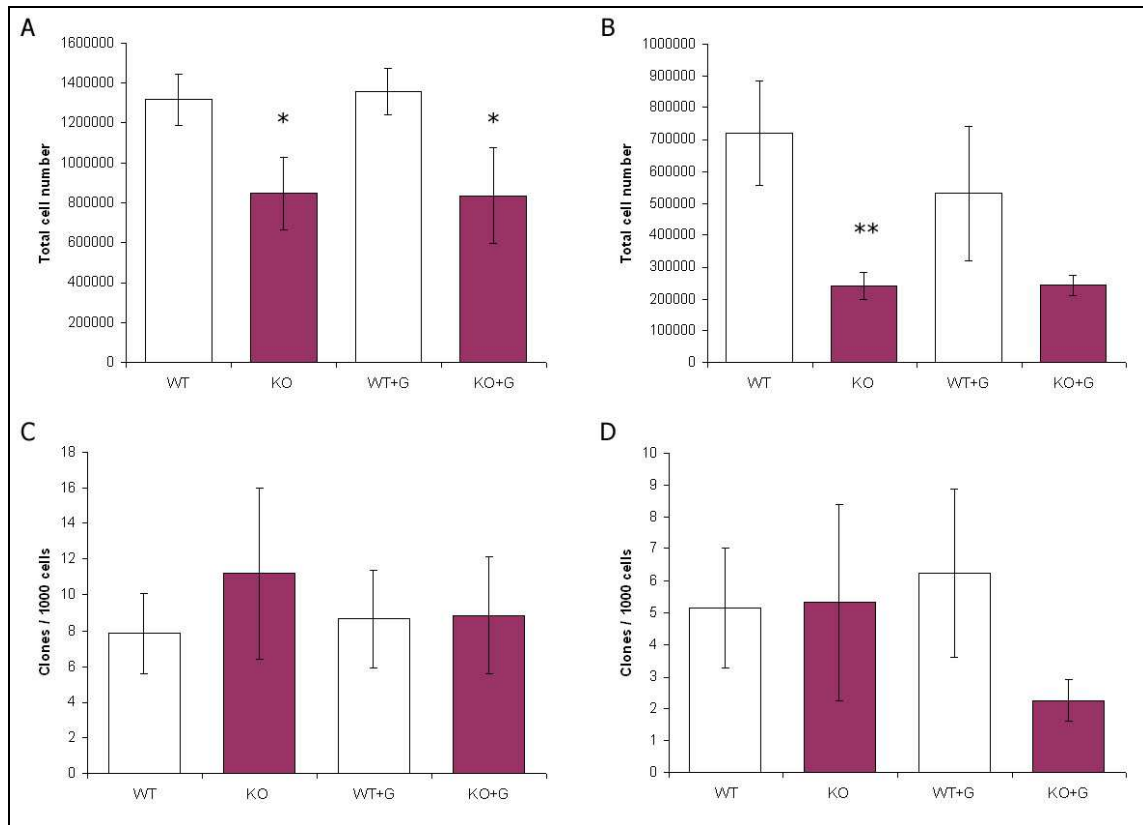


Fig. 3.4.1- GDF15 significantly affects neural precursor proliferation. (A, B) Quantitative analysis of the total cell number generated after a week in hippocampus derived neurosphere cultures from E14 (A) and E18 (B) WT and GDF15^{-/-} grown in the presence or absence of GDF15 (WT+G and KO+G). (C, D) Quantitative analysis of the clone forming capacity of E14 and E18 (respectively C and D) hippocampal clonal primary cultures from WT and GDF15^{-/-} animals grown in the presence or absence of exogenous GDF15 (WT+G and KO+G). Data represent the means \pm SEM of at least three independent experiments.

Thus, as observed in the GE, lack of GDF15 also leads to a reduced proliferation of hippocampal neurosphere cultures without affecting the size of the neural stem cell pool.

3.4.2- Absence of GDF15 leads to a decrease in EGFR expression in hippocampal NPC.

Similar to the GE, at E18, a subset of hippocampal NPC express high levels of EGFR whereas at E14, a 48 hour exposure to FGF-2 is required to promote EGFR expression in NPC (Ciccolini and Svendsen, 1998). I previously found that in the GE absence of GDF15 leads to a downregulation of EGFR expression in NPC that is probably responsible for the decreased ability of these cells to proliferate *in vitro* (see Fig. 3.3.1 A and B). I therefore next investigated whether EGFR expression is also downregulated in GDF15^{-/-} hippocampal NPC. To this end, I analysed expression of EGFR in NPC by

FACS. As previously mentioned for the GE, I sorted dissociated cells from E18 hippocampi, whereas E14 derived samples were exposed to FGF-2 for 48 hours prior to sorting (DIV 2).

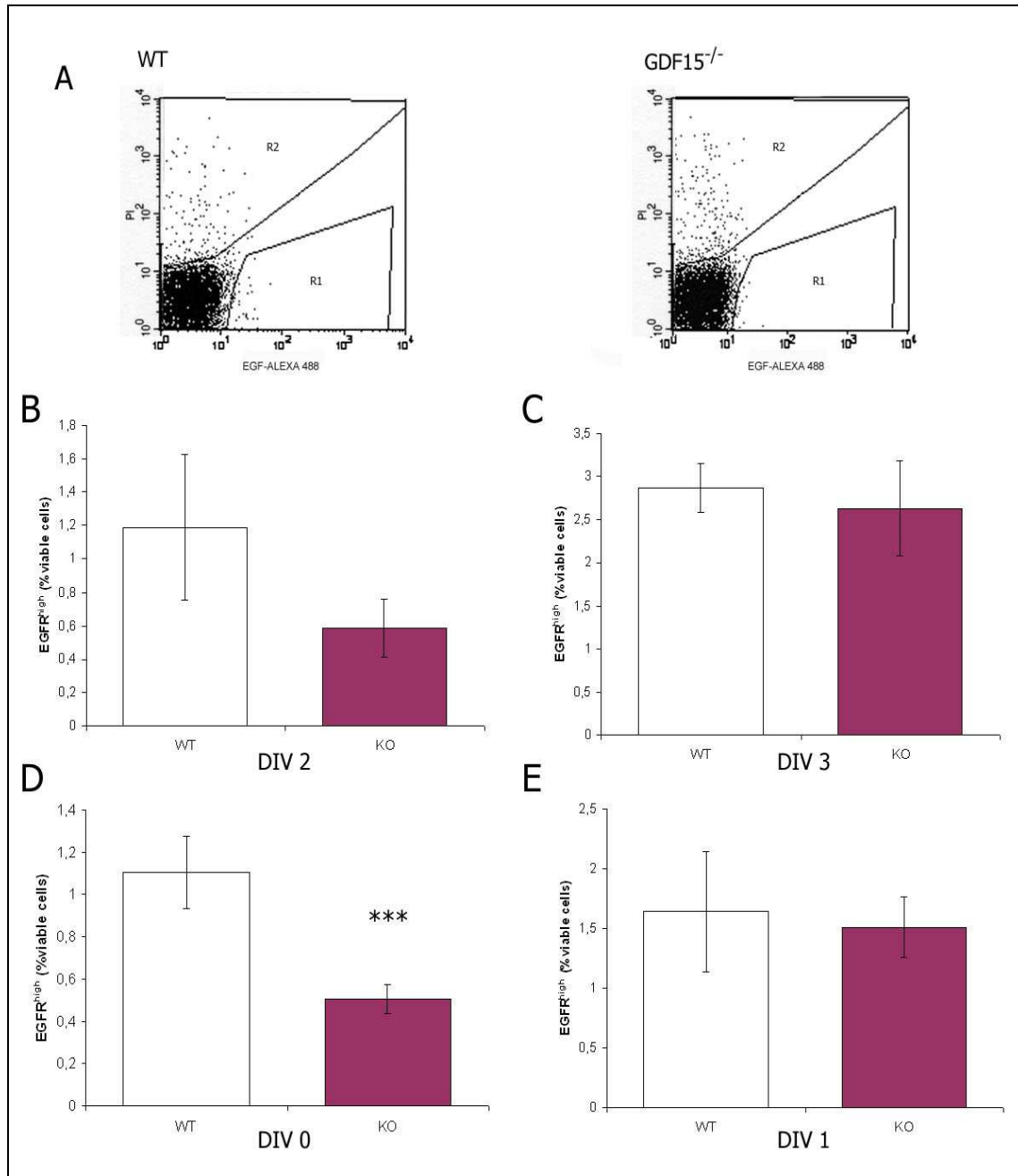


Fig. 3.4.2- Absence of GDF15 affects expression of EGFR in NPC but not their absolute number. (A) Representative FACS plots of dissociated E18 hippocampal cells derived from WT and GDF15^{-/-} animals after staining with EGF-Alexa 488 and PI to reveal EGFR^{high} cells (R1) and dead cells (R2). (B-E) Quantitative analysis of the number of EGFR^{high} cells isolated from the hippocampus of E14 (B and C) and E18 (D and E), WT and GDF15^{-/-} animals analysed by FACS at the indicated DIV. Data represent the means \pm SEM of at least three independent experiments.

Results

In both populations the number of EGFR^{high} cells was reduced in the hippocampus of GDF15^{-/-} mice (Fig. 3.4.2 B and D). Furthermore, this difference was not observed after further treatment with FGF-2 (Fig. 3.4.2 C and E). However, no difference was observed between WT and GDF15^{-/-} animals with respect to the percentage of clone forming cells either in the EGFR^{high} (Fig. 3.4.3) or EGFR^{low} subset (Fig. 3.4.3 E and F).

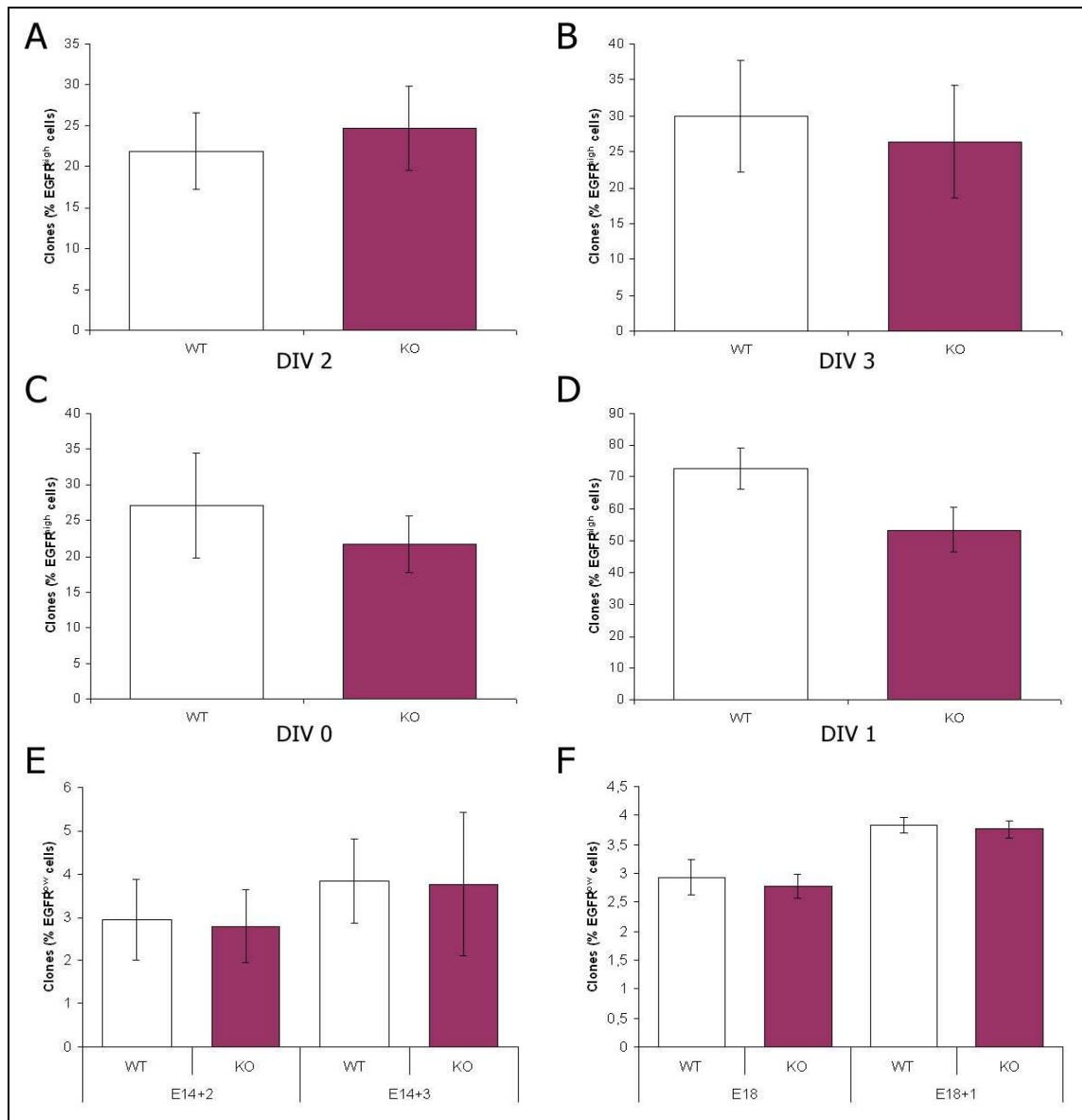


Fig. 3.4.3 Clone formation of sorted hippocampal precursors is not affected by the lack of GDF15. Sorted EGFR^{high} cells from E14 (A, B) and E18 (C, D), and EGFR^{low} cells from E14 (E) and E18 WT and GDF15^{-/-} hippocampi were plated at clonal density (EGFR^{high} cells 1cell/well; EGFR^{low} cells 10 cells/well) immediately after dissection (DIV 0) or after FGF-2 exposure as indicated in the figure. Percentage of clones was counted after one week in culture. Data represent the means \pm SEM of at least three independent experiments.

Since it is known that FGF-2 promotes EGFR expression also in hippocampal NPC (Bull and Bartlett, 2005), I exposed dissociated hippocampal cells to exogenous FGF-2 (24 hours for E18 cultures and 48/72 hours for E14 cultures) before analysis of EGFR by FACS. After FGF-2 treatment I found no difference in the number and ability to form clones of EGFR^{high} cells between WT and GDF15^{-/-} cultures (Fig. 3.4.2).

As for EGFR^{high} cells isolated from the GE the proliferative ability and the self-renewal of EGFR^{high} cells isolated from the E14 and E18 hippocampus were also not affected by the absence of GDF15 (Fig. 3.4.4). In addition, as for the GE analysis, PI exclusion revealed no significant difference in the number of dying cells between WT and GDF15^{-/-} cells (Fig. 3.4.4), indicating that the decrease in the number of EGFR^{high} cells in the hippocampus of GDF15^{-/-} animals was not due to differences in cell viability.

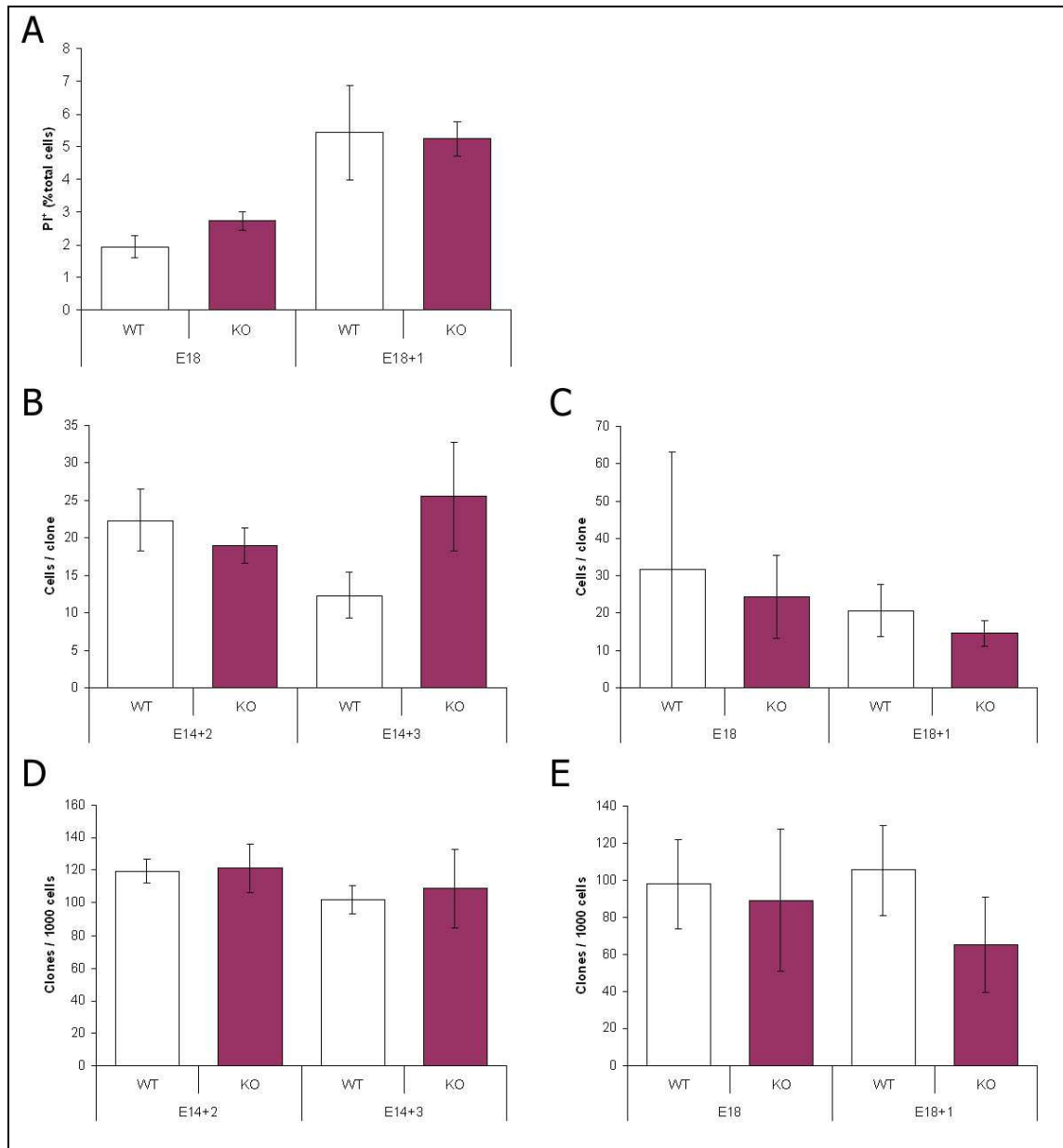


Fig. 3.4.4- Absence of GDF15 does not affect cell viability, proliferation and self renewal of EGFR^{high} cells. (A) Quantitative analysis of cell death of E18 hippocampal dissociated cells assessed as PI positive cells and shown as a percentage of the total sorted cell population. (B, C) Quantitative analysis of the number of cells per clone derived from EGFR^{high} E14 (B) and E18 (C) cells. (D, E) Quantitative analysis of the number of secondary clones obtained per 1000 cells of E14 (D) and E18 (E) primary clones. Data are given as means \pm SEM of at least three independent experiments.

To investigate whether exogenous GDF15 rescued the decrease in EGFR expression observed in the hippocampus of GDF15^{-/-} animals, dissociated tissue was incubated overnight in the presence of different concentrations of FGF-2 (1, 5 and 10 ng/ml) with or without GDF15, before analysing EGFR levels by FACS. I observed no effect on the

number of EGFR^{high} cells at any FGF-2 concentration tested, neither in the presence nor in the absence of GDF15 (Fig. 3.4.5 D, E). Furthermore, unlike in the GE (see figure), a difference was not observed upon shorter (6 hours) treatment times (3.4.5 F, G) suggesting that GDF15 does not directly affect EGFR expression in hippocampal NPC.

Taken together, these observations indicate that GDF15 indirectly affects expression of EGFR in hippocampal NPC, but does not affect the size of the NPC pool.

3.4.3- Decrease on EGFR expression is not mediated by a change in FGF-2 signaling.

I next investigated the expression of FGF-2 and FGFR1 and 2 in the hippocampus of WT and GDF15^{-/-} perinatal mice by quantitative rt-PCR (Fig. 3.4.5, A-C respectively). This analysis did not reveal significant differences between WT and GDF15^{-/-} animals.

Results

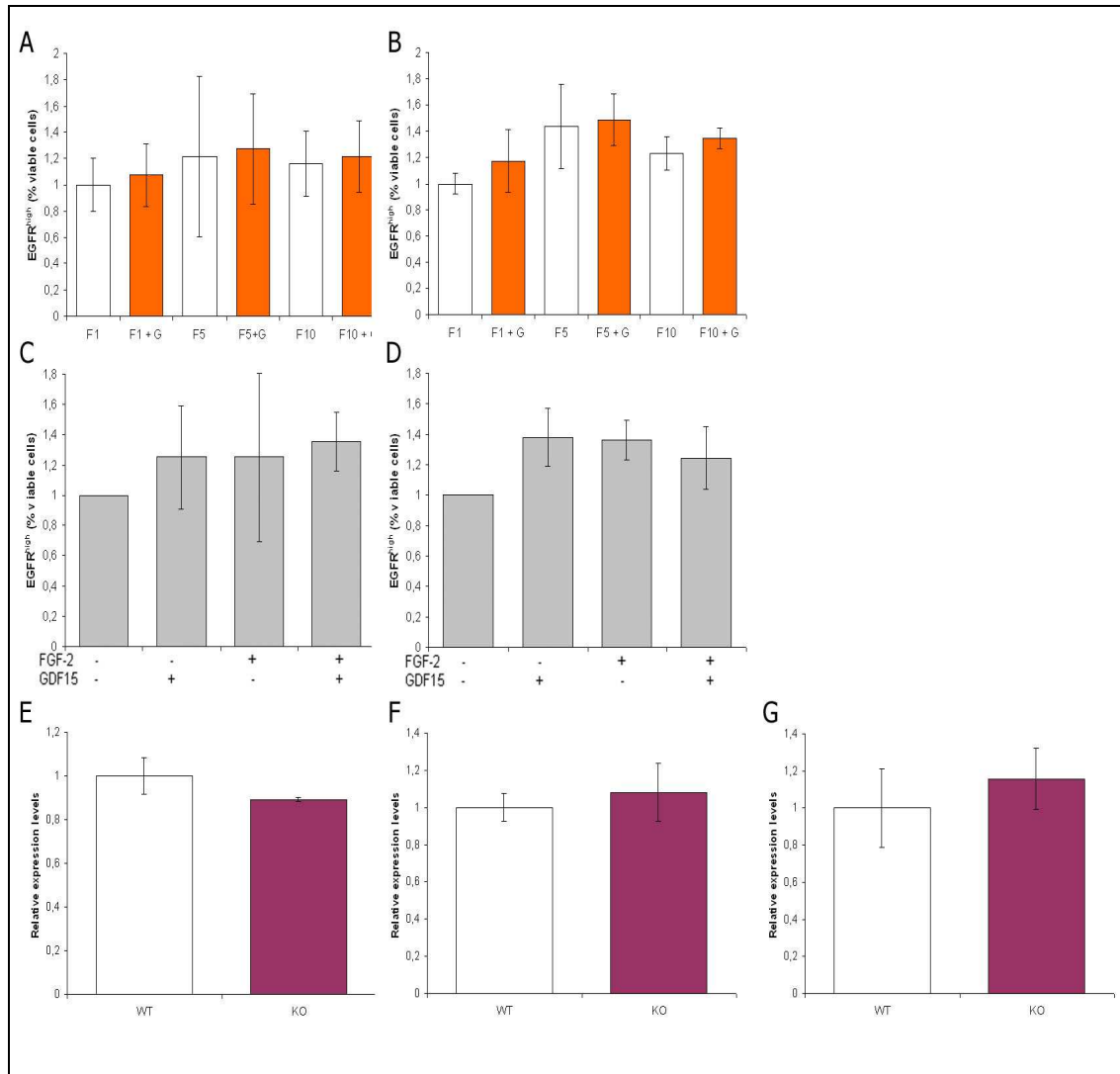


Fig. 3.4.5- GDF15 modulates EGFR expression in NPC independently of FGF signalling. (A, B) Quantitative analysis of the number of EGFR^{high} cells isolated from the E18 hippocampus of WT (A) and GDF15^{-/-} (B) embryos analysed by FACS after overnight exposure to different concentrations of FGF-2 in the presence and/or absence of GDF15. (C, D) Quantitative analysis of the number of EGFR^{high} cells isolated from E18 hippocampus of WT (C) and GDF15^{-/-} (D) after 6 hours of incubation in the presence and /or absence of FGF-2 and GDF15. Data analysed by one-way ANOVA. (E-G respectively) Analysis of FGF-2, FGFR1 and 2 expression in the perinatal hippocampus by Real Time PCR. Comparison of FGF-2, FGFR1 and 2 mRNA levels, relative to GAPDH and β -Actin mRNAs, of hippocampi dissected from perinatal WT and GDF15^{-/-}. Data are given as means \pm SEM of at least three independent animals.

These data indicate that the reduction in EGFR expression observed in the hippocampus of GDF15^{-/-} embryos does not depend on an apparent alteration in FGF-2 signalling.

3.4.4- Analysis of EGFR expression in vivo.

I next investigated EGFR expression in the hippocampus using immunohistochemistry with EGFR antibodies on coronal sections of E18 WT and GDF15^{-/-} hippocampi. In the WT hippocampus EGFR immunoreactive cells were found mainly in the subependymal region adjacent of the CA1 area, the hSVZ. EGFR expression was downregulated at increasing distance from the lateral ventricle and very few immunopositive cells were observed in the CA3 region and in the DG (Fig. 3.4.6).

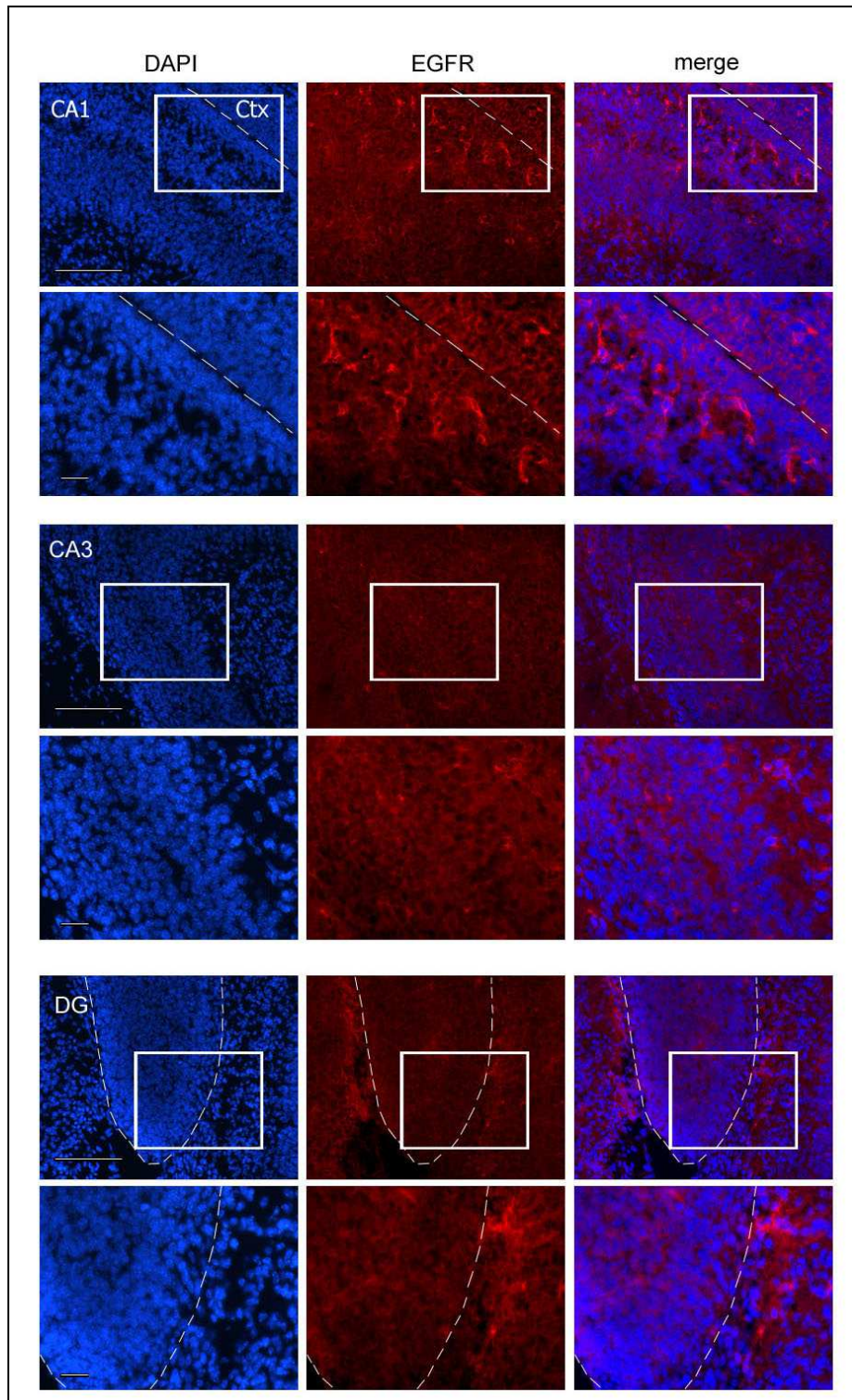


Fig. 3.4.6- Localization of EGFR^{high} cells in the E18 hippocampus. . Fluorescent micrographs showing representative examples of coronal sections of CA1, CA3 and DG regions immunostained with EGFR and counterstained with DAPI. For each hippocampal region lower row panels show a higher magnification view of the area enclosed by the square .Note that immunopositive cells are mainly found in the subependymal region of CA1. Scale bar is 100 μm at low magnification, and 20 μm at higher magnification.

In addition, compared to the WT, EGFR expression was decreased in the corresponding CA1 region of knock-out animals, consistent with our FACS-based analysis of EGFR expression (Fig. 3.4.7).

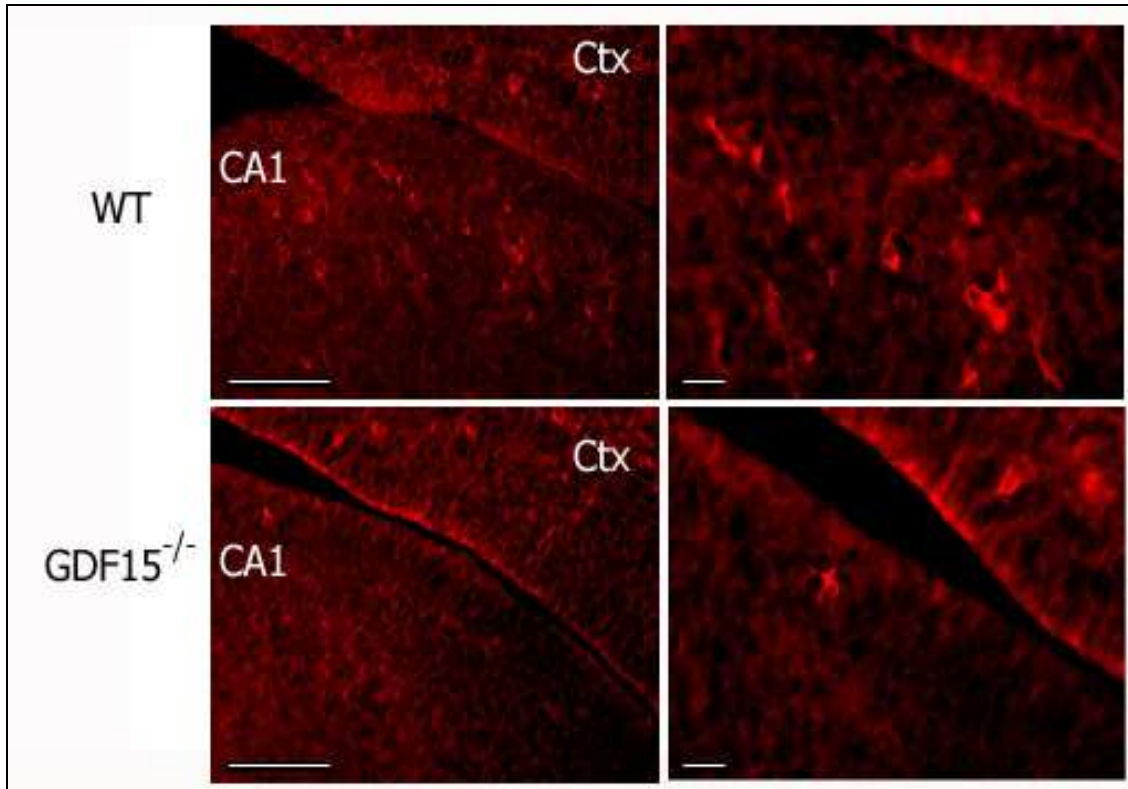


Fig. 3.4.7- EGFR expression is decreased in $GDF15^{-/-}$ animals *in vivo*. Coronal sections showing representative examples of EGFR expression in E18 CA1 areas from WT and $GDF15^{-/-}$ brain slices. Scale bar is 100 μm (lower magnification micrographs), and 20 μm (higher magnification micrographs).

3.4.5- Role of GDF15 in the regulation of hippocampal NPC *in vivo*.

Previous results had shown that GDF15 affects NPC proliferation *in vitro*. To investigate whether GDF15 was also relevant *in vivo*, I first analysed the expression of phospho histone H3 (PHH3), a cell cycle marker which labels cells in late G2 and M phases of the cell cycle. PHH3 has a defined temporal expression in actively cycling cells, labelling only mitotic cells, and it is not expressed after cell cycle exit. Immunostaining of WT and $GDF15^{-/-}$ coronal telencephalic sections with antibodies to PHH3 revealed that the vast majority of PHH3 immunoreactive cells in the hippocampus were located in the CA1 region, mostly in the subependymal area

underlying the ventricle (hSVZ) (Fig. 3.4.8 A). CA1 immunopositive cells were counted within a region of interest (for details see Material and Methods), whereas in the CA3 and the DG cells were counted throughout the entire area of these regions. Interestingly, in the DG most mitotic cells were found in the subregion corresponding to the hilus (Fig. 3.4.8 B-D). Comparative quantification of WT and GDF15^{-/-} PHH3 immunoreactive cells in the hippocampus revealed a reduction in GDF15^{-/-} animals in all the three areas, i.e. CA1, CA3 and DG (Fig. 3.4.8 B-D).

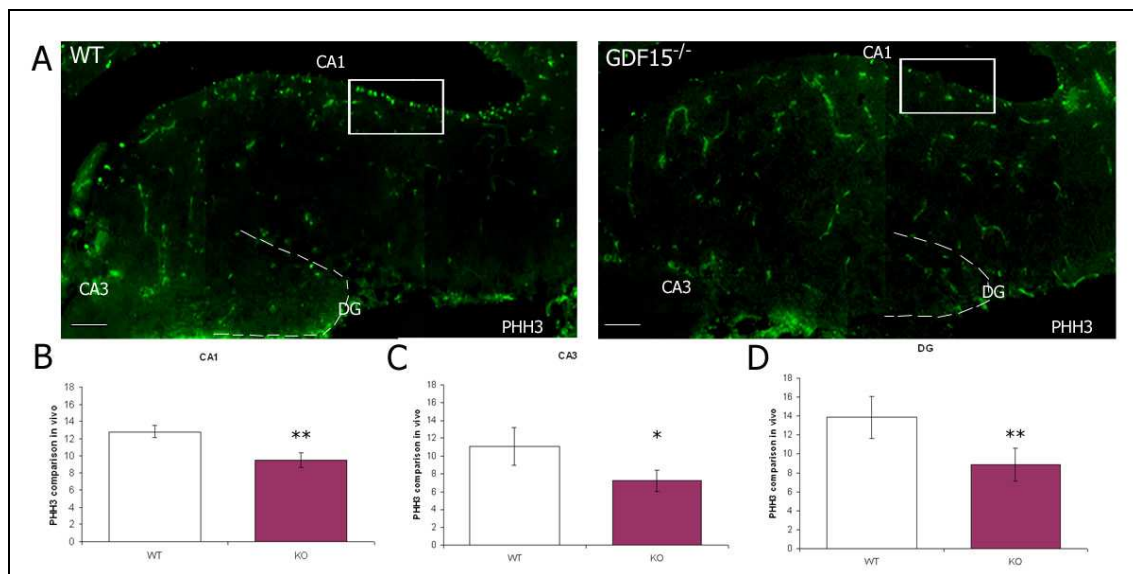


Fig. 3.4.8- Analysis of mitotic cells *in vivo* in WT and GDF15^{-/-} animals. (A) Coronal sections showing representative examples of PHH3 immunolabelling in E18 WT and GDF15^{-/-} hippocampi. Scale bar is 100 μ m. (B-D) Quantitative analysis of the number of PHH3 positive cells undergoing mitosis in CA1 (B), CA3 (C) or in the hilus of the DG (D) of WT and GDF15^{-/-} animals. Data represent the means \pm SEM of at least three independent experiments.

To further investigate this issue, I analysed BrdU incorporation *in vivo* in WT and GDF15^{-/-} animals. To this end I set up heterozygous matings and time mated females were injected intraperitoneally with BrdU and sacrificed 2 hours after the injection. Brains of embryos were removed and one hemisphere was processed for immunohistochemistry, while the hippocampus of the other hemisphere was dissected, mechanically dissociated, and cells were plated out shortly on polyornithine coated chamberslides and processed for BrdU immunocytochemistry. Analysis of dissociated hippocampal cells revealed a significant reduction (-28,8%) in the number of proliferating cells in the GDF15^{-/-} hippocampus compared to its WT counterpart (3.4.9

C). Furthermore, immunohistochemistry of coronal sections showed that, compared to WT, GDF15^{-/-} embryos displayed less BrdU immunopositive cells (-16.7%) in the subependymal area of the CA1 region (3.4.9 D).

Immunohistochemistry for PHH3 had revealed that absence of GDF15 leads to a reduction in the number of mitotic cells in all analysed areas of the hippocampus (Fig. 3.4.8). In contrast, after a 2 hour BrdU pulse, I found reduced proliferation only in the CA1 subependymal region of GDF15^{-/-} embryos. This difference between the two sets of experiments may be due to the fact that proliferating CA1 cells may represent a migratory population (Navarro-Quiroga et al., 2006). To investigate this possibility, I analysed BrdU immunopositive cells after a 6 hour BrdU pulse, since a longer pulse allows the labelling of most proliferating precursors and monitoring of their putative migration. Analysis of the dissociated tissue after a 6 hour BrdU pulse revealed again a 27,9% reduction in the number of proliferating cells in GDF15^{-/-}-cells compared to WT (3.4.9 E). Furthermore, this reduction was also found after comparative quantification of coronal hippocampal sections. However, this time the decrease in BrdU incorporation, amounting to around 34%, was found in the hilus, and not in the CA1 region as previously observed after a 2 hour BrdU pulse (3.4.9 F). Furthermore, in WT animals the number of BrdU incorporating cells increased with prolonged exposure time to BrdU in all hippocampal subregions except the CA1 (Table 1; compare also panels D and F of Fig. 3.4.9).

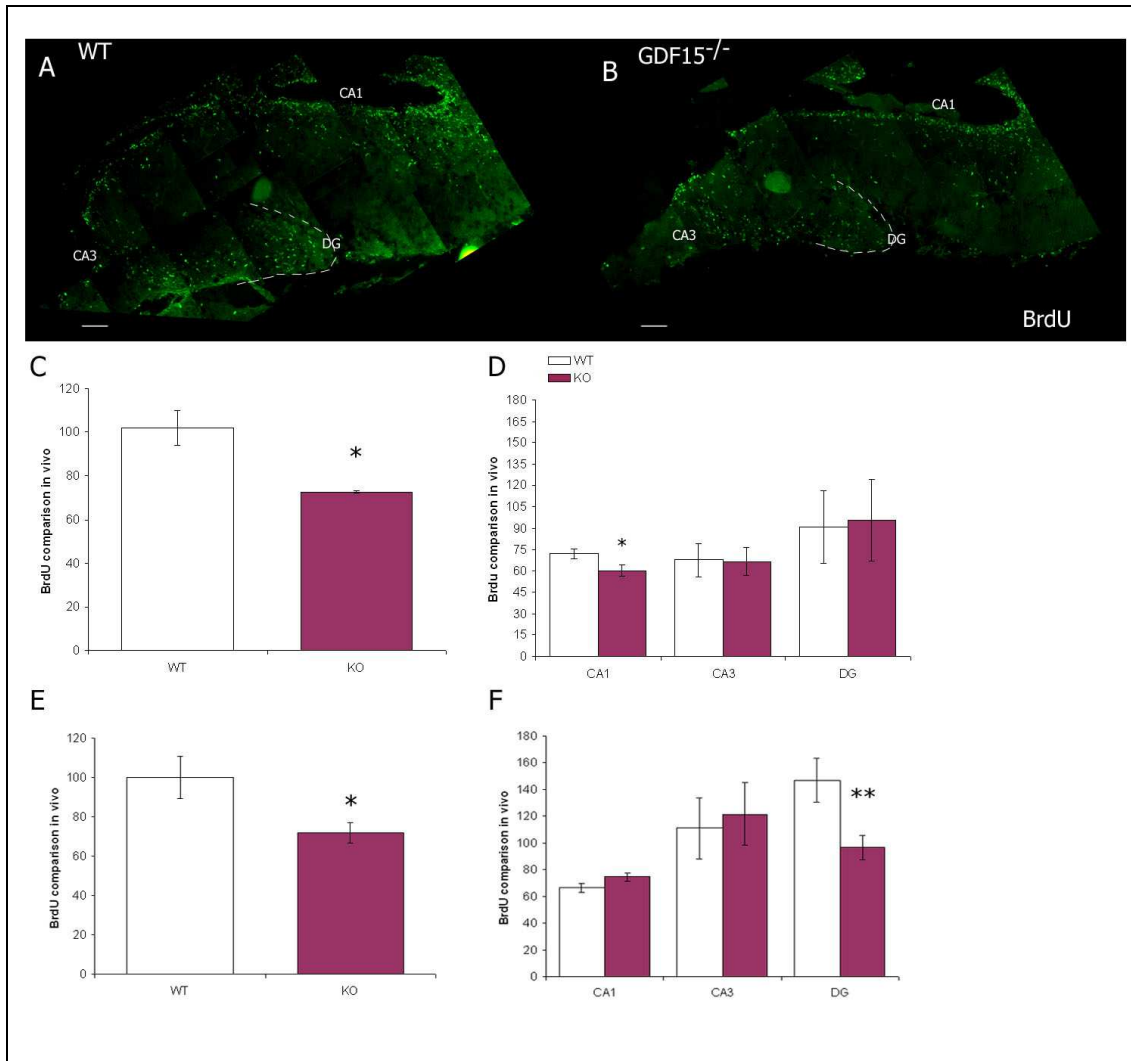


Fig. 3.4.9- Effect of GDF15 on NPC proliferation *in vivo*. (A and B) Coronal sections showing representative BrdU immunolabelling of WT (A) and GDF15^{-/-} (B) hippocampi after a 6 hours BrdU pulse. Scale bar is 100 μm. (C and E) Quantitative analysis of the percentage of BrdU immunopositive cells in WT and GDF15^{-/-} dissociated hippocampus after 2 (C) and 6 (E) hours BrdU pulse. (D and F) Quantitative analysis of the percentage of BrdU positive cells found in the different hippocampal subregions of WT and GDF15^{-/-} embryos after 2 (D) and 6 (F) hour BrdU pulse. Data represent the means ± SEM of at least three independent experiments.

I also compared the number of dividing cells quantified after BrdU pulses of 2 and 6 hours. I observed that in WT embryos, independently of the time, the amount of positive cells in the CA1 region was similar, suggesting that dividing cells do not accumulate in this region but migrate out of the CA1. However, the same comparison in the GDF15^{-/-} animal showed a significant increase (P=0.016) in the number of BrdU⁺ cells after a longer BrdU exposure time, suggesting a delay of the NPC on the timing of departure from the CA1.

Results

	CA1		CA3		DG	
	WT	GDF15 ^{-/-}	WT	GDF15 ^{-/-}	WT	GDF15 ^{-/-}
2 hours BrdU	72 ± 3,74	60 ± 3,94*	67,6 ± 11,87	66,4 ± 9,90	90,8 ± 25,64	95,8 ± 28,63
6 hours BrdU	66,6 ± 3,59	74,5 ± 3,43	111 ± 22,83	121,5 ± 23,61	146,8 ± 16,55	96,8 ± 9,27*

3.4.10- Table 1 BrdU incorporation in hippocampal subregions after 2 and 6 hour BrdU pulses in WT and GDF15^{-/-} E18 embryos. Areas of identical sizes were analysed on equivalent anatomical regions. Data represent the means ± SEM of at least three independent experiments. * indicates significantly different from corresponding WT region.

Taken together, these data indicate that at least some cells undergoing mitosis in the CA3 and DG derive from CA1 cycling migratory precursors. This is consistent with previous evidence indicating the existence in the hippocampus of a stream of precursors migrating from CA1 to the DG (Navarro-Quiroga et al., 2006). My data also show that GDF15 is important for the regulation of the proliferation of CA1 precursors.

3.4.7- Comparison of EGFR and PHH3 expression in vivo.

I next investigated whether EGFR expressing cells represent NPC. To this end, I performed double immunohistochemistry for PHH3 and EGFR to investigate the relation between both markers on E18 WT and GDF15^{-/-} hippocampus. I observed that in the periventricular area the vast majority of PHH3 immunoreactive cells were also positive for EGFR, suggesting that in germinal epithelia EGFR expression is closely associated with proliferation. I also observed that PHH3 in the CA3 and DG displayed progressively lower levels of EGFR expression, suggesting that cycling precursors downregulate EGFR expression in these regions. This observation is consistent with the hypothesis that some EGFR immunopositive precursors may initiate cell division in the CA1 subependymal region and then migrate through the CA3 to the DG.

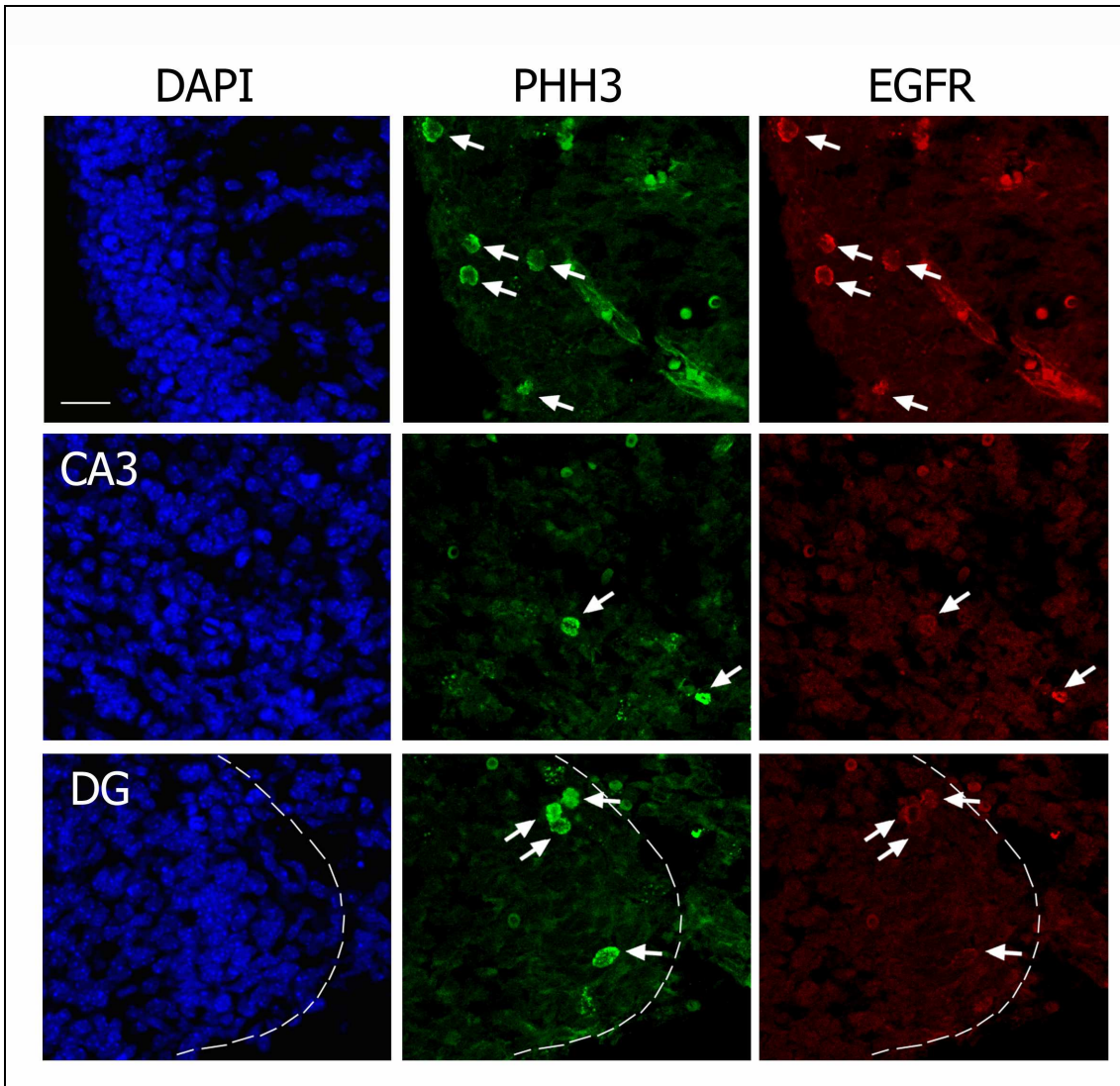


Fig. 3.4.12- Comparison of EGFR and PHH3 expression *in vivo*. Confocal images showing coronal sections of the hippocampi of E18 WT mice immunostained with PHH3 antibodies (central panels) and EGFR (right panels). Left panels show DAPI staining of nuclei. Scale bar: 37.5 μm .

Chapter 4: Discussion.

4.1- GDF15 is expressed by NPC from embryonic and adult GE/SVZ and hippocampus.

In the first part of this study, I provide evidence that GDF15 is expressed in particular by late development NPC derived from neurogenic areas of the embryonic and adult telencephalon. Several lines of evidence support this conclusion. Firstly, in the GE and adult SVZ, where I micro-dissected the germinal epithelium, I observed that GDF15 mRNA levels increase with age. Instead, in the hippocampus where this analysis was performed on whole tissue, levels of GDF15 mRNA decrease in adult animals. This pattern shows a correlation between levels of GDF15 mRNA and the abundance of NPC in the analysed tissues. Secondly, I found that GDF15 is expressed in differentiating neurosphere cultures derived from the GE but not from the hippocampus and that this correlates with the differences in the number of proliferating NPC between the two sets of cultures. This difference likely depends on the fact that GE but not hippocampal neurosphere cultures are derived from NSC. Previous studies have shown that neural precursors in the hippocampus and in the anterior telencephalic germinal epithelium differ in the ability to long term self-renew and generate neurons. This had suggested that SC are very rare or virtually absent in the hippocampus. Consistent with this observation I found that GE derived neurospheres, upon differentiation, generated more neurons than the hippocampal counterpart. More importantly, I observed a several fold higher expression of GDF15 mRNA in sorted EGFR^{high} NPC compared to the remaining cells expressing low levels of EGFR (EGFR^{low}) in both GE and hippocampus (GE seven-fold; hippocampus five-fold). Since it has previously been shown that the incidence of clone forming NSC is thirty-fold higher in EGFR^{high} than in EGFR^{low} cells (Ciccolini et al., 2005; and my own results), these experiments strongly indicate that clone-forming neural precursors represent the main source of GDF15 in the telencephalic germinal epithelium. In the present study I have investigated the expression of GDF15 mRNA and not protein; detection of the protein by immunohistochemistry was not possible, most likely due to the low expression levels of GDF15 in the brain. However, previous studies have analysed expression of GDF15 protein in the postnatal rat brain by immunohistochemistry. This analysis revealed that

GDF15 is expressed at very low levels in the rat brain under normal conditions and that GDF15 protein is localized in close proximity of the neurogenic areas, particularly around the lateral ventricle (Böttner(a) et al., 1999; Schober et al., 2001). Taken together, my results and those from previous studies indicate that NPC not only express GDF15 mRNA but also synthesise GDF15 protein.

4.2- GDF15 does not directly affect NPCs proliferation in vitro.

Members of the TGF β superfamily have been involved in the regulation of multiple aspects of NPC behaviour (Munoz-Sanjuan and Brivanlou, 2002; Mishra et al., 2005; Golestaneh and Mishra, 2005; Falk et al., 2008). In particular, TGF β and BMPs have been shown to affect the regulation of NPC proliferation (Munoz-Sanjuan and Brivanlou, 2002; Falk et al., 2008). In the present study I found that, although GDF15 is particularly expressed by clone-forming cells, it does not affect their maintenance, proliferation, or viability. Although, compared to WT, I observed a reduction of cell number in bulk cultures derived from GDF15^{-/-} animals, my data indicate that this is likely due to the fact that absence of GDF15 leads to downregulation of EGFR in NPC both in the hippocampus and the GE. As mentioned in the introduction (Chapter 1.3), it is well established that early-embryonic NPC (E14) are FGF-2 but not EGF responsive, while late-embryonic NPC (E18) are responsive to both factors (Ciccolini and Svendsen, 1998; Lillien and Raphael, 2000). NPC initially responding to FGF-2 become responsive to EGF later during development. This change in growth factor responsiveness is promoted by FGF-2 and leads to the appearance of a population of precursor cells responding to both EGF and FGF-2. Late development neural precursors display increased levels of EGFR expression, and *in vitro* exposure to exogenous FGF-2 leads to upregulation of EGFR expression in EGFR^{low} NPC (Ciccolini and Svendsen, 1998; Lillien and Raphael, 2000). In the present study, I have shown that, independent of age analysed, the number of EGFR^{high} cells in cultures that had been previously exposed to FGF-2 did not differ significantly in cultures derived from wild type and GDF15^{-/-} mice, respectively. However, when cells were analysed directly after dissection, compared to WT counterparts, the number of EGFR^{high} cells in both GDF15^{-/-} GE and hippocampus cells was significantly decreased. Taken together, these data show that immediately after dissection, compared to WT counterparts, less GDF15^{-/-} NPCs will be capable to proliferate in response to EGF. Although it has been

shown that at E18 FGF-2 and EGF are equally potent in promoting proliferation of neurosphere precursors (Ciccolini et al., 2003), in this study I used half of the concentration of FGF-2 normally required to promote neurosphere proliferation. Under these conditions EGF is a more potent mitogen than FGF-2 for NPC (Tropepe et al., 1999; Kelly et al., 2005). Therefore, I conclude that the delayed expression of high levels of EGFR in GDF15^{-/-} NPC is likely responsible for the decreased cell numbers in neurosphere cultures. BMP4, another member of the TGF β superfamily, has been shown to regulate EGFR signalling in embryonic NPC. Whether this effect is due to a direct alteration of EGFR expression or by antagonism between BMP4 and FGF-2 it is not clear (Lillien and Raphael, 2000). Other TGF β s regulate Trk (tropomyosin receptors kinase) neurotrophin receptors in the CNS. For example, it has been shown that TGF β 1 upregulates expression of TrkB and one of its ligands, BDNF, thereby mediating neuronal survival in cultured cortical neurons (Sometani et al., 2001).

Interestingly, I found that GDF15^{-/-} EGFR^{high} cells freshly isolated from the GE were less clonogenic than their WT counterparts. This effect could be due to the fact that mutant mice not only have lower numbers of EGFR^{high} cells but also a general decrease in the levels of EGFR expression. Thus, EGF may not sufficiently stimulate EGFR signalling to reach a threshold level required for proliferation. However, I provide several lines of evidence that this is unlikely. Firstly, analysis of the FACS plots revealed no significant variation in the levels of fluorescence indicating EGFR expression. Secondly, western blot analysis showed no significant difference of EGFR expression levels between WT and GDF15^{-/-} mice (data not shown). Most importantly, I found that although the number of EGFR^{high} cells is reduced also in the hippocampus, as in the GE of mutant mice, unlike the GE counterpart, the clone formation ability of hippocampal EGFR^{high} cells is not affected by the absence of GDF15. Therefore, I conclude that the defect in clone-forming ability of EGFR^{high} cells derived from the GE of GDF15^{-/-} mice is not due to a general reduction in EGFR expression but rather to the fact that this cell population contains less clone forming cells than the WT counterpart. In light of my observations that in vivo EGFR expression is associated with actively proliferating cells and that in GDF15^{-/-} GE there is an increase in the proliferation of secondary progenitors, I propose that these extra-proliferating secondary progenitors are overrepresented in the population of EGFR^{high} cells derived from GDF15^{-/-} mice. This interpretation is supported by the pattern of EGFR expression observed in the GE

germinal epithelium. I found that compared to WT EGFR GDF15^{-/-} mice displayed lower levels of EGFR throughout the VZ and especially at the apical borders. Instead, in the SVZ overall levels of EGFR expression were similar in WT and GDF15^{-/-} mice. Furthermore, whereas in the GE of wild type mice EGFR expressing precursors were often organized in radial columns extending from the VZ into the SVZ, in GDF15^{-/-} mice EGFR expressing precursors formed rather small clusters dispersed within the SVZ. Taking these observations together, I conclude that compared to WT, the incidence of clonogenic primary precursors in EGFR^{high} cells isolated from GDF15^{-/-} mice is decreased.

4.3- GDF15 directly regulates EGFR expression in NPC and not by modulation of FGF-2 signalling.

It is well known that EGFR acquisition in NPC is mediated by FGF signalling. In particular, it has been shown that FGF-2 and not other FGF family members, are involved in this regulation (Ciccolini and Svendsen, 1998; Lillien and Raphael, 2000). Since it has been shown that members of the TGF beta superfamily may also modulate FGF signalling (Lillien and Raphael, 2000; Falk et al., 2008), I here investigated the possibility that the effect of the lack of GDF15 on EGFR acquisition is due to an effect in FGF-2 signalling. My data clearly show no overt differences in levels of FGF-2 expression between WT and GDF15^{-/-} mice. Similarly, mRNAs levels of FGFR1 and FGFR2, the principal receptor types mediating the response to FGF-2 in the brain (Raballo et al., 2000; Maric et al., 2007; Saarimaki-Vire et al., 2007) were not overtly different in WT and GDF15^{-/-} mice. Furthermore, I found no difference in the response to FGF-2, as measured by the ability to promote EGFR expression, between cells derived from WT and GDF15^{-/-} mice. This suggests that also the signalling ability of FGF-2 is not compromised in GDF15^{-/-} mice. Instead, I found that exogenous GDF15 could significantly promote EGFR expression in a subset of cells derived from the GE. This effect on EGFR expression was observed already after 6 hours of GDF15 application, suggesting that GDF15 may promote EGFR expression by directly acting on NPC.

4.4- GDF15 promotes cell cycle exit of GE derived progenitors in vitro.

I found that in the absence of GDF15 more proliferating cells were present in differentiating GE neurosphere cultures. This was paralleled by a decrease in neuronal differentiation in GDF15^{-/-} cultures, compared to WT counterparts. Both changes were temporary and were not observed at later stages of differentiation. In particular, at all time points analysed I observed similar levels of proliferation in WT cultures. Extra proliferating cells in GDF15^{-/-} cultures were only observed at the earliest time point analysed after induction of proliferation (DAP 2). This indicates that GDF15 is only affecting the proliferation of progenitors that divide at early time points after induction of differentiation or that at later stages of differentiation other factors may compensate for the absence of GDF15. Interestingly, it has been previously suggested that neural stem cells while differentiating in vitro give rise first to neurons whereas they generate glia cells at later stages of differentiation (Shen et al., 1998; Qian et al., 2000). Indeed, in our analysis we found that the only cell lineage affected by the absence of GDF15 was the neuronal lineage. However, since we found that at later time points there is no difference in the number of neurons between cultures differentiating in the absence or in the presence of GDF15, is unlikely that the extra proliferating cells in GDF15^{-/-} cultures represent neuroblasts uniquely committed to the neuronal lineage.

4.5- GDF15 provides a feed forward signal regulating the cell cycle of proliferating progenitors in the developing GE.

Analysis of cell proliferation in vivo indicates that GDF15 affects cell cycle exit of secondary precursors not only in vitro but also in vivo. The number of proliferating cells is increased in the VZ of the GDF15^{-/-} mouse in vivo. I also found that the supernumerary cells are apically dividing precursors, and they are not resident in the germinal epithelium but within 6 hours they migrate towards the striatum. These characteristics suggest that the extra proliferating cells represent intermediate progenitors. The proliferation dynamics of this cell population has been studied in detail in the developing cortex (Noctor et al., 2002; Noctor et al., 2004; Martinez-Cerdeno et al., 2006; Noctor et al., 2008; Attardo et al., 2008). Cells proliferating within this area derive from radial glia precursors undergoing asymmetric cell division. They generate another radial glia that maintains the contact with the apical surface of the germinal

epithelium and a precursor that migrates into the SVZ. Here these secondary precursors undergo their last division that generally results in the generation of two neurons that will migrate out of the germinal epithelium to reach their final destination (Noctor et al., 2004; Noctor et al., 2008). After a six hour BrdU pulse I did not observe an increase in BrdU incorporation in the VZ/SVZ of GDF15^{-/-} mice but instead a decrease. This suggests that in the absence of GDF15 the secondary precursors undergo only an extra round of cell division before migrating outside of the germinal area. This is consistent with the in vitro analysis of the effect of GDF15 in differentiating NPC.

To confirm the identity of the supernumerary cells as secondary progenitors, I analysed Mash1 expression, which has been described to be expressed by intermediate progenitors in the GE. I observed an upregulation of Mash1 in the GE of GDF15^{-/-} mice in vivo compared to their WT littermates. Thus, in the absence of GDF15, secondary precursors do not exit the cell cycle but undergo an extra cell division, showing that in vivo as in vitro GDF15 regulates the timely exit from cell cycle in these cells.

Taking these observations together, I propose the following model (Fig. 4.5.1) to describe the role of GDF15 in EGFR acquisition of NPC, and in the regulation of the cell cycle exit of secondary progenitors.

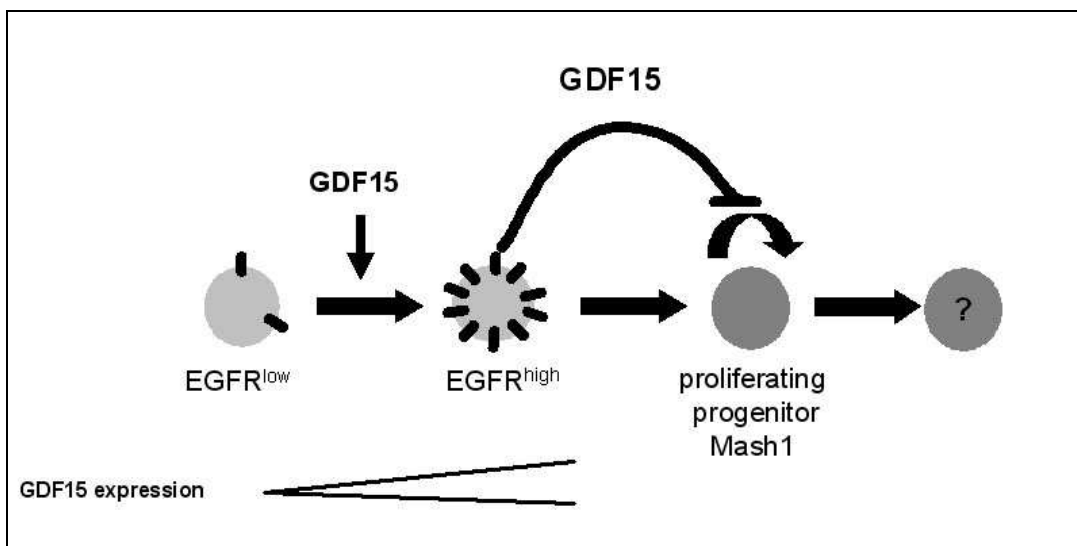


Fig 4.5.1- Scheme summarizing my findings about the role of GDF15 in mouse GE. I have found that GDF15 is expressed mainly by NPC in the developing GE. This factor affects EGFR expression in late developmental neural precursor cells and exerts a feed-forward signal regulating cell cycle exit of Mash1⁺ secondary progenitors.

Although the role of GDF15 in neural stem cells was not investigated before, previous studies have shown a role of this factor in regulating the cell cycle of other cell types. An inhibitory role of GDF15 in marrow progenitor proliferation has been proposed, showing a more active effect against later myeloid progenitors (Hromas et al., 1997). In tumour cells, GDF15 has been shown to inhibit cell growth acting as an anti-tumorigenic factor, as for example in colorectal and breast cancer cells (Tan et al., 2000; Baek et al., 2001).

Other GDF subfamily members have also been described to exert an effect on cell cycle. GDF8 and GDF11 inhibit growth by promoting the cell cycle exit in myoblasts or intermediate neuronal progenitors respectively. The authors propose an effect of those factors arresting cell cycle, nevertheless the progenitors are not retained in the cycle but they exit it generating myotubes and neurons respectively. Both factors exert an inhibitory feedback signal in progenitor cells which promote the cells to exit the cell cycle, thus regulating myogenesis and neurogenesis (Thomas et al., 2000; Wu et al., 2003). These observations suggest that the mechanisms by which GDF8 and GDF11 control cell cycle exit of progenitors is conserved between muscle and neuronal lineages. Mutant mice for GDF11 and GDF15 show an increase in the number of progenitor cells. For the GDF11 mutant, an increase in the number of neurons has also been documented. In the GDF15^{-/-} mouse this has not been analysed yet, although I have described an increase in Mash1 progenitors. Mash1 labels progenitor cells that give rise to neuronal and glial lineages depending on the developmental stage (Torii et al., 1999). Thus, GDF15 and GDF11 both act as signals that control cell cycle exit of neural progenitors although they act in different systems, the SVZ and olfactory epithelium, respectively, and are produced by different cell types. While GDF11 is produced by olfactory neurons and their progenitors, exerting a feed-back signal which promotes the cell cycle exit of neuronal progenitors, my data show that GDF15 is produced mainly by primary neural precursor cells, thus exerting a feed-forward signal which controls the cell cycle length and exit of secondary progenitor cells (Fig 4.5.2).

Finally, regulation of cell cycle exit of neural precursors may also be a role exerted by other members of the TGF β superfamily. In particular, a role of TGF β signalling in regulating the choice of neuroepithelial cells between NSC maintenance versus differentiation by modifying their cell cycle length and exit has recently been described. It has been proposed that TGF β exerts a negative effect on self-renewal by promoting the differentiation of NE cells (Falk et al., 2008).

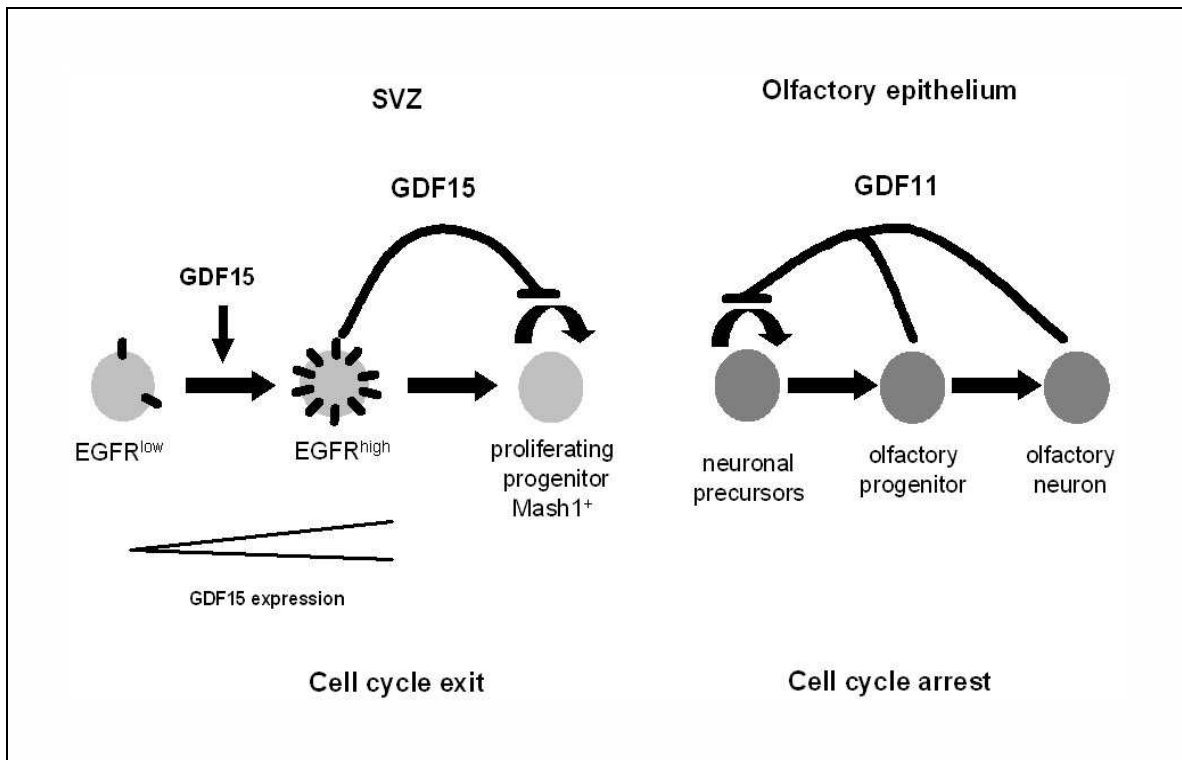


Fig 4.5.2- Scheme depicting the effects of GDF15 and GDF11 on neurogenesis in the SVZ and olfactory epithelium.

Thus, this is the first study which describes GDF15 as a new regulatory molecule of the neuronal lineage in the developing mouse telencephalon. Analysis of the effect of GDF15 in the adult SVZ and in cell fate determination of secondary progenitors will provide further insight into the regulatory function of this factor on embryonic and postnatal neurogenesis.

4.6- Impaired EGFR expression in GDF15^{-/-} hippocampal NPC leads to a decrease in proliferation in the hippocampal subependyma in vivo.

The in vitro analysis of the role of GDF15 in hippocampal NPC has revealed that GDF15 does not affect the number and proliferation capacity of hippocampal clonogenic NPC but rather promotes EGFR expression on these cells. In vivo, compared to WT, GDF15^{-/-} mice show a reduction in mitotic cells in all three different areas of the hippocampus analyzed (i.e. CA1, CA3 and hilus of the DG). Furthermore, the analysis of the number of the proliferating cells after 2 and 6 hour BrdU pulses

indicates not only a reduction of proliferation but also that such a decrease reflects mainly a reduction in the number of NPC in the hippocampal subventricular zone (hSVZ) which is localized within the anatomical CA1 region. Indeed, two hours after BrdU injection a decrease in proliferation was only observed in this area. Instead, in the DG BrdU incorporation was affected by the absence of GDF15 only after a 6 hour BrdU pulse. A possible explanation for these results is that at least a subset of precursors proliferating in the hSVZ migrates towards the DG. Therefore, a reduction in the pool of NPC proliferating in the hSVZ with time results in a decrease in the number of BrdU immunopositive cells in the DG. This hypothesis is consistent with recent studies on the migration of NPC in the developing hippocampus (Navarro-Quiroga et al., 2006; Han et al., 2008).

Recent studies have characterized a migratory pathway within the developing hippocampus, the so called hippocampal migratory stream (HMS). In the HMS, cells from the hSVZ migrate through the CA3 towards the hilus which will evolve into the subgranular zone (SGZ) of the adult dentate gyrus (DG) (see Fig. 4.6.1) (Navarro-Quiroga et al., 2006). In line with the hypothesis that at least some proliferating cells in the hSVZ migrate to the DG, I observed that in the DG proliferating cells were localized in the hilus and not in the SGZ where adult neurogenesis takes place. Furthermore, in WT embryos a prolonged BrdU pulse led to an increase in the number of BrdU immunopositive cells in both the CA3 and in the DG but not in the hSVZ, suggesting that proliferating precursors do not accumulate in this region.

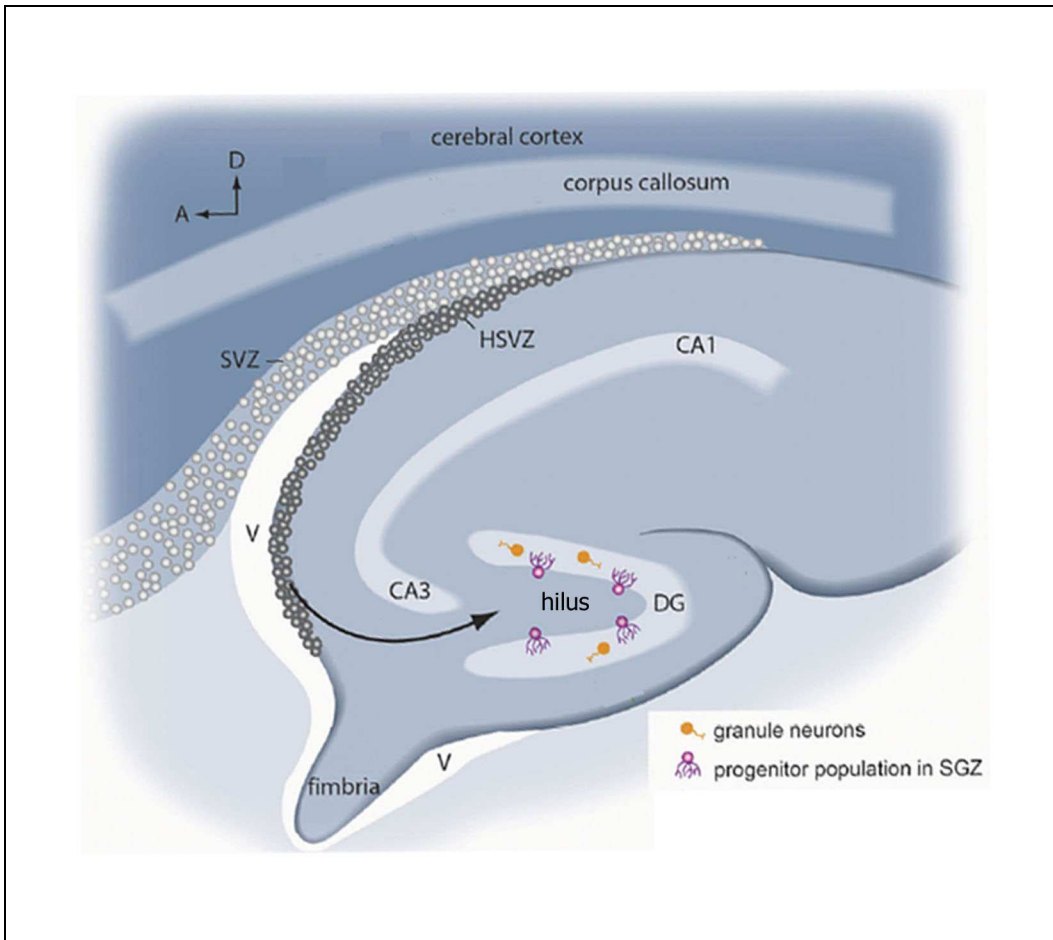


Fig. 4.6.1- Schematic representation of the hippocampal migratory stream (HMS). NPCs from the hSVZ migrate through the CA3 towards the hilus of the DG which will form in adult mice the SGZ. Cartoon modified from (Navarro-Quiroga et al., 2006).

In contrast, in $GDF15^{-/-}$ animals, after a 6 hour BrdU pulse the number of dividing cells in the hSVZ was significantly greater than after a two hour BrdU pulse. Estimation of cell cycle length, as measured by the ratio between the number of BrdU⁺ cells (after a two hour BrdU pulse) and the number of mitotic cells, indicates that absence of GDF15 does not affect the cell cycle length, neither in hippocampal nor in GE NPC (data not shown). Taken together these data suggest that not only less progenitors proliferate in the hSVZ of the hippocampus but also that the departure of these cells from this region in mutant mice is delayed. EGFR expression has been shown to affect both proliferation as well as migration of NPC in the developing and postnatal brain (Burrows et al., 1997; Lillien and Raphael, 2000; Caric et al., 2001; Ciccolini, 2001; Ciccolini et al., 2005; Aguirre et al., 2005), although these previous studies have mainly focused on the effect of EGFR in regulating migration in the GE and cortex. Caric et al have shown that cells

expressing EGFR at a certain threshold level migrate by chemotactic mechanisms towards a source of ligand. Thus, they propose that the timing of departure from proliferative zones as well as their migration routes is regulated in part by EGFR expression (Caric et al., 2001). The EGFR null mouse is not viable, but studies of overexpression of EGFR have been conducted on non migratory progenitors. Those studies show the acquisition by those cells of migratory properties in response to the expression of high levels of EGFR (Aguirre et al., 2005). Here I show that NPC up-regulate EGFR during mitosis. Since expression of EGFR in NPC in the absence of GDF15 is downregulated I can conclude that proliferation within the hSVZ is affected by the impairment of the EGFR expression in NPC. Instead it is not clear whether the decrease in the number of precursors migrating to the DG is only a consequence of the decrease proliferation in the hSVZ of $GDF15^{-/-}$ embryos or if also there is a delay in the migration of the precursors. Although I observed that in the absence of GDF15 more BrdU immunopositive cells are retained in the hSVZ, along the migratory stream, i.e. in the CA3, the number of BrdU immunopositive cells is similarly increased upon a prolonged pulse both in wild type and $GDF15^{-/-}$ animals. This suggests that along the migratory stream migration is not affected and that the cells stuck in the hSVZ represent a separate set of precursors that are not directed to the dentate gyrus. This interpretation is consistent with the pattern of EGFR expression in the E18 hippocampus showing that it is downregulated in precursors proliferating outside the hSVZ. .

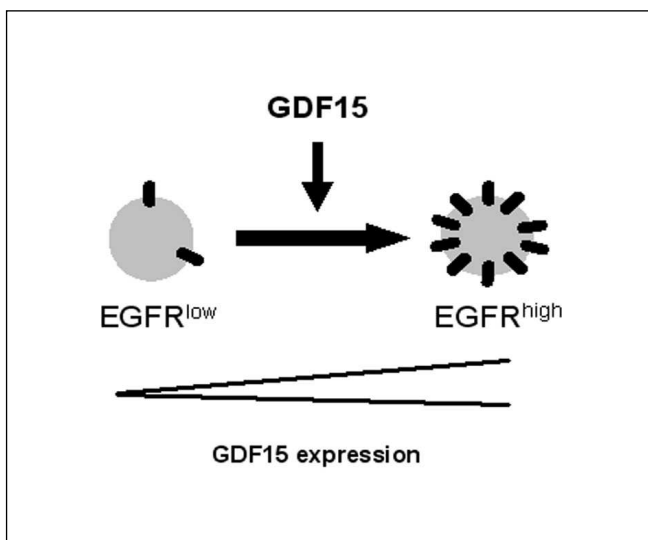


Fig 4.6.2- Scheme summarizing my findings about the effect of GDF15 in hippocampal derived NPCs. I have shown that late NPCs are the main source of GDF15 in the hippocampus during development and that this factor regulates EGFR expression in neural precursors.

Further studies on postnatal and adult WT and $GDF15^{-/-}$ hippocampi will help to clarify the consequences of the lack of GDF15 on the hippocampal formation.

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Abbreviations:

aa	amino acid
aSVZ	anterior sub-ventricular zone
BLBP	brain lipidic binding protein
BMP	bone morphogenetic protein
BP	basal progenitor cell
BrdU	5-bromo-2-deoxyuridine
BSA	bovine seroalbumin
CA	cornu ammonis
CNS	central nervous system
DAP	day after plating
DAPI	4', 6-diamidino-2-phenylindole
DG	dentate gyrus
DIV	day in vitro
DVR	decapentaplegic-Vg-related
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDF	growth/differentiation factor
GDNF	glial cell line-derived neurotrophic factor
GE	ganglionic eminence
GFAP	glial fibrillary acidic protein
Glast	glutamate aspartate transporter
HMS	hippocampal migratory stream
hSVZ	hippocampal sub-ventricular zone
mash1	mammalian achaete-schute homolog 1
NE	neuroepithelial cell
NPC	neural precursor cell

Abbreviations

NSC	neural stem cell
O/N	overnight
O4	oligodendrocyte 4 marker
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PHH3	phospho-histone H3
PI	propidium iodide
PLO	poly-ornithin
RC2	radial glia cell marker
RG	radial glia cell
RT-PCR	real time-PCR
SC	stem cell
SGL	sub-granular layer
Shh	sonic hedgehog
SVZ	sub-ventricular zone
TGF β	transforming growth factor beta
Trk	tropomyosin receptors kinase
TuJ1	type III tubulin
VZ	ventricular zone
WT	wild type

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