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HANNA BERGLIND

In vitro regulation of neuronal markers in differentiating adult mouse neural stem cells

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Author

Hanna Berglind

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Abstract

In an attempt to learn more about the development of adult neural stem cells, this study focuses on the *in vitro* characterisation of the neuronal markers doublecortin, ßIII tubulin and microtubule associated protein type 2. Their regulation has been analysed in a time dependent manner in adherent adult mouse neural stem cells following control versus platelet-derived growth factor (PDGF) treatment. In addition, 5'-bromo-2'deoxyuridine studies have been performed to provide information about expression of neuronal markers in newly differentiated stem cells. PDGF was found to have a proliferative effect on neuronal progenitors. This study shows that it is possible to measure, in a semi- quantitative way and in a time dependent manner, *in vitro* proliferation and differentiation of adult mouse neural stem cells.

Keywords

Neural, adult stem cell, neuronal marker, PDGF, differentiation, proliferation

Supervisors

Cesare Patrone
NeuroNova AB

Scientific reviewer

Olof Zachrisson
NeuroNova AB

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Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

In vitro regulation of neuronal markers in differentiating adult mouse neural stem cells

Hanna Berglind

Sammanfattning

Tidigare trodde man att nervceller inte kunde nybildas hos vuxna individer. Nyligen har man dock funnit stamceller i hjärnan som kan bilda nya nervceller. Man vill utnyttja dessa celler vid behandling av neurologiska sjukdomar. De skulle kunna ersätta skadade nervceller via transplantation eller genom att stimulera cellerna på plats. Vid läkemedelsutveckling för neurologiska sjukdomar utförs ofta anlyser i cellkulturer innan man studerar effekter hos försöksdjur. I cellkulturerna vill man kunna identifiera de olika celltyperna. Under neurala stamcellers utveckling uttrycks olika proteiner varav vissa är specifika för celltypen och används därför som markörer.

I denna studie analyserades de nervcellspecifika markörerna doublecortin, ßIII tubulin samt mikrotubulin-associerat protein 2, för att ta reda på i vilken ordning de uttrycks i nybildade nervceller. Analyserna gjordes i cellkulturer av stamceller från hjärnan hos vuxna möss. Cellerna behandlades med tillväxtfaktorn platelet-derived growth factor (PDGF), som visats kunna påverka utvecklingen av neurala stamcellerna så att de bildar nervceller. För att kunna bestämma fenotypen av de celler som stimulerats till celldelning med PDGF, utfördes även analyser där cellerna inkuberades med en cellcykelmarkör (5'-bromo-2'deoxyuridine, BrdU). Celler positiva för både BrdU och neuronala markörer undersöktes sedan. All detektion utfördes med immunocytokemi, som är en metod där immunologiska interaktioner detekteras med hjälp av fluorescerande molekyler. Resultaten från denna studie kan sedan användas för *in vivo* försök.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet Februari 2004

Abbreviations

bFGF - basic fibroblast growth factor
BrdU - 5'-bromo-2'deoxyuridine
Dcx - doublecortin
EGF - epidermal growth factor
ICC - immunocytochemistry
Map2 - microtubule associated protein 2
PDGF - platelet-derived growth factor
Tub - ßIII tubulin

1 Introduction

1.1 Neural stem cells

A stem cell is a cell that has the potency to differentiate towards several types of cells and has the capacity to regenerate itself unlimitedly through asymmetric cell divisions.[1] The source of stem cells includes for example embryos, fetal tissue, bone marrow and blood. Recently stem cells have also been identified in the adult brain and these are referred to as neural stem cells.[1]

When stem cells proliferate, one of the two daughter cells remains in a stem cell status and the other one is referred to as a neural progenitor cell and can undergo further differentiation. Neural progenitor cells can generate the three major cell types of the brain: neurones, astrocytes and oligodendrocytes. The final phenotype that a progenitor cell obtains depends on which stimuli the cell receive from its surrounding environment.[2]

The two most prominent regions where neural stem cells have been identified in the adult rodent brain are the subventricular zone lining the lateral ventricle and the dentate gyrus of the hippocampus[1] (fig. 1). There are indications of the existence of neural stem cells in other areas of the mammalian brain as well.[2] The newly generated neural progenitors in the wall of the lateral ventricle migrate through a pathway called the rostral migratory stream mainly to the olfactory bulb where they undergo their final differentiation.[1]

External factors can be used to drive the differentiation of neural stem cells towards a specific fate. For example in rodents, the administration of brain derived neurotrophic factor into the lateral ventricle increases the number of newly generated neurones in the olfactory bulb.[3]

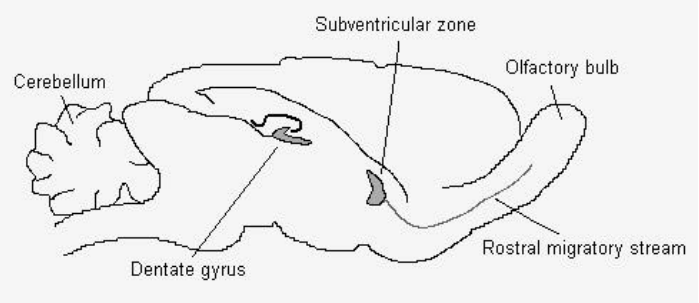


Figure 1. Neural stem cell regions. Sagittal section of a rodent brain showing the subventricular zone lining the lateral ventricle and the dentate gyrus of the hippocampus, which are regions where stem cells have been identified.

1.2 Potential use of neural stem cells in therapies

Since the existence of neural stem cells in the adult human brain has been established, the important issue now is whether it is possible to use these stem cells for treatment of various neurological disorders like Parkinson's, Alzheimer's and Huntington's diseases. There are different strategies in which neural stem cells could be used in therapy, once we have increased our knowledge of how to control their proliferation, differentiation, migration and survival. One way is to deliver a proliferative drug directly to the neural stem cells in the brain of the patient in the attempt to increase the number of neurones in that specific region. Another way is to remove neural stem cells from the patient and trigger them to differentiate into a specific phenotype *in vitro*, e.g. dopaminergic neurones for the treatment of Parkinson's disease, which thereafter are transplanted back into the damaged brain region of the patient.[4] The increasement of the number of neurons in the damaged brain area by stimulation of neurogenesis or through transplantation of stem cells might be a functional way to improve treatment of neurodegenerative disorders like Parkinson's disease in the future.

1.3 Neural stem cells *in vitro*

The ability of culturing neural stem cells *in vitro* is of great importance when trying to identify external factors regulating their proliferation and differentiation. Neural stem cells can be cultured and expanded *in vitro* in the presence of epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF), which are both mitogenic.[1] The proliferating cells grow in suspension and form cell clusters called neurospheres with each sphere originating from a single cell[1] (fig. 2). In this state they are still considered to be undifferentiated cells. If the cells are plated on an adhesive surface, EGF and FGF are withdrawn and serum is added, the cells adhere and start to differentiate into different phenotypes.[1]

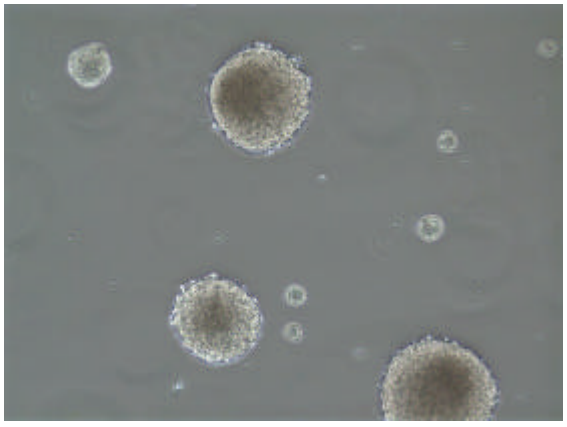


Figure 2. Neurospheres in culture. Neural stem cells can be cultured and expanded *in vitro* in the presence of EGF or bFGF. The proliferating cells grow in suspension and form cell clusters called neurospheres.

Studies performed *in vitro* have shown that it is possible to influence the phenotypic outcome by treating the progenitor cells with different factors that promote a glial fate or a neuronal fate. For example, it has been shown that platelet-derived growth factor (PDGF) stimulates neural stem cells into a neuronal fate.[5]

1.4 PDGF

PDGF and its receptors are expressed in both the central and peripheral nervous system. The growth factor plays an important role in the development and maintenance of the central nervous system.[6] PDGF has been identified as a factor that stimulates progenitor cells into a neuronal fate *in vitro*.[5]

PDGF is a 30kD dimeric molecule consisting of two polypeptide chains linked together with disulfide bonds. PDGF can be either homodimeric or heterodimeric depending on the isoform: PDGF-AA, PDGF-BB or PDGF-AB. PDGF acts on target cells through tyrosine kinase receptors dimers named a- and ß-receptors.[7] PDGF-BB activates all three receptor isoforms, aa, aß and ßß, while PDGF-AA only activates the aa-receptor. Binding of PDGF to its receptor causes dimerisation of the receptor and the elicited signalling cascade works through several pathways, which often ends with transcription regulation. The biological responses include proliferation, differentiation and chemotaxis. PDGF is also believed to have a neuroprotective effect.[6]

PDGF-AA is thought to be associated with the development of the cells of the central nervous system[6] and during differentiation of neural stem cells its receptor is constantly expressed while the receptor for PDGF-BB is almost absent.[8]

1.5 Neuronal markers

As mentioned above, neural progenitor cells can differentiate into three different phenotypes: neurones, astrocytes and oligodendrocytes. When performing studies with neural progenitor cells, it is often desirable to be able to distinguish specific subsets of these cells. During neural progenitor differentiation, the cells express various proteins. Some of these proteins are specifically related to certain phenotypes and are therefore used as identifiers i.e. markers. This study has focused on the regulation of the neuronal specific markers doublecortin (Dcx), ßIII tubulin (Tub) and microtubule associated protein 2 (Map2).

2 Aim of the project

2.1 Aim

The aim of this project was to study the *in vitro* expression of the neuronal markers Dcx, Tub and Map2 in relation to each other. Their regulation was analysed in a time dependent manner in adult mouse neural stem cells during differentiation induced by the growth factor PDGF. In order to also provide information regarding expression of neuronal markers in newly differentiated progenitor cells, analysis of the cell cycle marker 5'-bromo-2'deoxyuridine (BrdU) was also included in the experiments. The detection was performed using immunocytochemistry (ICC).

2.2 Study plan

The experiments were set up as described in figure 3. Briefly, neural stem cells were plated and grown in culture for a few days and neuronal markers were analysed at different time points. In addition, cells were labelled with BrdU for analysis of the phenotype in newly differentiated neuronal cells.

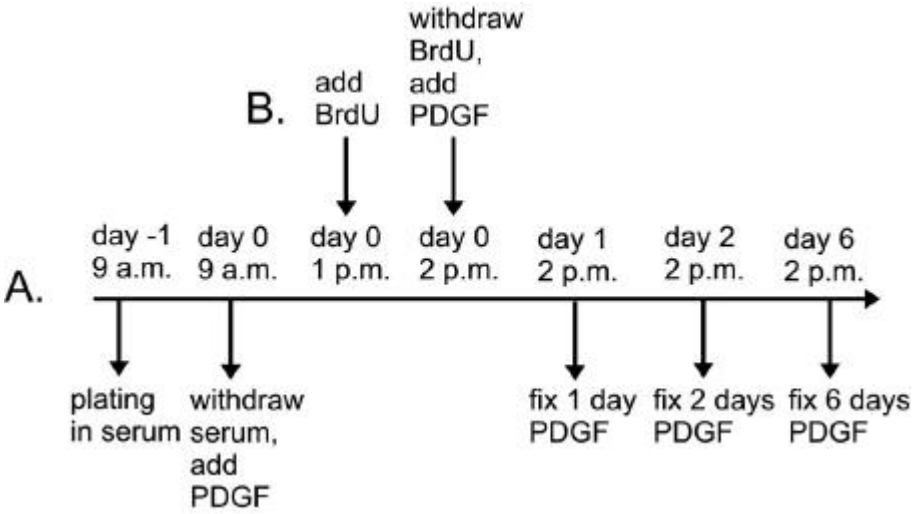


Figure 3. Study plan. A**.** Neural stem cells were plated in 1% serum over night. The next day, serum was withdrawn and the adherent cells were incubated with PDGF for 1, 2 and 6 days. B. BrdU experiments were also performed and in this case, the adherent cells were incubated in PDGF for 4 hours and then pulsed with BrdU for 1 hour. The cells were then fixed after 1, 2 and 6 days of PDGF incubation as before.

3 Methodology

3.1 ICC

ICC is a technique where labelled antibodies are used to localise specific cellular antigens and it is performed on cells grown in culture.[8] Primary antibodies are raised against the antigen in various animals and can be either mono- or polyclonal (monoclonal antibodies recognise only one antigen epitope whilst polyclonal recognise several different epitopes on the antigen). For the detection of primary antibodies it is common to use secondary antibodies conjugated to an enzyme or a fluorescent molecule. This system gives an amplification of the detection signal since several secondary antibodies can attach to the same primary antibody. It is also possible to use directly conjugated primary antibodies, although the signal will be weaker than following usage of secondary antibodies.

When performing ICC, the cells need to be fixed to keep their morphology and for the epitopes to stay intact. If the antigen is located within the cell, the membrane has to be permeabilised prior to incubation with the antibody. This can be achieved by using a detergent like Tween. Before labelling the cells with primary antibodies, it is common to include an incubation step with blocking buffer containing serum from the same animal as the secondary antibody is raised in. This is done to reduce background signals. After incubation with the primary antibody followed by the secondary, detection is made with epifluorescence microscopy. The molecules are excited and the emitted light is detected as a coloured image of the specific structure labelled by the antibodies. It is possible to use more than one antibody in the same experiment if the fluorescent molecules are chosen so that they emit light in non-overlapping wavelengths. As a negative control for the ICC, the primary antibody is omitted and only the secondary antibody is used, which should result in a completely dark image.

3.1.1 Primary antibodies

In order to detect the neuronal markers Tub, Dcx and Map2 the following primary antibodies were used:

- **anti-ßIII tubulin.** This antibody is reactive to neuron specific class III ß-tubulin. It labels cell bodies, dendrites and axon.
- **anti-Doublecortin.** Doublecortin is highly expressed in the developing brain and is a phosphoprotein associated with microtubules.[9] The antibody labels neuronal extensions and cell bodies.
- **anti-Microtubule associated protein 2.** Map2 promotes microtubule assembly and interacts with the cytoskeleton. The antibody associates with axons.

3.1.2 Secondary antibodies

In order to detect the primary antibodies described above, the following secondary antibodies were used:

- **Fluorescein Isothiocyanate (FITC)-conjugated**
  Excitation wavelength: 495 nm, emission wavelength: 515 nm.
- **Texas Red-conjugated** (Sulforhodamine 101)
  Excitation wavelength: 595 nm, emission wavelength: 615 nm.

3.2 Proliferation detection with BrdU

BrdU is a thymidine analogue that can be used for detection of proliferating cells. Therefore, it is common to use BrdU analysis to detect newborn cells in studies performed *in vivo* or *in vitro*. The BrdU is incorporated in the DNA strands during replication i.e. during the S-phase of the cell cycle. The BrdU labelled cells can then be detected with ICC. The signal of BrdU is very stable and can be analysed a long time after injection.

4 Materials and Methods

4.1 Neurosphere cultures

The lateral ventricle walls of 4-5 weeks old mice (C57, Scanbur B&K) were dissociated in 0.8 mg/ml hyaluronidase and 0.5 mg/ml trypsin in DMEM with Glutamax containing 4.5 mg/ml glucose and 4000 units/ml DNase at 37°C for 2x10 min. The cells were triturated and mixed with three volumes of Neurosphere medium (DMEM/F12, B27 supplement, 8 mM HEPES, Invitrogen) to stop the enzymatic reaction. After passing through a 70 µm strainer, the cells were pelleted at 250x g for 4 min. The supernatant was removed and the cells resuspended in Neurosphere medium supplemented as above, plated in a 100 mm culture dish adding EGF (3 nM), 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37ºC. After 2-3 days the cells were transferred to two new culture dishes adding new media (supplemented as above) and EGF.

Neurospheres were ready for the first split approximately 7 days after plating. The spheres were collected by centrifugation at 250x g for 4 min. The supernatant was removed and the neurospheres were resuspended in 0.5 ml Trypsin-EDTA, incubated at 37ºC for 2x2 min and triturated to aid dissociation. After adding three volumes of B27-medium to stop the enzymatic reaction, the cells were pelleted at 250x g for 4 min, resuspended in Neurosphere medium and placed in flasks. EGF (3 nM), bFGF (3 nM), 100 U/ml penicillin and 100 µg/ml streptomycin was supplemented and cells were incubated at 37ºC.

4.2 *In vitro* differentiation of neural stem cells

After passage 2, 3 or 4, neural stem cells were plated as single cells in Neurosphere medium (DMEM/F12, B27 supplement, 8 mM HEPES) with 1-2% fetal bovine serum into a 24-well Poly-D-Lysine plate (250 000 cells/well) to become adherent. When the cells had adhered after 24 h, the medium was changed to serum free Neurosphere medium with factors PDGF-AA (1nM) or EGF (3 nM). Some wells were kept as controls and contained Neurosphere medium without factors. Every second day PDGF and EGF were added as described. The cells were incubated for 1, 2 and 6 days and ICC was performed to detect different neuronal markers.

4.3 BrdU labelling

The proliferative effect of PDGF was identified using BrdU on adherent cells, prepared and cultured as described above. The cells were given a pulse of 1mM BrdU after 4 h of incubation with PDGF (1nM). The BrdU was withdrawn after 1 h by changing the medium to Neurosphere medium with PDGF. The cells were incubated for 1, 2 and 6 days, adding PDGF every second day. ICC was performed to simultaneously detect cells positive for BrdU and different neuronal markers.

4.4 ICC

After the incubation with PDGF, the cells, prepared and cultured as described above, were fixed in ice cold 4% paraformaldehyde for 10 min followed by washing in PBS. For ICC detection with BrdU-antibody, DNA was denatured by treating cells with 2 M HCl at 37°C for 30 min. After washing in PBS 3-4 times the cells were permeabilised in 0,1% Tween in PBS (PBS-T), supplemented with 10% serum, for 1-2 h at room temperature. The cells were then double stained with different combinations of primary antibodies: rat anti-BrdU 1:100 (Immunologicals Direct), mouse anti-βIII tubulin 1:1000 (Promega), rabbit anti-βIII tubulin 1:1000 (Biosite), mouse anti-Map2 1:1000 (Sigma), goat anti-doublecortin 1:100 (Santa Cruz), over night at 4°C. After 1-2 h of thorough washing in PBS-T, the cells were labelled with secondary antibodies conjugated to fluorescent molecules (FITC 1:200, Texas Red 1:200) for 1-2 h, room temperature. All antibodies were diluted in PBS-T with 10% serum. In experiments without HCl-pretreatment, the cell nuclei were labelled with Hoechst 33342 (10 µM, for 10 min, room temperature). Another washing step with PBS-T was performed during 1 h, the cells were then mounted in a few drops of Vectashield and detection was made by epifluorescence microscopy. Positive cells were counted manually in 5 randomly chosen fields at x40 magnification. The nuclear stain Hoechst was included in the ICC to determine the total number of cells in the fields.

5 Results

In order to study the relative expression of the neuronal markers Tub, Map2 and Dcx, neural stem cells were plated as adherent and incubated with PDGF for 1, 2 and 6 days as shown in the study plan (fig. 3A). The expression of the neuronal markers in relation to proliferation was also studied using BrdU double labelling experiments. In this case, neural stem cells were plated as adherent and incubated with PDGF for a few hours prior to a 1 hour BrdU pulse followed by further PDGF incubation (fig. 3B). The detection of the markers and BrdU was simultaneously made by ICC (see materials and methods).

The markers were analysed in double staining experiments: Tub and Map2, Dcx and Map2, Dcx and Tub, BrdU and Map2, BrdU and Tub. Approximately three experiments were run for each double staining. The results shown are from one representative experiment for each double staining.

5.1 Expression of neuronal markers

Neural stem cells were treated with PDGF for 6 days as described above. In general after 6 days, untreated cells decreased in number, while the number of PDGF treated cells was higher than in the control (fig. 4). This suggests that PDGF was able to induce cell proliferation.

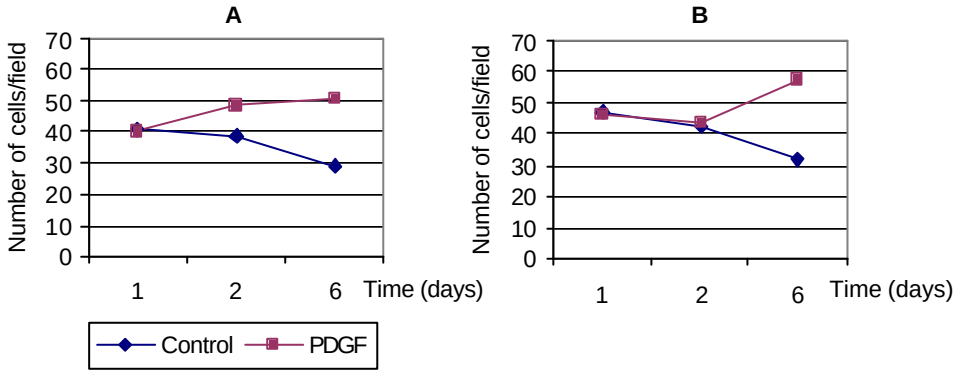
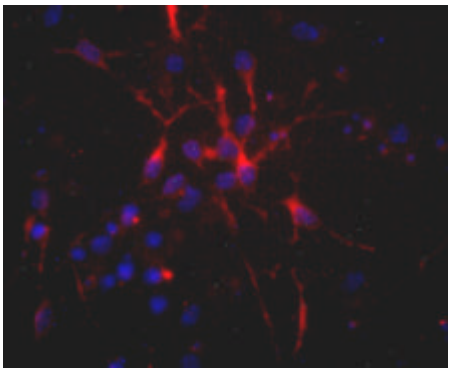


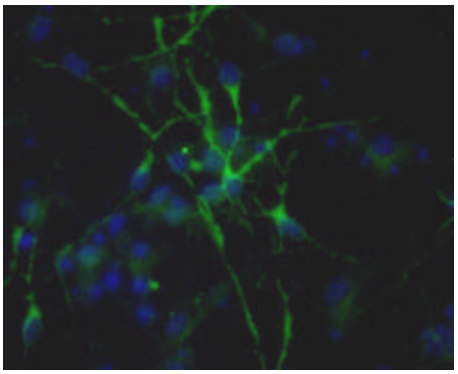
Figure 4. Total number of cells. Neural stem cells were plated as adherent and incubated with PDGF for 1, 2 and 6 days. ICC was performed and Hoechst positive nuclei were counted manually in 5 randomly chosen fields at x40 magnification in control and PDGF treated cells. Results from two representative double staining experiments are shown: A. Total number of cells in Map2/Dcx experiment, B. Total number of cells in Tub/Dcx experiment.

5.1.1 Map2 and Tub

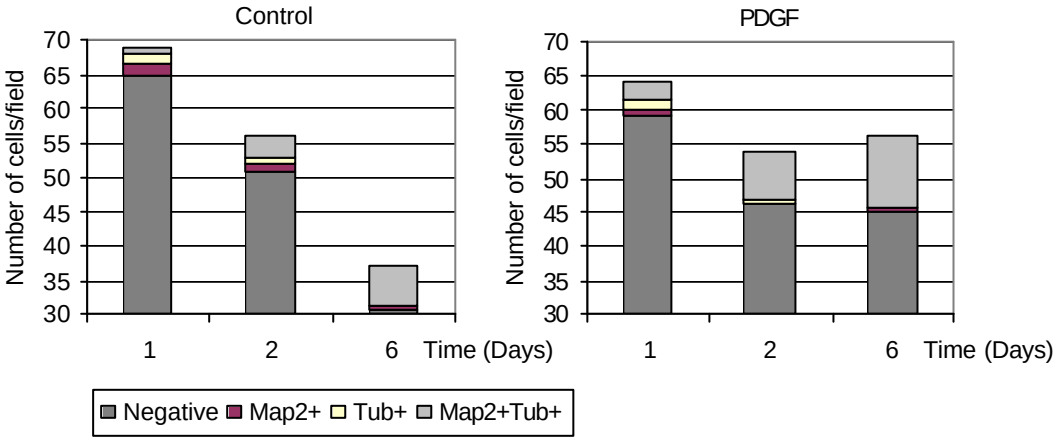
In control cells, the total number of cells clearly decreased as described above (section 5.1). When comparing the subset of Map2 and Tub double positive neurons at the different time points, their number increased in a time dependent manner (fig. 5C, left panel). At day 1, the expression of Map2 and Tub was low and there were only a few double positive cells present. At day 2, the number of cells expressing only one of the two markers was still low, but there was an increase of the number of double positive cells. At day 6 a further increase of the number of double positive cells occurred. Very few cells expressed only one of the two markers.



A. Tub



B. Map2



C.

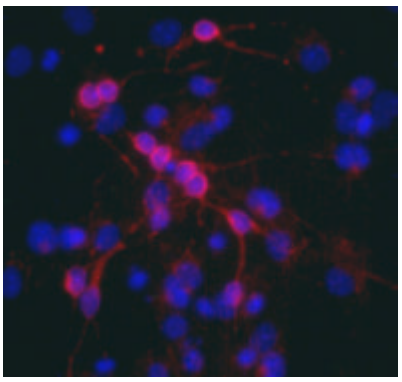
Figure 5. Double staining of Map2 and Tub. Neural stem cells were plated as adherent and incubated with PDGF for 1, 2 and 6 days. ICC was performed and cells positive for Map2 and/or Tub were counted manually in 5 randomly chosen fields at x40 magnification in control and PDGF treated cells. A. ICC of 6 days control cells, Tub (red) and Hoechst (blue). B. ICC of 6 days control cells, Map2 (green) and Hoechst (blue). C. Cell count of Tub and/or Map2 positive cells.

In the PDGF treated cells, the subset of Map2 and Tub double positive neurons increased with time similarly to the control treated cells (fig. 5C, right panel). The number of Map2 and Tub double positive cells was also higher compared to untreated cells at all different time points. At day 1, the number of cells expressing either Map2 or Tub was similar to the control. However, the number of cells double positive cells for Map2 and Tub was higher. At day 2, the number of cells expressing the two markers increased compared to day 1 and the colocalisation resulted to be higher than the control. At day 6, the Map2 and Tub double positive cells increased even further as in the control. This suggests that PDGF was accelerating the expression of neuronal markers in a time dependent manner.

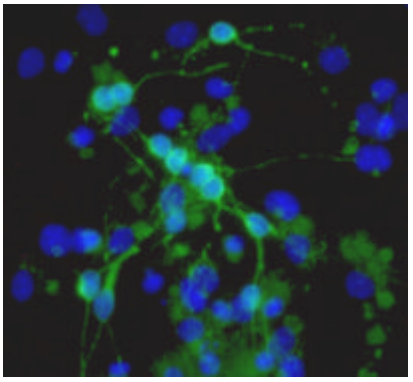
In conclusion, PDGF seems to increase the number of Map2 and Tub double positive cells as compared to untreated cells with time in culture. At day 1, Map2 and Tub appeared to be expressed by two different subsets of neuronal progenitor cells, while this effect seemed to disappear after a few days in culture and the two markers seemed to colocalise completely. This suggests that PDGF increases the colocalisation process of the two markers Map2 and Tub.

5.1.2 Map2 and Dcx

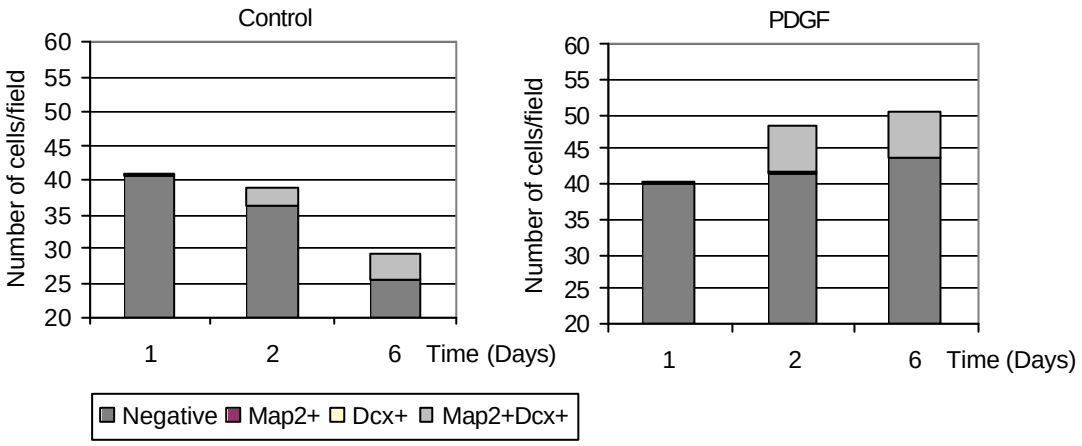
In control cells, similarly to the experiment with Map2 and Tub expressions, the total number of Map2 and Dcx double positive cells increased in a time dependent manner (fig. 6C, left panel). At day 1, the expression of Map2 and Dcx resulted to be low, but the few cells that did express the markers were found to be double positive. At day 2, the expression of the two markers increased and they still seemed to colocalise entirely. The number of cells expressing both markers increased slightly at day 6 compared to day 2.



A. Map2



B. Dcx



C.

Figure 6. Double staining of Map2 and Dcx. Neural stem cells were plated as adherent and incubated with PDGF for 1, 2 and 6 days. ICC was performed and cells positive for Map2 and/or Dcx were counted manually in 5 randomly chosen fields at x40 magnification in control and PDGF treated cells. A. ICC of 6 days PDGF cells, Map2 (red) and Hoechst (blue). B. ICC of 6 days PDGF cells, Dcx (green) and Hoechst (blue). C. Cell count of Map2 and/or Dcx positive cells.

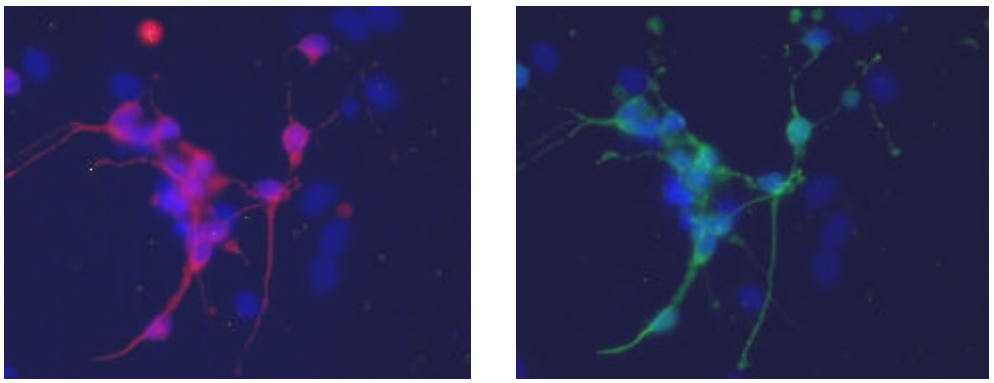
In PDGF treated cells, as well as in the control treated cells, the subset of Map2 and Dcx double positive cells increased with time (fig. 6C, right panel). However, the number of Map2 and Dcx double positive cells was higher as compared to untreated cells at the different time points similarly to the Map2 and Tub double expression. As in the control, the two markers showed low expression at day 1 and all the cells resulted to be double positive. At day 2, both markers were expressed and they still colocalised completely as in the control. There was no apparent change in the number of positive cells at day 6 compared to day 2.

In conclusion, as in the experiment with Map2 and Tub, PDGF seems to increase the number of cells expressing the two markers Map2 and Dcx compared to control in a time dependent manner. In contrast to the regulation of Map2 and Tub, where the markers increased their colocalisation process with time in culture, here, the markers Map2 and Dcx appeared to be expressed in the same subset of cells already from day 1.

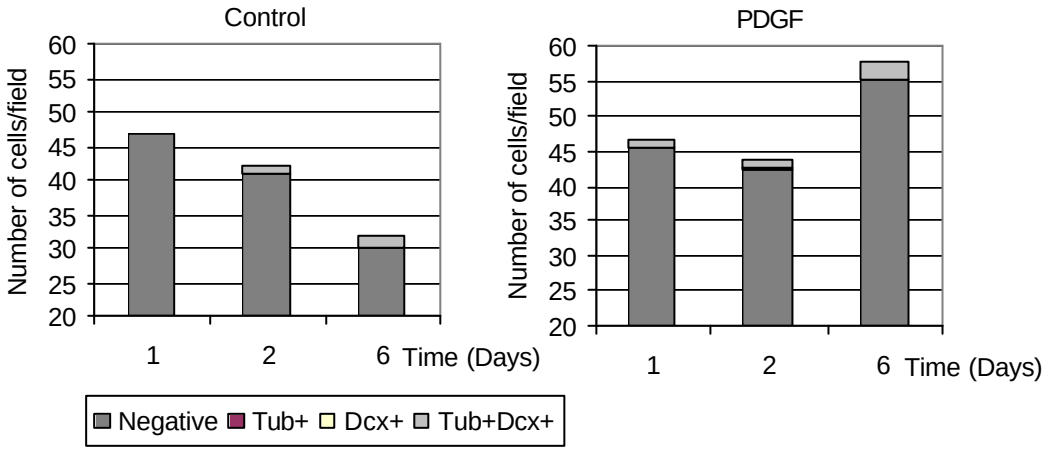
5.1.3 Tub and Dcx

The expression of Tub and Dcx resulted to be low at day 1 in both control and PDGF treated cells (fig. 7) and cells expressing the markers were found to be double positive. At day 2, the expression increased slightly in both control and PDGF cells and the two markers still seemed to colocalise entirely. The expression of Tub and Dcx at day 6 was only increased moderately compared to day 2 in both control and PDGF treated cells.

In conclusion, the expression pattern of Tub and Dcx resulted to be similar to the expression pattern of Map2 and Dcx i.e. the markers seemed to be expressed by the same subtype of cells already from day 1.



A. Tub **B. Dcx**



C.

Figure 7. Double staining of Tub and Dcx. Neural stem cells were plated as adherent and incubated with PDGF for 1, 2 and 6 days. ICC was performed and cells positive for Tub and/or Dcx were counted manually in 5 randomly chosen fields at x40 magnification in control and PDGF treated cells.
A. ICC of 6 days PDGF cells, Tub (red) and Hoechst (blue). B. ICC of 6 days PDGF cells, Dcx (green) and Hoechst (blue). C. Cell count of Tub and/or Dcx positive cells.

5.2 Proliferation analysis

In order to analyse neural stem cell proliferation in relation to neuronal differentiation, a BrdU assay for adherent cells was established and control cells versus cells treated with PDGF were double stained for BrdU and neuronal markers. Adherent neural stem cells incubated with PDGF were pulsed with BrdU followed by further incubation with PDGF for 1, 2 and 6 days (see study plan, fig.3). ICC was performed and cells double positive for BrdU and neuronal markers Tub or Map2 were counted (see materials and methods). Data of double positive cells were only obtained at 6 days.

As shown in figure 8, the number of BrdU positive cells was not modified in the control experiment at the different time points. However, following PDGF treatment, the number of BrdU positive cells increased with time. At day 2 and 6, more than a two-fold increase of the number of BrdU positive cells compared to the control was observed. This indicates that PDGF does indeed induce neural stem cell proliferation as already suggested by the data in figure 4.

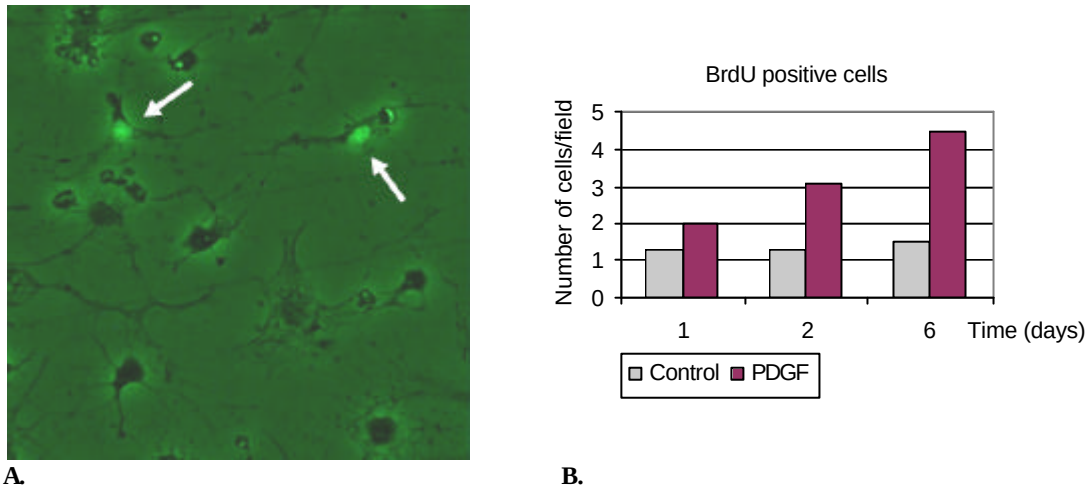
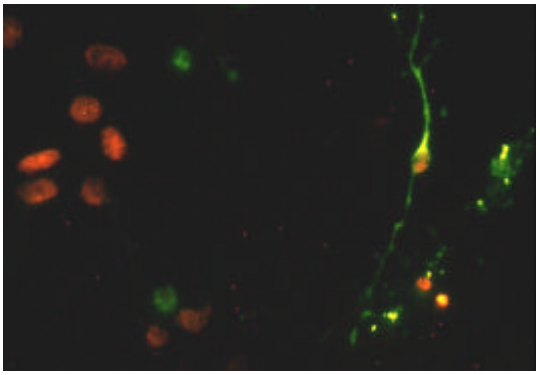


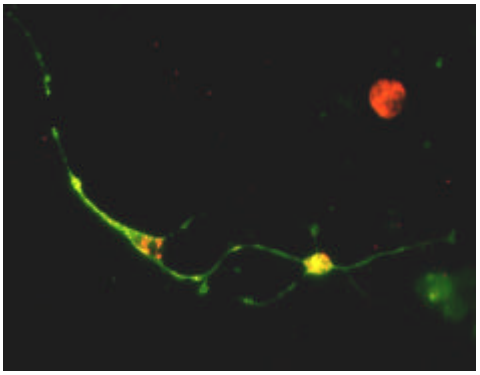
Figure 8. Number of BrdU positive cells. Adherent neural stem cells incubated with PDGF were pulsed with BrdU followed by further incubation with PDGF for 1, 2 and 6 days. ICC was performed and cells positive for BrdU were counted manually in 5 randomly chosen fields at x40 magnification. A. BrdU positive cells after 2 days of PDGF (indicated by arrows). B. Cell count of BrdU positive cells.

When studying the phenotype of the BrdU positive cells at day 6 (fig. 9), a slight increase of cells double positive for Map2 and BrdU and for Tub and BrdU was observed in the PDGF treated cells compared to the control. Interestingly, this suggests that PDGF has a proliferative effect on the sub population of cells that express Map2 and Tub.

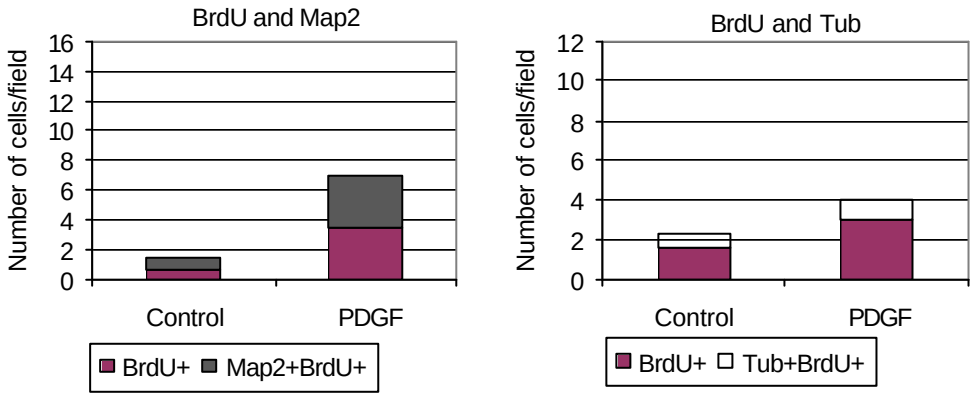
In conclusion, the data showed that PDGF was able to significantly increase neural stem cell populations *in vitro* and some of these cells maintained their potential to become neurons.



A. Map2 and BrdU



B. Tub and BrdU



C.

Figure 9. Cells double positive for BrdU and neuronal markers. Adherent neural stem cells incubated with PDGF were pulsed with BrdU followed by further incubation with PDGF for 6 days. ICC was performed and cells double positive for BrdU and Map2 or BrdU and Tub were counted manually in 5 randomly chosen fields at x40 magnification. A. ICC of 6 days PDGF cells, Map2 (green) and BrdU (red). B. ICC of 6 days PDGF cells, Tub (green) and BrdU (red). C. Cell count of BrdU and Map2/Tub positive cells.

6 Discussion

Compared to embryonic stem cells, little is known about the regulation and differentiation of adult stem cells *in vitro* and *in vivo*. However, recent studies have shown the potential of the adult stem cells, which makes them attractive for further research.[1,2] One advantage with the adult stem cells is that endogenous tissue would reduce the risk of graft rejection after transplantation. Another aspect is that adult stem cells are not as controversial to work with as the embryonic, which hopefully makes future applications more acceptable for ethical reasons.

This study suggests that it is possible to measure, in a semiquantitative way and in a time dependent manner, *in vitro* proliferation and differentiation of adult neural stem cells. In particular, this can be performed in control treated cells versus cells that have been treated with an exogenous factor e.g. a possible drug candidate. In order to establish this assay, the relative expression of the neuronal markers Map2, Tub and Dcx was studied during adult mouse neural stem cell proliferation and differentiation. The system was set up with untreated cells vs. cells treated with the growth factor PDGF, since this factor is known to stimulate neural progenitor cells into a neuronal phenotype.[5,8] When looking at the regulation of Map2, Tub and Dcx, all three markers were expressed at day 1, although the expression of Dcx was somewhat weaker and less frequent. Interestingly, two subsets of cells expressing specific combinations of neuronal markers were observed during early neural stem cell differentiation. At a later stage, neuronal cells expressed all three markers, suggesting that neuronal differentiation occurs starting from different neural stem cell populations.

Thus, the results illustrated in the result section showed that PDGF increased the population of cells expressing Map2, Tub and Dcx with increasing incubation time. Map2 and Dcx seemed to be coexpressed by the same subset of cells from day one, which was also the situation for Tub and Dcx. However, Map2 and Tub did not seem to be coexpressed from the beginning. This suggests that at an early stage of the differentiation of neural progenitor cells, it is possible to distinguish two different subsets of cells, one expressing Map2 and Dcx the other expressing Tub and Dcx. In a later stage, the two subsets of cells start expressing the marker that was initially absent. This hypothesis is illustrated in figure 10.

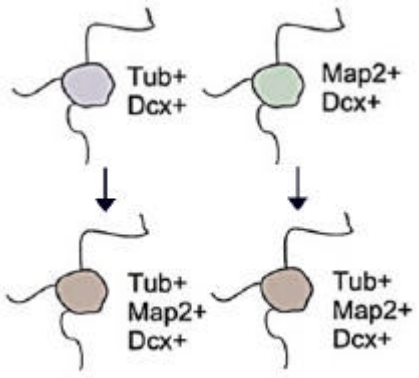


Figure 10. Development hypothesis. At an early stage of differentiation of neural progenitor cells, it is possible distinguish two different subsets of cells, one expressing Tub and Dcx, the other expressing Map2 and Dcx. In a later stage, the cells express all three markers.

In order to investigate if an external factor has a proliferative effect on the neural stem cells that later differentiate into neurons, BrdU analysis can be included in the assay and simultaneous detection of both BrdU and neuronal markers can be performed. In this study, PDGF was shown to increase the proliferation of neural stem cells compared to untreated cells. If PDGF also has a proliferative effect at the level of the neuronal precursor subpopulation, this should increase the number of cells double positive for BrdU and the neuronal marker of interest. Interestingly, this was found to be the case, since cells double positive for Map2/Tub and BrdU were identified.

The majority of the BrdU positive cells were found to be negative for Map2 or Tub, suggesting that PDGF was able to induce proliferation of other neural progenitor sub populations as well. These might consist of glial cells and/or oligodendrocytes. Alternatively, the PDGF stimulated BrdU cells might need further time in culture to develop into their neuronal phenotype.

In the attempt to develop a drug, *in vitro* models are often an advantage compared to *in vivo* studies. In particular, when several compounds need to be screened to find possible candidate drugs, the *in vitro* methods are quicker and easier to handle. The results obtained from this study suggest that this *in vitro* method can be applied to *in vivo* studies.

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9 Litterature

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