Generation of a NG2-EYFP mouse for studying the properties of NG2-expressing cells.

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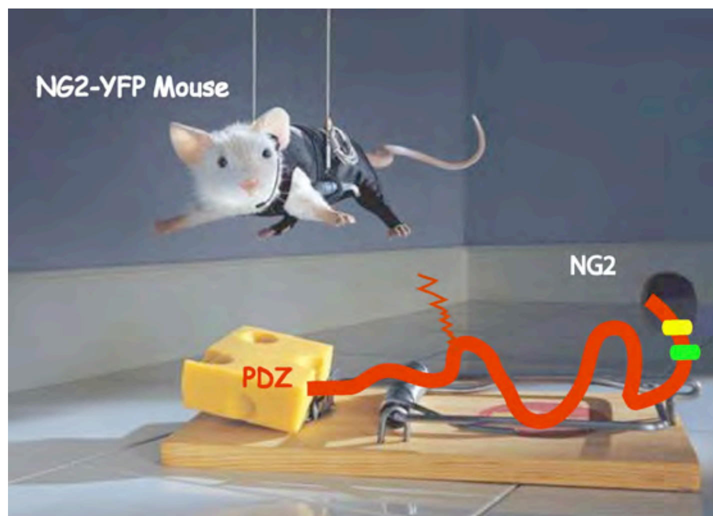
An exciting new field of study has blossomed in the last few years, which revolves around mysterious cells that express the Nerve-Glia antigen 2 (NG2) protein. Many different cell types of the body express this protein. They represent one of the largest proliferating populations in the Central Nervous System (CNS) during early development and adulthood. Their functional roles still eludes researchers, and their true cell identity is still unknown. The NG2 field is calling for a new tool, to aid the progression of studies to unravel the mystery of this elusive protein and the cells that express it. In this study a knockin mouse was generated to help clarify the mystery of the NG2 protein and the cells that express this protein.



1. Introduction:

1.1 Central Nervous System

The vertebrate nervous system consists of the Central Nervous System (CNS), and Peripheral Nervous System (PNS). The CNS is primarily composed of the brain and the spinal cord, while the PNS encompasses the rest of the nervous system. The brain provides the integrative power that constitutes complex behavior, movement, and reactions of all animals, whilst the spinal cord integrates responses to different types of stimuli that are conveyed to and from the brain. The monitoring of the internal environment is carried out by the PNS, which in turn relays this information to the CNS. Anatomically the adult CNS consists of six parts: the Telencephalon (cerebral cortex, white matter, basal nuclei), the Diencephalon (thalamus, hypothalamus, epithalamus), the Mesencephalon (midbrain), the Metencephalon (pons, cerebellum), the Myelencephalon (Medulla oblongata), and the spinal cord. The latter is mainly composed of bundles of axons and glia cells: astrocytes, microglia and oligodendrocytes.

1.1.1 Neurons

In the human CNS, there are approximately 1 X 10^{12} neurons. Each neuron on average makes about one thousand contacts or synapses with neighboring cells (Brose, 1999). Neurons vary considerably in size and shape from uni-polar to multi-polar, each consisting of a cell body, a single axon, and multiple dendrites, where information is received and processed. Axons are slender cylindrical processes with a smooth uniform diameter that is specialized to conduct electrical signals, known as nerve impulses, away from the cell body. Axons can vary from unbranched to highly branched and terminate at axon terminals, where the synaptic cleft begins. At the synapse electrochemical signals in the form of neurotransmitters are transmitted from neuron to neuron across the synaptic clefts. This results in a highly specific network of synapses, which governs all brain functions from simple motor controls to sophisticated emotional and cognitive behavior (figure 1.1.1).

1.1.2 Neuroglia

Neuroglia cells, which outnumber neurons by ten fold, have often been viewed as mere bystanders giving structural support for cells in the neuronal network. However in the recent years this view has been considerably altered. Neuroglia cells are recognized to have a central function in catalyzing formation of synapses and modulation of synaptic activity.

1.1.2.1 Astrocytes

Astrocytes are star-like cells that are commonly found in-between neurons and blood vessel and their development parallels that of the blood vessel network (Bertossi et al., 1993; Virgintino et al., 1993; Bertossi et al., 2003). They make up approximately 20% to 50% of the volume of most brain areas and appear to be a more heterogenous group of cells. The degree of diversity of astrocytes is still unclear, but there are at least two different types that can be distinguished by morphology: fibrous and protoplasmic (Lazzarini et al., 2004). An important characteristic shared by all classes of mature astrocytes is their expression of glial fibrillary acidic protein (GFAP) (Bignami et al., 1972; Uyeda et al., 1972). At this point in time the definition of an immature astrocyte is unclear. Astrocytes are arranged in complex networks often contacting each other through GAP junctions resulting in coupling between cells (Chan-Ling and Stone, 1991; Levison and Goldman, 1993; Tout et al., 1993). They have multiple functions that include structural support for the CNS, maintenance of neurons and other cell types by releasing trophic factors, trafficking of nutrients from the blood to neurons, and release of neuractive substances such as ATP in response to synaptic stimulation through an increase in intracellular calcium (Fields and Stevens-Graham, 2002; Nedergaard et al., 2003). In addition astrocytes secrete thrombospondins and cholesterol bound to apolipoprotein E, that promote synaptic formation and function (figure 1.1.1,Goritz et al., 2002; Pfrieger, 2002; Ullian et al., 2004; Allen and Barres, 2005). A subtype of astrocyte is the radial glia cell, which is considered to be the stem cell of the developing CNS (Gotz and Steindler, 2003). Furthermore it provides scaffolding for the migration of neural cells in the developing brain (figure 1.1.3, Rakic, 2003).

1.1.2.2 Microglia

Microglia are small mobile cells, which are specialized macrophages of mesodermal origin. They are scattered throughout the CNS and they defend the CNS against microorganisms and clear debris from damaged cells via phagocytosis. Their numbers increase when there is an infection in the CNS due to inflammation. Upon activation, microglia cells produce a large number of pro-inflammatory substances, like cytokines and proteases, which may promote repair, but can also cause neuronal cell death (figure 1.1.1,Diemel et al., 1998; Barron, 2003; Dringen, 2005).

1.1.2.3 Oligodendrocytes and Oligodendrocyte Precursor Cells (OPC)

The myelinating cells in the CNS are oligodendrocytes and in the PNS, Schwann cells. Oligodendrocytes, resemble astrocytes morphologically, but are smaller in shape and have fewer processes. They often reside in the CNS in rows (so called the interfasicular oligodendrocytes), where they myelinate axons. In the CNS, during myelination, a single oligodendrocyte has the potential to ensheath as many as 50 axons, while in the PNS a Schwann cell ensheathes a single axon (Blakemore and Murray, 1981). Myelin, which is rich in lipids, insulates the axonal surface between Nodes of Ranvier. At the nodes, sodium channels are concentrated allowing a rapid propagation of the action potential that jumps from node to node by saltatory conduction. In the juxtaparanodal region potassium channels are concentrated allowing K+ to exit the axon to restore the resting membrane potential after depolarization (Ritchie and Rogart, 1977; Waxman, 1977; Fields and Stevens-Graham, 2002). Electrical activity appears to stimulate and regulate myelination in the CNS (figure 1.1.1, 1.1.2, Fields and Stevens-Graham, 2002).

Oligodendrocytes develop from precursor cells, which migrate from the subventricular zone (SVZ) into grey and white matter, making contact to axonal tracts in the early stages of development (Marshall et al., 2003). Oligodendrocyte precursor cells (OPC) differentiate to oligodendrocytes through a series of intermediate stages. OPC express many immature antigens like A2B5 (ganglioside antigen), NG2 (Nerve-glia Antigen 2), PDGFα-R (Platelet Derived Growth Factor) and Vimentin (an intermediate filament). Upon differentiation to oligodendrocytes, the early markers are down regulated and typical myelin proteins MAG (Myelin Associated Glycoprotein), MOG (Myelin Oligodendrocyte Glycoprotein), and PLP (Proteolipid Protein) are expressed (refer to

figure 1.1.4). OPC that are present in the adult CNS retain their proliferative capacity and can undergo mitosis prior to remyelinating-demyelinated lesions (Ffrench-Constant and Raff, 1986; Miller et al., 1989; Gensert and Goldman, 1997; Marshall et al., 2003). OPC express voltage-gated channels, GluR receptors (AMPA, Kainate), GABAA and NMDA receptors, and exhibit delayed rectifying-K+ channels and sometimes voltage active Na+ channels (Kettenmann et al., 1991; Von Blankenfeld et al., 1991; Borges et al., 1994; Karadottir et al., 2005; Salter and Fern, 2005). OPC proliferation and differentiation can be influenced in the CNS, by the stimulation of GluR receptors (Gallo et al., 1996; Steinhauser and Gallo, 1996; Yuan et al., 1998). Upon differentiation, OPCs lose their expression of these GluR and GABAA receptors.

1.1.2.4 Ependymal cells

Ependymal cells are cubical cells that have cilia that form the inner lining of the central canal that extends downward through the spinal cord. They also cover the inside of the ventricles within the brain. They regulate the composition and flux of the cerebrospinal fluid. At one point in time they were to be considered the stem cell in the developing nervous system, but through lineage tracing experiments this was ruled out (figure 1.1.1, Johansson et al., 1999).

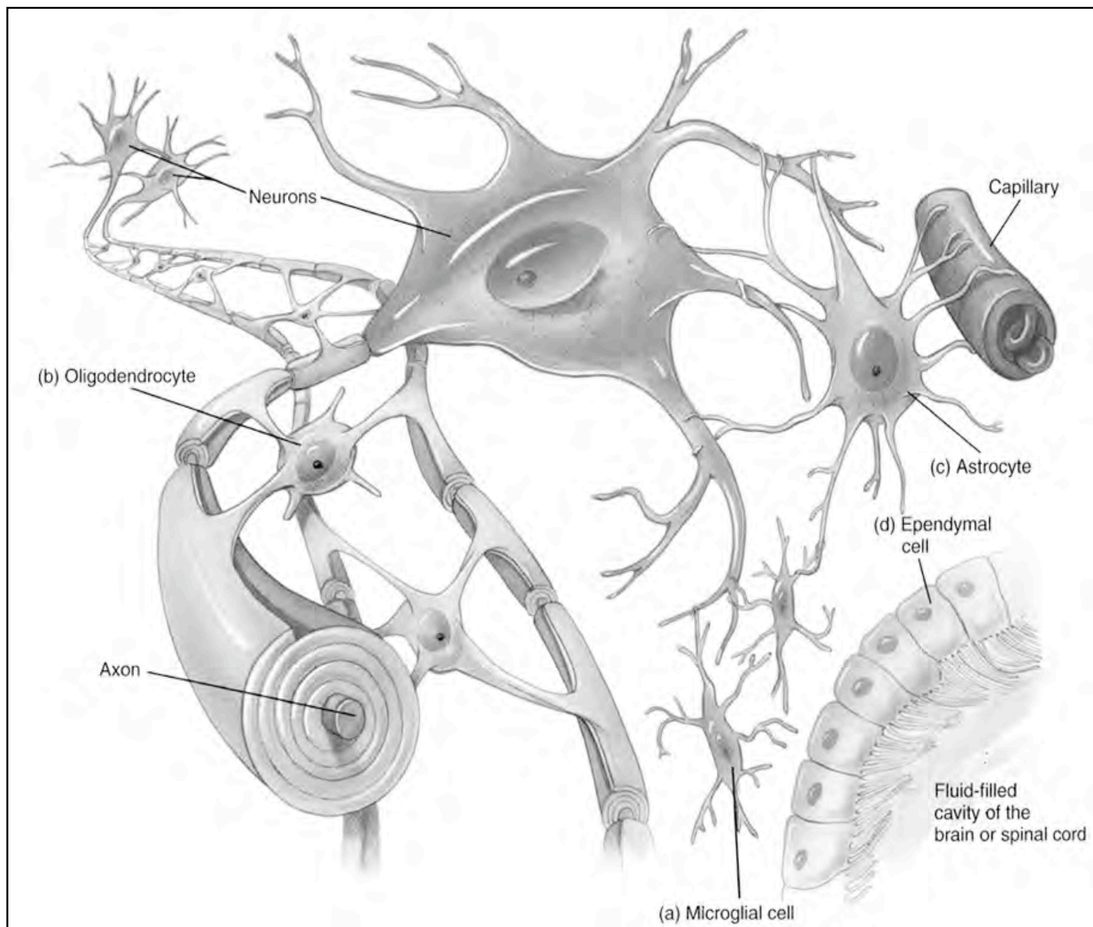


Figure 1.1.1: The different types of cells in the CNS of the developing and adult mammalian brain other than neurons. a) Microglia cells, b) Oligodendrocytes, c) Astrocytes, and d) Ependymal cells. Modified from Chapter 10, pg 352, Human Anatomy and Physiology, McGraw-Hill 1999 (Shier et al., 1999).

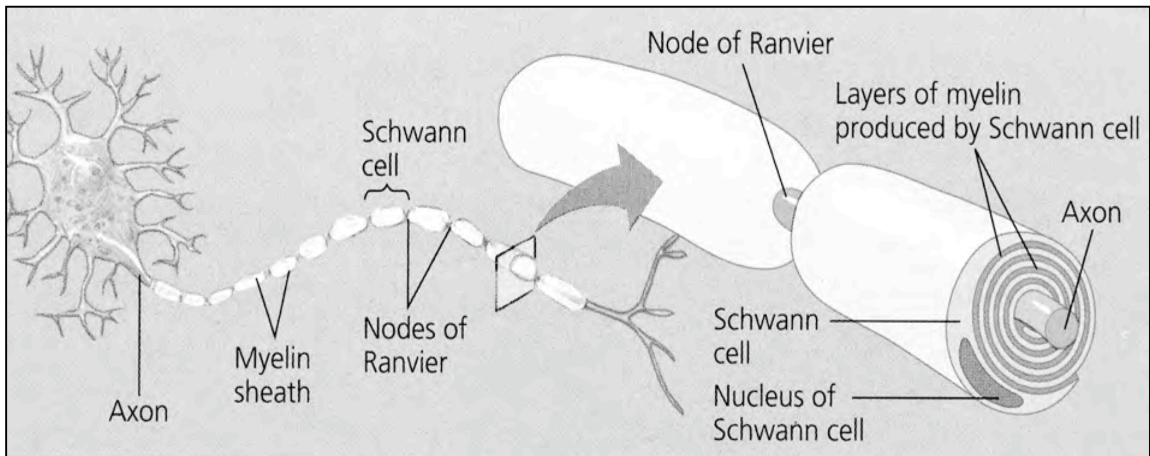


Figure 1.1.2: In the PNS there are Schwann cells instead of oligodendrocytes. A simple overview of a neuron that is myelinated by a Schwann cell. Modified from Chapter 48, pg 1026, Biology, Benjamin-Cummings 2002 (Campbell and Reece, 2002).

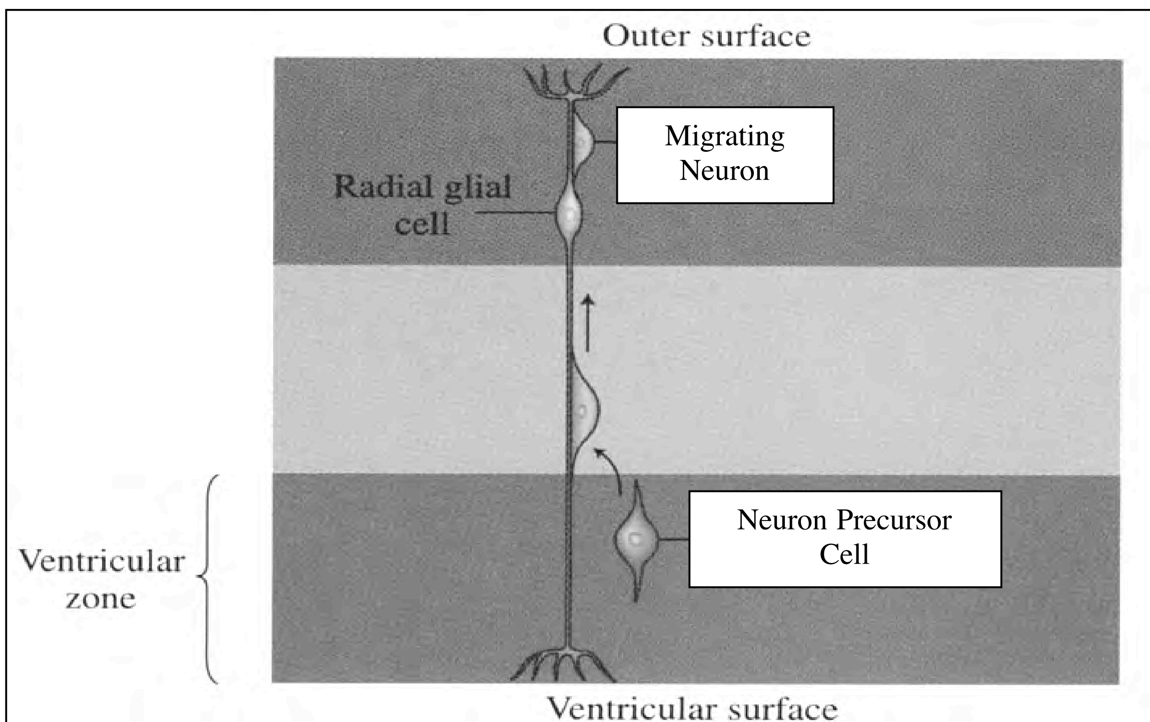


Figure 1.1.3: In the CNS neuronal precursors use radial glial cells as scaffolding to migrate into the different layers of the developing brain.
Modified from Chapter 19, pg 441, Neurobiology, Blackwell Science 2001 (Matthews, 2001)

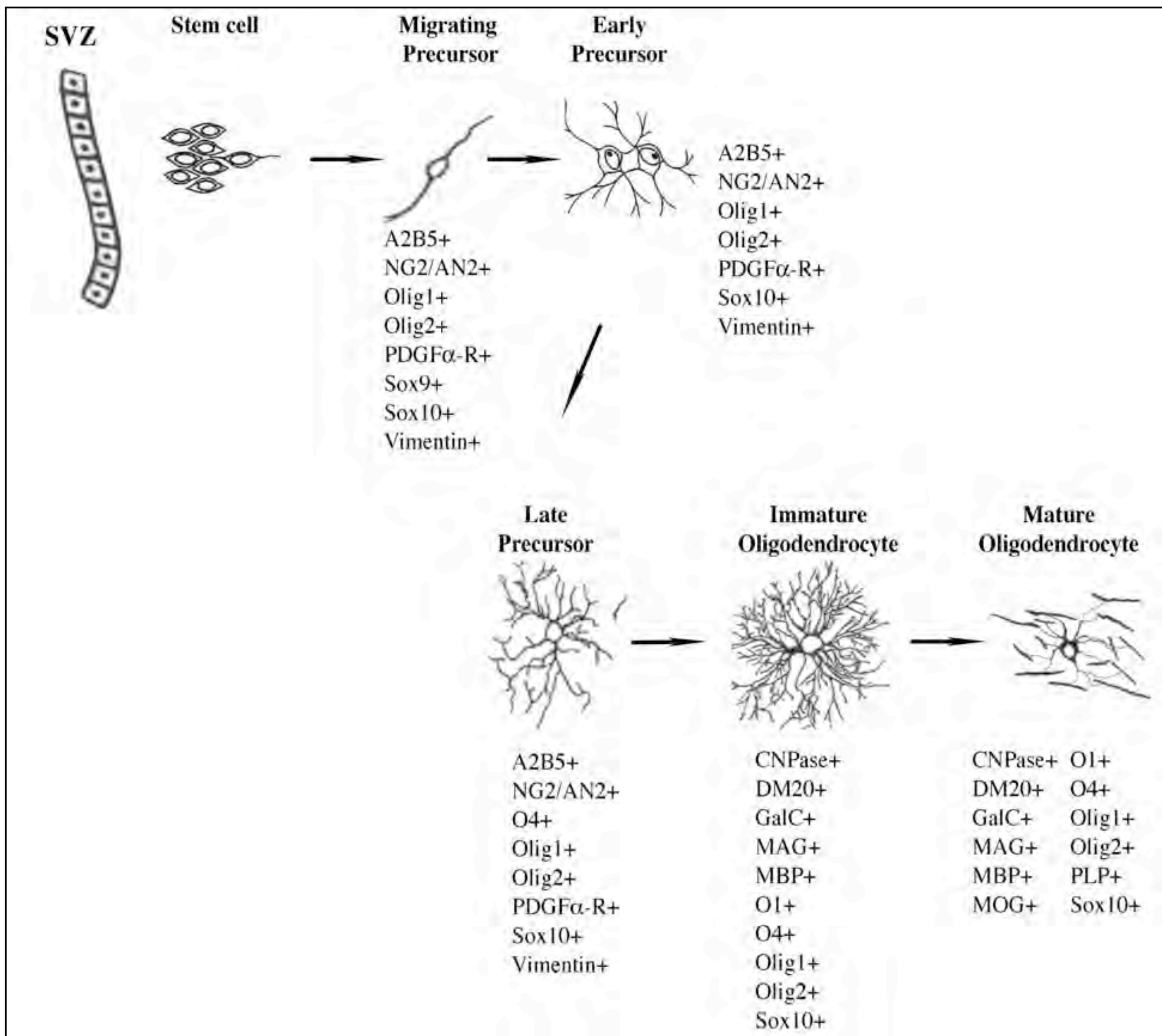


Figure 1.1.4: Schematic summary of Oligodendrogenesis *in vivo*

Modified from Chapter 2 pgs 29-56, Myelin Biol., Elsevier, 2004(Trapp et al., 1997; Stolt et al., 2003; Lazzarini et al., 2004)

1.2 Development of the Central Nervous System

1.2.1 Neural Induction

During gastrulation of the vertebrate embryo three cellular layers develop. The outer layer known as the ectoderm which later gives rise to the CNS, PNS, and epidermis of the animal, the middle layer known as the mesoderm which gives rise to the skeleto-muscular system, blood, urinogenital system, connective tissue, and internal organs such as heart and kidneys and the inner layer known as the endoderm which gives rise to the inner organs. Within the mesoderm is an area termed “the organizer” from which signals are directed toward the ectoderm, which in turn leads to the development of the neural plate in the process known as neural induction (Spemann and Mangold, 2001). Some of the major signals that inhibit neural induction are the Bone Morphogenic Proteins (BMP) 2, 4, and 7 (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). At the time of neural induction antagonists such as noggin, chordin, and follistatin are expressed in the neuroectoderm that are released by the organizer region. These antagonists bind directly to BMP2, 4, and 7, preventing them from interacting with their receptor(s). Therefore, these antagonists are prime candidates for initiating the process of neural induction (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994; Mehler et al., 2000; Kuroda et al., 2005; Reversade and De Robertis, 2005; Reversade et al., 2005). Following neural induction the neural plate is restricted to the production of neural cells.

1.2.2 Neurulation

Neurulation in vertebrates results in the transition of the neural plate into the neural tube, an epithelial structure composed of ectoderm, which later develops into the brain and spinal cord. It is further shaped by the notochord, a derivative of the organizer. The convergent extension of the neural plate is associated with a change in tissue morphology, gradually giving rise to raised edges above the surface of the neural plate. This results in two parallel neural folds with a depression (the neural groove) between them. The marginal areas of the neural groove contain the neural crest cells, that latter migrate away from the neural plate giving rise to dorsal root ganglia, the sympathetic ganglia, and neurons whose cell bodies are located in the PNS. With time, the folds

start to close to form a cylinder and with the eventual fusion of the margins, the neural tube is on the dorsal midline of the developing embryo (Schoenwolf, 1984, 2001). Along the line where the neural tube begins to pinch off from the future epidermis, some ectodermal cells detach from the epithelium and migrate out through the mesoderm. The neural crest cells will later form part of the PNS, which then separates from the adjacent ectoderm. The surface of the ectoderm becomes epidermis. A series of folds, swellings and constrictions of the neural tube results in the formation of the different CNS regions: the forebrain, midbrain and hindbrain at the rostral end and the spinal cord at the caudal end of the tube (figure 1.2.1).

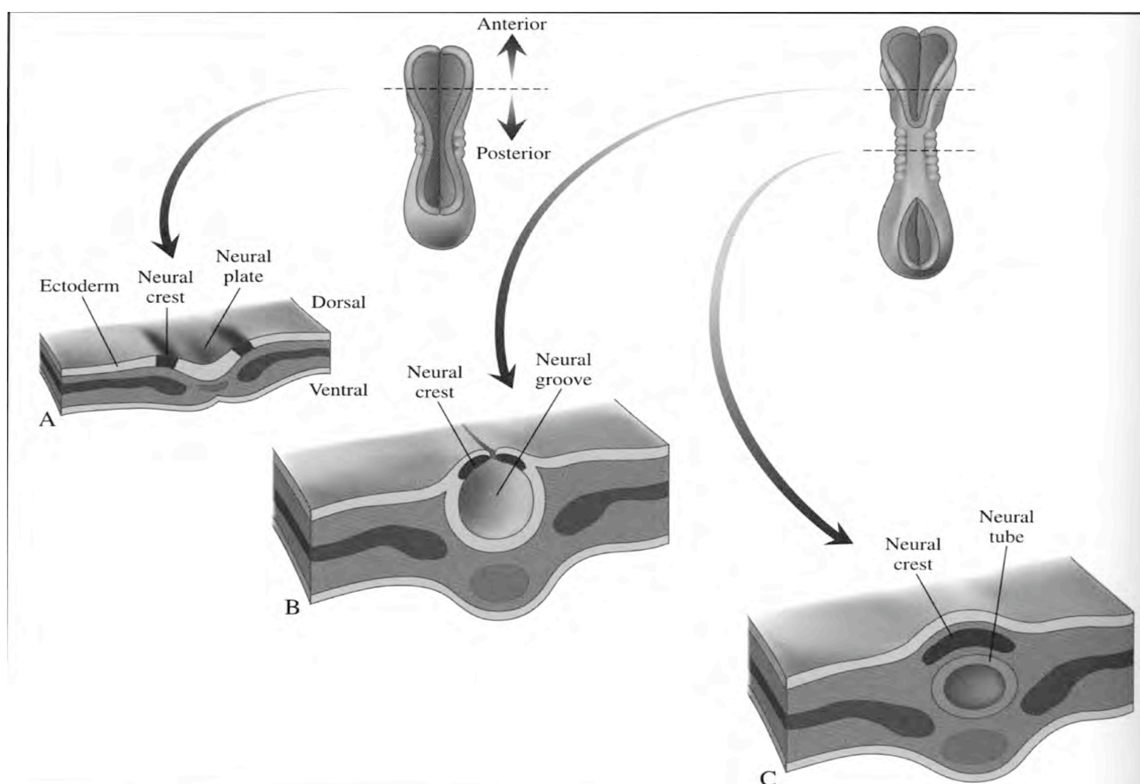


Figure 1.2.1: The development of the neural tube in the vertebrates. Modified from Chapter 19, pg 434, Neurobiology, Blackwell Science 2001 (Matthews, 2001)

As neural development proceeds, the initially formed neural tube undergoes differential expansion and regionalization to form the different subdivisions of the brain. Initially, there are three primary vesicles: the forebrain, the midbrain, and the hindbrain. Later in development the vesicles start to differentiate into subdivisions. The forebrain divides into the Telencephalon and the Diencephalon. The Telencephalon, gives rise to the olfactory lobes, the hippocampus, and the cerebrum, while the Diencephalon gives rise to the retina, thalamus, and hypothalamus. The Mesencephalon that latter gives rise to the midbrain regions. The rhombencephalon or hindbrain differentiates further into the medulla and the cerebellum. The caudal tube does not under go a further differentiation, but does become larger and gives rise to the spinal cord. Each of these brain regions has physical boundaries, which restricts cellular movement, and allows the different brain regions to develop independently (Sanes et al., 2000).

The precursor cells of the neural tube ultimately give rise to all the different cell types of the CNS. They do not proliferate uniformly; hence during maturation a single cell layer develops into a structure many centimeters thick in the adult brain. New neural cells proliferate at the inner surface of the neural tube, at the edge of the lumen, and migrate outwards. The neural tube will eventually become the ventricle of the brain and the central canal of the spinal cord.

The single layer of cells known as the ventricular zone consists of pseudo-stratified neuroepithelium containing the neuroepithelial cells. The first neural precursors proliferate quickly within this area next to the fluid-filled ventricle. The ventricular zone eventually gives rise to the three major cell types of the CNS: neurons, astrocytes, and oligodendrocytes. As the neural cells proliferate, they slowly migrate out of this zone into the adjacent zone known as the marginal zone. In order for this to happen the neuroepithelial cells divide symmetrically at first to expand the pool of stem cells. Around embryonic day 11 in mice, the neuroepithelial cells start to divide asymmetrically to generate neuroepithelial cells and neuronal progenitors (Caviness and Takahashi, 1995; Caviness et al., 1995; Takahashi et al., 1995a, b; Nowakowski et al., 2002). Neuroepithelial cells that are initially a homogenous population differentiate to generate all the neurons and glial cells that constitute the adult brain. After the onset of neurogenesis neuroepithelial cells give rise to a distinct, but related cell type “ radial glia cells”. The radial glia cells are more fate restricted and replace the neuroepithelial cells

overtime. These cells are restricted to generate certain cell types: astrocytes, oligodendrocytes, or neurons. A majority of the neurons are either directly or indirectly derived from these cells. When neurogenesis starts, neuroepithelial cells and radial glia cells divide rapidly either symmetrically or asymmetrically. When the cells divide symmetrically, they generate two equal daughter cells, but on asymmetrical division one cell inherits more of the apical-basal complex and the other cell does not. This type of division derives a daughter cell; to generate more daughter cells, or an intermediate cell is derived which eventually generates a neuron. Further characterization of the neuroepithelial cells revealed that these cells are devoid of radial glia marker such as RC1, RC2 (cytoskeletal antigens), vimentin (an intermediate filament) GLAST, GFAP, and S100ß (figure 1.2.2) (Gotz and Huttner, 2005).

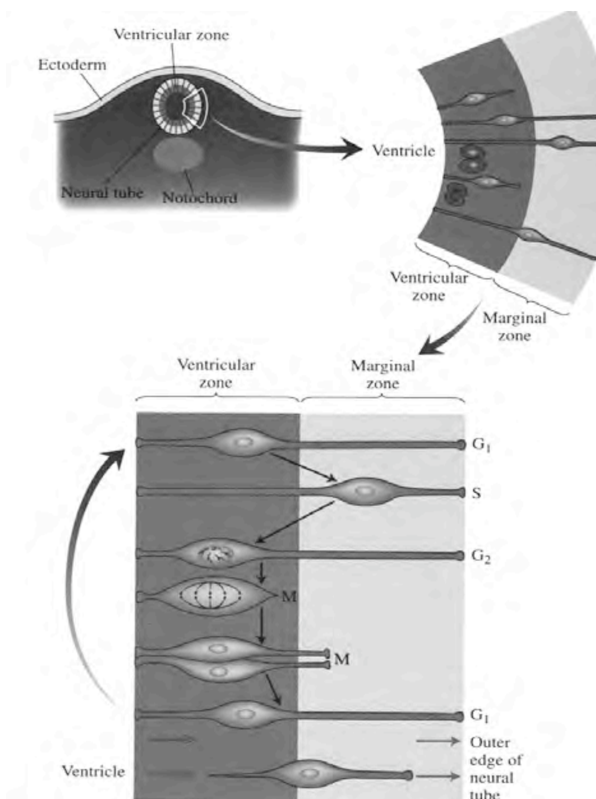


Figure 1.2.2: Proliferation and migration of neural stem cells from the ventricular zone into the marginal zone. Modified from Chapter 19, pg 436, Neurobiology, Blackwell Science 2001 (Matthews, 2001)

1.3 Neural Cell Specification

Numerous models have been suggested to define the origin of the different cells in the nervous system. One model states that the neuroepithelial cells consists of committed stem cells, where each cell type in the nervous system has a unique stem cell. However, retroviral labeling and dye tracing studies have shown, that the neuroepithelial cell can give rise to multiple phenotypes in chick embryos (Bronner-Fraser and Fraser, 1988; Sanes, 1989; Leber et al., 1990; Artinger et al., 1995). Furthermore by the use of these techniques it was shown that the neuroepithelial cells are multipotent, giving rise to the different cells of the CNS such as neurons, and glia. This has been further confirmed in rodents. Thus, these results argue against the model where neuroepithelial cells are committed stem cells giving rise to only one cell population. There are restricted precursors that are derived from the neuroepithelial cells that generate particular cell types in the developing CNS, for example glial restricted precursors (GRP), neuronal restricted precursors (NRP), motorneuron-oligodendrocyte precursors (MNOP), and oligodendrocyte type-2 astrocyte precursor (O-2A, Lazzarini et al., 2004; Richardson et al., 2006).

1.3.1 Glia Restricted Precursor (GRP) / Neuronal Restricted Precursors (NRP)

In the developing embryonic spinal cord there are glial restricted precursors (GRP) and neuronal restricted precursors (NRP) which give rise to astrocytes and oligodendrocytes and neurons respectively *in vitro*. GRPs can be identified at embryonic day 12 by their expression of A2B5 and nestin (a neuroepithelial stem cell marker, Rao and Mayer-Proschel, 1997; Rao, 1999). They initially lack the expression of PDGFα-R, and are located more ventrally from the proliferating neuroepithelium. When the GRP starts to differentiate they can generate different types of glia cells, for example oligodendrocytes, type-1 astrocytes and type 2 astrocytes (Lee et al., 2000). In the case of the NRPs, these cells make only neurons *in vitro.* The NRPs express transcription factors like Olig1/2, before they start to differentiate to neurons, which is associated with an upregulation of Pax 6 (figure 1.3.2) (Hack et al., 2004).

1.3.2 Motorneuron-Oligodendrocyte Precursor (MNOP)

Motorneuron-Oligodendrocyte precursors are cells that derive either motorneuron or oligodendrocytes within the developing mouse. Two genes that belong to the same family that are important for their development are Olig 1 and Olig2, and mice lacking these genes display an absence of motorneurons and oligodendrocytes (Arnett et al., 2004; Xin et al., 2005). Mature Oligodendrocytes still express Olig2, while neurons do not. These genes are expressed in the motorneuron domain of the spinal cord. Wu et al., argue against this concept of a common precursor for the motorneuron-oligodendrocytes. They show using cell ablation to kill Olig-expressing cells, that all differentiated neurons and oligodendrocytes are eliminated, but there is a continuous generation of the precursors. When the motorneuron precursors are eliminated, normal amount of oligodendrocytes precursors are still generated, supporting the concept of a sequential generation of both cell types from neuroepithelial stem cells *in vivo* (figure 1.3.2 ,Wu et al., 2006).

1.3.3 Oligodendrocyte-Type-2 Astrocyte (O-2A)

Another restricted precursor is the O-2A (oligodendrocyte-type 2 astrocyte) cell, which represents one of the earliest defined glial precursors of the CNS, but has largely been defined by *in vitro* studies. The cells were first initially isolated from rat optic nerve (Raff et al., 1983a, b). They have a default pathway of differentiating into oligodendrocytes, and *in vitro* this can be manipulated by growth factors and culture conditions. In the presence of serum, the O2A precursor differentiates into a type 2- astrocyte that expresses GFAP and A2B5 (Raff et al., 1983c; Lee et al., 2000). In culture conditions, where serum is lacking, the O2A precursor differentiates to oligodendrocytes. At this point in time the evidence for the existence of these cells *in vivo* is still scanty.

1.3.4 Radial Glia are stem cells in the CNS

A model that has received a lot of attention in the last few years is the concept that radial glial cells are stem cells that derive the different cell types of the developing CNS (Johansson et al., 1999). Two areas in the developing and adult brain that were studied quite well are the subventricular zone and the dentate gyrus in the hippocampus. These two areas are known to continue with neuronal genesis throughout development and

into adulthood (Eckenhoff and Rakic, 1984; Rickmann et al., 1987; Cameron et al., 1993). It has been shown that the location of the radial glia cell will determine the type of neurons it generates (Malatesta et al., 2000; Noctor et al., 2002; Kriegstein and Gotz, 2003; Malatesta et al., 2003). The model states that a slower dividing radial glia cell (type-B cell) can self renew, differentiate to an astrocyte, or divide to a more restricted transit amplifying precursor (type-C cell). The type-C cell is a faster dividing precursor that is more restricted in its differentiation potential, not being able to generate another slower dividing type-B cell.

The type-C transient amplifying precursor cell expresses a helix-loop helix transcription factor, known as Olig2 (Hack et al., 2004). This particular transcription factor has been shown to be important for the development of neuroblasts and oligodendrocytes. This type-C cell is a rapidly dividing cell that gives rise to oligodendrocytes and type-A cells known as the neuroblast. The neuroblasts generate further neuroblasts and eventually neurons of the developing CNS. When the type-C cell commits to a neuronal fate, then it down regulates Olig2 and up regulates other transcription factors such as Pax6, Ngn2, Mash1, and Gsh1 transcription factor that are unique to neuronal development (Gotz, 2003; Gotz and Steindler, 2003; Buffo et al., 2005; Gotz and Barde, 2005). Once committed to the neuronal fate, the type-C cell is no longer able to differentiate to an oligodendrocyte (figure 1.3.1).

1.3.5 Transcription factors important for oligodendrocyte development

Transcription factors, which are vital for the development of oligodendrocytes within the CNS, are Olig1, Olig2, Nkx2.2, Sox8, Sox9 and Sox10. Olig2 and Nkx2.2 are expressed early around embryonic day 3 in distinct adjacent precursor domains like (motorneuron domain) pMN and p3, but around embryonic day 6 their expression begins to overlap when oligodendrocyte precursors start to migrate out of these domains (Zhou et al., 2001). It has been shown that these 2 transcription factors are important for the maturation of oligodendrocytes, and knocking out either one of these transcription factors causes complete loss of mature oligodendrocyte (Lu et al., 2002). However, oligodendrocyte precursors are still present judged by the expression of the NG2 antigen, indicating that the oligodendrocytes are not completely lost, but just hindered in their ability to reach the myelin forming stage (Liu and Rao, 2004).

Another transcription factor, which is important for glial determination is Sox9, which plays a role in astrocyte and oligodendrocyte determination in the developing CNS. The heterozygous mouse is an embryonic lethal. However, Sox9 is expressed not only in precursors of oligodendrocytes and astrocytes, but also in other cell types in the developing mouse. It is down regulated during glia cell maturation (Stolt et al., 2003; Wegner and Stolt, 2005).

Distinct transcription factors are expressed both in OPC and mature oligodendrocytes. For example the transcription factor Sox10, which is unique to oligodendroglia in the CNS is up regulated in the oligodendrocyte precursor and maintained throughout the existence of the oligodendrocyte (Kuhlbrodt et al., 1998; Stolt et al., 2002). It is important for oligodendrocyte maturity and myelin formation. Sox 10 knockout mice die at embryonic stages due to the lack of mature oligodendrocytes, even though they have oligodendrocyte precursors. Sox8 is a transcription factor that belongs to the same family as Sox10 and appears to play an equivalent role to the Sox10 transcription factor. In knockout mice lacking the expression of Sox8 retardation in myelination is seen, but the mice are viable. This indicates that Sox10 and Sox8 may play roles in the CNS during terminal myelination, but Sox10 has a more important role. In the absence of Sox8, Sox10 can compensate for the lack of Sox8, however vice versa is not possible (Stolt et al., 2004).

2.Materials and Methods:

2.1 Chemicals and Materials

All of the following materials were used from the following firms: Amersham Pharmacia, Roche, Carl Roth, Serva, Falcon, Fluka, Eppendorf, Merck, Sigma, Stratagene, Qiagen, MBI, NEB, BD bioscience, Invitrogen, Nunc, Biometra, Biorad, and Promega.

Acrylamid (30%)	ROL
Agarose	Biorad/Invitrogen/Sigma
Ampicillin	Merck
BSA	Sigma/Merck
Cell culture plates	Falcon/Nunc
Chloroform	Merck/Sigma
Cyro-tubes	Nunc
DAPI (4,6-diamidino-2-phenylindol)	Roche
Diethlprocarbonate (DEPC)	Sigma
Dulbeccos modified Eagle Medium (DMEM)	Gibco
EDTA	Sigma
Ethidium bromide	Roth/Sigma
FCS (fetal calf serum)	Gibco
Filter paper	Whatman
Formaldehyde	Sigma
Gelatin	Sigma
Glycerol	Sigma/Merck
HBSS	Gibco
Hepes	Gibco/Sigma
Hybond-N	Amersham Pharmacia
Isoamyl alcohol	Roth
Kanamycin	Merck
LIF (ESGRO)	Chemicon
MEM (non-essential amino acids)	Gibco
ß-Mercaptoethanol	Sigma
Mitomycin C	Sigma

Na-Pyruvate	Gibco
Paraformaldehyde	Sigma
Penicillin/Streptavidin	Gibco
Phenol	Roth/Gibco
Poly-L-lysine	Sigma
Polypropylene tubes (15, 50 ml)	Falcon, Nunc
Polypropylene tissue culture dishes	Falcon, Nunc
2-propanol	Sigma/promega
Reaction tubes (.2, .5, 1.5, 2.0ml)	Eppendorf
Slides	Menzel-glass
SDS	Merck
TEMED	Merck
Trypsin	Gibco

2.2 Equipment

Cell incubator	Heraeus/Baker
Centrifuge	Heraeus/Eppendorf/Sigma
Concentrator	Eppendorf
Electroporation apparatus (Gene pulser)	Birorad
Gel chamber for agarose	Biorad, Biometra
Gel documentation machine	Ray-test/Biorad
Hybridization oven	Biorad
Lab scale	Sartorius
Microscope	Lecia inverse Lecia Lecia LSM Stemi (Zeiss) LSM 510 Axiovert 20 (Zeiss)
PCR machine	Biometra
Perestaltic pump	Heraeus
Phosphoimager	Fuji/Ray-test
Photometer	Biorad
Pipettes	Gilson

Power supply	Ray-test/Biorad/Pharmacia
Southern blot apparatus	Biomaterial
Sterile hood	Heraeus/Baker
Thermo mixer	Eppendorf/Fisher
UV illuminator (Stratalinker)	Stratagene
UV hand held light	Biorad
Vacuum blotter	Biometra
Vibratome	Lecia VT 1000S
Vortexer	Sigma
Western blot apparatus	Gibco

2.3. Solutions, Buffers and Media

2.3.1 Microbiology and Protein chemistry

Chloroform-is amyl alcohol

24:1(v/v)

DEPC-H_2O

H_2O	1000 ml
DEPC	1 ml

Overnight at 37°C stand then autoclave.

DNA probe buffer

Bromo-phenol blue	0.25%
Xylen cyanol	0.25%
Ficoll (type 400)	15%

In H_2O.

High SDS pre-hybridization buffer (1000 ml)

Na_2HPO (1M)	500 ml (fact. 0.5M)
SDS (20%)	350 ml (7%)
EDTA	10mM

Fill to 1000 ml with H_2O.

Homogenization Buffer

$NaHCO_3$	1mM
$CaCl_2$	3mM
$MgCl_2$	2.5mM
Spermidine	1mM
Phosphate inhibitor	
Protease inhibitor	

LB-Medium

Bacto Trypton	10 g
Bacto Yeast extract	5 g
NaCl	10 g

Fill to 1000 ml with H_2O and pH to 7.4 with NaOH. For selection Ampicillin was added (f.c. 75 μg/ml); for LB agar plates, 15 g of agar was added to the bottle before autoclaving. The bottle was cooled down and Ampicillin was added.

PBS/Tween 20

PBS (10x)	100 ml
Tween 20	1 ml (0.1%)

Fill to 1000 ml with H_2O

Phosphatase inhibitors

Na_3VO_4	100μM
Sodium Fluoride	100mM

Protease Inhibitors

Antipain	1mg/ml
Aprotinin	1mg/ml
Benzamidine-HCl	26mg/ml
Iodoacetamide	18mg/ml
Leupeptin	5mg/ml
Pepstatin	5mg/ml
PMSF	100mM

Southern Blott-Depurineration solution

HCl	0.125M

Southern Blott-Depurineration buffer

NaCl	1.5M
NaOH	0.5M

Southern Blott-Neutrealization buffer

NaCl	1.5M
Tris-HCl	0.5M

SSC (20x, 1000 ml)

NaCl	3M
Sodium Citrate	0.3M

TAE buffer (50X, 1000 ml)

Tris-HCL-Base	242g (2M)
Acetic Acid	57.1 ml (1mM)
EDTA (0.5M, pH 8)	100 ml

Fill to 1000 ml with H_2O

TB-Medium

Bacto Trypton	12 g
Bacto Yeast extract	24 g
Glycerin	4 ml
Fill to 900 ml and then autoclave	
KH_2PO_4(0.17M)/ K_2HPO_4(0.7M)	100 ml

TBS (1000 ml)

Tris-Base (1M, pH 8)	50 ml (50 mM)
NaCl (5M)	30 ml (150 mM)

Fill to 1000 ml with H_2O and pH to 7.4 with HCl.

TE-buffer

Tris-Cl (pH 7.4)	10 mM
EDTA (0.5 m)	1 mM

Transfer Buffer I

0.3 M Tris	36.3g
20% Methanol	200ml
pH 10.4	add1000ml with water

Transfer Buffer II

0.025 M Tris	3g
20% Methanol	200ml
pH 10.4	add 1000ml with water

Transfer Buffer III

0.025 M Tris	3g
0.04 M amino-n-capronic acid	5.2g
20 % Methanol	200 ml
pH 9.4	add 1000ml with water

Transformation buffer (TB jap, 1000 ml)

PIPES	3.03 g (10 mM)
$CaCl_2$	2.21 g (15 mM)
KCl	18.64 g (250 mM)
With KOH pH to 6.7	
$MnCl_2$	8.91 g (55 mM)

Fill to 1000 ml with H_2O and then filter with 0.45 μm pore filter.

Western Blott running buffer

Glycine	1.9 M
Tris	0.25 M
SDS	1%
PH 8.8	

Western blot transfer buffer

Tris	0.3%
Methanol	20%
PH 10.4	

2.3.2 Cell Culture

Poly-L-lysin coated coverslips

Coverslips were autoclaved and then washed and sterilized with EtOH and fire. They were placed at 37°C in poly-l-lysine solution (0.01%) and then washed with PBS 3 times.

PBS (10X stock solution)

NaCl	100 g
KCl	2.5 g
$NaHPO_4$*2 H_2O	7.2 g
KH_2PO_4	2.5 g

Fill to 900 ml with H_2O, and then pH was adjusted to 7.2 with NaOH. Then the buffer was filled to the 1000 ml with H_2O. The buffer was diluted 1:10 and then autoclaved for use.

FCH/HS

50 ml aliquots were made and heat inactivated at 56°C for 45 minutes.

EMFI-Medium

FCS (ES-tested)	50 ml
L-glutamine (100 X)	5.5 ml
Pen/Strep (100 X)	5.5 ml

HBSS+ (500 ml HBSS)

$MgSO_4$	7.5 ml (0.15%)

BME/HS (500 ml)

Pen/Strep	5 ml
HS	50 ml

Sato-medium (100 ml) for primary oligodendrocytes

DMEM (High glucose, $NaHCO_3$, Glutamine, Pyruvate	97 ml
Transferrin (5 mg/ml in DMEM)	100 μl (1μg/ml)
Insulin (1 mg/ml in 0.01 M HCl in DMEM)	1 ml (10 μg/ml)
Putrescine (10 mM in DMEM)	1 ml (100 μM)
Progesterone (2 mM in EtOH)	10 μl (200 nM)

Tri-iodo-thyronine (500 mM in EtOH)	100 µl (500 pM)
Na-Selenite (300 µM in H_2O)	74 µl (220 nM)
L-Thyroxine (4mM in 0.13 M NaOH in 70% EtOH)	13 µl (520 nM)
Gentamycine (50 mg/ml, Sigma)	50 µl (25 µg/ml)

Sterile filter with 0.2 µm syringe filter and then add 1% (f.c.) HS

Mitomycin C (2 mg/bottle)
Dissolve in 0.1 ml of DMSO and 1.9 ml of PBS; 100 µl/10 ml EMFI-medium (f.c. 10µg/ml)

ES cell medium (633 ml)

DMEM (4.5 g glucose/l)	500 ml
Non essential amino acids (100x)	6.3 ml
Sodium Pyruvate (100x)	6.3 ml
Penicillin/Streptomycin (100x)	6.3 ml
L-glutamine (100x)	6.3 ml
β-Mercaptoethanol, 10 mM in PBS	6.3 ml
LIF (10^7 U/ml)	6.3 ml
FCS (ES cell tested)	95 ml

Trypan Blue (0.08% in 1x PBS)

Trypan Blue	0.16 g

Fill to 200 ml with 1x PBS.

EMFI freezing medium (10 ml, 2x)

DMSO	2 ml (20%)
FCS	2.5 ml (25%)
DMEM	5.5 ml

ES cell freezing medium

DMSO	2 ml (20%)
FCS	5 ml (50%)
ES cell medium	3 ml

Methylene blue (100 ml)

Methylene blue	2 mg (2%)

Fill to 100 ml with H_2O

Hoechst

Dissolve 1 mg in 1 ml of methanol, store at –20 in the dark.

For staining dilute stock solution 1:100 in PBS or TBS

Gelatin (500 ml)

Gelatin	0.5 mg

Fill to 500 ml with H_2O and autoclave

G418 (10 ml)

G418	0.5 g (50 mg/ml)

Fill to 100 ml with H_2O, sterile filter and keep at –20 for use.

2.3.3 Histology and Immunohistochemistry

Fixation for immunohistochemistry

Paraformaldehyde	40 g (4%)

Fill to 900 ml with heated 1x PBS and then pH to 7.2 mix until clear and then add PBS to 1000 ml. Filter solution with vacuum filter.

Tris buffer (stock solution 0.5 M pH 7.6)

TRIS	60.57 g
H_2O	500 ml

For pH of 7.6 add 390 ml of 1M HCl in 1000 ml of the buffer.

Tris buffer (working solution 0.05 M pH 7.6)

NaCl	9 g
Tris (0.5 M pH 7.6)	100 ml

Fill to 1000 ml with H_2O.

2.4 Antibodies, Enzymes and Reaction kits

2.4.1 Antibodies

Anti-AN2 (monoclonal, rat)	Ag trotter
Anti-CNP (monoclonal, mouse)	Sigma
Anti-Cre- recombinase (polyclonal, rabbit)	BabCO
Anti-double-cortin	Chemicon
Anti-GFAP (Monoclonal, mouse)	Roche/Chemicon
Anti-GFAP (polyclonal, rabbit)	Dako
Anti-MBP (monoclonal, mouse)	Dako
Anti-Neun (monoclonal, mouse)	Chemicon
Anti-O1 (monoclonal, mouse)	Schachner/Roche
Anti-olig 1	Gift from Dr. Rovitch
Anti-olig 2	Gift from Dr. Rovitch
Anti-OMGP	Gift Dr. Miko
Anti-S100 Beta	Sigma
Anti-sox 10	Gift from Dr. Wegner
Anti-sulfatide (O4, monoclonal, mouse)	Schachner, Roche

2.4.2 Secondary antibodies

Goat anti-mouse Cy2	Dianova
Goat anti-mouse Cy3	Dianova
Goat anti-mouse Fitc	Dianova
Goat anti-mouse Rhod.	Dianova
Goat anti-rabbit Cy2	Dianova
Goat anti-rabbit Cy3	Dianova
Goat anti-rabbit Fitc	Dianova
Goat anti-rabbit Rhod.	Dianova
Goat anti-rat Cy2	Dianova
Goat anti-rat Cy3	Dianova
Goat anti-rat Fitc	Dianova
Goat anti-rat Rhod.	Dianova

2.4.3 Enzymes

Restriction Endonucleases (5-20 U/ml) were ordered from Roche, New England biolabs, MBI, and Promega. Each of the enzymes was delivered with 10 X reaction buffer for their use.

For double restriction digests manufacture's suggestions were used to ensure proper cutting of the desired DNA

CIP (Alk. Phosphatase) 1 U/ml	Roche
Proteinase K 10 mg/ml	Qiagen
Red-Taq-polymerase 5 U/μl	Sigma
PFU taq-polymerase 2 U/μl	Qiagen, MBI
Vent-taq-polymerase 2 U/μl	MBI
Ultra-PFU-taq-polymerase 3 U/μl	Stratagene
T4-DNA-Ligase 3 U/μl	MBI, Promega

2.4.4 Reaction kits

Mycoplasma-detection-kit	Stratagene
Qiaprep-kits	Qiagene
Qiaamp-tissue-kit	Qiagene
Gel extraction Kit	Qiagene
Plasmid-prep kit	Promega
Maxi-prep kits	Qiagene, Cat

2.5 Amino Acids and Nucleotides

2.5.1 Vectors and Constructs

pBluescript II KS+	Stratagene
peYFP	Clonetech
pGEM-T Easy	Promega
pMC-Cre	R. Sprengel
pSP72	Promega

The NG2 start codon was cloned by Judith Stegmuller and used for generating a BAC for the fusion PCR between the start codon and the inserted gene of interest.

2.5.2 Other Amino acids and Nucleotides

dNTPs	Roche, Peq labs
Large DNA marker Lambda/HindIII	Promega, MBI

Small DNA marker ΘX/Hae III	Promega, MBI
Large DNA marker Gene Ruler 1Kb ladder	MBI
Small DNA marker Gene Ruler 100 bp ladder	MBI

2.6 Eukaryotic cell lines and Bacterial cells

2.6.1 ES cell lines

R1 embryonic stem cells	A. Nagy Toronto
129 OLA embryonic stem cells	Nils Brose, Gottingen

2.6.2 Bacterial cells

E.coli XL1-Blue	Stratagene
Top 10 cells	Stratagene

2.7 Animals

2.7.1 Wild-type mice strains: C57BL/6J and SV/129.

Mouse line	Short description of genetic change	Reference
NGYF	Expression of eYFP under the NG2 promoter. Uses endogenous poly-a tail.	This work
ANYF	Expression of eYFP under the NG2 promoter. Uses artificial poly-a tail.	This work

2.8 Primers for cloning and sequencing the AN2eYFP and AN2Cre constructs

AN2I s: 5’-GCAAGTGAAAACACCAAG-3’

AN2I as: 5’-CATCGCGGCGGGGCTGGGTGC-3’

AN2-YFP s: 5’-CAGGCACCCAGCCCCGCCGCGATGGTGAGCAAGGGCGAGGAG-3’

AN2-YFP as: 5'-GTGGTCGGGGTAGCGGGC-3'

AN2.2 s: 5'-CCAGATTTCCAGGAGGTG-3'

AN2.3 s: 5'-GGTAGGAGCTAAATCCAG-3'

AN2-Cre s: 5'-CAGGCACCCAGCCCCGCCGCGATGCCCAAGAAGAAGAGGAAG-3'

PLPCre as: 5'-TTCGGATCCGCCGCATAAC-3'

AN2LA s: 5'-GCTAGGCCGGCCGACACCCGCTGTCAGCTCCAGCC-3'

AN2LA as: 5'-GAGTCTCGAGCAGGTGCATGCTCTCACACTCAG-3'

gIPA as: 5'-GAATAGCGGCCGCGCATATGTTGCCAAACTCTAAAC-3'

gIPA s: 5'-GAATAGCGGCCGCATCCTCTAGACTGAGAACTTC-3'

gIPA2 as: 5'-GAATAGCGGCCGCAATACGCAAACCGCCTCTC-3'

Intron-5516 as: 5'-CTGAAGTTCTCAGTCTAGA-3'

Link1 s: 5'-
CGGATCCCTGCAGATTTGCGGCCGCCATATGGGCCGGCCGCCTCGAGCCCCCGGCC
GC-3'

Link2 as: 5'-
GGCCCGGGGGCTCGAGGCGGCCGGCCCATATGGCGGCCGCAAATCTGCAGGGATCC
GGTAC-3'

LACP s: 5'-AGCCTACCCAGAAGTATATGA-3'

LA s1 s: 5'-ATACTTGGGTGGCTCCCC-3'

LA as1 as: 5'-AGTCTGTGAGGGCAGGCC-3'

LA-linker s: 5'-TCGAGCGGCGAGCTCGAATAGGGCCGGCCG-3'

LA-linker2 as: 5'-AATTCGGCCGGCCTTATTCGAGCTCGCCGC-3'

LA s2 s: 5'-GCAACCATCTGGACCAAC-3'

LA 2 as: 5'-GAGTCTCGAGCTGGCCATGAACTTTC-3'

LA-4134 s: 5'-TACCCTGTGCCAGACAGCTTC-3'

LA-4135 s: 5'-AGGAAGGCTTGACTGTAACCC-3'

LA-4136 s: 5'-GCTGTTTGCTGGAACTAGGAG-3'

LA-4137 s: 5'-ACACAGAATTGCCTTCATTGC-3'

LA-4138 s: 5'-TGACCACAGATACTCTGAGCC-3'

LA-4139 s: 5'-ACATTTGTCTCTGATATGCGG-3'

LA-4140 s: 5'-TCAGCCTACATCTTTCAGGAC-3'

LA-5039 as: 5'-ACAGCTTTCCTTCCAGAC-3'

LA-5040 s: 5'-TGCTGTGAAGTGAGACTC-3'

LA-5041 s: 5'-AGCAGAGCCTGAGTGAAG-3'

LA-5042 s: 5'-TGCAGGCCCTACCTGCTTG-3'

LA-5043 s: 5’-TGCAGCCGAGCCCTGGT-3’

LA-5044 s: 5’-TGCATTCCAGAGGATCCAG-3’

LA-5045 s: 5’-ATACAAGAAGAGAATCAG-3’

LA-5046 s: 5’-TGGCCCCGTTTTACAG-3’

LA-5047 s: 5’-AATTTTGCACCCAGGG-3’

Lox P Neo as (4937): 5’-ATGGCCGGCCGCCAGTGCCAAGCTACTCGCGAC-3’

Lox P Neo s (4938): 5’-ATCATATGGCGGCCGCAGACCTACTTCACTAACAACCGG-3’

FRT Neo as: 5’-AAATATGGCCGGCCCTGCAGGGTCAGATCTGTC-3’

FRT Neo s: 5’-GGAATTCCAATATGGGCGGCCGCCCGGTACAGTTCGAAGTTC-3’

2.9 Primers for cloning control plasmid

KP 1 s: 5’-CGGGGTACCGCCTCAGTTTCTCTATCG-3’

KP 2 as: 5’-CGGGGTACCTCCAGACCCTCAGCCTGG-3’

2.10 Primers for verifying homologous recombination in ES cells

AN2C1 s (3305): 5’-GAAGAGAGGAACGGGAGTGTT-3’

AN2C2 s (3306): 5’-GCCAAACACAGGCACGGGGAA-3’

AN2C3 s(3307): 5’-GCTCCTGGTTGGGACTAGGCA-3’

NI Cre2 as: 5’-CATCAGGTTCTTGCGAAC-3’

Cre-5002 as: 5’-TGCTCAGAAAACGCCTGGCG-3’

Cre-5003 as: 5’-TTCAACTTGCACCATGCCGC-3’

YFPC1 as (3311): 5’-CATGGGCACCACCCCGGTGAA-3’

YFPC2 as (3312): 5’-CGCTGAACTTGTGGCCGTTTA-3’

YFPC3 as (3313): 5’-GCGGTTCACCAGGGTGTCGCC-3’

2.11 Primers for verifying EIIa-Cre-mediated removal of Neo^R cassette in the knockin mice

LA-5039 as: 5’-ACAGCTTTCCTTCCAGAC-3’

YFP-4362 s: 5’-CCCGCGCCGAGGTGAAGT-3’

2.12 Primers of genotyping of NGYP and ANYP mouse lines

LA-5039 as: 5’-ACAGCTTTCCTTCCAGAC-3’

AN2g s: 5'-ATTGCGACTTGCGACTTG-3'

YFPC2 as: 5'-CGCTGAACTTGTGGCCGTTTA-3'

AN2-7383 s: 5'-TGACCTTGGATTCTGAGC-3

2.13 Molecular Cloning

2.13.1 DNA digestion with Type II Restriction Endonuclease

DNA was digested with the suggested amount of type II restriction endonuclease depending on the company. The standard for a restriction digest is normally 1unit of the type II restriction endonuclease digests 1 µg of DNA at 37°C for 1 hour. Normal volumes of digestion were 30-100 µl.

2.13.2 Generation of Blunt Ends

For the generation of blunt ends, a PCR was used to put T overhangs at the end of the product. By using 5 units of T4 DNA-polymerase for every 1µg of DNA. In the reaction mix dNTPs containing the four different bases at a concentration of 100 µg, 5 X PCR buffer. The reaction mix was placed at 37°C for 5 minutes of incubation. The reaction was stopped with placing the mix at 75°C for 10 minutes.

2.13.3 Dephosphorylation of DNA Ends

The 5'-phosphate groups in the vectors that were cut, were removed by incubating the vectors at 37°C with an enzyme taken from the stomach from the cow (CIP; 3U/10 µg DNA). This enzyme removes exposed phosphate groups, so that the vector does not ligate again, without the proper insert.

2.13.4 DNA ligation

Digested DNA fragments were ligated with T4 ligase in a 10µl volume overnight at 4°C. The vector: insert concentration were in a molar ratio of 1:3(Crouse et al., 1983).

X µl of vector-DNA (50-100 ng)
x µl of DNA fragment (150-300ng)
1 µl of T4 DNA Ligase Buffer (10x)
1 µl of T4 DNA Ligase (3U)
Fill to a final volume of 10 µl with H_2O

2.13.5 Cloning from PCR Products

PCR fragments were cleaned with a rapid PCR cleaning kit from Qiagen or were extracted from the gel and then cleaned. A phenol or a qiagen extraction method was used. Then the PCR products were either cut with proper restriction enzymes or not. Then they were ligated with the proper vector as described in the DNA ligation section.

2.13.6 Cloning from Oligonucleotides

For the generation of new multiple cloning sequences in the pSp72 or the PKS-bluescript. Invitrogen synthesized oligonucleotides with the desired proper order. After the removal of the original multiple cloning sequence the vector was dephosphorylated according to the protocol. The oligonucleotides were allowed to anneal with each other. By placing the sense and the anti-sense at a concentration of 10pmol/μl in a 50 mM NaCl. The reaction was then placed at 90°C for 2 minutes to get rid of secondary structures from the reaction. Then the annealing started at 72°C for 8 minutes. After the annealing process 10-30ng of the annealed product was used to ligate into the cut vector plasmid.

2.13.7 Making competent bacterial cells

Bacteria (E. coli XL1-Blue) was grown in 250 ml of TB medium (+30 μg/ml tetracycline) at 18°C until the OD600 0.6 (24-40 hours). (Inoue et al., 1990) After the incubation step the bacterial suspension was placed on ice for 10 minutes. The suspension was then centrifuged at 4°C for 10 minutes at 2500 rpms in a Sorvall GS3-rotor. The supernatant was removed and the cells were resuspended in 80 ml of cold TB jap-medium. The cells were incubated on ice for 10 minutes and then centrifuged as described above. After the last centrifuging step, the supernatant was removed and the bacterial cells were resuspended in 18.6 ml of cold TB Jap-medium and 1.4 ml of DMSO. The cells were incubated on ice for 10 minutes. Hundred μl aliquots were frozen in liquid nitrogen and then stored at –80°C.

2.13.8 Transformation of Bacteria

An aliquot containing 200μl of the competent cells E.coli XL1-Blue were thaw from the –80°C on ice. The bacteria, was then incubated with 3.4 μl β-Mercaptoethanol which was diluted 1:10. During this incubation step the bacteria was shaken every few minutes to ensure proper mixing. After this step 5μl of the ligation mix was placed into the tube and then placed on ice for 30 minutes to allow the bacteria to pick up the plasmid. The tube containing the bacteria and the ligation mix were heat shocked in a hot water bath set to 42°C for 30

seconds. The mixture was placed on ice for 5 minutes. After the ice incubation, 800μl of warmed LB media was added to the bacteria/ligation mixture. The bacteria was allowed to develop a resistance by growing for 45 minutes at 37°C. During this period LB agar plates were prepared for plating out the bacteria. The bacteria, was then plated out at different concentrations. The plates were placed overnight at 37°C and on the next day bacterial clones were picked for further processing of the plasmid.

2.14 Preparation and Analyses of DNA

2.14.1 Plasmid preparation from Bacteria

For the preparation of the plasmid from bacteria (Birnboim and Doly, 1979), a kit from Qiagen was used. Clones were picked from bacterial plates that were grown overnight. The clones were then grown overnight in 3 ml of LB media containing 100μg/ml of Ampicillin. On the next day the bacterial soup should appear murky. 2 ml of the bacterial soup was centrifuged down at low speed to allow the bacteria to be resuspended later. The LB media was removed and the buffers from the kit were added to lyse the cells. After the lyses of the bacterial cells another buffer was used to neutralize the mixture. The mixture was centrifuged at high speed for 10 minutes to separate the cell derby. After this step the supernatant was taken and placed over a special Silica column trap the plasmid DNA (Vogelstein and Gillespie, 1979). The column was then washed with washing buffers that were provided in the kit. Then the plasmid DNA was eluted with buffer or H_2O.

2.14.2 Preparation of genomic DNA from tissue

Genomic DNA was prepared out of 0.5 cm fragments of mice tails. A kit from Qiagen was used to prepare the tails. Tails were digested overnight with proteinase K and on the next day the mixture was taken through the protocol that was provided by the tissue kit. After the tails were ready, they were stored at 4°C. For PCR use 1-5μl of the genomic DNA was used and for southern blots 70μl of digested DNA was used.

2.14.3 Phenol/Chloroform extraction of DNA

A Phenol/Chloroform method was used to extract DNA. For the extraction, one part DNA to one part Phenol/Chloroform was taken. The mixture was shaken, then vortexed to ensure a

proper mixture. The mixture was centrifuged for 1 minute at 13,000 rpms. and only the supernatant was taken for the next step. To the supernatant one part Chloroform/ isoamyl alcohol (24:1) was added, once again the mixture was shaken and vortexed to ensure proper mixing. The mixture was centrifuged down at 13,000 rpms and the supernatant was taken for the next step. DNA was then precipitated with 2 parts ice cold 100% Etoh and 0.1 part 3M NaAc (pH 5.2). the mixture was then placed at –20 °C for 30 minutes. After the precipitation step the DNA was centrifuged down at 13,000 rpms for 30 minutes. By this time the pellet could be seen. The supernatant was removed and the DNA pellet was washed with 70% Etoh. The DNA was then centrifuged at 13,000 rpms and the Etoh was removed. The DNA pellet was allowed to air dry. After air drying the pellet was resuspended in either 11µl water or tris buffer. 1µl was loaded onto an agarose gel to confirm extracted product.

2.14.4 Measuring DNA concentration

The DNA solution concentration was, with the help of a spectral photometer measured. The solution was diluted 1:100 and the absorption was measured at 260 nm in a quartz cuvette. For double stranded DNA an OD_{260}=1 has a concentration of 50µg/ml. The measuring of the absorption at 280 nm tells how clean the solution of DNA is, where a measurement of 1.5-2.0 tell the ratio of the $OD_{260:280}$ is clean. The molarity of the oligonucleotide solution was established with the help of the extinction coefficient E ($M^{-1}cm^{-1}$) with the formula M= OD_{260}/E.

Guanine: E = 12010	Adenine: E = 15200	Thymine: E = 8400	Cytosine: E = 7050

2.14.5 Agarose gel electrophoresis of DNA

For the visualization of DNA fragments between 0.5 and 15 KB an agarose gel between 0.7% and 2% was used. The agarose was cooked in 1 X TAE buffer to allow it to dissolve. 1µg/ml of ethidium bromide was given to the dissolved agarose. The mixture was poured into a gel chamber containing a comb for the DNA pockets to polymerize. After the agarose gel was polymerized, it was submerged in a chamber containing 1 X TAE buffer. Before loading the probes, 0.1 volume of loading buffer was added. The probes were then loading into the agarose pockets. The DNA is negatively charged so it will run toward the positive end of the voltage pole. The voltage amount used to run the probes depend on how big the gel chamber was. The bigger the chamber the higher the voltage needed to run the DNA through the gel. Through the interaction of the ethidium bromide with the DNA, it was

visualized by using an UV light. A photograph was taken of the results. Markers were loaded next to the DNA probe to determine proper sizes. DNA from the bacterial phage λ was digested with the restriction enzyme Hind III giving bands ranging from 0.5-23 KB and from the Phage ΦX174 DNA was digested with Hae III giving bands ranging from 70 –1350 bp.

2.14.6 Elution of DNA from agarose

The agarose gel containing the DNA bands were visualized by placing the gel under a UV light, which had wavelength of 356 nm. By using this wavelength, it made sure that no additional mutations were added to the DNA probes. The desired DNA fragment was cut out with a disposable scalpel. The fragment was placed into an Eppendorf tube. With the help of a blue pipette tip, the agarose containing the fragment was crushed as small as possible. The tubes were then frozen in liquid N_2 (freeze shock method). After the freezing the tube were remove and were allowed to thaw at 37°C. 500μl of phenol was added and then mixed well. The tube was then placed back into liquid N_2 for freeze shocking. The tube was allowed to thaw at room temperature in a centrifuge at 13,000 rpms for 15 minutes. The supernatant, which is the aqueous phase held the extracted DNA, was removed and placed into a new tube that contained 500μl of Chloroform/ isoamyl alcohol (24:1). The tube contents were mixed and centrifuged at 13,000 rpm for 5 minutes. To precipitate the DNA, the supernatant was removed and placed into a new tube containing 2 parts ice cold 100% Etoh and 0.1 part 3M NaAc (pH 5.2), the mixture was then placed at –20 °C for 30 minutes. The precipitated DNA was centrifuged at 13,000 rpms for 30 minutes. The Etoh was removed and the DNA pellet was washed once with 70% Etoh. After the washing step the Etoh was removed and the pellet was allowed to air dry. Placing the pellet in a speed vac for 10 minutes speeded up air-drying of the pellet. The pellet was then dissolved in 11μl of H_2O or tris buffer. 1μl of the dissolved DNA pellet was loaded onto an agarose gel to ensure the DNA was retrieved by the extraction method. The rest of the DNA was stored at –20°C for latter use.

2.14.7 Radioactive labeling of DNA fragments

The radioactive labeling of DNA fragments with α-dCTP was done by using a 'Prime-it-II'®((Stratagene) by following the directions supplied by the kit (Crouse et al., 1983). To the kit, 25ng of the desired DNA fragment to be labeled was used. After the DNA was labeled, the unused nucleotides were separated from the DNA labeled fragments. This was done by

running the labeled fragments over a spin column (Bio-Spin 30 column) that only allowed large fragments to pass through. To test the activity of the probe, a radioactive detector was used. Then the DNA was prepared for hybridization, by denaturing the probe for 10 minutes at 90°C.

2.14.8 Southern Blot

5-10μg of DNA was digested overnight at 37°C and then run on an 0.7% agarose gel by a voltage between 90-110V for 5 hours, to allow a proper separation. To check if everything ran properly, a ruler was documented with the gel. Then the gel was depuriniated for 20 minutes, denatured for 30 minutes and then neutralized for 15 minutes. The DNA in the gel was then transferred onto a Nylon-membrane (hybond^{tm}-N, BioRad) with the help of a vacuum blotter (Appligene). After the transfer of the DNA from the gel to the Nylon-membrane, the gel was checked to see, if the DNA was transferred. The membrane and DNA were then fixed by stratolinker at 120 mJ. The membrane was subjugated to pre-hybridization for 20 minutes at 68°C and then hybridization at 68°C in 10 ml of high-SDS hybridization buffer in a round flask (BioRad) overnight. For the hybridization, 10^6cpm/ml of randomly labeled radioactive DNA probe was added to the membrane. After the overnight incubation the membrane was washed 3 times stringently for 10 minutes.(60-80°C; 2X SSC/0.5% SDS-0.1%X SSC/0.1% SDS). After the washing of the membrane, it was then exposed to a Phosphoimager plate for 2-12 hours. The hybridization signal was seen with a phosphoimager with the program McBas2.0. Then the membrane was exposed to an x-ray film for 3-5 days at –80°C.

2.14.9 Polymerase Chain Reaction (PCR)

For the polymerase chain reaction (Mullis et al., 1986) kits from Promega, Statagene, Qiagen and Sigma were used. The standard reaction was done in a final volume of 50μl.

Final concentration	Volume
	X μl of DNA material
1x	5 μl of PCR buffer Mg^{2+}free
1-2 mM	0-4 μl of $MgCl_2$
200 μM	5 μl of dNTP(each nucleotide 2mM)
300 nM	1 μl of 5'-primer (15 μM)
300 nM	1 μl of 3'-primer (15 μM)
2.5 U	0.5 μl of Taq DNA polymerase PFU (5U/μl)

Add to final volume 50 µl	H_2O

Standard amplification protocol (36 cycles):

3 min.	***95°C***	***denaturing***
30 sec.	56°C	'annealing' (cycle)
1 min.	72°C	extension (cycle)
1 min.	95°C	denaturing (cycle)
1 min.	56°C	end 'annealing'
10 min.	72°C	end extension

The optimization of the reaction depends on changing the annealing and extension temperatures and time of the primers being used. Changing the concentration of $MgCl_2$, and adding different substances, like Substance-Q from Qiagen usually lead to a better PCR result.

2.14.10 DNA sequencing

DNA sequencing analysis was don by Fritz Benseler (department 501, Max-Planck-Inst. Experimental Medicine, Göttingen). The sequence uses a linear PCR, where the dNTPs are labeled to give a result.

2.14.11 Western blot

Western blot samples were denatured at 95°C for 10 minutes in 4 x sample buffer before loading onto gel. 5-20µg were loaded onto gels ranging from 8-12%.

The Blot Chamber is packed **from the plus pole** as follows:

3 layers Whatman paper soaked in Transfer Buffer I

2 layers Whatman paper soaked in Transfer Buffer II

PVDF-membrane activated by briefly putting in methanol and than in water and Transfer Buffer II , then add the gel

3 layers Whatman paper soaked in Transfer Buffer III

Blotting is performed at 250mA constant for 2 hr. Remember: The current flows from - to + and with it all the proteins which have bound SDS – so they are negatively charged. After blotting the membrane can be stained with Poinceau S for 10 min (no shaking required) and destained with water in order to check successful protein transfer and eventually cut the blot in pieces/stripes.

2.15 Cell Culture Methods

2.15.1 Culture and Analysis of Embryonic Stem Cells (ES cells)

Methods for culturing ES cells were taken from Joyner et al. (1993). ES cells were cultured on embryonic feeder layer in media containing LIF, in an incubator set to 37°C with 5% CO_2.

2.15.2 Trypan Blue Live Staining

For the live staining of ES cells 10 μl of dissociated cells were mixed with 10 μl of trypan blue for 2 minutes at room temperature. The cells that were stained blue were not counted, because trypan blue only stains dead or dying cells. Normally the chances of cells surviving are more then 20%.

2.15.3 Serum Testing on ES cells

To test the quality of the serum, 300-3000 ES cells were grown in medium containing 10%, 15%, and 20% serum for 5-7 days. The cultures were observed daily to see which serum charge was best suited for their growth.

2.15.4 Antibiotic Concentration Testing

To check the concentration of antibiotic G418 need to select ES cells in 8-10 days, ES cells were plated at a density of 4×10^4cells onto a 6 cm tissue culture plate. 0-500 μg/ml of G418 was added everyday for 8 days. The viability of the cells was checked on the surviving clones.

2.15.5 Mycoplasma Testing

The testing of mycoplasma on the ES cells and embryonic feeders was done with the use of a kit from Stratagene. (Mycoplasma-Detection-Kit)

2.15.6 Preparation of Mouse Embryonic Fibroblasts

For the preparation, mouse embryos between E13-E14 were used. The mother was killed by anesthesia of overdosing with chloroform. The uterus was removed and placed into a dish

containing 1 X PBS. This was all done in a tissue culture hood, to keep the cells sterile. Thu embryos were removed from the uterus horns. The head, legs, arm, and all internal organs from the embryo were removed and discarded. With a small scalpel, the rest of the carcass was cut into smaller pieces to allow better dissociation of the tissue. The tissue was then placed in a 50 ml tube containing 25 ml of trypsin, at 37°C for 15-30 minutes.0A 5 ml pipette was used to dissociate the tissue even further, and then a flame polished glass pipette was used to get a single cell suspension. The cell suspension was then centrifuged down at 900 rpms for 5 minutes. The supernatant was discarded and the pellet was resuspended in embryonic feeder medium. The cells were counted and 1 X 10^6 cells were plated in a 10 cm dish and incubated for 3-4 days until sub-confluent. The cells were either passaged further 1:4 and expanded, or frozen down in liquid N_2 for latter use.

2.15.7 Passaging, Freezing down and Thawing of Embryonic Feeders

For passaging of the feeder cells, they were washed with PBS 3 times. 5 ml of trypsin was added to the dish, and the dish was placed at 37°C for 5 minutes. Trypsin activity was stopped by adding 2 x the volume of medium. The cells were then centrifuged down for 5 minutes at 900 rpms. The cells were then resuspended and 1:4 expanded. For the freezing of cells, first they were counted and concentrated to 1 x 10^7 cells/ml. The cells were then mixed with 1 part freezing medium and 1 part cell suspension. The mixture was then aliquot in cyro-tubes in 1 ml portions and placed at –80°C in a Styrofoam box overnight. Then the cyro-tubes were placed into liquid $N_2$for longer storage. For thawing of feeder cells, they were removed from the liquid N_2 and warmed up quickly. The cells were then removed from the cryo-tube and mixed with fresh medium. The tube was centrifuged at 900 rpms for 5 minutes. The supernatant was removed and the cell were resuspended with 10 ml of fresh medium and plated in a 10 cm dish.

2.15.8 Preparation of Mitomycin C treated Fibroblasts for ES cells Plating

1 cyro tube was thawed and 5 x 10^6 feeders were plated on four 15 cm tissue culture dishes. The cells were expanded after 3-4 days of being culture. The embryonic feeders were treated for 2-3 hours with mitomycin C in the medium (10 μg/ml), and aliquots were then frozen down (5 x 10^6cells/ml). To test if the embryonic mitomycin C treated fibroblasts (EMFI) were clean; cells were thawed and plated for a week before plating ES cells on top. The fibroblast could be held in culture for about a week if not need.

Tissue culture plate size (cm)	Medium (ml)	Fibroblasts #
10	10	2.5 x 10^6
6	4	1 x 10^6
3.5 (6 well plate)	3	3 x 10^6
1.5 (24 well plate)	1	2x 10^6
0.9 (96 well plate)	0.3	4 x 10^6/plate; 4 x 10^5/well

2.15.9 Growing, Passaging, and Freezing of ES cells

ES cells were grown onto fibroblasts that were treated with mitomycin C (EMFI) in ES cell medium at high density. After, a maxim of three day in culture the ES cells were at 80% sub-confluence. The ES cells were expanded at 1:3-5. Every 2 days the medium was changed. For freezing of the cells they were plated onto a 24 well plate in freezing medium. The dish was then placed on ice and 250 µl of cold freezing medium was added. The plate was then wrapped tightly with paraffin, and placed at –20°C for 2 hours. After the 2 hour freezing, the cells were then stored for longer period of time at –80°C.

Tissue culture plate size (cm)	Cell #*	Cell #°	Final cell #
10	2 x 10^6	10 x 10^6	10-15 x 10^6
6	1 x 10^6	5 x 10^6	5-7 x 10^6
3.5 (6 well plate)	0.5 x 10^6; 3 x 10^6/plate	2.5 x 10^6	2.5 x 10^6/well
1.5 (24 well plate)	0.2 x 10^6; 5 x 10^6/plate	1 x 10^6	1 x 10^6/well

*:for passaging, °:after thawing

2.15.10 Electroporation and Antibiotic selection of ES cells

For the electroporation of ES cells 50 µg of vector containing the desired DNA construct was used. First the vector was linearized with the restriction endonuclease XhoI. The construct was extracted with the phenol, chloroform/isoamyl alcohol and precipitated with ice cold 100% Etoh and 0.1 part 3M NaAc (pH 5.2), the mixture was then placed at –20 °C for 30 minutes. The precipitated DNA was centrifuged at 13,000 rpms for 30 minutes. The Etoh was removed and the DNA pellet was washed once with 70% Etoh. After the washing step the Etoh was removed and the pellet was allowed to air dry. Placing the pellet in a speed vac

for 10 minutes sped up air-drying of the pellet. The pellet was then dissolved in 100 μl of tris buffer. 1 μl of the dissolved DNA pellet was loaded onto an agarose gel to ensure the DNA was retrieved by the extraction method. The DNA went through another round of cleaning by running it over a C-30 column. The final amount of DNA was measured with a mass spectrometer, and 1 μl of the DNA was placed on an agarose gel, to make sure that the vector containing your plasmid was digested completely with the restriction endonuclease. Once the DNA concentration was determined, it was ready to be used for the electroporation of the ES cells. 1-2 hours before the electroporation, the medium of the ES cells (70% confluent) was changed. ES cells (passage 8-12) were removed from the fibroblasts. The ES cells and the fibroblasts were first washed 3 x with PBS. Trypsin was added and the cells were suspended into a single cell suspension. The cell suspension containing ES cells and fibroblast were centrifuged at 900 rpms for 5 minutes. The supernatant was removed and the cells were suspended in fresh ES cell medium. The cells were plated on tissue culture plates, that were coated with 0.1% gelatin and the plate was then placed back into the incubator set for 37°C for 45 minutes. This step separates the ES cells from the fibroblast. The fibroblast plate down faster compared to the ES cells, which need 24 hours to plate down. After the separation step, the ES cells were washed off with a little pressure with the medium from the plate. The ES cells were easily removed from the gelatin plate and counted. The plate containing the fibroblasts was discarded. 10 μl of cell suspension was mixed with 10 μl of trypan blue to count and check the viability of the ES cells. The cells were prepared for electroporation, by getting the electroporator ready. The settings used for the electroporation were 240 volts, 500 μF and a time constant of 9 ms. These are ideal settings for ES cell electroporation. After the counting of the ES cells, the cells were centrifuged at 900 rpms for 5 minutes. The supernatant was removed and the cells were resuspended in cold PBS or warm medium at a concentration of 1-14 x 10^7ES cells/ml. For a better survival rate of cells after electroporation, warm ES cell medium was used. The vector containing the desired DNA was mixed with cold PBS with a final volume of 100 μl. 700 μl of ES cells was mixed with the 100 μl of the vector/DNA construct. The mixture was placed in a pre-cooled cuvette and electroporated with the proper settings. After the electroporation the cells were placed on ice for 20 minutes. The cells were then plated onto 3 x 10 cm plated containing EMFI. 24 hours, after the electroporation the selection of the positive ES cell clones was started. By adding fresh medium containing 300 μg/ml of G418. old medium was exchanged with fresh medium containing G418 daily. Since a lot of cells were dying it was better to change the medium daily. The selection process usually took 8-10 days to see nice clones. Clones were then picked, starting day 8 of selection.

2.15.11 Isolation and Analysis of G418 resistant ES cell Clones

In a time period between 8-10 days of selecting ES cells with G418, macroscopic colonies could be seen on the dish. The colonies on the dish were washed 3 times with PBS and then the final wash was done in PBS containing I Pen/Strep. The cells were kept in this final wash for picking. The ES colonies were picked under a microscope with a yellow tip of a Gilson-pipette from the bottom of the dish in 60µl of PBS by scraping the colony with the tip. The colony that was picked was then dissociated in a round bottom 96 well dish containing 150µl of PBS/trypsin mixture (100µl/50µl). The colony was checked to make sure it was dissociated under the microscope. From the colony 1/3 was placed in a well of a flat bottom 96 well dish containing EMFI and 2/3 of the colonies was taken for the DNA extraction for the PCR screening. The ES cells were then grown for 2-3 days on the flat bottom 96 well dish. The 2/3 of the ES cell colony was then pooled in a group of 8 colonies for the DNA extraction. The pooled cells were placed into a 2ml eppendorf tube, containing medium to neutralize the trypsin. The DNA should be extracted within 24 hours after pooling the cells. For the DNA extraction, the tubes were first centrifuged at 12,000 rpm for five minutes to pellet the cells down. The supernatant was removed from each tube. The pellet was washed with 100µl of 1X PBS. The tubes were then centrifuged for 1 min at room temperature. The supernatant was removed and the pellet was resuspended in 50µl H_2O, then the tubes were placed in a heating block set for 95° C for ten minutes. The tubes were then shortly centrifuged and then placed on ice. 1µl of proteinase K (10 mg/ml) was added to the tubes, and the tubes were place on a heating block at 50°C for 30 minutes. The tube were quickly centrifuged and then placed in a heating block at 95°C for 10 minutes to heat inactivate the enzyme. For the nested PCR, 5µl of the DNA was taken. The following protocol was used for the screening of the AN2-YFP and AN2-Cre constructs:

Control PCR AN2-YFP Part 1 (20µl reaction)	Control PCR AN2-YFP Part 2 (20µl reaction)	Control PCR AN2-Cre Part 1 (20µl reaction)	Control PCR AN2-Cre Part 2 (20µl reaction)
5 µl DNA	5 µl of PCR part 1	5 µl DNA	5 µl of PCR part 1
1 µl AN2 C1s	1 µl AN2 C3s	1 µl AN2 C1s	1 µl AN2 C3s
1 µl YFP C3as	1 µl YFP C1as	1 µl Cre-as 905	1 µl Cre-as 845
2 µl dNTPs	2 µl dNTPs	2 µl dNTPs	2 µl dNTPs
2 µl Q-solution	2 µl Q-solution	2 µl Q-solution	2 µl Q-solution
6 µl H_2O	6 µl H_2O	6 µl H_2O	6 µl H_2O
1 µl Red Taq-DNA-Polymerase	1 µl Red Taq-DNA-Polymerase	1 µl Red Taq-DNA-Polymerase	1 µl Red Taq-DNA-Polymerase
2 µl of 10X PCR Buffer	2 µl of 10X PCR Buffer	2 µl of 10X PCR Buffer	2 µl of 10X PCR Buffer

Part 1 and part 2 of the nested PCR was done with the following programs:

Part 1 of nested PCR (20 cycles)	Part 2 of nested PCR (40 cycles)
1. 94°C -pause	1. 94°C -pause
2. 94°C -3 minutes	2. 94°C -3 minutes
3. 58°C -30 seconds	3. 58°C -30 seconds
4. 72°C –1 minute	4. 72°C –1 minute
5. 94°C –1 minute (cycle to step 3)	5. 94°C –1 minute (cycle to step 3)
6. 58°C –1 minute	6. 58°C –1 minute
7. 72°C –10 minutes	7. 72°C –10 minutes
8. 4°C -pause	8. 4°C -pause

The PCR product was visualized on an agarose gel then the positive clone was checked by southern blotting. This checks that homologous recombination actually occurred. The southern blot was done with an oligonucletide probe, where it hybridizes in the short arm of the AN2 gene. The digestion of the ES cell DNA was done with the HindIII restriction. With homologous recombined clones, two bands should appear in the southern blot. One band for the wild-type DNA and one band for the homologous recombined DNA.

Once a clone is found, then the clone is expanded from the flat bottom 96 well dish to a flat bottom 24 well dish containing EMFI. Once the cell density on the dish has reached 80% the ES cell clone is further passaged to a 6 well dish. At this point 1/3 of the positive clone is frozen down, as back up. From the 6 well dish the ES cell clone is further expanded onto a 10 cm dish. At this point ES cells could be taken for DNA extraction for the southern blot or the cell could be prepared for blastocyst injection. Some ES cell, are frozen down in liquid Nitrogen.

2.15.12 Injection of Blastocysts and Embryo transfer

To prepare ES cell clones for injection, the EMFI were first separated from the ES cell clone. This was done as described in the *electroporation section* of Material and methods. The ES cells and the EMFI were plated onto coated dishes for 45 minutes. Since the EMFI require only a short time to plate down, this method is a quick and easy way of getting the ES cells EMFI free. The ES cells are then washed off the plate with normal medium. The ES cells were then centrifuged and then taken up in medium at a concentration of (1 X 10^3 cells/ml). 15-20 ES cells were injected into one blastocyst (3.5 day old). The blastocysts were then transferred to a mouse black C57Bl6. Normally 40-80 blastocysts are injected in a session and 2-5 mice are injected with the blastocysts. The blastocysts were taken also from a C57Bl6 mother. The ES cells originate from 129 ola cells. Monika did the blastocyst injection and transfer in the mouse house of the Max-Planck inst. Göttingen.

2.16 Histological and Immunohistochemical methods

2.16.1 Whole mouse fixation and perfusion

The mice were anesthetized with chloroform and the breast cavity was opened to expose the heart. A butterfly needle was inserted into the left ventricle of the heart and the right atrium was cut. The needle was connected to a peristaltic pump. The blood was flushed out with 50 ml Hanks Balanced Salt Solution (HBSS) which was heat to 50°C. After the flushing of the blood, 4% PFA (Para formaldehyde) was pumped through the mouse. This fixes the brain within the animal. The brains were taken out from the mouse and post-fixed for 2 hours at 4°C. At this point, the brains could be directly cut on a vibrotome. For longer storage of the brains, they were immersed in 30% sucrose until they sunk to the bottom of tube. This sucrose immersion is used to protect the brains from freezing. After the sucrose treatment, the brains were frozen on dry ice, and then placed at –80°C for longer storage. The brains could either be cut on a vibrotome or a cryostat.

2.16.2 Immunohistochemistry on vibratome sections

Frontal or sagitial cut sections (20-50 μm) of paraformaldehyde fixed brains from mice of different ages and genotypes were first permeablized with 0.4% tritonX-100 in Phosphate buffer (PBS) for 30 minutes on a shaker at low speed. The sections were then blocked in 0.4%horse serum (HS) and PBS/0.2% tritonX-100 for 30 minutes at room temperature. The sections were incubated in 1% HS in PBS/0.05% triton X-100 containing the desired diluted antibody over night at room temperature or at 4°C for longer periods of time. After the incubation of the primary antibody sections were then washed with 1 X PBS for 15 minutes each wash. Sections were incubated in species specific secondary antibodies that are coupled to different chlorofores for 2 hours at room temperature in 1.5% HS in PBS. The sections were then washed 3 times with 1 X PBS for 15 minutes. After the last wash sections were then stained with Dapi for 10 minutes in PBS to see the cell nucleus. Sections were washed again with 1 X PBS 3 times for 15 minutes. The sections were then placed in a beaker containing PBS for mounting on slides that were coated with poly-l lysine. When the sections were mounted, they then were placed in water to wash the PBS and then mounted with Aqua poly mount. The section, were dried over night. The sections were then imaged with a confocal and the pictures were adjusted with Photoshop 7.0.

Primary antibody	Dilution
Anti-AN2	1:20
Anti-CNP	1:100
Anti-Double Cortin	1:800
Anti-EYFP	1:1000
Anti-F4/80	1:2
Anti-GFAP	1:500
Anti-Neun	1:500
Anti-O4	1:5
Anti-Olig1	1:1000
Anti-Olig2	1:1000
Anti-OMGP	1:1000
Anti-PDGFα-R	1:200
Anti-S100-Beta	1:2500
Anti-Sox 10	1:1000
Anti-Tuj 1	1:100

Secondary antibody	Dilution
Goat anti-mouse -biotin	1:200-1:400
Goat anti-mouse CY2/Fitc	1:200
Goat anti-mouse CY3/Trit	1:1000
Goat anti-rabbit -biotin	1:200-1:400
Goat anti-rabbit CY2/Fitc	1:200
Goat anti-rabbit CY3Trit	1:1000
Goat anti-rat -biotin	1:200-1:400
Goat anti-rat CY2/Fitc	1:200
Goat anti-rat CY3/Trit	1:1000

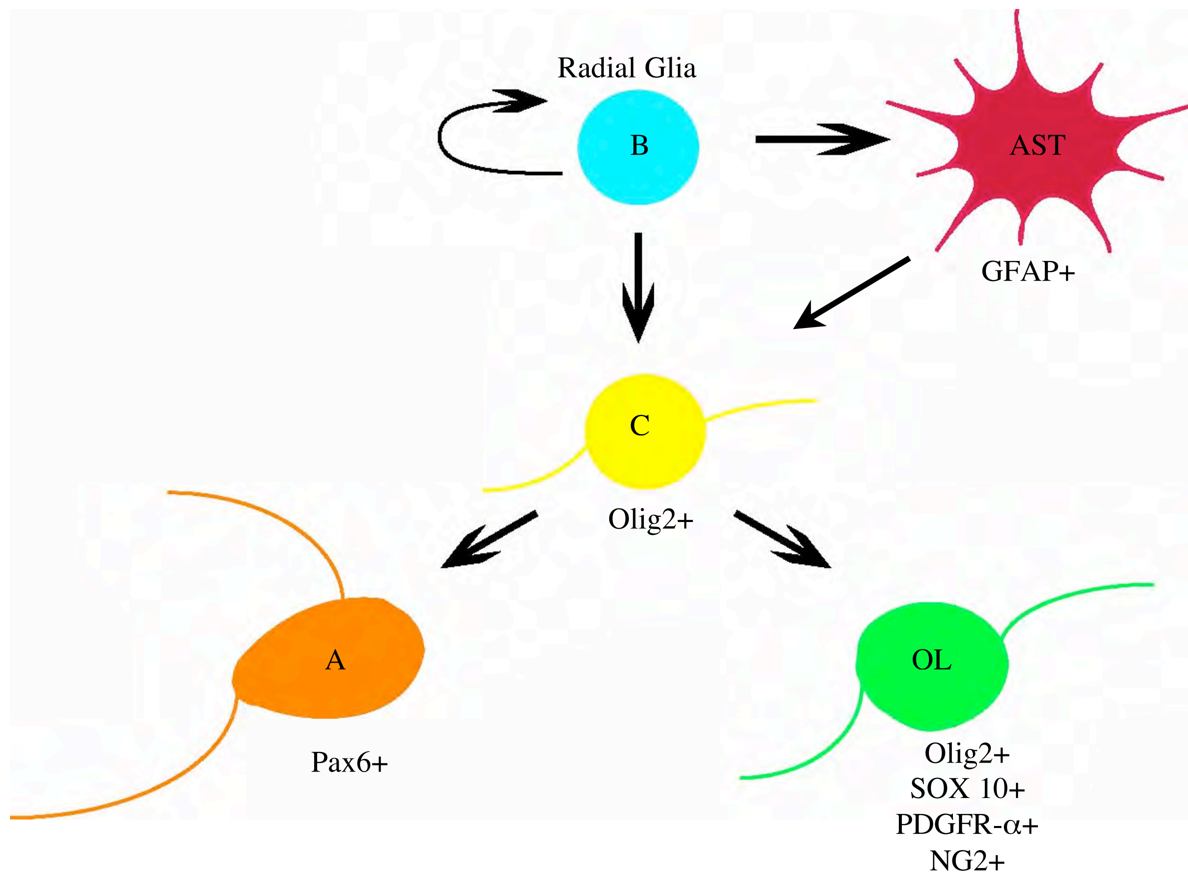


Figure 1.3.1: Schematic lineage diagram of Radial Glia:

This diagram depicts the progression from multipotent precursors to a more differentiated progeny via lineage-restricted precursors. This particular diagram incorporates the idea that in the developing brain stem cells are actually radial glia cells, and in the adult brain they remain as astrocytes. These radial glia cells are termed type-B cells, they differentiate to a transit-amplifying precursors (type-C cell), which give rise to neuroblasts (type-A cell) or Oligodendrocytes (OL).

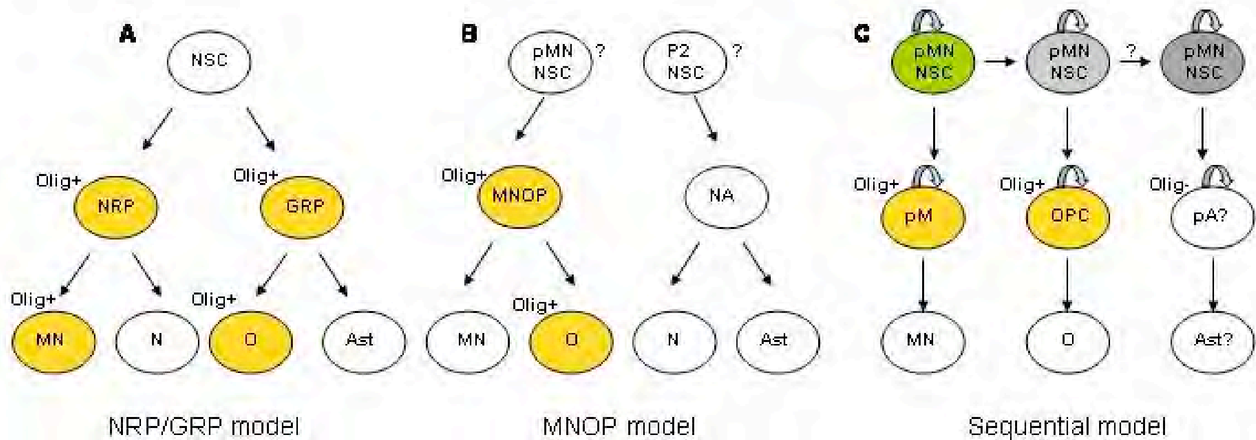


Figure 1.3.2: Different models explaining Neural stem cell differentiation in the Motorneuron domain (pMN):

A) GRP/NRP model depicting restricted precursors for glia and neurons, B) MNOP model depicting that oligodendrocytes and motorneurons have a common lineage precursor, and C) Sequential model depicting that oligodendrocytes and neurons are sequentially generated. Ast, astrocyte; MN, motorneuron; MNOP, motorneuron and oligodendrocyte common precursor; N, neuron; NA, neuron and astrocyte common precursor; OPC, oligodendrocyte precursor; O, oligodendrocyte; pA, astrocyte precursor; pM, motorneuron precursor (Wu et al., 2006).

1.4 Nerve-Glia Antigen 2 (NG2)

1.4.1 NG2 gene

NG2 is a transmembrane glycoprotein expressed by many different immature and adult cell types including oligodendroglia precursor cells, immature glia, subpopulations of perisynaptic glia, immature Schwann cells, pericytes, developing cartilage, immature muscle cells, skeletal myoblast and human melanoma cells, throughout development and adulthood (Levine et al., 1986; Niehaus et al., 1999; Diers-Fenger et al., 2001; Schneider et al., 2001; Stallcup, 2002; Tillet et al., 2002; Karram et al., 2005). The NG2 gene is located on chromosome 8 in the rat, chromosome 9 in the mouse, chromosome 15 in human and chromosome 2L in Drosophila. The gene extends over 33 KB and has eight exons in the human, mouse and rat. The details of the NG2 promoter in particular the starting point and the end are still unclear. Between exon 1 and exon 2 there is a span of 18,000 nucleotides: this very large intron could play an important role in promoter regulation. Exon 1 consists of 110 nucleotides that contains the start codon of the NG2 gene. The LNS domains are encoded by exon 2 and exon 3. LNS1 starts in exon 2 and ends in exon 3, while the complete LNS 2 domain is encoded in exon 3. At this point in time there are no known splice variants for the NG2 protein. It was first characterized as a high molecular weight type 1 membrane protein of about 330 kDa in rats (Stallcup, 1981; Nishiyama et al., 1991; Levine and Nishiyama, 1996). The NG2 amino acids sequence is conserved between species. The human MCSP (Melanoma Chondrotin Sulphate Proteoglycan) has 82.85% homology, the mouse NG2 has 95% homology, the drosophila NG2 known as CG10275 has 45% homology and in C.elegans NG2 known as C48E7.6.p has 23% homology, when all are compared to the rat NG2 homologue (Pluschke et al., 1996; Schneider et al., 2001; Yang et al., 2004).

1.4.2 Structure of NG2

NG2 comprises of 2327 amino acids (aa) with a very large extracellular domain containing two LNS (Laminin G/Neurexin/Sex-hormone binding-globulin) domains near the NH_2 terminus (aa47-179 and aa224-364). The LNS domains are typical adhesion domains, but at this point in time no known receptor for the LNS domains is known. Even though the protein contains multiple sites for possible glycosylation, only a single site at

999 is known to be glycosylated (Stallcup and Dahlin-Huppe, 2001). It has a single transmembrane helix of 25 aa followed by an intracellular domain which contains a PDZ-binding (Postsynaptic density protein-95, Discs-large, Zona occludens-1) motif, which specifies binding to a class II PDZ domain binding motif at the C terminus. NG2 has several extracellular and intracellular binding partners. The extracellular binding partners include Collagen 5 and 6, PDGFα receptor, PDGF-AA ligand and Galectin 3 (Nishiyama et al., 1996; Tillet et al., 1997; Goretzki et al., 1999)(Fukushi et al., 2004). Published intracellular binding partners are GRIP, and Mupp1 (Barritt et al., 2000; Stegmuller et al., 2002; Stegmuller et al., 2003). The biological significance of the Mupp1/NG2 interaction in glial cells is still not clear, but Mupp1 is located at the Schmidt-lanterman incisures that are channels of cytoplasm in the myelin sheath of neurons that lead back to the Schwann cell body (Poliak and Peles, 2003). In neurons Mupp1 interacts with the NMDA receptors playing a possible role in signal regulation (Krapivinsky et al., 2004).

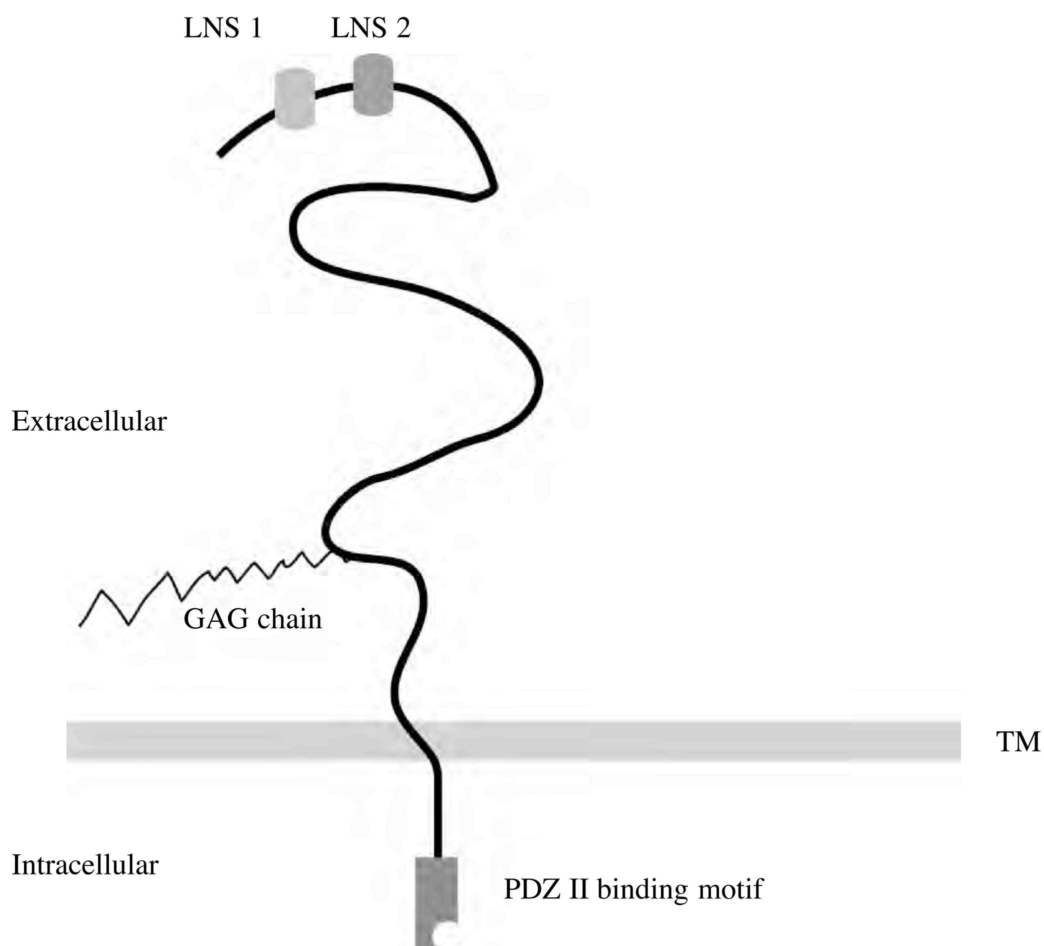


Figure 1.4.2: Schematic Diagram of the NG2 Transmembrane Glycoprotein

1.4.3 Function of NG2 Protein

1.4.3.1 Cell Migration and Cytoskeleton Reorganization

The NG2 protein promotes the motility of endothelial cells through cross-talk with the NG2+ pericytes. Human melanomas that over express MSCP, a homolog to NG2 are highly invasive. The NG2 protein appears to be involved in cell migration and spreading, but not proliferation (Eisenmann et al., 1999; Fukushi et al., 2004). To further characterize the function of NG2 involvement in cell motility, the immortalized highly motile glial derived cell line Oli-*neu* generated by the Trotter lab was studied. This line was developed by infecting primary cultures of glial cells with a retrovirus containing a t-*neu* tyrosine kinase oncogene immortalized the cells (Jung et al., 1995). Oli-*neu,* express the NG2 protein and have characteristic glial precursor like properties. Using polyclonal antibodies directed against immunopurified NG2 protein in Oli-*neu* and primary oligodendrocyte precursor cells, Niehaus et al., were able to show a dose dependent reduction in cell migration *in vitro*. However, a monoclonal antibody directed to the middle of the protein, away from the N-terminal LNS domains failed to hinder the migration of these cells (Niehaus et al., 1999). Similarly, polyclonal anti-NG2 antibodies inhibited the migration of an immortalized Schwann cell clone. Schwann cells express NG2 protein at early stages of development (Schneider et al., 2001).

Other groups have shown that NG2 is essential for the reorganization of the actin cytoskeleton, possibly through binding adaptor proteins (Lin et al., 1996a; Lin et al., 1996b) (Stallcup and Dahlin-Huppe, 2001). Using NG2 constructs lacking the cytoplasmic portion, Fang et al., were able to demonstrate compromised cell spreading in astrocytomas (Fang et al., 1999). Therefore this lead to the conclusion that the cytoplasmic tail of NG2 is involved in actin cytoskeleton reorganization and cell migration. The presence of a PDZ II binding motif at the c-terminal cytoplasmic tail of NG2 alludes to the presence of an adaptor protein that would link the NG2 protein via binding to cytoskeletal elements. At this point in time no direct association has been described between the NG2 protein and cytoskeleton.

1.4.3.2 Role of NG2 at the Synapses between Neuron and Glia

A new class of synaptic interaction has been recently identified where immature glia cells, in particular glia cells that express NG2, form glutamatergic and gamma-amino butyric

synapses with neurons. These NG2+ cells engage in rapid signaling with glutamatergic and gamma-amino butyric acid (GABA) ergic neurons through direct neuron-glia synapses in various brain regions (Lin and Bergles, 2004b; Lin et al., 2005). Quantal release of transmitter from neurons at these sites triggers rapid activation of glial AMPA or GABAA receptors (Bergles et al., 2000; Lin and Bergles, 2004a; Lin et al., 2005). This rapid form of signaling between NG2+ cells and the neurons may play important roles in adapting the behavior of the NG2+ cells to the needs of surrounding neurons in vivo (Gallo et al., 1996; Steinhauser and Gallo, 1996; Yuan et al., 1998; Lin and Bergles, 2004b). The NG2 protein has been linked to the glial AMPA receptors by a scaffolding protein known as glutamate receptor interaction partner (GRIP) implying that NG2 could be involved in the alignment and formation of these neuron-glia synapses. The NG2 protein may thus play a role at clustering and orienting AMPA receptors towards the sites of transmitter release at the neuron-glia synapse (Stegmuller et al., 2002; Stegmuller et al., 2003).

1.4.3.3 Guiding growing neurites

There is still some confusion as to whether NG2 is a repulsive molecule or an attractive molecule for neurite outgrowth. Several studies have shown that there is an accumulation of NG2-expressing cells in the glial scar that forms at the site of injury in the CNS. This accumulation of the NG2 protein has been proposed to create a physical and biochemical barrier for axon regeneration (Ughrin et al., 2003; Tan et al., 2005). In lesions in the spinal cord where the NG2 protein was neutralized by blocking antibodies, neurites were able to penetrate the lesion. In lesions without antibodies neurite penetration was reduced (Tan et al., 2006). A recent study by Yang et al., however showed that NG2 expressing cells provide an adhesive substrate, and even promoted axonal growth. Neurons preferred growing on the NG2+ cells compared to other substrates indicating an attractive property of the cells (Yang et al., 2006). The role the NG2 protein plays in guiding or repelling neurite outgrowth is still undetermined: it may have repulsive or growth-promoting properties dependent on time or site of the lesion.

1.4.4 Lineage of the NG2+ cells in the CNS

A large fraction of cells expressing NG2 retain the ability to divide throughout development. This suggests that NG2 expressing cells have a precursor like nature

(Levine and Nishiyama, 1996) (Stallcup, 2002). *In vitro* experiments have confirmed that NG2 expressing cells have an O2A (oligodendrocyte-type 2 astrocyte) precursor quality. Antibodies to NG2 label O-2A cells (Raff et al., 1983a, b,c), which give rise to oligodendrocytes and type 2 astrocytes. When NG2 expressing cells from mouse brains using immunoaffinity isolation are grown in the presence of serum the cells differentiate into astrocytes, and when grown in the absence of serum they have the ability to differentiate into oligodendrocytes (Diers-Fenger et al., 2001). Under both culture conditions no neurons were seen. These experiments suggest that NG2 cells are exclusively committed to a glial fate. Belachew et al. claim that the NG2 cell type has a multipotent phenotype and is able to generate astrocytes, oligodendrocytes and neurons *in vitro,* and *in vivo* (Belachew et al., 2003). They used a transgenic mouse that expresses GFP under the CNPase promoter and sorted the GFP+ cells by FACS. They showed that a majority of the sorted cells expressed the NG2 antigen. Isolation of these cells via polyclonal antibodies to NG2 and culture of the sorted cells showed that these NG2+ cells have a multipotent quality *in vitro*. The GFP cells were also seen in the hippocampus and subventricular zone, where they expressed the NG2 antigen, TOAD-64 (Turned On After Division), Doublecortin, and Tuj-1 giving rise to functional GABAergic interneurons *in vivo* and *in vitro* (Aguirre et al., 2004). Furthermore these sorted NG2+ GFP+ cells gave rise to functional neurons when transplanted into the mouse hippocampus (Aguirre et al., 2004). Using the same transgenic mouse, they showed that the majority of the NG2+ cells also express Olig2, a transcription factor that is important for motor neuron and oligodendrocyte development (Aguirre et al., 2004). Dayer et al. showed that in the neocortex some NG2+ cells on rare occasions co-express Neun, a marker for post mitotic neurons in the mammalian brain (Dayer et al., 2005).

In vivo NG2 cells express the PDGF-α receptor, but have never been shown to express PLP/DM-20 protein. Mallon et al. generated a transgenic animal expressing EGFP under the PLP/DM20 promoter and demonstrated that there are two populations of NG2 expressing cells. One population consists of cells, which are most likely an earlier oligodendrocyte precursor and do not have an active PLP/DM20 promoter, while the second population of cells have an active PLP/DM20 promoter, indicating that these cells should be the source of myelinating oligodendrocytes in the developing brain. They migrate into the cortex from the subventricular zone and stay in an undifferentiated state for up to three weeks until myelination starts (Mallon et al., 2002).

NG2+ cells could be an intermediate step between radial glia and neurons: this idea has not yet been confirmed by experimental analysis. In the developing mouse brain there are many different types of radial glia, it is known that radial glia are stem cells, which have the ability to develop into various subpopulations of neurons. A further study has shown that a subpopulation of NG2 cells in P6-P20 transgenic mice under the influence of a human GFAP promoter expressing GFP, also expresses S100-β, mRNA for excitatory amino acid carrier 1 (EAAC1), glutamate/ aspartate transporter (GLAST), and glutamate transporter 1 (GLT-1). S100-β is a calcium binding protein, which is unique to glia cells. EAAC1 is a neuronal glutamate transporter. GLAST is a glia associated glutamate transporter expressed by radial glia and GLT-1 is also glia associated. These cells express AMPA receptors and all the AMPA receptor subunits GluR 1-4. It is possible that these GluR cells could be the transit-amplifying type-c precursors that give rise to neurons and oligodendrocytes (Matthias et al., 2003). Therefore, proof for the generation of the NG2 expressing cells from radial glia is still lacking and thus this fate story still remains inconclusive.

1.4.5 NG2 cells in the adult brain

NG2-expressing cells make up one of the largest populations in the adult brain after gliogenesis (Ong and Levine, 1999; Reynolds et al., 2002). Their role in the normal adult and the activation of these cells in response to injury and the following repair is poorly understood. There is a limited amount of evidence to suggest that a subpopulation of this NG2 expressing cell still plays a precursor role in the adult CNS by continuing to divide. However, it is still unclear what function they play during myelination.

The NG2 expressing cells represent about 5-8% of all glia in the adult CNS. (Levison et al., 1993; Levison and Goldman, 1993). Interestingly some of these cells retain their proliferation ability, maintaining a close association to PDGFα-R expression. These adult cells have clear differences to the embryonic cells in migration, cell-cycle length and lineage restriction. Thus, they have an antigenic phenotype of oligodendrocyte precursor cells, while their morphology and distribution represents astrocytes. They are antigenetically different from astrocytes, microglia, myelin-producing oligodendrocytes and neurons. The general consensus being that some of these NG2 positive cells are

oligodendrocyte progenitors, which lose their NG2 antigencity as they differentiate into mature oligodendrocytes. Yet another little understood role of NG2+ cells is their close association or contact to synapses in the grey matter and nodes of Ranvier in the white matter (Butt et al., 1999; Chekenya et al., 1999; Bergles et al., 2000). Under the electron microscope these cells are clearly distinct from any other type of cell in the adult CNS (Peters, 2004). NG2 cells in the adult CNS have 2 distinct morphologies; one morphology has an oblong nucleus with limited cytoplasm, which contain either a bipolar or unipolar processes. These cells resemble and show the common phenotype of dividing protoplasmic astrocytes.

The second type of cell has the morphologic feature of a multipolar stellate cell that resembles microglia or premyelinating oligodendrocytes. These particular cells do not express any markers for microglia and mature oligodendrocytes, but they do appear to make contacts at the nodes of Ranvier (Horner et al., 2002). Thus these cells are a unique population in the adult CNS. It is not yet clear at this point in time whether NG2 cells exclusively generate oligodendrocytes or whether they also give rise to neurons. Even though there is little evidence to support this latter contention, one could argue that NG2+ cells could indeed be the transit amplifying type-c cells in the adult brain.

1.4.6 NG2 and Disease

1.4.6.1 Tumors

The high expression of NG2 in human tumors implies a role for the NG2 in tumorgenesis. The NG2 protein could play a number of multiple roles in cell adhesion, cell motility and as a repulsive or attractive molecule for neurite outgrowth. Oligodendrogliomas, one of the most common CNS gliomas often expresses the NG2 protein (Shoshan et al., 1999). NG2+ tumors have the ability to migrate and invade non-tumorigenic tissue (Ferrone and Kageshita, 1988; Pluschke et al., 1996; Chekenya et al., 1999; Shoshan et al., 1999; Chekenya and Pilkington, 2002). NG2 is also expressed by some leukemias (Behm et al., 1996; Smith et al., 1996). Therefore, this makes NG2 an ideal candidate for the novel drug development process or maybe a target antigen for melanomas.

1.4.6.2 Multiple Sclerosis

Multiple sclerosis (MS) is a disease that is characterized by the loss of myelin, oligodendrocytes and loss of axons, corresponding to damage that progresses over a number of years. The disease is characterized by the loss and repair of myelin, however as the disease progresses this ability to repair myelin is lost (Blakemore, 1974, 1981; Ludwin, 1997; Scolding and Franklin, 1997; Lassmann, 2005). Studies in humans and mice allude to a population of precursor cells that are thought to remyelinate in MS, by differentiating to mature oligodendrocytes. The inability to remyelinate with increasing age and progression of the disease has been attributed to the depletion of the oligodendrocyte precursor, or their inability to differentiate (Franklin and Blakemore, 1997; Franklin et al., 1997; Wolswijk, 1998; Franklin, 2002).

Possible therapies for remyelination of lesions could be stem cell transplantation or the manipulation of endogenous oligodendrocyte precursors. In multiple sclerosis lesions there are many putative OPC cells abound which express NG2 or O4+ (Chang et al., 2000; Watanabe et al., 2002; Wolswijk, 2002). These might be oligodendrocyte precursor cells, but this is still under debate. NG2+ cells in the proximity of the MS lesion do not express markers for neurons, astrocytes, mature oligodendrocytes, and microglia, and appear to have two morphologies, a stellate shape and an elongated shape. Demyelinated lesions generated in the rat spinal cord showed that NG2+ cells could be detected around the periphery of the lesion on post lesion day 2-3, followed by a decrease of NG2+ cells with the onset of myelination (Keirstead et al., 1998; Watanabe et al., 2002). An increase of NG2+ cells in the center of lesion is accompanied by a decrease of cells at the periphery of the lesion. NG2+ cells may have migrated from the periphery to the center of the lesion, and the gradual decline of the NG2+ cells with time within the lesion could be due to their differentiation into myelin forming oligodendrocytes (Watanabe et al., 2002). Niehaus et al., showed that patients with active relapsing remitting MS contain antibodies against NG2 in the cerebrospinal fluid (CSF), whereas patients with non-active disease had no antibodies against NG2 within the CSF. Furthermore, repeated lysis of oligodendrocytes precursors in myelinating aggregate cultures with the NG2 antibody hindered the expression of myelin proteins *in vitro,* providing strong evidence that oligodendrocyte development proceeds via an NG2+ precursor cell stage (Niehaus et al., 2000; Trotter, 2005).

1.5 Generation of Knockouts and Conditional Knockouts

1.5.1 Knockout animals

The generation of null mouse mutants through homologous recombination of a targeting vector that disrupts the endogenous gene in embryonic stem cells is a very useful technique to study gene function *in vivo* (Capecchi, 1989a, b). One major problem arises when using this strategy: disrupting the gene with a targeting vector affects all cells within the mouse. This sometimes leads to early death in embryos, and developmental defects, which hinders the study of individual tissues in the adult animal (Joyner and Guillemot, 1994). For example in the Olig2 knockout, the mouse dies at early embryonic stages. Since motorneurons and oligodendrocytes develop from a common precursor in early stages of embryonic development, disruption of the Olig2 has a detrimental affect on both cell types (Lu et al., 2002; Takebayashi et al., 2002).

1.5.2 Conditional knockout animals

One way to avoid embryonic lethality is to disrupt a specific gene only in distinct cell types. One of these systems, the Cre-Lox P from the bacteriophage P1 allows for cell-type specific gene expression through sequence specific DNA recombination (Abremski et al., 1986; Sternberg et al., 1986) (Stricklett et al., 1998; Nagy, 2000). The Cre recombinase is a 343 amino acid peptide that catalyzes site-specific recombination between two 34-base pair Lox P DNA sequences. The Lox P site consists of two 13 base pair inverted repeats separated by an asymmetric 8 base pair region. This asymmetric region determines whether excision or inversion of an intervening DNA sequence occurs after recombination. If the Lox P sites are orientated in the same direction, then the DNA sequence will be excised. If the Lox P sites are oriented in opposite directions then the DNA sequence will be inverted (Figure 1.5.2a). Another recombinase that belongs to the λ integrase family like Cre is the Flp recombinase. Flp recombinase comes from *Saccharomyces cerevisiae*, and recognizes a 34 base pair FRT DNA sequence. The FRT site consists of two 13 base pair inverted repeats separated by an asymmetric 8 base pair region (Figure 1.5.2a, Qian et al., 1990; Nakano et al., 2001). Genetic regulation via the use of these Cre and Flp recombinases provides a very sophisticated strategy for cell type specific regulation of gene expression.

Cre and Flp can be used to inactivate a gene or activate a gene in a particular cell type. A targeting vector is generated, which includes a Cre gene driven by a specific promoter, from the gene of interest. Through homologous recombination this targeting vector is inserted into the mouse genome. Activation of this specific promoter results in the translation of Cre recombinase protein in the cell type of interest. The mouse strain expressing Cre under the cell-specific promoter is crossed to another mouse strain, where Lox P sites flank a gene, or Lox P sites flank a stop codon, in front of a reporter gene such as GFP. In the case of a gene flanked by Lox P sites, the offspring will have cut out the flanked gene specifically in cells, in which the promoter driven Cre expression is active. The Cre recombinase excises the floxed gene, this enabling a study of animals lacking the gene of interest expressed in a distinct cell type (figure 1.5.2b). In the latter case, the reporter gene is expressed when the stop codon is deleted through recombination. This particular model is particularly useful for lineage studies. This results furthermore in the continual expression of GFP in the daughter cells, even after the promoter is down-regulated (figure 1.5.2c).

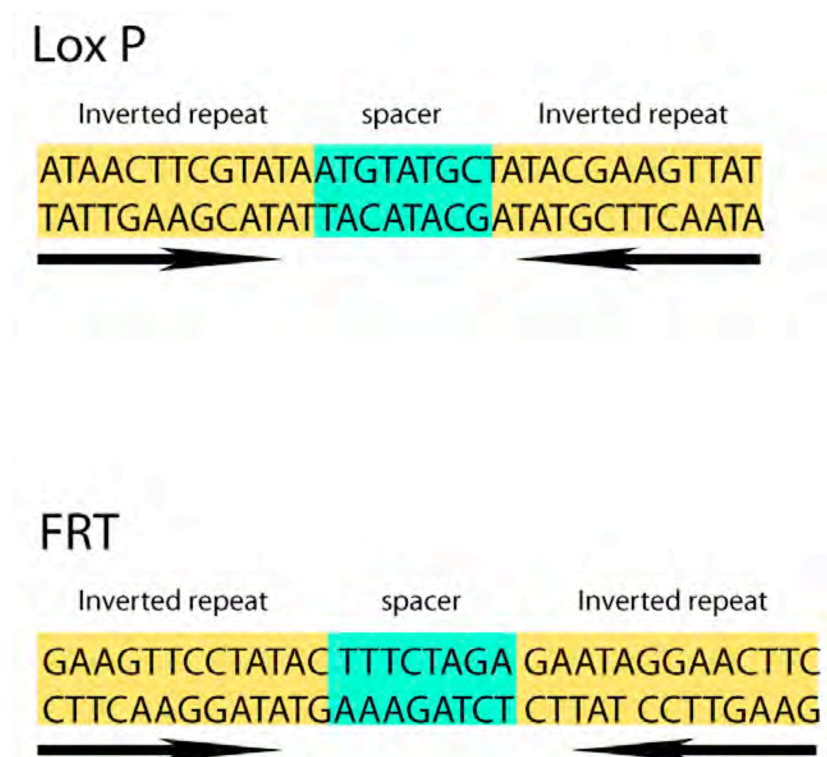


Figure 1.5.2a: Schematic representation of Lox P sites and Frt sites recognized by Cre and Flp recombinases respectively.

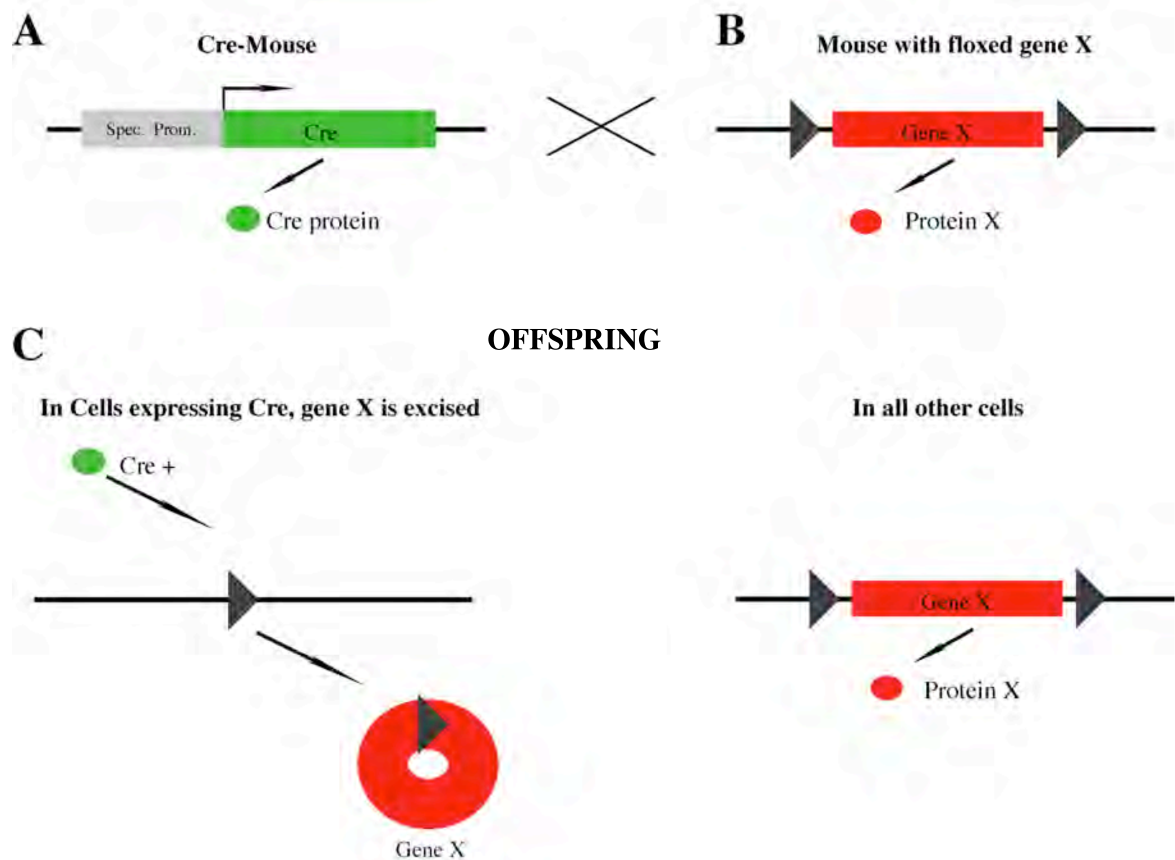


Figure 1.5.2b: Schematic diagram of specific gene excision in a mouse using Cre recombinase:
In mouse A) Cre recombinase protein is made in cells in which the specific promoter is active. Mouse B) contains Lox P sites flanking gene X. Offspring C) generated by breeding these two mouse strains contain cells in which Cre recombinase is expressed and which thus excise gene X resulting in the absence of protein X. In all other cells in which the specific promoter is not active, protein X is still synthesized.

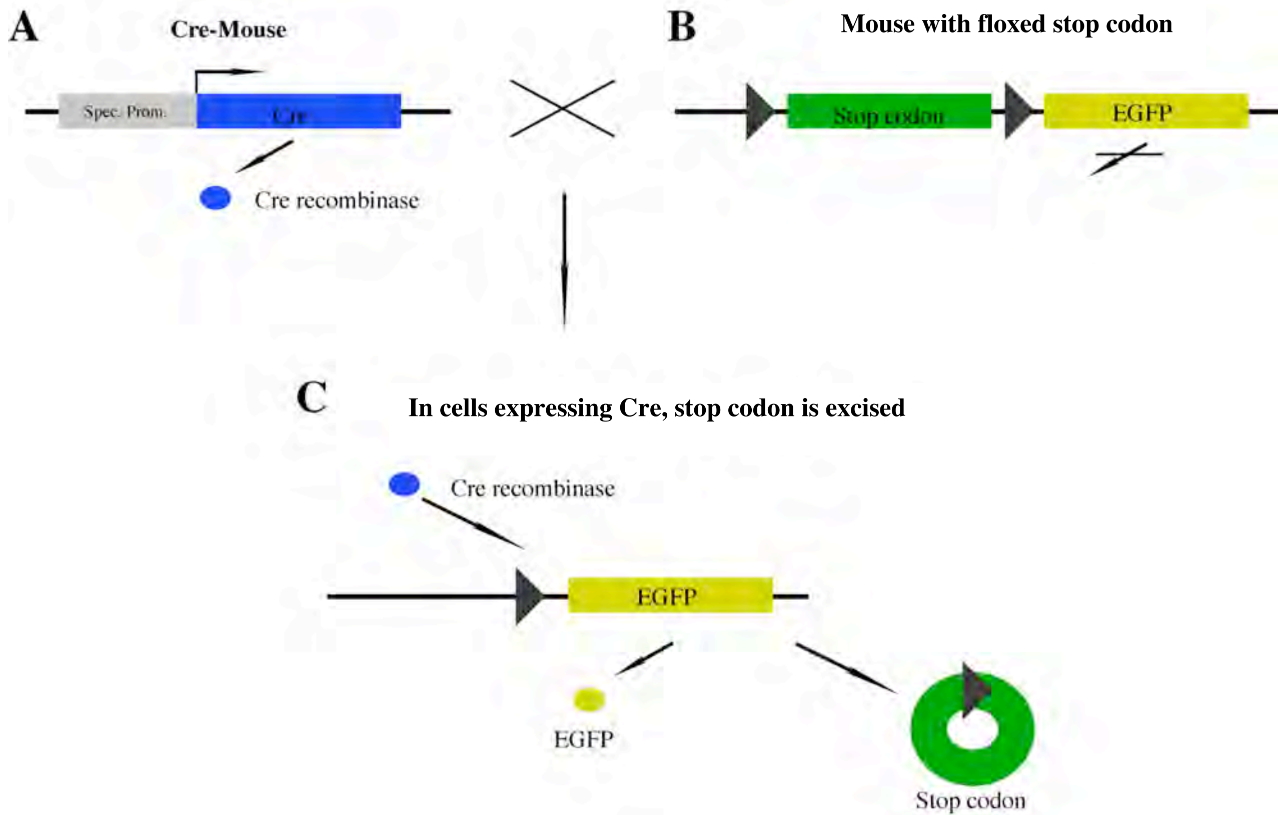


Figure 1.5.2c: Schematic diagram of a stop codon excision in a mouse using Cre recombinase:

In mouse A) Cre recombinase protein is made in cells in which the specific promoter is active. Mouse B) contains Lox P sites flanking a stop codon. Offspring C) generated by breeding these two mouse strains contain cells in which Cre recombinase is expressed and which thus excise the stop codon resulting in the expression of the reporter gene EGFP. In all other cells in which the specific promoter is not active, the EGFP protein is not synthesized.

1.5.3 NG2 Knockout

An NG2 null mouse line was generated that fails to produce the transmembrane Chondrotin sulfate Proteoglycan NG2. Grako et al. made a targeting vector where a Neo resistance gene was cloned into exon 3 of the NG2 gene, causing a disruption of the NG2 gene (Grako et al., 1999). Animals heterozygous for the NG2 gene (NG2+/-) and animals in which both alleles of the NG2 were disrupted showed no obvious phenotypic abnormalities. However, this null mutant showed abnormalities in smooth muscle cells. NG2 binds directly to PDGF-AA as well as associating with the receptor for PDGF-AA in cis (Grako and Stallcup, 1995; Goretzki et al., 1999). Aortic smooth muscle cells from the knockout mouse fail to proliferate and migrate in response to PDGF-AA, implying a defect in the signal cascade pathway that is normally activated by the PDGFα-R. There was also no observed autophosphorylation of the PDGFα-R in the knockout (Grako et al., 1999). However, Neomycin is known to interfere with gene expression and so a thorough analysis of the null mutant is required to better understand the role of the NG2 protein in the developing mouse.

1.6 Aim of Study

The aim of this study was to generate a knockin mouse where the NG2 expressing cells are labeled by EYFP to help clarify the function and lineage of the NG2+ cells in the developing and adult CNS. Since the knockin mouse replaces the endogenous NG2 gene with the EYFP gene, the homozygous mouse where both the NG2 alleles are disrupted by the EYFP gene allows the study of the labeled cells now lacking the NG2 protein. Another knockin mouse where EYFP is replaced by the Cre gene would also provide a powerful tool for lineage studies were the NG2+ cells and their progeny are permanently labeled with GFP, or to selectively delete specific genes in the NG2+ cells. Several approaches were undertaken to generate this knockin mouse in this study.

3. Results:

3.1 Generation of the mouse line containing EYFP in the NG2 gene

A knockin strategy was devised to label NG2 expressing cells with EYFP or to express Cre recombinase in the NG2+ cells, by targeting the NG2 gene in the start codon. A targeting vector containing either the EYFP gene or the Cre recombinase gene was designed for homologous recombination in mouse embryonic stem cell. Mice bred to homozygosity will lack the NG2 gene in cells in which the promoter is active, but these cells will express EYFP or Cre. The use of this "knockin" strategy has several advantages over the transgenic approach. These are 1) cells with an active NG2 promoter will only express EYFP or Cre, 2) this represents the closest to wild-type NG2 protein expression with up-regulation and down-regulation through out development and 3) the ability to study the NG2 cells lacking the NG2 protein in the homozygous mice.

3.2 Generation of the NG2-EYFP and NG2-Cre Targeting Vectors for Homologous Recombination

The NG2 ("Nerve-Glia antigen 2") vector was cloned from genomic DNA from mouse OLA-129 embryonic stem cells or from a BAC vector containing the NG2 start codon through PCR amplification. The targeting vectors are designed to target the start codon, in exon 1. A backbone vector was used to clone in the different fragments, to generate the complete targeting vector. The pKS-blue script II vector (2.96 Kb), was used as the backbone and original multiple cloning site was replaced by a multiple cloning site designed for this knockin mouse. The multiple cloning site was cut out by Kpn I / Sac II and through ligation a new multiple cloning site was inserted containing the following restriction enzymes in this order: Kpn I, BamH I, Pst I, Not I, Nde I, Fse I, Xho I, Xma I, and Sac II. A Neomycin resistance gene was inserted following the EYFP or Cre genes to allow selection of the cells containing the targeting vectors. In the event of low expression of EYFP and Cre, alternative vectors were generated in which an artificial intron containing a poly-A tail was cloned in directly behind the EYFP or Cre genes. This method of cloning in an artificial intron has been shown to increase expression levels in genes of interest.

3.3 NG2-EYFP Targeting Vector

The modified pKS-blue script vector containing the NG2-EYFP has the following elements (figure 3.3.1):

1. Homologous short arm and the EYFP coding sequence: The short arm, consists of two parts, a DNA fragment about 735 bp that is homologous to the NG2 gene upstream of the open reading frame (ORF) in exon 1 followed by the 5' end of 232 bp of the EYFP gene. There are no known splice variants to the NG2 gene. The promoter region is also very large, therefore the best option was to insert the EYFP directly into the ORF of the NG2 gene. The two fragments were fused together through a fusion PCR giving a fragment of approximately 967 bp. The fusion product was then cloned in the pKS-blue-script by using BamH I / Pst I restriction sites. The 3' fragment of the EYFP of about 514 bp was cut out of the EYFP vector with Pst I / Nde I and cloned into the pKS-blue script vector, with the same restriction sites. The over all size of the short arm and the complete EYFP gene is 1.46 KB. The identity of the short arm was checked with restriction digests and sequencing. No mutations were detected during sequencing. Through homologous recombination in the embryonic stem cells, the targeting should integrate into the start codon in exon 1 without interfering with the other exons further downstream. This resembles the closest expression pattern to that of the wild-type gene.
2. Neomycin-resistance gene (Neo^R): This Neo^R gene used was about 1.31 KB and was floxed on either side by Lox P sites for latter excision from the targeted allele. A thymidine-kinase promoter drives the Neo^R gene derived from the *Herpes simplex* virus. The original gene was first amplified by PCR from the pMCNeoPA vector (Stratagene) using primers AN2-Neo anti-sense and AN2-Neo sense. This PCR product was cloned into an intermediate vector containing Lox P sites. The Neo^R gene, which is floxed on both sides by Lox P sites was then further, amplified through PCR using primers 4937 (Neo anti-sense with Fse I restriction site) and primer 4938 (Neo sense with Nde I and Not I restriction sites). The final Neo^R was cloned in behind the EYFP gene in the backbone vector pKS-blue-script. Depending on its successful homologous recombination in the embryonic stem cell, the gene provides resistance against the drug G418 giving a positive selection technique to screen resistant clones.

3. <u>Homologous long arm:</u> A long arm of 5.3 KB was used which was made up of the 3' end of exon 1, directly behind the Neo^R gene. The long arm did not contain any part of exon 2 of the gene. A long distance PCR was first attempted to generate this large fragment by using the primers AN2-LA anti-sense and AN2-LA sense from genomic DNA. This long distance PCR method did not work as was expected, therefore an alternative method was used to get around this problem of amplifying such a large product. When looking at the homologous long arm nucleotide sequence, one major feature is clearly visible, which is right in the middle of the 5.3 KB long arm is a Sac I restriction site. This is unique only to the long arm and not the short arm. The Sac I restriction site cuts the long arm in half giving a fragment of 2.6 KB and 2.7 KB. It was easier first to amplify these smaller fragments by PCR. The fragments are then fused together to make up the homologous long arm. Another vector was designed for fusing the long arm together, by replacing multiple cloning site from the pSP72 vector (2.9 KB) and replacing it with an artificial multiple cloning site. The old multiple cloning site was removed by cutting with the restriction enzymes EcoR V and Xho I. The new multiple cloning sites contained the following restriction sites in the following order: EcoR V, Fse I, Sac I, and Xho I. The individual fragments were generated by PCR amplification using primers LA anti-sense 1 and LA sense 1 for the first fragment of 2.7 KB, so the primers restriction site Fse I and Sac I were brought in. For the second fragment LA anti-sense 2 and LA sense 2 were used and restriction sites Sac I and Xho I were brought in. After PCR amplification the fragments were cloned into the intermediate vector, the modified pSP72 vector. The homologous long arm was double checked through restriction digest and sequencing. Four different restriction digests were done to verify the identity of the long arm. It appeared that the homologous long arm was correct. The pSP72 vector was then sent for sequencing to check for mutations. Multiple primers were generated for primer walking. Five mutations were found in the homologous long arm, but the mutations were downstream from exon 1. Since the homologous long arm is mostly intron, it was used in the final targeting vector.
4. <u>Cloning vector backbone:</u> The pKS-blue-script II multiple cloning site was exchanged by restriction digestion with Kpn I and Sac II. Another multiple cloning site was cloned in, containing the unique restriction sites in the following order: Kpn I, BamH I, Pst I, Not I, Nde I, Fse I, Xho I, Xma I, and Sac II. The 3' end of the

EYFP was cloned in first followed by the short arm fused to the 5’ end of the rest of the EYFP gene. The homologous long arm was then cloned into the backbone vector. The final step was cloning in the Neo^R gene that is floxed by the Lox P sites. The final vector was checked through restriction digests with seven different restriction enzymes BamH I, Bsg I, Hind III, Nae I, Nhe I, Xmn I and Pst I. From these restriction digests the targeting vector appeared to be correct. The targeting vector was double checked by sequencing over the unique restriction sites to look for any mutations. No new mutations were observed within the target vector. The final targeting vector **pKS-AN2-EYFP** has a size of 10.2 KB. 250 μg of it were linearized before electroporation in embryonic stem cells with Xho I.

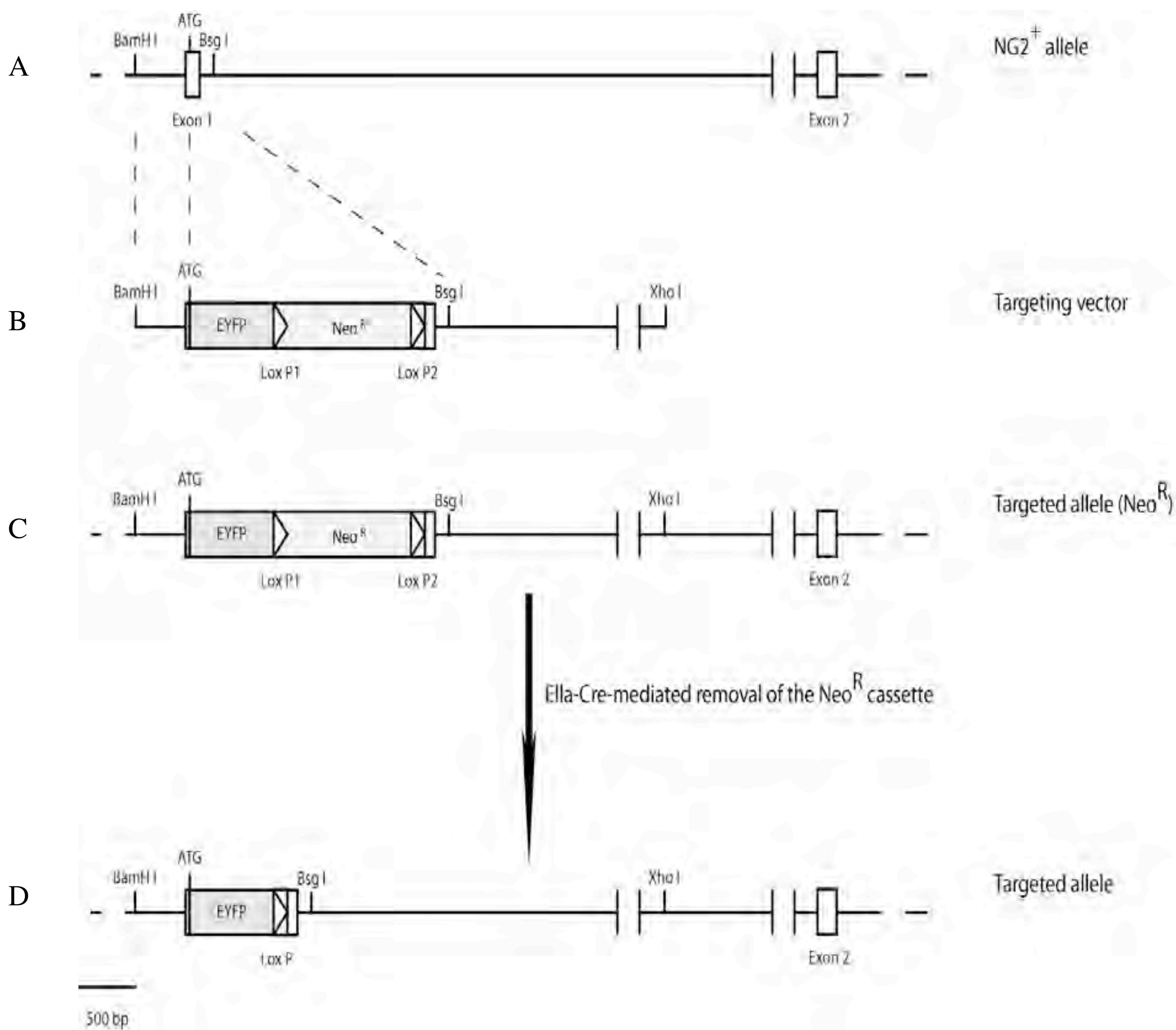


Figure 3.3.1: Schematic Diagram of NG2-EYFP Targeting Strategy for Homologous Recombination in Embryonic Stem Cells.

A) Wild-type NG2 allele, B) targeting vector containing the EYFP gene and the Neo^R gene, C) targeted allele after homologous recombination in embryonic stem cells, and D) targeted allele after the excision of the Neo^R gene by breeding F1 mouse generation with Ella-Cre mouse. F2 generation lacked the Neo^R gene. (Lox P sites in the diagram are not to scale)

3.4 NG2-EYFP Homologous Recombination in Embryonic Stem Cells

3.4.1 PCR identification of homologously recombined embryonic stem cell clones

For the identification of homologously recombined embryonic stem cell clones containing the targeting vector, PCR amplification was performed with the anti-sense primer 3313 that is located near the 5' end of the EYFP gene and the sense primer 3305 that is located upstream from the 5' end of the short arm of the targeting vector. A product of about 996 bp was observed in correctly recombined embryonic clones. No PCR product was observed if targeting vector inserted improperly. For optimization of this PCR a control plasmid was designed. A large fragment of 1.37 KB that contains exon 1 of the NG2 gene, the homologous short arm and 300 bp upstream from the short arm was PCR amplified and cloned into the original EYFP plasmid (clonetech) by Kpn I restriction sites. The anti-sense and sense primer for the EYFP targeting vector were optimized using this control plasmid.

To improve the specificity of the control PCR, a “nested” PCR was performed, where an intermediate product was first amplified. Then another set of primers were used on the in between product to give a second product which was seen on the agarose gel. The primers used are anti-sense 3311 that is upstream from 3313 and sense primer 3307 that is downstream from 3305. For the first reaction with primers 3313 and 3305, 19 cycles were done using an annealing temperature of 58°C. From the first PCR 1/5 of the product was used for the second PCR reaction for 39 cycles with an annealing temperature of 58°C. The final product of 996 bp is slightly smaller then the control PCR product of 1.15 KB. This ruled out any possible contaminations from the control plasmid. The nested PCR was optimized to where it was possible to detect 200 copies, 20 copies and sometimes 2 copies of the control plasmid. This was a powerful tool to determine how many copies of the targeting vector are present in the positively recombined embryonic stem cell clone. This nested PCR technique is required, because of a lack of starting DNA material. Normally an embryonic stem cell clone consists from anywhere between 50-1000 cells. Through the nested PCR method only a minute amount of starting material is required to visualize a positive clone. To improve the working quality of the nested PCR Q-solution (Qiagen) was added.

3.4.2 Isolation of homologously recombined embryonic stem cell clones

The OLA-129 embryonic stem cell line with a passage P8 was electroporated with 50 μg of linearized targeting vector **pKS-AN2-EYFP.** 24 hours after electroporation, G418 selection was started and continued for seven to ten days. The first three days of selection the embryonic stem cells were growing at a normal rate, very little cell death was observed in the first days of selection. G418 selection medium was changed every 24 hours. Between days four to six there was massive cell death observed among the embryonic stem cells indicating some cells did not integrate the targeting vector carrying the resistance gene. On day nine of G418 selection, clones were picked and transferred to a 96 well plate. 2/3 of the clone was used for DNA isolation and 1/3 was plated onto a 96 well plate containing EMFI feeders for further expansion. When a positive clone was found in a pool of eight clones, individual clones were expanded in the presence of G418 onto a 24 well dish. After every passage, the clone was double checked by PCR. The clone was expanded until it was large enough to freeze down and inject into blastocytes. DNA from eight clones were pooled and analyzed by PCR using the control plasmid (figure 3.4.1). 256 clones were selected and screened through this method. After the initial check two clones appeared to be positive, but after further passaging, one out of the two clones disappeared. In the end, out of 256 clones only one positive clone was found that had homologously recombined the targeting vector. Clone 19E, was further checked by southern blot and confirmed that it was homologously recombined (figure 3.4.2). For the southern blot embryonic stem cell DNA was extracted from wild-type cells and from clone 19E. The extracted DNA was digested overnight with a three-fold amount of Hind III restriction enzyme. The NG2 probe used is 450 bp long and corresponds to an area upstream from start codon in the NG2 gene. If the targeting vector homologously recombined, it brought in an extra Hind III restriction site. Digestion of clone 19E with Hind III gave two bands, one corresponding to the wild-type allele of 5.89 KB and a second band corresponding to the correctly incorporated targeting vector of 3.63 KB. This embryonic stem cell clone was used for establishing the NG2-EYFP mouse line.

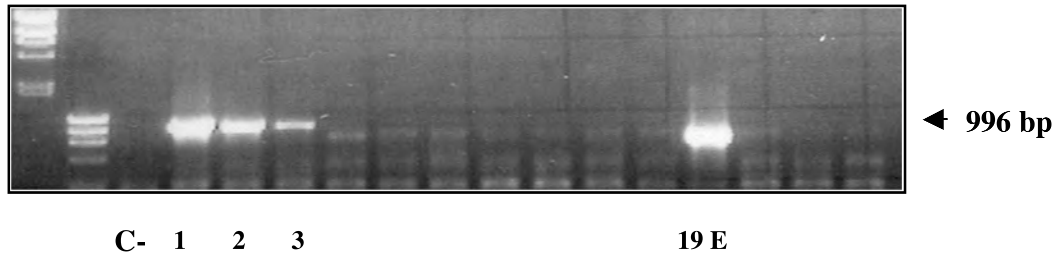


Figure 3.4.1: **PCR Screening for Homologous Recombination**
PCR screening of embryonic stem cells showed that one clone (19 E) integrated the targeting vector through homologous recombination giving a band at 996 bp. The numbers 1, 2, and 3 are bands of the control plasmid at 1.15 KB. 1) 200 copies of the control plasmid, 2) 20 copies, and 3) 2 copies. C-) negative control

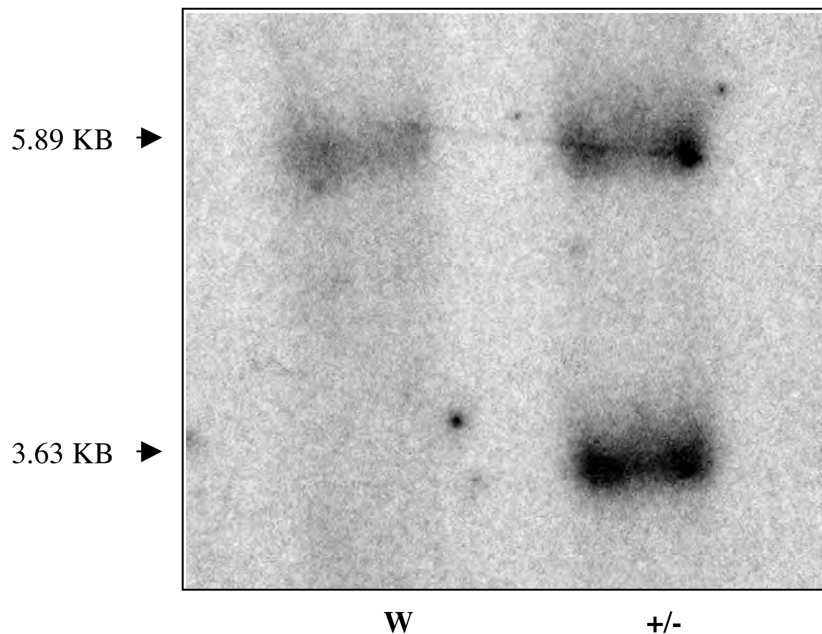


Figure 3.4.2: Southern Blot of Homologous Recombination
Southern blot of homologously recombined ES clone using an NG2 probe. Wild type ES cell (W) has one band at 5.89 KB. The heterozygous ES cell (+/-) has two bands one at 5.89 KB and one at 3.63 for the transgene.

3.4.3 Germ line transmission of embryonic stem cell clone 19E

The embryonic stem cell clone 19E that was identified through PCR and southern blot analysis was injected into blastocytes (3.5 dpc) from C57Bl6/J mouse line. After transferring the blastocytes into a pseudo-pregnant mouse mother from the mouse line NMRI, male chimeric mice were born (brown fur usually meant 100% chimeric). High chimeric male mice were taken for further breeding with C57Bl6/J female mice to establish the mouse line. About half of the F1 generation carried the mutated NG2 gene. Three mice from the F1 generation were histologically analyzed to see if there was expression of the EYFP. No expression was observed in the F1 generation. It was concluded that the Neo^R gene interfered with the EYFP expression. F1 males carrying the modified NG2 gene were bred to Ella-Cre female mice in order to selectively excise the Neo^R gene from the F2 generation (figure 3.4.3). EYFP+ cells were observed in the F2 generation. Heterozygous mice from the F2 generation, where the Neo^R was removed were inbred to obtain the NG2 knockout mutant. About 3/4 of the offspring express EYFP (1/4 homozygous, 2/4 heterozygous), and 1/4 were did not express EYFP (wild-type).

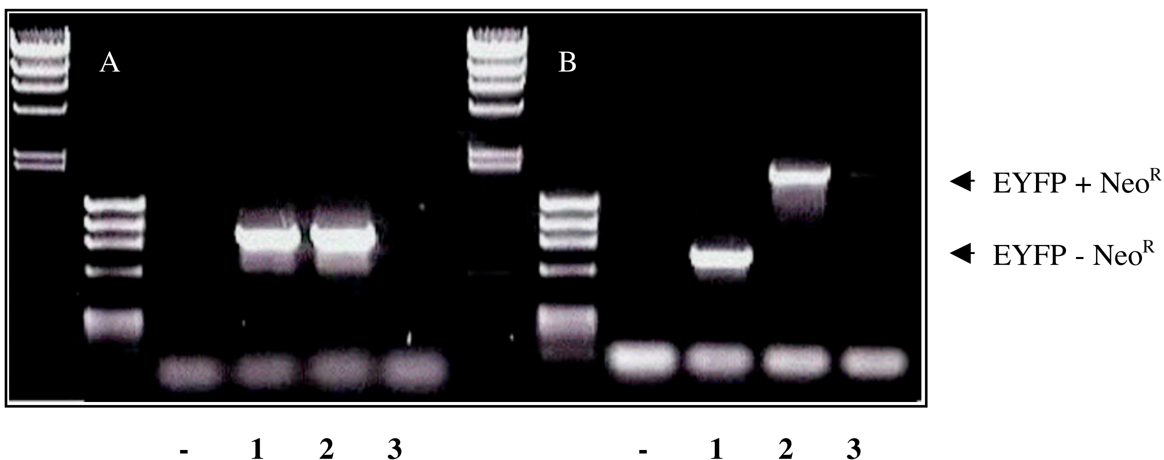


Figure 3.4.3: Germline Transmission and Removal of Neo^R by Ella-Cre Breeding

A) Genotyping of F2 offspring to check for the presence of the EYFP transgene (animals 1 & 2 are positive, animal 3 is negative).

B) PCR analysis showing, that not all animals, which had integrated the EYFP transgene had excised the Neo gene (animal 1 lacks neo, animal 2 contains neo, animal 3: control wild-type animal).

3.4.4 Histological analysis of the Heterozygous (+/-) F2 mouse generation

The F2 mouse generation, in which Neo^R has been excised was checked for the expression of EYFP in NG2+ cells. The F2 generation has two NG2 loci, one with the normal NG2 gene and one with the targeted gene containing the EYFP. Brain sections of 5-day-old mice were stained with the AN2 monoclonal antibody that recognizes mouse NG2 and co-expression of NG2 protein and EYFP was observed (Figure 3.4.4). There was almost a complete overlap between the NG2 protein and EYFP in all brain regions in the heterozygous mouse. The expression of the NG2 protein detected by the antibody was seen only on the surface and in the processes of the cells, while EYFP expression was seen in the cell body and processes. A very small fraction of the cells (less then 1%) did not express EYFP, but reacted with the NG2 antibody. These cells were termed “phantom cells”. In the CNS of the F2 generation blood vessels also expressed EYFP, since pericytes of blood vessels are NG2+. Cells that express EYFP were termed NG2-EYFP+ cells.

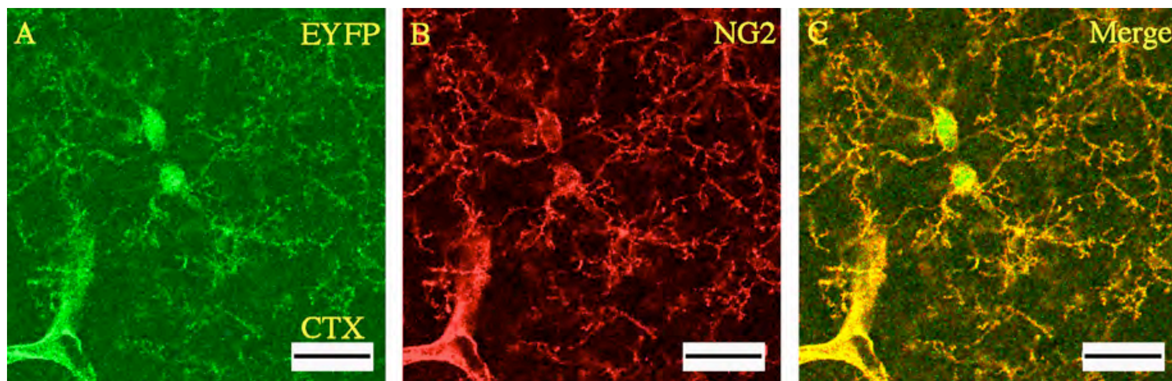


Figure 3.4.4: NG2 Expression in the F2 Generation of the EYFP Neonatal Animal

Confocal image of the cortex of a 5-day-old mouse expressing EYFP under the NG2 promoter (A, green), stained with the AN2 monoclonal antibody recognizing mouse NG2 (B, red). Merged image (C) shows that there is an overlap of EYFP and NG2 expression. (CTX = cortex, Scale bar = 20μm)

3.4.5 Molecular analysis of the heterozygous (+/-) versus the homozygous (-/-) NG2-EYFP mouse

Genotyping: Mice carrying the modified NG2 locus were mated to attain a homozygous mouse in which both alleles of the NG2 gene are disrupted (knockout). A tail biopsy was taken and a genotyping PCR was used to identify the wild type, heterozygous and the homozygous mice. PCR was done with three different primers: sense 7383, anti-sense 3312, and anti-sense 5039. Primer 7383 binds upstream before exon 1, primer anti-sense 3312 binds only in the EYFP and primer 5039 binds down stream from exon 1 in both the normal NG2 locus and the NG2-EYFP targeted locus. The primer pair 7383 and 5039 amplifies a product of 900 bp correlating to the normal NG2 locus. The primer pair 7383 and 3312 amplifies a product of 750 bp correlating to the NG2-EYFP allele. Wild-type animals would have one band of 900 bp, heterozygous animals two bands of 900 bp and 750 bp, and the homozygous animals would have one band at 750 bp. Bands were visualized on a 1% agarose gel containing ethidium bromide (figure 3.4.5).

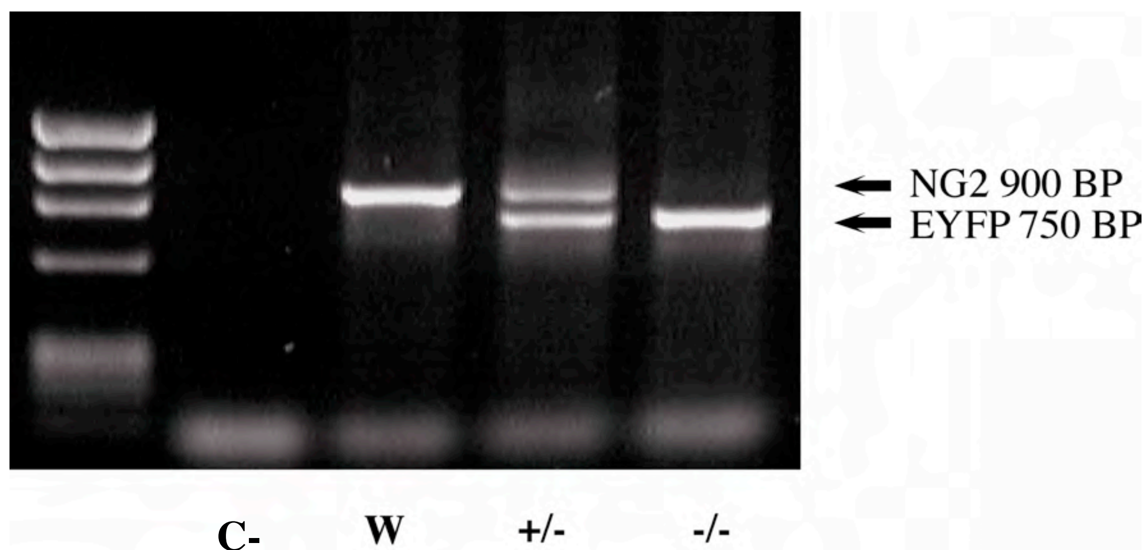


Figure 3.4.5: Genotyping PCR for the Identification of Mice Carrying the EYFP Gene in the NG2 Locus.

C-) negative control, W) wild-type, +/-) heterozygous, -/-) homozygous

Immunohistochemistry: Coronal brain sections were cut from heterozygous and homozygous mouse brains. These sections gave the best overall view of grey and white matter areas (figure 3.4.6). Heterozygous mice (+/-) and homozygous mice (-/-) were compared using the AN2 monoclonal recognizing mouse NG2. In the heterozygous mouse there was a strong immunoreactions against NG2 (figure 3.4.7), while in the knockout there was no reaction with the antibody (figure 3.4.8). Different areas of P28 day old mouse brains were examined and grey and white matter was compared in the heterozygous and the homozygous mice. The “phantom cells” disappeared in the homozygous mouse.

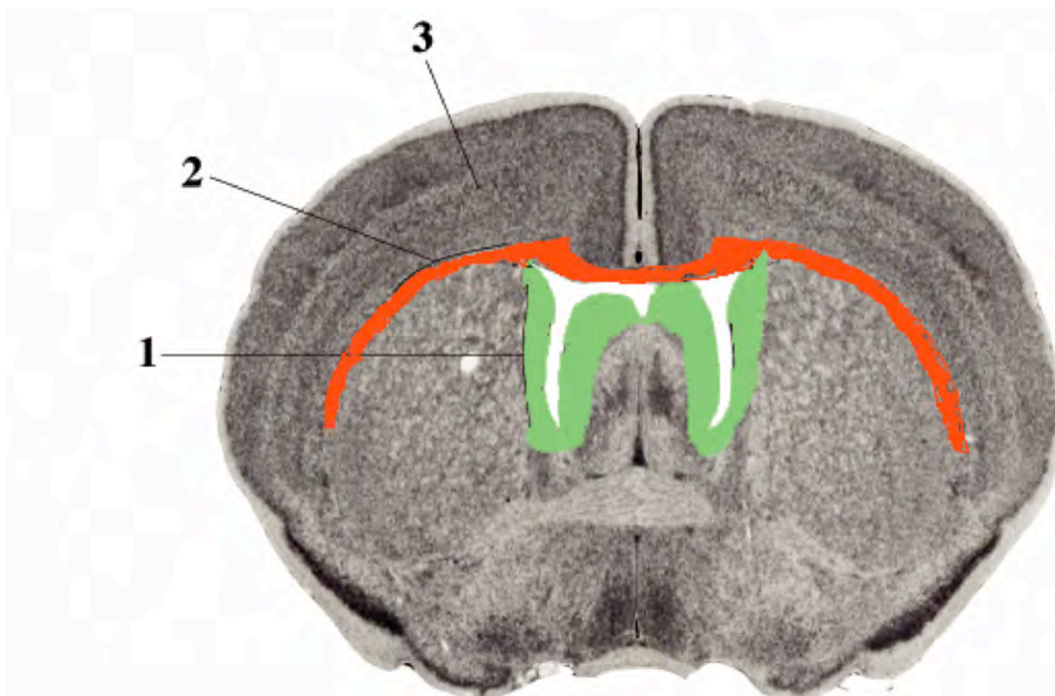


Figure 3.4.6: A 25μm Brain Section Demonstrating Areas of Grey and White Matter examined

1) Subventricular zone: area of proliferation (green)
2) Corpus callosum: area of white matter (red)
3) Cortex: area of grey matter (grey)

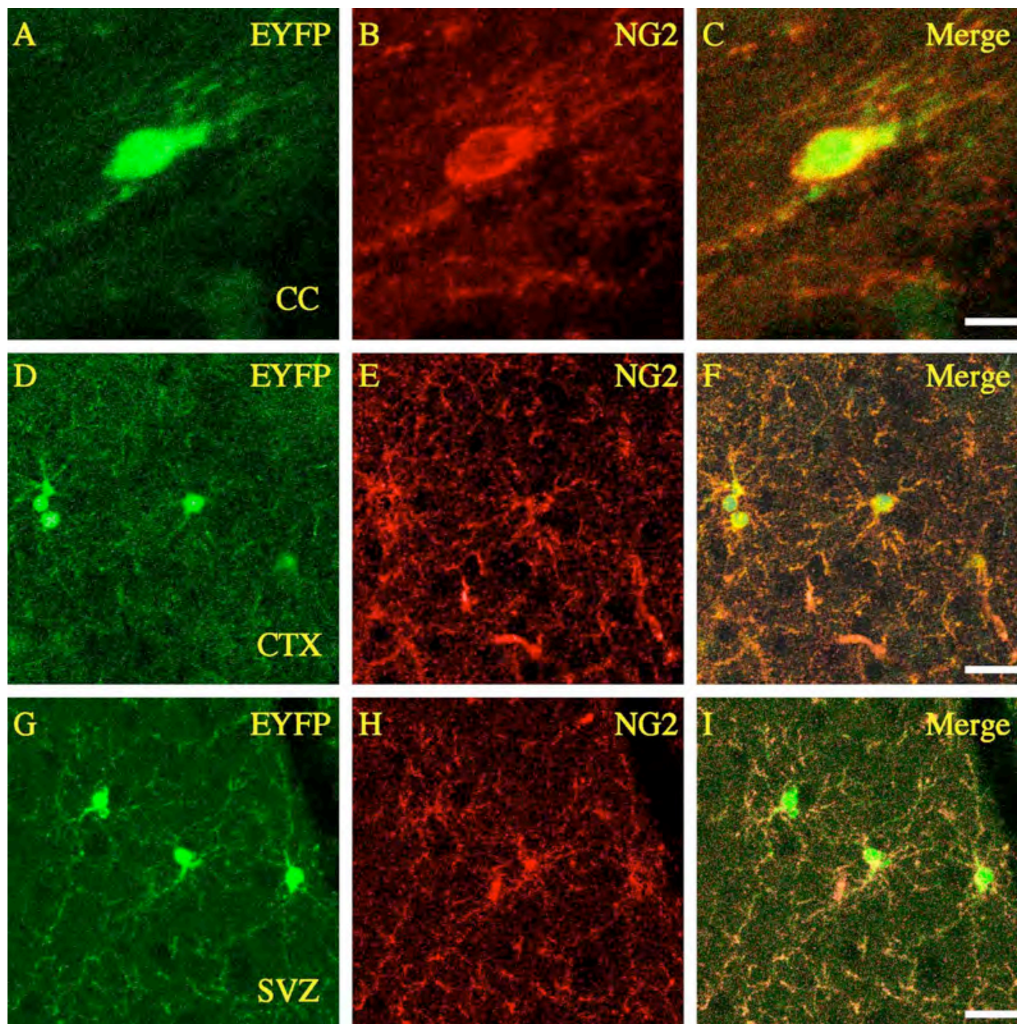


Figure 3.4.7: Expression of NG2 and EYFP in the Heterozygous Mouse (+/-)

Confocal image scan of a 28-day-old mouse brain expressing EYFP under the NG2 promoter (A, D, G), stained with the AN2 monoclonal antibody recognizing NG2 (B, E, H). Merged image (C, F, I) shows that there is an overlap of EYFP and NG2 protein expression. (CC = corpus callosum, CTX = cortex, SVZ = subventricular zone, Scale bars = 10μm - C, 20μm - F, - I).

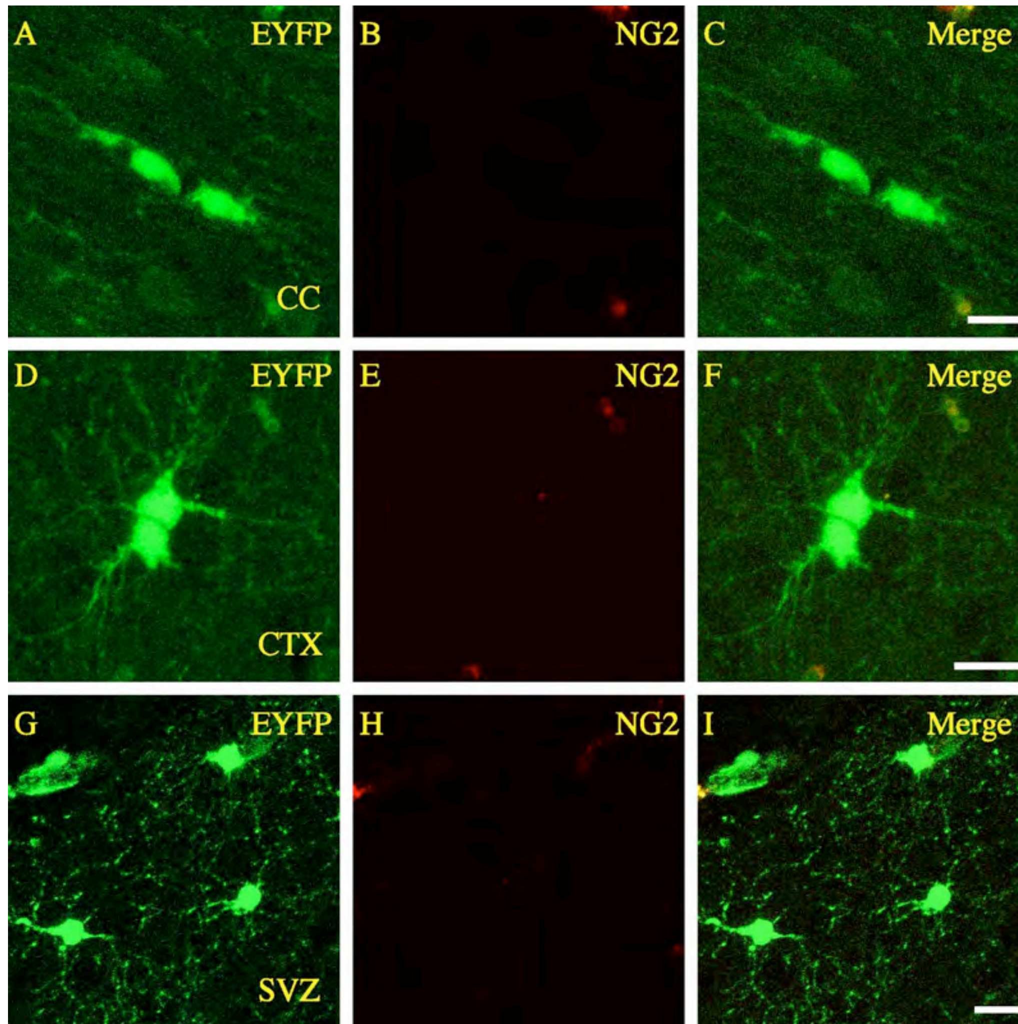


Figure 3.4.8: Expression of NG2 and EYFP in the Homozygous Mouse (-/-)

Confocal image scan of a 28-day-old mouse brain expressing EYFP under the NG2 promoter in grey and white matter (A, D, G), stained with the AN2 monoclonal antibody recognizing NG2 (B, E, H). Merged image (C, F, I) shows that there is EYFP expression, but no NG2 protein expression. (CC = corpus callosum, CTX = cortex, SVZ = subventricular zone, Scale bars = 20μm).

Biochemistry: The expression of NG2 protein was analyzed by western blotting with the AN2 antibody. Since NG2 expression peaks between neonatal day 6 and day 12, eight day old mouse brains were analyzed. Wild-type mice were compared to heterozygous, and homozygous animals carrying the modified NG2 locus. The protein F3 expressed by many different cells, was used as a loading control. The results show that there is less NG2 protein in the heterozygous mouse as compared to the wild-type mouse. In the homozygous mouse there was no NG2 protein present verifying that this mouse is a knockout. Mice were analyzed for EYFP expression. No EYFP was detected in the wild type, but a signal was seen in brains from homozygous and the heterozygous animals. As a loading control, tubulin was used (figure 3.4.9).

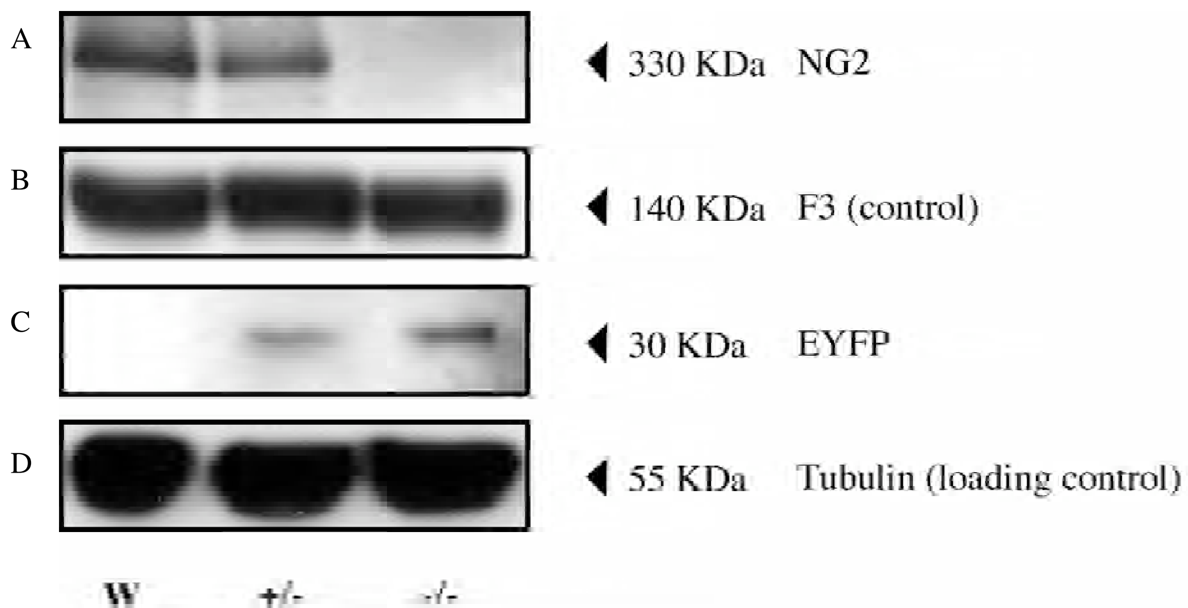


Figure 3.4.9: Western Blots analysis of proteins from brains of P8 Mice.
A) NG2 protein B) F3 loading control C) EYFP protein and D) Tubulin loading control. (W = wild type, +/- = heterozygous, -/- = knockout)

3.5 Characterization of NG2-EYFP+ cells in Neonatal and Adult Mouse Brain

The NG2-EYFP heterozygous mouse was used to help characterize the NG2 cells. NG2+ labeled cells appeared to have an elongated morphology in the white matter and a stellate morphology in the grey matter (figure 3.5.1). In the NG2-EYFP adult brain, there were many EYFP+ cells in both the grey and white matter (Figure 3.5.2). Some cells were wrapping neurons (neurons appear as black holes).

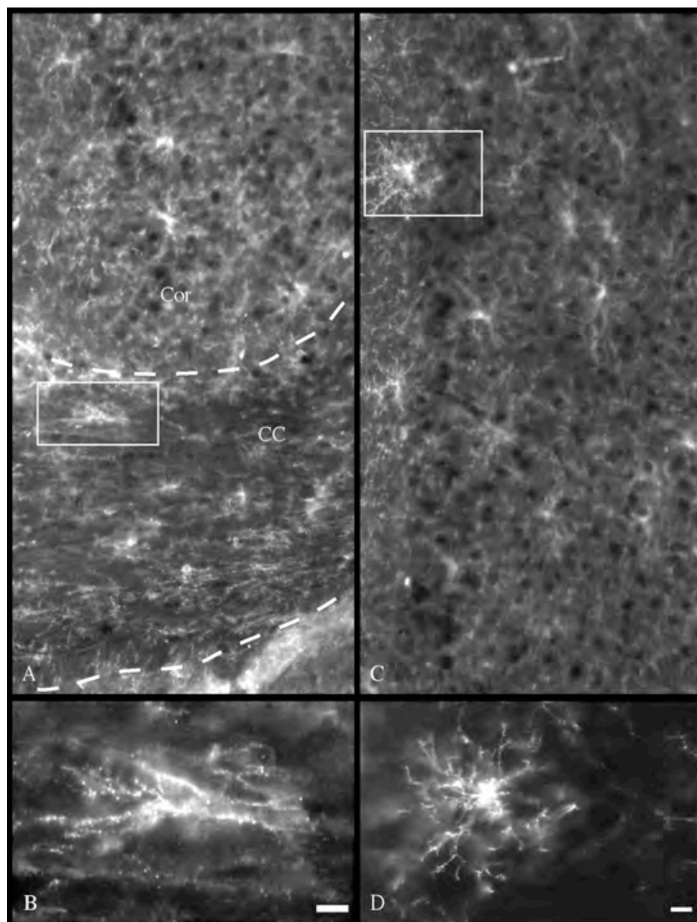


Figure 3.5.1: Section of adult mouse heterozygous (+/-) brain examined for the distribution and morphology of NG2 expressing cells.
A) Low magnification of NG2+ labelled cells stained with the AN2 monoclonal antibody in a 75 day-old mouse. **B)** High magnification of NG2+ labelled cells in corpus callosum **(CC)**. **C)** Low magnification of NG2 + labelled cell in the grey matter. **D)** High magnification of NG2+ labelled cell in the cortex **(Cor)**. Dashed lines signify separation between grey and white matter. (Scale bars = 10 μm)

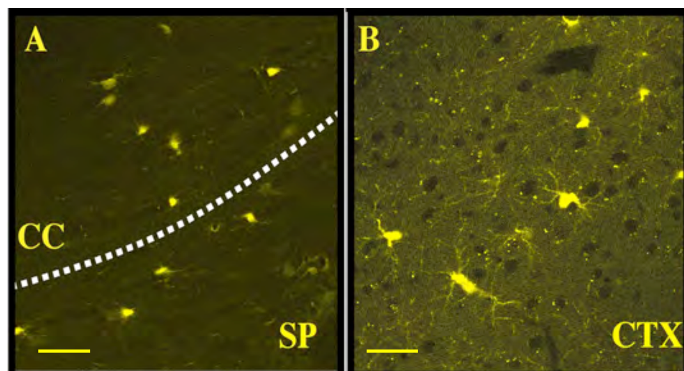


Figure 3.5.2: EYFP Expression in the CNS of a Heterozygous (+/-) Mouse
Confocal image scan of EYFP cells found in CNS of a 75-day-old mouse. A) Confocal image scans showing different morphologies of the EYFP cells in the corpus callosum (CC) and septum (SP). (B) Some EYFP positive cells appear to be enwrapping neurons in the cortex (CTX). Other EYFP cells have very long and complex processes. (Scale bar A = 40 μm, B = 20 μm)

3.5.1 Expression of Oligodendrocyte and Oligodendrocyte precursor specific antigens by the NG2-EYFP cells in the CNS

NG2-EYFP+ cells label for oligodendrocyte progenitor markers like O4, Olig-2 and PDGFα-R. There are some cells that do not express EYFP, but stain for antibodies against NG2. It appears that all NG2-EYFP+ cells express PDGFα-R in the neonatal and the adult brains (Figure 3.5.3, 3.5.4). No differences were seen in the expression of the PDGFα-R in the heterozygous and the homozygous mouse.

NG2-EYFP+ cells stain with the O4 antibody, recognizing predominantly sulfatide expressed by immature and mature oligodendrocytes. Not all NG2-EYFP cells are O4 positive and since the NG2 protein is expressed before the O4 antigen (Niehaus et al., 1999). NG2-EYFP O4- cells are at a more immature stage. In the adult brain, there are also NG2-EYFP+ cells that express O4, indicating presence of immature oligodendrocyte. No differences were seen in the distribution of O4 expressing cells between the heterozygous and homozygous mouse brains, in regions such as cortex and subventricular zone (figures 3.5.5, 3.5.6)

Olig1 and Olig2 are transcription factors expressed by oligodendrocyte precursors and oligodendrocytes. The NG2-EYFP+ cells, showed no overlap of Olig1 and EYFP expression, indicating that Olig1 is expressed at a later stage than NG2. Olig2 was observed in various brain regions like the cortex and the corpus callosum. NG2-EYFP+ cells expressed Olig2; however not all Olig2+ cells expressed EYFP. There was no difference in distribution of the Olig2 cells in the heterozygous and homozygous mouse at different ages (figures 3.5.7-3.5.10).

Sox 10 is a transcription factor that is expressed in early oligodendrocyte precursors and mature oligodendrocytes in the developing CNS. Heterozygous and homozygous NG2-EYFP mice were stained with an antibody against sox 10. No difference was seen between the heterozygous and homozygous animals. Almost all cells that expressed EYFP were also sox 10+. (figures 3.5.11 and 3.5.12).

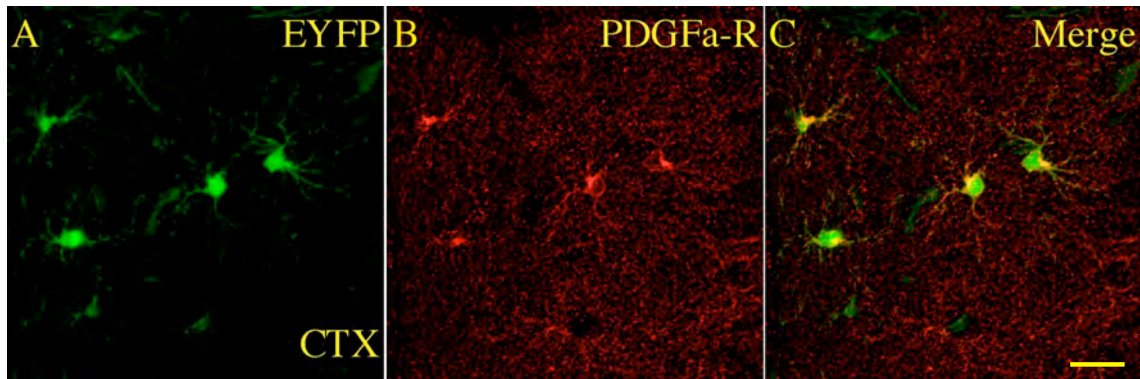


Figure 3.5.3: Expression of EYFP and PDGFa-R in the Mouse Cortex of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) of a 28-day-old mouse expressing EYFP (A) stained with an antibody that recognizes PDGFa-R (B). Merged image C shows an overlap. Scale bar = 20 μm

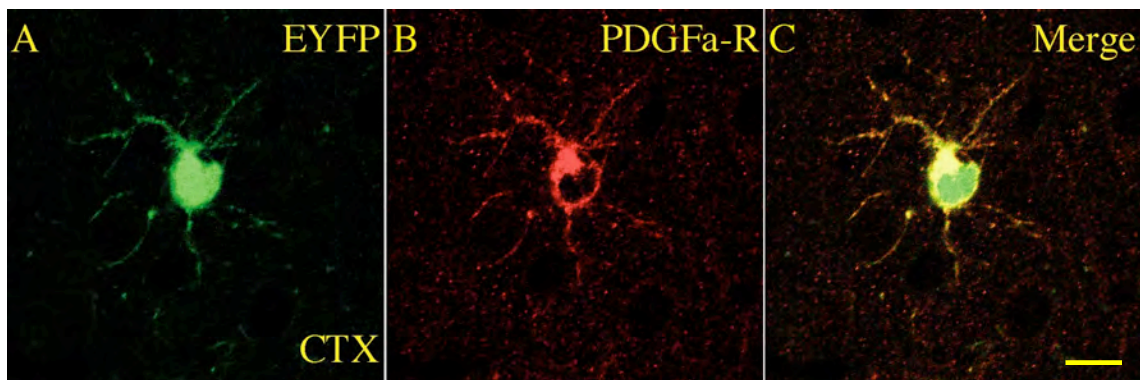


Figure 3.5.4: Expression of EYFP and PDGFa-R in the Mouse Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 75-day-old mouse expressing EYFP (A) stained with an antibody that recognizes PDGFa-R (B). Merged image C shows an overlap. Scale bar = 10 μm

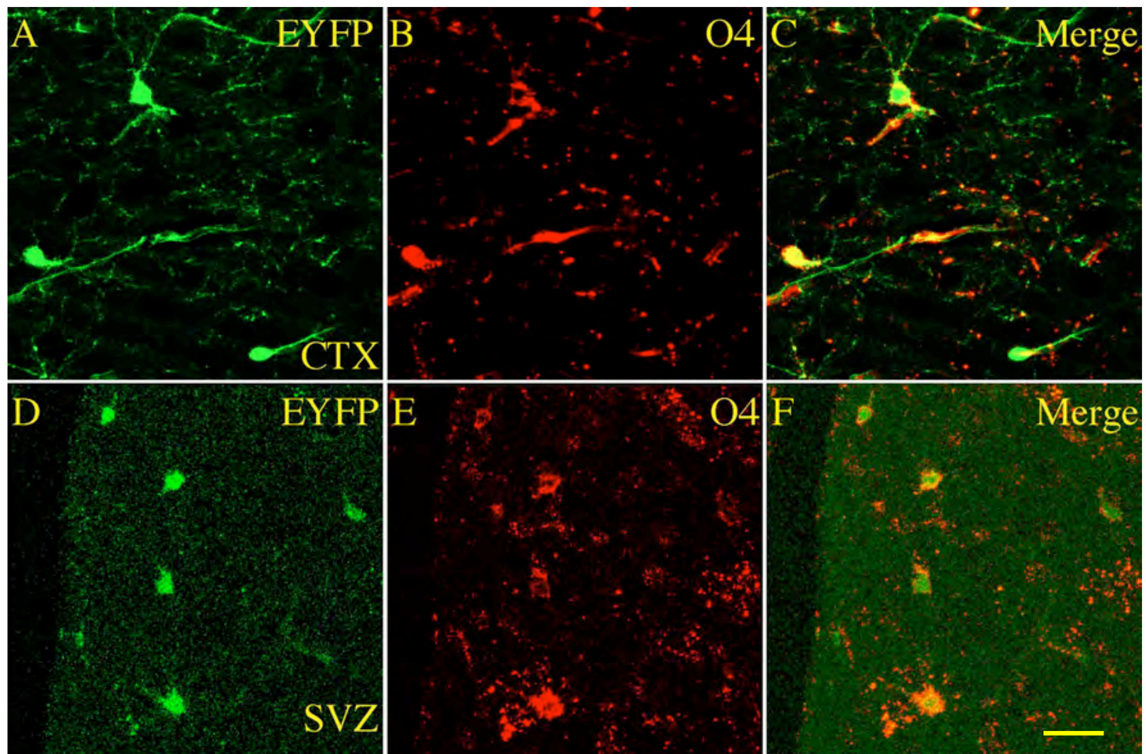


Figure 3.5.5: Expression of EYFP and sulfatide in the Mouse Cortex and Subventricular Zone of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) and subventricular zone (SVZ) of a 28-day-old mouse expressing EYFP (A, D) stained with the O4 antibody (B, E). Merged images C and F show an overlap. Scale bar = 20 μm

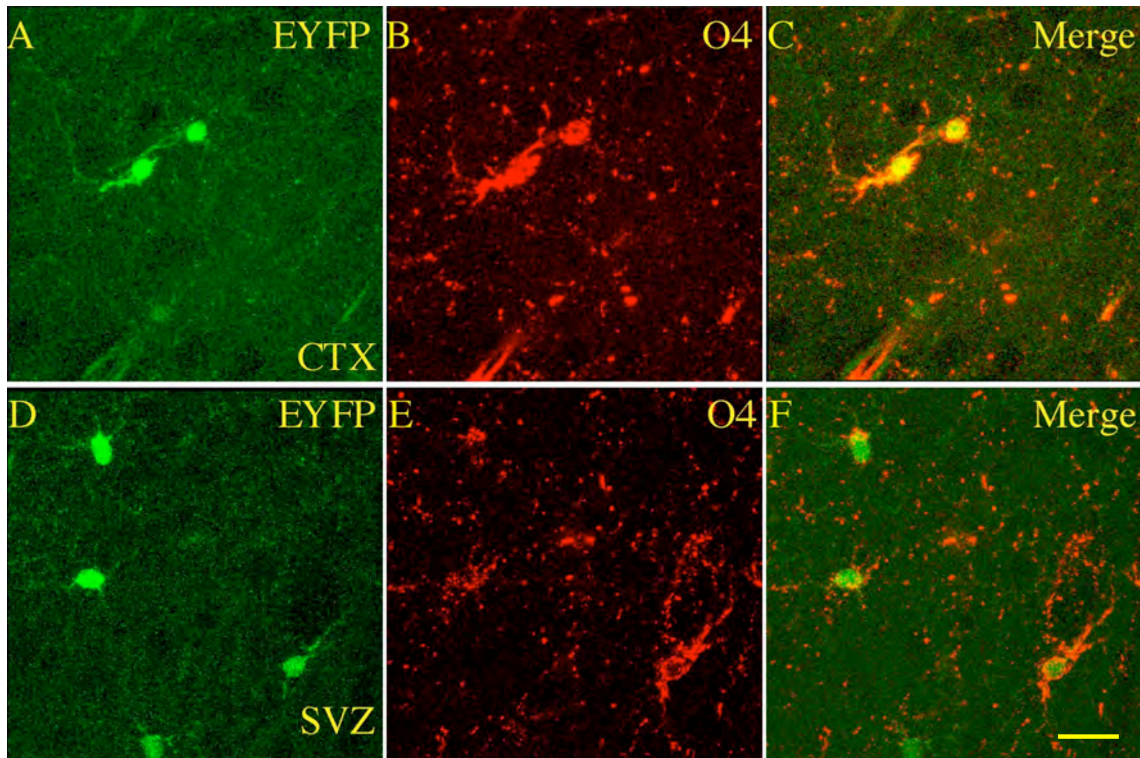


Figure 3.5.6: Expression of EYFP and sulfatide in the Mouse Cortex and Subventricular Zone of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) and subventricular zone (SVZ) of a 28-day-old mouse expressing EYFP (A, D) stained with the O4 antibody (B, E). Merged images C and F show an overlap. Scale bar = 20 μm

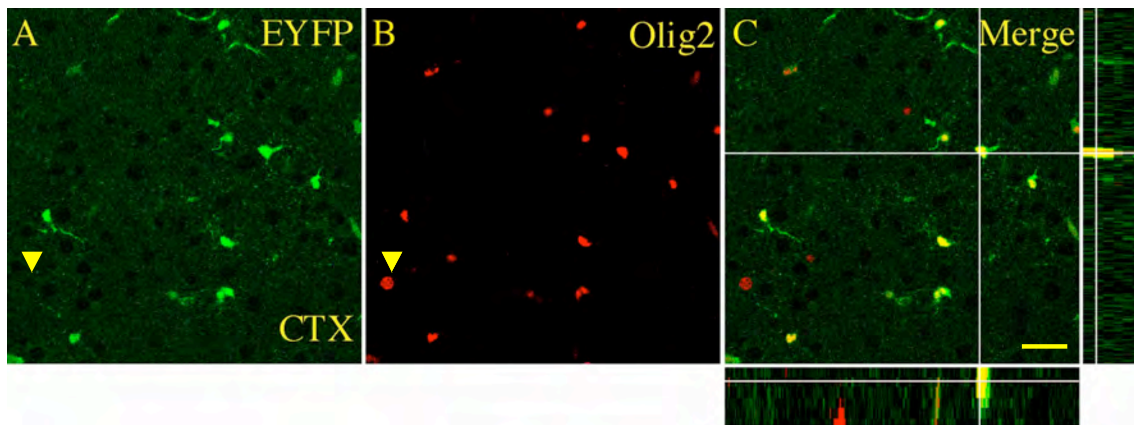


Figure 3.5.7: Expression of EYFP and Olig2 in the Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Olig2 (B). Merged image C shows an overlap. X, Y and Z axis are represented to show double labelled cells. Arrow points to Olig2+, EYFP- cell. Scale bar = 40 μm

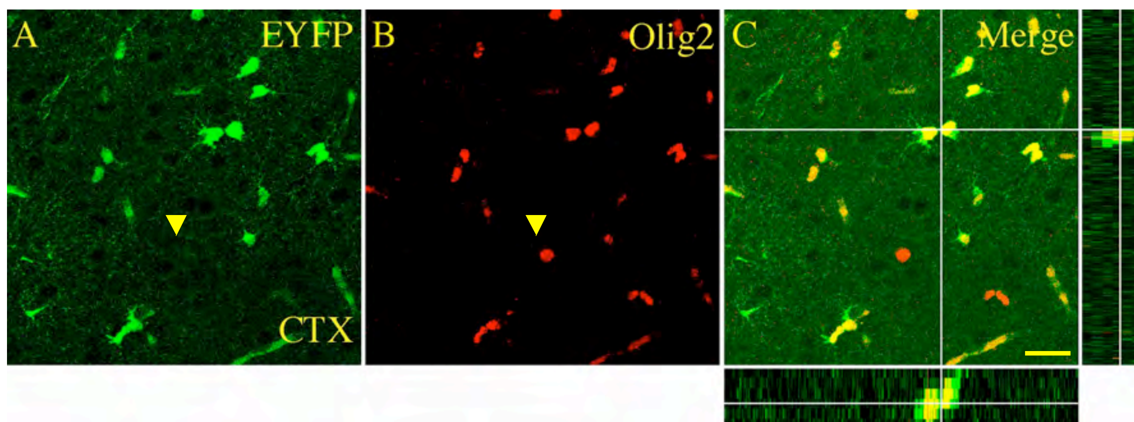


Figure 3.5.8: Expression of EYFP and Olig2 in the Cortex of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Olig2 (B). Merged image C shows an overlap. X, Y and Z axis are represented to show double labelled cells. Arrow points to Olig2+, EYFP- cell. Scale bar = 20 μm

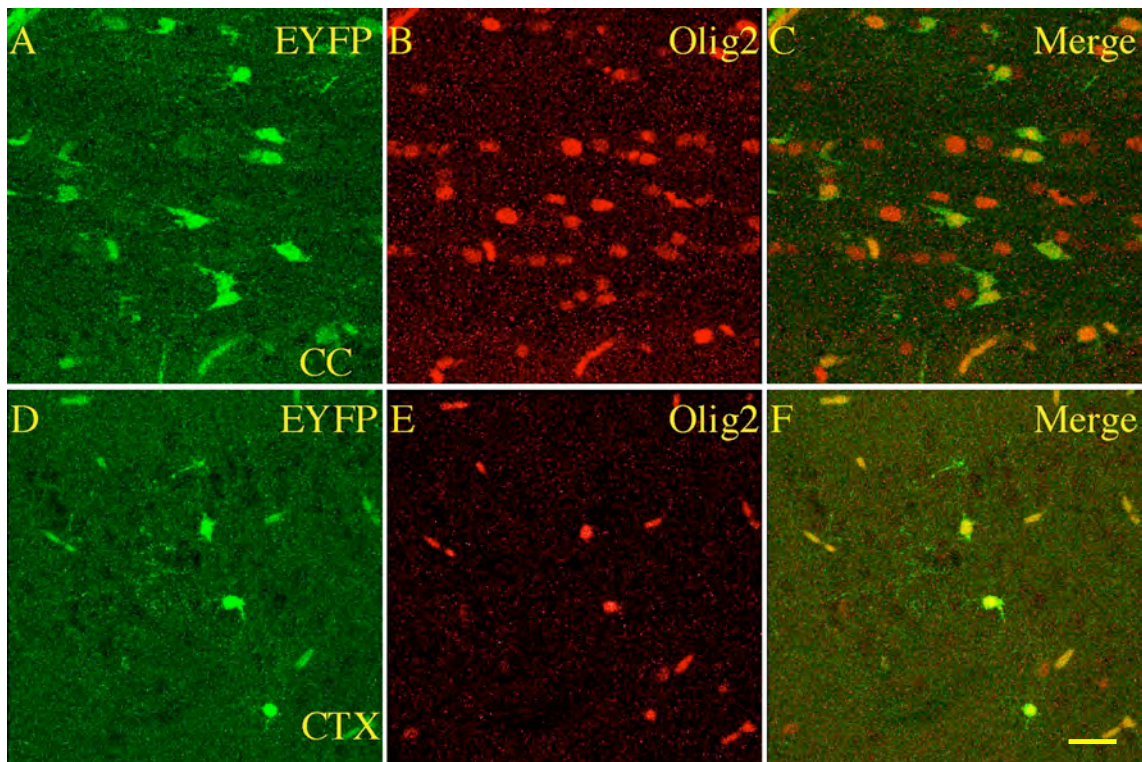


Figure 3.5.9: Expression of EYFP and Olig2 in Cortex and Corpus Callosum of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) and corpus callosum (CC) of a 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Olig2 (B, E). Merged images C and F show an overlap. Scale bar = 20 μm

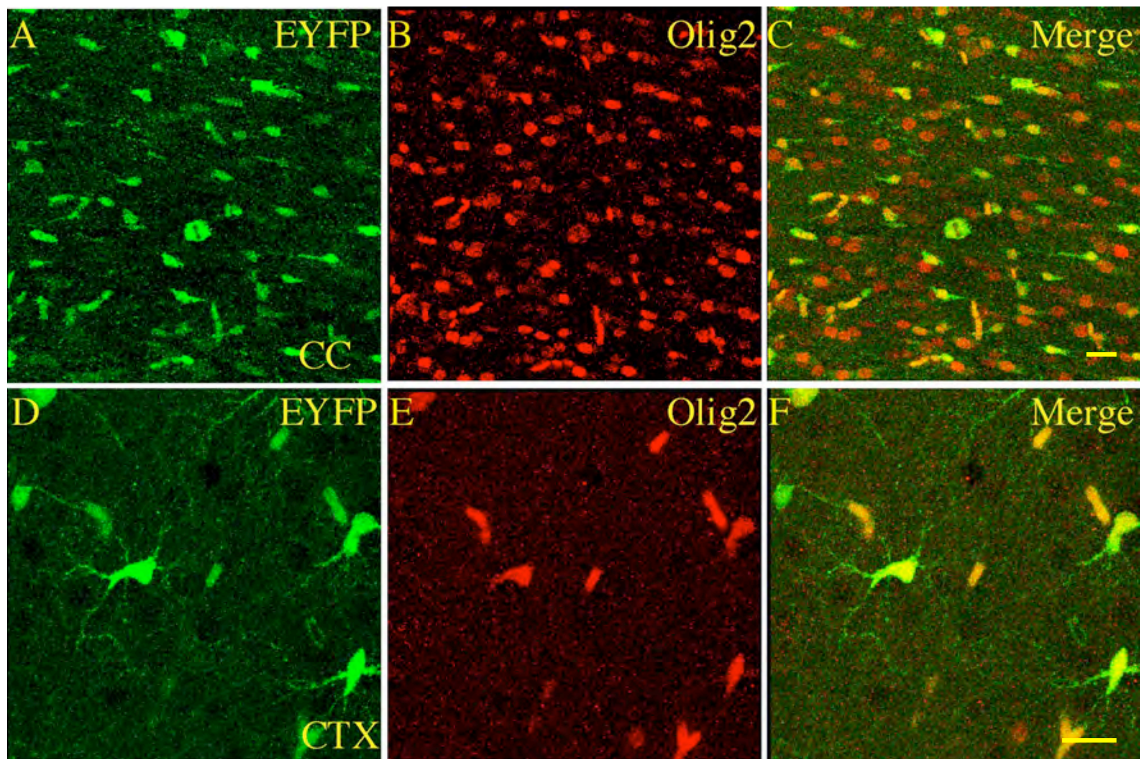


Figure 3.5.10: Expression of EYFP and Olig2 in the Cortex and Corpus Callosum of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) and corpus callosum (CC) of a 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Olig2 (B, E). Merged images C and F show an overlap. Scale bars = 20 µm

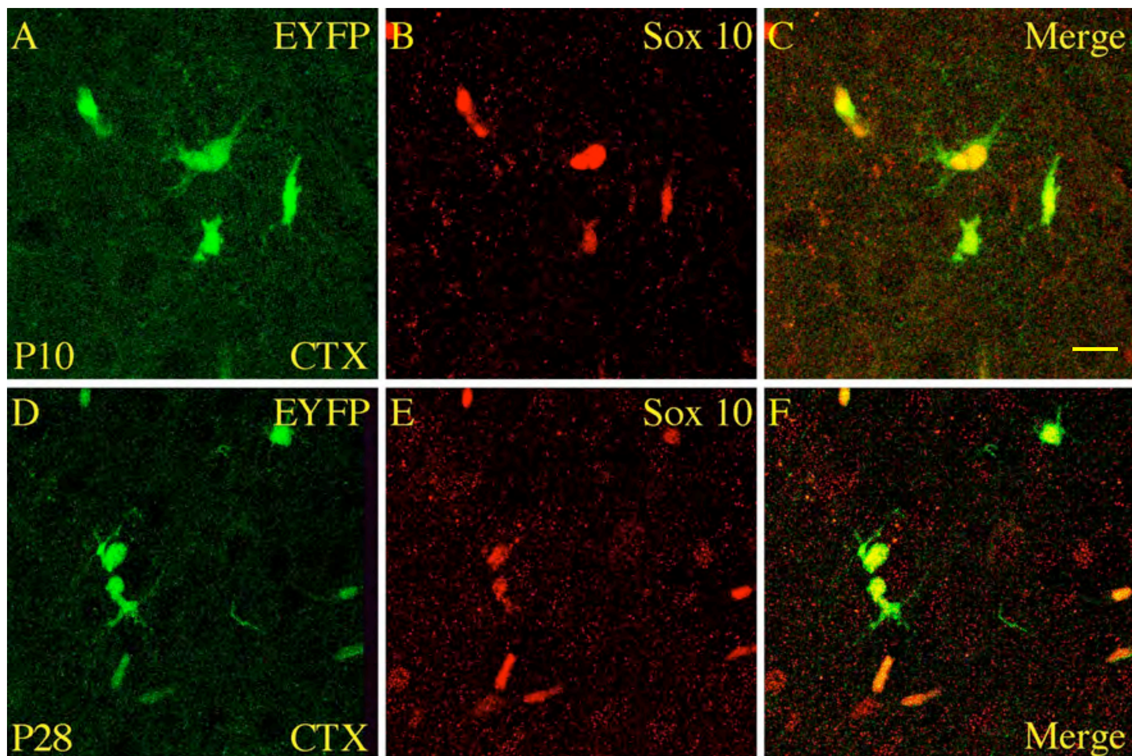


Figure 3.5.11: Expression of EYFP and Sox 10 in the Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old and 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes sox 10 (B, E). Merged images C and F show an overlap. Scale bar = 20 μm

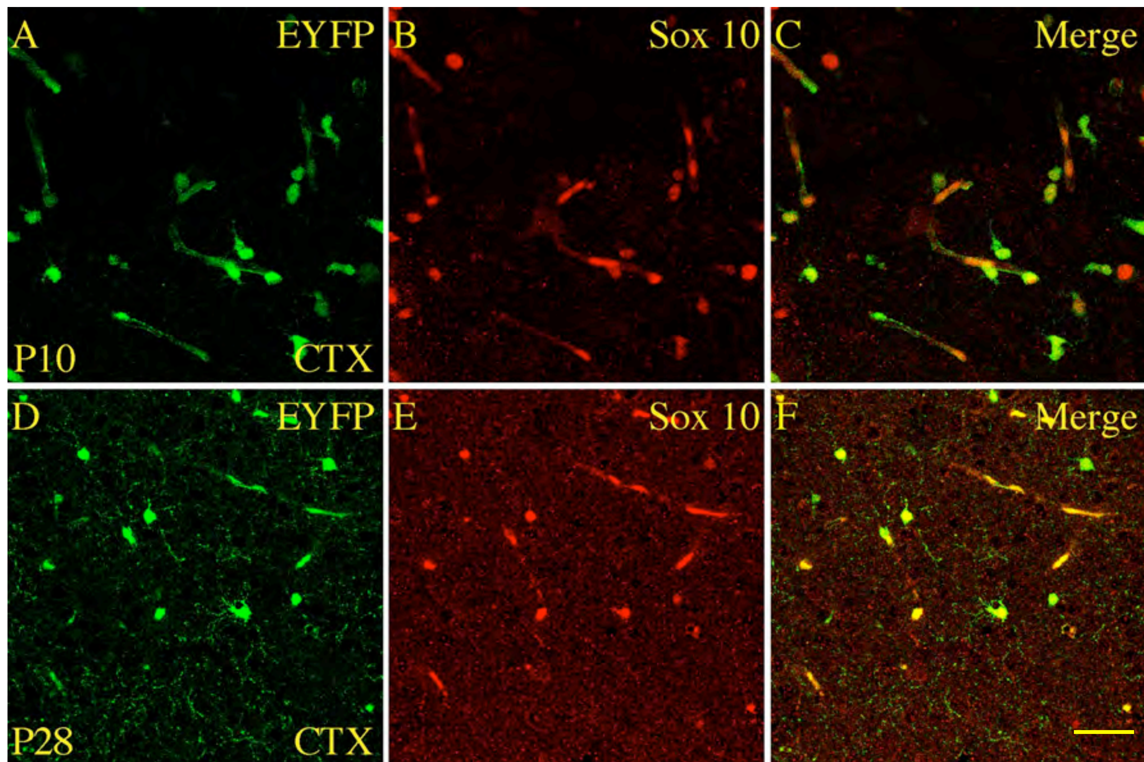


Figure 3.5.12: Expression of EYFP and Sox 10 in the Cortex of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old and 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes sox 10 (B, E). Merged images C and F show an overlap. Scale bar = 40 μm

3.5.2 Expression of Neuron specific antigens by the NG2-EYFP cells in the CNS

No expression was seen by the NG2-EYFP cells of any neuronal markers in either heterozygous or the homozygous animals. Three different neuronal markers were used: Double cortin, Neun, and Beta-III tubulin. Double cortin is a microtubule-associated protein, which is uniquely found in newborn neurons and recognizes all newborn and migrating neurons. Some post mitotic neurons, stain weakly. Developmentally, the antibody reacts with highly proliferative areas of the brain, which include the subventricular zone and the dendate gyrus. In the neonatal and developing mouse brain there was no overlap of expression between the NG2-EYFP+ cells and the Double cortin antibody staining in both the heterozygous and homozygous mouse (data not shown). In the adult mouse proliferation is still seen in these two areas: the subventricular zone and the dendate gyrus. These are areas where neurogenesis is still occurring. No double labeling of the NG2-EYFP+ cells was seen in either the heterozygous or homozygous animals, indicating that the NG2-EYFP cells are not newborn neurons (figures 3.5.13 and 3.5.14).

Beta III-tubulin is a microtubule-associated protein, which is normally expressed in adult neurons, but highly proliferative areas in the brain have been shown to react to this antibody. There was no expression of Beta III-tubulin by the NG2-EYFP+ cells in the heterozygous or homozygous animals (figure 3.5.15), indicating the NG2-EYFP+ cells are not neurons.

Neun is a vertebrate neuron-specific protein expressed by post-mitotic neurons. No Neun staining was observed in any proliferative zone of heterozygous or homozygous animals. There was no overlap of expression of Neun and the NG2-EYFP+ cells. There is a close association between the neurons and the NG2-EYFP cells in different brain regions in the neonatal and adult mouse. This phenomenon appears quite often in both the heterozygous and homozygous NG2-EYFP mice (figures 3.5.15 - 3.5.26). Multiple stainings were done to verify that the NG2-EYFP cells were not neurons. Some NG2-EYFP cells were so close to a neuron that it was difficult to tell them apart. The cell body of the NG2-EYFP cell appeared to be closely aligned to the neuron body. In the NG2-EYFP mouse, the complete cell and its processes are expressing EYFP, and in the neuron

stained for Neun, only the cell body was stained. There was no overlap of colors in these cells that were closely associated, thus indicating that they are really two different cells. To be completely sure, Propidium iodide (PI) was used to stain the DNA and RNA and label nuclei of all cells in the brain. The PI staining showed two closely aligned nuclei, thus confirming the NG2-EYFP+ cells and the Neun expressing cells are two distinct, but closely associated cells (figures 3.5.20 and 3.5.24).

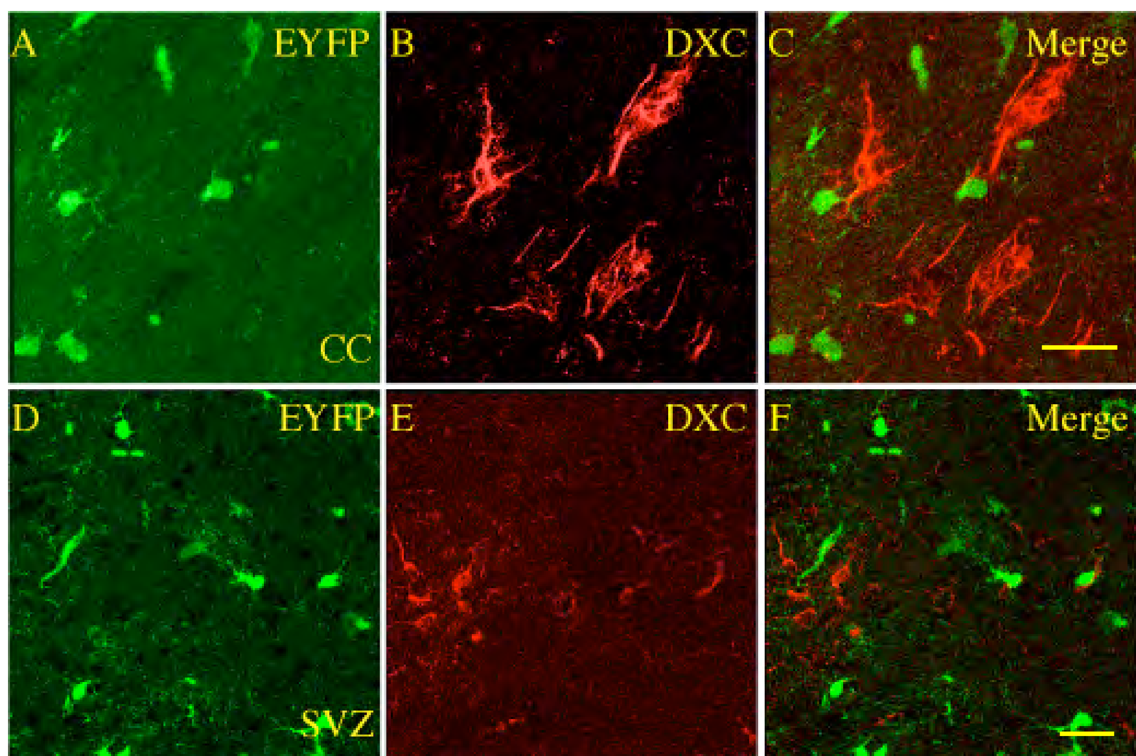


Figure 3.5.13: Expression of EYFP and Double Cortin in the Corpus Callosum and Subventricular Zone of the Heterozygous (+/-) Mouse.
Confocal image scan of the corpus callosum (CC) and subventricular zone (SVZ) of a 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes double cortin (DXC) (B, E). Multiple neuroblasts are seen that are closely associated with the EYFP expressing cells. Merged images C and F show no overlap between EYFP and DXC. Scale bars = 20 μm

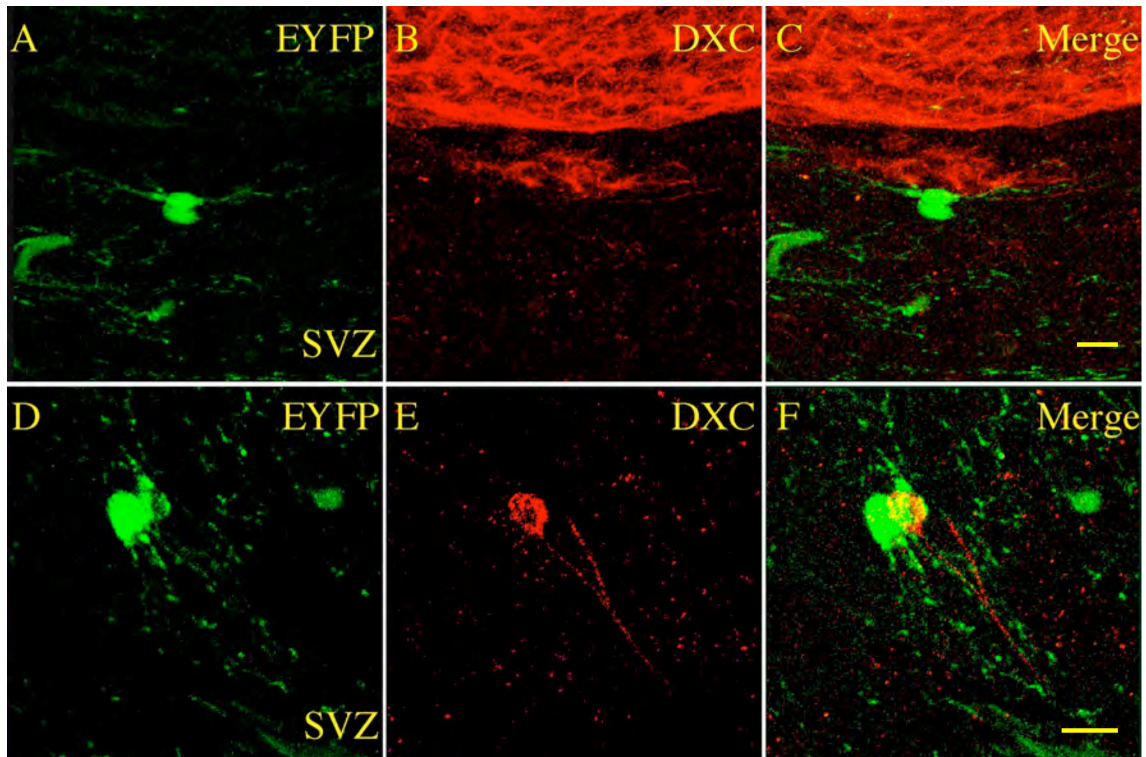


Figure 3.5.14: Expression of EYFP and Double Cortin in the Subventricular Zone of the Homozygous (-/-) Mouse.

Confocal image scan of the subventricular zone (SVZ) of a 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes double cortin (DXC) (B, E). Multiple neuroblasts are seen that are closely associated with the EYFP expressing cells. Merged images C and F show no overlap. C shows a group of newborn neurons closely associated with two EYFP+ cells at the rim of the subventricular zone. F shows a newborn neuron that is associated with 2 EYFP+ cells. Scale bars = 10 μm

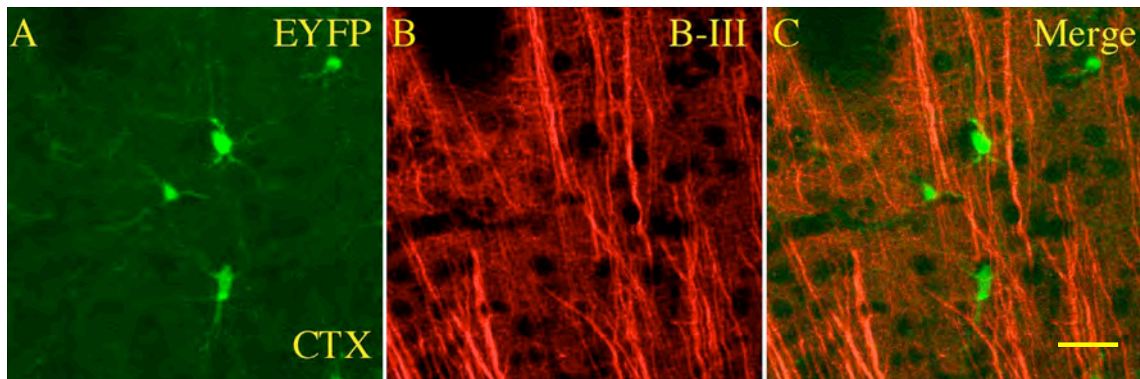


Figure 3.5.15: Expression of EYFP and Beta-III tubulin (microtubule-associated protein for neurons) in the Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 75-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Beta-III tubulin (B). Merged image C shows no overlap between the EYFP and the Beta-III tubulin, but close association. Scale bar = 20 μm

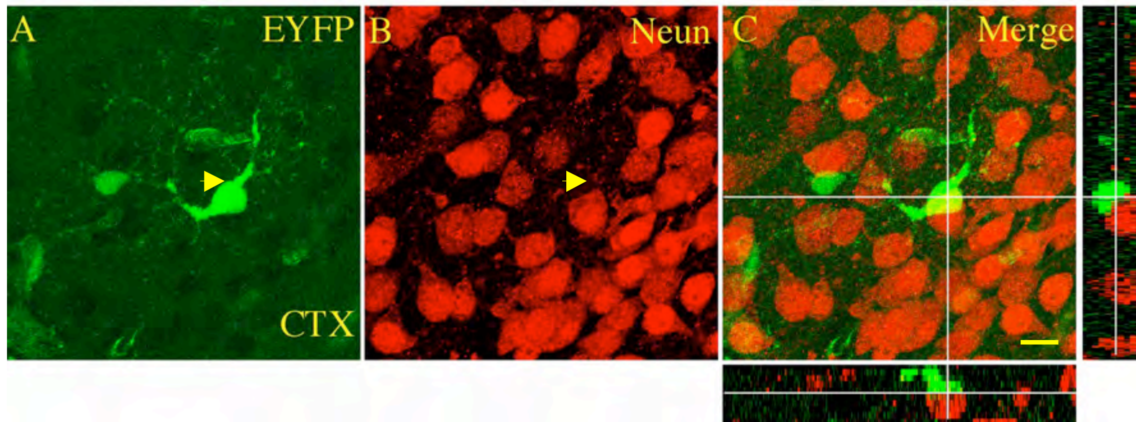


Figure 3.5.16: Expression of EYFP and Neun in the Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Neun (B). Merged image C shows no overlap. Arrow shows that the EYFP+ cell does not stain with the Neun antibody. X, Y and Z axis are represented to show that there is a close association between the EYFP+ cell and the Neun+ neuron. Scale bar = 10 µm

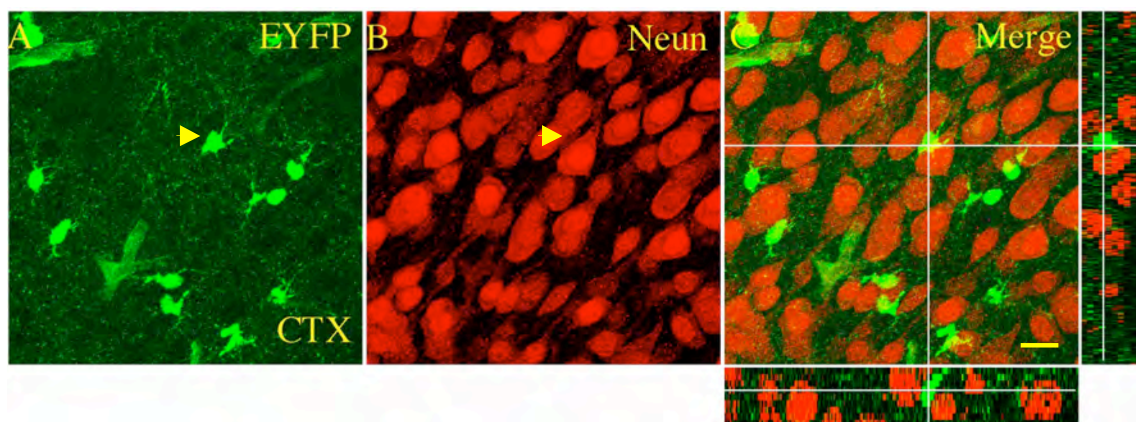


Figure 3.5.17: Expression of EYFP and Neun in the Cortex of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Neun (B). Merged image C shows no overlap. Arrow shows that the EYFP+ cell does not stain with the Neun antibody. X, Y and Z axis are represented to show that there is a close association between the EYFP+ cell and the Neun+ neuron. Scale bar = 20 µm

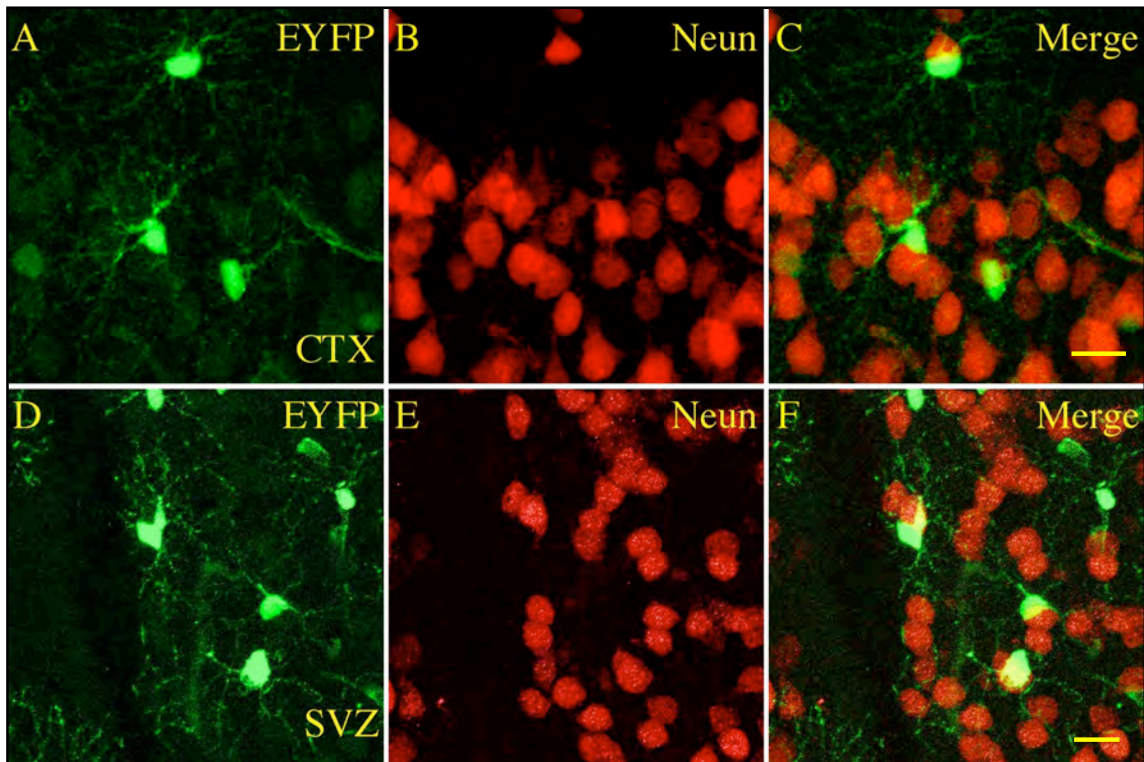


Figure 3.5.18: Expression of EYFP and Neun in the Cortex and the Subventricular Zone of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) and subventricular zone (SVZ) of a 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Neun (B, E). Merged images C and F show no overlap, but close association between the EYFP cells and neurons. Scale bars = 20 μm

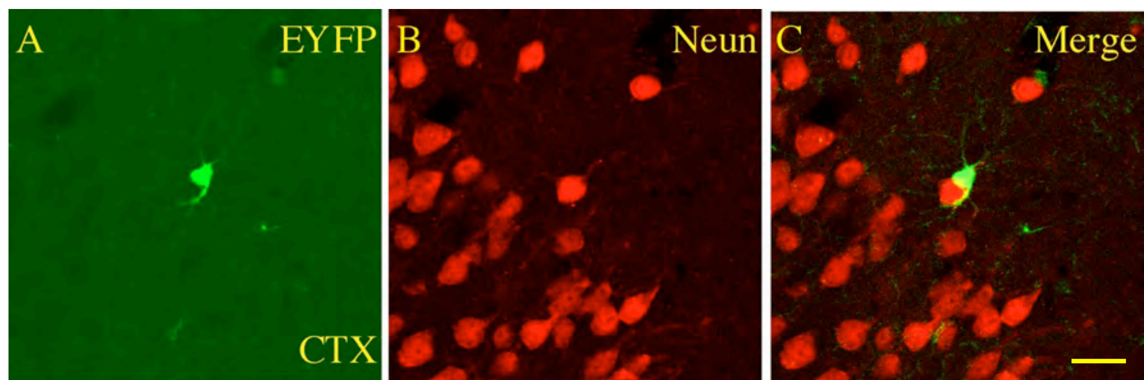


Figure 3.5.19: Expression of EYFP and Neun in the Cortex of the Heterozygous (+/-) Mouse Showing Intimate Contact

Confocal image scan of the cortex (CTX) of a 28-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Neun (B). Merged image C shows no overlap, but close association between an EYFP cell and a Neuron. Scale bars = 20 μm

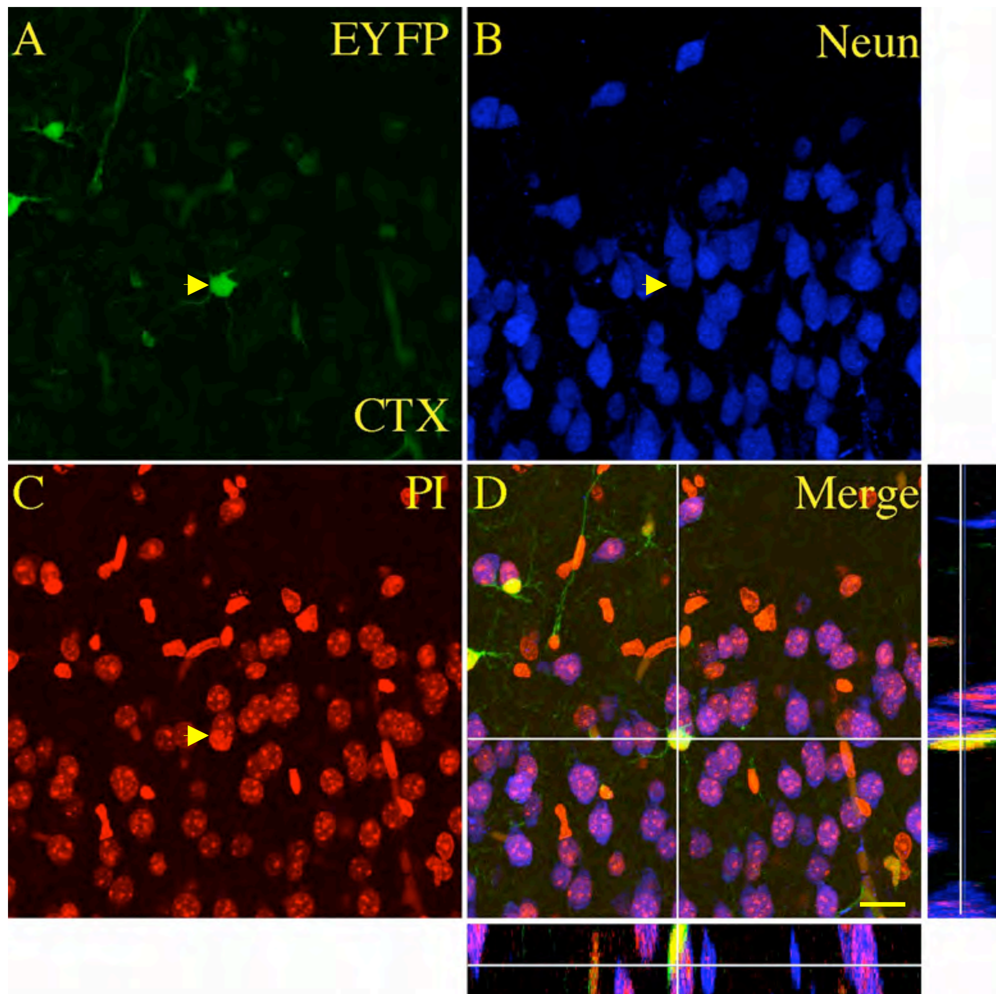


Figure 3.5.20: Expression of EYFP, Neun and Propidium Iodide in the Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 28-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Neun (B) and counterstained with Propidium Iodide (PI, C). Merged image D shows no overlap. Arrow shows that the EYFP+ cell does not stain with the Neun antibody. The PI staining demonstrates that these are two different cells and not one cell. X, Y and Z axis are represented to show that there is a close association between the EYFP+ cell and the Neun+ neuron. Scale bar = 20 μm

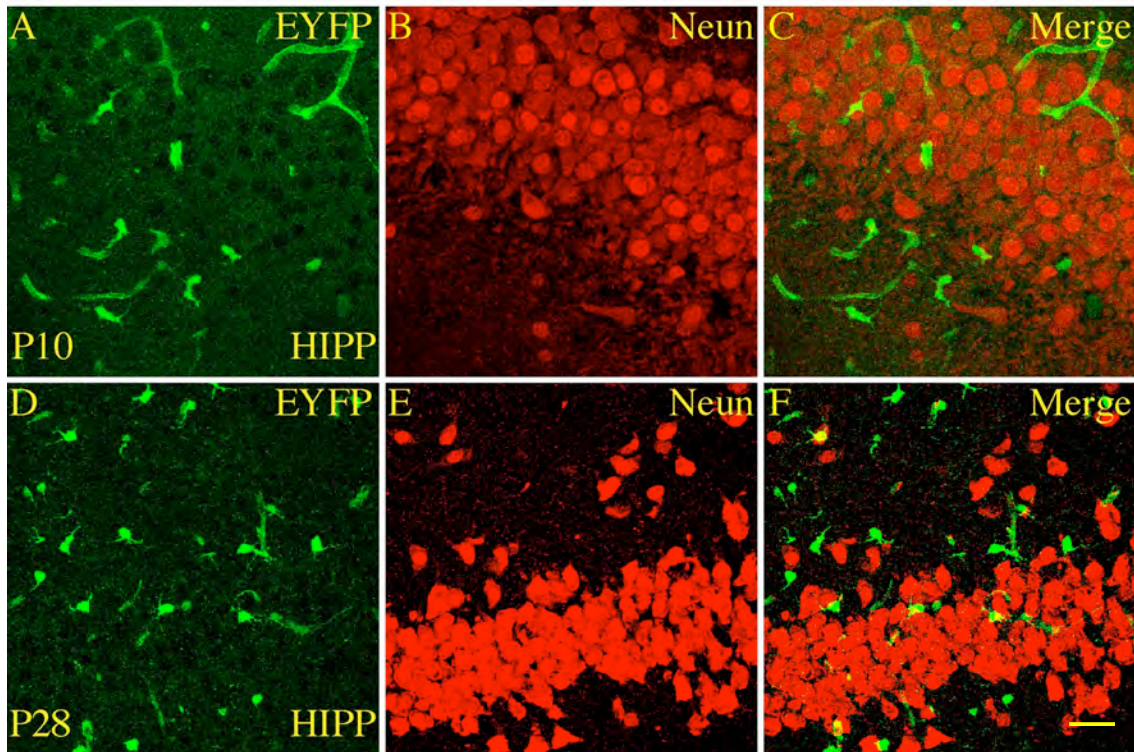


Figure 3.5.21: Expression of EYFP and Neun in the hippocampus of the Heterozygous (+/-) Mouse

Confocal image scan of the hippocampus (HIPP) of a 10-day old and 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Neun (B, E). Merged images C and F show no overlap, but close association between the EYFP+ cells and Neun+ Neurons.

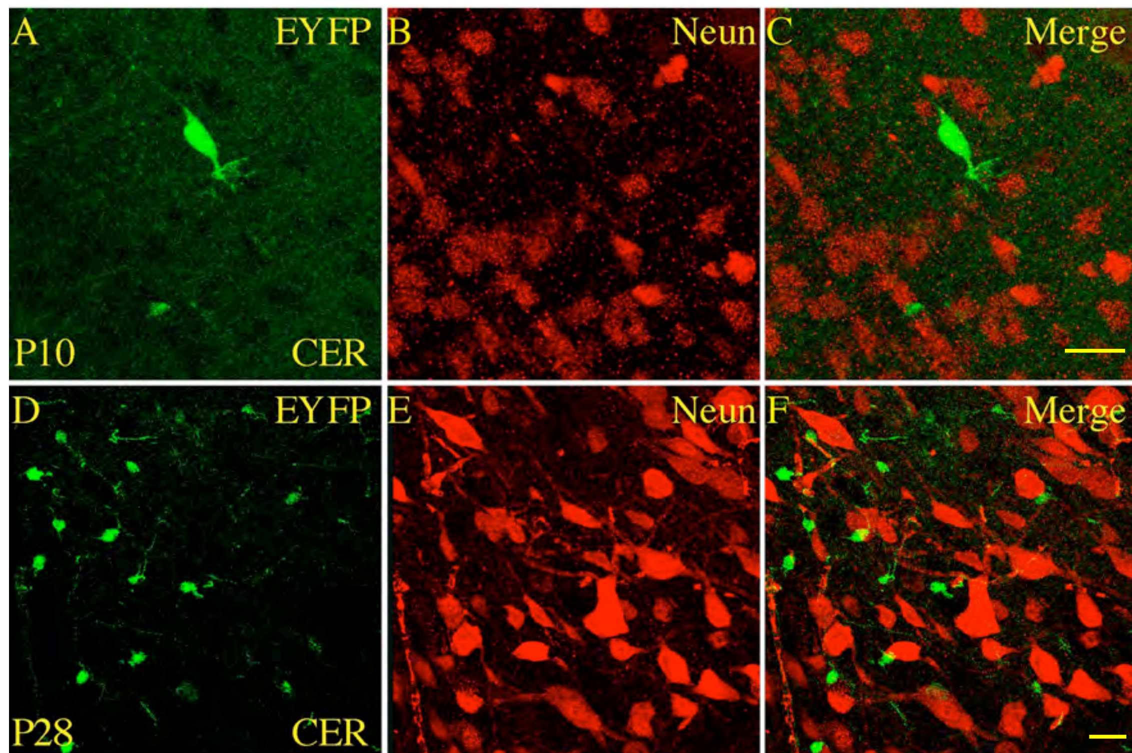


Figure 3.5.22: Expression of EYFP and Neun in the cerebellum of the Heterozygous (+/-) Mouse

Confocal image scan of the cerebellum (CER) of a 10-day old and 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Neun (B, E). Merged images C and F show no overlap, but close association between the EYFP+ cells and Neun+ Neurons.

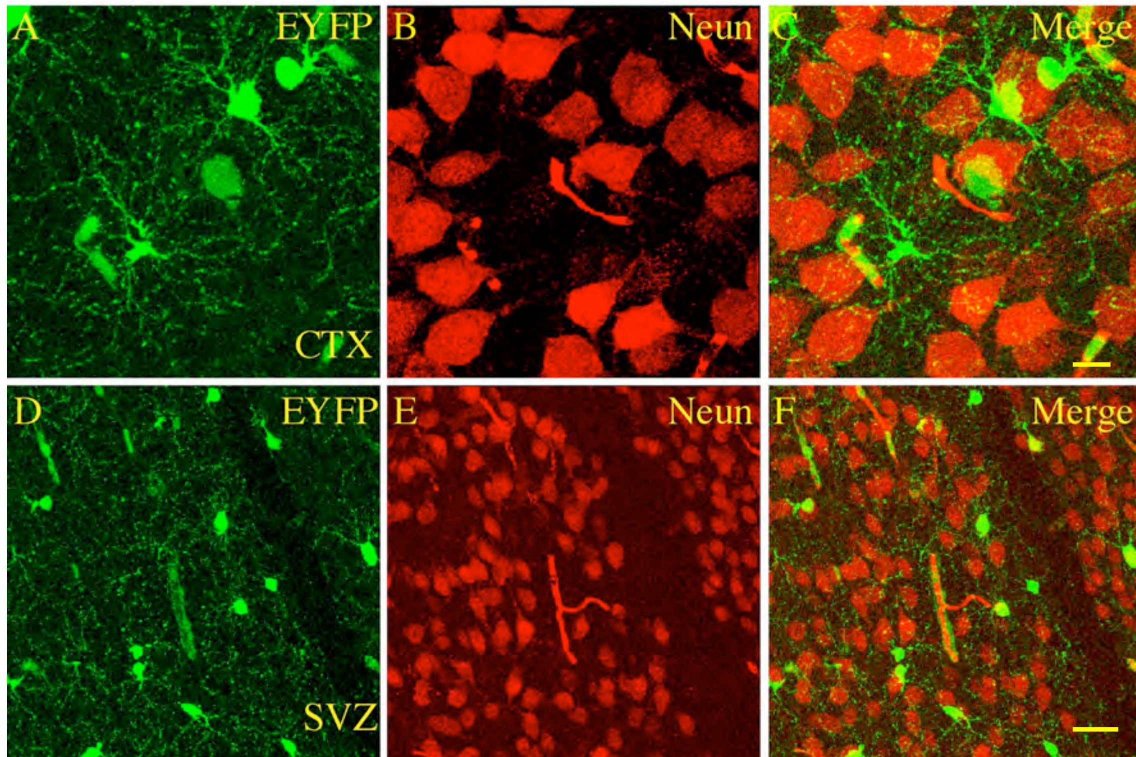


Figure 3.5.23: Expression of EYFP and Neun in the Cortex and the Subventricular Zone of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) and subventricular zone (SVZ) of a 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Neun (B, E). Merged images C and F show no overlap, but close association between the EYFP+ cells and Neun+ neurons.

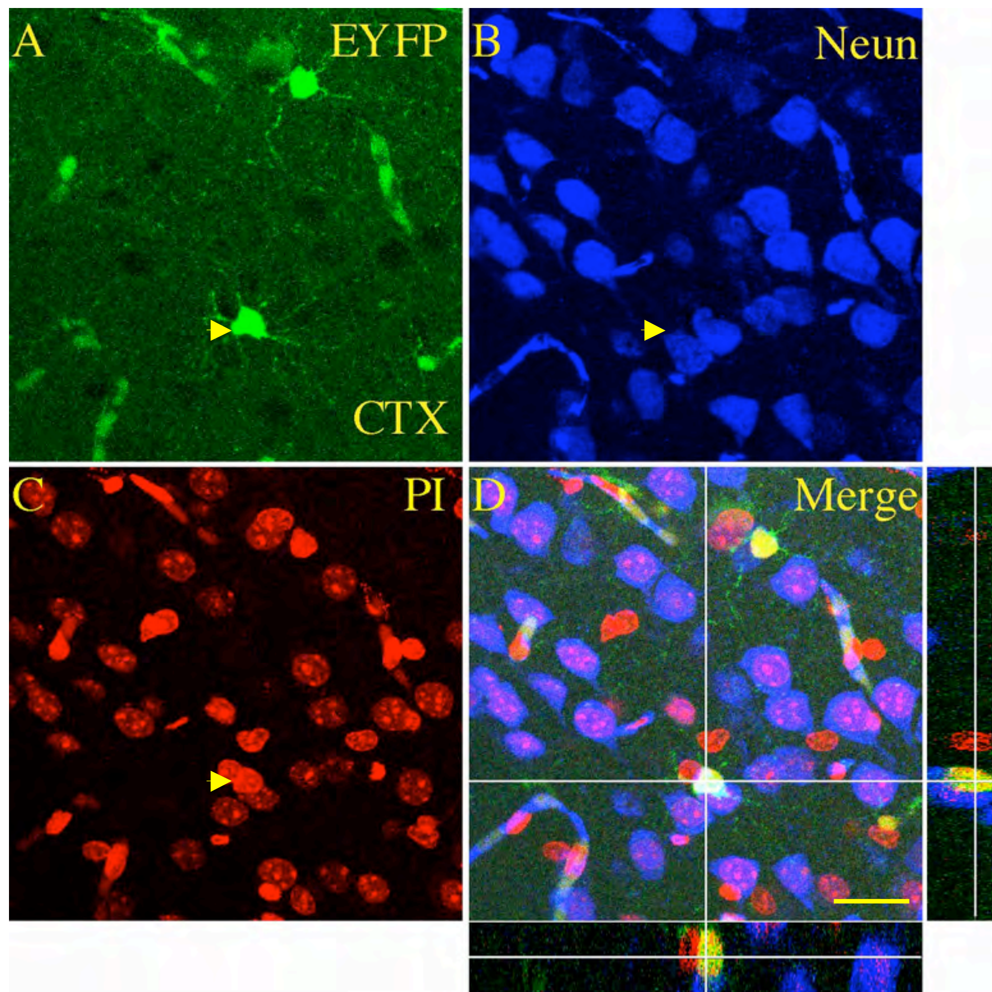


Figure 3.5.24: Expression of EYFP, Neun and Propidium Iodide in the Cortex of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) of a 28-day-old mouse expressing EYFP (A) stained with an antibody that recognizes Neun (B) and counterstained with Propidium Iodide (PI, C). Merged image D shows no overlap. Arrow shows that the EYFP+ cell does not stain with the Neun antibody. The PI staining demonstrates that these are two different cells and not one cell. X, Y and Z axis are represented to show that there is a close association between the EYFP+ cell and the Neun+ neuron. In this particular case there are three cells that are closely associated. A NG2-EYFP cell, a Neun + cell and a cell not labelled for either. Scale bar = 20 μm

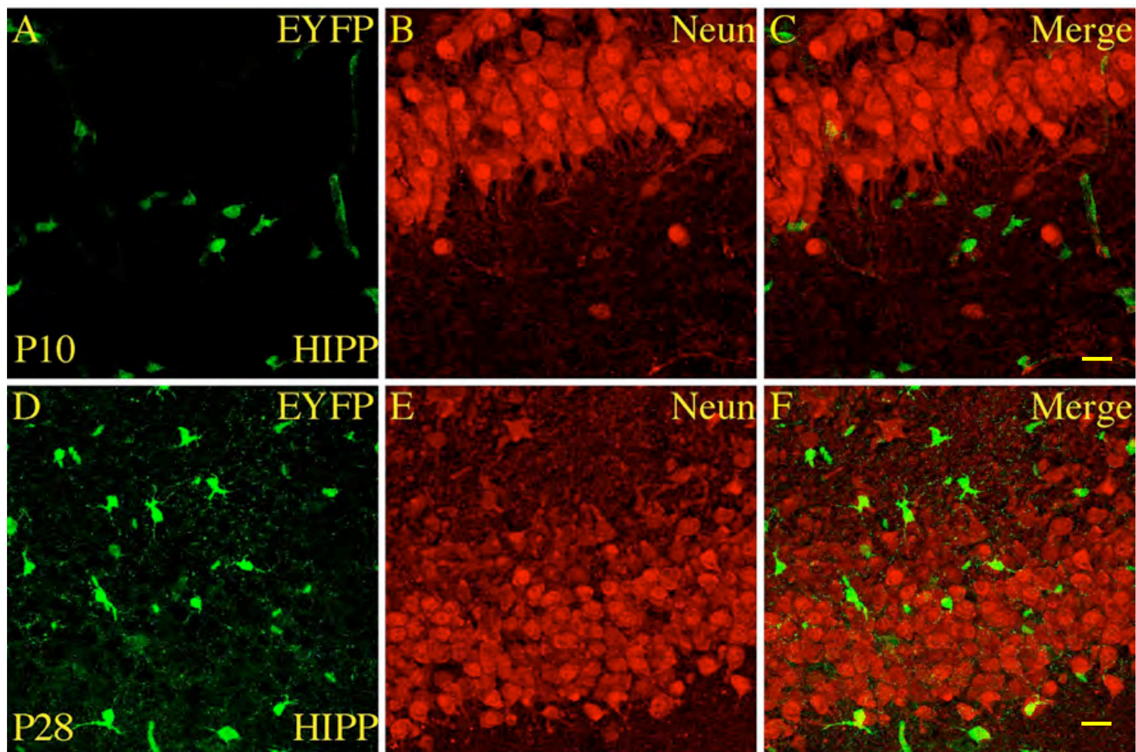


Figure 3.5.25: Expression of EYFP and Neun in the hippocampus of the Homozygous (-/-) mouse

Confocal image scan of the hippocampus (HIPP) of a 10-day old and 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Neun (B, E). Merged images C and F show no overlap, but close association between the EYFP+ cells and Neun+ Neurons. Scale bars = 20 μm

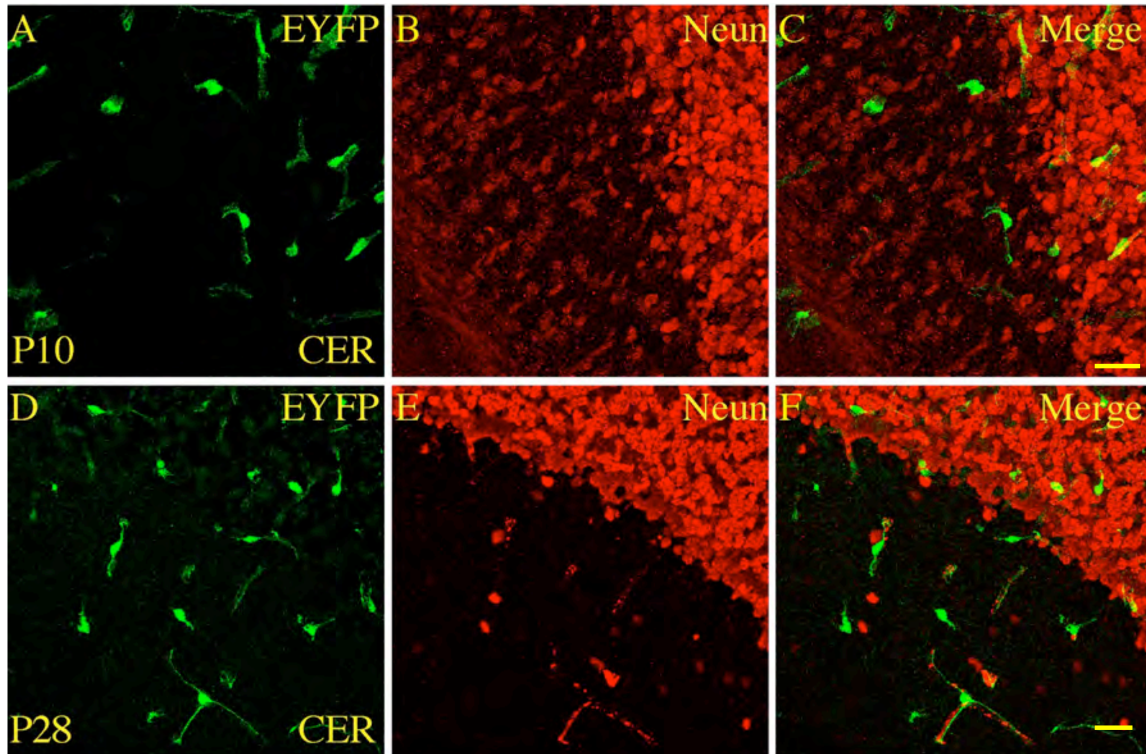


Figure 3.5.26: Expression of EYFP and Neun in the cerebellum of the Homozygous (-/-) mouse

Confocal image scan of the cerebellum (CER) of a 10-day old and 28-day-old mouse expressing EYFP (A, D) stained with an antibody that recognizes Neun (B, E). Merged images C and F show no overlap, but close association between the EYFP+ cells and Neun+ Neurons.

3.5.3 Astrocytes and Microglia

Glial fibrillary acidic protein (GFAP) is a member of the class III intermediate filament protein family. It is heavily and specifically expressed in mature astrocytes in the central nervous system. In addition, some neural stem cells such as radial glia express GFAP. There was no expression of GFAP by NG2-EYFP+ cells in heterozygous or homozygous animals, thus implying the NG2-EYFP+ cells are not astrocytes (figure 3.5.27-3.5.29). A close association between astrocytes and blood vessels was observed (figure 3.5.29).

S100- Beta is a calcium binding, neurotrophic protein produced by non-neuronal cells in the nervous system. There was some overlap of S100-Beta staining and EYFP in various regions of the heterozygous and homozygous animals (figure 3.5.30).

The F4/80 antigen, a 160kD glycoprotein, is expressed by murine macrophages. No expression was observed of F4/80 antibody by NG2-EYFP+ cells (figure 3.5.31).

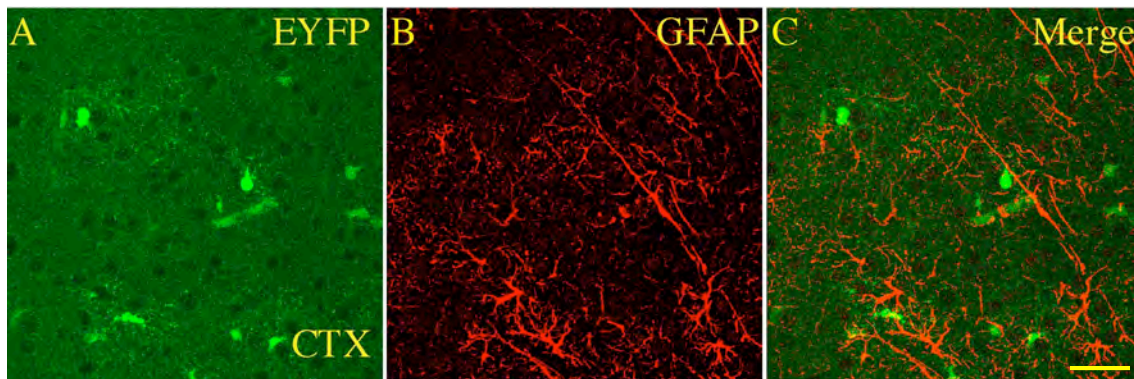


Figure 3.5.27: Expression of EYFP and GFAP in the Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old mouse expressing EYFP (A) stained with an antibody that recognizes GFAP (B). Merged image C shows no overlap. Scale bar = 40 μm

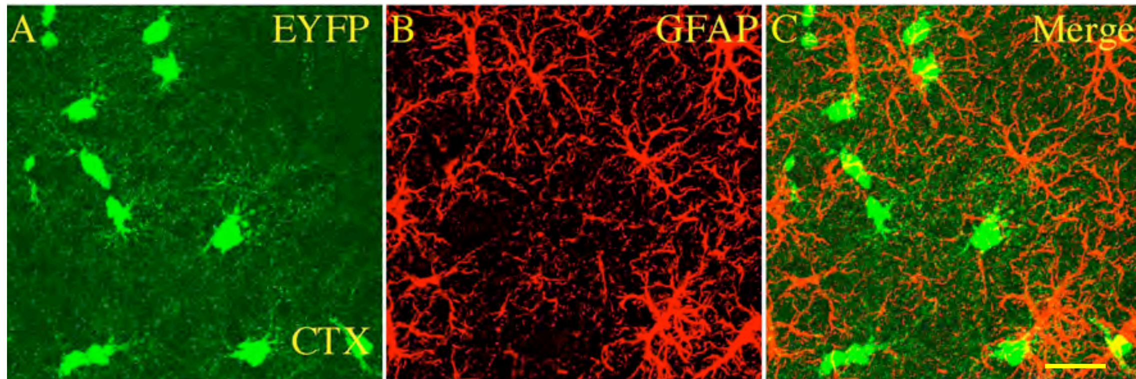


Figure 3.5.28: Expression of EYFP and GFAP in the Cortex of the Homozygous (-/-) Mouse

Confocal image scan of the cortex (CTX) of a 10-day-old mouse expressing EYFP (A) stained with an antibody that recognizes GFAP (B). Merged image C shows no overlap. Scale bar = 20 μm

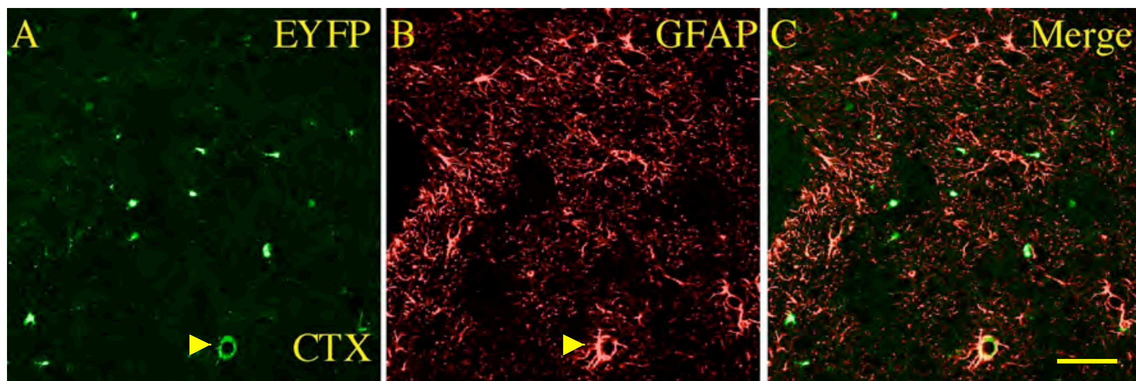


Figure 3.5.29: Expression of EYFP and GFAP in the Cortex (CTX) of a Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 75-day-old mouse expressing EYFP (A) stained with an antibody that recognizes GFAP (B). Merged image C shows no overlap. Arrowhead points to a blood vessel that is EYFP+ that is closely associated to GFAP+ processes. Scale bar = 40 μm

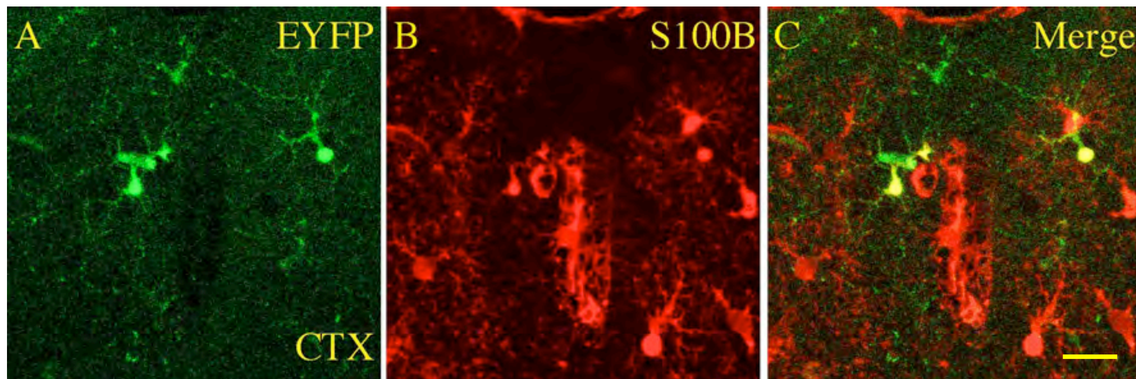


Figure 3.5.30: Expression of EYFP and S100-β in the Cortex of the Heterozygous (+/-) Mouse

Confocal image scan of the cortex (CTX) of a 75-day-old mouse expressing EYFP (A) stained with an antibody that recognizes S100-β (B). Merged image C shows some overlap of EYFP expression and S100-β staining. Scale bar = 20

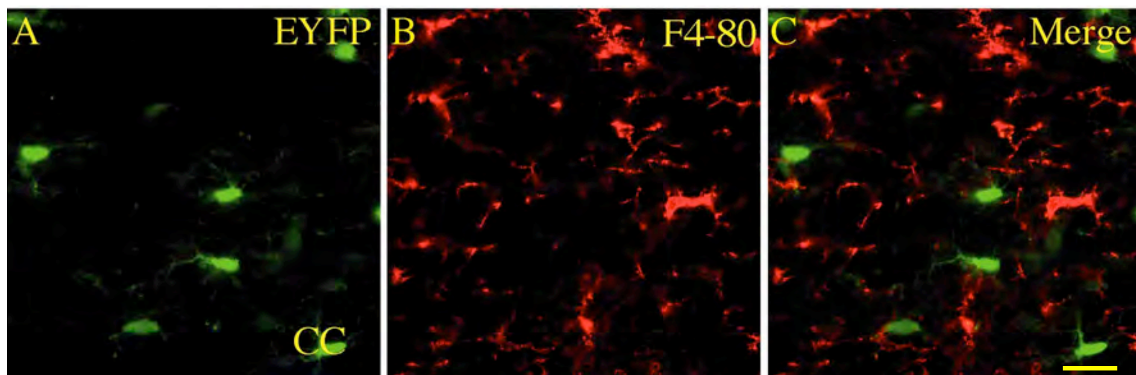


Figure 3.5.31: Expression of EYFP and F4/80 in the Corpus Callosum of a Heterozygous Mouse

Confocal image scan of the corpus callosum (CC) of a 75-day-old mouse expressing EYFP (A) stained with an antibody that recognizes F4/80 (B). Merged image C shows no overlap between EYFP+ expression and F4/80 staining. Scale bar = 20 μm

3.5.4 Schwann cells

NG2 has been reported to be expressed by immature Schwann cells (Schneider et al., 2001). In the NG2-EYFP mouse, multiple elongated cells were observed that expressed EYFP in the sciatic nerve. Stainings were performed with a p75-NTR antibody, as Schwann cells express p75-NTR (Song et al., 2006). It appeared that the EYFP+ were double labeled with the p75-NTR on the surface of the cell. Morphologically the NG2-EYFP+ cells look like Schwann cells.

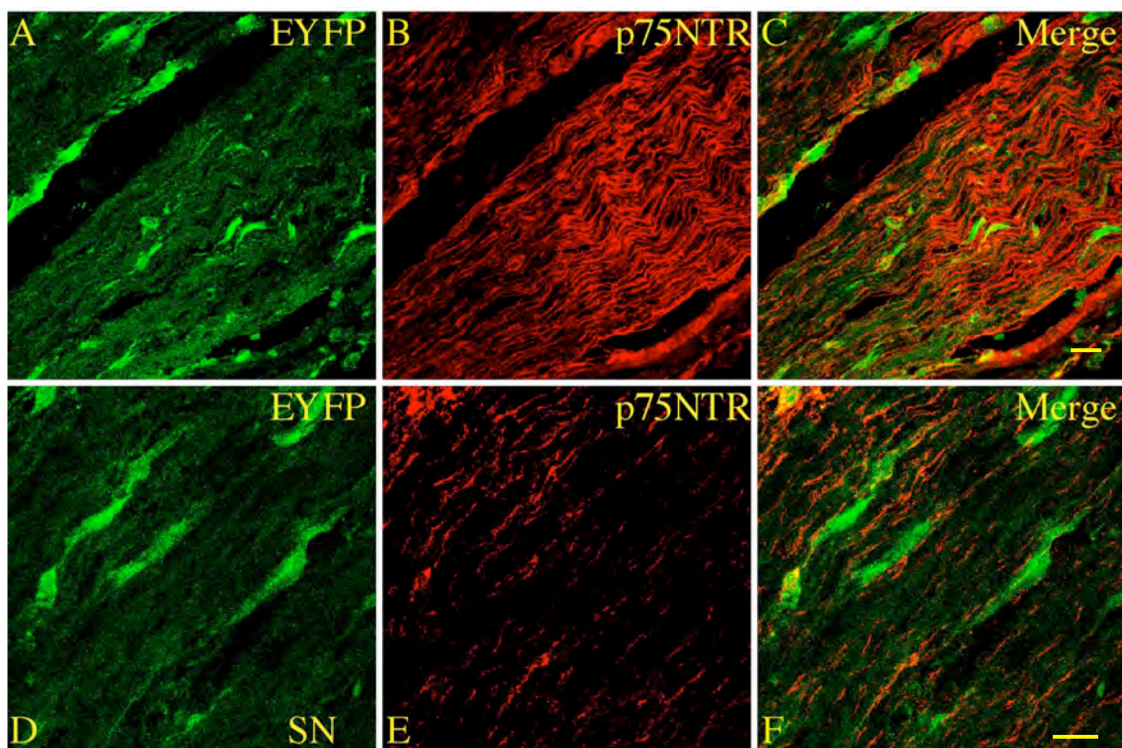


Figure 3.5.32: Expression of EYFP and p75-NTR in the sciatic nerve of the Heterozygous (+/-) mouse

Confocal image scan of the sciatic nerve (SN) of a 5-day old mouse expressing EYFP (A, D) stained with an antibody that recognizes p75-NTR (B, E). Merged images C and F show overlap of p75-NTR expression and EYFP at the cell surface.

3.6 Additional vectors and mouse lines generated

3.6.1 NG2-EYFP-Intron Targeting Vector

The modified pKS-blue script vector containing the NG2-EYFP intron has the following elements (figure 3.6.1):

1. Homologous short arm and the EYFP coding sequence: The short arm, consists of two parts, a DNA fragment about 735 bp that is homologous to the NG2 gene upstream of the open reading frame (ORF) in exon 1 followed by the 5' end of 232 bp of the EYFP gene. There are no known splice variants to the NG2 gene. The promoter region is also very large, therefore the best option was to insert the EYFP directly into the ORF of the NG2 gene. The two fragments were fused together through a fusion PCR giving a fragment of approximately 967 bp. The fusion product was then cloned in the pKS-blue-script by using BamH I / Pst I restriction sites. The 3' fragment of the EYFP of about 514 bp was cut out of the EYFP vector with Pst I / Nde I and cloned into the pKS-blue script vector with the same restriction sites. The over all size of the short arm and the complete EYFP gene is 1.46 KB. The identity of the short arm was checked with restriction digests and sequencing. No mutations were detected during sequencing. Through homologous recombination in the embryonic stem cells, the targeting gene should integrate into the start codon in exon 1 without interfering with the other exons further down stream.
2. Neomycin-resistance gene (Neo^R): The Neo^R gene used was about 1.31 KB and was floxed on either side by Lox P sites for latter excision from the targeted allele. A thymidine-kinase promoter drives the Neo^R gene derived from the *Herpes simplex* virus. The original gene was first amplified by PCR from the pMCNeoPA vector (Stratagene) using primers AN2-Neo anti-sense and AN2-Neo sense. This PCR product was cloned into an intermediate vector containing Lox P sites. The Neo^R gene, which is now floxed on both sides by Lox P sites was then further, amplified through PCR using primers 4937 (neo anti-sense with Fse I restriction site) and primer 4938 (neo sense with Nde I and Not I restriction sites). The final Neo^R was cloned in behind the EYFP gene in the backbone vector pKS-blue-script. Depending on its successful homologous recombination within the embryonic stem cell, the gene provides resistance against the drug G418 giving a positive selection technique to screen resistant clones.

3. Homologous long arm: A long arm of 5.3 KB was used which was made up of the 3' end of exon 1, directly behind the Neo^R gene. The long arm did not contain any part of exon 2 of the gene, due to the fact that the intron between exon 1 and exon 2 is over 13 KB. A long distance PCR was first attempted to generate this large fragment by using the following primers AN2-LA anti-sense and AN2-LA sense from genomic DNA. This long distance PCR method did not work as was expected therefore an alternative method was used to get around this problem of amplifying this large product. When looking at the homologous long arm nucleotide sequence, one major feature is clearly visible which is right in the middle of the 5.3 KB long arm is a Sac I restriction site. This is unique only to the long arm and not the short arm. The Sac I restriction site cuts the long arm in half giving a fragment of 2.6 KB and 2.7 KB. It is easier first to amplify these smaller fragments by PCR. The fragments are then fused together to make up the homologous long arm. For this to happen another vector was designed for this step. By removing the multiple cloning sites from the pSP72 vector (2.9 KB) and replacing it with an artificial multiple cloning sites designed to integrate the two fragments of the homologous long arm. The old multiple cloning site was removed by cutting with EcoR V and Xho I. The new multiple cloning sites contained the following restriction sites in the following order: EcoR V, Fse I, Sac I, and Xho I. The individual fragments were generated by PCR amplification using primers LA anti-sense 1 and LA sense 1 for the first fragment of 2.7 KB, with the primers restriction sites Fse I and Sac I are added artificially to the amplified product. For the second fragment LA anti-sense 2 and LA sense 2 were used and restriction sites Sac I and Xho I were brought in. After PCR amplification the fragments were cloned into the intermediate vector the modified pSP72 vector. The homologous long arm was double checked through restriction digests and sequencing. Four different restriction digests were done to verify the identity of the long arm. It appeared that the homologous long arm was correct. The pSP72 vector was then sent for sequencing to check for mutations. Multiple primers were generated for primer walking. Five mutations were found in the homologous long arm, but the mutations were downstream from exon 1. Since the homologous long arm is mostly intron, it was used in the final targeting vector.
4. Artificial intron containing a poly-A tail: An artificial intron containing a poly-A tail was cloned into the backbone vector as the final cloning step for the **pKS-AN2-**

EYFP-intron. The intron that contains a poly-A tail that comes from the rabbit Beta globulin gene. It was PCR amplified from the PUHG-17.1 vector with anti-sense primer gIPA2 and sense primer gIPA1, which gave a product of 1.21 KB. Not I restriction sites were added at the ends of the PCR product which allowed it to be cloned into the backbone vector. Problems arose due to the fact that the Not I restriction site did not cut well at the end of the PCR product. The PCR product was first cloned into an intermediate vector p-GMT with T-A overhangs. The product was then sent for sequencing for mutations. The sequencing results showed no mutations present, so the intron was cut out of the intermediate vector and cloned into the backbone vector. Since there was only one restriction site the Not I, orientation of the insert was checked by a double digest with restriction enzymes Xba I and Fse I. When the intron is correctly orientated six bands were seen on the agarose gel (4.5 KB, 3 KB, 2.5 KB, 864 bp, 665 bp, 473 bp), When the intron orientation is backwards also six bands were seen, but with different sizes as compared to the correct orientation (5.78 KB, 3 KB, 1.3 KB, 864 bp, 665 bp, 473 bp). Out of 16 clones picked, eight clones had the correctly orientated intron with poly-A tail (data not shown).

5. Cloning vector backbone: The pKS-blue-script II multiple cloning site was exchanged by restriction digestion with Kpn I and Sac II. Another multiple cloning site was cloned in, containing the unique restriction sites in the following order: Kpn I, BamH I, Pst I, Not I, Nde I, Fse I, Xho I, Xma I, and Sac II. The 3' end of the EYFP was cloned in first followed by the short arm fused to the 5' end of the rest of the EYFP gene. The homologous long arm was then cloned into the backbone vector. The final step was cloning in the Neo^R gene that is floxed by the Lox P sites. The final vector was checked through restriction digests with seven different restriction enzymes BamH I, Bsg I, Hind III, Nae I, Nhe I, Xmn I and Pst I. From these restriction digests the targeting vector appeared to be correct. The targeting vector was double checked by sequencing over the unique restriction sites to look for any mutations. No new mutations were observed within the target vector. The final targeting vector **pKS-AN2-EYFP-Intron** is 12.14 KB, 250 μg was linearized before electroporation in embryonic stem cells with Xho I.

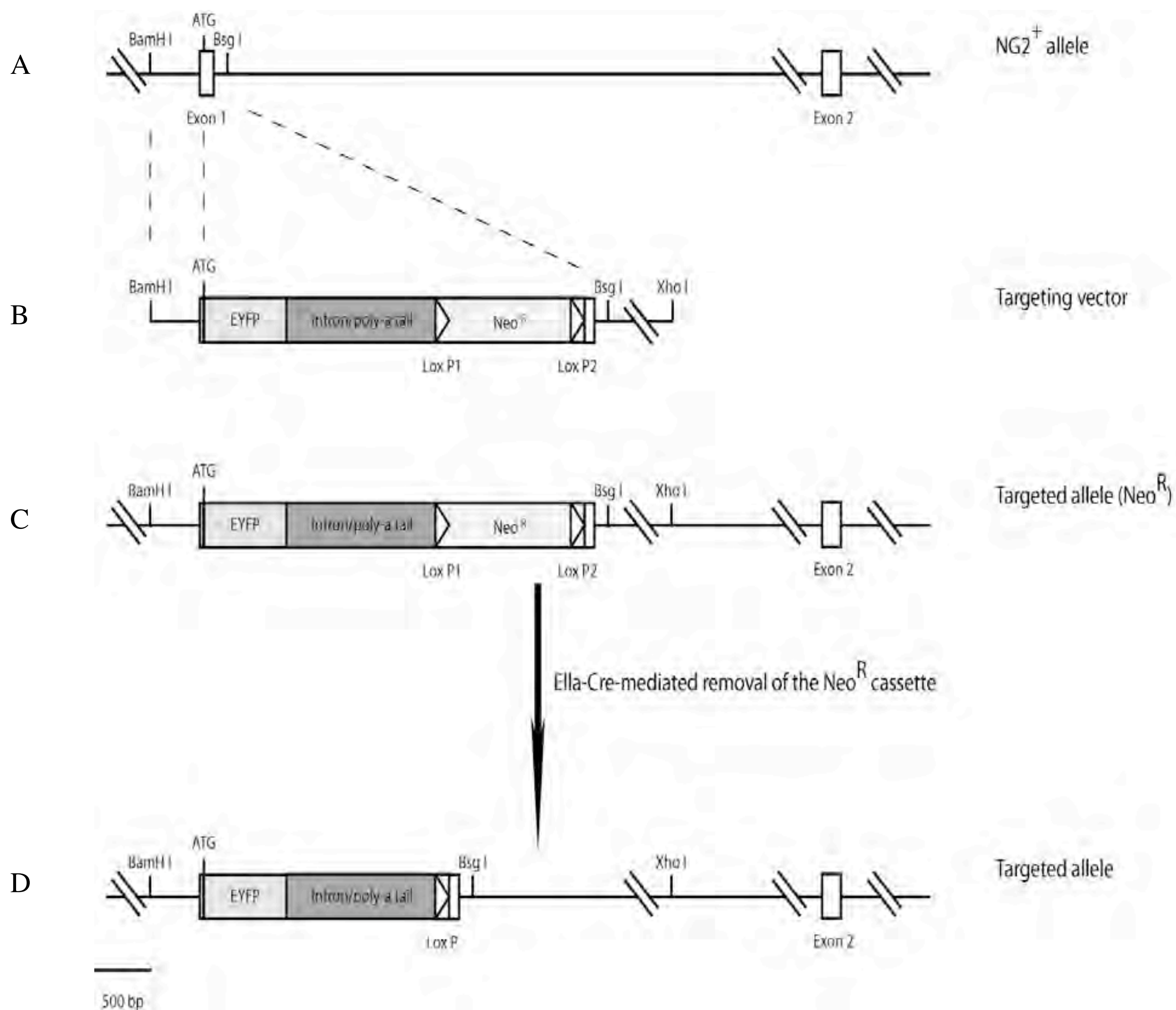


Figure 3.6.1: Schematic Diagram of NG2-EYFP-Intron Targeting Strategy for Homologous Recombination in Embryonic Stem Cells.

A) Wild-type NG2 allele, B) targeting vector containing the EYFP gene and the Neo^R gene, C) targeted allele after homologous recombination in embryonic stem cells, and D) targeted allele after the excision of the Neo^R gene by breeding F1 mouse generation with Ella-Cre mouse. F2 generation lacked the Neo^R gene. (Lox P sites in diagram are not to scale)

3.6.2 NG2-EYFP-Intron Homologous Recombination in Embryonic Stem Cells

3.6.2.1 PCR identification of homologously recombined embryonic stem cell clones

For the identification of homologously recombined embryonic stem cell clones containing the targeting vector, PCR amplification was done with the anti-sense primer 3313 that is located near the 5' end of the EYFP gene and the sense primer 3305 that is located upstream from the 5' end of the short arm of the targeting vector. A product of about 996 bp was observed in correctly recombined embryonic clones. If the targeting vector inserted anywhere else, this PCR would not have amplified a product. For optimization of this PCR a control plasmid was designed. A large fragment of 1.37 KB containing exon 1 of the NG2 gene, the homologous short arm and 300 bp upstream from the short arm was PCR amplified and cloned into the original EYFP plasmid (Clonetech) by Kpn I restriction sites. The anti-sense and sense primer for the EYFP targeting vector were optimized using this control plasmid.

To improve the specificity of the control PCR, a "nested" PCR was performed, where an intermediate product was first amplified. Then another set of primers was used on the intermediate product to give a second product that was seen on the agarose gel. The primers used are anti-sense 3311 that is upstream from 3313 and sense primer 3307 that is downstream from 3305. For the first reaction with primers 3313 and 3305, 19 cycles were done with an annealing temperature of 58°C. From the first PCR 1/5 of the product was used for the second PCR reaction for 39 cycles with an annealing temperature of 58°C. The final product of 996 bp is slightly smaller then the control PCR product of 1.15 KB. This ruled out any possible contaminations from the control plasmid. The nested PCR was optimized to where it was possible to detect 200 copies, 20 copies and sometimes two copies of the control plasmid. This was a powerful tool to determine how many copies of the targeting vector are present in the positively recombined embryonic stem cell clone. This nested PCR technique is required, because of a lack of starting DNA material. Normally an embryonic stem cell clone consists from anywhere between 50-1000 cells. Through the nested PCR method only a minute amount of starting material is required to visualize a positive clone. To improve the working quality of the nested PCR Q-solution (Qiagen) was added.

3.6.2.2 Isolation of homologously recombined embryonic stem cell clones

The OLA-129 embryonic stem cell line with a passage P8 was electroporated with 50 μg of linearized targeting vector **pKS-AN2-EYFP-Intron.** 24 hours after electroporation, G418 selection was started and continued for 7-10 days. The first three days of selection the embryonic stem cells were growing at a normal rate and very little cell death was observed in the first days of selection. G418 selection medium was changed every 24 hours. Between days four to six there was massive cells death observed among the embryonic stem cells indicating some cells did not integrate the targeting vector carrying the resistance gene. On day nine of G418 selection, clones were picked and transferred to a 96 well plate. 2/3 of the clone was used for DNA isolation and 1/3 was plated onto a 96 well plate containing EMFI feeders for further expansion. When the clone was found in a pool of eight cells, then individual clones were expanded in the presence of G418 onto a 24 well dish. After every passage, the clone was double checked by PCR. The clone was expanded until it was large enough to freeze down and inject into blastocytes. DNA from eight clones was pooled and analyzed by PCR using the control plasmid (figure 3.6.2). By this method, 384 clones were selected and screened. After the initial check eight clones appeared to be positive, but after further passaging, one out of the eight clones still remained. In the end, out of 384 clones only one positive clone was found that had homologously recombined the targeting vector. Clone 5F clone was used for establishing the NG2-EYFP-intron mouse line.

3.6.2.3 Germ line transmission of embryonic stem cell clone 5F

The embryonic stem cell clone 5F that was identified through PCR and southern blot analysis was injected into blastocytes (3.5 dpc) from C57Bl6/J mouse line. After transferring the blastocytes into a pseudo-pregnant mouse mother from the mouse line NMRI, male chimeric mice were born (brown fur usually meant 100% chimeric). High chimeric male mice were taken for further breeding with C57Bl6/J female mice to establish the mouse line. About half of the F1 generation carried the mutated NG2 gene (Figure 3.6.3). One mouse from the F1 generation was histologically analyzed to see if there was expression of the EYFP. Expression was observed in the F1 generation (data not shown). Since the Neo^R gene interferes with gene regulation, as a precaution it was removed from the F2 generation. F1 males carrying the modified NG2 gene were bred to Ella-Cre female mice in order to selectively excise the Neo^R gene from the F2 generation (data not shown).

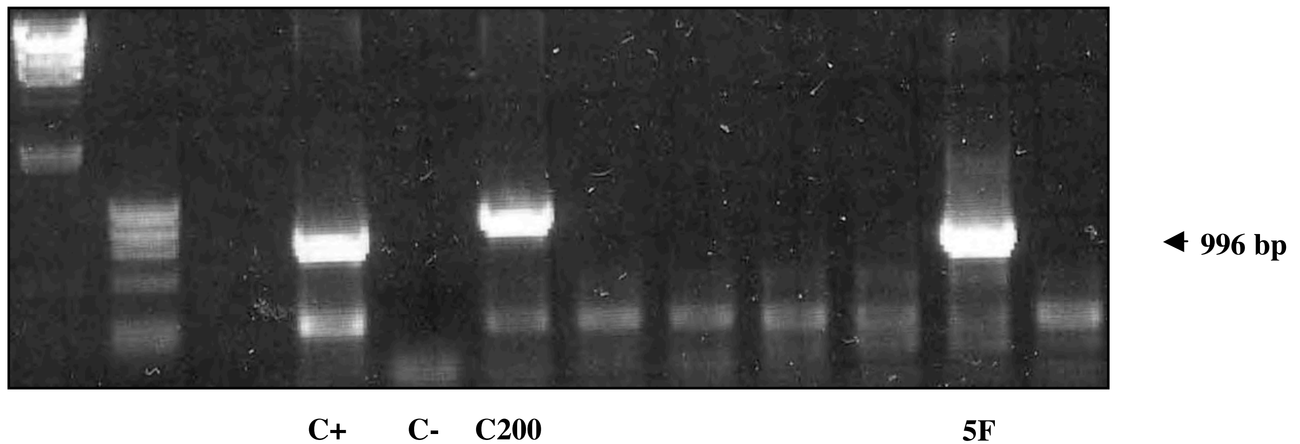


Figure 3.6.2: PCR Screening for Homologous Recombination
PCR screening of embryonic stem cells showed that one clone (5 F) integrated the targeting vector through homologous recombination giving a band at 996 bp. C+) positive ES cell clone (996 bp), C-) negative control, and C 200) control plasmid with 200 copies (1.15 KB).

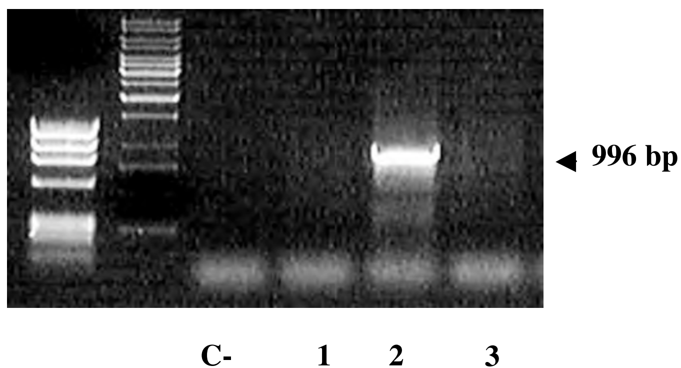


Figure 3.6.3: Germ line Transmission
A) Genotyping of F1 offspring to check for the presence of the EYFP transgene. Animal number 2 is positive indicating germ line transmission, while animal numbers 1 and 3 are negative. C-) neg. con.

3.6.3 NG2-Cre Targeting Vector

The modified pKS-blue script vector containing the NG2-Cre has the following elements (figure 3.6.4):

1. Homologous short arm and the pMC-Cre coding sequence The short arm, consists of two parts, a DNA fragment about 735 bp that is homologous to the NG2 gene upstream of the open reading frame (ORF) in exon 1 followed by the 5' end of 386 bp of the Cre gene. There are no known splice variants to the NG2 gene. The promoter region is also very large, therefore the best option was to insert the Cre directly into the ORF of the NG2 gene. The two fragments were fused together through a fusion PCR giving a fragment of approximately 1.1 KB. The fusion product was then cloned in the pKS-blue-script by using BamH I restriction site. The orientation of the NG2-Cre fusion product was checked with the following restriction enzymes: Kpn I and Xmn I. After the restriction enzyme digest, correct orientation was determined by the DNA band pattern. The orientation was correct when three bands with sizes of 1.8 KB, 1.9 KB and 958 bp were seen. Out of 24 bacterial clones picked, eight had the correct orientation. The 3' fragment of the Cre of 724 bp length was cut out of the pMC-Cre vector with BamH I / Nde I and cloned into the pKS-blue script vector, with the same restriction sites. The over all size of the short arm and the complete Cre gene is 1.835 KB. The identity of the short arm was checked with restriction digests and sequencing. One point mutation was detected during sequencing, upstream from the start codon, outside the exon 1. It was decided, that this mutation would not hinder the targeting vector. Through homologous recombination in the embryonic stem cells, the targeting should integrate into the start codon in exon 1 without interfering with the other exons further downstream. This resembles the closest expression pattern to that of the wild-type gene.
2. Neomycin-resistance gene (Neo^R): The Neo^R gene used was about 1.31 KB and was floxed on either side by Lox P sites for latter excision from the targeted allele. A thymidine-kinase promoter drives the Neo^R gene derived from the *Herpes simplex* virus. The original gene was first amplified by PCR from the pMC-Neo-PA vector (Stratagene) using primers AN2-Neo anti-sense and AN2-Neo sense. This PCR product was cloned into an intermediate vector containing Lox P sites. The Neo^R gene, which is now floxed on both sides by Lox P sites was then further, amplified through PCR using primers 4937 (neo anti-sense with Fse I restriction

site) and primer 4938 (neo sense with Nde I and Not I restriction sites). The final Neo^R could not be cloned in behind the Cre gene in the backbone vector pKS-blue-script. The problem was that the Cre recombinase gene was turned on in the bacteria cutting out the Neo^R. Multiple strategies were used to overcome this problem, but none worked properly. In the end, the solution was to replace the Lox P sites with Frt-sites. The same Neo^R was amplified from a vector called pCon-KO-true, where the gene was floxed on both sides by Frt-sites. The primers used are FRT Neo anti-sense that added restriction site Fse I and FRT Neo sense that added Nde I and Not I. This was successfully cloned into the vector backbone. Out of eight bacterial clones picked, all eight carried the new Neo^R that was flanked by Frt-sites. Depending on its successful homologous recombination with in the embryonic stem cell, the gene provides resistance against the drug G418 giving a positive selection technique to screen resistant clones.

3. Homologous long arm: A long arm of 5.3 KB was used which was made up of the 3' end of exon 1, directly behind the Neo^R gene. The long arm did not contain any part of exon 2 of the gene, due to the fact that the intron between exon 1 and exon 2 is over 13 KB. A long distance PCR was first attempted to generate this large fragment by using the following primers AN2-LA anti-sense and AN2-LA sense from genomic DNA. This long distance PCR method did not work as was expected, therefore an alternative method was used to get around this problem of amplifying such a large product. When looking at the homologous long arm nucleotide sequence, one major feature is clearly visible, which is right in the middle of the 5.3 KB long arm is a Sac I restriction site. This is unique only to the long arm and not the short arm. The Sac I restriction site cuts the long arm in half giving a fragment of 2.6 KB and 2.7 KB. It is easier first to amplify these smaller fragments by PCR. The fragments are then fused together to make up the homologous long arm. For this to happen another vector was designed for this step. By removing the multiple cloning site from the pSP72 vector (2.9 KB) and replacing it with an artificial multiple cloning site designed to integrate the two fragments of the homologous long arm. The old multiple cloning site was removed by cutting with EcoR V and Xho I. The new multiple cloning site contained the following restriction sites in the following order: EcoR V, Fse I, Sac I, and Xho I. The individual fragments were generated by PCR amplification using primers LA anti-sense 1 and LA sense 1 for the first fragment of 2.7 KB, so the primers

restriction site Fse I and Sac I were brought in. For the second fragment LA anti-sense 2 and LA sense 2 were used and restriction sites Sac I and Xho I were brought in. After PCR amplification the fragments were cloned into the intermediate vector, the modified pSP72 vector. The homologous long arm was double checked through restriction digests and sequencing. Four different restriction digests were done to verify the identity of the long arm. It appeared that the homologous long arm was correct. The pSP72 vector was then sent for sequencing to check for mutations. Multiple primers were generated for primer walking. Five mutations were found in the homologous long arm, but the mutations were down stream from exon 1. Since the homologous long arm is mostly intron, it was used in the final targeting vector.

4. Cloning vector backbone: The pKS-blue-script II multiple cloning site was exchanged by restriction digestion with Kpn I and Sac II. Another multiple cloning site was cloned in, containing the unique restriction sites in the following order: Kpn I, BamH I, Pst I, Not I, Nde I, Fse I, Xho I, Xma I, and Sac II. The 3’ end of the Cre was cloned in first followed by the short arm fused to the 5’ end of the rest of the Cre gene. The homologous long arm was then cloned into the backbone vector. The final step was cloning in the Neo^R gene that is floxed by the Frt sites. The final vector was checked through restriction digests with six different restriction enzymes BamH I, Bsg I, Hind III, Nae I, Xmn I and Pst I. From these restriction digests the targeting vector appeared to be correct. The targeting vector was double checked by sequencing over the unique restriction sites to look for any mutations. No new mutations were observed within the target vector. The final targeting vector **pKS-AN2-Cre** is about 11.3 KB long, 250 μg of it was linearized before electroporation in embryonic stem cells with Xho I.

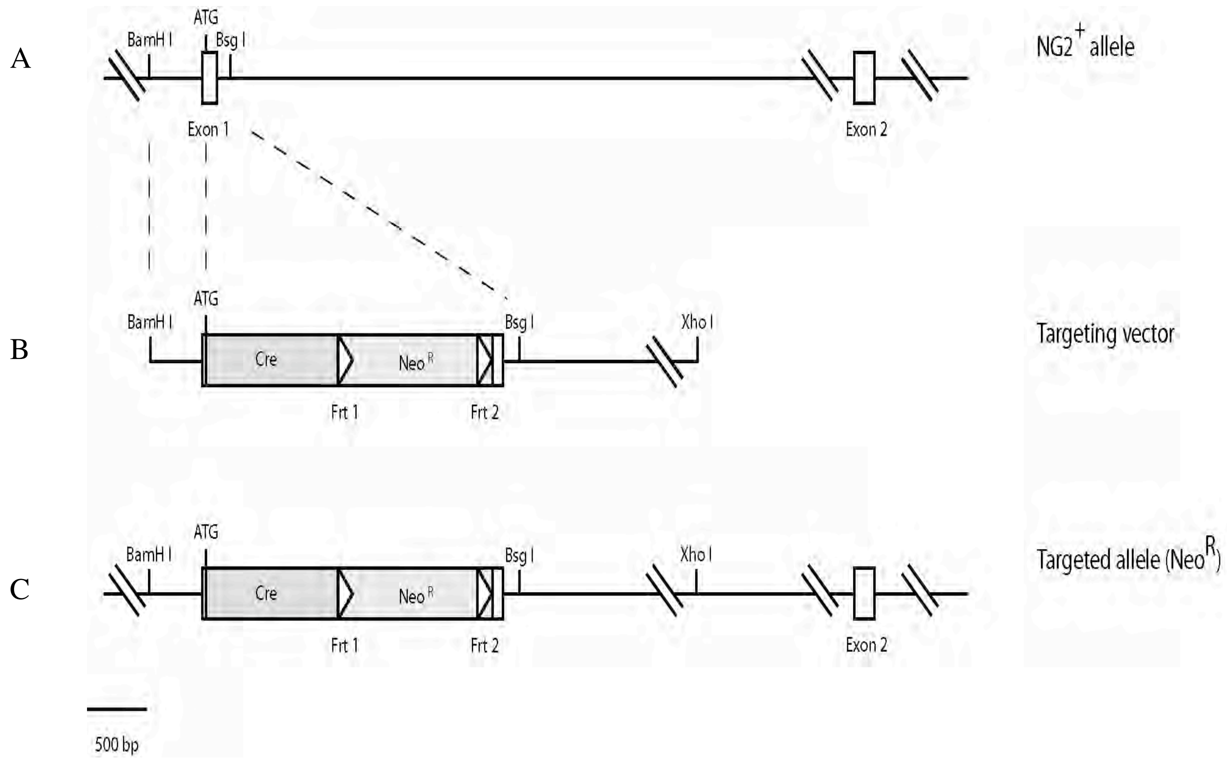


Figure 3.6.4: Schematic Diagram of NG2-Cre Targeting Strategy for Homologous Recombination in Embryonic Stem Cells.

A) Wild-type NG2 allele, B) targeting vector containing the Cre gene and the Neo^R gene, C) targeted allele after homologous recombination in embryonic stem cells, and (Frt sites in diagram are not to scale. Frt sites consist of 34 bp.)

3.6.4 NG2-Cre Homologous Recombination in Embryonic Stem Cells

3.6.4.1 PCR identification of homologously recombined embryonic stem cell clones

For the identification of homologously recombined embryonic stem cell clones containing the targeting vector, PCR amplification was done with the anti-sense primer 5003 that is located near the 5' end of the Cre gene and the sense primer 3305 that is located upstream from the 5' end of the short arm of the targeting vector. A product of about 1 KB was observed in correctly recombined embryonic clones. If the targeting vector inserted anywhere else, this PCR would not have amplified a product. For optimization of this PCR a control plasmid was designed. A large fragment of 1.37 KB containing exon 1 of the NG2 gene, the homologous short arm and 300 bp upstream from the short arm was PCR amplified and cloned into a modified Cre litmus-29 plasmid (by Sandra Goebbels) by Kpn I restriction sites. The anti-sense and sense primer for the Cre targeting vector were optimized using this control plasmid.

To improve the specificity of the control PCR, a "nested" PCR was performed, where an in-between product was first amplified. Then another set of primers was used on the in between product to give a second product which was seen on the agarose gel. The primers used are anti-sense 5002 that is up stream from 5003 and sense primer 3307 that is downstream from 3305. For the first reaction with primers 5003 and 3305, 19 cycles were done with an annealing temperature of 58°C. From the first PCR 1/5 of the product was used for the second PCR reaction for 39 cycles with an annealing temperature of 58°C. The final product of 1 KB is slightly smaller then the control PCR product of 1.18 KB. This ruled out any possible contaminations from the control plasmid. The nested PCR was optimized to where it was possible to detect 200 copies, 20 copies and sometimes two copies of the control plasmid. This was a powerful tool to determine how many copies of the targeting vector are present in the positively recombined embryonic stem cell clone. This nested PCR technique is required, because of a lack of starting DNA material. Normally an embryonic stem cell clone consists from anywhere between 50-1000 cells. Through the nested PCR method only a minute amount of starting material is required to visualize a positive clone. To improve the working quality of the nested PCR Q-solution was added.

3.6.4.2 Isolation of homologously recombined embryonic stem cell clones

The OLA-129 embryonic stem cell line with a passage P8 was electroporated with 50 μg of linearized targeting vector **pKS-AN2-Cre.** 24 hours after electroporation, G418 selection was started and continued for 7-10 days. The first three days of selection the embryonic stem cells were growing at a normal rate and very little cell death was observed in the first days of selection. G418 selection medium was changed every 24 hours. Between days four to six there was massive cells death observed among the embryonic stem cells indicating some cell did not integrate the targeting vector carrying the resistance gene. On day nine of G418 selection, clones were picked and transferred to a 96 well plate. 2/3 of the clone was used for DNA isolation and 1/3 was plated onto a 96 well plate containing EMFI feeders for further expansion. When the clone was found in a pool of eight cells, then individual clones were expanded in the presence of G418 onto a 24 well dish. After every passage, the clone was double checked by PCR. The clone was expanded until it was large enough to freeze down and inject into blastocytes. DNA from eight clones was pooled and analyzed by PCR using the control plasmid (data not shown). By this method, 528 clones were selected and screened. No homologously recombined clones were found.

3.6.5 NG2-Cre-Intron Targeting Vector

The modified pKS-blue script vector containing the NG2-Cre Intron has the following elements (figure 3.6.5):

1. Homologous short arm and the pMC-Cre coding sequence: The short arm, consists of two parts, a DNA fragment about 735 bp that is homologous to the NG2 gene upstream from the open reading frame (ORF) in exon 1 followed by the 5' end of 386 bp of the Cre gene. There are no known splice variants to the NG2 gene. The promoter region is also very large, so the best option was to insert the Cre directly into the ORF of the NG2 gene. The two fragments were fused together through a fusion PCR giving a fragment of approximately 1.1 KB. The fusion product was then cloned in the pKS-blue-script by using BamH I restriction site. The orientation of the NG2-Cre fusion product was checked with the following restriction enzymes: Kpn I and Xmn I. After the restriction enzyme digest, correct orientation was determined by the DNA band pattern. The orientation was correct when three bands with sizes of 1.8 KB, 1.9 KB, and 958 bp were seen. Out of 24 bacterial clones picked, eight had the correct orientation. The 3' fragment of the

Cre of about 724 bp was cut out of the pMC-Cre vector with BamH I / Nde I and cloned into the pKS-blue script vector, with the same restriction sites. The over all size of the short arm and the complete Cre gene is 1.835 KB. The identity of the short arm was checked with restriction digests and sequencing. One point mutation was found after sequencing, upstream from the start codon, outside the exon 1. Through homologous recombination in the embryonic stem cells, the targeting should integrate into the start codon in exon 1 without interfering with the other exons further down stream.

2. Neomycin-resistance gene (Neo^R): The Neo ^{R} gene used was about 1.31 KB and was floxed on either side by Lox P sites for latter excision from the targeted allele. A thymidine-kinase promoter drives the NeoR gene derived the *Herpes simplex* virus. The original gene was first amplified by PCR from the pMCNeoPA vector (Stratagene) using primers AN2-Neo anti-sense and AN2-Neo sense. This PCR product was cloned into an intermediate vector containing Lox P sites. The Neo^R gene, which is now floxed on both sides by Lox P sites was then further amplified through PCR using primers 4937 (neo anti-sense with Fse I restriction site) and primer 4938 (neo sense with Nde I and Not I restriction sites). The final Neo^R could not be cloned in behind the Cre gene in the backbone vector pKS-blue-script. The problem was that the Cre recombinase gene was turned on in the bacteria cutting out the Neo^R. Multiple strategies were used to overcome this problem, but none worked properly. In the end, the solution was to replace the Lox P sites with Frt-sites. The same Neo^R was amplified from a vector called pCon-KO-true, where the gene was floxed on both sides by Frt-sites. The primers used are FRT Neo anti-sense that added restriction site Fse I and FRT Neo sense which added Nde I and Not I. This was successfully cloned into vector backbone. Out of eight bacterial clones picked, all eight carried the new Neo^R that was flanked by Frt-sites. Depending on its successful homologous recombination with in the embryonic stem cell, the gene provides resistance against the drug G418 giving a positive selection technique to screen resistant clones.
3. Homologous long arm: A long arm of 5.3 KB was used which was made up of the 3' end of exon 1, directly behind the Neo^R gene. The long arm did not contain any part of exon 2 of the gene, due to the fact that the intron between exon 1 and exon 2 is over 13 KB. A long distance PCR was first attempted to generate this large fragment by using the following primers AN2-LA anti-sense and AN2-LA sense

from genomic DNA. This long distance PCR method did not work as was expected, therefore an alternative method was used to get around this problem of amplifying such a large product. When looking at the homologous long arm nucleotide sequence, one major feature is clearly visible which is right in the middle of the 5.3 KB long arm is a Sac I restriction site. This is unique only to the long arm and not the short arm. The Sac I restriction site cuts the long arm in half giving a fragment of 2.6 KB and 2.7 KB. It is easier first to amplify these smaller fragments by PCR. The fragments are then fused together to make up the homologous long arm. For this to happen another vector was designed for this step. By removing the multiple cloning site from the pSP72 vector (2.9 KB) and replacing it with an artificial multiple cloning site designed to integrate the two fragments of the homologous long arm. The old multiple cloning site was removed by cutting with EcoR V and Xho I. The new multiple cloning site contained the following restriction sites in the following order: EcoR V, Fse I, Sac I, and Xho I. The individual fragments were generated by PCR amplification using primers LA anti-sense 1 and LA sense 1 for the first fragment of 2.7 KB, through the primers the restriction sites Fse I and Sac I were brought in. For the second fragment LA anti-sense 2 and LA sense 2 were used and restriction sites Sac I and Xho I were brought in. After PCR amplification the fragments were cloned into the intermediate vector the modified pSP72 vector. The homologous long arm was double checked through restriction digests and sequencing. Four different restriction digests were done to verify the identity of the long arm. It appeared that the homologous long arm was correct. The pSP72 vector was then sent for sequencing to check for mutations. Multiple primers were generated for primer walking. Five mutations were found in the homologous long arm, but the mutations were down stream from exon 1. Since the homologous long arm is mostly intron, it was used in the final targeting vector.

4. Artificial intron containing a poly a tail: An artificial intron containing a poly-A tail was cloned into the backbone vector as the final cloning step for the **pKS-AN2-Cre-Intron.** The intron that contains a poly-A tail comes from the rabbit Beta globulin gene. It was PCR amplified from the PUHG-17.1 vector with anti-sense primer gIPA2 and sense primer gIPA1, which gave a product of 1.21 KB. Not I restriction sites were added at the ends of the PCR product which allowed it to be cloned into the backbone vector. Problems arose do to the fact that the Not I

restriction site did not cut well at the end of the PCR product. The PCR product was first cloned into an intermediate vector p-GMT. The product was then sent for sequencing to look for mutations. The sequencing results showed no mutations present, so the intron was cut out of the intermediate vector and cloned into the backbone vector. Since there was only one restriction site the Not I, orientation of the insert was checked by a double digest with restriction enzymes Xba I and Fse I. When the intron is correctly orientated six bands were seen on the agarose gel (4.5 KB, 3 KB, 2.5 KB, 864 bp, 665 bp, 473 bp), when the intron orientation is backwards also six bands were seen, but different then the first (5.78 KB, 3 KB, 1.3 KB, 864 bp, 665 bp, 473 bp). Out of 16 positive clones picked, eight clones had the correctly orientated intron with poly-A tail.

5. Cloning vector backbone: The pKS-blue-script II multiple cloning site was exchanged by restriction digestion with Kpn I and Sac II. Another multiple cloning site was cloned in, containing the unique restriction sites in the following order: Kpn I, BamH I, Pst I, Not I, Nde I, Fse I, Xho I, Xma I and Sac II. The 3’ end of the EYFP was cloned in first followed by the short arm fused to the 5’ end of the rest of the EYFP gene. The homologous long arm was then cloned into the backbone vector. The final step was cloning in the Neo^R gene that is floxed by the Lox P sites. The final vector was checked through restriction digests with 7 different restriction enzymes BamH I, Bsg I, Hind III, Nae I, Nhe I, Xmn I and Pst I. From these restriction digests the targeting vector appeared to be correct. The targeting vector was double checked by sequencing over the unique restriction sites to look for any mutations. No new mutations were observed within the target vector. The final targeting vector **pKS-AN2-Cre-Intron** is 10.2 KB long and was linearized before electroporation in embryonic stem cells with Xho I.

A

B

C

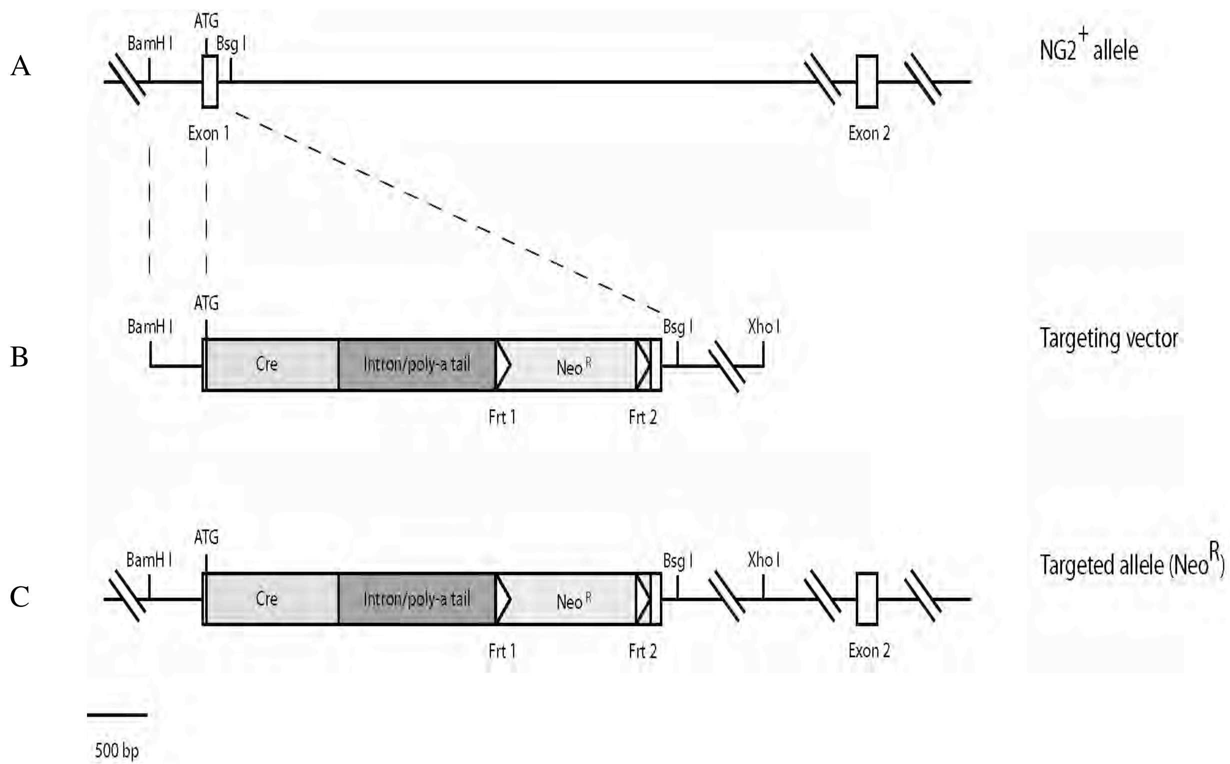


Figure 3.6.5: Schematic Diagram of NG2-Cre-Intron Targeting Strategy for Homologous Recombination in Embryonic Stem Cells

A) Wild-type NG2 allele, B) targeting vector containing the Cre gene and the Neo^R gene, C) targeted allele after homologous recombination in embryonic stem cells, and (Frt sites in diagram are not to scale. Frt sites consist of 34 bp.)

3.6.6 NG2-Cre-Intron Homologous Recombination in Embryonic Stem Cells

3.6.6.1 PCR identification of homologously recombined embryonic stem cell clones

For the identification of homologously recombined embryonic stem cell clones containing the targeting vector, PCR amplification was done with the anti-sense primer 5003 that is located near the 5’ end of the Cre gene and the sense primer 3305 that is located upstream from the 5’ end of the short arm of the targeting vector. A product of about 1 KB was observed in correctly recombined embryonic clones. If the targeting vector inserted anywhere else, this PCR would not have amplified a product. For optimization of this PCR a control plasmid was designed. A large fragment of 1.37 KB containing exon 1 of the NG2 gene, the homologous short arm and 300 bp upstream from the short arm was PCR amplified and cloned into a modified Cre litmus-29 plasmid (by Sandra Goebbels) by Kpn I restriction sites. The anti-sense and sense primer for the Cre targeting vector were optimized using this control plasmid.

To improve the specificity of the control PCR, a “nested” PCR was preformed, where an in-between product was first amplified. Then another set of primers were used on the in between product to give a second product which was seen on the agarose gel. The primers used are anti-sense 5002 that is up stream from 5003 and sense primer 3307 that is downstream from 3305. For the first reaction with primers 5003 and 3305, 19 cycles were done with an annealing temperature of 58°C. From the first PCR 1/5 of the product was used for the second PCR reaction for 39 cycles with an annealing temperature of 58°C. The final product of 1 KB is slightly smaller then the control PCR product of 1.18 KB. This ruled out any possible contaminations from the control plasmid. The nested PCR was optimized to where it was possible to detect 200 copies, 20 copies and sometimes two copies of the control plasmid. This was a powerful tool to determine how many copies of the targeting vector are present in the positively recombined embryonic stem cell clone. This nested PCR technique is required, because of a lack of starting DNA material. Normally an embryonic stem cell clones consist from anywhere between 50-1000 cells. Through the nested PCR method only a minute amount of starting material is required to visualize a positive clone. To improve the working quality of the nested PCR, Q-solution was added.

3.6.6.2 Isolation of homologously recombined embryonic stem cell clones

The OLA-129 embryonic stem cell line with a passage P8 was electroporated with 50 μg of linearized targeting vector **pKS-AN2-Cre-Intron.** 24 hours after electroporation, G418 selection was started and continued for 7-10 days. The first three days of selection the embryonic stem cells were growing at a normal rate and very little cell death was observed in the first days of selection. G418 selection medium was changed every 24 hours. Between days four to six there was massive cell death observed among the embryonic stem cells indicating some cell did not integrate the targeting vector carrying the resistance gene. On day nine of G418 selection, clones were picked and transferred to a 96 well plate. 2/3 of the clone was used for DNA isolation and 1/3 was plated onto a 96 well plate containing EMFI feeders for further expansion. When the clone was found in a pool of eight cells, then individual clones were expanded in the presence of G418 onto a 24 well dish. After every passage, the clone was double checked by PCR. The clone was expanded until it was large enough to freeze down and inject into blastocytes. The DNA from eight clones was pooled and analyzed by PCR using the control plasmid (data not shown). By this method, 488 clones were selected and screened. No homologously recombined clones were found.

4. Discussion:

NG2 is a 330kDa transmembrane glycoprotein that is expressed in multiple different cell types in the developing and adult mammal (Stallcup, 1981; Nishiyama et al., 1991; Niehaus et al., 1999; Stegmuller et al., 2002). The gene that encodes for NG2 has 8 known exons with no as yet described splice variants. The promoter of the NG2 gene is quite large, but still not completely defined. The NG2 gene is highly conserved between rat, human and mouse. NG2 has been used as a marker for immature oligodendrocytes and Schwann cells. In the developing mouse brain, NG2 is first observed as early as embryonic day 13. Expression of NG2 peaks between neonatal day 6 and day 12, with a down regulation at day 15. This observation strongly correlates with myelination in the mouse, starting at day 6 and continuing up to adult. During development, NG2 staining overlaps completely with PDGFα-R and partly with early myelin markers like O4 and CNPase. NG2 is absent from differentiated oligodendrocytes expressing myelin proteins like MAG (Myelin Associated Glycoprotein), and MOG (Myelin Oligodendrocyte Glycoprotein). NG2+ cells are still present in the grey and white matter of the adult brain, but their function is still undetermined. The original NG2 knockout mouse, which uses a neomycin selection cassette to disrupt the NG2 gene, has no apparent phenotype.

To better understand the role that NG2 and NG2+ cells play in the developing CNS, a knockin mouse was generated, where EYFP was inserted into the start codon of the NG2 gene. Targeting vectors containing the Cre gene under the regulatory influence of the NG2 promoter were also generated.

4.1 The Generation of the NG2-EYFP and NG2-EYFP-Intron Mouse Line

4.1.1 Transgenic mice compared to Knockin mice

The exact NG2 promoter is ill defined, hence the generation of a transgenic mouse, in which a construct containing a chromophore under the NG2 promoter is randomly inserted in the genome is unsatisfactory. A knockin mouse is much better, than a transgenic mouse in that the expression of the reporter gene is under the regulation of the endogenous promoter. The NG2-EYFP knockin mouse allows the study of the NG2+ cells in situ. Furthermore, the breeding of the mouse to homozygosity yields a knockout

mouse in which cells lacking the NG2 express EYFP. This permits the analysis of the knockout cells in situ. Even though the expression level of the transgenic animal is much stronger, due to multiple insertions of the transgene, in transgenic animals expression is sometimes seen in other cell types that normally do not express the promoter (Matthias et al., 2003). In contrast, in the knockin there is only one copy of the targeting vector inserted through homologous recombination with expression determined by the endogenous promoter, thus allowing expression patterns comparable to the wild-type gene. The EYFP gene was homologously recombined into the start codon of the NG2 loci. This is an established method used to generate mouse lines (Capecchi, 1989a). The passage number and the culture conditions of the embryonic stem cells are an important factor that determines the efficiency and success in generating chimeric mice (Nagy et al., 1993). If the embryonic stem cells are passaged too often, they lose their pluripotenticy and the resulting ES cells cannot insert the targeting vector at the right point during the cell cycle, influencing the efficiency of recombination (Fedorov et al., 1997; Udy et al., 1997).

The targeting vector containing the start codon of NG2, followed by the EYFP gene and a lox P floxed neomycin resistance gene was electroporated into the OLA-129 embryonic stem cell line. The embryonic stem cells were grown on an embryonic mouse feeder layer with the addition of LIF (Leukemia inhibitory factor) that hindered the embryonic stem cells from differentiating and selected by G418 for 7-10 days (Takahama et al., 1998). The NG2-EYFP targeting vector used the endogenous poly A tail in the NG2 gene allowing splicing equivalent to the wild-type situation. To improve the expression level of the targeting vector, it was modified slightly to include an intron containing poly A tail directly following the EYFP gene. In transgenic animals an artificial intron is known to improve expression, by stabilizing the pre-mRNA allowing for proper expression. PCR and Southern blot analysis detected one homologously recombined clone for the EYFP and the EYFP-intron, with recombination frequencies of 1 in 256 and 1 in 384 respectively.

Injection of the clones in blastocyts generated highly chimeric animals that were selected according to fur color. For the EYFP homologously recombined embryonic stem cell clone, 13 chimeric male animals ranging from 10% to 95% chimerism and 8 chimeric female animals ranging from 10% to 50% were born. For the EYFP-intron homologously

recombined embryonic stem cell clone, 9 chimeric male animals ranging from 10% to 80% and 10 chimeric female animals ranging from 10% to 70% were born. For the EYFP embryonic stem cell clone 3 highly chimeric male and for the EYFP-intron embryonic stem cell clone 2, highly chimeric male animals were used for further breeding. Germ line transmission was checked through PCR analysis and coat color. A brown coat color signified that the offspring carried the modified NG2 gene.

4.1.2 EYFP expression in the NG2-EYFP and NG2-EYFP-intron mouse lines

The NG2-EYFP F1 generation failed to express EYFP, probably due to interference in pre-mRNA splicing by the Neo resistance cassette. The NG2-EYFP F1 generation was bred to ELLA-Cre mice, which express Cre recombinase in all cell types. Strong expression of EYFP was observed in the F2 generation in various brain regions including blood vessels, verifying the interference of the Neo gene in the F1 generation (figure 4.1.2). Proper expression was verified by immunohistochemistry with an antibody against the NG2 protein. There was almost a complete overlap in the CNS between the antibody staining and the EYFP expression, in cells with typical morphology and also in pericytes of the blood vessels. Pericytes express high levels of NG2 in the CNS (Grako and Stallcup, 1995).

The NG2-EYFP-intron mouse F1 generation expresses EYFP, since an artificial intron containing the poly A tail is cloned in between the EYFP gene and the Neo gene, allowing expression of EYFP. This mouse line is not equivalent to the normal wild-type situation, when compared to the NG2-EYFP mouse line. The NG2-EYFP mouse undergoes normal-splicing equivalent to the wild-type situation making it a better model to study NG2 cells *in vivo.* Hence, for further studies the NG2-EYFP mouse strain was used and embryos of the NG2-EYFP-Intron strain were frozen.

4.1.3 The NG2-EYFP Knockout mouse line

The original NG2 knockout mouse was viable and showed no striking phenotype apart from the muscle cells (Grako et al., 1999). The NG2 transmembrane glycoprotein is expressed by oligodendrocyte precursors: no hindrances of oligodendrocyte development were observed in this knockout. Strong EYFP expression was seen in areas of the CNS

in the NG2-EYFP heterozygous mouse. Stronger expression of EYFP was observed in the NG2-EYFP homozygous mouse, and the NG2-EYFP+ cells in the homozygous animals did not react with the NG2 antibody, indicating the lack of NG2 protein. To confirm that the homozygous animal is a true knockout of the NG2 protein, a western blot was done on wild type, heterozygous and homozygous brains from 8-day-old animals to check for the presence or absence of the NG2 protein. In the western blots NG2 is a 330-kDa protein showing two bands, a strong NG2 glycosylated band at 330-kDa and a minor band running a little below it at 315 kDa. The western blot showed clear evidence that the wild-type animal expressed large amounts of the NG2 protein, while the heterozygous animal brain had half the amount of the NG2 protein, and the homozygous animal lacked NG2 protein completely. An antibody for EYFP was used to check for the expression of EYFP in the brains of the wild type, heterozygous, and homozygous mice. Expression was seen in the homozygous and the heterozygous, but not in the wild-type brain. The advantage of this NG2-EYFP knockout mouse is that the cells lacking NG2 protein expression can be studied *in situ*, due to their EYFP expression, thus providing a powerful tool to understand the function and role of these cells in the CNS.

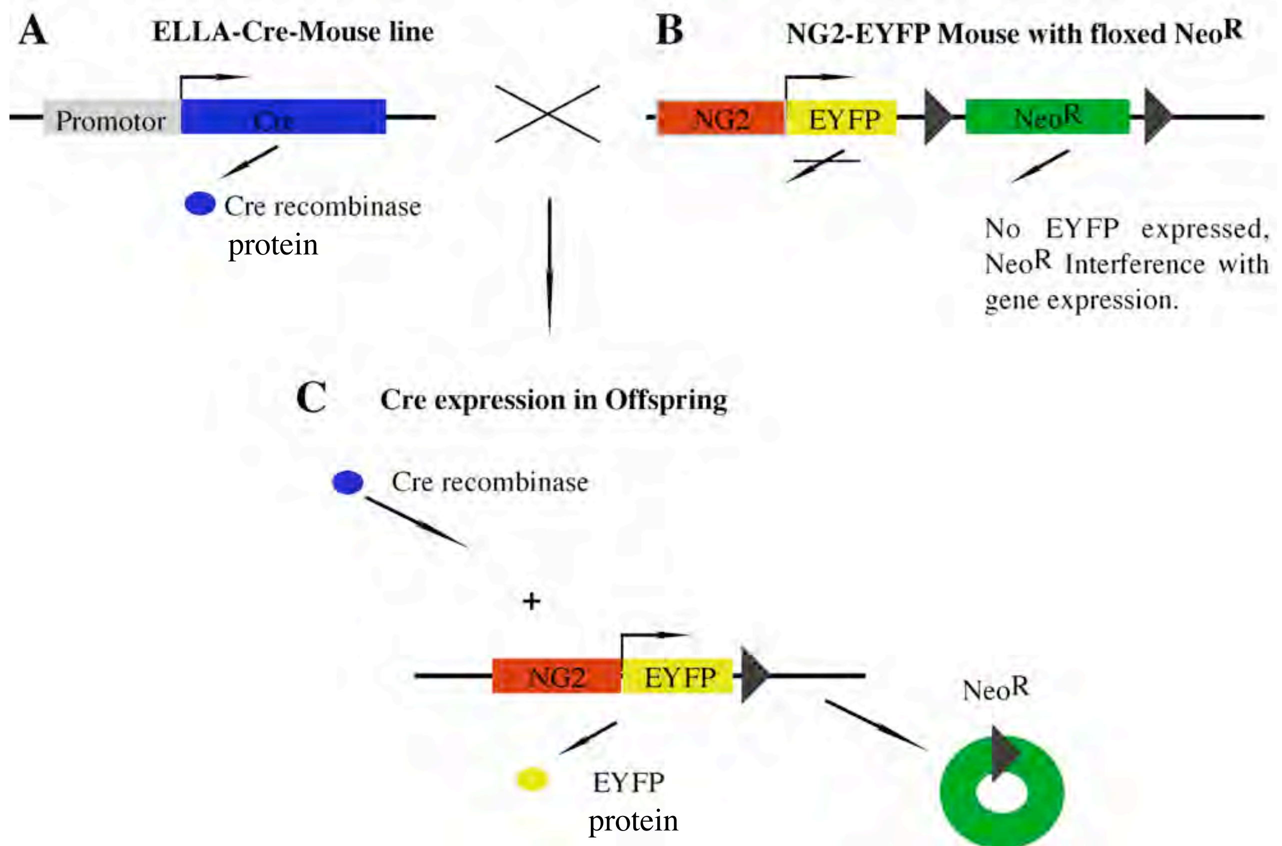


Figure 4.1.2: Schematic representation of Neo excision by Cre recombinase

In mouse A) Cre recombinase protein is made in all cells due to the ubiquitous promoter used. B) NG2-EYFP F1 generation contains Lox P sites flanking Neo^R. Offspring C) generated by interbreeding these two mouse strains contain cells in which Cre recombinase is expressed and which thus excise the Neo^R resulting in the expression of EYFP.

4.2 Lineage commitment of the NG2+ cells

NG2+ cells are still present in the adult brain and represent the largest proliferating population (Dawson et al., 2003). What role they actually play in the adult CNS is still not clear. Since oligodendrocyte precursor cells express NG2 in the adult brain it is possible that these cells can replenish oligodendrocytes in the adult (Reynolds et al., 2002; Dawson et al., 2003). In the developing and mature CNS, NG2+ cells are thought to be responsible for generating oligodendrocytes and thus myelin (Levison et al., 1993; Niehaus et al., 2000; Watanabe et al., 2002; Windrem et al., 2004). There is still an ongoing debate, whether NG2+ cells in the developing brain represent a homogenous or heterogenous population. Two groups have provided evidence that NG2+ cells represent a homogenous population belonging to the oligodendrocyte lineage in the developing and adult brain, by the expression of well-established early oligodendrocyte markers and expression of mRNA for myelin proteins (Dawson et al., 2003; Ye et al., 2003). Two common markers used in the first study were O4, and PDGFα-R. The O4 antibody recognizes sulfatide that is expressed early in oligodendrocyte development and is maintained on mature cells. PDGFα-R is expressed by early oligodendrocyte precursors but is down regulated when the oligodendrocyte begins to differentiate to produce myelin-forming cells. The first study showed an overlap of NG2, O4 and PDGFα-R expression in the developing mouse that extended into the adult brain. Some of the NG2+ cells co-expressed CNPase, a later marker of oligodendrocyte development. Different morphologies were observed in CNS grey and white matter. In the grey matter of the developing brain the cells had a stellate morphology having highly branched processes. In the white matter of the adult brain the cells appeared to be elongated between tightly packed myelinated axons. They stated that the NG2+ cells belong to the oligodendrocyte lineage in the adult brain. (Dawson et al., 2003). The second study used laser capture microdissection to isolate mRNA from NG2+ cells in the adult mouse brain. From the laser-captured cells reverse-transcription PCR was performed to determine the profile of mRNA expression. These experiments demonstrated that all adult NG2+ cells expressed mRNA for proteolipid protein (PLP), myelin basic protein (MBP), and 2', 3'-cyclic nucleotide 3' –phosphodiesterase (CNPase), but not mRNA for DM20, a PLP splice variant which an immature form of PLP. This study shows that NG2+ cells express mRNA for various myelin proteins, indicating that the cells belong to the oligodendrocyte

lineage (Ye et al., 2003). This is in contrast to results from the Macklin group, using a PLP-EGFP transgenic mouse, in which some NG2+ cells are EGFP-.

Other studies demonstrate that the NG2+ cells represent a heterogenous population that is present in the embryonic, neonatal and adult brain. In one study, a transgenic mouse was made with a proven PLP promoter in which EGFP was fused to the 3' UTR of the PLP gene. By using this transgenic mouse it was possible to show that in the developing subventricular zone there are two populations of NG2+ cells. One population expressed EGFP indicating that the PLP promoter was active and another population had no expression of EGFP. This study showed that the NG2+ cells are heterogenous and suggested that at any given time point there is one population that is geared toward generating oligodendrocytes and another population that stays in an immature state. The NG2+ cells that are EGFP negative could possibly be the adult oligodendrocyte precursor cells that remain in an immature state until they receive a signal to start myelination (Mallon et al., 2002). Taking a similar approach, Matthais et al., used a transgenic mouse expressing EGFP under the promoter for human GFAP and showed that weakly labeled EGFP+ cells were also NG2+. When the weakly positive EGFP cells were labelled with NG2+ and S100 ß antibodies, there was some overlap of expression of the markers. This indicated that the NG2+ cells express S100 ß (Matthias et al., 2003). S100 ß is a calcium binding protein that has been used previously as a marker for astroglia but recently it is thought that the S100ß is also a marker for oligodendroglial cells, depending on the region of the CNS studied. When a transgenic mouse line was made where EGFP was expressed under the S100 ß promoter, EGFP expression was not only seen in astrocytes, but also in oligodendrocytes and their precursors. In this study some of the EGFP cells expressed CNPase, a unique oligodendrocyte marker implying that oligodendrocytes are S100ß+ (Hachem et al., 2005). NG2+ cells have been given different names according to their characterization in different experiments. They have been given the name synantocytes because they make contact to neuron and axons (Butt et al., 2002). Another given name to the NG2 cells is polydendrocytes, representing that they are a unique glial population in the CNS (Nishiyama et al., 2002). In some cases NG2+ cells contact the node of Ranvier, a behavior thought to be unique to astrocytes (Butt et al., 1999). These results highlight how difficult it is to characterize these cells, indicating that the cells could comprise a heterogenous group of precursors with distinct developmental potentials.

4.2.1 Developmental Fate of NG2+ cells in the NG2-EYFP Mouse Knockin Line

In the NG2-EYFP heterozygous mice, EYFP expression was seen in the cell body as well as the processes, and in the homozygous mice the EYFP expression level was higher, making the cells brighter. The mice studied varied in ages from 10 day old, 28 day old, and 75 days old. No apparent abnormal phenotype was observed in any of the heterozygous and homozygous animals studied, comparable to the first NG2 null mouse. NG2 cells in the grey matter and white matter appear stellate, multiple elongated and bipolar processes (Horner et al., 2002). Our observation is that stellate EYFP expressing cells with multiple processes were located in the grey matter and the elongated bipolar cells were associated more with the white matter.

In the mice, EYFP appeared to be expressed by all of the NG2+ cells as there was almost a complete overlap in the heterozygous animal of EYFP expression and staining with the antibody against NG2. EYFP expression was seen in pericytes around blood vessels. There was one exception, a minute quantity of cells that labeled with the NG2 antibody that did not express EYFP. We called these cells “phantom cells”. They were located in the grey matter of the brain, in particular in the cortex. These cells made up less then 1 percent of the total population and the reason for their lack of EYFP expression is undetermined. One possible reason is interference from the Neo resistance gene that was not excised from a few cells in the F2 generation. Another possibility is that the NG2 promoter is inactive thus causing a loss of EYFP expression, while maintaining NG2 protein expression. A third possibility is that the phantom cells bind proteolytically cleaved NG2 protein that is recognized by the antibody. In the homozygous animals, NG2-EYFP+ cells, showed no co-labeling with the NG2 antibody, verifying it as a knockout and the phantom cells also disappeared. No migration defects in the CNS were seen when comparing the heterozygous and the homozygous animals, indicating a compensating factor for the loss of the NG2 protein. It is known that the NG2 transmembrane glycoprotein plays a role in migration (Fang et al., 1999; Niehaus et al., 1999; Stegmuller et al., 2002; Fukushi et al., 2004).

4.2.2 NG2 Cells are not Astrocytes or Microglia

NG2 cells in the developing brain do resemble astrocytes, but NG2 cells do not express GFAP protein even in demyelinating lesions where there is an up regulation of NG2, OX-42, and GFAP around the lesions (Keirstead et al., 1998). OX-42 is an antigen unique to microglia. It has been shown that transgenic mice that express EGFP under the GFAP promoter, some NG2 cells are EGFP+ in the developing and adult brain (Matthias et al., 2003). Since this mouse is a transgenic mouse, it cannot be excluded that the promoter is active in cell types other than the intended astrocyte population. Also, GFAP protein expression may be visualized later than the promoter activity. In the NG2-EYFP mouse, there was no overlap of EYFP expression and expression of the astrocyte protein GFAP in the CNS. When looking at different areas of the brain, considerable GFAP staining was seen in grey matter and less labeling was seen in the white matter. The white matter consists mostly of myelinating oligodendrocytes. In the grey matter, which consists of the cortex, hippocampus, septum, and the subventricular zone, multiple cells stained with the GFAP antibody were seen, but none of the cells expressed EYFP, indicating the NG2-EYFP expressing cells are not astrocytes. In the white matter, which consists of areas of the cerebellum, and the corpus callosum, multiple NG2-EYFP+ cells, which do not stain with the GFAP antibody are present. This holds true for the NG2-EYFP heterozygous and the NG2-EYFP homozygous mouse. *In vitro,* it has been shown that the NG2 cells behave as O2A precursor cells, giving rise to astrocytes or oligodendrocytes, depending on the culture conditions (Diers-Fenger et al., 2001). The S100 ß antibody was used to further examine the properties of the NG2-EYFP+ cells. This antibody has also been shown to label oligodendrocytes (Hachem et al., 2005). In the NG2-EYFP mouse line some of the cells that expressed EYFP were also co-labeled for S100ß in the white matter and the grey matter of CNS. Double-labeled cells were widespread in the cortex and the subventricular zone in the NG2-EYFP mouse line. No differences were observed between heterozygous and homozygous animals of the NG2-EYFP mouse line at various ages. The majority of the EYFP expressing cells did not label for the S100 ß protein. These results do not rule out the possibility that the NG2 cells could make astrocytes *in vivo*, but up to now all evidence points against the NG2 cells thought to be astrocytes.

Reynolds et al., show that microglia are not normally NG2+ (Reynolds and Hardy, 1997). The NG2-EYFP+ cells did not co-label with microglia markers like F4/80 in any of the

CNS areas observed through out this study, indicating that the EYFP expressing cells are not indeed microglia, in a normal situation. These results do not rule out the fact that NG2 cells could make microglia cells in lesion models, but may bind cleaved ectodomains (Yokoyama et al., 2006).

4.2.3 Evidence that NG2 Cells have the Potential to Generate Oligodendrocytes from the NG2-EYFP Mouse

In vitro NG2 cells stain with oligodendrocyte markers like PDGFα-R, and O4 (Niehaus et al., 1999). *In vivo*, some of the NG2+ cells express O4, PDGFα-R and there is a slight overlap with CNPase expression, indicating that they are from the oligodendrocytes lineage (Dawson et al., 2003). In the NG2-EYFP heterozygous and homozygous mouse, all EYFP expressing cells in the brain labeled with PDGFα-R antibodies. PDGFα-R has been used as a marker for oligodendrocyte precursor cells, but it has been reported that some neuronal precursors express PDGFα-R (Vignais et al., 1995; Oumesmar et al., 1997). In contrary, not all PDGFα-R positive cells are expressing EYFP in the NG2-EYFP mouse. These could possibly be neuronal precursors in the CNS, in which the NG2 promoter is inactive.

In the NG2-EYFP mouse line, multiple EYFP expressing cells were labeled with the O4 antibody, but many cells did not stain for O4. NG2-EYFP+ cells lacking O4 labeling are either early oligodendrocyte precursors before the onset of O4 expression or another cell type. Sulfatide (O4 staining) is still expressed by mature oligodendrocytes in the CNS. This phenomenon was seen in the grey and white matter of the heterozygous and homozygous mice of different ages. The NG2-EYFP+ cells thus appear to be heterogenous when looking at their expression profile of O4 in 10 day old, 28 day old and 75 day old animals.

CNPase is a protein expressed later in the oligodendrocyte lineage, after O4 expression. Studies have shown that there are some cells that express CNPase and NG2. Theses cells are very rare in the developing and adult brain. In the NG2-EYFP mouse cell line, no overlap was seen between CNPase and the EYFP expression.

4.2.4 Expression of transcription factors by the NG2+ cells

Antibodies for Olig1 and Olig2 were used to characterizes the cells. Olig1 and Olig2 are helix-loop-helix transcription factors that affect oligodendrocyte and motor neuron development (Lu et al., 2002). Olig2 knockout mice die at an early age due to the lack of maturation of oligodendrocytes and motor neurons (Takebayashi et al., 2002). Olig2 is expressed in oligodendrocytes throughout development and adulthood. Olig1 has been shown to play a role in early stages of myelination and myelin repair. Olig1 knockout mice either die early at embryonic stage or live for three weeks depending on which knockout (Arnett et al., 2004; Xin et al., 2005). A unique study involving demyelinating lesions was done on the first Olig1 null mutant generated by the insertion of a PGK-neo cassette to disrupt the gene. In a normal mouse, after myelination has occurred, the Olig1 transcription factor is translocated out of the nucleus of oligodendroglia cells into the cytoplasm. Demyelination and subsequent remyelination resulted in translocation of Olig1 back into the nucleus. Even though the knockout was able to initially myelinate under normal circumstances, under demyelinating conditions the mouse was unable to induce the repair mechanism of remyelination (Arnett et al., 2004). This knockout model did not have a strong phenotype: due to the PGK (phosphoglycerate kinase) promoter, which contains a strong enhancer region that has the capacity to influence the expression of neighboring genes. Since Olig1 and Olig2 are located on the same chromosome in close proximity and that the Olig2 gene encodes a protein that is 80% homologous to the Olig1 gene, it can be postulated that the Olig2 gene compensated for the lack of the Olig1 gene (Balabanov and Popko, 2005). This was demonstrated with another Olig1 knockout mouse, where the PGKneo gene was excised from the Olig1 gene with Flp recombinase (Xin et al., 2005). This resulted in an embryonic lethal knockout, indicating that Olig1 is vital for the first stage of myelination. It was postulated that the PGKneo gene influenced expression of the Olig2, which could partially compensate for the lack of Olig1.

In the NG2-EYFP mouse, all of the EYFP+ cells expressed the Olig2 transcription factor in both heterozygous and homozygous animals. Some cells did not express EYFP, indicating that the Olig2 antibody also labeled active NG2- more mature oligodendrocytes in the brain. Olig2 is a transcription factor for oligodendrocytes and motor neurons: hence theoretically the NG2-EYFP cells could give rise to either oligodendrocyte precursors or

motor neuron precursors, this possibility cannot be ruled out at this point in time. Mukouyama et al. showed that Olig2+ cells isolated from mouse spinal cord at embryonic day 9.5 and then transplanted in to chick spinal cords gave rise to both motor neurons and oligodendrocytes. On the other hand if they transplanted Olig2+ cells from embryonic day 13.5 into chick embryos, these cells gave rise to only glial derivatives (Mukouyama et al., 2006). Taking this into account, it is thus possible that the NG2-EYFP cells are oligodendrocyte precursors that will give rise to oligodendrocytes when needed. In brain injury studies, Buffo et al. showed that in a brain lesion, Olig2 is first up regulated. When a retroviral vector containing a dominant negative form of Olig2 was injected into the lesioned cortex 2 days after a stab wound, the Olig2 function was antagonized resulting in a significant number of infected cells up regulating Pax6 generating immature neurons, that were not observed after injection of the control virus (Buffo et al., 2005). None of the NG2-EYFP+ cells in 10 day old and 28 day old mice expressed the Olig1 transcription factor, indicating that these cells are not myelinating or the Olig1 protein expression is trivial. It is possible that the NG2+ cells express Olig1 only when they are determined to start myelination (Ligon et al., 2006a).

Another group of transcription factors that are important for the glia maturation and development is the Sox protein family mainly the Sox 8, Sox 9 and Sox 10. Sox 9 is an important regulatory factor for glial determination, which is expressed by early oligodendrocyte precursors, and by astrocytes. Sox 9 is down regulated when Sox 10 is upregulated in oligodendrocytes. We examined Sox 10 expression in the NG2-EYFP mouse. Almost all EYFP expressing cells in the heterozygous and the homozygous animals labeled for the Sox 10 transcription factor. So a majority of the NG2-EYFP cells appear to be determined to become oligodendrocytes, but when will they start myelinating? One can also postulate that the minor population of the NG2-EYFP+ cells in various CNS regions that did not express Sox 10 could be the cells that give rise to neurons.

4.2.5 Do NG2 Cells make Neurons in the NG2-EYFP Mouse CNS?

NG2 cells in the developing and adult brain have been termed transient amplifying precursors, implying that these cells could give rise to different cell types like astrocytes,

neurons, and oligodendrocytes (Aguirre et al., 2004). NG2 cells do express the Olig2 transcription factor that is furthermore essential for their development, as shown in studies in the Olig2 null mutant, where lack of Olig2 leads to almost complete loss of the NG2 cell population (Ligon et al., 2006b). Multiple groups have shown that isolated NG2 cells appear to be able to differentiate to neurons *in vitro* and *in vivo* (Belachew et al., 2003; Aguirre et al., 2004; Dayer et al., 2005). The groups showed that NG2+ cells label with neuronal markers like Neun, TUJ-1, Double cortin, and TOAD-64. Neun is a nuclear protein unique to postmitotic neurons. TUJ-1 recognizes Beta-III tubulin, which is abundant in the central and peripheral nervous systems (CNS and PNS), where it is prominently expressed during fetal and postnatal development. As exemplified in cerebellar and sympathoadrenal neurogenesis, the distribution of Beta III tubulin is specific to neurons. In adult tissues, the expression of Beta III tubulin is almost exclusively in neurons. However, transient expression of this protein occurs in the subventricular zones of the CNS comprising putative neuronal- and/or glial precursor cells. Thus Beta III tubulin is not an ideal marker to define the neuron population. Double cortin is a microtubule-associated protein found in newborn and migrating neurons (Liour and Yu, 2003). TOAD-64 (Turned On After Division) is a protein expressed by early post-mitotic neurons (Liu et al., 2003). Heterozygous and homozygous NG2-EYFP animals yielded identical results, where none of the EYFP expressing cells expressed neuronal markers.

The Neun antibody labeled all neurons, but none of the EYFP expressing cells were Neun positive. Some cells are very closely associated with neurons; making it difficult to clearly conclude if one was observing one double-labeled cell or two closely opposing cells. By using a marker that labeled all the nuclei of all cells, it was evident that these were two cells and not one. In the NG2-EYFP expressing cells, the cell body and the processes expressed EYFP. The EYFP cells did not contain Neun+ nuclei. Multiple brain regions were checked, including the neocortex and the hippocampus. Our observations are thus in contrast to those of the other groups, who observed double-labeled cells in these areas (Belachew et al., 2003; Aguirre et al., 2004; Chittajallu et al., 2004; Dayer et al., 2005).

To identify newborn or migrating neurons, we used an antibody recognizing double cortin instead of TOAD-64. Double cortin is a microtubule-associated protein specific for

newborn and migrating neurons. In the NG2-EYFP mouse, none of the double cortin expressing cells expressed EYFP. Multiple newborn neurons and neuroblasts were seen in the subventricular zone in 10 day old and 28 day old heterozygous and homozygous mice. None of the neuroblasts expressed EYFP, but once again we did see a very close association between some EYFP expressing cells and newborn neurons. From these observations we can conclude that the EYFP cells are not neurons, but we cannot rule out the fact they might have the potential to generate neurons, at a time when EYFP expression has been down regulated.

4.2.6 Dedifferentiation of NG2 cells?

It has been reported that O2A precursors can be reprogrammed to become astrocytes, neurons and oligodendrocytes (Kondo and Raff, 2000). In actuality the O2A precursors can be induced to become oligodendrocytes or astrocytes depending on the culture conditions. If the O2A precursor is pushed toward an astrocytic fate, by culturing in 15% serum for 3 days, then given growth factors like FGF, the cells were reported to have the potential then to generate the three different cell types (Kondo and Raff, 2000). In the developing brain it is known that radial glia cells can give rise to neurons, but in the adult brain these cells disappear and are replaced by astrocytes, which maintain a stem cell quality (Malatesta et al., 2000; Kriegstein and Gotz, 2003; Mori et al., 2005). Thus, conceivably the O2A precursor is first pushed to an astrocytic fate, where it gains a stem cell quality to generate the other three cell types. NG2 cells have an O2A precursor quality, where they can generate oligodendrocytes and astrocytes depending on culture conditions (Diers-Fenger et al., 2001). One can argue that NG2- cells derived astrocytes attain a stem cell potential that can then derive astrocytes, neurons, and oligodendrocytes *in vitro*. Just because NG2-EYFP+ cells that are labeled with neuronal markers are not seen does not rule out the fact that these cells could have the potential to generate neurons by a pathway of first generating multipotent astrocytes (figure 4.2.1).

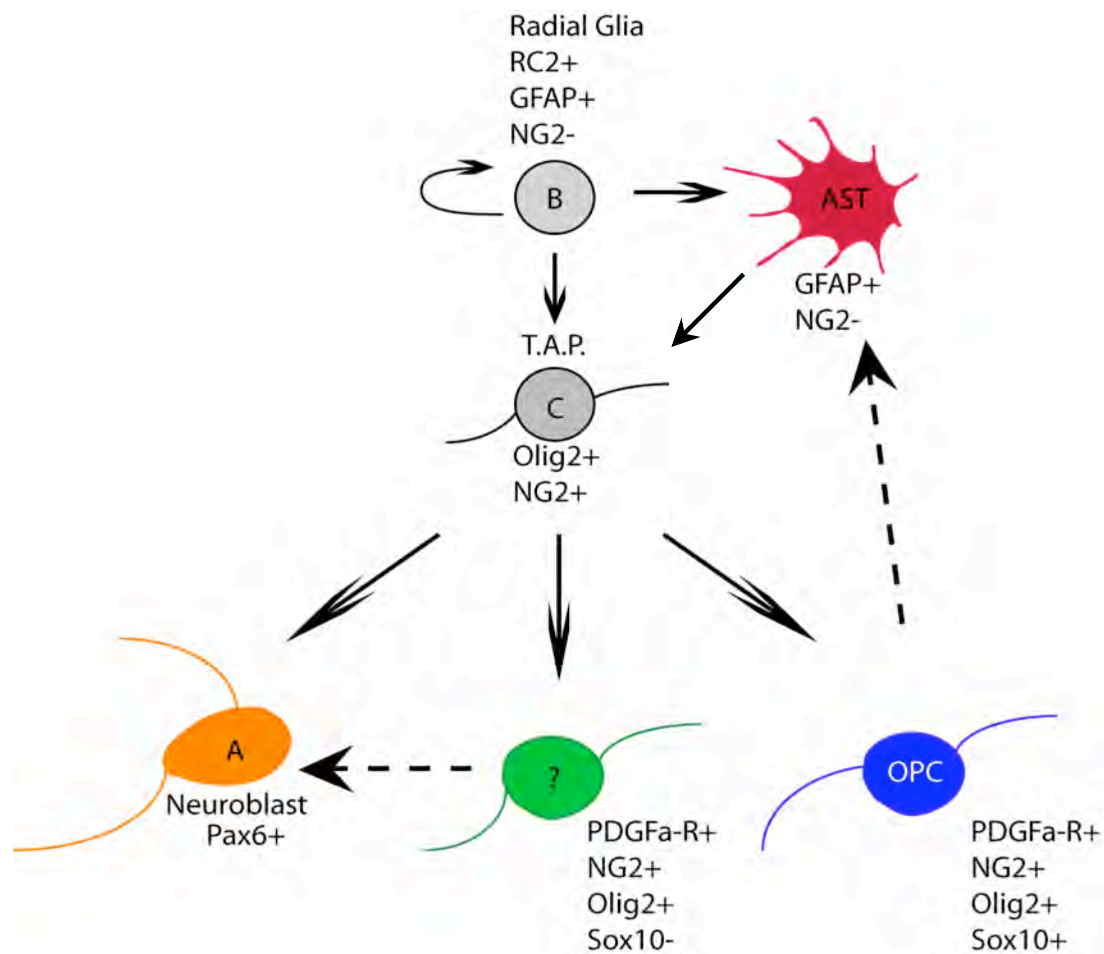


Figure 4.2.1: A Schematic Representation of How Neurons could derive from NG2+ cells.

NG2+ cells represent a heterogenous population, which could give rise to oligodendrocytes, astrocytes and possibly neurons *in vitro and in vivo*. (OPC-Oligodendrocyte Precursor Cell, T.A.P.- Transient Amplifying Precursor)

4.3 The Role of NG2 cells at Synapses

Multiple groups have shown that immature glia express AMPA and GABA receptors in the CNS during development and adulthood (Kettenmann et al., 1991; Von Blankenfeld et al., 1991; Gallo et al., 1996; Steinhauser and Gallo, 1996)(Williamson et al., 1998; Nguyen et al., 2003). It is known that some NG2+ cells make identifiable synapses with neurons within the hippocampus and cerebellum of the brain, where they respond to neuronal stimulation and neuronal release of GABAA and glutamate via their expression of AMPA and GABAA receptors (Bergles et al., 2000; Seifert et al., 2000; Lin and Bergles, 2002; Matthias et al., 2003; Lin and Bergles, 2004a, b; Lin et al., 2005; Ge et al., 2006). Our group has shown that the NG2 transmembrane glycoprotein interacts with AMPA receptors via a complex with the PDZ-protein GRIP (Glutamate Receptor Interaction Partner). A complex of NG2, GRIP, and the GluR B/C subunit of the AMPA receptor could be immunoprecipitated from primary OPCs and from whole brain (Stegmuller et al., 2003). The majority of the AMPA receptors in the CNS are permeable to Ca+, which is known to play a role in cell proliferation and lineage progression of oligodendrocytes (Gallo et al., 1996; Steinhauser and Gallo, 1996; Yuan et al., 1998). Ca+ could have possible effects on gene regulation and expression within the NG2+ glial cells similar to the role in neurons. NG2 could be clustering glial the AMPA receptors toward the presynaptic neuron, which is thus ideally positioned to respond to released glutamate (Stegmuller et al., 2003). Since NG2 is expressed by early oligodendrocyte precursor cells, signaling via the NG2-AMPA receptor complex may keep these cells at an immature state, to provide support for neurons. How NG2 cells support neurons is still not clear: however a release of neurotrophic factors could play a role in this regard. The question arises as to how the NG2 glycoprotein interacts with the presynaptic neuron. This could possibly be through a receptor that interacts with the LNS domains of the NG2 protein (Figure 4.3.1). Such a neuronal receptor still remains elusive. In the NG2-EYFP mouse a majority of the EYFP expressing cells are associated with neurons, making intimate contact in brain regions additional to the hippocampus and the cerebellum. Since AMPA and GABAA receptors are expressed on the cell body and processes of NG2 cells, the NG2-EYFP+ cell may conform to the cell body of the neuron waiting for neurotransmitter release. This enwrapping of the cell body is easy visible in both the NG2-EYFP heterozygous and the NG2-EYFP homozygous mouse. Furthermore, the NG2 cells could play a role in modulating the synapse through the release of factors like

BDNF, which are known to influence synaptic formation (Komitova et al., 2006). This conformation of the cell body of the NG2 cell around the neuron has been observed in electron micrographs in rats and monkey (Peters, 2004). Electron dense areas were seen between the two cells, indicating the formation of synapse between the two cells. It is thus likely that these neuron-glia synapses are much more widespread and not just restricted to the hippocampus and the cerebellum as has been reported to date by electrophysiology and EM analysis (Bergles et al., 2000; Lin et al., 2005). It is still not clear if the NG2 cells actually integrate into the neuronal signaling network. Earlier studies using biotin have shown that NG2 cells do not couple with other cells (Matthias et al., 2003). What impacting role NG2 cells play in the neuronal network is still elusive.

Chittajallu et al. has shown that NG2 cells in different brain regions of the developing mouse first have different morphologies and elicit different types of action potentials. This observation that the NG2 cells have different morphologies can also be seen in the NG2-EYFP mouse line. Furthermore, they also demonstrated that NG2+ cells elicit immature action potentials within the grey matter, but not within the white matter. This could indicate that these cells could be immature neurons derived from the NG2+ cells (Chittajallu et al., 2004). This observation is still not completely understood, but it is certain that the NG2+ cell population is a heterogenous population in intimate contact with neurons

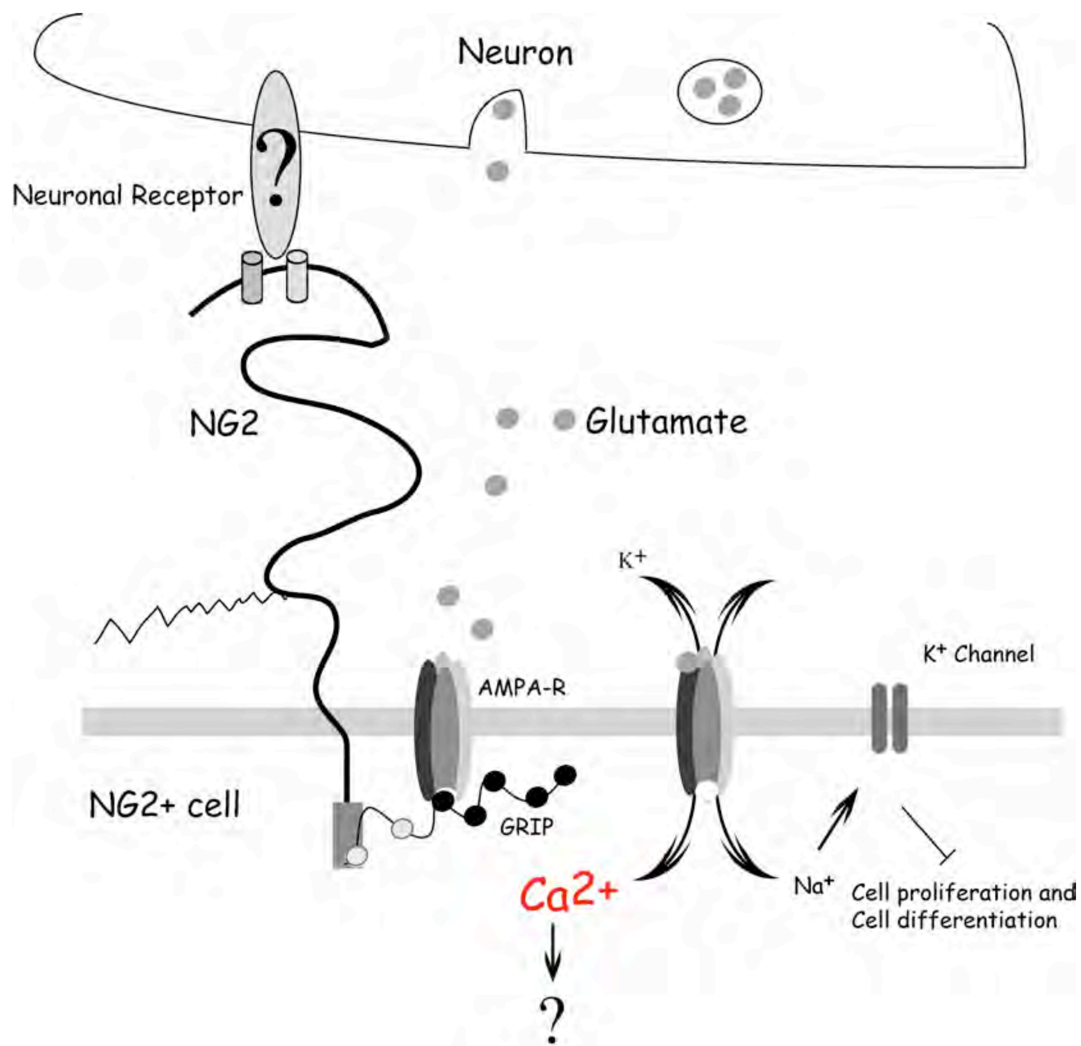


Figure 4.3.1: A Schematic Diagram of a possible Role of NG2 at the Neuron-Glia Synapse

The NG2 protein could play a role in clustering the AMPA toward the neuron for glutamate release. After the glutamate release NG2 cells could still maintain their immature precursor quality, due to an influx of Ca^{2+} that influences gene expression.

4.4 The Generation of the NG2-Cre and NG2-Cre-Intron Targeting Vectors

Targeting vectors were made where the Cre recombinase gene replaced the EYFP gene in the start codon of the NG2 locus. Difficulties arose in the final Cre targeting vectors, where the Cre gene was translated into protein causing the excision of the lox P floxed Neo resistance cassette resulting in loss of the vector in bacterial transformation (figure 4.4.1). The lox P site is an asymmetric region determining whether excision or inversion of an intervening DNA sequence occurs after recombination (Abremski et al., 1986; Sternberg et al., 1986; Stricklett et al., 1998; Nagy, 2000). The lox P floxed Neo was subsequently replaced by an Frt floxed Neo, bypassing this problem of excision by the Cre recombinase. Both Cre recombinase and Flp recombinase belong to the same family, but recognize different asymmetric sites (Qian et al., 1990; Nakano et al., 2001).

The targeting vector containing the start codon of NG2, followed by the Cre recombinase gene and an Frt floxed neomycin resistance gene was electroporated into the OLA-129 embryonic stem cell line. The original targeting vector was modified slightly to include an intron containing poly A tail directly following the Cre gene. PCR analysis of cells surviving G418 selection detected no homologously recombined clone, indicating a lower frequency of recombination when compared to the NG2-EYFP vectors.

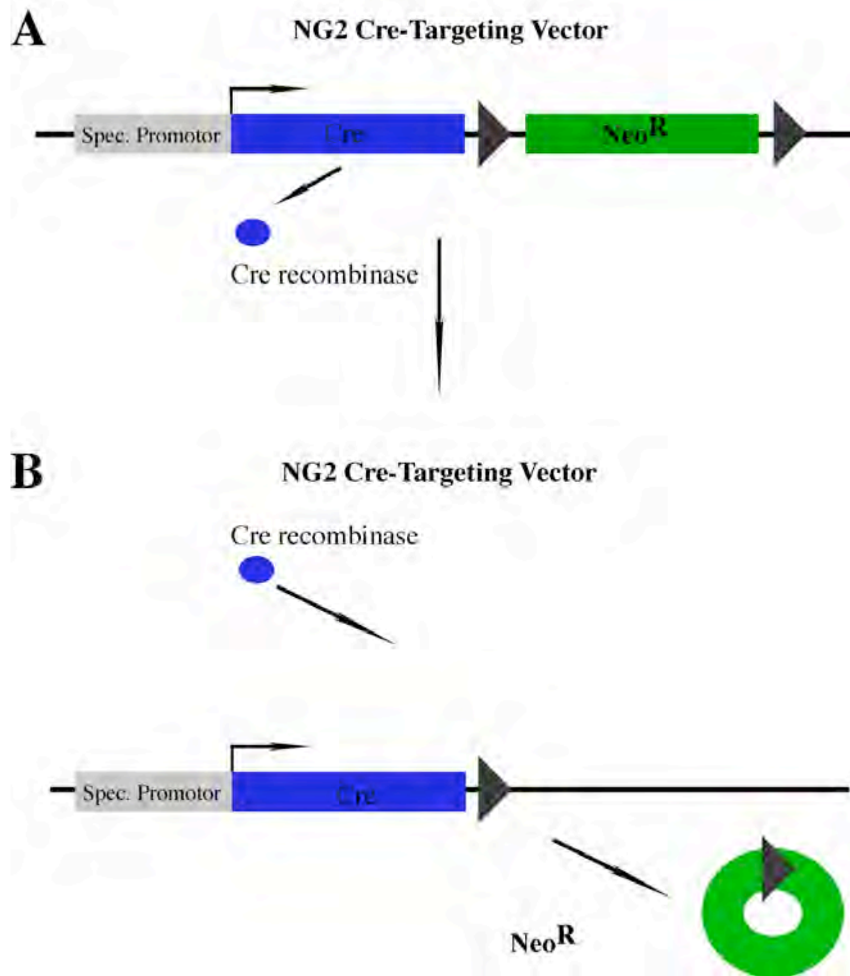


Figure 4.4.1: Schematic representation of Cre recombinase excising the lox P floxed Neo cassette in the targeting vector

4.5 Outlook

The NG2-EYFP heterozygous and homozygous mice are tools to examine:

- Neuron-glial signaling at the specialized synapses in the NG2 cells (eg. Electrophysiology).
- Secretion of neurotrophic factors by NG2 cells (eg. Apo E and BDNF).
- Neuron-glial signaling in mice lacking the NG2 protein.
- Heterogeneity of the NG2 cells (eg. Chip analysis on FACS sorted cells)
- Behavioral response (eg. Stress) in mice lacking the NG2 protein.
- The response of NG2+ cells to lesions including demyelination.
- Whether NG2+ cells have the ability to differentiate to neurons, is still an unanswered question. The only way to answer this question is by making this NG2-Cre mouse. With this mouse we can label all NG2+ cells and their offspring by crossing this mouse to reporter mice.
- Deletion of specific genes in the NG2 cells by Cre recombinase to determine their importance in the cell development.

5. Summary:

A range of vectors were made in which the EYFP gene or the Cre gene were inserted in the start codon of the NG2 gene. The NG2-EYFP vectors were used to generate NG2-EYFP "knockin" mice by homologous recombination. The F1 generation showed lack of EYFP expression, due to Neo^R cassette interference. Excision of the Neo^R, by breeding the F1 generation to ELLA-Cre mice allowed proper expression of EYFP. NG2-EYFP heterozygous mice were characterized in detail for astrocytic, neurogenic and oligodendrocytic properties through antibody labeling. NG2-EYFP+ cells did not label for the astrocyte marker GFAP, but some cells did express S100 Beta. The cells did not label with any neuronal markers like Beta III tubulin, Neun, and double cortin, but many of the NG2-EYFP+ cells made intimate contacts to the neurons. These contacts are widespread throughout the grey and white matter of the brain. The NG2-EYFP+ cells did label for oligodendrocyte markers like PDGFα-R, NG2, Olig2, O4, and Sox 10. There were a few cells termed phantom cells that did label for NG2, but had no EYFP expression. This could have been caused by improper excision of the Neo^R cassette in the F2 generation. The heterozygous mouse is a tool to allow the characterization of the *in vivo* properties of the NG2+ cells.

Breeding of these mice to homozygosity yielded an NG2-knockout mouse, which was also subjected to initial characterization. The NG2-EYFP homozygous showed equivalent cell labeling results to the NG2-EYFP heterozygous mouse, but the phantom cells disappeared in the knockout. The results show that the NG2 cells are a heterogenous population that does not express astrocytic or neuronal markers. The homozygous mouse is an ideal tool to further dissect the properties of the cells, lacking NG2 *in vivo*.

Vectors where Cre was inserted into start codon of the NG2 gene did not homologously recombine into the embryonic stem cell. The targeting vectors were thoroughly examined for mutations, but none were found. More rounds of embryonic stem cells electroporation need to be done to attain an ES cell clone where homologous recombination has taken place.

6. Abbreviations:

AMPA _-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
APS Ammonium-persulphate
BrdU Bromo deoxy Uridine
CC Corpus callosum
CNP 2’, 3’ Cyclic nucleotide phosphodiesterase
CNS Central nervous system
CSPG Chondroitin sulphate proteoglycan
CTX Cortex
DAPI 4', 6-Diamidino-2-phenylindole
DMEM Dulbecco’s modified Eagle’s medium
DTT Dithiothreitol
DXC Double cortin
EAE Experimental Allergic Encephalomyelitis
EGFP Enhanced green fluorescent protein
ERK Extracellular signal regulated kinase
FACS Fluorescence activated cell sorter
FGF Fibroblast growth factor
GABA g-amino-butyric acid
GAG Glycosaminoglycan
GFAP Glial Fibrillary Acidic Protein
GLAST glutamate/ aspartate transporter
GLT-1 glutamate transporter-1
GRIP Glutamate receptor interaction protein
GRP Glial restricted precursor
HBSS Hanks balanced salt solution
HEK Human embryonic kidney
Hipp Hippocampus
KAR Kainic acid receptor
LNS Laminin-Neurexin-Sex Hormone binding globulin
MAG Myelin associated glycoprotein
MBP Myelin basic protein
MCSP Melanoma Chondroitin Sulphate Proteoglycan
MOG Myelin oligodendrocyte glycoprotein
MS Multiple Sclerosis
MUPP1 Multi-PDZ domain protein 1
Neun Neuron nuclear protein
NG2 Nerve glia antigen 2
NMDA *N*-methyl-D-aspartic acid
O2A Oligodendrocyte type 2 astrocyte
OPC Oligodendrocyte precursor cell
PBS Phosphate buffered saline
PDGF-alpha-R Platelet-derived-growth-factor alpha receptor
PDZ PSD-95, Disc-large protein, Zona occludens
PFA para-formaldehyde

PKC Protein kinase C
PLL Poly L-lysine
PLP Proteolipid protein
PNS Peripheral nervous system
SDS Sodium dodecyl sulphate
SEP Septum
STR Straitum
SVZ Subventricular zone
TEMED N, N, N', N'-Tetramethylethylenediamine
TOAD-64 Turned on after division

7. References:

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Publications during the course of the current thesis

Karram K, Chatterjee N, Trotter J. (2005) NG2-expressing cells in the nervous system: role of the proteoglycan in migration and glial-neuron interaction. J Anat 207:735-744.

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Conference Presentations

Poster presentation at EUROGLIA 2005, 7th European Meeting on Glial Cell Function in Health and Disease, May 17-21 2005, Amsterdam.

Poster presentation at Society for Neuroscience 2005, Session: Gila, Radial glia and Astrocytes, November 12-17 2005, Washington D.C. “ Generation of a NG2-EYFP mouse for the studying the *in vivo* properties of the NG2-expressing cells”.

Poster presentation at Synapse Conference, May 29-31 2006, Paris, “ The role of glia in the synapse”.

8. Acknowledgement: