Mouse embryonic stem cells as a discovery tool in neurobiology

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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March 2012

Genehmigt von der Philosophisch
Naturwissenschaftlichen Fakultät

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Basel den 10. November, 2009

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Acknowledgements

I would like to thank Yves for being a very inspiring and caring teacher. I appreciate greatly his support, his trust in me and the many discussions which influenced my thinking immensely.
Also thanks to all the members of the lab, past and present for their help.
I am especially grateful to Chantal Rencurel for providing me with excellent technical assistance. I also thank Miriam Bibel for developing the ES cell differentiation assay that has been key in my work, Heiko Lickert and Patrizia Giallonardo for the tetraploid complementation assay and Magdalena Gotz for her essential contribution.
Special thanks to Mihai Ionescu for being always so helpful, and to Ruben Deogracias, Mori Yazdani, and Vincent Bischoff for the countless coffees that significantly contributed to my work and made life in the lab fun…

Dedicated to my Emir.
Με πολλη αγαπη στην γιαγια και τον παππου μου, στον αδερφο μου Διονυση και στους γονεις μου.

SUMMARY

Mouse ES cells can recapitulate, under suitable tissue culture conditions, early events in neurogenesis. As wildtype or genetically modified ES cells can be grown in unlimited quantities, their differentiation into neurons represents an attractive model for studying the function of genes involved in early development, such as those controlling neuronal specification and survival. A few years ago, our laboratory established a robust differentiation protocol leading to the generation of well-defined and virtually pure populations of Pax6-positive radial glial (RG) cells with a profile and developmental potential characteristic of Pax6-positive RG cells of the cortex. Like their in vivo counterparts, these progenitors generate homogeneous populations of glutamatergic neurons. In my thesis work, I first addressed the role of Pax6 in the generation, specification and developmental potential of RG cells, by analyzing the progeny of ES cells isolated from homozygote *Pax6*-mutant embryos. I found that while Pax6 is not required for the generation of neurogenic RG, it is both sufficient and necessary for specifying them into a glutamatergic lineage. RG cells lacking Pax6 express genes specifying an interneuron fate, like Mash1, and generate GABAergic inhibitory neurons. These cells die prematurely due to an aberrant over-expression of the neurotrophin receptor p75. I could verify these findings in the cortex of mutant embryos lacking Pax6. This work led to new insights as to the regulation of neuronal specification and survival during neurogenesis.

In the second part of my thesis, I used this ES cell-based differentiation system to test any potential instructive roles of the 3 neurotrophin tyrosine kinase receptors TrkA, TrkB and TrkC, after recombining them into the neuron-specific *mapt* locus. This approach led to the surprising observations that TrkA and TrkC cause neuronal death when not activated by their neurotrophin ligands, whereas TrkB does not. Both the death inducing activity of TrkA and TrkC and the lack of death-inducing activity of TrkB were explained by differential distribution of these receptors with p75. The TrkA and TrkC-induced death involves their segregation together with p75 in lipid rafts, and the subsequent proteolysis of the latter

signals to the apoptotic machinery. By contrast, TrkB is not recruited to lipid rafts and it does not result in p75 proteolysis. Subsequent analyses of *TrkA* and *ngf* mutants, as well as of embryos lacking both TrkA and p75 receptors confirmed the relevance of this novel death triggering mechanism during the development of the peripheral nervous system. These findings also point to a major, and so far un-described, difference in the way growth factors regulate the survival of neurons in the developing peripheral versus central nervous system. It is the receptors themselves that cause neurons to become growth factor dependent in the peripheral, but not in the central nervous system.

Taken together, my results demonstrate that the differentiation of mouse embryonic stem cells into defined neuronal populations represents a useful tool allowing observations to be made that are relevant to the development of the nervous system.

1.INTRODUCTION

1.1) DEVELOPMENT OF THE PRE-IMPLANTATION EMBRYO

Mammalian development starts with the fusion of two highly specialized, transcriptionally silent germ cells, leading to the formation of a totipotent zygote. The zygote, or fertilized egg, undergoes several cleavage events leading to the production of an 8-cell morula. This early embryo is the basis for the first lineage decisions, underlined by the instructive role of certain transcription factors. During this process, the morula undergoes compaction and polarization to transform into the blastocyst, a cavity-filled structure consisting of the founder fetal tissue, the epiblast, as well as two extraembryonic structures: The first is a layer of epithelium called the trophectoderm (TE), which is specified early on by the transcriptional regulators Tead4, a TEA domain family transcription factor and its downstream target caudal-related homeobox 2 (Cdx2) (Nishioka et al., 2008). Cdx2 protein starts in eight-cell blastomeres and is gradually upregulated in future TE cells located toward the exterior of the embryo that finally enclose the epiblast and blastocoel. The second, the primitive endoderm (PrE), is consequently specified within the inner cell mass by GATA6 and eventually forms a distinct layer at the late blastocyst-stage embryo lining the blastocoel side of the epiblast (see Fig.1).

The epiblast is a transient structure, characterized by the molecular signature of 3 key transcription factors: Oct4, Sox2 and nanog. Although Oct4 is ubiquitously expressed prior to the segregation of fetal and extraembryonic tissues and as early as the unfertilized egg and in the zygote, its expression is then restricted to

epiblast cells of the pre-implantation embryo. Later in development, Oct4 is expressed by migratory primordial germ cells (PGCs) where it persists throughout the formation of the genital ridges in both sexes. A member of a group of transcription factors that bind the octamer sequence ATGCAAAT or to alternative A/T-rich sites (Saijoh et al., 1996), Oct4 is a crucial component of pluripotency, as demonstrated by the compromised ability of mutant embryos to generate primitive endoderm and to develop further (Nichols et al., 1998).

Sox2, a member of the transcription factors containing a high mobility group (HMG) DNA binding motif, acts synergistically with Oct4 to regulate target genes, often by binding to adjacent sites on their promoters (Chew et al., 2005). Interestingly, Sox2 mutant embryos lack Oct4-expressing cells and cannot sustain an epiblast component. However, in contrast to Oct4 mutants, the formation of the primitive endoderm is not compromised (Avilion et al., 2003). The key distinguishing contribution of Sox2 appears to be in maintaining *Oct4* expression. Consistent with this view, enforced expression of *Oct4* can rescue ES cells from differentiation induced by the loss of *Sox2* (Masui et al., 2007).

Nanog, like Oct4, it is a homeodomain (HD) containing protein. However, its HD sequence is not more than 50% homologous to the homeodomains of other mouse proteins (Kappen and Ruddle, 1993), indicating that it is a divergent HD protein. Nanog protein is first detected in morulae being most obvious in cells in the interior of the embryo.

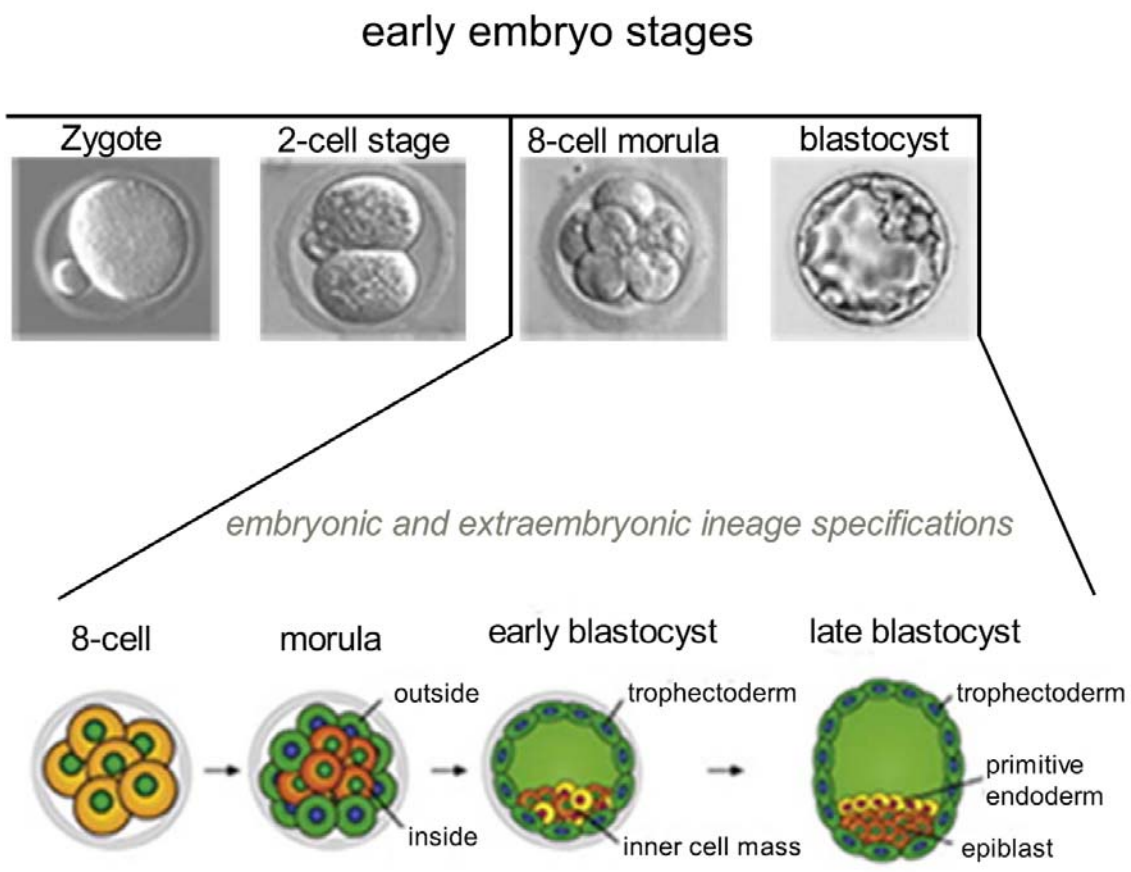

Figure 1. Early embryo stages. Images (top row) and schematics (bottom row) of the early stages of embryo development, from the zygote to the late blastocyst.

High levels of Nanog mRNA persist in the early blastocyst, but its expression declines prior to implantation. This dynamic expression pattern could indicate that downregulation of Nanog may be important to avoid an uncontrolled expansion of pluripotent cells. Following implantation, Nanog mRNA is expressed in a subset of epiblast cells with highest levels in the proximal posterior region; upon entry into the primitive streak, Nanog mRNA is rapidly downregulated (Hart et al., 2004) and is subsequently detected in migratory PGCs and those in genital ridges between days 9 and 13 of gestation (Chambers et al., 2003) While at 3.5 dpc *nanog* null embryos are indistinguishable from control, when cultured on gelatin-coated plate, however, the inner cell mass (ICM) of *nanog* null blastocysts fails to proliferate (Mitsui et al., 2003).

1.2) ORIGINS AND CHARACTERISTICS OF EMBRYONIC STEM CELLS

Early work with mouse teratocarcinomas paved the road for the derivation of embryonic stem (ES) cell lines (Smith, 2001). These germ cell tumors in addition to multiple differentiated tissues found in benign teratomas also contain undifferentiated stem cells, termed embryonal carcinoma (EC) cells (Damjanov and Solter, 1974; Dixon and Moore, 1952; Kleinsmith and Pierce, 1964). The discovery that male mice of the SV129 strain have a high incidence of spontaneous testicular teratocarcinomas (Stevens and Little, 1954) was key to making EC cells amenable to experimental analysis and conditions were soon after established to culture these cells in vitro (Kahan and Ephrussi, 1970).

The cellular origin of teratocarcinoma-forming cells in the pregastrulation embryo was shown to be the epiblast (Diwan and Stevens, 1976). However, as

pluripotent cells of the intact early embryo proliferate only for a limited period of time, it was not immediately obvious that pluripotent cell lines could be established without undergoing a malignant transformation. The striking finding that embryonic genital ridges and peri-implantation stage embryos transplanted into ectopic sites of adult mice, such as under the kidney capsule, gave rise to teratocarcinomas with a high frequency and in strains that did not spontaneously produce these tumors (Solter et al., 1970; Stevens, 1970), fueled attempts to directly derive pluripotent cell lines from pre-implantation embryos without the teratocarcinoma step (Evans and Kaufman, 1981; Martin, 1981). The success of these attempts demonstrated that embryo founder cells can become deregulated to support indefinite self-renewal, indicating that they have an intrinsic propensity for extended proliferation, a property also shared by EC and germ cells.

The origin of murine ES cell lines lies in the founder cells of the blastocyst stage pre-implantation embryo, known as the epiblast. ES cell lines established from the epiblast of pre-implantation mouse embryos can be propagated under culture conditions similar to those used for EC cells, consisting of a feeder layer of fibroblasts and the presence of serum. However the principal difference between the two is that even after expanded propagation and manipulation in vitro, ES cells remain capable of re-entering embryogenesis: EC cells contribute poorly to chimeric fetuses when injected into blastocysts and as they are usually aneuploid, they cannot undergo meiosis and fail to produce germ cells. By contrast, ES cells retain their full developmental potency as they contribute well

to chimeras including to germ cells in the genital ridges, thus allowing for further germline transmission.

The landmark of deriving mice from cultured ES cells was reported by the Evans laboratory in 1984 (Bradley et al., 1984). Consistent with their epiblast origin, ES cells contribute very poorly to extraembryonic endoderm and almost never to trophoblast (Beddington and Robertson, 1989). However, ES cells are self-sufficient for the generation of the entire fetus, as shown by Nagy and co-workers by introducing them into tetraploid recipient embryos, known as the “tetraploid complementation assay” (Nagy et al, 1991, 1993) (Figure 2). In tetraploid embryos extraembryonic structures develop normally, whereas the fetal component fails to grow. In the chimeras generated between tetraploid embryos and diploid cultured ES cells, the entire fetus is derived from the diploid cells, demonstrating that they are competent of generating the entire fetus.

With regards to establishing culture conditions for ES cells, Smith and Hooper demonstrated in 1987 that medium conditioned from the buffalo rat liver cell line was able to sustain ES cell propagation and further fractionation of this medium identified leukemia inhibitory factor (LIF) as the active component (Smith et al., 1988). LIF is a member of the IL6 family of cytokines that signal through receptor complexes always including the transmembrane receptor gp130 as a transducing subunit, reviewed in (Heinrich et al., 2003). The signaling cascade downstream of this receptor involves the JAK-kinase mediated activation and recruitement of the transcription factor STAT3. While LIF signaling is known to operate in multiple tissues, like for example myeloid cells, its output in inhibiting

differentiation is unique to the ES cellular context. Inactivation of LIF or its receptor in mice (Stewart et al., 1992; Ware et al., 1995; Yoshida et al., 1996) indicated that these molecules are not required for development prior to gastrulation. However, further investigation revealed that these mutations compromise the ability of the embryo to undergo diapause, a process whereby blastocyst stage embryos are halted from implanting into the uterus until mother estrogen levels are restored, typically following the weaning of a preceding litter. It is worth noting that almost all mouse ES lines have been derived from diapause embryos, since this greatly improves the efficiency of the derivation. External signals like LIF support the undifferentiated state of ES cells by regulating the “pluripotent genes”.

There is considerable evidence that a “trinity” of transcriptional regulators, Oct4, Sox2 and Nanog, is responsible for maintaining ES cells in a pluripotent state both in vitro and in vivo. Surprisingly, however, Oct4 and Sox2 are also responsible for the progressive extinction of pluripotency by activating the transcription of FGF4. This growth factor then acts in an auto- and para-crine fashion to propel ES cells towards a general state of differentiation by activating downstream signaling through the mitogen-activated protein kinase Erk1/2 (Kunath et al., 2007). Consequently, ES cells in vitro or epiblast cells in vivo lacking FGF4 or components of the Erk1/2 signaling are impaired for lineage commitment. As maintaining ES cells pluripotency means to interfere with their developmental progression towards differentiation, Austin Smith and colleagues

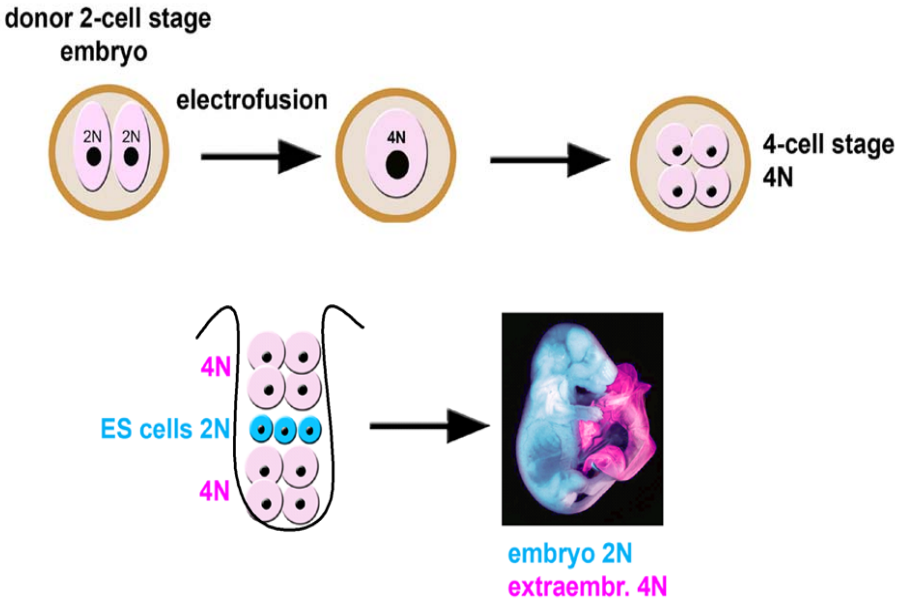

Figure 2. Schematic of tetraploid complementation assay. Diploid donor embryos at the 2-cell stage are fused to a tetraploid state. Tetraploid embryos develop in vitro to the 4 cell stage. Subsequently, the zona pellucida is removed and tetraploid cells are aggregated in wells together with the diploid ES cells of interest, then transferred to pseudopregant females.

demonstrated that genetic disruption or pharmacological inhibition of the endogenous FGF4-Erk1/2 signaling axis is sufficient to maintain ES cell pluripotency in the absence of exogenous LIF and STAT3 activation (Ying et al., 2008). However, growth and viability are impaired under these conditions, either as a direct consequence of loss of Erk activity or possibly due to off-target effects of the chemical inhibitors. This “collateral damage” can be avoided by inhibition of glycogen synthase kinase-3 (GSK3). The fact that inhibition of the one and same signaling axis can capture the pluripotent state of both ES and epiblast cells suggests that this pathway may be conserved between mammalian species and could therefore facilitate the derivation of authentic ES cells from other mammals including humans. Indeed, using the 2-inhibitors described above the first pluripotent rat ES lines were isolated, which were so far impossible to derive with conventional means (Buehr et al., 2008; Li et al., 2008). To date, while ES lines have also been isolated from human pre-implantation embryos, the necessary culture conditions and even the identity of true human ES cells remain unclear. Moreover, whether the same FGF-Erk signaling axis is operative during early human development is under investigation with considerable efforts, including in our own laboratory, focusing on deriving “ground state” hES lines in the presence of the 2 inhibitors.

1.3) NEURAL INDUCTION IN THE EARLY EMBRYO

Morphogenesis of the peri-implantation mouse embryo proceeds with the transformation of the compact inner cell mass (ICM) of the late blastocyst into a hollow structure, the egg cylinder: Shortly after implantation, the pro-amniotic

cavity forms in the core of the ICM and, by E6.0, the embryonic ectodermal cells that line the cavity differentiate into a pseudostratified columnar epithelium. The trophectoderm-derived extraembryonic ectoderm undergoes a similar process at a slightly later stage. The process by which these changes occur is known as cavitation, and similar changes also underline the formation of other hollow (tubular) structures that arise from compact primordia, such as the ducts of various exocrine glands. Cavitation prepares the embryo for gastrulation, the process by which the three germ layers are formed, for review see (Beddington and Smith, 1993).

During gastrulation, primitive ectoderm in the anterior distal quadrant of the egg cylinder expands proximally and differentiates to establish the ectodermal germ lineage, which gives rise to the non-neural ectoderm, the neurectoderm and the neural crest.

1.3.1) The neurectoderm

Neuroepithelial progenitor cells of the neurectoderm differentiate to the major cell types of the *central* nervous system (CNS), neurons and glia. Establishment of the neurectodermal lineage has been relatively well characterized at the level of gene expression, such that cell differentiation and tissue morphogenetic events can be correlated with the temporal regulation of several genes.

Formation of the neural plate from definitive ectoderm is coincident with up regulation of *Sox1* (Pevny et al., 1998), and expression of *Gbx2 (*Wassarman *et al., 1997)*. With closure of the neural tube, *Gbx2* expression is downregulated within the neural epithelium (Wassarman et al., 1997). The undifferentiated

neurectoderm, or neuroepithelial progenitors, within the neural tube are characterized by the expression of several markers including *Sox1*, *Sox2*, *nestin* (Lendahl et al., 1990; Pevny et al., 1998; Wood and Episkopou, 1999), musashi1 (Kaneko et al., 2000), and N-CAM (neural cell adhesion molecule) (Ronn et al., 1998; Rutishauser, 1992). These neuroepithelial cells can either differentiate directly into neurons, but more often they do so by generating progenitor intermediates: These are the so called radial glial and basal progenitors. Radial glia are more abundant and widely distributed throughout the neural axis, whereas basal progenitors, which could be generated directly from neuroepithelial cells, or indirectly from radial glial cells, are less abundant in rodents, compared to primates, and restricted to the cortical region of the telencephalon. Radial glial cells have a bipolar morphology, with one extension and broad endfoot sited at the luminal surface and a longer process extending in the opposite direction through to the basement membrane adjacent to the pia mater, reviewed in (Gotz and Barde, 2005). Similar to neuroepithelial cells, they also exhibit an ovoid cell body and have a nucleus situated in the ventricular zone, adjacent to the lumen that undergoes interkinetic nuclear migration. Ultrastructural studies performed using electron microscopy revealed that radial glia function as a substrate/guide upon which newly generated immature neurons migrate (Rakic, 1971a, b). Radial glia display astrocyte characteristics, such as electron lucent processes, abundant intermediate filaments, and glycogen granules condensed at their end-feet (Choi, 1981; Rakic, 1971a). They also express several astrocyte markers, such as astrocyte specific glutamate

transporter (GLAST) (Shibata et al., 1997), brain lipid-binding protein (BLBP) (Feng et al., 1994), and glial fibrillary acid protein (GFAP) (Levitt and Rakic, 1980), although in rodents GFAP is not expressed by radial glia (Choi, 1981; Sancho-Tello et al., 1995). Radial glia are also immunoreactive to the RC2 and Vimentin antibodies (Houle and Fedoroff, 1983; Misson et al., 1988). Together these astrocytic features distinguish radial glia from their neuroepithelial ancestors.

1.3.1.1) Neuronal specification

After induction, the neurectoderm is intricately subdivided, or patterned, with respect to both the dorsoventral and anterior-posterior axes, manifested in regionalized gene expression within the neural tube. Positional specification in the neural tube is determined in response to signals emanating from neighboring tissues. For example, concentration gradients of sonic hedgehog (Shh) from the notochord and floor plate and BMP4 from the overlying surface ectoderm result in dorsal-ventral specification (Sasai and De Robertis, 1997). In conjunction with anterior-posterior specification (see Beddington and Robertson, 1999), this results in regionally restricted gene expression and restriction of developmental potential. For example, the expression of *Hoxc5* and *Hoxc6* delineates areas of posterior neural tube fated to contribute to the spinal cord (Wichterle et al., 2002), whereas dorsal-ventral specification can be recognized by expression of genes like *Nkx6.1* and *Olig2* (ventral) and *Dbx1*, *Irx3*, and *Pax6* (dorsal) (Wichterle et al., 2002).

1.3.1.2) The homeodomain transcription factor Pax6

Pax6 is a highly conserved member of the *pax* gene family, and it encodes a protein containing a homeodomain and a paired domain. In the mouse, Pax6 begins to be expressed during the eighth day of development in a restricted area, the dorsal telencephalon, that gives rise to the cerebral cortex (Walther and Gruss, 1991), in a rostro-lateral-high to caudo-medial-low gradient. This is before the generation of radial glial cells, the first defined progenitors that can be distinguished from other neuroepithelial cells (for review, see (Gotz and Huttner, 2005). Pax6 positive radial glial cells are the progenitors of most glutamatergic neurons in the rodent cortex (Malatesta et al., 2003). Pax6 is though to exert its patterning activity by directly activating the bHLH neurogenin 1 and 2 genes, which in turn inhibit the expression of genes such as Mash1, responsible for a ventral telencephalic identity. In the absence of Pax6, the cerebral cortex is markedly smaller (Schmahl et al., 1993), the number of neurons is reduced at midgestation (Heins et al., 2002), and the cortex is ventralized (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). However, as cell lineage analyses cannot be readily performed in vivo, it remained unclear for a long time whether Pax6 controls the generation of radial glial cells, their neurogenic potency or specification. In the first part of my thesis, I used ES cells lacking Pax6 to explore their developmental potential by comparison with wildtype ES cells (see Results section2.a).

1.3.2) The neural crest

The neural crest, a transient structure present only in vertebrates, is derived from the cells located at the border between the non-neural ectoderm and the neurectoderm. The formation of this structure involves several signaling events, including signals emanating from the newly formed neurectoderm, the non-neural ectoderm, as well as the underlying paraxial mesoderm. The main gene families involved in the early steps of induction include, in particular, secreted growth factors of the bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wingless/INT-related (WNT) families, for review see (Knecht and Bronner-Fraser, 2002). During neurulation, the border region containing the "pre-migratory neural crest cells" bends to form the neural folds. Upon closure of the neural tube, neural crest cells are localized to the dorsal aspect of the tube, for review see (Gammill and Bronner-Fraser, 2003). Neural crest cells are migratory, and depending on the organism and the axial level they initiate their migration either from the closing neural folds or from the dorsal neural tube. Changes in cadherin-mediated cell adhesion play a role in the process by which neural crest cells escape from the neural tube, but the precise nature of that involvement remains unclear (Taneyhill, 2008). It has long been known that neural crest cells give rise to an amazingly diverse set of derivatives, including smooth muscle and cartilage, and indeed most of the *peripheral* nervous system (PNS), including neurons and non-neuronal cells (Le Douarin, 1982).

1.4) TROPHIC SUPPORT OF NERVOUS SYSTEM SURVIVAL

Early work with chick and amphibians demonstrated that interactions of neurons with their environment are crucial for their survival. As early as 1909, Shorey reported that unilateral ablation of the limb bud of the chick resulted in severe hypoplasia of both the ipsilateral sensory ganglia and spinal cord, including the motor column in the ventral horn (Shorey, M. L. 1909. J. Exp,. Zool. 7: 25-63). These results were later replicated by Hamburger (1934) and set the foundation for the target-derived neurotrophic theory. The purification of nerve Growth Factor (NGF), first from sarcoma lines and subsequently from snake venom (Cohen and Levi-Montalcini, 1956, 1957; Cohen et al., 1954), followed by the extraordinary demonstration that NGF has all the characteristics of a target-derived molecule necessary for the survival of defined populations of neurons during development (Levi-Montalcini, 1966), allowed the understanding of the target-derived neurotrophic theory at the molecular level. Not only is NGF expressed in the tissues that are innervated by NGF-responsive neurons and axons (Heumann et al., 1984), but also the degree of its expression is proportionate to the density of innervation (Korsching and Thoenen, 1983; Shelton and Reichardt, 1984). NGF is now well recognized to be required for the survival of all sympathetic and many sensory neurons, as demonstrated by the death of these neurons after axotomy, and the ability of NGF to rescue them, or prevent the death of sympathetic neurons whose nerve endings have been destroyed by 6-hydroxy-dopamine at birth, in a model known as chemical sympathectomy (Levi-Montalcini et al., 1975).

Neurotrophic factors comprise several gene families of ligands and their receptors, with Neurotrophin and GDNF (Glial cell line Derived Neurotrophic Factor) families being the best characterized with regard to their activity as target derived trophic molecules supporting the survival of neurons in the peripheral nervous system.

1.4.1) The Neurotrophin family

Neurotrophins (NGF, BDNF, NT3 and NT4) and their receptors are components of a signaling system known to play major and diverse functional roles in the nervous system of vertebrates (Reichardt, 2006). They signal by activating specific Trk receptors expressed at the cell surface as progenitors exit the cell cycle and begin to grow axons (Bibel and Barde, 2000). The Trk receptors belong to the family of receptor tyrosine kinases, and three *trk* genes have been identified in mammals. The TrkA proto-oncogene was first identified as an NGF receptor (Kaplan et al., 1991; Klein et al., 1991) followed by TrkB and TrkC (for review, see (Barbacid, 1994). NGF is the preferred ligand for TrkA, BDNF and NT4/5 are preferred for TrkB, and NT3 for TrkC (Barbacid 1994). These specificities are not absolute, as NT3 is also a ligand for TrkA and TrkB (Figure 3). Binding of the neurotrophins to the Trk receptors leads to receptor tyrosine phosphorylation (for review, see (Friedman and Greene, 1999). This triggers the activation of pathways leading to the prevention of programmed cell death and neuronal differentiation.

Decades of work with avian and rodent embryos has clarified some of the basic functions of the neurotrophin signaling system, such as the ability of target derived neurotrophins to regulate the survival of large numbers of neurons in the peripheral nervous system (PNS) including most sympathetic and sensory neurons (Reichardt, 2006; Bibel and Barde, 2000). The exquisite ability of neurotrophins to act as messengers between neurons and their targets is explained by their selective expression pattern: Trk receptors are expressed by sensory and sympathetic neurons (Tessarollo et al., 1993), while neurotrophin ligands are primarily localized in the target areas innervated by their responsive axons (Davies, 1994) and released in limiting amounts. TrkA is expressed in all sympathetic neurons, as well as in small and middle size dorsal root gangion neurons. Mice mutant for *ngf* or *TrkA* genes exhibit loss of all sympathetic neurons (Fagan et al., 1996) and of the selective DRG neuronal populations expressing TrkA. Similarly, TrkC is expressed in large size proprioceptive neurons of the DRG, which are lost in *TrkC* or *nt3* mutants. Unexpectedly, NT3 was also shown to be required for the survival of about half the number of cranial sympathetic neurons. This conclusion was reached both with antibodies blocking the function of NT3 (Gaese et al., 1994; Zhou and Rush, 1995) and with *nt3*−/− mutants (Ernfors et al., 1994; Farinas et al., 1994). There is now good evidence that NT3 exerts its survival action through TrkA (Brennan et al., 1999; Wyatt et al., 1997). A small population of DRG mechanoreceptors innervating Merkel cells and deep-hair cells express TrkB and depend on target derived BDNF and

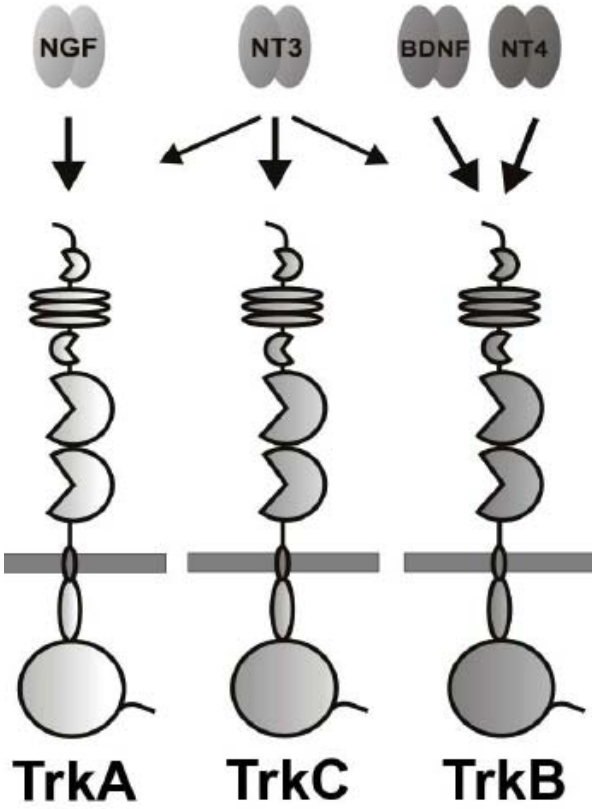

Figure 3. Schematic structures of Trk receptors.

In their extracellular domains, three tandem repeat leucine-rich motives are flanked by two cystein clusters. The main contacts between the Trk receptors and their ligands occur within two Ig-like C2 type domains. The intracellular, enzymatically active, tyrosine kinase domains are highly conserved among the three Trk receptors.

NT4 respectively for their survival (Carroll et al., 1998). In the PNS, TrkB is also expressed in the vestibular, petrosal and nodose ganglia, often together with TrkC, and its activation is required for the survival of these neurons (Ernfors et al., 1994; Jones et al., 1994; Silos-Santiago et al., 1997).

In addition to having a pro-survival role for PNS neurons, neurotrophins are also required for inducing the expression of genes required for the establishment of proper connections. This is best exemplified by the requirement of NT3 signaling for the induction of the ETS transcription factor ER81 in proprioceptive sensory neurons, which is necessary for the formation of the appropriate connections to motor neurons (Patel et al., 2003).

In the CNS, only very few neurons express TrkA (mostly the basal forebrain cholinergic neurons), whereas by contrast TrkB is expressed at comparatively high levels by the majority of CNS neurons. TrkC is typically expressed in the CNS early in development and at lower levels (Tessarollo et al., 1993). The view that neurotrophin dependency for survival would also be applicable to neurons of the central nervous system is mainly based on early classical observations that motorneurons die after removing the target cells they innervate (Shorey 1909; Hamburger, 1934). This view was further supported after it was realized that the death of motor-neurons can be prevented after axotomy by neurotrophins, such as BDNF applied to the cut end of the motor axons (Sendtner et al., 1992). However, it was soon after realized that motor neuron survival is only supported by neurotrophins in lesion paradigms such as axotomy or limb removal (see above). Doubts about the survival promoting role of neurotrophins in the CNS

during NORMAL development begun to arise following the generation of the first *bdnf* knockout animals. They showed very little, if any, cell death in the CNS, in marked contrast to what had been previously observed in the PNS following NGF removal experiments.

In addition to Trk receptors, all neurotrophins also bind to p75, a member of the TNF family, which lacks catalytic activity. As other members of this family, p75 has a death domain in its cytoplasmic tail and signals both in ligand dependent and independent fashion by recruiting a number of interacting molecules. Although p75 over-expression or activation by neurotrophins classically results in cell death, the outcome of its activation often varies considerably depending on the cellular context. P75 is often co-expressed in the same cell together with a Trk receptor, where it has the capacity to modulate neurotrophin signaling (for review see (Barker, 1998).

1.4.2) The glial cell line-derived neurotrophic factor family

Members of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) are crucial for the development and maintenance of distinct sets of peripheral neurons, as well as specific non-neuronal cells, for reviews see (Airaksinen and Saarma, 2002; Baloh et al., 2000; Enomoto, 2005; Takahashi, 2001). Four different ligand-receptor pairs exist in mammals, GDNF-GFRα1; neurturin (NRTN)-GFRα2; artemin (ARTN)-GFRα3; and persephin (PSPN)-GFRα4. For all these pairs, signaling requires the RET tyrosine kinase receptor. In any given RET/GFRα receptor complex, two distinct tasks, namely ligand

binding and intracellular signaling, are performed separately by GFRα and RET, respectively (Figure 4). Mice deficient for *GFLs*, *GFRαs* or *Ret* have been shown to exhibit a number of neuronal deficits, establishing these ligands as a novel and bona fide family of neurotrophic factors acting *in vivo* (Baloh *et al.*, 2000). In addition to nervous system development, they play crucial roles in the development of the urogenital system, including the kidney and testis (Meng et al., 2000; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994).

Highlighting the importance of this signaling system, inactivating mutations in the *Ret* gene have been identified as one of the major causes of Hirschsprung disease (Edery et al., 1994), characterized by congenital absence of enteric neurons in the digestive tract. The development of the enteric nervous system relies on the proper migration, proliferation and differentiation of neural crest cells emanating from the dorsal region of the embryo at the vagal, sacral and trunk levels. Mice deficient for *GDNF*, *GFRα1* or *RET* lack all neurons in the gut distal to the stomach and die within 24 h after birth (Schuchardt *et al*., 1994; Moore *et al*., 1996; Pichel *et al*., 1996; Sanchez *et al*., 1996; (Cacalano et al., 1998); Enomoto *et al*., 1998). In GDNF signaling mutants, the initial deficits are discernible as early as embryonic day (E) 10.5, when EN precursors begin to enter the midgut (Taraviras et al., 1999).

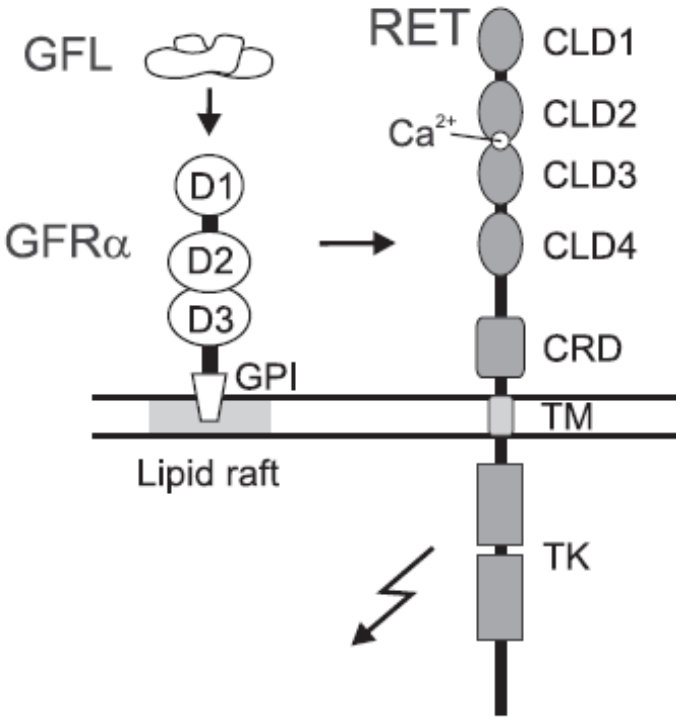

Figure 4. Schematic structures of GDNF family ligand (GFL), GDNF family receptor alpha (GFRa) and RET proteins.

The GFL dimmer first binds a GFRa receptor that is attached to the membrane with a GPI-anchor that keeps the receptor in lipids rafts. GFLs are cystine- knot proteins forming a distant subgroup in the TGF-b superfamily (Eigenbrot and Gerber, 1997), whereas GFRa s are composed of three homologous globular GFR domains (D1–D3) that have a novel fold [Leppänen et al., 2004]. The vertebrate RET extracellular domain contains a single cysteine-rich domain (CRD). A calcium-binding site in RET is important for its activation.

All post-ganglionic sympathetic neurons are located in close proximity to vertebral bodies, meaning that the axons of these neurons have to travel across significant distances to reach their target tissues. During this process, sympathetic neurons target blood vessels as intermediate guidance routes and extend their axons along these vascular pathways. Recent studies have provided evidence that ARTN signaling via GFRα3/RET is the central regulator for the growth of sympathetic axons in alignment with blood vessels. This migration and axon growth are severely impaired in animals lacking ARTN signaling ((Nishino et al., 1999); Enomoto *et al*., 2001; (Honma et al., 2002) followed by increased death of sympathetic neurons later in development. Because a failure in axon growth precedes this cell death, it has been suggested that the abnormal cell death occurs because of the unavailability of some target-derived survival factor, such as nerve growth factor (NGF). Therefore, it seems that during sympathetic neuron development, GFL and NGF provide signals sequentially to support neuronal migration/axon growth and cell survival, respectively. Experimental evidence also indicates that GFL signaling is also required for the survival of parasympathetic neurons during development: At early stages, signaling of GDNF via GFRα1/RET is essential for the proper migration and proliferation of cranial parasympathetic neuron precursors (Enomoto *et al*., 2000; (Rossi et al., 2000)). Later in development, NRTN signaling via GFRα2/RET is required for the survival of parasympathetic neurons and innervation of target organs.

Perturbations in GDNF family signaling also affects different populations of sensory neurons. *GDNF* deficient mice exhibit a substantial loss of neurons in

the petrosal ganglia, which innervate the carotid body. The same neuronal population is affected by a *BDNF* loss-of-function mutation, indicating requirements for both GDNF and BDNF in the development of these viscerosensory neurons (Erickson et al., 2001). The biological roles of the GDNF ligands in trigeminal and dorsal root ganglion neurons is less obvious, as they seem to function in the maintenance of these neurons postnatally. In rodents, approximately two-thirds of the neurons in the spinal sensory ganglia express TrkA at birth. As they mature, half these neurons lose their TrkA expression and begin to express RET and IB-4 (Molliver et al., 1997). In adult animals, the population of *Ret*-expressing neurons includes both large and small-sized cells expressing GFRα1–3 receptors in complementary or overlapping patterns (Bennett et al., 1998; Orozco et al., 2001). Adult *neurturin*-deficient mice lose approximately 50% of their *GFR*α*2*-expressing DRG neurons (Heuckeroth et al., 1999). Similarly to the neurotrophins, the prosurvival activity of GDNF family members is almost exclusive to peripheral neurons and neural crest derivatives. By contrast, CNS neurons do not depend during development on these or any other soluble factors known to date, in order to survive and to establish proper innervation patterns. However, GDNF can have other effects on central neurons. For example, GDNF synthesized first in the plexus of the developing limb and later on in two muscles of the limb, the cutaneous maximus and the latissimus dorsi, is necessary for the induction of the transcription factor Pea3 in the motor neurons innervating these targets, which in turn regulates their correct pattern of connectivity (Haase et al., 2002). In GDNF knockout animals, the induction of

Pea3 fails, and the projections of the relevant motor neurons are misrouted into the spinal cord (Haase et al., 2002).

1.5) NEURONAL DIFFERENTIATION OF EMBRYONIC STEM CELLS

As amply demonstrated with the mouse system, ES cells cultured in vitro, even for extended periods of time, retain the capacity of re-entering embryogenesis, and share with epiblast cells the ability to differentiate into cell types representing all 3 definite germ layers. A realistic goal for neurobiologists interested in developmental mechanisms in vertebrates is then to use ES cells as a tool to delineate lineage commitment decisions and to generate in vitro relevant populations of neurons. Such undertakings, together with the ability to introduce specific genetic alterations, create the possibility of generating in vitro models for studying early steps of neural development that are difficult to access in vivo, because of limited quantity of materials, cellular heterogeneity and developmental gradients.

In order to differentiate ES cells, the cell culture medium is altered such that LIF and BMP/serum are replaced by alternative inductive signals. It was early on appreciated with embryonic carcinoma (EC) cells (Jones-Villeneuve et al., 1982) and later with ES cells (Bain et al., 1995) that culturing them as free-floating aggregates, known also as embryoid bodies, without the addition of LIF and in the presence of retinoic acid and serum, resulted in a differentiation towards the neuronal lineage. Modifications to this protocol were developed soon after in which exposure to retinoic acid was minimized and neural precursor populations

could be induced and subsequently enriched by selective survival in a serum free basal media (Okabe et al., 1996). Other distinct protocols were developed which rely on co-culture with a stromal cell line PA6, or exposure to conditioned media (Kawasaki et al., 2000; Rathjen et al., 1999). Ying and colleagues developed an alternative protocol in which Sox1 expressing neuroepithelial cells can be generated in adherent monolayer at higher efficiencies (>60%) in a process that requires FGF signaling (Ying et al., 2003). However, this protocol, like the preceding ones, results in only partial neural induction and relies on sorting of neurons from a cell mixture. In addition, the neuronal population obtained represents a heterogeneous sum of very different subtypes of neurons, presumably due to the diversifying effect of FGF.

More recently, efforts have been directed towards restricting the ES cell-derived progeny to specific neuronal types. In particular, work from the Jessell laboratory demonstrated that motor neurons can be generated from ES cells by treatment of RA-exposed aggregates to diffusible sonic hedgehog (shh), a morphogen known to play a crucial role in vivo during motor neuron specification (Wichterle et al., 2002). Work from the Vanderhaeghen laboratory demonstrated that in the absence of any morphogens, shh inhibitors facilitated the differentiation of ES cells into an array of neurons with a forebrain-like identity. Interestingly, ES cell-derived neurons corresponding to distinct cortical subtypes appeared in vitro through a coordinated sequence, as observed in vivo (Gaspard et al., 2008).

We recently described an ES cell-based culture system that closely recapitulates the early steps of cortical neurogenesis in vitro. This comparatively simple, but

robust differentiation protocol, based on the treatment of ES cell aggregates with retinoic acid, leads to the generation of neural progenitors identified as Pax6-positive radial glial (RG) cells (Bibel et al., 2004 and Bibel et al., 2007). These progenitors undergo few cell divisions and then go on to terminally differentiate into glutamatergic neurons, the progeny of Pax6-positive RG cells in the developing mouse cortex (Malatesta et al., 2003). Transplants of these cells have revealed some developmental restrictions (Plachta et al., 2004). Upon injection in chick embryos they generate neurons in the spinal cord and dorsal root ganglia (DRG). However, only in the spinal cord do they acquire an appropriate regional identity. This contrasts with the injection of non RA-treated ES cell aggregates that are able to generate both spinal cord neurons and DRG neurons (Plachta et al., 2004). Thus, our in vitro differentiation protocol appears to restrict the global CNS–PNS differentiation potential of ES cell derived progenitors to a CNS fate. The success of this procedure, the first to allow the reproducible generation of well-defined and homogeneous progenitor populations, as opposed to a mixture of different neural types, is thought to lie on the selection of rapidly dividing ES cells. As it is well recognized that ES cells slow down their rate of division as they start to differentiate, a uniformly pluripotent ES cell population can be maintained by simply splitting ES cells frequently, thereby diluting out cells that have begun to differentiate. We now understand (see above) that this is due the fact that 2 of the key pluripotency genes also regulate the expression of FGF4 that causes differentiation.

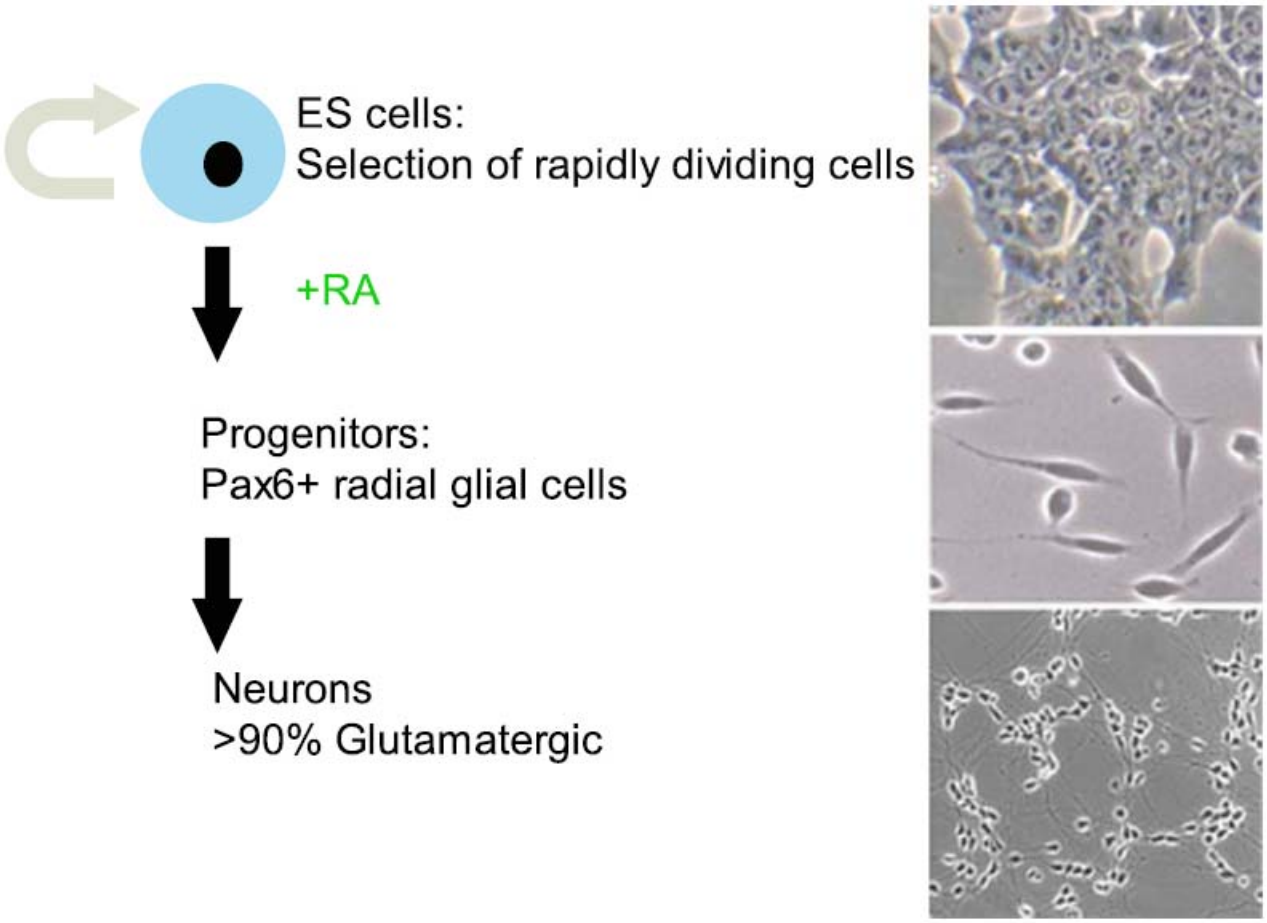

Figure 5. Outline of ES cell neuronal differentiation protocol. Briefly, rapidly dividing ES cells are selected on feeders, then cultured in feeder free conditions in the presence of LIF. ES cells are subsequently cultured as free-floating aggregates in the absence of LIF and treated with retinoic acid to induce neural fate. Following dissociation of aggregates, Pax6-positive radial glial cells are obtained, which rapidly go on to terminally differentiate into glutamatergic neurons.

2. RESULTS

In the first part of my thesis I investigated the role of the transcription factor Pax6 in the specification of cortical progenitors during development, using ES cells lacking Pax6 and comparing their developmental potential to that of wildtype ES cells. In the second part of the thesis, I used ES cells engineered to expressed the neurotrophin receptors TrkA, TrkB or TrkC from a neuron-specific locus, in order to study possible instructive functions of these receptors in developing neurons.

2.1) THE ROLE OF PAX6 IN NEURONAL SPECIFICATION

Radial glial (RG) cells expressing the transcription factor Pax6 are the progenitors of most glutamatergic neurons in the developing cortex of the mouse (Malatesta et al., 2003). Early work revealed the essential role of Pax6 in cortical development, as the small-eye “Sey” mutants lacking functional Pax6 protein display an array of neural defects, including a massive reduction in the number of neurons in the cortex. Pax6 begins to be expressed at embryonic day 8 in the pallium, before the generation of RG progenitors (Walther and Gruss, 1991). As lineage analyses are difficult in vivo at such early developmental stages, the role of Pax6 in RG generation and specification remained elusive. In the first part of my thesis, I used ES cells lacking Pax6 (thereafter referred to as Sey or mutant cells) and compared their developmental potential to that of wildtype cells.

2.1.1) Pax6 mutant embryonic stem cells generate mash1-positive radial glial cells

Two distinct ES cell lines isolated from the blastocysts of homozygote Sey mutants and wild-type (WT) mouse ES cells were cultured following a protocol involving treatment of ES cell aggregates with retinoic acid (RA) to trigger neural commitment (Bibel et al., 2004). Similar to WT ES cells after RA treatment, mutant cells also uniformly expressed the neuroepithelial antigen nestin as well as RC2 and GLAST, an astrocyte-specific glutamate transporter, but not Pax6. Both WT and mutant aggregates were negative for Pax7, Isl1, and MNR2 that mark spinal cord progenitors (data not shown; Plachta et al., 2004) but both expressed Emx2 (Figure 6A) at similar levels (Figure 6B), a marker for cortical progenitors (Simeone et al., 1992). Like previously reported for Pax6 (Bibel et al., 2004), Emx2 was also downregulated during the course of neuronal differentiation (Figure 6B). Following dissociation of RA-treated aggregates, mutant cells generated spindle shaped cells characteristic of RG cells (Bibel et al., 2004). However, they all had larger cell bodies than WT RG cells and one branched process (Figure 6C), a morphology reminiscent of RG cells in the ventral telencephalon. Mutant ES cells generated RC2- and GLAST-positive progenitors with equal efficiency compared with WT ES cells (Figure 6D) and most expressed the basic helix-loop-helix (bHLH) transcription factor Mash1 that marks subventricular zone progenitors of the ventral telencephalon (Porteus et al., 1994), while Emx2 expression was maintained (Figure 6D). Significantly higher levels of Mash1 were detected in mutant than in WT progenitors (Figure 6E), and Mash1 was not detected in neurons (Figure 6E). RT-PCR analysis also

revealed that Ngn2, a marker of dorsal telencephalic progenitors, and Tbr1, a marker of dorsal telencephalic neurons, were present in the WT ES-derived progenitors and neurons, respectively (Figure 6F). By contrast, Ngn2 and Tbr1 were barely detectable in mutant progenitors and neurons, respectively (Figure 6F). Loss of Pax6 Causes a Neurotransmitter Switch While neurons derived from WT ES cells express the vesicular glutamate transporter (vGLUT1) as early as 4 days in vitro (Figure 7A), neurons generated from mutant ES cells expressed instead typical markers of GABA-ergic neurons such as the vesicular transporter vGAT (Figures 7B and 7C) and glutamic acid decarboxylase (GAD) (Figures 7B and 7D). These characteristics were already apparent at day 4 (data not shown) and by day 6, over 80% of the neurons could already be labeled with both markers (Figures 7C and 7D). Subpopulations of GABAergic neurons can be identified on the basis of the expression of the calcium binding proteins (DeFelipe, 1997; Kubota and Kawaguchi, 1994), and the majority of the mutant neurons were positive for calretinin (Figures 7B and 7E), while calbindin-positive neurons were not observed (data not shown). A small number of glutamatergic WT neurons also expressed calretinin (Figures 7B and 7E). Western blot analyses confirmed that, unlike WT neurons, mutant neurons express vGAT and higher levels of calretinin compared to WT cells (Figure 7F). To determine whether the switch of neurotransmitter phenotype can be causally attributed to the lack of Pax6, we reintroduced it in mutant ES cells with a retrovirus encoding either Pax6-IRES-GFP or IRES-GFP alone (Hack et al., 2004). Because only few cells are infected and non-infected cells die prematurely (see below), we co-

cultured mutant with WT progenitors. While at day 6 most neurons derived from progenitors infected with GFP alone expressed vGAT (Figure 7G), the vast majority of the neurons derived from Pax6-infected progenitors did not (Figure 7G), but expressed vGLUT1 instead (Figure 7H).

2.1.2) Transplantation of embryonic stem cells in the chick telencephalon

We next tested the differentiation potential of ES cells and of ES cell-derived neurogenic progenitors after transplantation in the chick telencephalon. At about E1.5 (12-somite), most of the cells were removed in the areas corresponding to either the prospective ventral or dorsal telencephalon (see Figure 8A). Aggregates of RA-untreated (‘‘naive’’) ES cells or of progenitors (RA-treated WT or mutant ES cells) were then used to fill up these areas and their progeny was analyzed 8 days later. To facilitate the visualization of ES cell-derived neurons, WT and mutant ES cell lines were used with a reporter GFP cDNA inserted in the tau locus (Bibel et al., 2004). In agreement with the in vivo and in vitro lineage of Pax6-positive RG cells, almost all WT progenitors transplanted in the prospective dorsal telencephalon differentiated into glutamatergic neurons, as assessed by the expression of the vesicular glutamate transporter (vGLUT1) (73.9% ± 3.12), with only a small fraction expressing the vesicular GABA transporter (vGAT) (2.1% ± 0.92) (Figure 8B). To test whether the environment can override the intrinsic commitment of the Pax6-positive progenitors to a glutamatergic fate, we also transplanted WT RA-treated ES cells in the prospective ventral telencephalon of the developing chick, a region where GABAergic neurons are normally generated. While these cells successfully differentiated into neurons

that populated the ventral telencephalon, the majority of these neurons expressed vGLUT1, and only a few expressed vGAT (vGLUT1 56.4% ± 4.94; vGAT 5.4% ± 2.81) (Figure 8C). In addition, with the exception of a few cells, they failed to follow a tangential migratory route, as ventrally generated neurons do, and to populate the cortex. The control experiment with RA-untreated, naı̈ve, and pluripotent ES cell revealed that mouse ES cells are able to respond to the environmental cues of the ventral telencephalon. They differentiated mostly into GABA-ergic neurons (vGAT 66.9% ± 3.71; vGLUT1 10.1% ± 2.44), and most of them successfully migrated tangentially into the cortex (Figure 8D). Mutant RA-treated ES cells transplanted in the prospective ventral telencephalon were also able to integrate into the host environment and to differentiate into neurons. However, by contrast with WT RA-treated ES cells, almost all of these neurons expressed vGAT (vGAT 92.7% ± 1.27; vGLUT1 1.0% ± 0.82). In addition, the cells followed a tangential migratory route and populated the cortex (Figure 8E). Mutant RA-treated ES cells transplanted in the prospective dorsal telencephalon exhibited a clustering behavior, reminiscent of Pax6 mutant cells in the WT cortex of mice (Talamillo et al., 2003), and integrated much less successfully in the cortex (Figure 8F). Many cells expressed very low levels of GFP and exhibited apoptotic features, such as a pyknotic nuclear morphology (data not shown), and virtually all survivors differentiated into vGAT-positive neurons (vGAT 93.0% ± 2.6; vGLUT1 0%).

2.1.3) Neurotrophin receptor expression and death of neurons derived from Pax6-mutant embryonic stem cells

ES cell-derived mutant neurons failed to develop normal processes as revealed with b-III tubulin antibodies (Figure 9A). They remain thinner, less branched, and shorter than those of WT neurons (Figure 9A), and by day 8, the cell bodies exhibited features of apoptotic death (Figure 9A), including a pyknotic nuclear morphology (Figure 9A, insert). With regard to cell death, no significant differences between WT and mutant cultures were observed before day 6 (Figure 9B). However, at day 7 a significant fraction of mutant neurons became apoptotic, and they were all dead by day 8 (Figure 9B). These results were obtained with two independent clones of Pax6 mutant ES cells. As the neurotrophin receptor p75NTR has recently been shown to cause the death of processes and of cell bodies when overexpressed in ES cell-derived neurons (Plachta et al., 2007), we examined its levels in both WT and mutant progenitors (Figure 9C). While p75NTR was rapidly downregulated in WT neurons during differentiation of the progenitors (see Bibel et al., 2004), it remained expressed at high levels in mutant neurons (Figure 9C). By contrast, Trk receptors, that are activated by neurotrophins to allow the survival of developing neurons in the peripheral nervous system (Huang and Reichardt, 2003), failed to be expressed at detectable levels (Figure 9C). To test if the death of the mutant neurons is a direct consequence of the lack of Pax6, we reintroduced it in mutant ES cells (see above). As only between 10 to 30 cells per 5 3 105 cells were infected, we again co-cultured the neurons derived from infected RG cells with WT neurons to overcome potential problems resulting form low density cultures. Mutant RG cells

infected with GFP alone generated neurons (Figure 9D) with short, unbranched processes, a morphology characteristic of mutant neurons (see above). After 8 days in vitro, these neurons lost their processes and the vast majority of them died (survival rate 1.5% ± 0.69) (Figures 9D and 9E). By contrast, mutant RG cells infected with Pax6 generated neurons with longer processes, similar to that of wild-type cells (Figure 9D). In addition, these cells survived (survival rate 75.4% ± 4.9 at day 8) (Figure 9E) and could be maintained in vitro as long as WT neurons. With time, they developed into mature neurons with long processes and very extensive branching patterns (Figure 9D).

2.1.4) Neurotrophin receptor expression and cell death in the Pax6-mutant cortex

In view of these results, we examined the cortex of Pax6 mutant (Sey/Sey) animals for the expression patterns of p75NTR and TrkB as well as for evidence of cell death. In the WT cortex, p75NTR expression was restricted to the subplate neurons (arrows) as previously reported (Allendoerfer et al., 1990) (Figure 10A). By contrast, p75NTR was ectopically expressed in the mutant cortex at E12, particularly in the marginal zone and upper cortical layer (Figure 10A). The ectopic expression of p75NTR in the mutant cortex did not persist until later developmental stages, and already by E14, its expression pattern was similar to that of the WT cortex (Figure 5A). TrkB was present in the WT cortex at E12, and its expression levels increased by E14 (Figure 10A). Although neurofilament staining indicated the presence of neurons in the mutant cortex, almost no TrkB expression was detected, both at E12 and at E14 (Figure 10A). This defect was

restricted to the cortex, and ventral structures, where Pax6 is not expressed (Stoykova and Gruss, 1994), exhibited normal TrkB expression (Figure 10A, yellow arrow). In addition, the mutant cortex contained significantly more apoptotic cells at E12 compared to WT, as assessed by TUNEL (Figure 10B) and active-caspase 3 staining (Figure 10B). The distribution of apoptotic profiles corresponded to cells ectopically expressing p75NTR, predominantly in the cortical plate and marginal zone (mutant 78.7% ± 7.4; WT 29.9% ± 5.8, Figure 10C). Notably, increased cell death in the mutant cortex was less pronounced at later developmental stages (E14, see Figure 10B), and both the ectopic expression of p75NTR and increased apoptosis were restricted to the cortex and were not observed in the ventral telencephalon (Stoykova and Gruss, 1994; Figure 10D). In addition, ectopic expression of p75NTR and of active caspase 3 were also detected in the olfactory bulb-like structure (OBLS) (indicated by arrows in Figure 10D), a structure formed near the lateral pallium of the Sey cortex (Jimenez et al., 2002; Stoykova et al., 2003). Immunostaining of E12WTand mutant brain sections for vGAT, GAD, and Calretinin revealed that the mutant cortex contained about 3-fold more neurons expressing these markers compared to WT littermates that, as expected, contained only few GABAergic neurons at this stage (Figures 11A and 11B). These GABAergic neurons were primarily localized in the cortical plate of the Sey cortex. Only very few cells expressed Calbindin in the mutant cortex, as observed in the WT cortex (data not shown). The majority of these GABAergic neurons in the mutant cortex showed signs of apoptosis, as many vGAT-positive cells expressed high levels of

p75NTR, co-localized with active caspase 3, and exhibited nuclear pyknosis (Figure 11C). As p75NTR expression could also be detected close to the ventricular zone in the Pax6 mutant cortex (for example see arrow in Figure 7C), we performed co-immunostainings for p75NTR and the RG marker Glast and for doublecortin, an antibody that recognizes newborn neurons. Only few Glast-positive cells co-localized with p75NTR, while the majority of p75NTR-positive cells near the ventricular zone expressed doublecortin (data not shown). Taken together, these results indicate that, while cortical RG cells remain largely unaffected in the mutant, GABAergic neurons upregulate p75NTR soon after their birth and undergo apoptosis. Staining for the nuclei and for doublecortin indicated that the number of newborn neurons was significantly increased at E12 in the mutant cortex (Figure 11E) and that many doublecortin-positive cells were located in the vicinity of the ventricular zone, as indicated by the arrows (Figure 11D). At midgestation (E14), the number of doublecortin-positive cells in the mutant cortex was decreased compared to WT by about 40%. This finding is in line with the increased number of GABAergic cells in the early mutant cortex and with recent findings indicating that the loss of Pax6 prevents the reentry of cortical progenitors into S-phase, thus resulting in a greater proportion of differentiating neurons at E12.5 (Quinn et al., 2007).

2.1.5) Downregulation of p75NTR decreases neuronal death in vitro and in vivo

To test if the death of GABAergic neurons is due to the over-expression of p75NTR, mutant ES cells were electroporated with three different shRNA

constructs directed against mouse p75NTR mRNA and with a scrambled sequence with no match in the mouse genome. Stable clones were selected using neomycin and analyzed for p75NTR downregulation by western blot analysis (Figure 12A). While mutant neurons expressing the scrambled sequence died prematurely (data not shown), mutant neurons expressing shRNA against p75NTR survived for at least 30 days (Figure 12B). In addition, they uniformly expressed vGAT (Figure 12C), indicating that downregulation of p75NTR did not have any effect on the identity of the mutant neurons. Moreover, mutant neurons expressing sh-p75NTR seem to form synaptic contacts, as indicated by the distribution of synaptophysin immunoreactivity (Figure 12C). We also noted that those mutant neurons that managed to survive in the chick telencephalon downregulated the levels of p75 expression (see Figure 13). The reasons for the reduced expression of p75NTR after transplantation are unclear. To assess the role of p75NTR overexpression in the death of GABAergic neurons in the Pax6 mutant cortex, heterozygote Sey/+ mutants were crossed with p75NTR heterozygote animals (von Schack et al., 2001). Homozygote double mutants contained significantly fewer TUNEL-positive cells in the cortex at E12 compared to Pax6 homozygote mutants that lacked only one copy of p75NTR (Sey/Sey;p75+/-) or that were WT for p75NTR (Sey/Sey) (Figure 12E). This reduction in cell death was accompanied by increased survival of misspecified GABAergic neurons resulting in increased numbers of GAD-positive neurons at E14 in the Sey/Sey;p75-/- cortex compared to WT and Sey/Sey;p75+/- (Figure 12E). While most GAD-positive neurons were localized in

the cortical plate and marginal zone of the double mutant, far fewer were observed in the WT cortex, most of which were localized in the subventricular zone (Figure 12E, arrows) and only few in the marginal zone (Figure 12E, arrowheads).

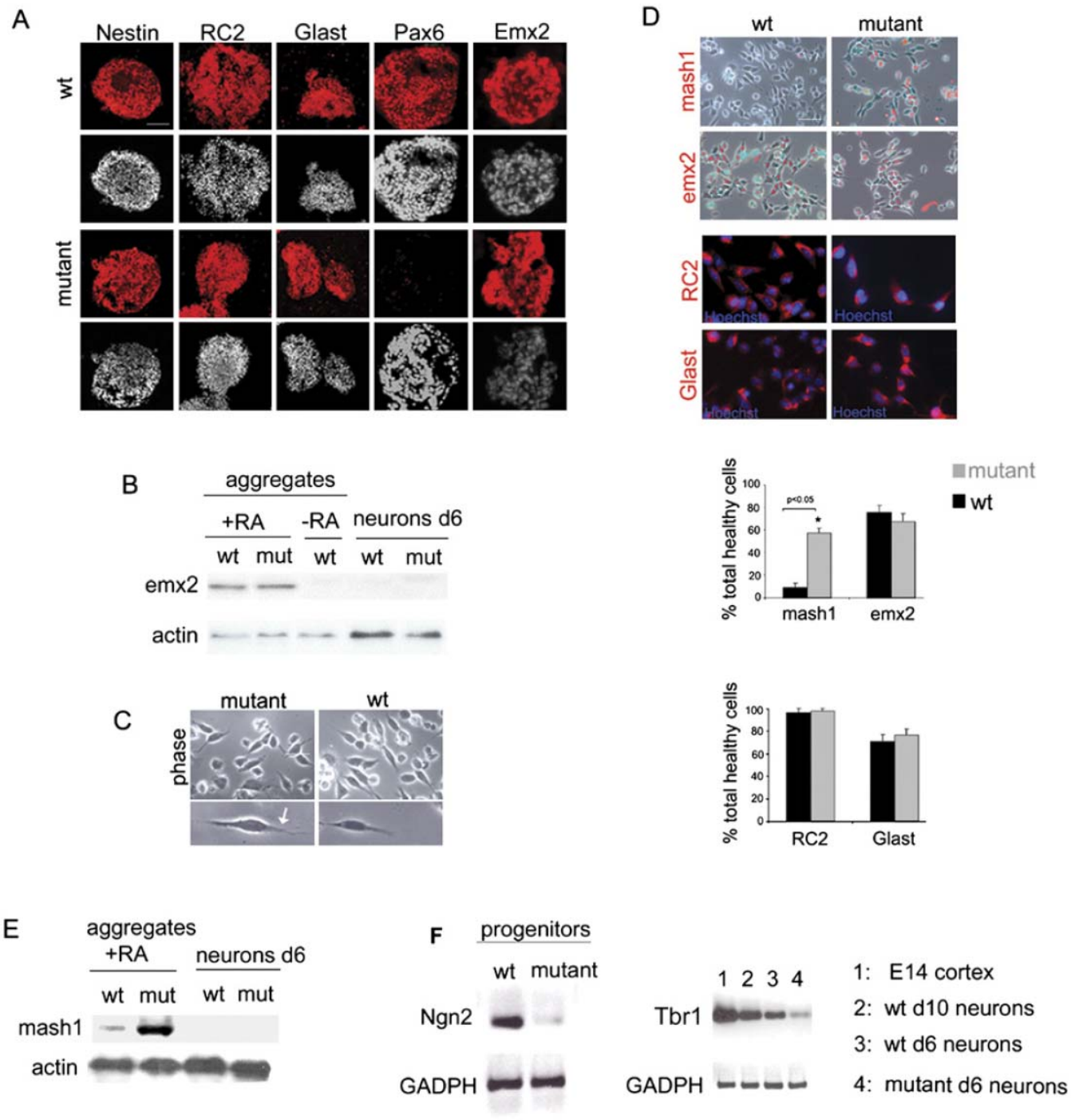
A
Nestin
RC2
Glast
Pax6
Emx2
wt
mutant
D
wt
mutant
mash1
emx2
RC2
Glast
Hoechst
B
aggregates
+RA
-RA
neurons d6
wt
mut
wt
wt
mut
emx2
actin
mutant
wt
% total healthy cells
p<0.05
mash1
emx2
C
mutant
wt
phase
RC2
Glast
E
aggregates
+RA
neurons d6
wt
mut
wt
mut
mash1
actin
F
progenitors
wt
mutant
Ngn2
GADPH
1 2 3 4
Tbr1
GADPH
1: E14 cortex
2: wt d10 neurons
3: wt d6 neurons
4: mutant d6 neurons

Figure 6

Figure 6. Loss of Pax6 Impairs the Specification, Not the Generation, of RG Cells from ES Cells

(A) RA-treated, WT, and mutant aggregates express the neuroepithelial marker Nestin, its modification recognized by RC2, the RG marker Glast, and the transcription factor emx2. Pax6 is not detected in mutant aggregates.
(B) WT and mutant RA-treated aggregates express Emx2, as shown by western blot. Emx2 is no longer expressed in neurons (normalized for actin).
(C) Dissociated aggregates generate spindle shape progenitors 2 hr after plating. Note the larger size and branched process of mutant progenitors (arrow).
(D) Immunostaining of ES cell-derived progenitors for mash1, emx2, RC2, and Glast. Equal percentages of RC2+ and Glast+ progenitors are generated by WT and mutant ES cells. WT progenitors only express emx2, while mutant progenitors express mostly both emx2 and mash1. More mutant progenitors express mash1 (56.8% ± 4.0) compared to WT (9.1% ± 4.7) (*$p < 0.05$).
(E) Western blot reveals that mutant progenitors express higher levels of Mash1 compared to WT. Mash1 is not expressed in neurons (normalized for actin).
(F) RT-RCR for Ngn2 and Tbr1 reveals that expression of both genes is dramatically decreased in mutant progenitors and neurons respectively, compared to WT.

Scale bars: (A), 100 μm; (C), 50 μm (top row), 25 μm (bottom row); (D), 100 μm (top rows), 50 μm (bottom rows).

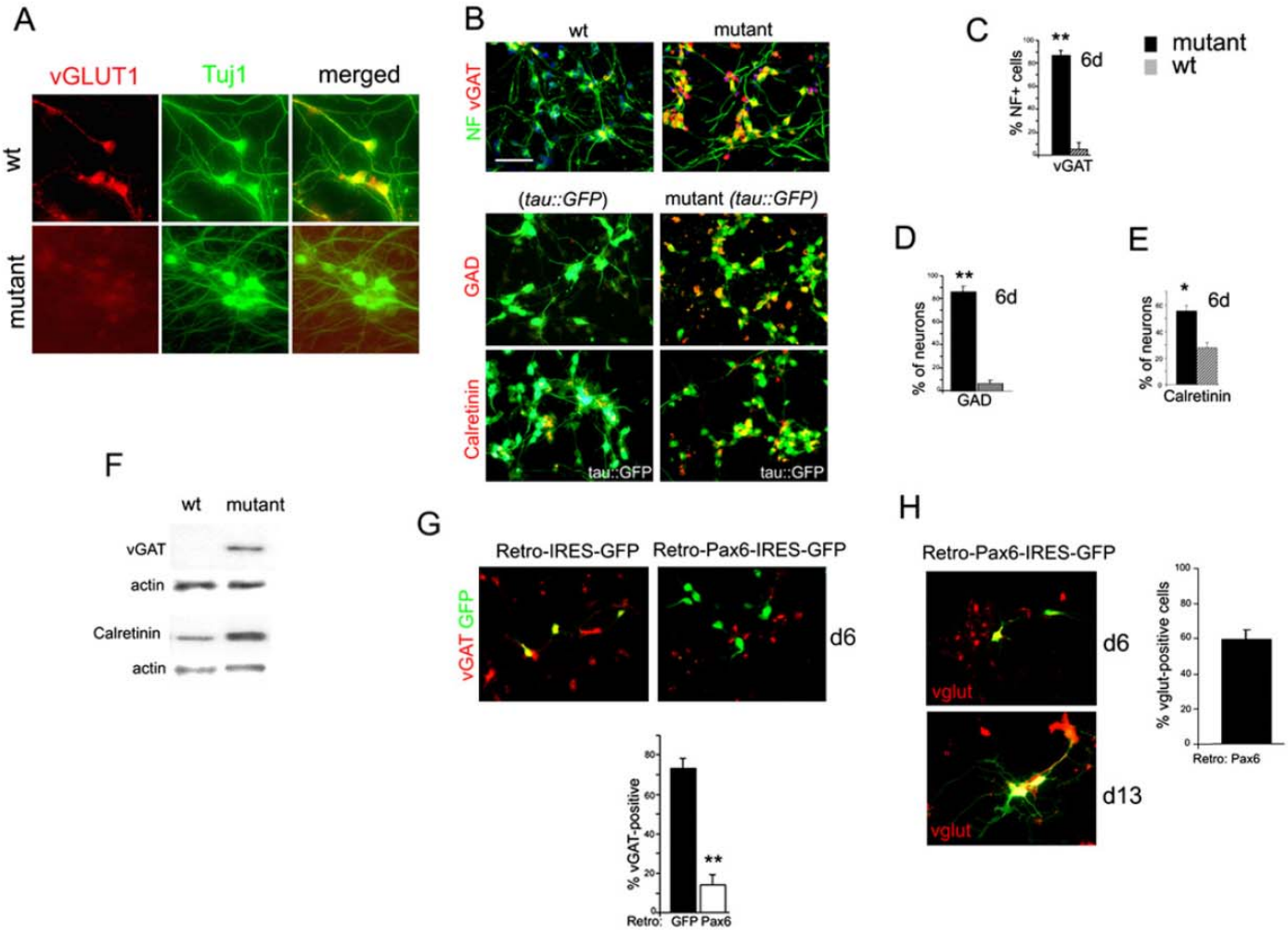
A
vGLUT1
Tuj1
merged
wt
mutant
B
wt
mutant
NF vGAT
(tau::GFP)
mutant (tau::GFP)
GAD
Calretinin
tau::GFP
tau::GFP
C
mutant
wt
% NF+ cells
6d
vGAT
D
% of neurons
6d
GAD
E
% of neurons
6d
Calretinin
F
wt
mutant
vGAT
actin
Calretinin
actin
G
Retro-IRES-GFP
Retro-Pax6-IRES-GFP
vGAT GFP
d6
% vGAT-positive
Retro: GFP Pax6
H
Retro-Pax6-IRES-GFP
d6
vglut
d13
vglut
% vglut-positive cells
Retro: Pax6

Figure 7

Figure 7. Pax6 Specifies the Glutamatergic Phenotype of ES Cell-Derived Neurons

(A) Staining of WT and mutant ES cell-derived neurons after 4 days in vitro for vGLUT1 and Tuj1. Most WT neurons express vGLUT1, but mutant neurons do not.

(B) Six-day-old ES cell-derived neurons co-stained for vGAT, neurofilament, and a nuclear dye (top row), and for GAD or Calretinin.

(C–E) Quantification of the number of cells expressing vGAT (C), GAD (D) and calretinin (E) reveals that almost all mutant neurons express these markers, while the majority of WT neurons do not (*$p < 0.05$, **$p < 0.01$).

(F) Western blot also indicates that mutant neurons express higher levels of vGAT and Calretinin compared to WT (normalized for actin).

(G and H) Neurons derived from mutant ES cells infected with a GFP or Pax6-IRES-GFP retroviral vector were co-cultured in a 1:1 ratio with WT neurons and co-stained with antibodies against vGAT and GFP (G), or for vGLUT1 (H).
The majority of GFP+ mutant neurons express vGAT (G), while neurons expressing Pax6 do not express vGAT, but express vGLUT1 (G and H).
Significantly fewer neurons expressing Pax6 are vGAT+ (16.3% ± 4.36) as compared to neurons expressing GFP only (73.4% ± 4.76) (*$p < 0.01$).
(H)
The percentage of mutant neurons infected with Pax6-IRES-GFP expressing vGLUT1 was quantified based on the total number of GFP+ cells.

Scale bars: (A) 100 μm (top row), 50 μm (bottom two rows); (F) 50μm.

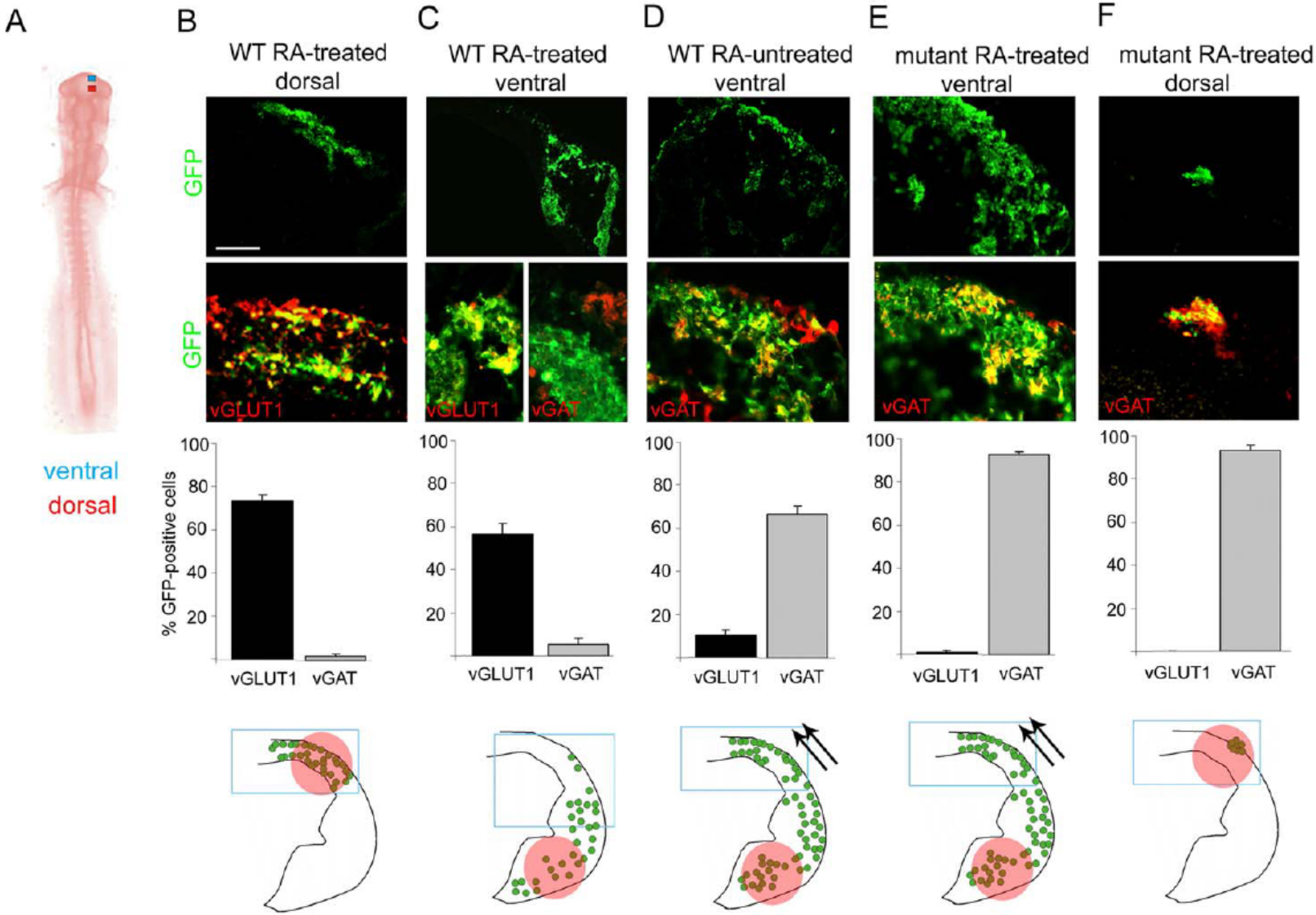
A
B
C
D
E
F
WT RA-treated dorsal
WT RA-treated ventral
WT RA-untreated ventral
mutant RA-treated ventral
mutant RA-treated dorsal
GFP
GFP
vGLUT1
vGLUT1
vGAT
vGAT
vGAT
vGAT
ventral
dorsal
% GFP-positive cells
100
80
60
40
20
vGLUT1
vGAT

Figure 8. Differentiation Potential of ES Cells and of WT and Mutant ES Cell-Derived Progenitors in the Chick Telencephalon

(A) Schematic of the chick embryo (10–12 somite stage) with a lesion in the prospective dorsal (red) or ventral (blue) telencephalon.

(B–F) The diagrams indicate where the lesion was made and the aggregate placed (red area). The blue box indicates where the picture was taken. All experiments were immunostained for vGLUT1 and vGAT.

(B) RA treated WT (mapt::GFP) aggregates implanted in the prospective dorsal telencephalon. Most GFP+ cells express vGLUT1 (n = 4) and are incorporated into the cortex.

(C) RA treated WT (mapt::GFP) aggregates implanted in the prospective ventral telencephalon. Most GFP+ cells express vGLUT1 (n = 4) and fail to migrate to the cortex.

(D) RA untreated (naive) WT (mapt::GFP) aggregates implanted in the prospective ventral telencephalon. Many GFP+ cells express vGAT (n = 4), populate the ventral telencephalon, and migrate to the cortex.

(E) RA-treated mutant (mapt::GFP) aggregates implanted in the prospective ventral telencephalon. The vast majority of GFP+ cells express vGAT (n = 4) and migrate to the cortex.

(F) RA-treated mutant (mapt::GFP) aggregates implanted in the prospective dorsal cortex. Mutant cells do not integrate well in the cortex, but exhibit a clustering behavior. Most express p75NTR at high levels (Figure S1). The percentage of GFP-positive cells expressing vGLUT1 or vGAT was quantified (n = 4).

Scale bars: (B)–(F), 100 μm (top rows), 50 μm (bottom rows).

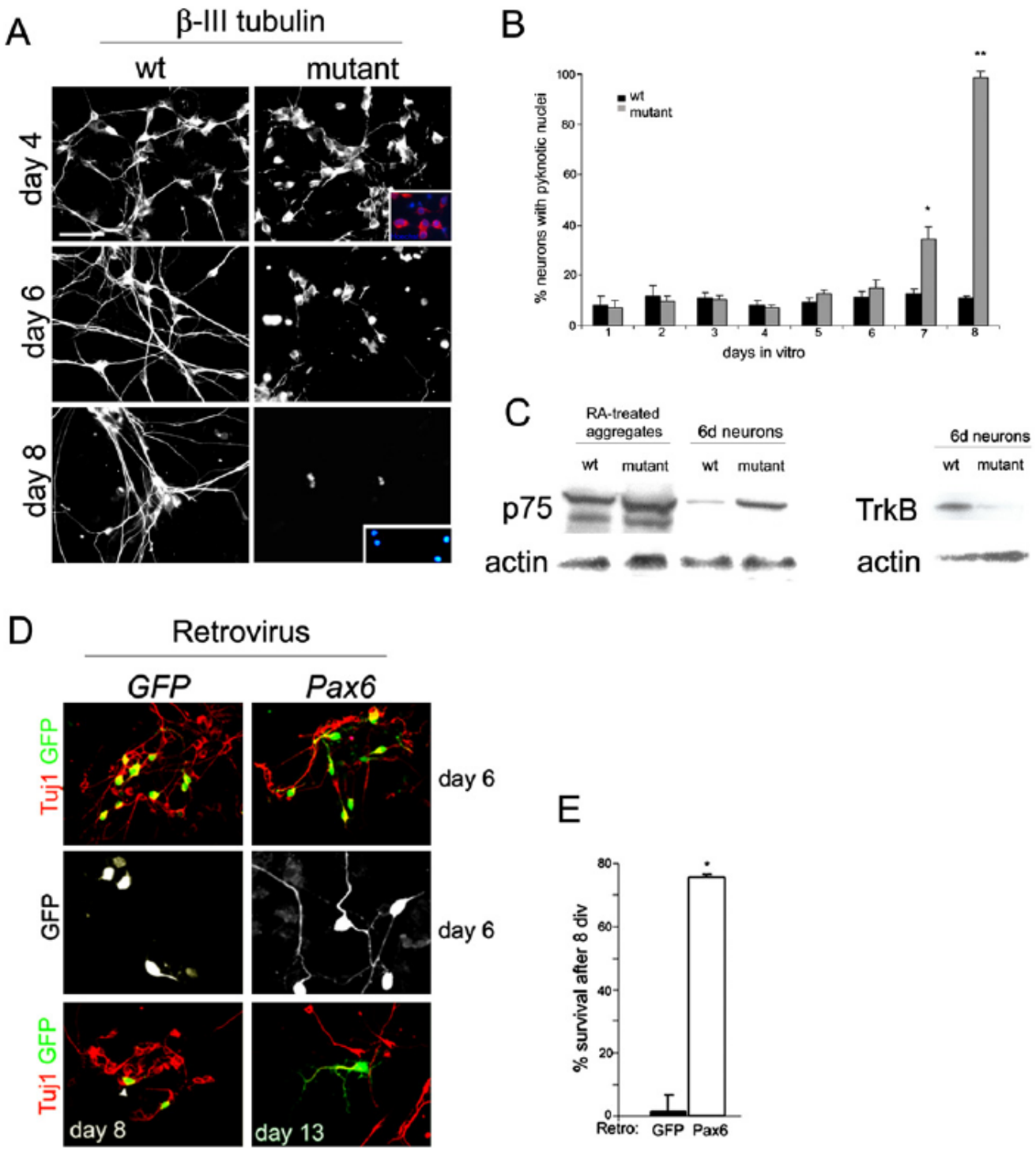
A
β-III tubulin
wt
mutant
day 4
day 6
day 8
B
wt
mutant
% neurons with pyknotic nuclei
days in vitro
C
RA-treated aggregates
6d neurons
wt
mutant
p75
actin
TrkB
D
Retrovirus
GFP
Pax6
Tuj1 GFP
GFP
day 6
day 8
day 13
E
% survival after 8 div
Retro: GFP Pax6

Figure 9

Figure 9. Mutant Neurons Exhibit Aberrant Expression of p75NTR and TrkB and Die Prematurely

(A) Staining of WT and mutant ES cell-derived neurons for Tuj1 and a nuclear dye. After 4 days in vitro, mutant neurons exhibit shorter and thinner processes compared with WT neurons and die prematurely after 8 days.

(B) Cell death in WT and mutant neuronal cultures from day 1 to day 8 in vitro as the percentage of neurons exhibiting a pyknotic nuclear morphology. Most mutant neurons die between day 7 and 8 in vitro (*$p < 0.05$, **$p < 0.01$).

(C) Mutant ES cell-derived neurons fail to upregulate TrkB and express higher levels of p75NTR, as shown by western blot.

(D) Neurons derived from mutant ES cells infected with a retroviral vector expressing either GFP alone or Pax6-IRES-GFP were co-stained for Tuj1 and GFP. Infected neurons were co-cultured with WT ES cell-derived neurons. While mutant neurons expressing GFP alone fail to survive longer than 8 days, mutant neurons expressing Pax6 survive for at least 13 days and develop a complex morphology with branching and filopodia (arrow).

(E) The survival of mutant neurons infected with Pax6-IRES-GFP or GFP alone was quantified after 8 days in vitro (*$p < 0.01$).

Scale bars, 50 μm in all panels.

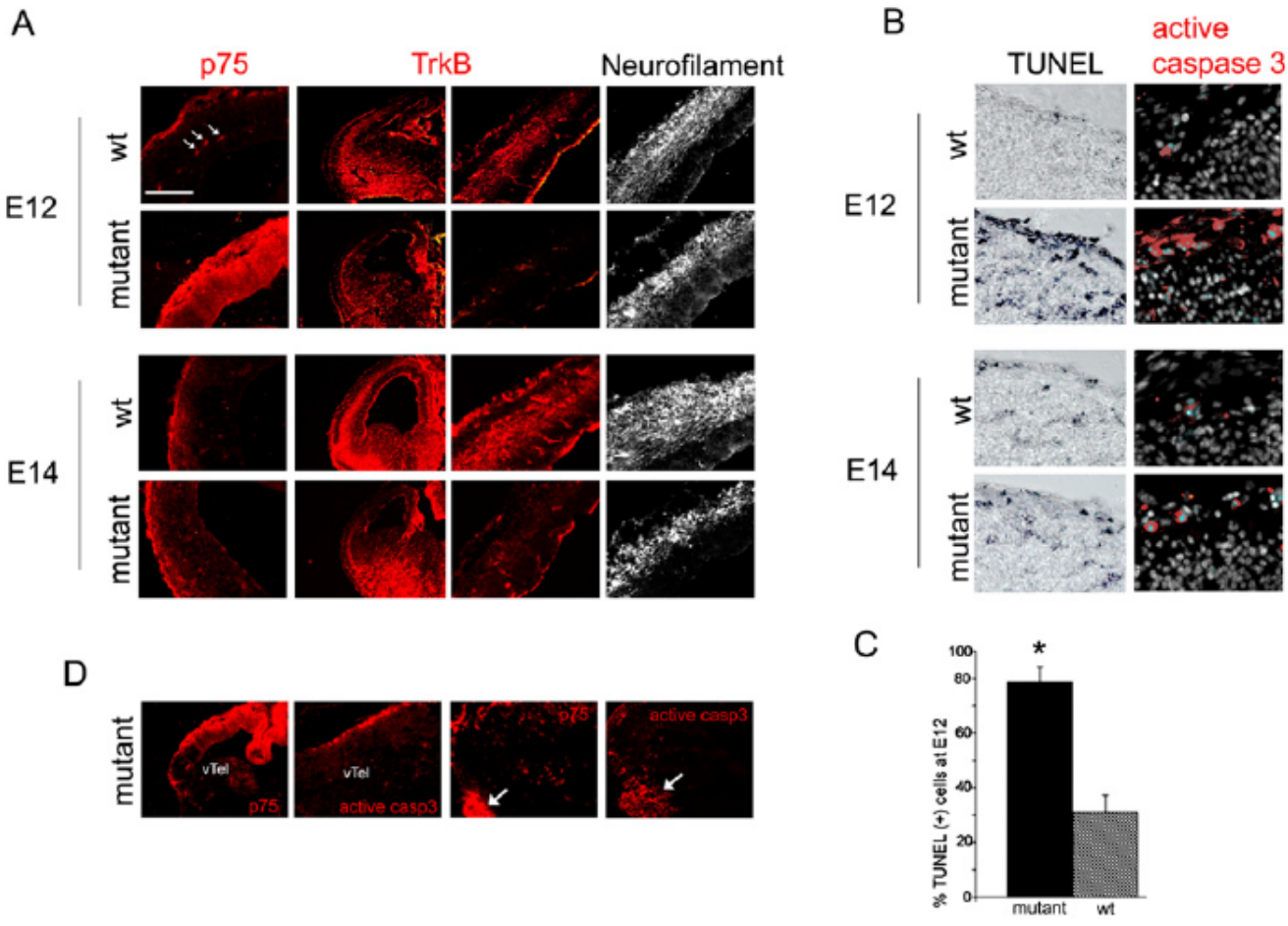
A
p75
TrkB
Neurofilament
E12
wt
mutant
E14
wt
mutant
B
TUNEL
active caspase 3
E12
wt
mutant
E14
wt
mutant
C
% TUNEL (+) cells at E12
100
80
60
40
20
0
*
mutant
wt
D
mutant
vTel
p75
vTel
active casp3
p75
active casp3

Figure 10

Figure 10. Neurotrophin Receptor Expression and Cell Death in the Pax6 Mutant Cortex

(A) Immunostaining of E12 and E14 WT and Pax6 mutant (Sey/Sey) brains for p75NTR, TrkB, and neurofilament. The mutant cortex exhibits ectopic overexpression of p75NTR at E12 and lacks TrkB protein both at E12 and E14.

(B) The mutant cortex contains more apoptotic cells at E12, as revealed by TUNEL, active caspase 3, and nuclear staining of cortical sections. These defects are less prominent at E14.

(C) The E12 mutant cortex contains significantly more apoptotic cells (78.7% ± 7.4) compared with WT (29.9% ± 5.8) (*$p < 0.01$).

(D) Ectopic overexpression of p75NTR and increased numbers of caspase3 positive cells are observed only in the cortex and in the olfactory bulb-like structure (arrows) of the mutant, but not in the ventral telencephalon (vTel).

Scale bars: (A), 100 mm (first, third, and fourth columns); 200 μm (second column); (B), 50 μm; (D), 200 μm.

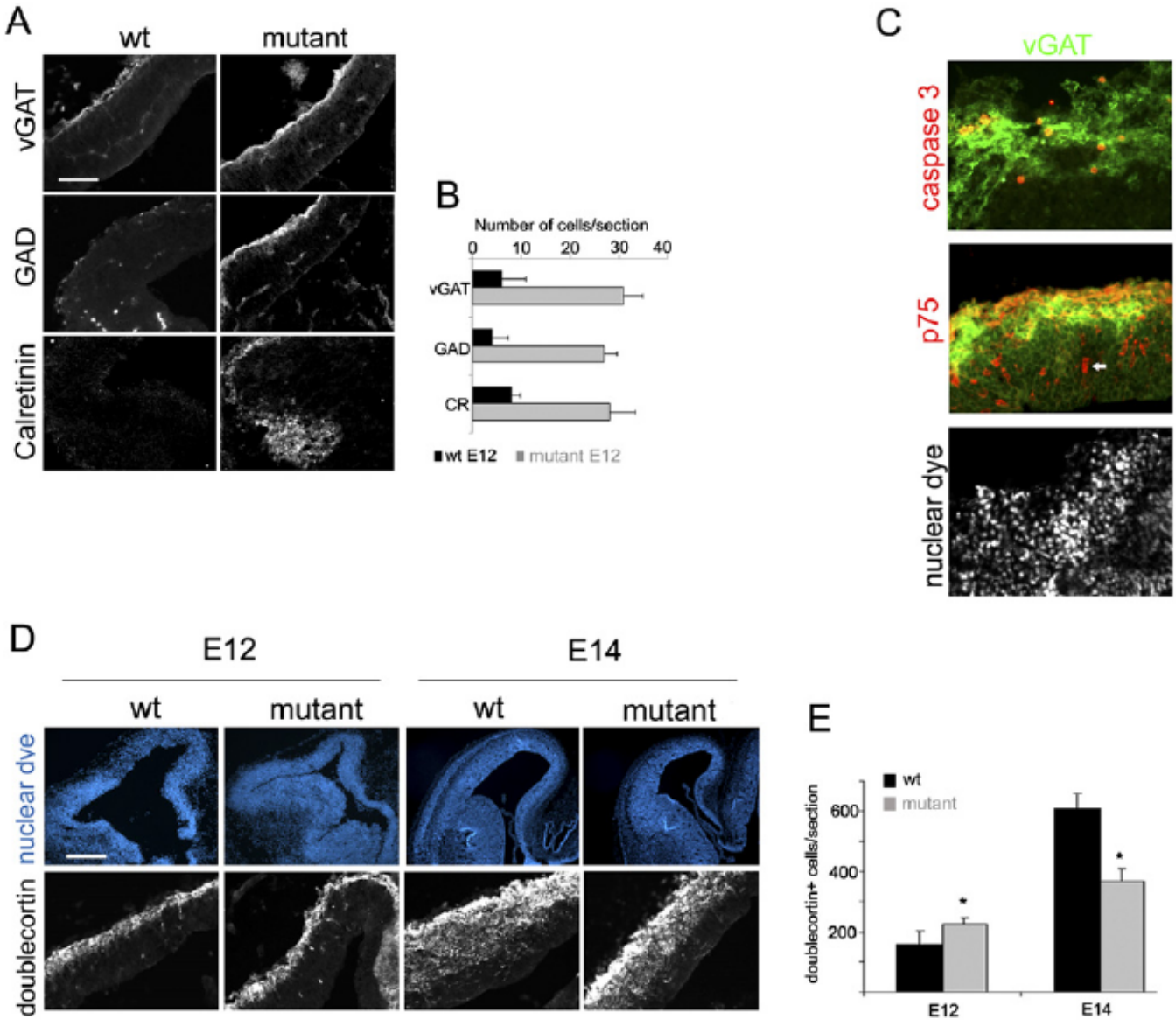
A
wt
mutant
vGAT
GAD
Calretinin
B
Number of cells/section
0
10
20
30
40
vGAT
GAD
CR
wt E12
mutant E12
C
vGAT
caspase 3
p75
nuclear dye
D
E12
E14
wt
mutant
wt
mutant
nuclear dye
doublecortin
E
wt
mutant
doublecortin+ cells/section
600
400
200
E12
E14

Figure 11

Figure 11. Increased Numbers of Transient GABAergic Cells in the Pax6 Mutant Cortex at E12

(A) Immunostaining of E12 WT and mutant cortex for vGAT, GAD, and calretinin.

(B) Quantification revealed increased numbers of vGAT-, GAD-, and calretinin-positive cells in the mutant cortex compared to WT.

(C) Co-staining of E12 mutant cortex for vGAT and active caspase3 or p75NTR. Note that a large number of vGAT-positive and that these cells also express p75NTR and active caspase3. Nuclear staining revealed a pyknotic nuclear morphology of cells in the cortical plate.

(D) Immunostaining of E12 and E14 WT and mutant cortex for doublecortin and a nuclear dye.

(E) Significantly more doublecortin-positive cells are present in the mutant cortex at E12 ($*p < 0.05$), but significantly fewer at E14 ($*p < 0.01$) as compared to WT.

Scale bars: (A), 100 μm; (C), 50 μm; (D), 400 μm (top row), 100 μm (bottom row)

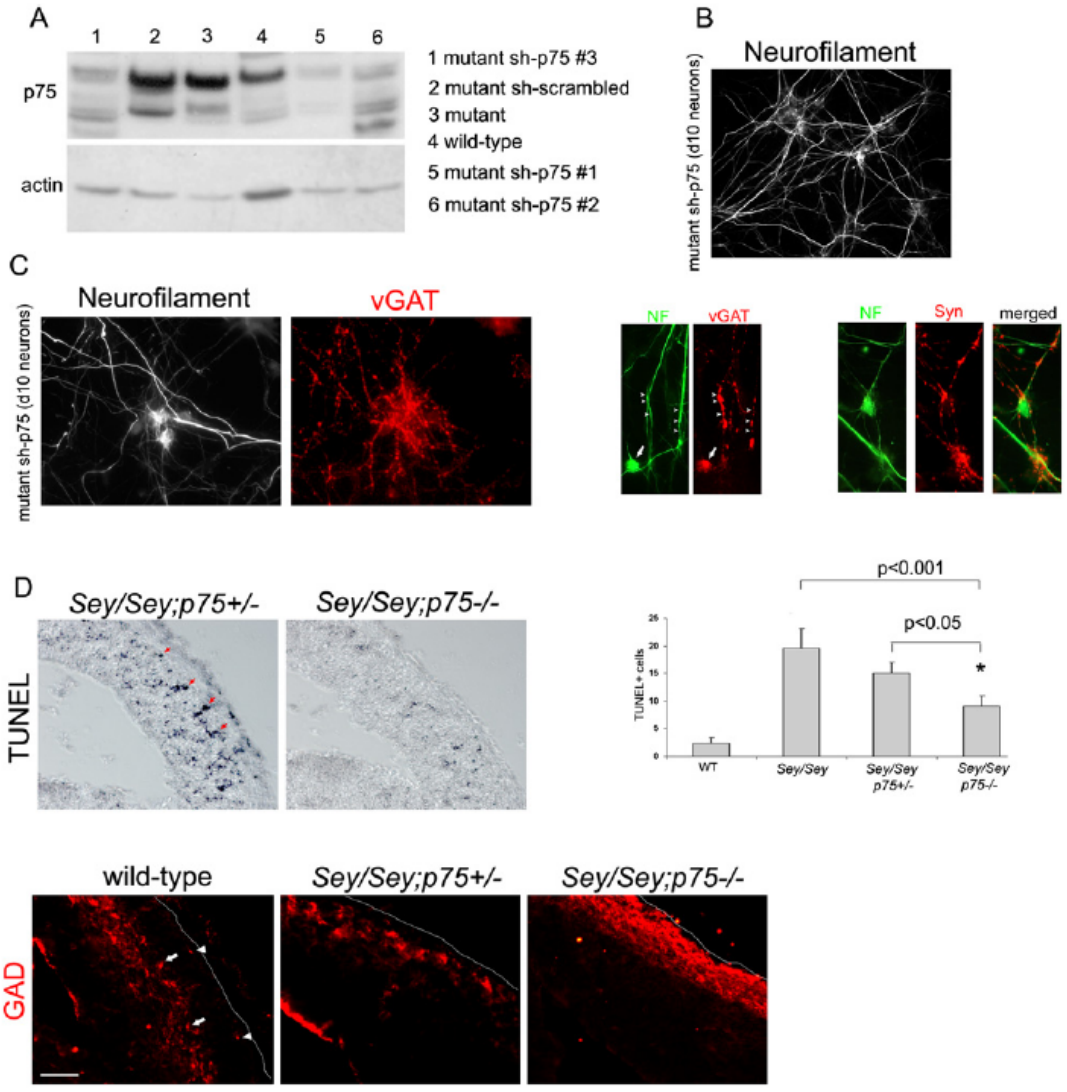
A
1 2 3 4 5 6
p75
actin
1 mutant sh-p75 #3
2 mutant sh-scrambled
3 mutant
4 wild-type
5 mutant sh-p75 #1
6 mutant sh-p75 #2
B
Neurofilament
mutant sh-p75 (d10 neurons)
C
Neurofilament
vGAT
mutant sh-p75 (d10 neurons)
NF
vGAT
NF
Syn
merged
D
Sey/Sey;p75+/-
Sey/Sey;p75-/-
TUNEL
p<0.001
p<0.05
*
TUNEL+ cells
WT
Sey/Sey
Sey/Sey p75+/-
Sey/Sey p75-/-
wild-type
Sey/Sey;p75+/-
Sey/Sey;p75-/-
GAD

Figure 12

Figure 12. Downregulation of p75NTR Prevents the Death of Pax6-Deficient Neurons

(A) Neurons derived from mutant ES cells stably expressing a shRNA construct against p75NTR (3 different constructs) downregulate p75NTR after 2 days in vitro as shown by western blot analysis. Stable expression of a scrambled construct in mutant ES cells does not cause a downregulation of p75NTR in neurons.

(B) Mutant neurons stably expressing shRNA constructs against p75NTR develop elaborate processes and survive, as shown by Neurofilament staining after 10 days in vitro.

(C) Immunostaining of 10 day old mutant sh-p75 neurons for Neurofilament, vGAT, and Synaptophysin (Syn). Mutant sh-p75 neurons express vGAT in their cell bodies (arrow) and processes (arrowheads), and mature to form punctuate structures accumulating synaptophysin immunoreactivity.

(D) Cortical sections of E12 WT (n = 3), Sey/Sey (n = 3) Sey/Sey;p75NTR+/- (n = 3) and Sey/Sey;p75NTR-/- (n = 2) brains were stained for TUNEL. Fewer TUNEL+ (red arrows) cells are present in the Sey/Sey;p75NTR-/- cortex as compared to Sey/Sey;p75NTR+/-.

(E) Increased number of GAD-positive neurons in the E14 Sey/Sey;p75NTR-/- cortex, as compared to WT or Sey/Sey;p75NTR+/-. Arrow indicates GAD-positive neurons in the subventricular zone and arrowheads in the marginal zone.

Scale bars: (B), 100 μm; (C), 50 μm; and (D) and (E), 100 μm.

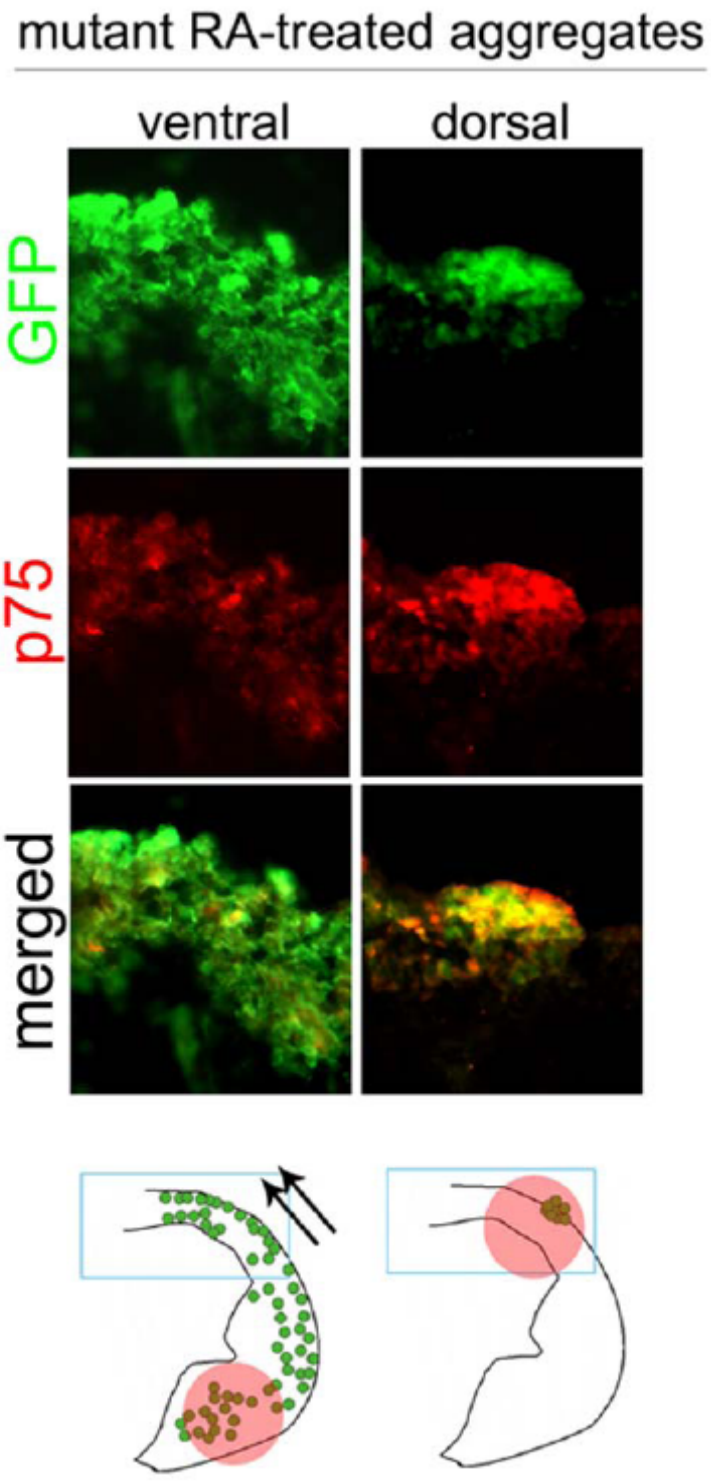
mutant RA-treated aggregates
ventral
dorsal
GFP
p75
merged

Figure 13

Figure 13. Expression of p75 in mutant ES-derived neurons following transplantation in the developing chick telencephalon

Sections were stained for GFP and p75 8 days after transplantation of mutant, RA-treated aggregates into the prospective ventral or dorsal telencephalon. The schemes indicate the implantation sites of the aggregates (red area) and the sites where the pictures were taken (blue box). Most mutant ES-derived neurons downregulate p75 expression when implanted in the ventral, but not in the dorsal telencephalon.

2.2) THE ROLE OF NEUROTROPHIN TYROSINE KINASE (TRK) RECEPTORS IN DEVELOPING NEURONS

Tyrosine kinase receptors of the Trk family expressed by growing axons bind neurotrophins selectively, resulting in the activation of their kinase domain (for review see Bibel and Barde, 2000). This process is well recognized to account for the ability of target-derived neurotrophins to regulate the survival of large numbers of neurons in the peripheral nervous system, during the period of naturally occurring cell death. Why PNS neurons become exquisitely dependent on neurotrophins for survival has remained a mystery. In addition, this feature is not shared by most CNS neurons. While CNS neurons respond to neurotrophins by regulating neurotransmitter release and synaptic function, neurotrophin signaling is dispensable for their survival. Virtually all CNS neurons express TrkB at relatively high levels, compared with TrkA and TrkC (see suppl. Fig.20), and yet in the absence of the TrkB ligands BDNF and NT4, the survival of most CNS neurons is not affected. Hitherto, comparing the activity of the 3 Trk receptors in the absence of ligand, when they are not activated, in one and the same neuronal context has been virtually impossible as acute cell transfection typically leads to receptor auto-activation and uneven number of receptors. In the second part of my thesis, I used the neuronal differentiation of ES cells in order to circumvent this difficulty and compared the instructive functions of the 3 Trk receptors in an identical neuronal context.

2.2.1) TrkA and TrkC trigger neuronal death, TrkB does not

The neuron-specific *mapt* (tau) locus was targeted in ES cells (Plachta et al., 2007) with cDNAs encoding TrkA, TrkB or TrkC with or without an additional 30 nucleotide sequence corresponding to a hemagglutinin (HA) epitope (Bibel et al., 1999). These ES cells were subjected to a differentiation protocol resulting in the generation of virtually pure progenitors expressing Pax6, a transcription factor instructing them to synchronously differentiate into glutamatergic neurons (Bibel et al., 2004; Nikoletopoulou et al., 2007). Previous experiments have shown that the *mapt* locus begins to be expressed soon after the progenitors exit the cell cycle4 and quantification of TrkA, TrkB and TrkC receptors using HA antibodies indicated indistinguishable levels of expression already 3 days after plating the progenitors (days in vitro, div, Fig. 14A, B). This strategy allows a precocious, but moderate expression of Trk receptors, as endogenous TrkB is expressed at significant levels only later (Fig. 14C). By 7 div, the endogenous levels of TrkB are clearly higher than those observed at 3 div when Trk receptor expression is under the control of the *mapt* promoter (Fig. 14C). TrkB and p75 are the only neurotrophin receptors detected with antibodies in this ES cell-based differentiation system (Bibel et al., 2004). We observed that neurons expressing TrkA and TrkC, but not TrkB, all died between 4 and 6 div (Fig. 14D). Identical results were obtained with HA-tagged and unmodified Trk cDNAs (data not shown). As expected, neurons expressing TrkA or TrkC could be rescued by the addition of NGF or of NT3 respectively (Fig. 14D, E). The possibility that neurons expressing TrkB ahead of schedule may survive as a result of secretion of

endogenous BDNF was excluded by incubating neurons with a monoclonal antibody blocking the activity of BDNF (Fig. 14F). K252a, an inhibitor of Trk tyrosine phosphorylation, failed to cause the death of *mapt::TrkB* neurons (Fig. 14F) or of wild-type neurons (data not shown). When used at identical concentrations, K252a completely blocked the rescue activity of NGF and NT3 observed with *mapt::TrkA and mapt::TrkC* neurons (Fig. 14G, H). K252a alone failed to prevent the death-inducing activity of TrkA or of TrkC. Targeting the second *mapt* allele with GFP did not cause any measurable changes compared with wild-type *mapt* alleles or with one allele targeted with HA-tagged or unmodified TrkB cDNAs.

2.2.2) *Mapt::TrkA* and *Mapt::TrkC* embryos lose their nervous system

Large scale death of the developing nervous system has been reported to be incompatible with the development of viable embryos, therefore preventing the generation of animals capable of germ line transmission (Gao et al., 1998). To test the relevance and generality of our in vitro findings in the context of the developing nervous system, we used an alternative approach based on tetraploid complementation allowing the generation of mouse embryos entirely derived from ES cells (Nagy et al., 1993). To this end we used ES cells expressing GFP, TrkA, TrkB, or TrkC from one of the 2 *mapt* alleles. GFP expressed from the second *mapt* allele was used to facilitate the visualization of phenotypes during neurogenesis. As previously reported, this strategy allows an early and comprehensive visualization of the developing central and peripheral nervous system (Tucker et al., 2001). When examined at E11.5, no obvious differences

were observed between any of the 4 genotypes examined, indicating that the initial steps of neurogenesis remain largely unaffected. In particular, peripheral ganglia could readily be observed in all 4 cases (Fig. 15A). However, 2 days later, while embryos expressing GFP or TrkB still appeared normal, TrkA and TrkC-expressing embryos exhibited a widespread and massive loss of the GFP signal (Fig. 15A). Cross-sections stained with antibodies against β-III tubulin revealed that most neurons were eliminated at E13.5 in both TrkA- and TrkC-expressing embryos (Fig. 15B). In spite of the lack of gross morphological effects, quantification of caspase-3 activity at E11.5 revealed a significant increase in the number of apoptotic neurons in TrkA and TrkC embryos compared with either TrkB-expressing or control embryos (Fig. 15C). These findings indicate that the death-inducing function of TrkA and TrkC has the potential to operate in all developing neurons, regardless of their identity, location and developmental history. Increased levels of developmental cell death observed in the dorsal root ganglia (DRG) of TrkA and TrkC embryos suggest that higher than normal levels of TrkA and TrkC are sufficient to cause the death of sensory neurons. However, unlike in the CNS, some sensory neurons do escape cell death (Fig. 15B, arrows), presumably when their axons come in contact with sufficient quantities of the corresponding neurotrophin.

2.2.3) TrkA- and TrkC-induced death requires p75 cleavage

Neurons generated in our ES cell-based differentiation system express the neurotrophin receptor p75 at significant levels and moderate p75 over-expression has previously been shown to cause their rapid death (Plachta et al. 2007;

Nikoletopoulou et al. 2007). As there is evidence that Trk receptors interact with p75 (Bibel et al. 1999), we then tested the possibility that p75 may be involved in the death caused by TrkA and TrkC. We found that cell death is decreased in neurons derived from *mapt::TrkA* ES lines stably expressing shRNA against p75 (Nikoletopoulou et al. 2007), compared to lines expressing scrambled shRNA sequences (Fig. 16 A,B). An involvement of p75 in the death phenotype observed with the *mapt::TrkA* and *mapt::TrkC* neurons is also suggested by the visualization by Western blots of p75-related peptides corresponding to the C-terminal fragment (CTF) and intra cellular domain (ICD) of p75 (Fig. 16C). These proteolytic products were not observed in the lysates of *mapt::TrkB* (Fig. 16 C,D). Previous work with p75 revealed that CTF and ICD are generated by the sequential activity of membrane-bound proteases (Jung et al., 2003; Kanning et al., 2003; Weskamp et al., 2004). A “sheddase” first cleaves off the extracellular domain of p75, leaving behind the membrane bound CTF further processed by the γ-secretase complex to release the death-inducing ICD (see below) (Frade, 2005; Kenchappa et al., 2006). To begin to understand how this initial proteolytic event can be triggered in *mapt::TrkA* and *mapt::TrkC* cells soon after the initiation of neuronal differentiation, we examined the corresponding lysates with antibodies to activated protein kinase C (PKC). Indeed, previous work showed that the proteolysis of a variety of membrane-bound proteins, including p75, is initiated by PKC-mediated activation of “sheddases”13**.** Western blot analysis of TrkA- and TrkC-expressing neurons revealed increased levels of phosphorylated PKC (pPKC) already after 2 div, which was largely prevented by the addition of

NGF (Fig. 16E) or of NT3 (data not shown). pPKC levels were not increased in TrkB-expressing neurons. To test the causal involvement of PKC activation in TrkA and TrkC-induced death, we then incubated the neurons with the PKC inhibitor Go6983 and found that it delayed neuronal death (Fig. 16F). As PKC activation and extracellular cleavage of p75 was previously shown to be a prelude to further cleavage of p75 leading to ICD release and cell death (Weskamp et al., 2004), we then tested the inhibitor LY411575, one of the most potent inhibitors of γ-secretase activity (Abramowski et al., 2008). Treatment of *mapt::TrkA* and *mapt::TrkC* neurons with LY411575 abolished the generation of p75 ICD fragment, as did incubation with the corresponding neurotrophin (Fig. 16G). LY411575 also prevented the death induced by TrkA and TrkC, to an extent similar with that observed following incubation with NGF or NT3 (Fig. 16H).

2.2.4) Sensory neurons death is prevented by inhibition of γ-secretase

TrkA activation by NGF in sympathetic and sensory neurons is well known to be required in order to prevent cell death during development (Smeyne et al., 1994). In view of the death-inducing role of TrkA in the absence of NGF and the proteolytic cascade TrkA rapidly initiates we then examined the response of chick dorsal root ganglia (DRG) cultured in the presence of the γ-secretase inhibitor LY411575 and in the absence of NGF. At 8 days of development, the majority of DRG neurons expresses both TrkA and p75 and depends on NGF for survival. NGF is also well known to cause massive axonal elongation in DRG explants (Levi-Montalcini et al., 1954). We found that LY411575 significantly increased

the survival of DRG neurons and that it caused substantial axonal outgrowth from DRG explants (Fig.17 A,B). This result is also consistent with previous findings indicating that down-regulation of Trk receptors in transgenic animals with a precocious expression of the transcription factor EWS-Pea3 in sensory neurons leads to neurotrophin-independent survival and axonal outgrowth (Hippenmeyer et al., 2005). As the finding that TrkA expression causes cell death suggests that it is causally involved in the dependency on NGF for survival in vivo, we then quantified the levels of programmed cell death in lumbar DRGs in mice lacking TrkA (Moqrich et al., 2004). While *ngf-/-* mutants already exhibit a 7-fold increase in the number of apoptotic neurons at E 11.5, the levels of cell death in TrkA mutants was not significantly different compared to wild-type animals (Fig.18 A,B). As previous reports indicate that *trkA-/-* neurons die at later time points (White et al. 1996) and because TrkA neurons are known to express high levels of p75, we then tested if the expression of p75 in the absence of TrkA may be sufficient to explain delayed cell death in the DRG, as previously observed with sympathetic neurons (Majdan et al., 2001). We found that at E14.5, mice lacking both TrkA and p75 (von Schack et al., 2001) display significantly less cell death in their lumbar DRG compared with single *trkA* mutant animals (Fig.18 C,D). Neurons of the superior cervical ganglia (SCG) constitute a population well known to express TrkA and to depend on NGF for its survival. We then compared the survival of SCG neurons isolated from E17 wildtype and *trkA* mutant embryos and cultured in the presence or absence of NGF (Fig.18 E). We found that *trkA* mutant neuronal cell bodies readily survive the absence of NGF

(Fig.18 E, arrows) but that they only extend short processes. By contrast, virtually all wildtype neurons die within 24 hours in the absence of NGF. In addition, while wildtype neurons respond to NGF by surviving and extending elaborate processes, TrkA mutants die rapidly in the presence of NGF, presumably because NGF activates p75 leading to apoptosis. In line with this, preliminary results indicate that SCG neurons lacking both TrkA and p75 receptors are able to survive in the presence as well as in the absence of NGF (data not shown). In addition, they do extend processes, presumably because of the known inhibitory activity of p75 in process elongation by modulation of RhoA activity (Yamashita et al., 1999).

2.2.5) TrkA, TrkC, not TrkB, in p75-, flotillin-rich fractions

As the 2-step proteolysis of p75 is reminiscent of the processing of amyloid precursor protein in cholesterol-rich domain, often referred to as lipid rafts (Cheng et al., 2009; Vetrivel et al., 2005), we then fractionated detergent-resistant membranes of 4 div ES-derived neurons and monitored cholesterol enriched fraction with flotillin-1 as surrogate marker (Bronfman, 2007). Western blot analyses revealed that 90.3% of TrkA and 94.5% of TrkC protein localized in flotillin-1-rich fractions. By contrast, most of TrkB was found in the pellet devoid of flotillin-1. Similarly, flotillin-1-rich fractions contained 92.7% of p75 in TrkA- and 73.2% in TrkC-expressing neurons, whereas in TrkB expressing neurons only 34.1% of p75 was in these fractions. Control neurons expressing GFP from the *mapt* locus exhibited an intermediate segregation of p75, with 67.4% in the upper fractions and 32.4% in the pellet (Fig.19).

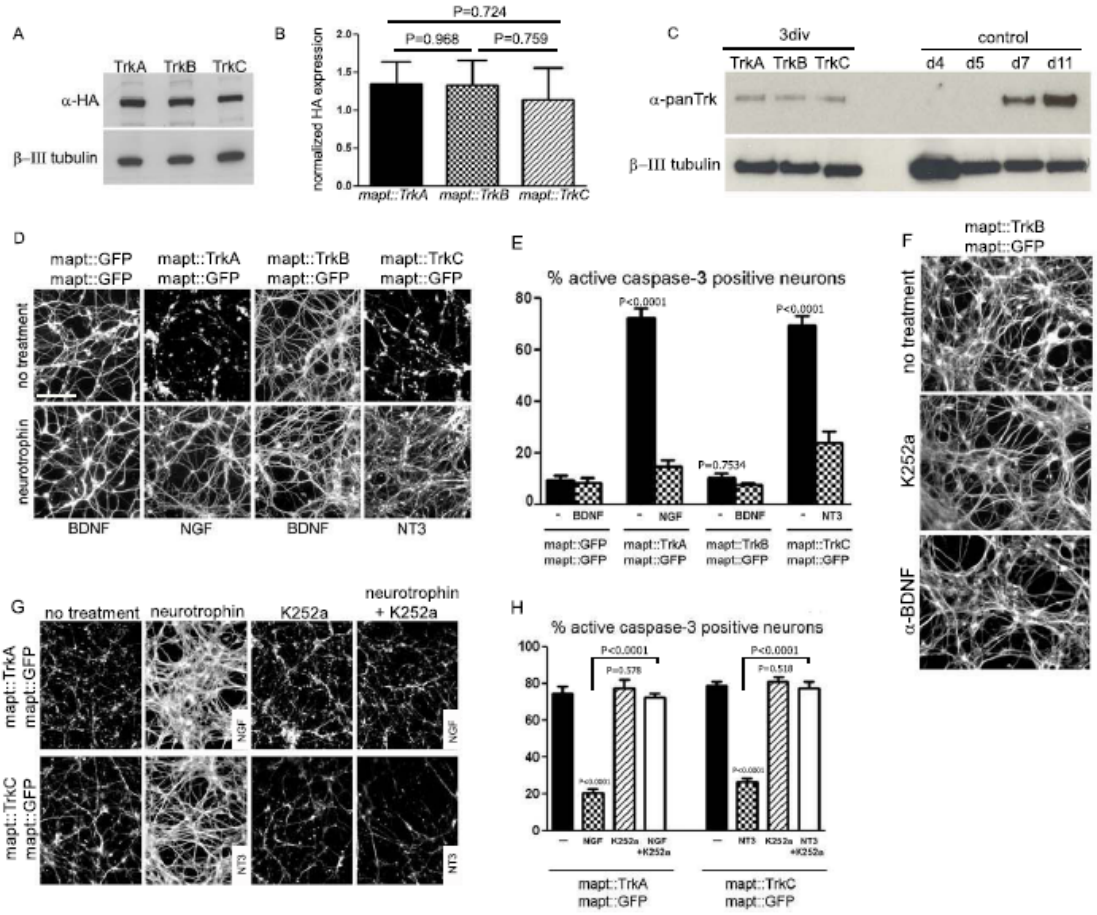
A
TrkA TrkB TrkC
α-HA
β–III tubulin
B
P=0.724
P=0.968
P=0.759
normalized HA expression
mapt::TrkA
mapt::TrkB
mapt::TrkC
C
3div
control
TrkA TrkB TrkC
d4 d5 d7 d11
α-panTrk
β–III tubulin
D
mapt::GFP mapt::GFP
mapt::TrkA mapt::GFP
mapt::TrkB mapt::GFP
mapt::TrkC mapt::GFP
no treatment
neurotrophin
BDNF NGF BDNF NT3
E
% active caspase-3 positive neurons
P<0.0001
P=0.7534
P<0.0001
BDNF NGF BDNF NT3
mapt::GFP mapt::GFP
mapt::TrkA mapt::GFP
mapt::TrkB mapt::GFP
mapt::TrkC mapt::GFP
F
mapt::TrkB
mapt::GFP
no treatment
K252a
α-BDNF
G
no treatment
neurotrophin
K252a
neurotrophin + K252a
mapt::TrkA mapt::GFP
mapt::TrkC mapt::GFP
NGF
NT3
H
% active caspase-3 positive neurons
P<0.0001
P=0.578
P<0.0001
P=0.518
— NGF K252a NGF +K252a
— NT3 K252a NT3 +K252a
mapt::TrkA mapt::GFP
mapt::TrkC mapt::GFP

Figure 14

Figure 14. TrkA and TrkC, but not TrkB, cause neuronal death in ES cell-derived neurons

(A) Trk protein levels in *mapt::TrkA*, *mapt::TrkB* or *mapt::TrkC* neurons (3 div). Staining is with a monoclonal antibody against the HA epitope.

(B) Quantification of HA expression (N=2, mean ± SEM, t-test).

(C) Trk expression from the *mapt* locus (left) or from the endogenous promoter (right). Western blot is with a panTrk antibody.

(D) β-III tubulin staining of control and Trk-expressing neurons cultured without (top) or with the indicated neurotrophins (bottom). Control, *mapt::TrkB* and *mapt::TrkC* cultures (6 div), *mapt::TrkA* (4 div).

(E) Percentage of active caspase-3 positive neurons (N=7, mean ± SEM, t-test. P values shown on black bars refer to comparison with wild-type. t-test between NGF or NT3 treatments and the corresponding non-treated cells, $P<0.0001$).

(F) β-III tubulin staining of *mapt::TrkB* neurons (8 div) treated at 2 div with either K252a or with a monoclonal antibody blocking BDNF activity.

(G) β-III tubulin staining of *mapt::TrkA* and *mapt::TrkC* neurons cultured with or without neurotrophin, K252a or both.

(H) Percentage of active caspase-3 positive *mapt::TrkA* and *mapt::TrkC* neurons treated with neurotrophins, K252a or both (N=9, mean ± SEM, t-test. P values refer to comparison with untreated samples).

Scale bars: 100 μm.

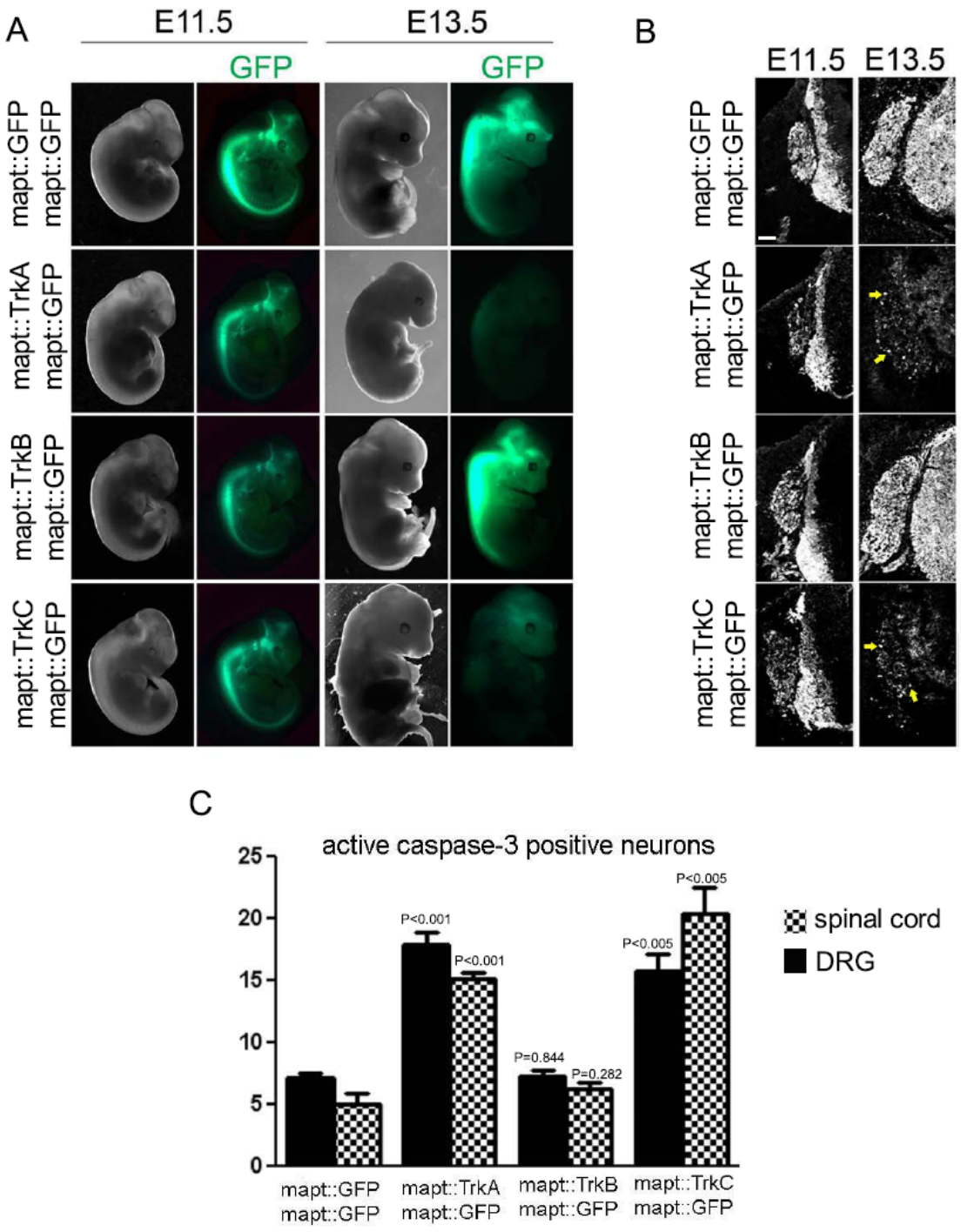
A
E11.5
E13.5
GFP
GFP
mapt::GFP
mapt::GFP
mapt::TrkA
mapt::GFP
mapt::TrkB
mapt::GFP
mapt::TrkC
mapt::GFP
B
E11.5
E13.5
mapt::GFP
mapt::GFP
mapt::TrkA
mapt::GFP
mapt::TrkB
mapt::GFP
mapt::TrkC
mapt::GFP
C
active caspase-3 positive neurons
25
20
15
10
5
0
P<0.001
P<0.001
P=0.844
P=0.282
P<0.005
P<0.005
spinal cord
DRG
mapt::GFP
mapt::GFP
mapt::TrkA
mapt::GFP
mapt::TrkB
mapt::GFP
mapt::TrkC
mapt::GFP

Figure 15

Figure 15. Embryos generated from TrkA, TrkB, TrkC or GFP expressing ES cells

(A) Visualization of the nervous system by GFP expressed from the endogenous *mapt* locus.

(B) β-III tubulin staining in transverse sections at day 11.5 and 13.5 of embryonic development (E11.5 and E13.5). Note the loss of GFP signal in TrkA and TrkC embryos between E11.5 and E13.5.

(C) Quantification of active caspase-3 positive neurons in the spinal cord (SC) and dorsal root ganglia (DRG) at E11.5 (N=3, mean ± SEM, t-test, P values refer to comparison with *mapt::GFP*).

Scale bars: panel B,100μm

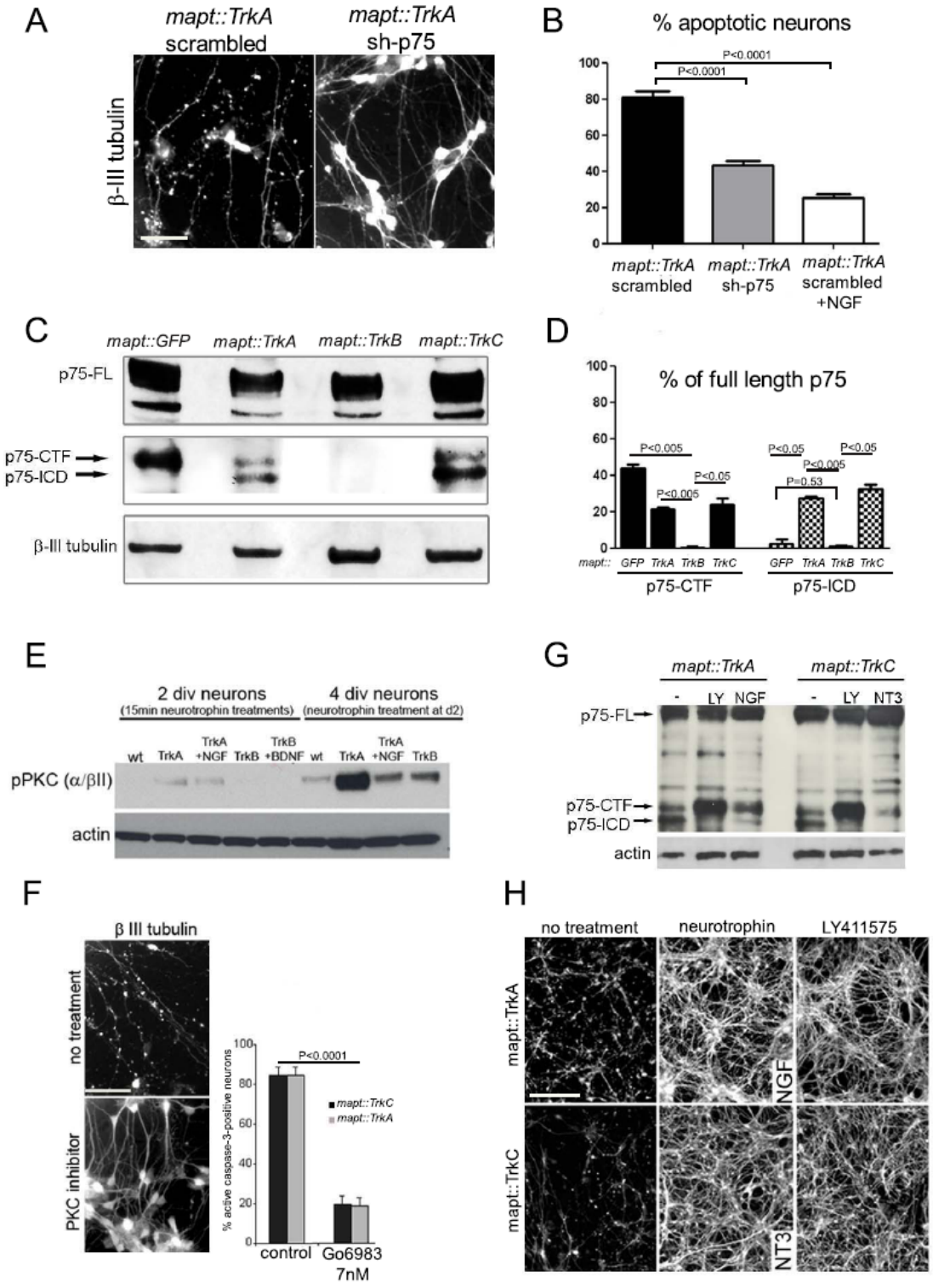
A
mapt::TrkA scrambled
mapt::TrkA sh-p75
β-III tubulin
B
% apoptotic neurons
P<0.0001
P<0.0001
mapt::TrkA scrambled
mapt::TrkA sh-p75
mapt::TrkA scrambled +NGF
C
mapt::GFP
mapt::TrkA
mapt::TrkB
mapt::TrkC
p75-FL
p75-CTF
p75-ICD
β-III tubulin
D
% of full length p75
P<0.005
P<0.05
P<0.005
P<0.05
P<0.05
P<0.005
P=0.53
mapt:: GFP TrkA TrkB TrkC
p75-CTF
p75-ICD
E
2 div neurons (15min neurotrophin treatments)
4 div neurons (neurotrophin treatment at d2)
wt TrkA TrkA +NGF TrkB TrkB +BDNF wt TrkA TrkA +NGF TrkB
pPKC (α/βII)
actin
G
mapt::TrkA
mapt::TrkC
- LY NGF - LY NT3
p75-FL
p75-CTF
p75-ICD
actin
F
β III tubulin
no treatment
PKC inhibitor
% active caspase-3-positive neurons
P<0.0001
mapt::TrkC
mapt::TrkA
control
Go6983 7nM
H
no treatment
neurotrophin
LY411575
mapt::TrkA
mapt::TrkC
NGF
NT3

Figure 16

Figure 16. Proteolysis of p75 mediates the death of *mapt::TrkA* and *mapt::TrkC* ES cell-derived neurons

(A) β-III tubulin staining of *mapt::TrkA* neurons stably expressing a scrambled shRNA sequence, or a shRNA against p75 (5 div).

(B) Percentage of apoptotic neurons (Hoechst staining) after 5 div (N=7, mean ± SEM, t-test,).

(C) p75 expression in lactacystin-treated control, *mapt::TrkA*, *mapt::TrkB* and *mapt::TrkC* (lysates at 3 div).

(D) Quantification of CTF and ICD as a percentage of full length p75 using Image J software (mean ± SEM, N=2, t-test).

(E) Detection of phosphorylated PKCα/βII in *mapt::TrkA* and *mapt::TrkB* neurons (2 and 4 div).

(F) β-III tubulin staining of *mapt::TrkA* neurons untreated or treated with Go6983 (2 div) and percentage of active caspase-3 positive *mapt::TrkA* and *mapt::TrkC* neurons treated with Go6983 or untreated (N=7, mean ± SEM, t-test).

(G) p75 expression in lactacystin-treated *mapt::TrkA* and *mapt::TrkC* neurons. H. β-III tubulin staining of *mapt::TrkA* and *mapt::TrkC* neurons treated with NGF and NT3 respectively or with LY411575 (8 div).

Scale bars: panels A and E, 50 μm; panel G,100μm.

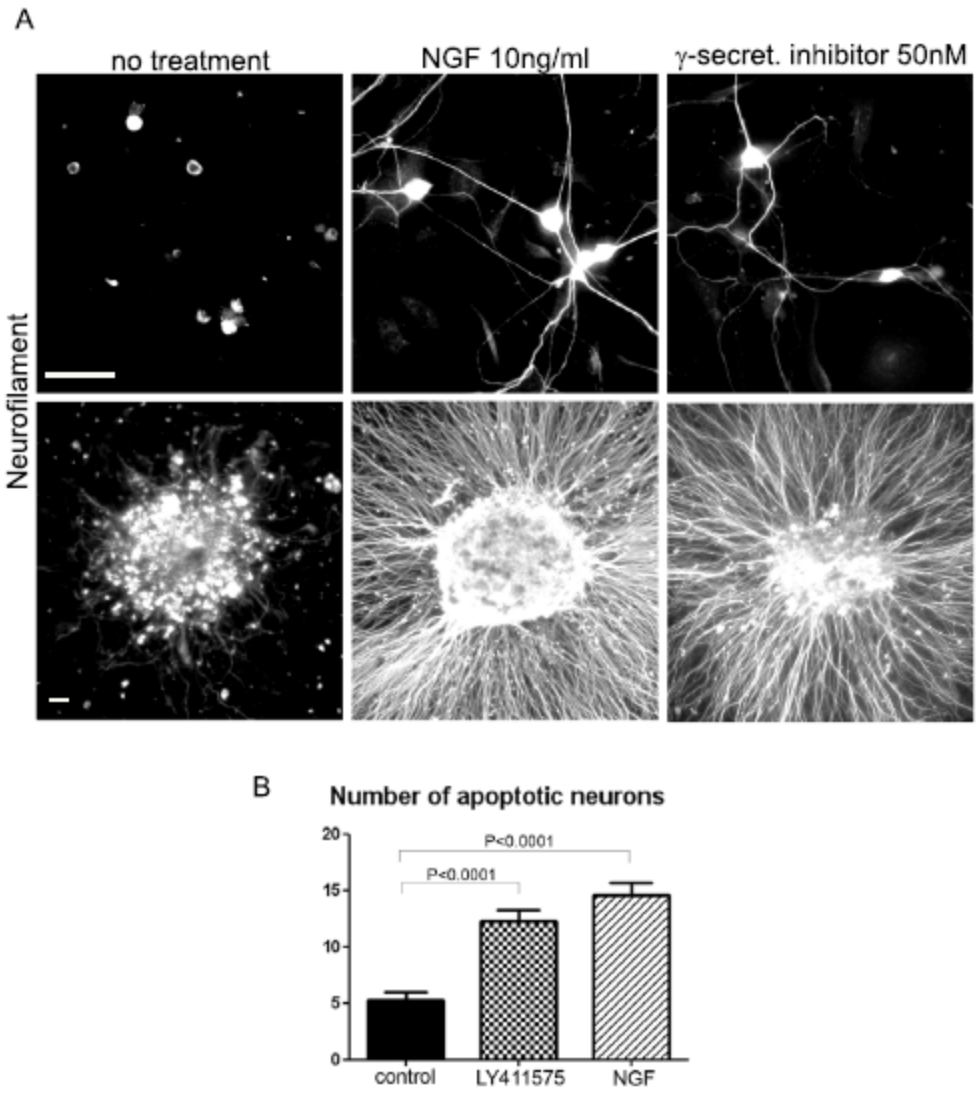
A
no treatment
NGF 10ng/ml
γ-secret. inhibitor 50nM
Neurofilament
B
Number of apoptotic neurons
P<0.0001
P<0.0001
20
15
10
5
0
control
LY411575
NGF

Figure 17

Figure 17. γ-secretase-dependent proteolysis is involved in the death of sensory neurons.

(A) Neurofilament staining of dissociated (top row) or whole (bottom row) dorsal root ganglia isolated from E8 chick embryos cultured with or without NGF or LY411575.

(B) Quantification of 3 div apoptotic neurons (two-tail t-test, N=18 per condition).

Scale bars: 100μm.

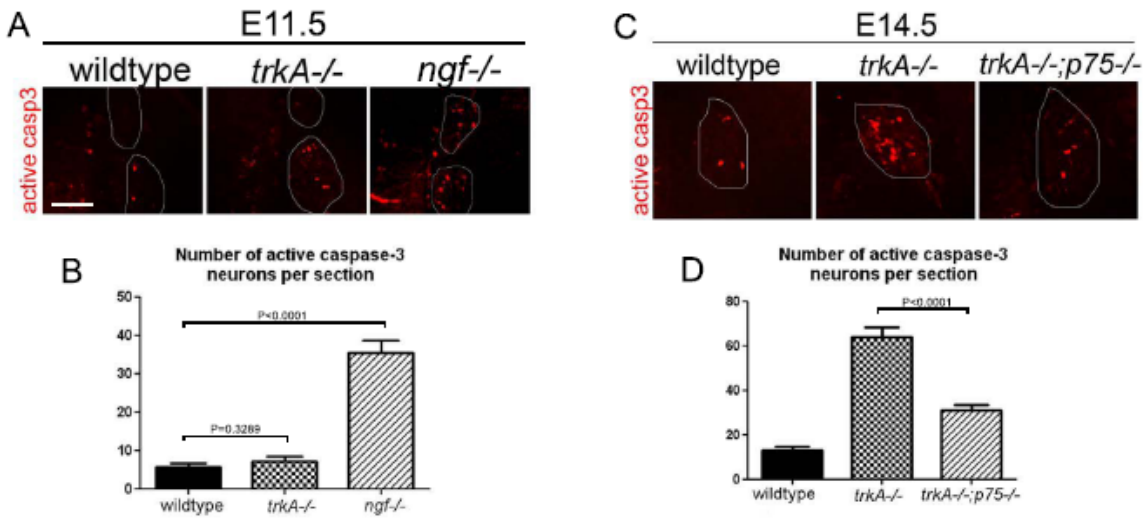
A
E11.5
wildtype
trkA-/-
ngf-/-
active casp3
B
Number of active caspase-3 neurons per section
P<0.0001
P=0.3289
wildtype
trkA-/-
ngf-/-
C
E14.5
wildtype
trkA-/-
trkA-/-;p75-/-
active casp3
D
Number of active caspase-3 neurons per section
P<0.0001
wildtype
trkA-/-
trkA-/-;p75-/-

Figure 18

Figure 18. Analysis of sensory neuron death in *ngf*, t*rkA* and t*rkA/p75* mutant embryos

(A) Active caspase-3 staining of spinal cord and lumbar dorsal root ganglia (DRG) sections.

(B) Number of active caspase-3 positive DRG neurons in E11.5 embryos of the indicated genotypes (t-test, N=4 embryos per genotype).

(C) Active caspase-3 staining of spinal cord and lumbar DRG cross sections.

(D) Number of active caspase-3 positive DRG neurons in E14.5 embryos of the indicated genotypes (t-test, N=3 embryos per genotype).

Scale bars: 100 μm.

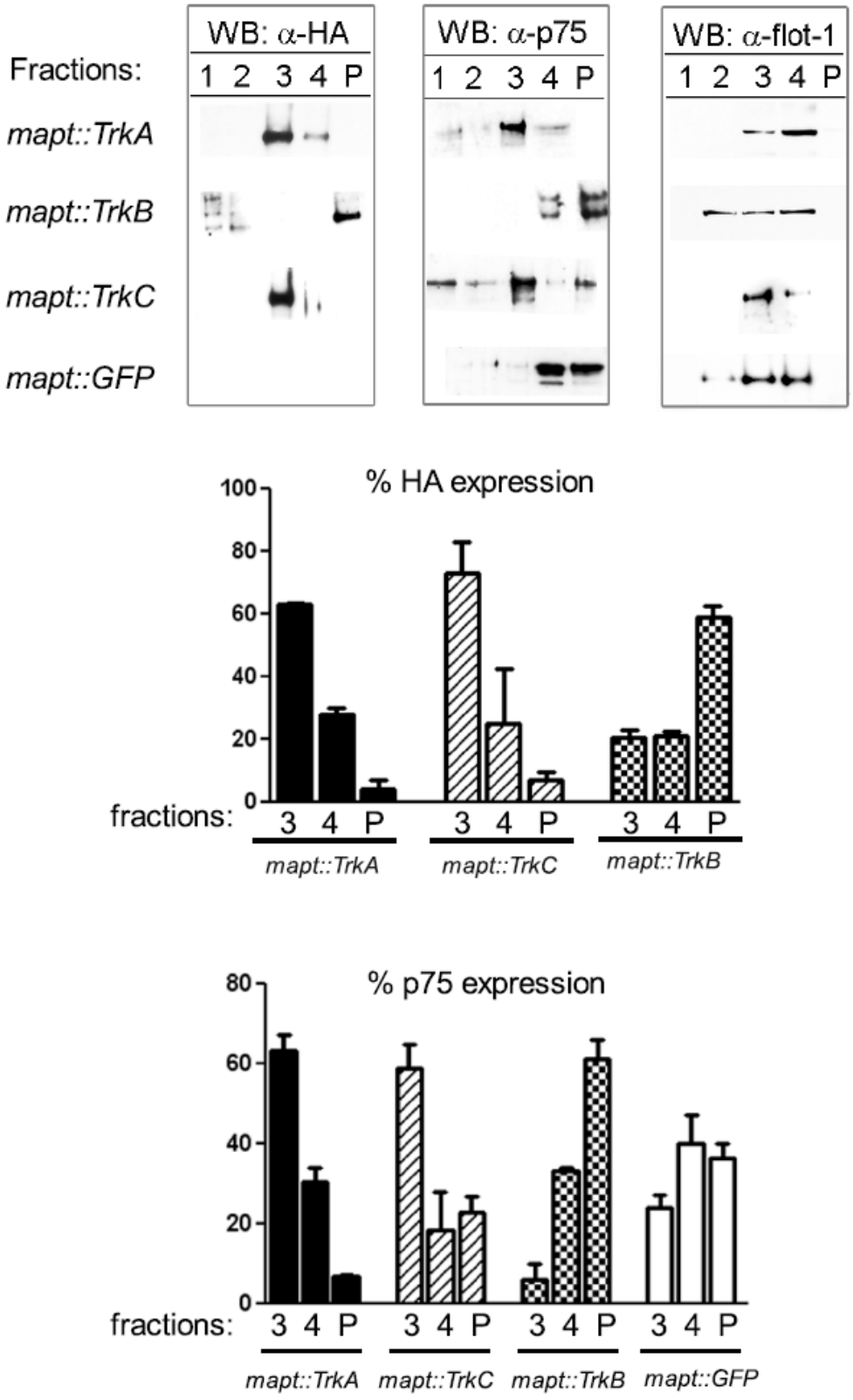
Fractions:
1 2 3 4 P
mapt::TrkA
mapt::TrkB
mapt::TrkC
mapt::GFP
WB: α-HA
WB: α-p75
WB: α-flot-1
% HA expression
100
80
60
40
20
0
fractions:
3 4 P
mapt::TrkA
mapt::TrkC
mapt::TrkB
% p75 expression
80
60
40
20
0
mapt::GFP

Figure 19

Figure 19. Segregation of p75 and Trk receptors in flotillin-rich factions

Western blot with antibodies to HA, p75 and flotillin-1 in detergent-resistant following fractionation of neuronal lysates by sucrose gradient. Lysates are from ES cell-derived neurons expressing TrkA, TrkB, TrkC or GFP from the *mapt* locus. Quantification of HA and p75 bands is presented as a percentage of total expression in the upper fractions plus the pellet.

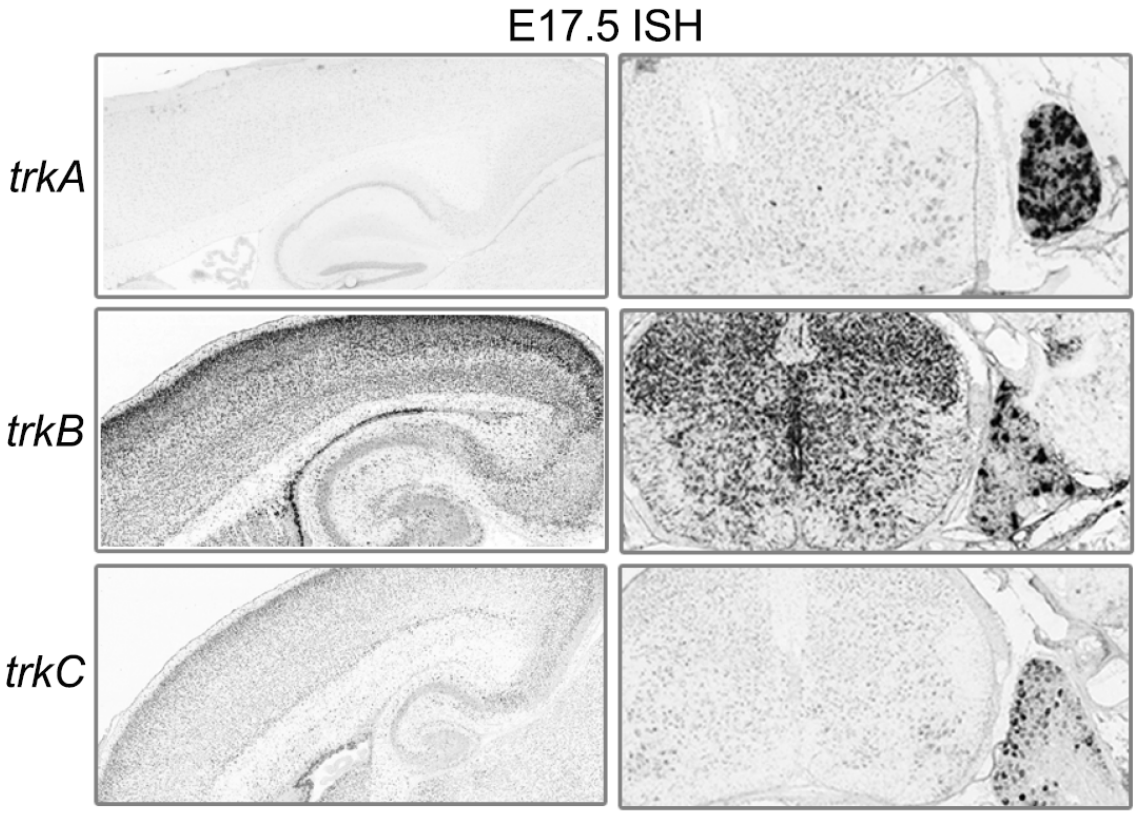
E17.5 ISH
trkA
trkB
trkC

Figure 20. Trk receptor expression in the developing nervous system.

In situ hybridizations with probes against TrkA, TrkB or TrkC are from the Allen Brain Atlas.

3. DISCUSSION

In my thesis, I provided two examples of using genetically modified ES cells as a tool in neurobiology. First, I will discuss the results obtained with ES cells carrying a mutation in the *Pax6* gene, and then the ones obtained with ES cells engineered to expressed the 3 Trk receptors under the endogenous *mapt* promoter.

3.1) THE ROLE OF PAX6 IN CORTICAL NEUROGENESIS

3.1.1) ES cells as a tool to study the specification of neural progenitors during development

The discovery that uniform populations of Pax6-positive RG cells can be generated from mouse ES cells makes it now possible to study early developmental events that have been previously very difficult to access in the mammalian CNS. As ES cell-derived progenitors become glutamatergic neurons in vitro when cultured under minimal conditions, we interpret these results to mean that Pax6-positive progenitors are intrinsically committed to become glutamatergic neurons, and that under permissive conditions in vitro, they follow the differentiation pathway adopted by their in vivo counterparts (Malatesta et al., 2003). In the present study, we tested if ES cell-derived progenitors are also committed to become glutamatergic neurons after implantation in the developing chick telencephalon. Following implantation in the prospective dorsal telencephalon, we found large numbers of vGLUT1- positive neurons in the developing cortical plate of the chick (Figure 8B). The commitment of these cells to become glutamatergic neurons was revealed by their implantation in the

prospective ventral telencephalon, as most cells failed to become GABA-ergic neurons as they normally should in this location or else after migration in the dorsal telencephalon. They instead adopted a glutamatergic phenotype and failed to migrate to the cortex (Figure 8C). The critical control experiment with RA-untreated, naive ES cells also implanted in the prospective ventral telencephalon revealed large numbers of mouse cells in the chick cortex that were positive for the vGAT (Figure 8D). These results are in line with the view that most cortical GABA-ergic neurons originate from the ventral telencephalon (Cobos et al., 2001) and indicate that pluripotent mouse ES cells can be instructed by the host cues to become GABA-ergic neurons and to adopt a migratory phenotype. The results obtained following transplantation of WT progenitors prompted us to examine the behavior of mutant progenitors. We found that when transplanted in the ventral telencephalon, they uniformly generated GABAergic neurons that successfully migrated in large numbers and colonized the chick cortex (Figure 8E). These findings indicate that the neurogenic potential of Pax6 mutant progenitors is not compromised, and that they can generate GABA-ergic neurons that survive and adopt a migratory behavior similar to that of the endogenous interneurons. However, implanting the progenitors derived from Pax6 mutant ES cells in the prospective dorsal chick telencephalon led to the generation of only small numbers of neurons that were GABA-ergic but failed to integrate, aggregated, and died (see Figure 8F). While these results confirmed the view that progenitors derived from Pax6 mutant ES cells are committed to become GABAergic neurons, they also raise the question

of why the survival of neurons derived from mutant progenitors differs depending on where they are implanted in the chick embryo. All we know at this point is that, unlike in vitro, some ES cell-derived progenitors do survive after transplantation and that this is accompanied by a downregulation of p75NTR expression (Figure 13).

3.1.2) Mechanisms of neural progenitor misspecification

While the progenitors generated from ES cells lacking Pax6 were found to express the transcription factor Emx2, they expressed the bHLH factor Mash1 that characterizes ventral telencephalic progenitors and lacked expression of neurogenin2 that characterizes cortical (Fode et al., 2000) and WT ES derived progenitors. This result fits well with previous observations on the cortex of the Pax6 mutants showing that the Mash1-positive domain extends to the dorsal pallium (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001), while neurogenin2 expression is drastically reduced (Scardigli et al., 2003). Reintroduction of Pax6 in mutant ES cells with a retrovirus indicated that Pax6 cell autonomously prevents the expression of ventral genes in the progenitors, thereby specifying a glutamatergic lineage and suppressing a GABA-ergic phenotype. In addition to the ventralization of the pallium, it has long been recognized that the cortex of mammals lacking Pax6 is grossly abnormal (Schmahl et al., 1993), including in particular the loss of almost half the number of neurons (Heins et al., 2002). Though we expected Pax6 to be required for the generation of RG cells and neurons (Heins et al., 2002), we found that mutant ES cells generate neurogenic RG cells as efficiently as WT ES cells. These

experiments thus indicate that Pax6 cell autonomously specifies the ES cell-derived RG cells to generate glutamatergic neurons and that, in its absence, Mash-1 is upregulated, and that their progeny has the characteristics of GABAergic neurons.

3.1.3) Mechanisms of neuronal elimination in the developing cortex

In light of the premature death of the mutant ES cell derived neurons, we reexamined the question of neuronal death in the Pax6 mutant (Sey/Sey) cortex as none of the previous studies (Quinn et al., 2007; Warren et al., 1999) retained cell death as an explanation for the neuronal losses in the Pax6 mutant cortex. At E12, i.e., earlier stages than in most previous studies, we readily found cohorts of apoptotic GABA-ergic neurons. In cultures, the GABAergic neurons generated by mutant progenitors massively upregulate the neurotrophin receptor p75NTR, well known to cause cell death, and fail to express significant levels of TrkB, the ‘‘trophic’’ receptor progressively upregulated in WT progenitors during the course of their neuronal differentiation (Bibel et al., 2004). In line with the possibility of imbalance between the levels of expression of p75NTRand TrkB in mutant cells, we found that three different mutant lines expressing shRNA targeting p75NTR survived like WT cells. These results further showed that the loss of TrkB is not per se the cause of the death of these neurons, as suppression of p75NTR was sufficient to significantly decrease neuronal death both in vitro and in vivo in line with previous observations indicating that TrkB null mutants do not exhibit significant levels of cell death in the developing cortex (Silos-Santiago et al., 1997). This result is also in agreement with previous studies indicating that

overexpression of p75NTR in neurons in vivo (Majdan et al., 1997) or in ES cell-derived neurons (Plachta et al., 2007) is sufficient to cause neuronal death. p75NTR overexpression and cell death was found to mostly affect the population of GABAergic neurons that was inappropriately generated in the cortex, most likely as a result of premature exit from the cell cycle of misspecified cortical RG cells (see also (Kroll and O'Leary, 2005). As noted, there is an early wave of increased neurogenesis in the Pax6 mutant cortex (Quinn et al., 2007 and our own results with doublecortin staining), consistent with a 3-fold increase in the number of GAD- and vGAT-positive neurons in the mutant cortex at E12 as compared to WT (Figure 11). In a recent study, Pax6 was suggested not to have an early role in neuronal specification based on an in situ hybridization experiment indicating that GAD65 mRNA was not upregulated, but rather downregulated at E13.5 in the Pax6 mutant cortex, as compared to WT (Schuurmans et al., 2004). Our results indicate that the misspecified GABAergic neurons generated very early are also rapidly eliminated, suggesting that most have already died when the mutant cortex is examined at E13.5. In line with this, our own in situ hybridization experiments indicate that GAD65 is indeed expressed at E12 in the Pax6 mutant cortex (data not shown). These results are reminiscent of those recently obtained in the retina, where Pax6 was proposed to play a very early role in the specification of retinal neurons. Like in the developing cortex, in the absence of Pax6, misspecified retinal neurons differentiate prematurely and die (Philips et al., 2005).

3.2) BIOLOGICAL FUNCTIONS OF TRK RECEPTORS DURING DEVELOPMENT

3.2.1) Physiological role of death inducing activity of TrkA and TrkC during naturally occurring neuronal death

Our findings indicate that TrkA and TrkC cause the death of newly born neurons throughout the developing nervous system in the absence of ligand-mediated activation. By contrast, TrkB fails to do so. As TrkB is expressed in the CNS at far higher levels and more widely compared with TrkA and TrkC (Figure 20), our observations invite the speculation that cell numbers in the PNS are regulated by mechanisms that differ from those operating in the CNS, where cell contact-based mechanisms appear more likely to regulate normally occurring cell death than those based on soluble growth factors. The appealing view that neuronal numbers and innervation density are regulated by target tissues releasing limiting amounts of trophic molecules (Barde, 1989; Davies, 1988; Oppenheim, 1991; Purves, 1988) is based on observations made in the PNS, where there is ample evidence that elimination of any of the neurotrophin signaling component results in massive neuronal losses in the developing sensory and sympathetic ganglia (Crowley et al., 1994; Ernfors et al., 1994; Farinas et al., 1994; Klein et al., 1994; Klein et al., 1993; Minichiello et al., 1995; Pinon et al., 1996; Smeyne et al., 1994). Elimination of other growth factors such as glia cell line-derived neurotrophic factor (GDNF) and its receptors similarly leads to the loss of the enteric nervous system (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). By contrast, the elimination of growth factors or of their receptors in the CNS fails to cause such dramatic cell losses during

normal development (Jones et al., 1994; Silos-Santiago et al., 1997), in the absence of lesion.

Obviously, the lack of death-inducing activity of TrkB does not imply that its activation is not required to prevent neuronal death during development of PNS neurons. For example, the lack of BDNF causes the substantial loss of cranial sensory neurons and these neurons are known to require TrkB activation to survive (Ernfors et al., 1994; Jones et al., 1994; Silos-Santiago et al., 1997). However, mechanisms other than TrkB expression are most likely to predispose neurons to die, including high levels of expression of death-inducing receptors such as p75, NRH2 and TrkC (Ernfors et al., 1988; Murray et al., 2004; Stenqvist et al., 2005).

3.2.2) Mechanisms of TrkA- and TrkC- induced death

While our findings point to a key role of p75 as an effector of the death mechanisms initiated by TrkA and TrkC, it appears unlikely that p75 is the only molecule involved in this process. The death of almost all neurons in the transgenic embryos expressing TrkA or TrkC suggests that related molecules are likely to play a role similar to p75. Indeed, p75 is but one member of a large family of molecules, including the closely related NRH2 that can also associate with Trk receptors. NRH2 is expressed at high levels in several areas of the developing nervous system, including some where p75 is not detectable (Murray et al. 2004).

As TrkA was identified as a receptor for NGF already in 1991 (Kaplan et al., 1991; Klein et al., 1991) it may seem surprising that its death-inducing function

has not been previously reported. However, Trk receptors readily auto-activate in the absence of ligand when expressed at high levels following acute transfection in cultured cells, and it is exceedingly difficult to control receptor expression in terms of timing, levels and evenness of expression unless endogenous promoters are used. Not only does the mapt promoter drive moderate and equal levels of expression of the 3 receptors tested, but also it ensures expression within hours after exit of the cell cycle and the onset of neuronal differentiation. It is typically at this time that neurons are eliminated in the developing nervous system during the process of naturally occurring cell death (Oppenheim, 1991) and our findings indicate that other key molecules such as p75 also need to be present at that time as effectors of the killing process.

In line with our findings, a previous report indicated that acute over-expression of TrkC in HEK293 cells leads to increased levels of cell death in an activation-independent manner (Tauszig-Delamasure et al., 2007). Yet, this study failed to reach similar conclusions upon TrkA over-expression, presumably due to the limitations inherent to the use of un-controlled expression in non-neuronal tumor cells.

Beyond new insights in nervous system development, our findings also help to better understand different prognoses associated with tumors of the nervous system, including neuroblastomas in particular. These tumors originate from neural crest derivatives and the long recognized better prognosis associated with tumors expressing TrkA or TrkC is now easier to rationalize (Lavoie et al., 2005;

Yamashiro et al., 1997). By contrast, TrkB-expressing neuroblastomas are linked with poor prognosis (Eggert et al., 2000), in line with our finding that this receptor does not induce cell death when expressed in the very same cell in which TrkA and TrkC both induce cell death.

3.3) CONCLUSIONS AND PERSPECTIVES

The findings of my PhD thesis suggest that ES cells represent a valuable tool to dissect relevant mechanisms operating during the development of the vertebrate nervous system. The quality of the predictions obtained with this system could be verified both in the case of Pax6 and of the Trk receptors by the analyses of the corresponding mutant embryos. How useful this differentiation system is can be illustrated by the fact that Pax6 and the Trk receptors have been reported about 20 years ago to be expressed in the developing mouse nervous system, and yet their role has remained very difficult to assess simply based on the analysis of the corresponding mouse mutants. The analytical power of our ES cell-based system lies in the still incompletely understood fact that ES cell-derived progenitors faithfully recapitulate in vitro early steps of corticogenesis. In addition to published data from our laboratory (Bibel et al., 2007; Bibel et al., 2004), we also profiled these cells in collaboration with the laboratory of Magdalena Goetz, which isolated endogenous cortical radial glial cells by fluorescence activated cell sorting at different developmental stages. The comparative transcriptome of ES cell-derived and endogenous E12 neurogenic progenitors indicate that in spite of their different origins both types of progenitors are quite similar, while neurospheres or progenitors derived from ES cells by

different methods (Conti et al., 2005) show a transcriptome profile clustering more closely with what is seen with the E18 cortex, a time point when glia are generated in vivo. Thus, as demonstrated with the transcription factor Pax6, this system can facilitate the study of genes controlling early aspects of neurogenesis, such as the generation, patterning, and developmental potential of cortical progenitors.

In addition to the isolation of ES cells from existing mutants such as Pax6, another obvious, but particularly useful feature of ES cells is that they can be readily manipulated. In particular, homologous recombination into a specific locus, such as *mapt* in my case, allows the control of both the levels and timing of expression of cDNAs. As all progenitors and neurons are derived from a single ES clone, they carry identical genetic information. This is in contrast to acute cDNA transfection often driven by ubiquitous promoters, resulting in uncontrolled over-expression in terms of numbers of copies and timing and cell type expressing the constructs.

In addition, this system allows the effects of mutations or other genetic manipulations to be studied not only in an identical neuronal background, but also in cells that are essentially synchronous. As the progenitors undergo only one or two cell divisions before their terminal differentiation, neurons are born and mature together and therefore respond to the genetic manipulations they carry in a coordinated manner, making the identification of phenotypes easier. All these aspects were key to the observation that TrkA and TrkC, but not TrkB, kill developing neurons, perhaps the most surprising aspect of my work. As ES

cell-derived neurons can be generated in essentially unlimited numbers, I could also generate sufficient numbers of neurons carrying TrkA, TrkB or TrkC to perform the necessary biochemical analyses. In particular, the results presented in Fig.19 necessitated about 60 million neurons per condition.

Since genetically modified ES cells can re-enter embryogenesis, even after being extensively expanded in vitro (see introduction), observations obtained with ES cell-derived progenitors and neurons can then be readily verified in vivo by the generation of mouse embryos. With the tetraploid complementation assay as an alternative method to the classical generation of chimeras, the entire embryo is derived in one step from the ES cells of interest, making possible the generation of mutants with dominant lethal phenotypes. An example was demonstrated in the second part of my thesis, with the generation of embryos expressing TrkA or TrkC under one mapt allele and which would have been impossible to obtain with other methods as they are embryonic lethal.

Beyond the use of mouse ES cells as described in my work, it can be hoped that similar developments may take place with human ES cells. Given the isolation of human ES cell lines (Thomson and Marshall, 1998), it would appear in principle possible to address in the future questions related to neuronal specification and survival using human neural progenitors and neurons. Such studies would greatly enhance our molecular and cellular understanding of how neural progenitor diversity is achieved in humans, including in particular why the human cortex is so much larger than that of the mouse.

However, the culture conditions necessary to maintain hES cells, and indeed even the identity of hES cells remain unclear at this point. While mouse ES cells are typically propagated in the presence of LIF to maintain them pluripotent, hES cells are cultured instead by most laboratories in the presence of FGF, and it remains very unclear whether LIF signaling is necessary for the regulation of the key genes controlling pluripotency. The difficulties with establishing satisfactory conditions for the propagation of pluripotent hES cells lines, are likely to arise from their identity and authenticity. Recent work indicated that mouse pluripotent lines, defined as “epiblast stem cells” (epiSCs), isolated from embryos at later stages of development, require FGF instead of LIF to be maintained in culture (Tesar et al., 2007). This observation invites the speculation that if signaling cascades operating at early stages of embryogenesis are conserved among mammals, the hES lines cultured by most laboratories may represent a mixture of authentic stem cells and of pluripotent epiSCs. As long as these basic problems have not been sorted out, it is unlikely that useful, robust neuronal differentiation protocols can be established with human ES cells.

Re-programming of somatic cells offers yet another source of human pluripotent cells. This spectacular achievement takes its origin in early work, when Gurdon and his colleagues were successful in generating adult frogs after transferring nuclei isolated from intestine cells of the tadpole into enucleated unfertilized *Xenopus laevis* eggs (Gurdon, 1962a, b, c). This finding demonstrated that the mature cell’s nucleus retains the capacity of re-activating genes necessary for pluripotency and is returned to a state from which it can go on to generate the

entire animal. However, when the same experiment was performed with nuclei isolated from adult cells, they were able to generate tadpoles but not adult frogs, raising the concern that the more differentiated cells are the more limited their efficiency of being reversed. The next big step was by Wilmut and colleagues with the generation of the ewe “Dolly”, by fusing cultured mammary gland cells with enucleated eggs (Wilmut et al., 1997). This stunning experiment, completed by the demonstration of germline transmission by the generation of several lambs that were born to Dolly, demonstrated beyond doubts that reprogramming from somatic cells can be very extensive in mammals.

These experiments led to a search of re-programming factors that can directly convert differentiated cells into the pluripotent state, that culminated in 2006 with the report by Takahashi and Yamanaka that the epigenome of embryonic or adult differentiated cells can be erased by the introduction of initially 4 factors, Oct4, Sox2, Klf4 and c-Myc using viral vectors (Takahashi and Yamanaka, 2006). The resulting induced pluripotent cells (iPS) do not seem to be substantially different from ES cells and 2 months ago several reports indicated that mice can be generated from such iPS cells (Boland et al., 2009; Zhao et al., 2009). Even if work with human ES and iPS cells is not yet as far advanced as often assumed, it appears likely that not before long the impact of mutations affecting the human nervous system will be studied and much better understood using such strategies. This type of work will be particularly important in cases where genes function differently or have no real equivalent in rodents (for a recent review see (Lengerke and Daley, 2009)

4. MATERIALS & METHODS

Materials
ES cell culture medium and ingredients were as described in details in Bibel et al., 2007.

Pax6 mutant mouse ES Cells
Mice heterozygous for the Pax6 gene (Sey/+) were crossed, and two independent homozygote Sey/Sey ES cell lines were isolated from the inner cell mass of pre-implantation blastocyst-stage embryos. WT and homozygote Sey/Sey mouse ES cells were differentiated as described in Bibel et al. (2004). To facilitate the detection of ES cell-derived neurons in chick embryos after transplantation, we used WT and (Sey/Sey) ES cells engineered to express green fluorescent protein (GFP) from both or one tau alleles, respectively (Bibel et al., 2004).

Targeting of the mapt locus in ES cells
Targeting of the endogenous *mapt* locus was performed as previously described7. A cassette containing the EGFP (Clontech, Palo Alto, California), TrkA, TrkB or TrkC cDNA, the *Pgk-1* polyadenylation signal and the G418-selectable marker *Pgk-Neor* was inserted between the PmeI and NotI sites of a plasmid containing exon 1 of the tau locus and 8.0 kb flanking genomic sequence. The linearized targeting vector was electroporated into J1 embryonic stem (ES) cells as described39 . 200 G418-resistant colonies were picked for each line and analyzed by Southern blot. The 5' external probe consisted of a 500-base pair (bp) *Sma*I−*Eco*RI genomic fragment whose 5' end was located 2.8 kb upstream of exon 1, and the 3' external probe consisted of a 600-bp *Bam*HI−*Eco*RI genomic fragment whose 5' end was located 6.0 kb downstream of exon 1.

RT-PCR
Following RNA isolation and reverse transcription, Ngn2, Tbr1, and GADPH mRNA was amplified using the following primers: Ngn2, TCCAACTCCACGTCCCCATACCandGCTGCCAGTAGTCCACGTCTGA (primers corresponding to positions 658–678 bp and 709–730 bp, product size 72 bp); Tbr1, GAGGCTCTGGAAACACGAAG and ACTCGACTCGCCTAGGAACA (primers corresponding to positions 3033–3053 and 3192–3212, product size 160 bp); GADPH, TGAGGCCAGTGCTGAGTATG and CACATTGGGGGTAGGAACAC (product size 457 bp).

Chick transplantations
Fertilized White Leghorn eggs were incubated at 38.5^{o}C with 80% humidity for 36 hr (10–12 somites). Two ml of albumin was aspirated, and a small portion of the upper eggshell was discarded to access the embryo visualized with Pelikan ink (Pelikan drawing ink A) in phosphate- buffered saline (PBS) injected close to the blastoderm. The area of the prospective dorsal or ventral telencephalon was

removed with glass needles and filled with WT (tau::GFP) and Sey/Sey (tau::GFP) ES cell aggregates treated with RA for 4 days. Before implantation using tungsten needles, the aggregates were loosened by incubation in trypsin at 37°C for 10 min. WT ES cell aggregates, not treated with RA, were similarly implanted. The eggs were then sealed and incubated a further 8–10 days. The embryos were then removed from the egg, fixed in 4% PFA for 24–48 hr at 4°C, incubated in 30% sucrose for 24–48 hr at 4_C, embedded in Tissue-Tech, and stored at -80°C until cryosectioning.

Immunofluorescence
Cells or tissue sections were rinsed in PBS and incubated for 1 hr in blocking solution containing 10% horse serum and 0.2% Triton in PBS. Sections were incubated with primary antibodies in blocking solution for 12 hr at 4°C. PBS was substituted for the primary antibodies to test for unspecific labeling. Sections were rinsed in PBS and incubated with the following secondary antibodies (molecular probes) for 1 hr at room temperature: anti-rabbit Alexa 488 or 594, anti-mouse Alexa 488 or 594, anti-mouse IGg1 Alexa 594, anti-goat alexa 594, and anti-guinea pig (1:1000, gift from S. Arber). The nuclear dye Hoechst 33342 (10 mg/ml, Sigma) was used. Sections were rinsed in PBS and mounted.

TUNEL Staining
TUNEL staining on 12-mm-thick brain or spinal cord sections was performed as described by the manufacturer using the Roche In Situ Cell Death Detection Kit.

In vivo quantifications of cell numbers in the chick telencephalon
Cells transplanted in the chick telencephalon were stained with vGAT or vGLUT1 antibodies (both are mouse specific) and the nuclear stain Hoechst. Cells with the highest density of puncta over the cell body around mouse nuclei were counted (7 independent transplantations per condition). Cells expressing vGAT, GAD, and Calretinin in the WT and mutant brain at E12 were counted similarly. Doublecortin-positive cells in WT and mutant cortices were counted with the ‘‘Analysis’’ program. Quantifications were performed on 12-mm-thick sections.

Western Blot
Cells were lysed in 500 mM Tris-HCl pH 7.2, 1 M NaCl, EDTA, Triton 100-X, Na-deoxycholate, 10% SDS, supplemented with protease inhibitors (Roche) and 1 mM dithiothreitol (DTT), and placed for 20 min on ice, followed by 20 min centrifugation at 14,000 rpm. Samples were separated on a gradient (4%–12%) polyacrylamide gel (Invitrogen) and transferred to a polyvinylidene fluoride membrane (Immobilon- P, Millipore, IPVH00010). After blocking for 1 hr at room temperature in 5% skim milk, membranes were incubated in the primary antibodies overnight at 4_C. After three 5 min washes in TPBS (100mMNa2HPO4, 100mM NaH2PO4, 0.5N NaCl, 0.1% Tween-20), membranes were incubated for 1 hr at room temperature in secondary horseradish peroxidaseconjugated antibody (anti-mouse HRP and anti-rabbit HRP; Biorad).

Blots were developed by chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Retroviral vectors and infections
The Pax6-IRES-GFP viral vector contained the 2050 bp fragment with the entire coding region of Pax6 and parts of 50UTR and 30UTR inserted between the upstream LTR and the IRES sequence as described in Hack et al., 2004. Gpg helper-free packaging cells were used for viral production and resulted in titers of 3 3 106 and 2.5 3 106 of Pax6-IRES-GFP or GFP viral particles, respectively. For the infections of mutant ES cells, we used 5 ml of virus per ml medium and infected the cells for 12 hr at 37_C, 5% CO2. Subsequently, they were washed three times with warm PBS and were processed for aggregate formation.

Stable expression of shRNAs in ES cells
Plasmids containing a neomycin resistance cassette were purchased from Genescript expressing three different shRNA constructs against p75NTR or a scrambled sequence under the H1 promoter and GFP under the CMV promoter. Plasmids were linearized, phenol-chloroform extracted, and twice precipitated with ethanol. Mutant ES cells (53106 cells)were resuspended in 600 ml electroporation buffer (137mM NaCl, 20 mM HEPES pH 7.0, 5 mM KCl, 0.7 mM Na2HPO4 pH 7.0, 6 mM dextrose, 0.1 mM b mercaptoethanol) and electroporated with 30 mg of linearized plasmid in 0.4 cm cuvettes (BIORAD) at 400 volts and 25 mF. ES cells were subsequently plated on neomycin-resistant mouse embryonic fibroblasts. Two days after electroporation, 380 mg/ml G418 was added to the medium for 10 days to select for neomycin- resistant clones. Individual GFP-positive clones were expanded to establish a line.

Pax6 mutant animals
Pax6Sey mice were maintained as heterozygotes on a mixed C57BL/ 6JxDBA/2J background. P75 exon IV mutant mice were maintained on a C57BL/6 background and genotyped as described (von Schack et al., 2001).

In vivo tetraploid complementation assay
ES cell clones expressing TrkA, TrkB or TkC from one *mapt* allele and GFP from the second allele, or a control clone expressing GFP from both *mapt* alleles were aggregated with CD1 tetraploid embryos to generate ES cell-derived embryos as described11. Embryos were dissected at E11.5 and E13.5 and assessed for EGFP expression under fluorescence optics.

Sub-cellular fractionation
Neurons were homogenized in TNX buffer (25 mM Tris pH 7.5, 320 mM sucrose, 150 mM NaCl, 1% Triton-X100), supplemented with a protease inhibitor cocktail (Roche). Samples were centrifuged; the supernatant was collected and centrifuged again. The pellet was re-suspended in TNX buffer and centrifuged. The pellet was sonicated and its sucrose content adjusted to 45%. Samples were transferred into 2ml open top ultra clear BECKMAN centrifuge tubes and overlaid

with 35% and 5% sucrose solutions. Samples were ultra-centrifuged. Fractions were collected from top to bottom and analyzed by Western blot .

Chick DRG assay

Whole DRGs were plated as explants on coverslips without neurotrophin, or supplemented with either NGF (50 ng/ml) or LY411575 (50 nM). DRG explants were cultured for 48 h, then fixed and stained. Alternatively, DRGs were trypsinized and plated as single cells on coverslips at a density of 5,000 neurons per well of a 12-well plate. After 48 hours, cells were fixed and immunostained.

Antibodies and inhibitors

The following antibodies were used: GAD (1:1000, Chemicon), Glast (1:1000, Chemicon), active caspase 3 (1:1000, Chemicon), p75NTR (1:1000, Chemicon), vGAT (1:1000, Synaptic systems), vGLUT1 (1:3000, Synaptic systems), Tuj1 (1:1000, Sigma), Emx2 (1:500, Sigma), Pax6 (1:1000, an antibody that recognizes an epitope in first 223 amino acids, Developmental Studies Hybridoma Bank) RC2 (1:10, Developmental Studies Hybridoma Bank), Mash1 (1:200, PharMingen), GFP (1:1000, Molecular probes), and Doublecortin (1:1000, Santa Cruz). p75NTR intracellular domain (1:2000) (Gschwendtner et al., 2003), vGAT (1:1000, Synaptic Systems), and calretinin (1:500, Swant). mouse anti-BDNF#940, mouse anti-HA (1:1000, as described8), rabbit anti-p75 (1:1000, Promega), rabbit anti-p75 (1:1000, Freund I 41, raised against a bacterially expressed intracellular domain sequence), rabbit anti-Trk (C-14) (1:1000, Santa Cruz Biotechnology), mouse anti-actin (1:5000, Sigma), mouse anti-β−III tubulin (1:5000, Covance), rabbit anti-pPKCα/βII (Thr638/641) (1:500, Cell Signaling), mouse anti-flotillin-1 (1:100,BD transduction laboratories), rabbit anti-activated caspase-3 (1:1500, Chemicon). All HRP secondary antibodies were obtained from Dianova. Alexa fluorophore-conjugated secondary antibodies for immunohisto- and immunocytochemistry were obtained from Millipore. The following inhibitors were used at the indicated concentrations: K252a (100 nM, tyrosine kinase inhibitor, Calbiochem), Go6983 (7 nM, PKC inhibitor, Calbiochem), LY411575 (50 nM, γ-secretase inhibitor), lactacystin (5 μM, proteasome inhibitor, Cayman). Recombinant NGF and NT3 produced in Chinese hamster ovary cells (CHO) were a gift from Genentech, Inc. Recombinant BDNF was produced in *E. coli*. All neurotrophins were used at a final concentration of 50 ng/ml.

Statistical analyses

Statistical analyses were performed using Student's t test. Numbers represent mean values ± SEM.

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Education

1996: Graduated from Varvakeion Lyceum (Athens, Greece)
1998-2002: BSc in Biology (University of Ottawa, Canada)
2002-2003: Honors degree in Molecular Biology (University of Ottawa, Canada)
Neuro-endocrinology Laboratory of Dr. V. Trudeau
2003-2004: Research assistant in the laboratory of Dr. R. Slack
"*Role of Retinoblastoma proteins in neurogenesis"*
2004-2005: **Masters in Molecular Biology** (University of Basel, Switzerland)
Laboratory of Prof. Yves Barde
2005- 2009: **PhD in Cellular and Molecular Biology** (lab of Prof. Yves Barde)
"Using mouse embryonic stem cells to study cortical progenitor specification and mechanisms of neuronal apoptosis."
2009-2010: Postdoctoral Fellow, University of Basel, Switzerland
Laboratory of Prof. Yves Barde

2011 onwards: Post-doctoral Fellow, Institute of Molecular Biology and Biotechnology, Crete
Laboratory of Prof. Nektarios Tavernarakis on the project *"The role of Necdin in neural development and neurodegeneration"*

Funding

- EMBO long term post-doctoral fellowship
- Greek general secretariat for science (GGET) Postdoctoral Fellowship

Recent Presentations, courses and awards

2006:

- Annual Neurex Meeting, Basel, Switzerland

"Using mouse embryonic stem cells to study cortical development"
Invited speaker

- Practical course organized by EMBO, Sheffield, UK

Attended the one week course on the ***"Culture of human embryonic stem cells"***.

2007:

- Biozentrum Symposium, Basel, Switzerland

"Embryonic stem cells as a tool in neurobiology"
Invited speaker

- Stem cells, development and Regulation, Amsterdam, Netherlands.

Poster presentation

- Annual Meeting of European transcriptome, regulome and cellular commitment (EUTRACC) consortium, Antwerp, Belgium

Poster presentation

2008:

- Stem cells and regenerative medicine, Hydra, Greece

"Mouse embryonic stem cells as a discovery tool in neurobiology"
Poster presentation-1st poster award

2009:

- Annual Meeting of European transcriptome, regulome and cellular commitment (EUTRACC) consortium, Praque, Czech Republic

" Characterization of ES-cell derived neurons"
Invited speaker

2010:

- Friedrich Miescher Institute (FMI) 40th anniversary symposium

"Novel insights in neurotrophin signaling: Lesson from mouse ES cells"
Invited speaker

- Biovalley Life Sciences Meeting

"Novel insights in neurotrophin signaling: Lesson from mouse ES cells"
Poster presentation- 1st award

Publications

Vanderluit, J. L., Ferguson, K. L., **Nikoletopoulou, V**., Parker, M., Ruzhynsky, V., Alexson, T. ,McNamara, S. M., Park, D. S., Rudnicki, M., Slack, R. S. (2004).p107 regulates neural precursor cells in the mammalian brain. *J. Cell Biol.* **166**:853-863.

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** equal contribution*

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