Mouse embryonic stem cells as a new discovery tool in neurobiology

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SUMMARY

As mouse embryonic stem (ES) cells can be propagated in vitro in unlimited quantities and manipulated to generate homogenous populations of progenitor cells, they represent an attractive system to investigate questions that have been difficult to study in the developing mammalian nervous system. Here, we tested the differentiation potential of ES cell-derived progenitors that have the identity of radial glial (RG) cells, a population of cells that produce large numbers of neurons in the embryonic central nervous system of vertebrates. We implanted these cells into the neural tube of the chick embryo and examined their fate days later. The results indicate that the developmental potential of RG cells is restricted and they can only generate cell types that are expected from their developmental history.

Since ES cells are accessible for genetic manipulations, we also investigated the role of molecules involved in neuronal development in genetically-modified ES cell-derived neurons. Specifically, we tested the effects of preventing the down-regulation of the receptor for neurotrophins $p75^{NTR}$ during neuronal differentiation. This maneuver led first to the degeneration of all the neuronal processes (axons and dendrites), and to the later demise of the neuronal cell bodies. We found that the degeneration of these different neuronal compartments depends on distinct molecular mechanisms. In addition, we identified the endogenous lectin Galectin-1 as a player in the degeneration of neurons. Our results suggest that Galectin-1 acts as an effector of neuronal degeneration downstream of $p75^{NTR}$, both in vitro and in vivo. Together, the experiments presented here illustrate the advantages of working with homogenous cell populations derived from ES cells.

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Abbreviations

Aβ	amyloid β
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid
APP	amyloid precursor protein
BDNF	brain derived-neurotrophic factor
CNS	central nervous system
CRD	carbohydrate recognition domain
dLGN	dorsal lateral geniculate nucleus
DRG	dorsal root ganglia
DTT	dithiothreitol
EB	embryoid body
ES cells	embryonic stem cells
Gal-1	Galectin-1
IBO	ibotenic acid
JNK	c-Jun-N-terminal kinase
MAP2	microtubule binding protein-2
NMDA	N-methyl D-aspartate
NGF	nerve growth factor
NF-M	neurofilament-M
NT3	neurotrophin-3
NT4	neurotrophin-4
$p75^{NTR}$	neurotrophin receptor $p75^{NTR}$
PNS	peripheral nervous system
RA	retinoic acid
RG	radial glia
TNF	tumor necrosis factor
Trk	tropomyosin related kinase neurotrophin receptor
UPS	ubiquitin proteasome system
WGA	wheat germ agglutinin
Wlds	wallerian degeneration slow

Thesis outline

1. INTRODUCTION

1.1) Origin and characteristics of mouse ES cells

Following fertilization of a mouse egg, the newly formed diploid nucleus starts replicating and the egg is repeatedly cleaved, resulting in a rapidly growing cell aggregate generating two recognizable structures, the trophectoderm and the inner cell mass. The trophectoderm will become the placenta and the inner cell mass gives rise to the hypoblast and epiblast. The hypoblast in turn produces the yolk sac, which provides nutritional support to the embryo, while the epiblast gives rise to the embryo proper (Fig.1). Following gastrulation, the three primary germ layers –mesoderm, endoderm and ectoderm– are formed, from which all tissues of the body later arise. Early observations reported that embryos transplanted into adult mice readily generated teratocarcinomas, which are malignant tumors containing both differentiated and undifferentiated cells (Solter et al, 1970). Cells isolated from the undifferentiated fraction of teratocarcinomas exhibit extensive self-renewal in vitro and are also able to differentiate into cell types of all three primary germ layers. These cells are referred to as embryonic carcinoma (EC) cells, and it was established that they originate from the embryonic epiblast, since they cannot be isolated from teratocarcinomas derived from embryos that had already undergone gastrulation (for review see Smith, 2002). The differentiation of EC cells into mature cell types is however not frequent, and instead they readily form tumors. Moreover, if re-introduced into a blastocyst, EC cells can not contribute to the germ-lineage of chimeric mice and are therefore incapable of producing germ-line transmission, indicating that their developmental potential is limited. Nevertheless, these early results with EC cells suggested that cells with self-renewal and differentiation potential exist in the early embryo, and promoted the search for embryo-derived stem cells with larger developmental potentials.

An important realization was that culturing EC cells in the presence of mitotically inactivated mouse embryonic fibroblast (MEFs) improves both, their self-renewal and differentiation (Martin and Evans, 1975). One cell line isolated directly from

blastocyst-stage (pre-gastrulaiton) embryos exhibited similar self-renewal characteristics to EC cells and was referred to as embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981). Specifically, ES cells were isolated from the inner cell mass of the blastocyst by gentle dissociation, and then seeded onto MEFs. Like EC cells, ES cells divide rapidly and form colonies that can be easily distinguished by their morphology, which resembles a tightly packed potatoes-like structure. When these colonies were dissociated and re-platted onto inactive MEFs, individual clones generated ES cell colonies with the same morphology. By repeating this procedure, ES cells could be passed for unlimited times while retaining their undifferentiated state, and without signs of senescence such as telomere shortening (Smith, 2001).

When ES cells are cultured in the absence of MEFs and without added serum or growth factors, they rapidly start differentiating, suggesting that the maintenance of self-renewal is controlled by extrinsic factors. Notably, the differentiation-inhibiting activity of MEFs can be substituted by the cytokine leukemia inhibitory factor (LIF) (Smith et al, 1992; Williams, 1988). In the presence of ES cells, MEFs themselves produce LIF in substantial quantities (Rathjen et al, 1990), and MEFs deficient for the *LIF* gene cannot maintain ES cell self-renewal (Stewart et al, 1997). LIF binds to the gp130 receptor, which is expressed by mouse ES cells and is known to activate the intracellular JAK/STAT3 signaling pathway (Burdon et al, 1999). More recently, other factors in addition to LIF were shown to contribute to the maintenance of ES cell self-renewal. These include members of the BMP (Ying et al, 2003) and the Wnt families (Sato et al, 2004). While LIF signaling engages STAT3, BMP signaling engages as intracellular mediators the Id and Smad proteins, and Wnt signaling engages β-catenin and GSK-3.

What remains to be further investigated is how LIF, BMPs and Wnts, control intrinsic molecular determinants of self-renewal. The transcription factors Oct3/4 and Nanog were shown to be required for maintaining the undifferentiated state of ES cells (Shimozaki et al, 2003; Minucci et al, 1996; Niwa et al, 2002). *Oct3/4*- and *Nanog*- deficient ES cells are not capable of extensive self-renewal and spontaneously differentiate, even in the presence of LIF. It is likely then that LIF,

BMPs and Wnts, exert their effect on ES cells by controlling the expression and activity of transcription factors such as Oct3/4 and Nanog.

In addition to self-renewal under defined conditions, ES cells posses a remarkable differentiation potential. This is best demonstrated by experiments showing that when ES cells are re-introduced into mouse blastocysts, they contribute cells to tissues of all three primary germ layers, including the germ cell lineage of chimeric animals. More remarkably, the cells contributed to the germ cell lineage are often functional and can generate germ line transmission (Bradley et al, 1984). These attributes of ES cells, in combination with the ease of their genetic manipulation, have made possible the engineering of genetically modified mice carrying for example, deletions of specific genes (Capecchi, 1989). Based on these studies, ES cells are defined as pluripotent stem cells, as they can contribute to all cell types of the body.

While there is no question that ES cells are remarkably useful, it remains open if these cells really exist in vivo, or if they are an in vitro artifact made possible by the use of special culture conditions. Since ES cells are normally isolated from the epiblast, and this is a transient embryonic structure, one would expect that ES cells do not self-renew for too long, but instead differentiate into the cells of the three primary germ layers. One possibility is that epiblast cells have evolved mechanisms to delay their differentiation if development is for any reason halted. If this were the case, then the cells would self-renew without differentiating until development re-starts. Experimental evidence supporting this idea exists. The *LIF* and *gp130* genes are expressed in pre-gastrula embryos, and while removal of either gene does not cause overt phenotypes before gastrulation, it severely affects the maintenance of the epiblast during diapause (Nichols et al, 2001). The term diapause refers to a period during which the normal implantation of a blastocyst is delayed by 3-4 weeks, and during which epiblast cells are prevented from differentiating. Furthermore, isolation of ES cells from diapause-stage blastocysts is more efficient than from normal blastocysts (Gardner and Brook, 1997). Therefore, in vitro self-renewing ES cells may resemble epiblast cells in diapause blastocysts in vivo, and the use of differentiation-inhibiting factors such

as LIF may thus represent a natural mechanism for the maintenance of their undifferentiated state.

1.2) ES cells versus adult stem cells

Most tissues posses a certain number of stem cells, which are responsible for producing all mature cell types of the tissue where they reside. These cells are thus referred to as tissue stem cells or somatic stem cells. For example, hematopoietic stem cells continue to replenish all types of blood cells, including red blood cells, lymphocytes and macrophages. Mesenchymal stem cells produce cells of the bone, cartilage and muscle. Epidermal stem cells generate keratinocytes of the skin, and epithelial stem cells contribute different cell types in the lining of digestive tracts. In addition, somatic stem cells can be recruited by injury and they play an important role on tissue repair (for review see Rando, 2006). Stem cells have also been described in the adult nervous system. About 40 years ago it was observed that the adult mammalian brain exhibited active cell division in restricted regions (Altman, 1965). It was later shown that two regions contained neural stem cells, the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus of the hippocampus. SVZ stem cells generate neuroblasts that migrate towards the olfactory bulb to become inhibitory neurons. Neural stem cells in the dentate gyrus of the hippocampus generate granule cells that integrate within the local environment (for review see Gage, 2002).

While the extent of neurogenesis in the adult brain of rodents is somehow limited and its function is still under heavy investigation, work with songbirds demonstrated a remarkable physiological relevance. In some of these species, the capacity to learn new songs varies during adult life, and this correlates with the addition of new neurons in specific regions (Goldman and Nottebohm, 1983; Nottebohm, 1981). Interestingly, newly-generated neurons are short-lived, being used for learning of single songs and then discarded.

Early work by Reynolds and Weiss (1992) set out to isolate stem cells from the neurorogenic regions of the rodent brain. When the SVZ or dentate gyrus are dissociated and the cells are seeded without substrate, they form floating

aggregates that are reffered to as neurospheres (Reynolds and Weiss, 1992). These aggregates grow when the growth factors bFGF and EFG are added. If dissociated and re-seeded at clonal density under the same conditions, some of the cells can give rise to new neurospheres. When mitogens are removed, some of the cells within neurospheres differentiate and can produce astrocytes, oligodendrocytes and neurons (Reynolds and Weiss, 1992). Therefore, neurospheres are typically said to contain neural stem cells.

Given the interesting prospect of using neural stem cells for tissue repair, studies tested the differentiation potential of neurosphere-derived cells after transplantation in vivo. There, cells are confronted with signals pushing them into adopting different phenotypes. Interestingly, some of these studies suggested that neural stem cells posses a rather broad differentiation potential and can generate not only cells from the tissue where they reside, but also cells from other regions (for review see Frisen, 2002). For example, adult hippocampal stem cells transplanted into the hippocampus generated interneurons as expected, but when transplanted into other brain regions they could generate olfactory bulb neurons, retinal neurons and cerebellar cells (Suhonen et al, 1996; Takahashi et al, 1998). An even greater differentiation potential was ascribed to neural stem cells by studies claiming that these cells crossed lineage barriers to generate non-neural cell types. Two reports indicated that transplanted neural stem cells could differentiate into muscle cells (Galli et al, 2000) and into blood cells (Bjornson et al, 1999). Furthermore, Clarke et al (2000) suggested that neural stem cells transplanted into young embryos could also differentiate into cells of all primary germ layers. These studies however, need to be taken with caution for several reasons. First, the ex vivo expansion with growth factors prior to transplantation can affect the differentiation potential of cells in ways that they would not necessarily correspond to what stem cells do in vivo (for review see Anderson, 2001). For instance, when cultured with growth factors, oligodendrocytes can be “re-programmed” and generate neurons (Kondo et al, 2000). Furthermore, neurospheres typically contain a very heterogenous population of cells (Suslov et al, 2002), and the identity of the SVZ and

hippocampal stem cells has not been fully characterized yet. In addition, studies found that stem cells can also fuse with other cells (Ying et al, 2002), suggesting that some of the previous studies claiming that stem cells could cross lineage barriers might have been the result of stem cell fusion with the host cells (for review see Wurmser and Gage, 2002). Therefore, it remains difficult at present to precisely define the developmental potential of adult neural stem cells. In contrast to neural stem cells, ES cells do not seem to change their properties after long term expansion in vitro. In addition, the pluripotency of ES cells is hardly disputed, as the existence of ES cell-derived mice demonstrates their potential to produce all cell types of the body.

1.3) In vitro differentiation of ES cells into the neural lineage

An important aspect of ES cell biology is that upon removal of LIF, ES cells rapidly loose their undifferentiated state and start differentiating. Since ES cells are pluripotent when re-introduced in vivo, the possibility exists to direct ES cell differentiation into defined cell types in vitro by culture with a variety of pro-differentiation factors. Protocols were thus developed to obtain different cell types from ES cells, including for example cells of the skin, gut, immune system, bone, connective tissue and muscle (for reviews see Guan et al, 2001; Wobus and Boheler, 1999). Interestingly, when cultured without LIF and at very low density, some ES cells spontaneously differentiate into neurons (Ying et al, 2003). Because only low levels of neural differentiation are found during spontaneous differentiation of ES cells (Strübing et al, 1995; Schmidt et al, 2001), procedures to enhance their neurogenic potential were developed. Importantly, the ability of ES cells to differentiate into different cell types is enhanced when cultured without adhesive substrate. There, ES cells rapidly form cell aggregates referred to as embryoid bodies (EBs). EBs can be produced by ES cells even at clonal density and after 2-6 days of culture in the presence of LIF, they consist of thousands of undifferentiated ES cells.

Among the extracellular signals known to direct the differentiation of both EC cells and ES cells towards neurogenesis, retinoic acid (RA) has been particularly

successful (Rohwedel et al, 1999; Bain et al., 1995; Renoncourt et al, 1998; Li et al, 1998; Aubert et al, 2002). Retinoic acid is a low molecular weight (300 Da) lipophilic molecule and the biologically active metabolite of Vitamin A. Retinoic acid binds to cytosolic RA receptors, which then dimerize and activate RA-responsive genes in the nucleus. Studies reported that the addition of RA to ES cell aggregates directs their differentiation into the neural lineage, and when dissociated, some of the cells produce neurons (Bain et al., 1995; Okabe et al., 1996; Renoncourt et al, 1998; Li et al, 1998; Wichterle et al, 2002). However, most of these protocols lead to rather heterogeneous cell populations. For example, RT-PCR performed on RA-treated ES cells revealed the presence of transcripts for *Trk* receptors, *Glutamic acid decarboxylase*, *Tyrosine hydroxylase* and *Hb9*, which are genes expressed by different classes of mature neurons in the nervous system (Renoncourt et al, 1998). Immunostaining of ES cell aggregates showed cells expressing markers typical of neural progenitors such as Nestin, Pax6 and Pax7, and also of pan-neuronal markers like MAP2 and NF-145 (Renoncourt et al, 1998). More recently, Wichterle et al (2002) showed that motoneurons can be produced by treatment of ES cell aggregates with RA and sonic hedgehog, a molecule known to promote motoneuron differentiation in vivo (Roelink et al, 1995). This study was important as it showed that the difrentiation of ES cells can be directed into specific neuronal types. However, motoneurons constituted a small fraction of the cells within the aggregates, and FACS sorting was used to purify motoneurons from the rest of the cells (Wichterle et al, 2002). Other studies also employed methods for purifying the desired cells. One approach is the use ES cell lines carrying resistance cDNAs under the control of lineage-specific promoters, which are cultured with selection drugs (Klug et al, 1996; Li et al, 1998). Another alternative is the use of ES cells constitutively expressing transcription factors known to induce differentiation into specific neuronal types. For example, over-expression of the transcription factor Nurr1 in ES cells can enhance their differentiation into dopaminergic neurons (Kim et al, 2002). However, it remains unclear whether homogenous populations of progenitor cells and of neurons can be obtained using ES cell aggregates. This is

of importance since at present, obtaining large quantities of neurons by direct isolation from animals remains difficult. Moreover, the purity and homogeneity of the primary neuronal cultures typically established are not consistently satisfactory. Contamination with large numbers of non-neuronal cells and/or with different types of neurons troubles the analysis of results obtained for example, from gene or protein expression comparisons.

Recently, our laboratory established a new ES cell-based method leading to unlimited numbers of highly pure and homogenous neuronal cultures (Bibel et al, 2004). Briefly, ES cells are expanded first with and then without MEFs in the presence of LIF. Importantly, we now understand that special care has to be taken to select ES cells cultures where all ES cells exhibit an undifferentiated morphology, typically evidenced by a high nucleus/cytoplasm area ratio as well as by very salient nucleoli. In addition, only ES cell cultures that divide rapidly are used, as a high rate of cell division is essential for the subsequent differentiation into neural progenitors. ES cell aggregates are then formed and cultured for 8 days without LIF. In these aggregates, ES cells continue proliferating and the aggregates grow in size until the fourth day, when proliferation is balanced by extensive cell death. The aggregates are then exposed to RA for the last four days, and when gently dissociated and plated on poliornithine/laminin, the large majority of the cells develop into post-mitotic neurons within ~36 hours. Notably, the neurons have the antigenic profile of glutamatergic neurons isolated from the developing cerebral cortex. They express the glutamate transporter vGLUT-1, the receptors for neurotrophins TrkB and $p75^{NTR}$ and the amyloid precursor protein (APP). In addition, their electrophysiological characteristics are similar to those of glutamatergic cortical neurons. Interestingly, after plating the cells express the markers Pax6, BLBP and RC2. These markers are normally expressed during development by a population of cells in the neural tube called radial glial (RG) cells.

1.4) RG cells as neural progenitors

In vertebrates, RG cells are the first cell type that can be distinguished from neuroepithelial cells throughout the neural tube. They share some characteristics with neuroepithelial cells and also with mature astrocytes. Like neuroepithelial cells, they express the intermediate fillemant Nestin, the marker RC2 (likely to be a modified form of Nestin), and they have a bipolar morphology. Like astrocytes, they have glycogen granules and they express the glutamate transporter GLAST, the BLBP protein, and the intermediate filament GFAP (only in primates and adult rodents) (for review see Kriegstein and Gotz, 2003). Some RG cells also express the transcription factors Sox2 and Pax6 (see Discussion). It has traditionally been considered that the role of RG cells is to guide the migration of newly born neurons along their radial processes and to differentiate into astrocytes later in development (Rakic, 2003). However, recent work demonstrated that RG cells also generate neurons (Malatesta et al, 2000). Indeed, RG cells produce most of the pyramidal neurons in the developing dorsal telencephalon as well as many neurons in the spinal cord (Malatesta et al., 2003). Furthermore, there is increasing evidence suggesting that the cells that act as neural stem cells in the adult brain posses the characteristics of RG cells. Studies by Doescht et al (1999, 2003) first showed that cells belonging to the astroglial lineage acted as neural stem cells in the SVZ and the dentate gyrus (REF and see discussion). Cells in these regions express GFAP, and cell fate mapping experiments recently confirmed that these GFAP-expressing cells are the source of adult neurogenesis (Garcia et al, 2004). Moreover, the genetic ablation of GFAP-expressing cells dramatically decreased adult neurogenesis (Garcia et al, 2004). The same conclusion was also reached by another study using a virus-mediated cell fate mapping approach (Merkle et al, 2004). Interestingly, in several non-mammalian vertebrates RG cells are retained throughout life in their CNS. These animals are capable of extensively regenerating their adult CNS after lesion (for review see Doetsch, 2003), highlighting the potential use of RG cells for cell replacement therapies. Together, these studies show that RG cells play a major role as progenitors for neurons, both in the embryo as well as in the adult CNS. Therefore, it would be

of interest to test how broad the developmental potential of RG cells is, by for example, introducing these cells into an in vivo environment. Direct isolation of RG cells has been reported using transgenic mice in which the human *GFAP* promoter drives expression of *GFP* in RG cells, allowing these cells to be FACS-sorted (Malatesta et al, 2000). However, it remains difficult to obtain large numbers of homogenous RG cells using this approach. Given the generation of uniform RG cells from ES cells (Bibel et al, 2004), we thus set out to compare our ES cell-derived progenitors to in vivo-existing RG cells, and to test their developmental potential by implanting them into the nervous system of chick embryos. The results of these experiments are presented in section 2.1 and discussed in section 3.1 of this Thesis.

1.5) The neurotrophin receptor $p75^{NTR}$

In the second part of this Thesis (sections 2.2 and 3.2), we illustrate another use of ES cells to study mechanisms related to neural development. As in vivo over-expression of the neurotrohin receptor $p75^{NTR}$ is known to cause neuronal degeneration in the developing nervous system, we engineered ES cells to prevent the down-regulation of this receptor during neuronal differentiation. $p75^{NTR}$ is a single pass transmembrane glycoprotein that belongs to the TNF receptor superfamily (for review see Dechant and Barde, 2002). It has a death domain in its intracellular region (conserved among TNF receptor superfamily members), and four consecutive extracellular cystein-reach domains. $p75^{NTR}$ is expressed mostly in the developing nervous system by a large number of neurons, especially those extending long axons. In the adult nervous system, $p75^{NTR}$ expression is down-regulated in almost all neurons, with the exception of for example cholinergic neurons of the basal forebrain. In addition, a large number of neurons express $p75^{NTR}$ in primary cultures, including sensory, sympathetic, hippocampal and cortical neurons. Like is the case in vivo, these cells also down-regulate $p75^{NTR}$ expression as they mature in vitro. $p75^{NTR}$ binds to all the mammalian neurotrophins (NGF, BDNF, NT3 and NT4), and ligand binding leads to different outcomes, depending on the cellular context. Since

$p75^{NTR}$ lacks an intrinsic catalytic activity, it activates signaling by recruiting different intracellular interactors. It is generally thought that signaling via $p75^{NTR}$ leads to negative cell behaviours including cell death and inhibition of axonal elongation. For example, NGF acting via $p75^{NTR}$ induces programmed cell death in olygodendrocytes and in retinal ganglion cells (Frade and Barde, 1996; Cassacia-Bonnefil et al, 1996). *$p75^{NTR}$* null mice exhibit decreased apoptosis in the developing retina, spinal cord and sympathetic system (Frade and Barde, 1999; Bamji et al, 1998), and increased numbers cholinergic neurons in their adult basal forebrain (Naumann et al, 2002). In transgenic mice, over-expression of the intracellular domain of $p75^{NTR}$ using a neuronal-specific tubulin promoter leads to widespread decrease in neuronal numbers throughout the nervous system (Majdan et al, 1997).

$p75^{NTR}$ also acts as a negative regulator of axonal elongation by activating the small GTPase RhoA, and defects in the growth of spinal nerves (Yamashita et al, 1999) and in spine numbers (Zagrebleski et al, 2005), were reported in *$p75^{NTR}$* mutants. Also in cultured retinal ganglion neurons, $p75^{NTR}$-activation of RhoA restricts the elongation of fillopodia (Gehler et al, 2004). In addition to Trk receptors, $p75^{NTR}$ also interacts with the Nogo receptor and LINGO (for review see Gentry et al, 2004), and together these proteins mediate the inhibitory effects of various myelin-associated proteins.

ES cell-derived neurons also express $p75^{NTR}$ in our system (Bibel et al, 2004). Like is the case for neurons in vivo, the expression of $p75^{NTR}$ is down-regulated in ES cell-derived neurons in the first days after plating of the progenitors (Bibel et al, 2004). Here, we engineered ES cells to express a cDNA for *$p75^{NTR}$* from an endogenous neuronal-specific promoter. We differentiated these cells in vitro and found that this can be used as a new model system to study neuronal degeneration.

2. RESULTS

2.1) IN VIVO IMPLANTATION OF ES-CELL DERIVED PROGENITORS

2.1.1) Antigenic characterization of RA-treated ES cells

The goal of this set of experiments was to test the differentiation potential of homogenous populations of ES cell-derived neural progenitors. We used mouse ES cells pre-differentiated in vitro as described in Bibel et al (2004) and implanted them into the neural tube of chick embryos. Briefly, ES cells were cultured in non-adhesive dishes where they formed aggregates. After four days of treatment with RA we fixed and cryo-sectioned aggregates for immunostaining. The majority of the cells within the aggregates expressed several RG markers. These included the intermediate filament Nestin, the transcription factor Sox2, the RG marker RC2, the glutamate cell membrane transporter GLAST, and the homeodomain transcription factor Pax6 (Fig.2). Cells expressing these markers were evenly distributed throughout the aggregates. RA-treated aggregates did not contain cells expressing the markers Pax7 or Nkx2.2, which in the neural tube define progenitors located at more dorsal and ventral positions, respectively, of Pax6-positive cells (Ericson et al, 1997). Less than 1% of the cells in RA-treated aggregates expressed Lim1/2, Hb9 (recognized by the anti-MNR2 chick antibody), Isl1 or Olig2, which are markers normally expressed by differentiating neuronal precursors in the spinal cord and in the DRG. Also, no cells were positive for the sensory marker Brn3a (data not shown). The majority of the cells within RA-treated aggregates were found to still divide rapidly after 4 days, as assessed by a 3 hour pulse of BrdU prior to fixation (Fig. 2). ES cells in aggregates that were not treated with RA failed to express any of the markers mentioned in the above (data not shown). Instead, they expressed the transcription factor Oct3/4, indicating their pluripotent and undifferentiated state (Fig.6A). Taken together, these observations indicate that RA-treated ES cell aggregates contain a uniform population of progenitors with

the antigenic characteristics of RG cells found in developing neural tube (for reviewed see Kriegstein and Gotz, 2003).

2.1.2) Differentiation of RA-treated ES cell aggregates in the chick neural tube

To evaluate the differentiation potential of RA-treated ES cells, we partially trypsinised single aggregates and implanted them into the caudal CNS of stage 13-14 (E2) chick embryos in ovo. At these developmental stages the chick neural tube has just closed, neural crest cells started to migrate towards the periphery and the presomitic mesoderm adjacent to the neural tube differentiated into somites.

To follow their neuronal differentiation in ovo, we used for all the experiments a line of ES cells that had both *tau* alleles targeted with *GFP* (Bibel et al, 2004). Work from our laboratory has previously shown that the endogenous *tau* promoter drives expression of GFP soon, and long after cells become post-mitotic neurons (Tucker et al, 2001). In order to accommodate foreign cells, one neural fold was completely removed for 4 somites in length at level of the forelimb bud (somites 17 to 20) prior to transplantation (Fig.3A). The implanted cells occupied the slot made by the surgery. The eggs where then sealed and incubated for a further 6 days until E8, which is the time when all neuronal types have differentiated in the chick spinal cord. Embryos surviving the operation (Table 1) were removed from the egg and observed under a fluorescent microscope. A GFP signal found at the implant region indicated that the donor cells survived and differentiated into neurons in the chick host (see Table 1). Serial transverse sections were then analyzed and stained with the nuclear stain Hoechst, allowing mouse and chick cells to be unambiguously identified on the basis of their distinct nuclear morphologies (Fontaine-Perus et al., 1997 and Fig. 3B). Mouse neurons expressing GFP were detected in all consecutive sections of the implant area. Notably, the operated side of the spinal cord was extensively populated by mouse neurons and it closely resembled the adjacent non-operated side in size and morphology (Fig.3). Also, a bundle of GFP+ axons were often

observed projecting from the ventral spinal cord towards the periphery, resembling a ventral root (Fig. 3 and Table 1). Cell counts revealed that the majority of the nuclei exhibiting mouse morphology co-localized to GFP-expressing cells (see Table 2), indicating that the majority of the donor cells differentiated into post-mitotic neurons. Together, these observations show that donor cells survive in very large numbers for prolonged periods of time, and that they differentiate mostly into neurons in the chick spinal cord.

In the spinal cord, specific types of interneurons and motoneurons are generated from a pool of progenitor cells expressing markers that we also find in our RA-treated ES cells (Ericson et al., 1997; Graham et al., 2003; and also see scheme in Fig.4). Endogenous progenitor cells co-expressing Nestin, RC2, Sox2 and Pax6 are distributed in the central region of the neural tube that can be delineated by two borders, a Pax7+/Pax6- region lying dorsal to it, and a Nkx2.2+/Pax6- region lying ventral to it. Pax6+ progenitors generate interneurons that are located throughout the entire dorsal-ventral part of the central region of the spinal cord (Ericson et al, 1997). Some of these inhibitory neurons elongate axons ventrally that input into ventrally-located motoneurons to modulate motor activity, while others reach the ventral part of the spinal cord, cross the midline, and then project anteriorly through the white matter and towards higher central regions. Both of these interneuron subtypes can be recognized by the expression of the homeodomain transcription factor Lim1/2 (Ericson et al, 1997). Pax6+ progenitors also generate spinal cord motoneurons. These neurons have a larger cell body size than interneurons and are located ventrally with the exception of those occupying the column of Terni, that are located in the central region. Motoneurons express the transcription factors Hb9 and Isl1 and project their axons from the ventral spinal cord through the ventral roots into the periphery, where they bundle with sensory DRG axons to form spinal nerves (Wichterle et al, 2003).

We next asked whether the implanted progenitors had acquired these cell fates in vivo. GFP-expressing neurons were found located throughout the central

region of the spinal cord expressing the interneuron marker Lim1/2 and projecting axons ventrally (Fig.4A and Table 3). Ventrally-located GFP+ neurons expressed the motoneuron markers Hb9 and Isl1 (Fig. 4B,C), and extended long GFP+ axons from the ventral spinal cord towards the periphery (Fig. 4B). These cells also had larger cell body sizes than the GFP+/Lim1/2+ neurons (data not shown). Importantly, GFP+/Lim1/2+ cells located dorsal to the motoneuron domain of the spinal cord did not express Hb9 (Fig. 4J-K').

We then examined the expression of neurotrophins receptors by donor cells. In the chick embryo, expression of $p75^{NTR}$ becomes detectable in the ventral spinal cord at E2 (McKay et al., 1996), and is still detectable in motoneurons when they extend axons (Yamamoto and Henderson, 1999). By contrast, expression of TrkB becomes detectable in the spinal cord throughout the gray matter and also in motoneurons and their axons at E8 (McKay et al., 1996). Both because of the functional role of neurotrophin receptors and their characteristic expression pattern, we explored whether donor neurons would express neurotrophin receptors. We found that donor neurons in the spinal cord expressed both $p75^{NTR}$ and Trk receptors (Fig.4D-I). Expression of $p75^{NTR}$ was detected in GFP+ neurons throughout the spinal cord and high levels of expression were detected in cells located to the ventral motorneuron domain, which extended GFP+/ $p75^{NTR}$+ axons towards the periphery via the ventral root, in accord with their motoneuron identity. In constrast to $p75^{NTR}$, Trk receptor expression was widely detected in GFP+ neurons located throughout the dorsal-ventral axis of the spinal cord as well as in GFP+ axons projecting towards the periphery. Together, these results indicate that RA-treated ES cell aggregates can generate interneurons and motoneurons in a time- and position-dependent fashion in the host spinal cord.

2.1.3) RA-treated ES cell aggregates populate dorsal root ganglia, but fail to express sensory neuron markers and to elongate axons

In the chick embryo, neural crest cells delaminate from the dorsal neural tube and start migrating to the periphery to form the PNS at E2 (Le Douarin and

Kalcheim, 1999). Neural crest cells are a highly multipotent, transient cell population. These cells generate not only the entire PNS, but they also contribute non-neural cells to a remarkably large number of tissues (Le Douarin and Kalcheim, 1999). The dorsal root ganglia (DRGs) are composed of neural crest-derived sensory neurons that extend a bi-polar axon projecting both centrally through the dorsal horn of the spinal cord, as well as peripherally to innervate targets such as the muscle spindles. Since our progenitors were implanted at developmental stages when neural crest cells are migrating towards the DRG anlagen and starting to differentiate into sensory neurons, we examined if donor cells could also colonize the PNS. GFP-positive cells were frequently found in the host DRG (Fig.5A and Tables 1,2). However, unlike their chick counterparts in the DRG, mouse neurons never expressed the transcription factors Brn3a (Fig. 5B) or Isl1 (Fig. 5C), which are markers that define most neurons in that structure (Anderson, 1999 and references therein). In addition, we never found mouse neurons elongating axons outside the DRG, even though GFP expression indicated their neuronal identity. These cells expressed $p75^{NTR}$ at high levels (Fig. 5D), but they failed to express detectable levels of Trk receptors (Fig. 5E). It thus appeared that while the donor cells can differentiate into neurons in the host DRG, they are incapable of adopting a more mature DRG neuron phenotype and to elongate axons.

2.1.4) RA-untreated ES cell aggregates generate both spinal cord and DRG neurons in vivo

To test the pluripotent character of mouse ES cells in the chick embryo, we next implanted ES cells that were allowed to form aggregates for 36 hours in the presence of LIF and without RA, and subsequently implanted in the chick neural tube following the same procedure as described for RA-treated ES cells (Fig.3A). Prior to implantation, ES cells expressed the transcription factor Oct3/4 (Fig. 6A). Expression of this transcription factor is a reliable indication of the undifferentiated and pluripotent character of ES cells (Niwa et al, 2000; Boiani et al, 2002), and its expression is down-regulated when ES cells are deprived of LIF

(Fig.6A). RA-untreated ES cell aggregates completely failed to express Nestin, Sox2, Pax6, Pax7, RC2, GLAST, Lim1/2, Hb9, Isl1, Nkx2.2, Olig2, and Brn3a (data not shown). Like RA-treated ES cells, RA-untreated ES cells also survived in large numbers and differentiated into neurons in the host spinal cord (see Tables 1, 2). Mouse GFP-positive neurons located throughout the spinal cord expressed Lim1/2 (Fig.6B), suggesting their differentiation into interneurons. Ventrally located mouse neurons expressed Hb9 and Isl1 (Fig.6C,D) and elongated long axons towards the periphery, suggesting that they differentiated into motoneurons. These cells also expressed Trk and $p75^{NTR}$ neurotrophin receptors in their cell bodies and in their axons (Fig.6E,F). Like the progeny of RA-treated cells in the spinal cord, donor cells located dorsal to the motoneuron domain did not express Hb9 (data not shown).

Numerous GFP-positive neurons were also found in the DRG (Fig. 7A). Notably, these cells expressed Brn3a (Fig. 7C) as well as Isl1 (Fig. 7D), and they also elongated axons both towards the spinal cord and towards the periphery (Fig. 7A,B,H; see Table 3 for a quantitative comparison with RA-treated cells). ES cell-derived neurons expressed $p75^{NTR}$ (Fig. 7E), but in contrast to RA-treated cells in the DRG, they also expressed high levels of Trk receptors in their cell bodies and in their axons (Fig. 7F-H). Neither ES cells nor RA-treated cells ever expressed Hb9 (Fig. 7I) or Lim1/2 (Fig. 7J) when colonizing the DRG.Taken together, these results indicate that the failure of our ES cell-derived Pax6-positive progenitors to be fully responsive to chick derived development, is not due to a limitation of our implantation system.

2.2) AN ES-CELL BASED MODEL OF NEURONAL DEGENERATION

2.2.1) Engineering of *tau::p75^{NTR}* neurons

Since our results showed that cells that failed to extend axons in the DRG expressed high levels of $p75^{NTR}$, we were next interested to study the effects of this receptor on neuronal development using in vitro differentiation of ES cells. In

the ES cell-derived neurons obtained using our differentiation protocol, $p75^{NTR}$ is initially expressed at high levels (Bibel et al, 2004). But like is the case for many neurons in vivo, $p75^{NTR}$ is down-regulated as a function of neuronal maturation. To better understand the function of $p75^{NTR}$ in developing neuros, we designed a strategy to prevent its down-regulation as ES cell-derived neurons develop. A cDNA encoding rat $p75^{NTR}$ was targeted into the endogenous *tau* locus. These ES cells had their other *tau* allele previously targeted with *GFP*, as described in Bibel et al (2004). The *tau* gene is activated in all ES cells as soon as they become post-mitotic neurons, and GFP fluorescence starts to be visible ~24 hours after platting (data not shown). The increase in the number of GFP+ cells is accompanied by a reduction in the number of cycling cells within the first two days after platting. Cumulative BrdU experiments in which BrdU was applied to cultures at different times after platting, and its incorporation into cycling cells assessed at day 4, revealed that 48.3% incorporate BrdU applied at day 2, 31.2% at day 3 and 11.9 % at day 4 (data not shown). We next compared the differentiation of this ES cell line, hereafter referred to as *tau::p75*^{NTR}, with a control line that had both *tau* alleles targeted with GFP (Bibel et al, 2004).

2.2.2) Neuronal processes degenerate before cell bodies in *tau::p75*^{NTR} neurons

Both *tau::p75*^{NTR} and control neurons initially extended processes (Fig.8A), but on the third day after differentiation the processes of *tau::p75*^{NTR} neurons began to appear unhealthy as evidenced by the appearance of irregular swellings (Fig.8A,D), while the cell bodies remained indistinguishable from those in control cultures. By day 4, virtually all processes degenerated and became fragmented, while ever more branches developed in control cultures (Fig.8A,C). This morphological appearance is reminiscent of what has been observed with DRG neurons after axotomy or trophic factor deprivation of sympathetic neurons (Zhai et al, 2003 and see Discussion). The earliest signs of degeneration were consistently detected in the more distal secondary branches which were always eliminated first. This was followed by the degeneration of principal processes

(those growing directly from the cell body; Fig.8D). By day 5, virtually all neuronal processes in the culture had completely degenerated and at this time point, the cell bodies began to exhibit typical features of apoptotic death. They developed swellings at the cell surface that were visible by phase contrast, and their nucleus started to acquire a pyknotic morphology (Fig.8D).

We next examined the expression levels of $p75^{NTR}$ in our system. Western blots performed at day 2 revealed that *tau::p75^{NTR}* cells express about 50% higher levels of $p75^{NTR}$ than control cultures (Fig.9A).
To examine the localization of $p75^{NTR}$, we stained non-permeabilised neurons with an antibody directed against the extracellular domain of $p75^{NTR}$. We found that in both lines $p75^{NTR}$ was localized at the surface of cell bodies as well as along the processes, including the growth cones. Its distribution was not homogenous, but always appeared as clusters at the cell surface (Fig.9B). This pattern was confirmed by staining *tau::p75^{NTR}* neurons with the monoclonal antibody MC192, which is directed against the extracellular domain of rat $p75^{NTR}$ (data not shown). No positive staining was observed with neither of these antibodies in neurons carrying a full deletion of *p75^{NTR}* (data not shown). We further tested whether the clustering of $p75^{NTR}$ was specific for this protein by comparing it with staining for other proteins known to be associated with the cell surface of neurons. We chose the transferin receptor and the glutamate receptor interacting protein-1(GRIP1), which in early developing neurons is not present in clusters (Dong et al, 1999). Similarly to $p75^{NTR}$, the transferin receptor also appeared in clusters, but unlike the $p75^{NTR}$ clusters, these were more homogenously distributed along the processes (Fig.9C). GRIP-1 exhibited a very homogenous pattern and no clustering (Fig.9C). Interestingly, $p75^{NTR}$ clusters accumulated preferentially at sites where processes branch (Fig.9D).

2.2.3) Effects of neurotrophins and acute over-expression of *p75^{NTR}* deletion constructs

We next tested whether the degeneration of *tau::p75^{NTR}* neurons caused by the prevention of down-regulation of p75^{NTR}, could be affected by treatment with neurotrophins. Treatment with either NGF or BDNF at d2, neither accelerated nor delayed the degeneration of processes (Fig.10A), suggesting that their degeneration is independent of neurotrophin ligand binding.

To understand which of the domains of p75^{NTR} are necessary for causing degeneration, we performed acute transfection experiments with several rat *p75^{NTR}* constructs carrying deletions in different domains. We transfected wild type ES cells at day 3 with four constructs: full length *p75^{NTR}* (*p75^{NTR}-FL*), *p75^{NTR}* lacking the entire extracellular domain (p75$^{NTR}\Delta$ECD), p75^{NTR} lacking the intracellular death domain (*p75$^{NTR}\Delta DD$*), and *p75^{NTR}* lacking the entire intracellular domain (*p75$^{NTR}\Delta ICD$*). To visualize neurons that have been transfected with the p75^{NTR} constructs, we co-transfected with a plasmid encoding for GFP. We used an 8 fold lower concentration of the GFP plasmid and assumed that GFP-expressing neurons would also have incorporated the p75^{NTR} plasmids. We also checked this by staining with the MC192 p75^{NTR} antibody that recognizes rat p75^{NTR}, but not mouse p75^{NTR}. In all cells analyzed (n=12/12), expression of MC192 immunoreactivity co-localized with GFP expression (Fig.10C).

Unexpectedly, transfection with of all *p75^{NTR}* constructs caused the degeneration of processes (Fig.10A). As p75^{NTR} is know to activate signaling by recruiting intracellular interactors through its intracellular domain, these results suggest that the transfected mutant receptors might interact with endogenously-expressed p75^{NTR} in wild type cells. To test this hypothesis, we repeated these experiments using *p75^{NTR}*-/- neurons. While transfection with *p75$^{NTR}\Delta ECD$* could still cause the degeneration of processes in a *p75^{NTR}* null background, transfection with *p75$^{NTR}\Delta ICD$* was no longer able to caused degeneration (Fig.10D). Interestingly, deletion of the death domain in p75^{NTR} also cause degeneration (Fig.10D). This suggests that the killing activity of p75^{NTR} resides within its juxtamembrane intracellular domain and is consistent with a previous study showing that this domain, termed “Chopper”, is necessary for inducing cell death in other cellular

systems (Coulson et al, 2000). It should be noted also that in all cases where *p75^{NTR}* was acutely over-expressed, the degeneration of neurons occurred more rapidly than in *tau::p75^{NTR}* neurons, and the processes and cell bodies degenerated at the same time. These results further suggest that the acute over-expression of p75^{NTR} in this system is ligand-independent.

2.2.4) Involvement of caspase actvivity in p75^{NTR}-mediated neuronal degeneration

We next investigated the mechanism by which p75^{NTR} causes the degeneration of processes. First, we asked whether some of the same machinery typically involved in programmed cell death played a role, by looking at the distribution of caspase activity. We used an antibody that recognizes the active form of caspase-3, a central effector caspase in the apoptotic cascade. Strong immunoreactivity was observed in *tau::p75^{NTR}* neurons starting by day 4. It was localized to the cell body, but was never present in their processes (Fig.11A). By day 5, when all processes have completely degenerated, caspase-3 activity remained confined to the cell body, and it co-localized with the nuclei that, at this time point, showed signs of pyknosis (Fig.11A). Caspase-3 activity was never detected in control neurons (data not shown). To interfere with caspase activity, we treated *tau::p75^{NTR}* neurons starting at day 2 with the inhibitor fmk-Z-VAD, which blocks the activity of all caspases by preventing their activation by self-cleavage. The treatment prevented the death of cell bodies in *tau::p75^{NTR}* neurons, as assessed by quantifying cells exhibiting pyknotic nuclear morphologies (Fig.11B). However, fmk-Z-VAD did not prevent the degeneration of processes (Fig.11C), thus suggesting that the degeneration of processes does not require the activity of caspases.

2.2.5) Involvement of JNK in process degeneration

We next investigated the role of the c-Jun N-terminal kinase (JNK) in our system. JNKs are also referred to as SAPKs, for stress activated protein kinases. These enzymes are typically activated by cellular stress (for review see Davies, 2000).

JNK can phosphorylate several AP1 transcription factors such as for example c-Jun, as well as members of the Bcl2 family. Importantly, when acutely over-expressed in different cell types, $p75^{NTR}$ was shown to induce JNK activity (Bhakar et al, 2003). We first examined the distribution of JNK in *tau::p75^{NTR}* neurons at day 2 by immunostaining. While JNK was present both in processes and in the cell body, phosphorylated JNK was primarily located in processes (Fig.12A). Also in control neurons at day 2, activated JNK was localized to the processes exclusively (data not shown). Western blot analysis performed at day 2 revealed increased levels of phosphorylated JNK in *tau::p75^{NTR}* lysates compared with control lysates (Fig.12B).

As these observations suggest that JNK may play a role in processes at these time points, we used CEP-1347, an inhibitor of JNK signaling that acts by blocking the activity of Mixed Lineage Kinases upstream of JNK (Roux et al, 2002). Treatment of *tau::p75^{NTR}* neurons at day 2 with CEP-1347 prevented the degeneration of processes in a concentration dependent manner (Fig.12C,D).

Because JNK was previously shown to phosphorylate the pro-apoptotic protein BAD (Bhakar et al, 2003), we examined if this also occurred in our system. Western blotting revealed no phosphorylated BAD in control nor in *tau::p75^{NTR}* lysates at day 2 (Fig.12E). Interestingly, phosphorylated BAD was detected in control and *tau::p75^{NTR}* lysates at day 1 (Fig.12E), indicating that the absence of phosphorylated BAD at day 2 was specific. This early phosphorylation of BAD might be responsible for the basal level of cell loss detected in control cultures at day 1, perhaps as a result of the dissociation procedure that causes abundant cell death (data not shown).

Treatment with CEP-1347 not only prevented the degeneration of processes, but it also affected the morphology of neurons. By day 4, CEP-1347-treated *tau::p75^{NTR}* neurons exhibited increased branching, as assessed by the number of primary processes originating per cell body (Fig.13A).

To directly test the role of $p75^{NTR}$ in branching, we also compared control neurons to neurons deficient for both *p75^{NTR}* alleles. In line with the results obtained using CEP-1347, *p75^{NTR}* null neurons exhibited increased branching

than control (Fig.13B). Finally, we tested whether ligand binding affects branching in wild type cells. Treatment of control neurons with either BDNF or NGF resulted in increased branching (Fig.13C). Taken together, these experiments indicate that $p75^{NTR}$ plays a role as a negative regulator of process branching in ES cell-derived neurons.

2.2.6) Contribution of mitochondria and the ubiquitin-proteasome system to process degeneration

Given its prominent role in cell metabolism and its involvement in apoptotic cell body death, we examined the role of mitochondria in process degeneration in our system. To asses the distribution and integrity of mitochondria in neurons, we used Mitotracker-Orange, a cell permeable marker that selectively labels healthy and active mitochondria. Mitotracker-Orange was applied in vivo for one hour and neurons were then fixed and stained. Active mitochondria were present in the cell body and along processes of control and *tau::p75*^{NTR} neurons at day 2 (Fig.14A). To further confirm that mitochondria were active, we double stained with antibodies against cytochrome C (Fig.14B), which localizes exclusively to healthy mitochondria and is released upon mitochondria outer membrane polarization during programmed cell death. This revealed that the majority of Mitotracker-Orange-labeled mitochondria (~80%) co-localized with cytochrome C (Fig.14B). In processes, mitochondria clusters exhibited an elongate shape of about ~0.5-3 μm in length (Fig.14A). Clusters were evenly distributed along the process all the way to the growth cones, and were separated by 2±2 μm. By day 4, active mitochondria were still detected but they adopted a different morphology that was more compact (Fig.14A).

It has been shown that a compact mitochondrial morphology correlates with increased intracellular calcium levels (Murphy et al, 1996). Mitochondria can take up large quantities of calcium and this leads to their compact morphology and to increase production of ATP and of reactive oxygen species (ROS, Brookes et al, 2004). Thus, we asked whether calcium plays a role in process degeneration.

Treatment of *tau::p75*^{NTR} neurons with EGTA, an extracellular calcium chelator, could partially delay the degeneration of processes (Fig.14C).
We next tested whether increased mitochondrial activity would affect process degeneration. Treatment with creatine or sodium pyruvate, which are known to promote mitochondrial activity and to increase ATP levels respectively (Li et al, 2004), accelerated the degeneration of processes in *tau::p75*^{NTR} neurons (Fig.14C). Together, these results suggest that the levels of mitochondria-derived ATP contribute to the degeneration of processes.

As interference with the ubiquitin proteasome system (UPS) has been recently shown to prevent the degeneration of axons in vitro (Zhai et al, 2003) and in vivo (Watts et al, 2003), we asked next if this was also the case in our system. Treatment of *tau::p75*^{NTR} neurons with MG-132 could modestly prevent process degeneration at day 4 (Fig.14D). While this effect was observed with low concentrations of MG-132 (0.2 mM), higher concentrations of MG-132 (2 mM), which were shown to protect axotomized axons (Zhai et al, 2003), were toxic for our neurons (data not shown). It is conceivable that since our experiments were performed with early developing neurons, the UPS may be important for the survival of these cells. Indeed, pharmacological inhibition of the UPS has been shown to compromise the survival of cultured sympathetic neurons (Klimaschewski et al, 2003) and of motoneurons in spinal cord slice cultures (Tsuji et al, 2005).

2.2.7) Involvement of RhoA signaling in process degeneration

As essentially all cells behave similarly in our system, we next set out to identify early biochemical changes causing the degeneration of processes by proteomic analyses using 2-dimensional gel electrophoresis, a method that allows cell populations to be compared on the basis of their protein expression profiles. It is based on the separate labeling of lysates with different fluorescent markers, which allows proteins from each lysate to be resolved in the gels. Control and *tau::p75*^{NTR} cells were lysed at day 2, before any signs of process degeneration.

In 2 independent experiments, we identified 12 proteins that were reproducibly de-regulated in *tau::p75^{NTR}* neurons (Fig.15). Since several proteins were found in multiple spots on the gel, this suggests that some of the observed changes might be due to post-translational modifications, rather than to changes in the total levels of expression. The proteins identified included gelsolin, cofilin, destrin, calponin-3, tropomyosin, and caldesmon (Fig.15). All of these proteins have been directly linked with the regulation of actin filaments. The differential expression of all these proteins is consistent with previous observations indicating that p75^{NTR} is a major regulator of Rho activity, a small GTPase targeting the actin cytoskeleton (Jaffe and Hall, 2005). Thus, we asked whether in our system p75^{NTR} also controls the Rho pathway. We measured the levels of RhoA activity in day 2 neurons by pulling down GTP-bound RhoA (the active form of RhoA) using beads coupled with the Rho binding domain of the protein Rhottekin (Reid et al, 1996). This experiment indicated that Rho-GTP levels were higher in *tau::p75^{NTR}* compared with control cells (Fig.16A). The levels of total RhoA protein were not significantly changed between *tau::p75^{NTR}* and control neurons, as measured by Western blotting (Fig.16A).

Also at day 2, staining for F-actin revealed defects in the actin cytoskeleton in *tau::p75^{NTR}* cells compared with control neurons. The filaments became abnormally beaded throughout the processes as well as in the cell body (Fig.16B). This was likely due to rearrangements in the distribution of the filaments, and/or changes in the polymerization state of actin, as the total levels of actin measured by Western blot for β–actin were not changed (Fig.16C). At this time point, the microtubules of *tau::p75^{NTR}* neurons were neither beaded nor fragmented, and were indistinguishable from those in control cells (Fig.16B). These observations suggest that in our system, the actin cytoskeleton becomes affected before the microtubules. To test whether the increased Rho activity and the defects on F-actin have a causal role in the degeneration of processes, we interfered with the Rho pathway. Treatment with Y27632 or HA1077 at day 2, two inhibitors of the RhoA downstream effector Rho kinase, rescued process

degeneration in *tau::p75^{NTR}* neurons (Fig.16D). This rescue was less dramatic than the JNK signaling inhibitor (compare with Fig.12D).

As the activation of RhoA by p75^{NTR} has previously been shown to be linked with inhibition of process elongation, we also examined if the inhibitors of Rho Kinase would also have an effect on the branching of *tau::p75^{NTR}* neurons. The results shown in Figure 13A revealed that treatment with Y27632 promotes process branching in *tau::p75^{NTR}* neurons. This effect was less pronounced than the effect of CEP-1347 in process branching. One explanation for this is that CEP-1347 is a more efficient inhibitor of JNK signaling, than Y27632 is for the RhoA pathway. Alternatively, the JNK pathway may play a more significant role in process branching in *tau::p75^{NTR}* neurons.

2.2.8) Identification of Galectin-1 in *tau::p75^{NTR}* neurons

The analysis of proteins differentially expressed using proteomics also revealed a threefold up-regulation of a protein in *tau::p75^{NTR}* lysates that was identified by mass spectrometry as Galectin-1 (Fig.15). Galectin-1 is a member of the a family of lectins called the Galectins (for review see Liu and Rabinovich, 2005). Lectins are proteins that bind specific carbohydrates attached to other proteins or lipids. The Galectin family is highly conserved from *C. elegans* to human, consisting of about 15 family members that share a carbohydrate-recognition domain (CRD) with specificity for β-galactose. In order to confirm the up-regulation of Galectin-1 in *tau::p75^{NTR}* neurons, we performed Western blots on day 2 neurons. Galectin-1 was expressed at higher levels by *tau::p75^{NTR}* neurons as compared with control cells (Fig.17A). This up-regulation could be prevented by treatment of *tau::p75^{NTR}* neurons at day 1 with the JNK signaling inhibitor CEP-1347 and with the Rho kinase inhibitor Y27632 (Fig.17B). Reverse transcription-PCR also revealed a marked up-regulation of *Galectin-1* mRNA levels by *tau::p75^{NTR}* neurons at day 2 (Fig.17B), which was also prevented by CEP-1347 and Y27632 (Fig.17B).

Since a recent study showed that another family member, Galectin-3, facilitates the apical transport of p75^{NTR} in polarized cells (see Discussion), we tested the

possibility that Galectin-1 could also directly interact with $p75^{NTR}$. Immunoprecipitation with $p75^{NTR}$ antibodies pulled down endogenous Galectin-1, and substantially more Galectin-1 was precipitated from *tau::p75^{NTR}* lysates than from control lysates (Fig.17C).

2.2.9) Involvement of Galectin-1 in process degeneration

We then examined whether Galectin-1 participates in the degeneration of processes. Because increased Galectin-1 levels were detected in *tau::p75^{NTR}* neurons, and since this lectin likely interacts with the glycosylated domain of $p75^{NTR}$ that is situated in the ECD, we hypothesized that Galectin-1 may act extracellulary. In order to displace Galectin-1 from its binding sites, we used a recombinant protein consisting of the entire glycosylated ECD of $p75^{NTR}$ linked to a human Fc region. Treatment of *tau::p75^{NTR}* neurons at day 2 with soluble $p75^{NTR}$ protein prevented the degeneration of processes in a concentration dependent manner (Fig.18A,B).

We next asked whether Gallectin-1 may be an effector of neuronal degeneration. Control neurons were treated at day 3 with exogenously applied recombinant Galectin-1. This led to the degeneration of processes, which became evident already after 24 hours of treatment (Fig.18C-E), and was later followed by the death of the cell bodies. The morphology of degenerating processes was similar to that observed in *tau::p75^{NTR}* neurons (Fig.18D). Treatment of control neurons with Galectin-1 at day 9 also killed processes in a manner similar to day 3 neurons (data not shown). The killing effects of recombinant Galectin-1 could be prevented by co-treatment with soluble $p75^{NTR}$ (Fig.18C,E). To test if Galectin-1's killing effect depends on its ability to bind to carbohydrates, we also co-treated control cells with Galectin-1 and lactose, a disaccharide consisting of sucrose and galactose. Like soluble $p75^{NTR}$, lactose also prevented the killing effect of Galectin-1 (Fig.18C,E). This suggests that Galectin-1 may cause degeneration by binding to sugar residues present on cell surface molecules.

The sugar binding activity of Galectin-1 is known to depend on its state of oxidation. Indeeed, it seems to come out of the cells with cystein residues not

involved in the formation of disulfide bridges (Barondes et al, 1994). In our experiments we always used Galectin-1 that was prepared in the presence of DTT to avoid oxidation. We therefore compared the effects of Galectin-1 prepared with DTT (reduced) with that of Galectin-1 prepared without DTT (oxidized). This revealed that at the concentrations at which reduced Galectin-1 causes neuronal degeneration, oxidized Galectin-1 does not (FIG.18F).
Since the Galectins are a large family of proteins, we also compared the effects of Galectin-1 to that of Galectin-3 on control neurons, as these lectins have recently been shown to have similar effects on activated T cells (Stilmann et al, 2006). Interestingly, unlike Galectin-1, Galectin-3 failed to cause neuronal degeneration in control cells (Fig.18F), thus indicating that sugar binding alone may not be sufficient to trigger process degeneration.

2.2.10) The killing effect of recombinant Galectin-1 does not require $p75^{NTR}$

We next investigated the possibility that Galectin-1 would mediate its killing activity by binding to $p75^{NTR}$. Indeed, $p75^{NTR}$ is highly glycosylated in its ECD and interacts with Galectin-1 (see Fig.17C) as well as with another unrelated lectin, wheat germ agglutinin (WGA). WGA is a protein of 36,000 molecular weight isolated from *Triticum vulgaris* (Costrini & Kogan, 1981; Vale & Shooter, 1982; Buxser et al., 1983; Grob & Bothwell, 1983). Like with Galectin-1, we found that treatment of control neurons at day 2 with exogenous WGA caused the degeneration of processes (Fig.19). By contrast, neuronal processes of neurons with a full deletion of both $p75^{NTR}$ alleles escaped the killing effects of WGA (Fig.19). Treatment with Galectin-1 caused the death of both control as well as $p75^{NTR}$-/- neurons (Fig.19). These results show that while WGA acts primarily through $p75^{NTR}$, exogenous Galectin-1 does not require $p75^{NTR}$ expression to kill. In line with the view that exogenous Galectin-1 activates pathways additional to those triggered by $p75^{NTR}$, co-treatment of control neurons at day 2 with Galectin-1 and either the Rho kinase or the JNK signaling inhibitors, did not prevent the degeneration of processes (data not shown).

2.2.11) Recombinant Galectin-1 kills neurons isolated from the developing cerebral cortex

We tested if Galectin-1 could also cause the degeneration of primary neurons. As the ES cell-derived neurons used in our system resemble cultures of neurons isolated from the developing cerebral cortex (Bibel et al, 2004), we next used neurons isolated from embryonic day 16.5 mouse cortex and treated them with recombinant Galectin-1 at day 5 after plating. Like with ES cell-derived neurons, Galectin-1 caused the degeneration of their processes followed by the cell body (Fig.20). The first signs of process degeneration were observed after 36 hours of Galectin-1 treatment, compared with 24 hours in ES cell-derived neurons. When the first signs of degeneration were detected in processes, the time elapsed between this point and the death of the cell bodies was similar to that observed in ES cell-derived neurons, being roughly 24 hours. The killing effect of Galectin-1 could also be prevented by co-treatment with either lactose or the soluble $p75^{NTR}$ fusion protein (Fig.20).

2.2.12) Excitotoxicity increases Galectin-1 levels in vivo and is prevented by extracellular decoys

To test whether Galectin-1 plays a role during neuronal degeneration in vivo, we focused on a well-established model of neuronal degeneration caused by excitotoxicity. The medial septum is a region of the basal forebrain that contains large numbers of cholinergic neurons. These cells have projections towards different brain structures, including hippocampus, cortex and amygdala, and are involved in the regulation of cognitive function (Everritt and Robbins, 1997). Unlike most CNS neurons, basal forebrain cholinergic neurons continue expressing $p75^{NTR}$ at high levels even in the adult (Dechant and Barde, 2002). A previous study has shown that the delivery of exposure of the medial septum to excitotoxins leads to the loss of cholinergic neurons (Oh et al, 2000). In addition, this led to increased levels of $p75^{NTR}$ expression and the degeneration of the septal neurons could be prevented by antibodies against $p75^{NTR}$ as well as by inhibitors of protein synthesis (Oh et al, 2000).

We injected the N-methyl-D-aspartate (NMDA) glutamate receptor agonist ibotenic acid (IBO) stereotaxically onto the medial septum of adult rats, and assessed its effects 24 hours later (Fig.21A). Immunostaining revealed a local increase in Galectin-1 reactivity (Fig.21B).

We next quantified the number of cholinergic neurons by staining for $p75^{NTR}$, which labels both their processes and cell bodies. IBO injection resulted in a significant loss of $p75^{NTR}$-positive neurons as compared with sham (Fig.22A,C). High magnification images revealed that similarly to our in vitro system, the processes of cholinergic neurons degenerated before the cell bodies (Fig.22B); the majority of cholinergic neurons in IBO-injected animals were devoid of processes, their cell bodies appeared more compact and exhibited nuclear pyknosis, while sham neurons had several processes and healthy cell bodies. The staining intensity for $p75^{NTR}$ was stronger in degenerating neurons than in sham, suggesting an up-regulation in protein levels (Fig.22B). Co-injection of the soluble $p75^{NTR}$ fusion protein, lactose or an anti-Galectin-1 antibody prevented both the loss of cholinergic neurons and of their processes (Fig.22A,C,D). Given our in vitro results showing that CEP-1347 prevents Galectin-1 expression and the degeneration of *tau::p75*^{NTR} neurons, we tested its effects in our in vivo system. Co-injection of CEP-1347 and IBO resulted in decreased staining intensity for Galectin-1 (Fig.21B) and it also prevented cholinergic neuron degeneration (Fig.22A,C,D). These results thus indicate that endogenous Galectin-1 is up-regulated after excitotoxicity and is involved in the degeneration of neurons.

3. DISCUSSION

3.1) DEVELOPMENTAL POTENTIAL OF PROGENITOR CELLS

3.1.1) RA-treated ES cells generate uniform and defined progenitors

A key aspect of the work presened in this Thesis was the availability of a recently-developed system allowing the generation of uniform cell populations (Bibel et al, 2004). Unlike previous protocols leading to rather heterogenous and poorly defined cells, virtually all the progenitors we used have the identity of RG cells. Prior to implantation, all cells in the RA-treated aggregates expressed Nestin, Sox2, Pax6, RC2, and GLAST. Only very few cells (less than 1%) expressed the interneuron marker Lim1/2 and the motoneuron markers Hb9 and Isl1. No cells were found to be GFP-positive in intact aggregates or during the first hours following their dissociation (data not shown). These results indicate that RA-treated ES cell aggregates contained a homogeneous cell population with a profile of marker expression corresponding to in vivo existing RG cells. By contrast, previous studies using similar markers indicated cellular heterogeneity of RA-treated aggregates. For example, other differentiation protocols led to a mix of cells expressing Pax6, Pax7 and Nkx2.2 (Renoncourt et al., 1998; Wichterle et al., 2002). In the neural tube, expression of these markers is segregated; dorsal progenitors express Pax7, mid-dorsal to mid-ventral progenitors express Pax6 and ventral progenitors express Nkx2.2 (Ericson et al, 1997). These different populations generate distinct neuronal types. In our study, virtually all the cells expressed Pax6 prior to transplantation, while none expressed Pax7 or Nkx2.2.

The homogeneity of our RA-treated ES cells is likely to explain differences with other studies in the progeny obtained. For example, Wichterle et al (2002) failed to detect donor-derived motoneurons if their RA-treated ES cells were treated only with RA and without sonic hedgehog (Wichterle et al, 2002). By contrast, we observed significant numbers of motoneurons derived from our aggregates treated only with RA. As motoneurons constitute a fraction of the mouse-derived

cells, it can be expected that if the progenitor population is heterogeneous at the time of implantation, it likely contains fewer cells able to enter the motoneuron differentiation pathway. Presumably in our case, sonic hedgehog or other signalling molecules delivered by the host drive the generation of motoneurons at the appropriate location following implantation of sufficient numbers of competent cells.

Does the differentiation of ES cells with RA resemble an in vivo-existing mechanism? Retinoic acid plays important regulatory roles at different stages of embryonic development (Morriss-Kay and Sokolova, 1996). During gastrulation, after neural tissue has been induced by noggin- and follistatin-antagonism of BMPs, RA up-regulates the expression of pro-neural genes (for review see Maden, 2002). RA is later required for anterior neural progenitors to acquire a more posterior identity, and to subsequently differentiate into distinct neuronal subtypes of the hindbrain and spinal cord (Muhr et al, 1999; Pierani et al, 1999). The mechanisms by which RA exerts its effects on neural differentiation of ES cells remain unclear and may not be physiological, though we note that in ES cells RA induces the expression of a Wnt antagonist called Sfrp2 (Aubert et al, 2002). Interestingly, the addition of recombinant Sfrp2 mimmics the effects of RA (Aubert et al, 2002). Thus RA acts, at least in part, by interfering with one of the factors known to maintain ES cells undifferentiated (see Introduction).

3.1.2) Large numbers of ES cells differentiate into neurons in the host

Six days after implantation, ES cell-derived progenitors survived in very large numbers in the chick host and the vast majority of them differentiated into neurons. Donor cells differentiated into Lim1/2+ interneurons and into motoneurons that were Hb9+ and Isl1+. Motoneurons extended axons towards the periphery and expressed the neurotrophin receptors $p75^{NTR}$ and Trk in their cell bodies and axons. In addition to the identification of donor cells using GFP, we also monitored by nuclear staining the fate of other cells that survived but failed to differentiate into neurons. Both in the spinal cord and in the DRG, about 80% of the mouse nuclei localized to GFP+ cells. Previous studies indicated that

there is a complete overlap between cells expressing *GFP* from the *tau* locus and those positive for the antibody TuJ1 that recognizes a neuron-specific form of tubulin (Tucker et al., 2001). In addition, we also found that in the spinal cord, many GFP-negative donor cells expressed either Lim1/2 or Hb9 (data not shown). As progenitor cells differentiating into neurons start expressing these markers prior to exiting the cell cycle, it is likely that these Lim1/2+/GFP- and Hb9+/GFP- cells were on their way to become post-mitotic interneurons or motoneurons, respectively. While it may seem surprising that we only detected few cells not belonging to the neuronal lineage in the progeny of our precursor cells, it should be kept in mind that our experiments did not go beyond E8, which is before the time when large number of astrocytes are generated in the spinal cord. It is possible that some of the GFP-negative cells may later go on to differentiate into astrocytes. These results show that our progenitor cells generate mostly neurons after implantation, which is in line with recent evidence suggesting that Pax6-positive RG cells are neuronal progenitors (see Introduction). In addition, our results indicate that donor cells are able to respond to patterning signals in the spinal cord, generating spinal cord interneurons and motoneurons in a time- and in a position-dependent manner. At present, in vivo cell lineage studies have not yet rigorously proven that Pax6-positive RG cells in the spinal cord generate motoneurons and subtypes of interneurons, but this appears quite likely. The pattern of expression of RG markers and of Pax6 in the spinal cord, as well as the decrease of motoneuron numbers in the *small eye* mutant (Ericson et al., 1997) are compatible with this interpretation.

3.1.3) RA-treated ES cells fail to elongate axons in the DRG

Donor cells also exhibited a migratory behaviour and colonized the DRG, perhaps because they follow the stream of migratory neural crest cells. Strikingly, while most donor cells also differentiated into GFP+ neurons in the host DRG, they failed to elongate axons and to express Brn3a and Isl1, which are expressed by most neurons in that structure. These results can not be accounted by an intrinsic restriction of RA-treated cells to extend axons and to express

these markers, since the same cells were capable of doing so when located in the spinal cord. Moreover, while donor motoneurons expressed both $p75^{NTR}$ and Trk receptors, donor neurons in the DRG did not express detectable levels of Trk receptors. By contrast, these neurons expressed $p75^{NTR}$ at relatively high levels. Typically, signaling via Trk receptors supports neuronal survival and axonal elongation (Huang and Reichardt, 2003). By contrast, signalling via $p75^{NTR}$ leads to opposite effects (Dechant and Barde, 2002). It is thus possible that $p75^{NTR}$ expression by donor neurons in the DRG in the absence of detectable expression of Trk receptors, may account for their failure to elongate axons. During development, sensory neurons expressing $p75^{NTR}$ also express at least one type of Trk receptors (Wright and Snider, 1995). Our results suggest then that neurons located in the DRG do not extend axons by default even in the highly conducive environment provided by developing DRG. While we do not know why Trk receptors are not expressed when the ES cell-derived progenitors are located in the DRG, we note that they also failed to express the POU transcription factor Brn3a. It has been shown that the expression of all Trk receptors is compromised in the trigeminal ganglia of *Brn3a-/-* mice, while the expression of $p75^{NTR}$ is not affected (Huang et al., 1999). In addition, Brn3a has recently been shown to directly induce transcription of *TrkA* in the DRG (Ma et al., 2003). Thus, the absence of Brn3a expression by donor neurons in the DRG may be related with their failure to express Trk receptors. Why the expression of Brn3a is not turned on is unclear, but we note that in the DRG, the progenitors of Brn3a cells are not Pax6-positive, suggesting that Pax6 or other factors may repress the acquisition of alternative phenotypes.

In view of these results with the DRG, we performed similar implantation experiments with RA-untreated ES cells. Virtually all ES cells at the time of implantation expressed Oct3/4, one of the markers correlating with pluripotency of ES cells (Niwa et al., 2000; Boiani et al., 2002). We found that these cells also survived in large numbers after implantation. Like RA-treated cells, they also differentiated into Lim1/2+ interneurons, and into Hb9+ and Isl1+ motoneurons extending long axons towards the periphery that were positive both for Trk and

$p75^{NTR}$ neurotrophin receptors. We observed however that RA-untreated ES cells generated fewer motoneurons than RA-treated cells, as judged by the expression of Hb9 and Isl1. Recent studies indicated that somite-derived RA plays an early role in the acquisition of a neural fate by cells in the neural tube (Diez del Corral et al., 2003), and that the same molecule can further promote these cells into a motoneuron differentiation pathway, even in the absence of sonic hedgehog (Novitch et al., 2003). It is therefore conceivable that pre-treatment with RA may not only induce neural differentiation of ES cells, but also bring these cells closer to a motoneuron fate.

ES cells also colonized the host DRG, but in contrast to RA-treated cells, they expressed the markers Brn3a and Isl1. These cells also elongated axons outside the DRG both towards the spinal cord and the periphery, and they expressed Trk receptors in addition to $p75^{NTR}$. The proportion of ES cells colonizing DRG and differentiating into neurons in that structure was similar to that observed for RA-treated cells. Thus, the failure of RA-treated cells to express DRG markers and to elongate axons does not result from a higher number of cells colonizing the DRG and differentiating into neurons. We also note that this system of ES cell implantation into the chick embryo seems to work reliably in so far as neither RA-treated nor RA-untreated cells colonizing the DRG ever expressed spinal cord markers.

3.1.4) Testing the differentiation potential of progenitor cells using in vivo approaches

The differentiation potential of multipotent cells has been explored extensively in culture conditions using exogenous extracellular signals. However, the factors added might not be sufficient to explore the entire differentiation potential of these cells. A good test of the developmental potential of progenitor cells is to confront these cells with an in vivo system. The avian embryonic nervous system is ideally suitable for this as defined times and locations can be chosen to transplant cells when for example, active neurogenesis, cell migration, or gliogenesis are occurring (for review see Temple, 2001).

Previous studies have reported on the use of ES cell-derived progenitors for cell transplantation. In particular, Brustle and colleagues transplanted in vitro-differentiated neural progenitors from mouse ES cells into the brain ventricles of rats (Brustle et al, 1997). These cells populated the brain parenchyma where they differentiated into astrocytes and neurons. Similar results were also reported using human ES cell-derived neural progenitors implanted into the brains of mice or rats (Reubinoff et al, 2001; Zhang et al, 2001), and with mouse ES cell-derived neural progenitors implanted into the damaged spinal cord of rats (McDonald et al, 1999). While these studies showed that ES cell-derived neural progenitors responded to host signals, the extent to which they survived and differentiated in the host remains unclear and appeared to be limited. Furthermore, the in vitro-differentiated neural progenitors were not shown to correspond to a defined population of neural progenitors beyond Nestin positivity. Kim et al and Wichterle et al showed that mouse ES cells could differentiate in vitro into dopaminergic neurons and motoneurons respectively (Kim et al., 2002; Wichterle et al., 2002). These cells were even able to integrate as dopaminergic neurons in the striatum of hemiparkinsonian rats, and as motoneurons in the chick embryo spinal cord respectively. However, the frequency of these integration events was not reported in these studies. In our experiments, cells survived in large numbers, contributed to form a spinal cord of normal size and morphology and differentiated into spinal cord neurons. Therefore, our experimental approach offers now the possibility to examine the function of genes involved in neural cell fate specification and axonal elongation using genetically modified ES cells. An interesting prospect would be for example, to test the behaviour of neurons derived from ES cells engineered not to respond to myelin associated molecules, and ES cells deficient for the *Nogo receptor*, $p75^{NTR}$ and *LINGO*, could prove useful to test this suggestion. By implanting cells deficient for these receptor components, one could assess axonal elongation in an otherwise wild type environment. This would be of particular interest since several of these molecules serve many other roles in development, and thus the analysis of full knockout animals is often complex.

3.1.5) Restricted developmental potential of RA-treated ES cells

In the developing brain, neurons are generated from different types of progenitors. During early stages, only neuroepithelial cells are present, but as development progresses these cells differentiate into at least two distinct types of progenitor cells. The first type to appear is the basal progenitors, and is followed by RG cells (Kriegstein and Gotz, 2003). These different progenitor populations can be identified by their different morphology, location, and profile of marker expression. RG cells are distributed throughout the dorsal-ventral axis, but those located dorsally seem to differ functionally from those located ventrally. It has been proposed that only dorsally located RG cells are neurogenic (Malatesta et al, 2003), and cell fate mapping experiments indicated that these cells generate the majority of excitatory neurons in the cerebral cortex and in the hippocampus (Malatesta et al, 2003). By contrast, ventral RG cells do not substantially contribute to the neuronal population found in the striatum or to the interneuron population in the cerebral cortex, and these neurons seem to be the progeny of the basal progenitors (Gotz and Barde, 2005). Recently, this conclusion was challenged by Anthony et al. (2004) who suggested that RG cells are neuronal progenitors throughout the entire brain. In their study, the authors mapped the progeny of RG cells by crossing a line that expresses Cre recombinase from the BLBP promoter to reporter lines (Anthony et al, 2004). The caveat of this study however is that while BLBP expression in RG cells becomes detectable by E12, its mRNA is already present in neuroepithelial cells by E10, a time when RG cells are not yet present. In line with the view that the BLBP construct used by Anthony et al (2004) may have driven a premature expression of Cre, similar results to those obtained using the BLBP-Cre line were obtained using a Nestin-Cre line (Backman et al, 2005).

What are the molecular differences between dorsally- and ventrally located RG cells that could account for their different neurogenic potential? It appears that one key determinant of the neurogenic potential of dorsally-located RG cells is the expression of the transcription factor Pax6 (Heins et al., 2002; Malatesta et

al., 2003). During development, Pax6 expression is restricted to the dorsal telencephalon, where neurogenic RG cells are located, while it is absent from the ventral telencephalon (Heins et al, 2002). Moreover, fewer neurons are found in the cortex of *Pax6* mutant mice (Heins et al, 2002). Perhaps the most striking piece of evidence suggesting that Pax6 plays a major role as a neurogenic factor is that when over-expressed, Pax6 can instruct non-neurogenic mature astrocytes into neurogenesis (Heins et al, 2002). In addition, a recent study showed that Pax6 is essential for adult neurogenesis in the olfactory bulb (Hack et al, 2005).

The RA-treated ES cells used in our study have the antigenic profile of those found in the developing dorsal, but not ventral telencephalon. While previous work with these cells showed that in vitro they differentiate into neurons with the characteristics of pyramidal cells (Bibel et al., 2004), we now find that they can also respond to extracellular signals and differentiate according to their position in the embryo. However, their differentiation potential is restricted and they seem to generate only the neural progeny that is expected from their developmental history and location. In particular, they cannot acquire the antigenic and morphological features of peripheral sensory neurons. Our results then raise the question of the nature of the molecular determinants that restrict the differentiation potential of RG cells. One possibility is that Pax6 not only acts to commit RG cells to differentiate into specific neuronal subtypes, but that it also actively represses the differentiation into other different subtypes. In line with this notion, unpublished work from our laboratory suggests that in the absence of functional *Pax6*, RG cells are still generated, but their neuronal progeny differs from that obtained from RG cells wild type for *Pax6* (N. Nikoletopoulou and Y.-A. Barde, personal communication).

3.1.6) What are developmental restrictions good for?

Examples of developmental restrictions in the vertebrate nervous system were most extensively documented in the neural crest. These cells have an extensive differentiation potential, as there are few tissues of the vertebrate body to which

they do not contribute. The introduction of the quail-chick chimera system by Le Douarin and colleagues several years ago pioneered the use of avian transplantation experiments to probe the developmental potential of neural crest cells (see review by LeDouarin et al, 2004). This consisted on the isolation of neural crest cells from any region along the anterior-posterior axis of the quail embryo and the transplantation into chick donors. Moreover, these experiments allowed for crest cells to be transplanted at different locations and at different developmental time points. An important conclusion from these studies was that the differentiation of neural crest cells into different cell types depends on the environment they are confronted with (see for example Bronner-Fraser and Fraser, 1989). In addition, they indicated that neural crest cells are not restricted into generating specific cell types prior to their migration from the neural tube. However, studies that most critically assessed the potential of neural crest cells indicated that these cells become restricted as they migrate towards their targets (Sieber-Blum 1989; Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Raible and Eisen, 1994). Therefore, it appears that developmental restrictions are a commonality among progenitor cells throughout the nervous system. Because of the remarkably large number of different cell types in an organism, the purpose of restricting cell potential in terms of development, evolution and disease, may not be particularly obvious at first glance. A simple explanation however, is that evolution has found a way to prevent the production of inappropriate cell types, at the wrong place and time, by restricting the phenotypes that any given progenitor can generate.

Interestingly, while neural crest cells are a transient population in the embryo, it was suggested that some of them persist in adult tissues. Studies showed that cells with the characteristics of neural crest cells can also be isolated from adult peripheral tissues and that, if transplanted back into younger embryos, they are able to migrate and generate some of the cell types normally produced by neural crest cells (Stemple and Anderson, 1992; Morrison et al, 1999). Thus, some neural crest cells may not completely disappear after development is completed, but instead remain as neural stem cells in the PNS. This may also be the case

for RG cells in the CNS, as it was shown that while most of them differentiate into neurons and astrocytes during development, some of them become neural stem cells in the adult SVZ and dentate gyrus (Garcia et al, 2004).

3.2) NEURONAL DEGENERATION

Given our results showing that lack of axonal elongation in the DRG correlated with expression of $p75^{NTR}$, we examined the role of this receptor during neuronal differentiation of ES cell-derived neurons. As discussed below, we found that preventing the downregulation of $p75^{NTR}$ in these cells causes the degeneration of neuronal processes and the death of cell bodies, and that these result can be explained at least in part, by an up-regulation of the endogenous lectin Galectin-1.

3.2.1) Synchronous neuronal degeneration caused by the controlled expression of *p75^{NTR}*

In our system, all neuronal processes degenerate at day 4, and they are followed 24 hours later by the death of the cell bodies. Synchronicity of differentiation is a key aspect of our procedure, and experiments using a GFP cDNA introduced in the *tau* locus confirmed phase contrast observations indicating that the degeneration of processes is synchronous in our system. We also extensively tested whether the presence or absence of *tau* would alter process degeneration and never found any difference between wild type and mutant ES cells with one or both *tau* alleles eliminated (data not shown). The comparison of BrdU incorporation between day 2 and day 3 was a further confirmation of synchronicity.

3.2.2) Role of JNK in process degeneration

Immunostaining experiments with *tau::p75^{NTR}* neurons revealed increased JNK activity along their processes, but not in the cell body. Also, inhibition of the JNK pathway with CEP-1347 prevented process degeneration. CEP-1347 was initially developed as an analogue of the naturally-occurring, bacterially-expressed bis-

ethyl-thiomethyl small molecule, K252a. Unlike CEP-1347, K252a is a rather unspecific kinase inhibitor that interferes with the activity of serine/threonine kinases, phosphatidyl inositol 3-Kinase, protein kinase C, and the tyrosine kinase of all three Trk receptors (for review see Wang et al, 2004). By contrast, CEP-1347 does not interfere with Trk receptor kinase activity, but efficiently prevents the activation of JNK by blocking its upstream Mixed Lineage Kinase (Roux et al, 2002). Importantly, CEP-1347 has been shown to have a very efficient role in preventing the degeneration of neurons in different in vitro and in vivo paradigms. These include cultured sympathetic neurons deprived of NGF (Harris et al, 2002b), cerebellar granule neurons grown in low potassium levels (Harris et al, 2002a), dissociated spinal motoneurons grown at low density (Maroney et al, 1998), cortical neurons treated with recombinant Aβ (Bozyczko-Coyne et al, 2001), hair cells in an auditory-based in vivo degeneration paradigm (Pirvola et al, 2000), and dopaminergic neurons in an animal model of Parkinson's disease (Saporito et al, 2000). CEP-1347 also prevents the degeneration of cholinergic neurons of the rodent basal forebrain that were injured either by axotomy (Haas et al, 1998) as well as by exposure to IBO (Hass et al, 1998; Saporito et al, 1998), in a similar manner to our experiments here. Importantly, the protective effect of CEP-1347 in most of these systems, including ours, covered both the cell bodies as well as the processes of neurons. This is unlike the action of caspase-3 inhibition that only prevents the death of neuronal cell bodies.

While JNK and caspase activities are localized to different cellular compartments, we find that $p75^{NTR}$ is expressed at the surface of both processes and cell bodies. Cell body death could be triggered by a signal generated within processes and then transported towards the cell body. Inhibition of JNK signaling may prevent the generation of such signal. In addition, JNK activity might contribute both to programmed cell death and to process degeneration, but the levels of JNK activity in the cell body might be lower and hence more difficult to detect than in processes. Nevertheless, it seems that $p75^{NTR}$-induced neuronal degeneration proceeds in a way such that processes are more vulnerable than

the cell body, perhaps simply because the nucleus, ER and Golgi apparatus are contained within the cell body.

Our results also open the question of which JNK isoforms are activated and what their targets are in processes. Three different genes encode for JNK1, JNK2 and JNK3, and all of these can undergo differential mRNA processing, resulting in more than ten different JNK proteins (Davies, 2000). We do not know which JNK isofroms are controlled by $p75^{NTR}$ in our system since the JNK antibodies we used would recognize all JNK species. We note however that JNK1 is the predominant activated form in cultured cortical neurons (Coffey et al, 2000; Bjorkblom et al, 2005).

While we do not know the targets of JNK in neuronal processes, we did not find evidence of mitochondrial damage until very late stages of process degeneration, as assessed by staining for Mitotracker-Orange and for cytochrome C, or by western blot for phosphorylated Bad. This is unlike what was found by others using cell bodies (Bhakar et al, 2003). JNK may target components of the cytoskeleton, and a number of studies suggested that JNK1 controls microtubule dynamics in processes by regulating the phosphorylation state of microtubule associated proteins (Chang et al, 2003). We note that *JNK1-/-* mice have been reported to exhibit defects in the anterior commissure tract and a progressive loss of microtubules in axons and dendrites (Chang et al, 2003). JNK1 interacts with, and phosphorylates, both high molecular weight MAP2 (Chang et al, 2003) and doublecortin (Gdalyahu et al, 2004). MAP2 functions as a microtubule stabilizing protein and is expressed in dendrites mostly in mature neurons (Chang et al, 2003). DCX, which is expressed at high levels in the processes of developing neurons, is required for neuronal migration and *DCX-/-* mice exhibit lamination defects in the hippocampus (Corbo et al, 2002). It is unknown however, whether regulation of MAP2 and DCX by JNK1 directly controls microtubules. Recently, SCG10, a member of the stathmin family that is also known to regulate microtubules, was identified as a JNK1 target in neuronal processes (Tararuk et al, 2006). Phosphorylation of SCG10 is reduced in *JNK1-/-* cortex and JNK inhibition prevents SCG10 phosphorylation, reduces microtubule

polymerization and affects the morphology of processes of cortical neurons in vitro (Tararuk et al, 2006). Several studies showed that in degenerating processes, microtubules form beaded structures that accumulate in the same regions where swellings appear. These changes are likely due to spatial rearrangements rather than differences in the total levels of tubulin, since our results show that the total levels of tubulin monomers are not affected during early stages of process degeneration. While microtubules are essential for the maintaining of cell shape and for axoplasmic transport, it remains open if microtubule rearrangements play a causal role in the degeneration of processes or if it is a secondary event. It would therefore be interesting to further investigate if the effects of JNK signaling in our system may be accounted in part by the regulation of proteins that control the dynamics of microtubules. JNK may also contribute to the degeneration of processes by cross-talking with GTPase pathways, which we also find here to be involved in process degeneration. Indeed, when acutely over-expressed in oligodendrocytes, $p75^{NTR}$ causes cell death by activating the small GTPase Rac, which subsequently activates JNK1 and JNK3 (Harrington et al, 2002).

JNK is also likely to target transcriptional regulators in our system. We found that blocking the JNK or RhoA pathways prevents the transcriptional up-regulation of Galectin-1, suggesting that a $p75^{NTR}$/JNK-RhoA pathway also controls proteins involved in the regulation of gene expression. Several transcription factors were previously found to be targets of JNK, such as c-Jun, c-fos and Jun-D (Davis, 2000). It is thus possible that JNK phosphorylates some of these factors to mediate the transcriptional up-regulation of *Galectin-1*. However, it remains puzzling that at the time when *Galectin-1* mRNA levels were up-regulated, there was no detectable JNK activity in the cell body. One explanation for this is that after its activation in processes, a JNK target might be transported towards the cell body to act on DNA. Alternatively, JNK could be activated directly by $p75^{NTR}$ in the cell body but the levels of JNK activity, or the number of JNK molecules required for controlling gene expression, may be too low to be detected by immunostaining.

Our results suggest that the degeneration of processes can also be influenced to some degree by mitochondrial activity and by the availability of signaling second messengers, such as calcium and ATP. Mitochondria remained distributed along processes even during advance stages of their degeneration. They were labeled with Mitotracker-Orange and their compact morphology was characteristic of highly active mitochondria that has been correlated with increased production of ATP and ROS (Li et al, 2004). Treatment with EGTA could modestly delay the degeneration of processes, while creatine and sodium pyruvate precipitated degeneration. It remains to be tested whether these factors have an essential role in process degeneration or simply affect the rate of degeneration. A recent study by Wang et al (2005) showed that Wallerian degeneration could be prevented by treatment with NAD or sodium pyruvate. The authors examined the levels of ATP and NAD in DRG axons and showed a rapid decrease after axotomy (Wang et al, 2005). The differences between their study and ours may be accounted by the use of different cellular systems. In our model, the degeneration of processes is triggered by the activation of $p75^{NTR}$, while in their case it is caused by severing the axons. At present, RhoA is the only molecule to our knowledge shown to be activated after axotomy (Dubreuil et al, 2003; Madura et al, 2004; Yamagishi et al, 2005), and we also found its activity increased in *tau::*$p75^{NTR}$ neurons. It would be interesting to test whether the Rho pathway has an effect on the regulation of mitochondrial and of metabolic activity within processes. Another possibility is that the drop in ATP and in NAD levels reported by Wang and collegues are not due to halted axonal metabolism, but to the rapid leak of ATP, NAD and other factors, outside the axoplasm. This possibility could be tested by examining the levels of ATP and NAD in sympathetic neurons deprived of trophic support, as the degeneration of these cells is not caused by mechanical insult.

3.2.3) Galectin-1 as a novel signaling target and interactor of $p75^{NTR}$

Our results show that Galectin-1 expression is positively controlled by $p75^{NTR}$ via the JNK and RhoA pathways. Since inhibition of either the JNK or Rho pathways had similar effects in preventing the up-regulation of *Galectin-1* mRNA, this supports the idea that these pathways cross-talk. It would be interesting to examine if the *Galectin-1* gene contains regulatory sequences for transcription factors known to act downstream of these pathways. We also, found that Galectin-1 binds to $p75^{NTR}$, and interfering with Galectin-1 using a soluble $p75^{NTR}$ protein prevented degeneration of *tau::p75^{NTR}* neurons. These results suggested that the action of Galectin-1 can take place from outside of the cells. Once translated, Galectin-1 can be transported to different compartments within the neuron or externalize. Exactly how Galectins leave the cell is still unclear as these proteins lack a classical secretory signal (for review see Barondes et al, 1994). Muscle cells for example, externalize somehow Galectin-1 that binds to laminin in the basement membrane (Cooper and Barondes, 1990). This in turn diminishes attachment of differentiating myoblasts to laminin, presumably by preventing laminin-integrin interactions, and it seems to be necessary for their division and fusion into myofibres (Cooper et al, 1991). A recent study indicates that the externalization of Galectin-1 depends on its ability to bind to β-galactosides (Seelenmeyer et al, 2005). When over-expressed in CHO cells, Galectin-1 was detected at the cell surface and in the medium, and this localization was prevented by mutating its CRD (Seelenmeyer et al, 2005). Galectin-1 can also be externalized if expressed in yeast cells (Cleves et al, 1996), suggesting that the mechanisms responsible for its externalization are conserved among species.

Interestingly, Galectins were also shown to localize to specific regions of cell membranes such as lipid rafts (Delacour et al, 2005), which are specialized detergents-resistant membrane domains enriched in certain proteins and in the lipid cholesterol (Simons and Ikonen, 1997). Many functions have been attributed to lipid rafts, such as cholesterol transport, endocytosis and signal transduction, and a number of receptors were shown to accumulate in there (Paratcha and Ibanez, 2002). Interestingly, Delacour et al (2005) recently reported that Galectin-

4 localizes to lipid rafts in HT-29 cells. Treatment with an inhibitor of glycosylation prevented the localization to lipid rafts of Galectin-4 as well as of a number of other raft-associated proteins. A similar result was obtained by depletion of Galectin-4 by RNA interference, suggesting that a lectin-based mechanism mediated by Galectins is responsible for the transport of glycoproteins to specific membrane domains in polarized cells (Delacour et al, 2005). One such protein could be $p75^{NTR}$, which like many cell surface proteins is glycosylated (Baldwin and Shooter, 1995). Glycosylation is an enzymatic process that occurs both co- and post-translationally, and is essential for the proper folding, stability, transport and function of a large number of secreted and membrane bound proteins. There are two types of glycosylation, N- and O-linked. N-linked oligosaccharides are added to an asparagine residue occurring in the tripeptide sequence Asn-X-Ser or Asn-X-Thr, where X could be any amino acid except Pro, as the polypeptide chain translocates into the lumen of the ER. The glycoprotein formed is immediately subjected to further modifications by trimming of some of the sugar residues in the ER. After its transport into the Golgi apparatus, the protein can be O-linked glycosylated by the addition of N-acetyl-galactosamine to serine or threonine residues followed by other carbohydrates, such as galactose and sialic acid. Because of a large number of different oligosaccharide combinations are possible during glycosylation, a protein can be decorated by different sugars, allowing cells to diversify the localization, life-time and functions of proteins. Putative sites for both N- and O- glycosylation exist in the extracellular domain of $p75^{NTR}$, between the transmembrane region and the first cystein rich domain (Baldwin and Shooter, 1995). Breuza et al (2002) investigated whether glycosylation of $p75^{NTR}$ was necessary for its transport and cellular localization in MDCK cells, by over-expressing different $p75^{NTR}$ mutant constructs. MDCK cells are polarized cells having two distinct membrane domains, the basolateral and the apical domains, and when expressed in these cells, $p75^{NTR}$ localizes to the surface of the apical membrane (Monlauzeur et al, 1998; Breuza et al, 2002). Interestingly, it was found that O- but not N-linked glycosylation is essential for the correct targeting of $p75^{NTR}$ to the apical membrane. Since lectin proteins are

known to recognize specific sugar residues in glycoproteins, the authors suggested that a lectin might help $p75^{NTR}$ be transported to the cell surface (Breuza et al, 2002). In a more recent study, Galectin-3 was identified as a protein that binds to $p75^{NTR}$ directly and that helps its localization to the apical membrane (Delacour et al, 2006). It is possible that Galectin-1 in neurons plays a similar role to that of Galectin-3 in epithelial cells, aiding $p75^{NTR}$ intracellular transport to specific membrane regions.

In addition to participating in the transport of $p75^{NTR}$ to the cell surface, Galectin-1 may also help the clustering of the receptor. In its reduced form, Galectin-1 exists mostly as a dimer, having two unbound CRDs (Liu and Rabinovich, 2005; Barondes et al, 1994), and may thus facilitate the clustering of $p75^{NTR}$ by cross-linking receptor subunits. In preliminary experiments, we found immunoreactivity for Galectin-1 along the processes and in the cell body of non-permeabilized neurons, suggesting that Galectin-1 is externalized and associated with molecules at the cell surface (data not shown). Unlike $p75^{NTR}$, the staining for Galectin-1 did not exhibit an uneven pattern but was evenly distributed along the cell surface. This suggests that Galectin-1 binds not only to $p75^{NTR}$ but also to other cell surface receptors.

Unlike other TNF receptor superfamily members that form trimers upon ligand binding, studies reported that $p75^{NTR}$ bound to neurotrophins can occur either as a dimer or monomer. At present however, it is debated whether $p75^{NTR}$ activates signaling in its monomeric or dimeric form. Examining the crystal structure of $p75^{NTR}$ complexed with NGF, He and Garcia (2004) reported that $p75^{NTR}$ in its unliganded state exists either as a dimer or as a monomer, but a $p75^{NTR}$:NGF complex is composed of an NGF homodimer asymmetrically bound to a single $p75^{NTR}$ molecule. Binding of an NGF homodimer prevents monomeric $p75^{NTR}$ from binding another $p75^{NTR}$ molecule. By contrast, Aurikko et al (2005) recently suggested a 2:2 stoichiometry in the NGF:$p75^{NTR}$ complex. These different conclusions might be due to differences in the $p75^{NTR}$ preparations used. He and Garcia (2004) used tunicamycin to inhibit N-glycosylation, and this was previously shown to negatively affect of the affinity of $p75^{NTR}$ for NGF in vitro (He and

Garcia, 2004). In addition, treatment of PC12 cells with tunicamycin diminishes binding of NGF and prevents the elongation of processes (Baribault and Neet, 1985). As most cell surface receptors isolated from the mammalian nervous system typically exist as glycoproteins (Hoffmann et al, 1995), it is unclear whether unglycosylated $p75^{NTR}$ exists under physiological conditions. In the future it would be interesting to investigate how Galectins influence conformational changes in $p75^{NTR}$, and if this triggers signaling.

When testing the killing effects of exogenously-applied Galectin-1 and WGA, we also observed that neither of these lectins promoted the internalization of the receptor in control neurons. By contrast, both NGF and BDNF promoted the internalization of $p75^{NTR}$ from the cell surface of both processes and cell body (data not shown). Other studies also showed internalization of $p75^{NTR}$ upon neurotrophin binding. Bronfman et al (2003) reported that $p75^{NTR}$ was internalized with NGF via clathrin-coated pits in PC12 cells. It would be interesting to test the prediction that Galectins and WGA promote sustained signaling via $p75^{NTR}$ by preventing its internalization. If this were the case, then neurotrophin treatment should diminish signaling via $p75^{NTR}$ by promoting its removal from the cell surface. In line with this prediction, we found that treatment of control neurons with neurotrophins promoted branching of processes. However, neurotrophins failed to prevent the degeneration of processes in *tau::p75*^{NTR} neurons (data not shown). One possible explanation for this is that the expression levels of $p75^{NTR}$ in *tau::p75*^{NTR} neurons are sufficiently high so that the action and localization of the receptor become independent of neurotrophin binding. The different outcome of binding of lectins or neurotrophins to $p75^{NTR}$ are likely due to the different regions to which these ligands bind to. While all four mammalian neurotrophins bind to the cysteine-rich domains (Dechant and Barde, 2002), Galectins and WGA are likely to bind to the juxtamembrane extracellular domain which contains the N- and O-glycosylation sites (Baldwin and Shooter, 1995).

3.2.4) Killing function of Galectin-1

Our experiments using recombinant Galectin-1 showed that this lectin causes degeneration of control neurons. A killing role for Galectin-1 was first described in non-neuronal cells by Perillo et al (1995) using T cells. Galectin-1 is expressed by stromal cells in human thymus and lymph nodes, and is present at sites where T cells undergo programmed cell death during their development (Perillo et al, 1995). Interestingly, while exogenously applied Galectin-1 caused the death of activated human T cells and human T leukaemia cell lines, it did not kill resting T cells. Other Galectins were also shown to cause cell death. Like Galectin-1, Galectin-2 (Sturm et al, 2004), Galectin-9 (Wada et al, 1997; Kashio et al, 2003) and Galectin-3 (Stillman et al, 2006), kill activated T cells. Galectin-4 contributes to exacerbation of intestinal inflammation by killing activated intestinal T cells (Hokama et al, 2004). Galectin-7 (Bernerd et al, 1999; Kuwabara et al, 2002) and Galectin-12 (Hotta et al, 2001) were also shown to cause cell death, but unlike Galectin-1,-2-,9 and -3, Galectin-7 and -12 killing effects required their intracellular localization. It was suggested that certain tumors can escape the immune response by secreting Galectins that kill activated T cells. Galectins are often expressed at high levels by cancerous cells, and the levels of expression correlate with the aggressiveness of a number of tumors (see review by Liu and Rabinovich, 2005). Galectins may also help tumor cells proliferating by stimulating Ras-mediated mitogenic signaling pathways, and both Galectin-1 and Galectin-3 interact with oncogenic Ras and facilitate signaling (Paz et al, 2001; Elad-Sfadia et al, 2004).

Previous work by Horie et al (1999) proposed that Galectin-1 enhances the growth of regenerating axons after axotomy. In their experiments, the growth of processes was assessed from DRG explants that after dissection, retained part of their central and peripheral projections. Addition of recombinant Galectin-1 increased the number of regenerating processes (Horie et al, 2004). These experiments however, were performed with Galectin-1 in its oxidized form. Importantly, Galectins contain 6 cystein residues that do not form disulfide bridges, and can thus be rapidly oxidized in solution. In its oxidized form, Galectins loose their ability to bind to β-galactosides (Lopez-Lucendo et al,

2004). Also, the oxidation state of Galectins affects the balance between dimeric and monomeric forms, and significant conformational changes likely yield different surfaces accessible for binding to different ligands. In our study, we found that reduced, but not oxidized Galectin-1 kills processes. In addition, co-treatment with lactose prevented the killing effect of reduced Galectin-1, indicating that the oxidation status of Galectins determines the protein's function. It is therefore unclear how oxidized Galectin-1 increases the number of growing processes from DRG explants (Horie et al, 1999). One possibility is that this effect was rather indirect, as recent work by the same authors showed that oxidized Galectin-1 acts on macrophages to recruit these cells after nerve injury (Horie et al, 2004). Thus, while Galectin-1 in its oxidized form may function as a factor that indirectly regulates nerve regeneration by acting on macrophages, the function of Galectin-1 in its reduced form as well as its cellular target/s remain unknown.

The killing action of Galectin-1 kills in our system did not require $p75^{NTR}$, as processes of ES cell-derived neurons lacking both *p75*^{NTR} alleles were also eliminated. Galectin-1 may thus act by binding to other cell surface receptors, such as various members of the $p75^{NTR}$ family. Galectin-1 was also shown to bind to integrin receptors (Moiseeva et al, 2003). Future experiments will investigate the expression of these in different neurons. Indeed, it is of interest that while ES cell-derived neurons and cortical neurons were vulnerable to Galectin-1, hippocampal neurons were not (data no shown). This neuronal type specificity may be due to the differential expression of cell surface molecules and/or of interactor proteins that couple extracellular factors to intracellular signaling. Another possibility is that Galectin-1 mediates its effects indirectly, by interfering with the adhesion of neuronal processes to substrate. For example, Galectin-1 can bind to extracellular matrix components such as laminin and fibronectin. This might produce changes in signaling through receptors involved in cell adhesion that could also trigger a degeneration process. Indeed, such a mechanism has been shown for Galectin-8 on several immortalized cell lines (Hadari et al, 2000; Levy et al, 2001; Zick et al, 2004). Whether cortical neurons

are more sensitive than hippocampal neurons to contact deprivation with their substrate is unknown at this point.

3.2.5) Functional specificity of Galectin-1 and Galectin-3

Structural differences exist among Galectin-family members. Galectins 1,2,5,7,10,11,13,14 and 15 have only one CRD, while Galectins 4,6,8,9 and 12 have two CRDs connected by a ~70 amino acids linker region. The prevailing idea is that one-CRD Galectins form dimers, while two-CRD Galectins exist as monomers (Liu and Rabinovich, 2005). Galectin-3 has one CRD but differs from all other Galectin-family members in that it contains unique short stretches of amino acids tandem repeats in its CRD (Cooper, 2002). Since Galectins can form both bivalent and multivalent binding sites, they have the potential to cross-link cell surface glycoconjugates, leading to the formation of clusters or lattices. They also mediate cell-cell or cell-extracellular matrix interactions.

We compared the effects of Galectin-1 with that of Galectin-3 and found that unlike Galectin-1, Galectin-3 did not kill control neurons. Galectin-1 and Galectin-3 are the most widely expressed members of the Galectin family in the organism. Their expression can be either segregated or overlapping in different organs (see review by Rabinovich et al, 2002; Hsu et al, 2004). Previous studies have compared the functions of these Galectins in other systems and reported on a number of differences and similarities. For example, both Galectins can localize to the nucleus and were ascribed functions in RNA processing (Patterson et al, 2004). Galectin-1 can promote the attachment of the HIV virus to cells while Galectin-3 prevents this Galectin-1-mediated attachment (Ouellet et al, 2005). Only galectin-3, but not Galectin-1, can protect cells from nitric oxide-induced programmed cell death when expressed intracellulary (Song et al, 2002). This protective effect was suggested to stem from the fact that Galectin-3 shares sequence homology with Bcl2, and might interfere with the action of pro-apoptotic Bcl2 members (Akahani et al, 1997). Relevant to our study are experiments showing that both Galectin-1 and Galectin-3 cause the death of T cells in a carbohydrate binding-dependent manner (Perillo et al, 1995; Fukumori

et al, 2003). However, the killing effects of these two Galectins are not redundant, and they are mediated by binding to different cell surface receptors (Stillman et al, 2006). Galectin-1 causes T cell death by binding to CD7 and does not bind to the receptor for transferring CD71, while conversely, Galectin-3 does not bind to CD7 and causes T cell death by binding to CD71 (Stillman et al, 2006). Binding of Galectin-1 and Galectin-3 to CD7 and CD71 respectively, induces clustering of these receptors (Stillman et al, 2006). Thus, it is possible that neurons in our system express receptors for Galectin-1, but not for Galectin-3, and that this is why they differ in their vulnerability towards these different lectins.

3.2.6) Expression and function of Galectin-1 in vivo

At present, little is known about the functions of Galectins in the nervous system. Using a well established in vivo model of cholinergic neuron degeneration, we find here that Galectin-1 expression is up-regulated by excitotoxicity. We focused on cholinergic neurons of the medial septum, a structure of the basal forebrain that contains large numbers of these cells (for review see Colom, 2006). Basal forebrain cholinergic neurons are important for cognitive functions including memory and learning, and those located in the medial septum project axons that innervate the hippocampus and thalamus. Importantly, these cells degenerate early on during Alzheimer's disease, and it is generally thought that part of the cognitive decline in affected patients is due to the loss of cholinergic transmission (Whitehouse et al, 1981). Interestingly, unlike most neurons residing in other regions of the adult nervous system, cholinergic neurons of the basal forebrain continue expressing $p75^{NTR}$ throughout life, and are therefore a suitable system to study the biology of this receptor in adult neurons. It appears that $p75^{NTR}$ is involved in the survival of these cells, since increased numbers of cholinergic neurons were found in different animals carrying mutations in the *$p75^{NTR}$* gene (Naumann et al, 2002). It remains open however, whether $p75^{NTR}$ controls only the survival of these cells, or if it is also involved in other neuronal functions, such as for example synaptic transmission.

We were encouraged to focus on this system by a previous study reporting on the increase in $p75^{NTR}$ levels in the medial septum after injection of kainic acid (Oh et al, 2000). Kainic acid is a glutamate receptor agonist and its injection caused an up-regulation of $p75^{NTR}$ immunoreactivity both in the cell bodies and in processes of cholinergic neurons. Similar to our results, excitotoxicity led to the loss of cholinergic neurons within 24 hours. The authors also showed that interfering with $p75^{NTR}$ using an antibody that somehow blocks its function prevented cholinergic neuron degeneration (Oh et al, 2000). Together with the increased number of cholinergic neurons detected in *$p75^{NTR}$*-deficient animals, these results suggest that the up-regulation of $p75^{NTR}$ triggers the degeneration cascade in these cells. While kainic acid is an AMPA receptor agonist, we obtained similar results using IBO, which is an NMDA receptor agonist. The mechanisms linking glutamate signaling to $p75^{NTR}$ are unknown, but it is tempting to speculate that different forms of excitotoxicity might lead to the activation of the same molecular machinery involved in neuronal degeneration. Importantly, it was also shown that the use of inhibitors of protein synthesis prevented excitotoxicity (Oh et al, 2000), perhaps because the prevention of Galectin-1 up-regulation. This is likely to be the case, as our in vivo results revealed that CEP-1347 prevented IBO-induced Galectin-1 expression and cholinergic neuron degeneration. In addition, it has recently been shown that JNK is activated in cultured cholinergic neurons isolated from the basal forebrain (Volosin et al, 2006), and we find here that blocking the JNK pathway with CEP-1347 also prevents the up-regulation of Galectin-1 in tau::*$p75^{NTR}$* neurons in vitro. In the future, similar experiments could be performed in *$p75^{NTR}$* deficient mice to directly test if $p75^{NTR}$ is required directly for the up-regulation of Galectin-1.

Our results using Galectin-1 extracellular decoys indicated that this lectin acts as an effector of $p75^{NTR}$-mediated cholinergic neuron degeneration. Interestingly, a soluble $p75^{NTR}$ fusion protein was more effective in preventing the degeneration of these cells than lactose or a Galetin-1 antibody. It is possible that in addition to blocking Galectin-1, the soluble $p75^{NTR}$ fusion protein also prevents binding of pro-neurotrophins to $p75^{NTR}$. Pro-neurotrophins can be up-regulated in the adult

nervous system after injury, and they bind to $p75^{NTR}$ with higher affinity than mature neurotrophins (Lee et al, 2001; Harrington et al, 2004). In line with this idea, a recent report showed that blocking the actions of pro-neurotrophins prevents kainic acid-induced degeneration of cholinergic neurons in the medial septum (Volosin et al, 2006).

At present, a killing function of Galectin-1 in the nervous system has not been reported. Analysis of mutant mice deficient for the *Galectin-1* gene revealed no gross abnormalities (Poirier and Robertson, 1993). However, 15 genes have been identified to date that encode for different Galectin members. While some of them differ structurally (see above), all members have a conserved CRD. Since the killing function of Galectin-1 in our system is likely mediated by its binding to sugar, it is possible that other Galectins functionally compensate for the absence of *Galectin-1*. A number of experiments using *Galectin-1* null animals may reveal important insights into the biology of these proteins. For example, if cholinergic neurons were still lost upon excitotoxicity, this would speak in favor of gene complementation and should encourage the search for other Galectin members that may be involved. Importantly, since Galectin-1 interacts with $p75^{NTR}$ and may play a role in its transport to the cell surface, it will be crucial to examine if the expression of $p75^{NTR}$ is compromised in *Galectin-1* null animals. While a role for oxidized Galectin-1 in promoting axonal regeneration has been suggested based on experiments using *Galectin-1* mutants (see below), the degeneration of neurons and of their processes in these mutants has not been reported. Based on our results, the prediction is that after injury to neurons or their processes, the absence of the *Galectin-1* gene would result in a reduced spread of degeneration.

Interestingly, Galectin-1 expression is up-regulated in the adult nervous system after nerve transection. For example, axotomy of the rodent facial nerve, which innervates the whiskers and is composed solely of motor axons, leads to a rapid up-regulation of *Galectin-1* mRNA in facial motoneuron cell bodies (McGraw et al, 2004a). Similar observations were made in spinal cord motoneurons after axotomy of spinal nerves (McGraw et al, 2004b). Furthermore, increased levels

of Galectin-1 protein were detected in sciatic nerve homogenates after axotomy (Horie et al, 2004). In addition, injury of the central projection of DRG neurons led to an increase in Galectin-1 immunoreactivity in the dorsal horn of the spinal cord (McGraw et al, 2005). These results suggest that the up-regulation in Galectin-1 levels may occur both at the mRNA and protein level, and that Galectin-1 protein may localize not only to cell bodies but also along axons. While the up-regulation in *Galectin-1* mRNA in the cell bodies of motoneurons and DRG neurons suggests that Galectin-1 may be synthesized by neurons themselves, the source of production of Galectin-1 that is detected in the distal part of cut nerves remains unknown. One possibility is that Galectin-1 is delivered to axons by non-neuronal cells, such as Schwann cells. These cells can massively up-regulate expression of $p75^{NTR}$ after different types of nerve injury and work by Horie et al demonstrated Galectin-1 immunoreactivity in Schwann cells distributed along the distal part of axotomized nerves. Thus, it is possible that Galectin-1 is up-regulated after nerve injury, not only by neurons, but also by other cells.

In the adult PNS, the degeneration of axons after nerve axotomy can be followed by the regeneration of new axons that innervate pre-existing targets (Nguyen et al, 2002). In the facial nerve, regeneration after axotomy can be assessed functionally by monitoring the recovery of whisker movement (Gilad et al, 1996). Interestingly, it has been shown that whisker movement recovery is retarded in *Galectin-1* null mice as compare to wild type (McGraw et al, 2004), and it was proposed that Galectin-1 acts as a positive signal for axonal regeneration. However, based on the results presented here, we propose the possibility that the retardation of functional recovery in the *Galectin-1* mutants stems from the failure of transected axons to be rapidly eliminated. It will be important to test this possibility by examining the number of surviving axons after facial nerve axotomy in wild type and *Galectin-1* null animals. In line with this idea, we note that axonal regeneration is also retarded in Wlds mutant mice, in which the over-expression of a mutant protein somehow prevents the degeneration of axons after axotomy (see below and Perry et al, 1990; Brown et al, 1991; Chen and Bisby, 1993).

Like Galectin-1, the expression of p75^{NTR} was also shown to be rapidly up-regulated in facial motoneurons after nerve injury (Ferri et la, 1998). Mice deficient for *p75^{NTR}* exhibited faster recovery of whisker movement. This may seem contradictory with a role of p75^{NTR} as an activator of process degeneration upstream of Galectin-1. However, the initial observation of faster recovery in *p75^{NTR}* mutants has been difficult to reproduce by others. In addition, as p75^{NTR} can also restrict the growth of processes (Yamashita et al, 1999), one possibility is that in the absence of *p75^{NTR}*, regenerating axons would grow faster towards the whisker.

One additional interesting aspect of the role of Galectin-1 in the nervous system is that it has been linked with human nervous system pathology. Inclusions containing Galectin-1 were found in human spinal motoneurons from ALS patients (Kato et al, 2001). Increased expression of p75^{NTR} was detected in spinal cord of ALS patients as well as in transgenic mice over-expressing a mutant form of human *superoxide dismutase-1* (*SOD1*), which develop ALS-like pathology (Lowry et al, 2001; Kust et al, 2003; Turner et al, 2003; Pehar et al, 2004). Since the knock-down of *p75^{NTR}* in *SOD1* mice delays the onset of motoneuron degeneration (Turner et al, 2003), it would be interesting to test if increased levels of Galectin-1 are detected in SOD1 mutants, and if this is prevented by knock-down of *p75^{NTR}*. If this were the case, interfering with Galectin-1's function using extracellular decoys may prove useful to prevent neuronal degeneration in ALS-like disease.

3.2.7) p75^{NTR}, RhoA and process degeneration

Our proteomic analyses also revealed changes in the expression of several proteins linked with the regulation of actin filaments. These changes correlated with increased RhoA activity, as well as with defects on actin filaments, and inhibition of Rho kinase prevented the degeneration of processes. These results are in line with previous in vivo reports implicating the Rho pathway in the degeneration of axons. Studies showed that RhoA is activated after spinal cord injury (Dubreuil et al, 2003; Madura et al, 2004; Yamagishi et al, 2005).

Importantly, this activation was localized to axons and blocking the Rho pathway prevented their degeneration. Furthermore, Dubreuil et al (2003) showed that the activation of RhoA was dependent on $p75^{NTR}$ expression. The defects we observed in actin filaments were not due to changes in the overall actin levels, but to spatial re-arrangements. This is in line with the role of RhoA in controlling the polymerization state of actin, typically by rigidifying actin filaments (Jaffe and Hall, 2005). In future experiments, it will be interesting to investigate if the changes we observed in the distribution of actin filaments are also detected in axons after injury in vivo.

Previous studies suggested that the beading and the fragmentation of microtubules are the earliest events affecting the cytoskeleton of degenerating processes (Watts et al, 2003; Zhai et al, 2003). However, the integrity of microtubules was compared to that of neurofilaments and less attention was paid to actin filaments. By contrast, we detected defects in actin filaments 24 hours before the microtubules became beaded. We do not know how $p75^{NTR}$ might control actin filaments and microtubules. One possibility is that $p75^{NTR}$ controls actin filaments via the RhoA/Rho kinase pathway and microtubules through the JNK pathway. Actin rearrangements could contribute to process degeneration in several ways. Actin filaments are highly enriched in the distal regions of growth cone filopodia and thin branches, and are highly dynamic structures that can be rapidly rearranged (Dent and Gertler, 2003). One possibility is that they act as sensors, detecting the activation of signaling pathways that trigger process degeneration. Also, rapid changes in the distribution of actin filaments might allow molecules involved in signaling pathways to arrange differently, determining the nature of signaling events within a process. In this context, it will be interesting to investigate if the activation of RhoA occurs at the regions where the beading of actin filaments takes place.

3.2.8) Control of process branching by $p75^{NTR}$

We found here that $p75^{NTR}$ controls not only the degeneration of processes but also their growth and branching via the JNK and RhoA pathways. It is now well

established that activated Rho has 'negative' effects such as the retraction of processes in neuroblastoma cells (Jalink et al, 1994, Kozma et al, 1997), the inhibition process formation in cultured neurons (Bito et al, 2000), and the retraction of dendritic branches in cultured hippocampal neurons (Nakayama et al, 2000; Wong et al, 2000). The RhoA pathway also mediates the inhibitory effects of the myelin-associated molecules Nogo, Mag and Ompg on axonal elongation (He and Koprivica, 2004). Interestingly, inhibition of p190RhoGAP, a negative regulator for RhoA in *Drosophila*, results in retraction of mushroom body axons (Billuart et al, 2001), suggesting that neurons may have mechanisms that actively repress the RhoA pathway. On the other hand, the role of JNK in process branching is at present less clear. One possibility is that JNK affects branching by targeting components of the cytoskeleton, such as microtubule associated proteins (Chang et al, 2003). Treatment with JNK and Rho signaling inhibitors promoted growth and branching in *tau::p75^{NTR}* and in control neurons. In addition, we found that *p75^{NTR}*-/- neurons develop more processes than control neurons, and that treatment with neurotrophins can promote process branching in control neurons. These experiments suggest that p75^{NTR} controls the growth and the survival of processes by the same signaling pathways. It is interesting that while neurotrophins affect branching of control processes, they can not interfere with their degeneration. It is likely that since p75^{NTR} is expressed at higher levels in *tau::p75^{NTR}* as compare with control neurons, its actions may become independent of neurotrophin ligand binding. This is in line with our results using deletion construct of *p75^{NTR}* lacking the extracellular domain.

What then determines whether a process stops branching or degenerates? One possibility is that this depends on the extent of activation of theses pathways. For example, when JNK and RhoA reach a certain level of activation, branching is arrested, but if the activation levels are higher or last for longer periods, then the process degenerates. These mechanisms could serve a role during the time of developmental process elimination. For example, several neurons that elongate long axons in development express p75^{NTR}. During their elongation, the activity of a p75^{NTR}/JNK-RhoA mechanism would prevent axons from extending

branches towards inappropriate intermediate targets. In the case that an axon has miss-targeted a branch towards an ectopic location, increased signaling via $p75^{NTR}$/JNK-RhoA would lead to the degeneration of this branch.

We also cannot exclude the possibility that in addition to JNK and RhoA, other signaling molecules are recruited by $p75^{NTR}$, and that some of them mediate only branching inhibition or degeneration. A number of proteins previously identified to act as scaffolds linking $p75^{NTR}$ to intracellular messengers might be involved, and they include caveolin-1 (Bilderback et al, 1997), Bex3/NADE (Mukai et al, 2003), TRAF6 (Khursigara et al, 1999; Ye et al, 1999), and the zinc-finger domain containing proteins NRIF1/2 (Casademunt et al, 1999) and SC-1 (Chittka and Chao, 1999). Other $p75^{NTR}$ intracellular interactors include the MAGE homology domain members NRAGE (Salehi et al, 2000) and necdin (Tcherpakov et al, 2002), and these seem to be involved in the control of programmed cell death. $p75^{NTR}$ can also recruit proteins with intrinsic catalyitic activity including the serine–threonine kinases IRAK (Mamidipudi et al, 2002) and RIP2 (Khursigara et al, 2001) that control interleukin and NF-κB signaling, and the protein tyrosine phosphatase FAP-1 (Irie et al, 1999). $p75^{NTR}$ signaling has also been shown to induce sphyngomyelinase activity, which leads to the production of ceramide, a lipid involved in the regulation of cell death (Dobrowsky et al, 1994; Brann et al, 2002). Finally, $p75^{NTR}$ is also known to activate the transcription factor NF-κB in Schwann cells (Carter et al, 1996). This transcription factor has well established roles in promoting cell survival, and a recent study has linked it with the control of process branching (Gutierrez et al, 2005).

3.2.9) Comparison with other models of process degeneration

To gain insights into the cellular mechanism underlying process degeneration, it is useful to compare the in vitro system used here with other models. In vertebrates, the most commonly used in vitro model of process degeneration is the culture of chick or rodent DRG explants. After plating, axons are allowed to grow radially from the explant, and then they are mechanically injured by cutting them (for review see Raff et al, 2002). Axonal material then accumulates in the

axon stumps indicating that axonal transport was affected, and components of the cytoskeleton including microtubules and neurofilaments are broken. After a lagging period, axons start to appear unhealthy at the ultrastructural level and develop irregular swellings along the entire process. Many axonal components accumulate at these swellings and the axon subsequently fragments. The direction of degeneration usually starts at the two axon stumps generated by the cut, and progresses away from them. This process lasts for 24 to 48 hours between the time of axotomy and the final fragmentation. This series of events were initially described by Waller (Waller, 1850) and are referred to as Wallerian degeneration (for review see Raff et al, 2002). Interestingly, Wallerian-like degeneration is also observed when cultured sympathetic neurons are deprived of NGF, which is essential to maintain their survival (see for example Koike et al, 1989).

In vivo, the elimination of processes occurs by at least two different cellular mechanisms. Small scale elimination of axonal and dendritic branches occurs by retractions, in which branches are swelled back and fuse with the main process. This takes place in development during the reduction of poly-innervated synapses, and the best known example is the neuromuscular junction (for review see Luo and O'leary, 2005). The neuromuscular junction in a muscle fiber is initially innervated by axon branches from different motoneurons. Axons innervating the same fiber start a competition process that involves synaptic activity, in which the axon exhibiting a higher degree of correlated activity remains innervating the junction, while others retract their branches (Balice-Gordon and Lichtman 1994). Branch retractions also occur in the mammalian CNS during the formation of eye-specific connections (Luo and O'leary, 2005). Retinal ganglion cells project axonal branches towards the dorsal lateral geniculate nucleus (dLGN) in the midbrain, and neurons in the dLGN project axons to the V1 area of the neocortex. Initially, branches from the two eyes form overlapping arborizations in the dLGN, but as development progresses, these arborizations are reduced by branch retractions, until forming individual eye-specific domains. The same process occurs to the geniculocortical projections

that input into the V1 area, and like in the neuromuscular junction, these events are controlled electrical activity (Lichtman and Sanes, 2003). In contrast to small-scale retractions, large-scale elimination of long processes occurs by a different cellular mechanism, in which processes degenerate. Degeneration is first evidenced by the formation of swellings along the processes and is followed by a series of events similar to those described in our system, as well as in models of Wallerian degeneration in vitro. In all cases, the regions between the swellings thin down and fragment, and the process appears as a dotted line.

In the developing mammalian brain, large-scale process degeneration has been described for layer 5 neurons of the neocortex (O'Leary and Koester, 1993). These cells initially project a long axon with branches that input into visual and motor cortical areas and into subcortical areas including midbrain, hindbrain and spinal cord. By early postnatal days, some branches degenerate depending on the functional specification of each neuron (O'Leary and Koester, 1993). Cortical neurons involved in motor control eliminate their projections towards visual areas and retain their more caudal projections, while neurons involved in the processing of visual information retain their projection to visual areas and eliminate their projections to motor areas (O'Leary and Terashima, 1988). Through this mechanism neurons eliminate more than 50% of their original projections and form adult topographic maps that relate to the spatial organization of the parental neurons. Large-scale degeneration was also described for retinal ganglion axons that input into the mouse superior colliculus or chick optic tectum. When reaching the superior colliculus, axons overshoot their final termination areas and form an excessive number of small branches. During post-natal days in the mouse, these branches degenerate and only those located at the termination area are retained (McLaughlin et al, 2003). It is now well established that the formation of these topographic maps is mediated by the ephrin signaling system (Luo and O'Leary, 2003). However, the degeneration of processes seems to depend mostly on neuronal activity and like with the dLGN, correlated activity prevents degeneration. Large-scale elimination of neuronal processes also occurs in invertebrates. As they undergo metamorphosis,

neurons of the *Drosophila* mushroom body eliminate specific axonal and dendritic branches and they elongate new ones after metamorphosis (Watts et al, 2003). By doing so, these neurons can serve different functions in larval stages and in adult life.

In the lesioned adult nervous system, various pathological conditions also lead to large scale process degeneration. These include amyotrophic lateral sclerosis, spinal muscular atrophy, peripheral neuropathies, and AIDS (for review see Coleman, 2005). At present, it is not known what triggers the degeneration of processes in most of these neuropathies, and it would be important to investigate if a molecular mechanism is shared with processes degenerating in vitro.

Process degeneration may also be central to neurodegenerative diseases. Widespread loss of processes is observed in Alzheimer's, Parkinson's, Huntington's and prion diseases (Coleman, 2005). Because most of the analyses were performed in post-mortem brains, it is difficult to determine the precise time at which processes degenerated. Thus, it is unclear whether process degeneration represented a key early event contributing to the first symptoms of these diseases, or if it is a secondary accompanying event. It appears however that in many neuropathies and neurodegenerative diseases the degeneration of processes precedes the death of cell bodies. Important insights came from animal models. For instance, accumulation of Huntingtin protein and of amyloid deposits in mouse models of Hungtington's disease and Alzheimer's disease, respectively, were shown to cause the degeneration of axons before the death of cell bodies (Li et al, 1999a; Li et al, 1999b Tsai et al, 2004). Thus, interfering with the degeneration of processes may represent a useful approach for at least preventing the symptoms of some nervous system diseases.

At present, it remains unclear how many mechanisms participate in the degeneration of neuronal processes, and if it is likely that a process similar to programmed cell death may be central to all of them. We note however that in our system, the morphology of degenerating *tau::75*^{NTR} processes resembles that of processes undergoing large-scale degeneration in vivo. This is based on the formation of swellings at different lengths along the processes and their

subsequent fragmentation into several small fragments. The speed at which *tau::75*^{NTR} processes degenerate is also similar to what has been described for axotomized DRG neurons and for NGF-deprived sympathetic neurons in Wallerian degeneration models (Zhai et al, 2003). In all cases, 24 to 48 hours elapse between the time at which the first swellings form, and the time at which processes fragment. Little is know about the timing of large scale degeneration in vertebrates in vivo, and it is possible that the speed of degeneration varies depending on extrinsic variables such as the extent and type of myelination, the molecular and electrical interactions with other cells, the age of the neuron and the metabolic state of the cell. The work in *Drosophila* however (Watts et al, 2003), indicates that the elimination of processes of mushroom body neurons paralleles in its timing to those observed in systems including ours.

3.2.10) Process degeneration versus cell body death

Cells undergoing programmed cell death can be observed in real time in vivo, for example during the development of the worm *C. elegans* using Nomarski imaging techniques (see for example Hersh et al, 2002). Apoptotic cell bodies change light reflection, they shrink and form membrane blebbings that separate from the cell body and are referred to as apoptotic bodies (for review by Raff, 1998). By contrast, it is more challenging to visualize the degeneration of processes in vivo, as these are typically close to the limits of resolution of light microscopy. Transgenic flies and mice expressing fluorescent proteins in only a small number of neurons proved to be useful for imaging processes in their natural environment (Feng et al, 2000; Watts et al, 2003). In particular, expression of different GFP variants using the Thy1 promoter has helped understanding the cellular mechanisms underlying the post-natal process of synapse elimination at the mouse neuromuscular junction (see for example Lichtman and Sanes et al, 2003). However, while the role of genes predicted to play a role in process degeneration starts to be investigated (see for example Watts et al, 2003), it remains open whether the elimination of processes represents an active series of events. This is in contrast with the extensive

knowledge obtained in the last two decades regarding the molecular mechanism operating during programmed cell death (see reviews by Ellis and Horwitz, 1991; Raff, 1998). Programmed cell death is an active process, as it depends on the activation of a molecular machinery that seems to be present in virtually all cells of multicellular organisms (Raff, 1998). This includes the caspases, a family of cysteine proteases that cleave proteins after aspartic acid residues. Caspases are divided into two classes; initiator caspases which are activated first, and effector caspases that are activated by initiator capsases and directly cleave several substrates. Programmed cell death can be triggered by extrinsic mechanisms that involve activation of cell surface receptors such as Fas and TNF receptors. The intrinsic pathway involves molecules localized to the mitochondria membrane and regulates its permeability. Activation of cell surface receptors by pro-apoptotic ligands starts the extrinsic pathway. These receptors activate caspase-8 and subsequently the effector caspase-3. Typically, Bcl2 and BH3 family members regulate mitochondrial integrity and prevent the release of the pro-apoptotic factors cytochrome C and Smac/Diablo, which induce the formation of an apoptosome consisting of APAF and caspase-9. When complexed, the apoptosome activates the effector caspase-3. Finally, Caspase-3 is responsible for the execution phase which involves chromatin condensation, DNA fragmentation, protein cleavage and membrane permeability.

Most critical molecules involved in programmed cell death seem to have now been identified. However, they seem not to play a crucial role in Wallerian degeneration models. Finn et al (2000) reported that caspase activity was not detected in degenerating axons, while it was present in dying cell bodies. Moreover, broad spectrum caspase inhibitors failed to rescue the degeneration of axons while they prevented the death of cell bodies (Finn et al, 2000). These results are in line with the experiments reported here, as we find caspase-3 activity in cell bodies, but not in processes of *tau::p75*^{NTR} neurons, and pan-caspase inhibitors can only prevent the death of *tau::p75*^{NTR} cell bodies.

Similar results were obtained in other studies investigating the participation in process degeneration of molecules typically involved in programmed cell death.

For example, over-expression of Bcl2, a protein located in the outer mitochondrial membrane that protects against programmed cell death (for review see Yuan and Horwitz, 2004), prevented the death of motoneuron cell bodies, but not the degeneration of motor axons (Sagot et a, 1995). Moreover, over-expression of Bcl2 can support retinal ganglion cell survival even in the absence of processes and of added BDNF (Goldberg et al, 2002). Finally, the BH3 family members BAX and BAK also do not seem to play a role in process degeneration. Activation of these proteins induces programmed cell death by forming pores in the outer mitochondrial membrane, causing the release of cytochrome C into the cytoplasm (Yuan and Horvitz, 2004). Studies using *BAX* and *BAK* deficient animals found that axonal degeneration was not retarded after axotomy as compared to wild type animals (Whitmore et al, 2003).

3.2.11) Interfering with process degeneration: The Wlds mutant and the ubiquitin-proteasome system

If the mechanisms involved in programmed cell death do not participate in the degeneration of neuronal processes, what do we know about the later? The early discovery of a mouse mutant (OLA mouse) helped shedding light on this important problem. This was discovered accidentally and was initially thought to result from a delayed invasion of macrophages after axotomy. The analysis of the mutation in question, now referred to as *Wallerian degeneration slow* (*Wlds*), revealed that it results from a gene triplication leading to a fusion protein that consists of the first 70 amino acids of UFD2/E4, a protein involved in polyubiquitination, and the full length Nicotinamide mononucleotide adenylyltransferase (Nmnat) (Mack et al, 2001). The excitement about the *Wlds* mutant stands from the discovery that axonal degeneration is greatly delayed after axotomy, both in vivo as well as in cultured mutant neurons (Perry et al, 1990). Furthermore, axotomized *Wlds* axons remain electrically active for several days after separating them from the cell body and over-expression of *Wlds* in wild type axons protects them from degeneration after axotomy. The most striking results regarding the *Wlds* mutant is that the mutation protects axons in

several mouse models of disease. One interesting mutant is the *pmn* mouse, in which a mutation in a tubulin chaperone leads to the improper folding of tubulin and results in symptoms that are similar to human spinal muscular atrophy (Bommel et al, 2002). Crossing *Wlds* to *pmn* homozygous mice rescued the degeneration of motor axons and cell bodies and it increased lifespan (Ferri et al, 2003). The *Wlds* mutation also prevented the degeneration of axons in two other models. Injection of 6-hydroxydopamine into the nigrostriatal pathway of mice leads to the loss of dopaminergic axons and is often used as a model of Parkinson's disease. The dopaminergic axons of *Wlds* mutants were found to be less vulnerable to these manipulations (Sajadi et al, 2004). Axon degeneration is also observed in mice homozygous for a mutation in the myelin basic protein P0. Crossing of these mice to *Wlds* mice reduced the extent of axon loss and promoted motoneuron survival (Samsam et al, 2003). Since the UPS plays a role in process degeneration (Watts et al, 2003; Zhai et al, 2003), it was initially thought that the protective effect of the *Wlds* gene product has something to do with it. However, the first 70 amino acids of UFD2 do not contain the region of the full length protein that is required for polyubiquitination, and it turned out that it is only the Nmnat region that protects axons. Araki et al (2004) tested this by over-expressing different constructs in axotomized axons in vitro and found that Nmnat, but not UFD2, protected axons as efficiently as the full length mutant protein. One point that remains difficult to explain is that while over-expression of *Nmnat* did not increase the total levels of NAD, exogenously applied NAD could protect axons (Araki et al, 2004). Moreover, the localization of the Wlds protein remains unclear (Coleman, 2005). One possibility is that Wlds acts on targets within the cell body and that these transmit a pro-survival signal to the axons. Araki et al (2004) suggested that Sirt1, a protein involved in chromatin deacetylation could play such a role. A further investigation of the mechanism of action of the Wlds mutation could provide an important venue for preventing process degeneration in patients, for example by expressing it after injury.

So far, the claim that the degeneration of axons is an active process is largely based in results obtained with the Wlds mutant (Raff et al, 2002; Coleman,

2005). This however, is somehow misleading as *Wlds* is a fused gene and it is a dominant mutation. It is thus possible that its action remains non physiological and at present, the Wlds protein has not been shown to directly participate in a physiological mechanism required for process degeneration. Interestingly, previous studies showed that the normal up-regulation of $p75^{NTR}$ observed in Schwann cells after nerve injury, is greatly impaired in the *Wlds* mutant (Brown et al, 1991). This is of interest as it suggests that the Wlds mutation might interfere with a molecular mechanism leading to increased expression of $p75^{NTR}$. Along this line, it would be interesting to test if the expression of Wlds prevents degeneration in our system, as we now know some of the molecules involved. More specifically, it would be important to examine whether the expression Galectin-1 and the activity of the JNK and Rho pathways are affected by Wlds.

In addition to Wlds, the involvement of the ubiquitin-proteasome system (UPS), which controls the proteolysis of regulatory or aberrant proteins and is essential for cell viability (Ciechanover and Schwartz, 1993), has also been investigated in the context of process degeneration. Experiments by Zhai et al (2003) showed that pharmacological inhibitors of the UPS delay process degeneration in axotomized DRG and NGF-deprived sympathetic neurons. Genetic evidence supports a role for the UPS in process degeneration. In *Drosophila*, the axons of γ mushroom body neurons (MB), which are required for olfactory learning and memory are lost during metamorphosis (Lee et al, 1999). Watts et al (2003) demonstrated that the stereotypic degeneration of these axons is prevented in flies carrying mutations affecting different components of the UPS machinery. For example, homozygous mutants for the first enzyme involved in the UPS cascade, the E1 protein, do not degenerate their MB axons (Watts et al, 2003). The differentiation of these neurons and their axonal elongation was normal in these mutants, suggesting a specific role of the UPS in process degeneration. These data are important as they point to the UPS as part of the machinery underlying process degeneration. However, it is not known how early in a yet unidentified signaling cascade/s the UPS acts. Since it is expected that the degeneration of

processes involves widespread protein degradation, it is possible that the action of the UPS lies closer to the end of the signaling cascade that triggers degeneration.

4. Conclusions and prospects

We exploited here the combination of the use of unlimited quantities of homogenous ES cell-derived neurons with the expression of *p75*^{NTR} from the endogenous *tau* locus. This new cellular system allowed us to perform both, experiments based on predictions about the role of molecules known to participate in programmed cell death and in p75^{NTR} signaling, as well as unbiased analyses based on proteomic comparisons prior to the degeneration of processes. The main findings are that processes and cell bodies are eliminated by distinct molecular mechanisms, and the identification of a new role for Galectin-1 in neuronal degeneration (see working model on Figure.23).

Our findings also indicate that the degeneration of processes is an active process, as it depends on a series of events involving the activity of enzyme proteins and of proteins up-regulated by degenerating neurons themselves. It therefore appears that neurons may have evolved mechanisms ensuring their own elimination when these cells are malfunctioning or damaged. Future experiments should investigate how common the molecular mechanisms described here are in other models of neuronal degeneration. Furthermore, our results suggest that interfering with these signaling pathways by either blocking the JNK or Rho signaling pathways, or by using extracellular decoys against Galectin-1, could represent a venue for preventing process degeneration in disease.

5. METHODS

Neuronal differentiation of ES cells
All ES cell lines were cultured and differentiated into neurons as described in Bibel et al. (2004).

Engineering of mouse ES cell lines
All genetically modified mouse ES cells were engineered by inserting the following cDNAs into the genomic *tau* loci of J1 wild type ES cell line: The G30 (*tau*$^{egfp/egfp}$) line was generated by K.L..Tucker (University of Heidelberg, Germany) and is previously described in Bibel et al. (2004).
The the *tau::p75*^{NTR} (*tau*$^{rat\ p75NTR/eGFP}$) line was generated by M. Bibel (Novartis, Basel). A *p75*^{NTR} cDNA was targeted to exon 1 of the *tau* locus. In brief, PmeI restriction site followed by an optimized Kozak sequence at the start codon was added to *p75*^{NTR} cDNA and NotI was engineered just after the stop codon. Thus, the endogeneous initiation codon of *tau* as well as the stop codon in front were retained and three additional basepairs in between inserted for the Kozak sequence. The following primers were used (restriction sites in bold, Kozak sequence in italics, start and stop codon underlined): 5`-TCC AGG **GTT TAA AC***C GCC ACC ATG A*GG AGG GCA GGT GCT G-3` and 5`-GAT C**GC GGC CGC** TCA CAC TGG GGA TGT GGC AG-3`. The targeting vector for homologous recombination in ES cells was constructed by insertion of the engineered *p75*^{NTR} cDNA in the PmeI and NotI site of a plasmid containing *Pgk-1* polyadenylation signal, the G418-selectable marker *Pgk-Neo*^{r} and 8.0 kb flanking genomic sequence. The linearized targeting vector was electroporated into ES cells that were already targeted by a similar strategy with GFP on one allele of *tau* (Tucker et al., 2001) with the difference that the G418-selectable marker *Pgk-Neo*^{r} was flanked by *loxP* sites. After electroporation of this line with supercoiled plasmids encoding Cre recombinase, a clone that had the neomycin cassette deleted was selected (E3). Southern blot analysis of G418-resistant colonies was performed as described (Harada et al., 1994). We also tested ES cells with a *tau*$^{+/rat\ p75NTR}$ genotype and obtained similar results to those obtained with the *tau*$^{rat\ p75NTR/eGFP}$ line (data not shown). The *p75*^{NTR} -/- ES cell line was engineered by C. Anahaim (Biozentrum, University of Basel) and details are described in her Ph.D. thesis.

Staining of ES cell aggregates
Sixteen µm thick cross-sections were rinsed in PBS and incubated for 30 min in blocking solution containing 10% serum and 0.2% Triton in PBS (7% Triton was used for Oct3/4 staining). Sections were then incubated with primary antibodies in blocking solution for 12 hours at 4°C. The following antibodies were used at the indicated dilutions: Isl1 (1:500, gift from S Arber), Brn3a (1:10000, gift from E Turner), pan-Trk C-14 (1:1000, Santa Cruz), GLAST (1:1000, Chemicon), Oct3/4 N-19 (1:20000, Santa Cruz), Sox2 AB5770 (1:3000, Chemicon), BrdU (1:1000, Sigma), and a p75^{NTR} serum raised against the bacterially expressed cytoplasmic

domain of rat p75 (1:1000). The antibodies 40.3A4 (Isl1, 1:1500), 4F2 (Lim1/2, 1:500), 81.5 C10 (MNR2/Hb9, 1:500), Pax6 (1:1000), 74.5A5 (Nkx2.2, 1:50), Nestin (1:10), RC2 (1:10), 50.5A5 (LMX1, 1:50), and Pax7 (1:500) were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa. In all cases, PBS was substituted for the primary antibodies to test for unspecific labelling of secondary antibodies. Sections were rinsed in PBS repeatedly and incubated with the following antibodies for 1 hour at room temperature: anti-rabbit Rhodamine Red X-conjugated and anti-mouse Cy3 (1:1000, Jackson), anti-guinea pig (1:1000, gift from S Arber). Secondary antibodies were combined with the nuclear stain Hoechst 33342 (10 μg/ml, Sigma). Sections were rinsed in PBS and mounted. Sections used for BrdU staining were previously incubated in 2 N HCl for 30 min at 37°C, then neutralized in 0.1 M sodium tetraborate for 30 min and rinsed in PBS. Pictures were collected with a Zeiss Axioplan2 Imaging fluorescent microscope and processed with Adobe Photoshop 7.0.

Chick embryo experiments

Fertilized chick eggs were incubated at 38.5°C and 80% humidity for approximately 42 hours until they reached the 19-21 somite stage. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Two ml of albumen was removed from the egg and a portion of the upper eggshell was opened. To visualize the embryo, drawing ink (Pelikan, A17) was dissolved in PBS (16 μl/ml) and injected under the blastoderm. One neural fold was removed over a length of 4 somites at the level of the forelimb bud by tearing the tissue with glass needles. RA-treated aggregates were incubated with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) at 37°C for 10 minutes. ES cell aggregates are typically heterogeneous in size and those corresponding approximately to the size of the gap to be filled were selected for the implantation experiments. RA-untreated aggregates were trypsinized for 6 minutes. After incubation with trypsin, one aggregate was transferred with a pipette tip on top of the missing portion of the neural tube and implanted manually using tungsten needles. By the end of these manipulations, the aggregate had become a lose cell aggregate which helped accommodating it in the appropriate position. Trypsin-treatment of aggregates was found to be essential as untreated aggregates remained compact and did not integrate in the host environment. After sealing and incubation for 6 days the embryos were removed from the eggs, examined for GFP fluorescence and fixed in 4% paraformaldehyde for 4 hours. Following incubation in 30% sucrose for 36 hours, they were embedded in cryomedium and stored at -80°C for cryosectioning.

Western blots

Cells were washed twice in cold PBS, scraped and lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) for 30 min. After centrifugation at 14.000 rpm the supernatant was stored at -80^0. Samples were resolved on 10% SDS-PAGE gels semi-dry transferred into nitrocellulose. The following antibodies were used at the indicated dilution in 1% milk: p75 ICD

(Promega, 1:1000) JNK (Santa Cruz, 1:1000), phospho-JNK (Cell Signaling, 1:2000), phosphor BAD (Upstate, 1:2000); Tuj1 (Babco, 1:2000). Secondary antibodies conjugated to HRP were diluted 1:2000 in 1% milk. Blots were developed using the enhanced chemiluminescence detection method (Amersham).

Pull down assays

The detection of GTP-bound RhoA (Upstate) was performed following manufacturer's guidelines. For co-immunoprecipitation of Galectin-1, 50 μg of each lysate (prepared as described in the Western Blot section above) was mixed with 10 μl of anti-$p75^{NTR}$ antibody (Promega) and 200 μl RIPA buffer containing 100 μM DTT, and the mix was incubated for 2 hour at 4°C. Thirty μl of Protein G-Sepharose slurry was the added and the mix was incubated overnight at 4°C. After repeated washes in RIPA buffer, the mix was denature and processed for Western blot for Galectin-1 as described in the above.

Transfection of neurons with *$p75^{NTR}$* deletion constructs

The plasmids for over-expression of full length *$p75^{NTR}$*, *$p75^{NTR}$* ΔECD, and p75ΔDD were previously described in Bibel et al (1998). Four day-old cultures were co-transfected for 36-40 hours with 0.16 μg/ml of pcDNA3 plasmid (Invitrogen) for GFP expression, and with 1.6 μg/ml of one of the p75 plasmids, using Lipofectamine 2000 following manufacurer's instructions (Invitrogen). The pcDNA3 plasmid alone was used as control. Cells were then fixed and stained for microscopy.

In vitro treatments of ES cell-derived neurons

The following reagents were applied to cultures: CEP-1347 (kind gift from Phil Barker, McGill University, Canada), fmk-Z-VAD, Y27632, HA1077 (all from Calbiochem), creatine, sodium pyruvate, EGTA, lactose, MG-132 (all from Sigma), WGA (Vector laboratories). Dimethyl sulfoxide, PBS or bovine serum albumin (BSA) were used as controls when appropriate. Recombinant mouse Galectin-1 and Galectin-3 (R&D Systems) were diluted in PBS containing 0.1% BSA and 50 μM DTT to avoid oxidation and used immediately upon reconstitution. In some experiments, DTT was omitted to enhance oxidation of recombinant Galectin-1 and the mix was incubated for 1 hour at 37°C prior to adding it to the cultures. For the labeling of mitochondria, Mitotracker-Orange (200 nm, Molecular Probes) was added to the cultures for 1 hour prior to fixation. The soluble $p75^{NTR}$ protein consists of the entire extracellular domain of $p75^{NTR}$ fused with an Fc region of a human antibody, and it was previously produced in our laboratory (for details see Ph.D. Thesis of David von Shack).

Immunocytochemistry

Cells grown in nitric acid-treated glass coverslips were washed in PBS, fixed in 4% paraformaldehyde at 37^0 for 25 minutes, and washed three times in PBS. Cells were then incubated for 30 min in blocking solution containing 10% serum and 0.2% Triton in PBS, and for 1 hour with primary antibodies diluted in blocking

solution at the indicated concentrations: Tuj1/βIII-Tubulin (Babco, 1:2000), active-Caspase3 (Chemicon, 1:1000), JNK (Santa Cruz, 1:1000), phospho-JNK (Cell Signaling, 1:2000), p75ECD (Chemicon, 1:600), p75ICD (Promega, 1:1000), CytochromeC (Abcam, 1:1000), GRIP-1 (Cell signaling, 1:2000), NF-M (Chemicon, 1:1000). Cells were then washed and incubated with secondary antibodies conjugated to Rhodamine, Cy2, or Cy3 (Molecular Probes, 1:1000) for 1 hour and mounted for microscopy. In some cases the nuclear stain Hoechst 33342 (Sigma, 10 μg/ml) was combined with the secondary antibodies. Pictures were collected with a Zeiss Axioplan2 Imaging fluorescent microscope and processed using Adobe Photoshop 7.0.

Quantitative analysis of neuronal morphology and degeneration
Counts were performed manually on randomly-shot pictures of cells double labeled with Tuj1 and Hoechst. Neuronal cell body death was assessed by the presence of a pyknotic nuclear morphology, characteristic of apoptosis as well as the presence of membrane swellings in the cell body. Neuronal processes exhibiting signs of beading and swelling were classified as degenerating. To quantify the extent of branching, the number of principal processes originating from the cell body and having at least two cell body diameters in length were determined for each cell in randomly-shot images. Results are representative of three independent experiments performed with a minimum sample size of 3 per group. Statistical significance was determined by Student's *t*-test and data are presented as the mean ± standard error.

Reverse transcription-PCR. Following RNA extraction and reverse transcription, a 215 bp fragment of *Galectin-1* mRNA was amplified with the primers CTCTCGGGTGGAGTCTTCTG (sense) and GCCTACACTTCAATCCTCGC (anti-sense).

Proteomic analysis using difference gel electrophoresis (DIGE)
All proteomic analysis was performed by the laboratory of J. Voshol, Novartis, Basel. On the second day after plating, the cells were washed with cold PBS followed by 200 mM sucrose in water, and lysed with DIGE buffer containing 7M urea, 2M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5. 2D-DIGE using minimal labeling was performed as previously described[27] using triplicate gels of 2 independent cell preparations. Gels were digitized with the Typhoon imaging system (GE Healthcare) and transferred to the Progenesis Discovery V.2005 software package (Nonlinear Dynamics, UK) for spot detection and matching. Matchtables were then exported to Expressionist Pro V.2 (GeneData, Switzerland) for statistical analysis. Differentially expressed spots (t-test $p<0.05$ and two-fold up- or down regulation) were excised from the gels and identified by MALDI-TOF-TOF mass spectrometry.

Rat experiments
Sprague Dawley rats between 220-240 grams were anesthetized with isoflurene-oxygen gas and placed in a stereotaxic frame (TSE Systems, Germany). A 28

gauge injection cannula (Plastic One, Roanoke VA, USA) connected to a microinfusion pump (CMA, Sweden) via a 0.28 mm ID polythene tubing (SIMS, Portex) was filled with PBS or with 0.3 mg/ml ibotenic acid alone or in combination with 250 mM lactose or 0.5 mg/ml anti-Galectin-1 affinity purified goat antibody (R&D Systems). The cannula was directed towards the MS by using the coordinates from the Paxinos and Watson atlas from Bregma, AP: +0.7, ML: +0.4, DV: 6 from dura. 0.3 μl infusions were microinjected over a 5 min period and the cannula was left in place for another 5 min to allow for recovery. Twenty six hours after operation rats were perfused with 4% PFA, the brain was removed, post-fixed in cold 4% PFA for 4 hours, cryoprotected in sucrose gradients and frozen for cryosectioning. Twenty μm thick coronal sections were collected onto pre-coated slides, rehydrated in PBS, incubated for 1 hour with blocking solution containing 10% normal donkey serum, 0.2% bovine serum albumen and 0.3% Triton-X100 in PBS, and then incubated overnight with the following antibodies in blocking solution at the indicated concentrations: rabbit anti-$p75^{NTR}$ (1:250, Promega); goat anti-Galectin-1 (R&D Systems; RD). Sections were repeatedly rinsed in PBS and incubated with species-specific Cy3-conjugated antibodies (1:1,000; Molecular Probes) combined with Hoechst 33342 (10 μg/m, Sigma) in PBS and mounted. Pictures were collected with a Zeiss CCD digital camera connected to a Zeiss Axioplan2 Imaging fluorescent microscope and processed using Adobe Photoshop 7.0. Cholinergic neuron numbers and the presence of processes in each neuron were determined by direct counts using a 950 x 325 μm^2 grid corresponding to the medial septum in six sections per animal. This area lies directly above an imaginary line traced between the top of the anterior commissure traversed by the midline. Six sections were scored per animal. Results are representative of three independent experiments performed with a minimum sample size of 3 animals per group. Statistical significance was determined by Student's *t*-test and data are presented as the mean ± standard error.

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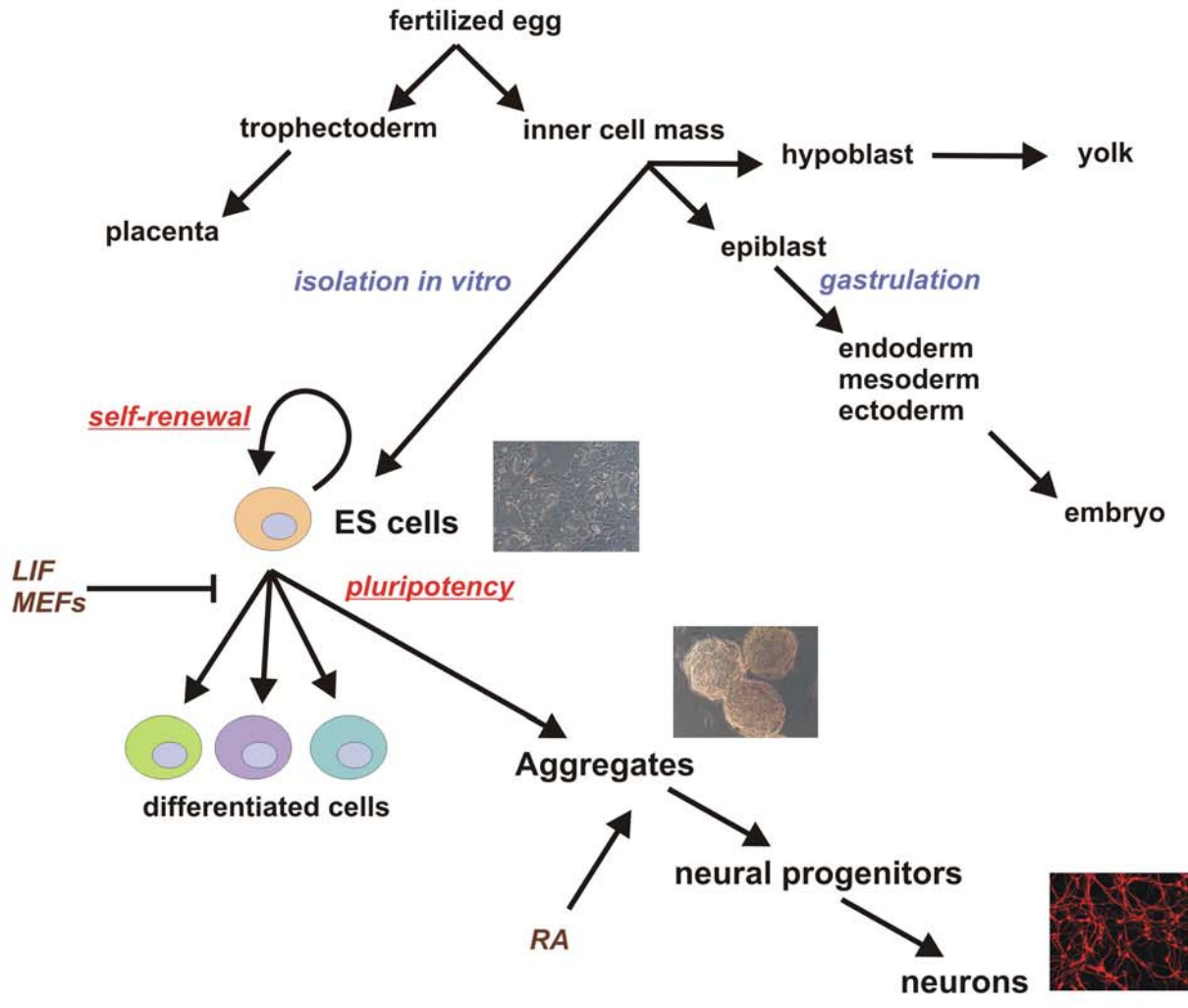
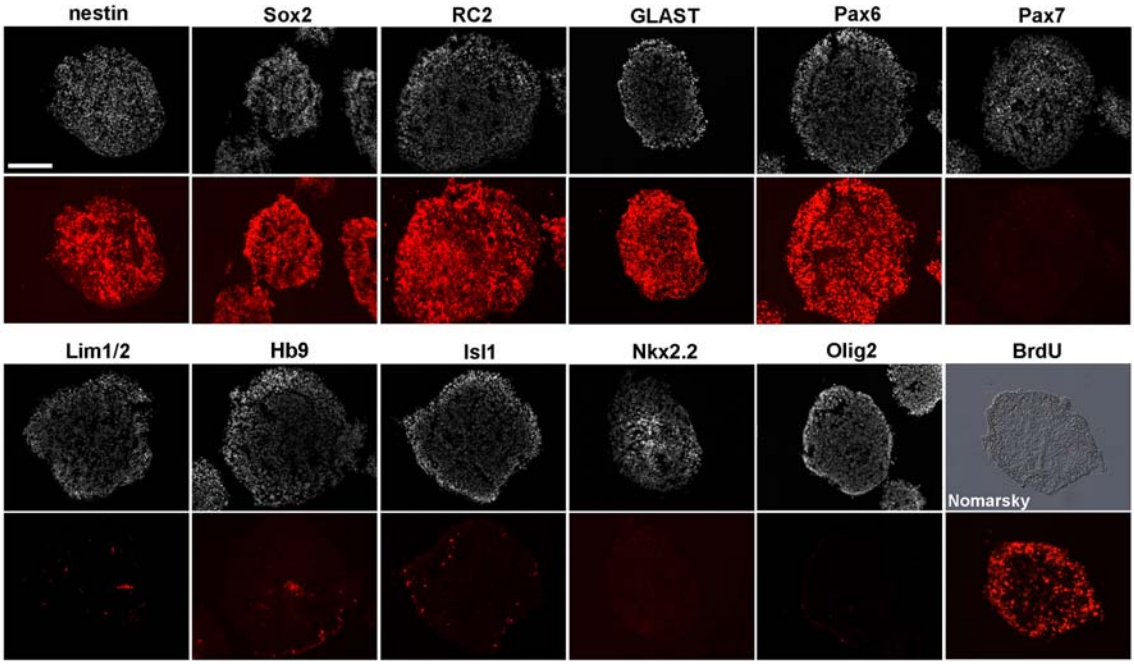
Fig. 1



Derivation and culture of mouse ES cells

ES cells are isolated from the inner cell mass (see Introduction). In vitro, culture of ES cells with LIF or mitotically inactivated MEFs supports their self-renewal and prevents differentiation. When these factors are removed, ES cells can differentiate into all cells types of the body. If grown without substrate, ES cells form aggregates, often referred to as embryoid bodies (EBs) in reference to their heterogeneity. However, in our case, virtually all cells seem to be identical and to remain undifferentiated until they are exposed to RA. This treatment shifts the entire population towards a radial glial cell phenotype (see Figure 2).

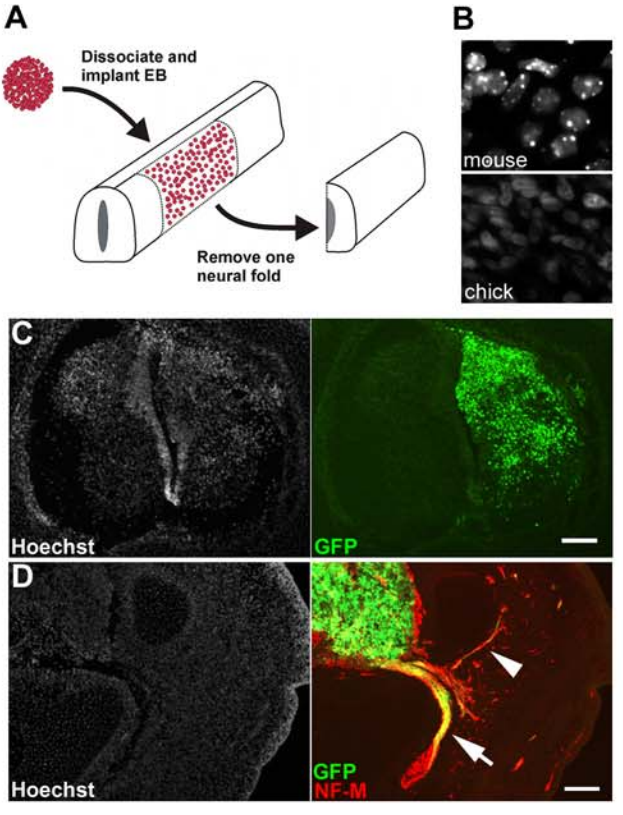
Fig.2



Antigenic characterization of RA-treated ES cells

Cryosections through ES cell aggregates four days after RA treatment, double labelled with the nuclear staining Hoechst (upper rows) and for the indicated markers (lower rows). The majority of the cells express nestin, Sox2, RC2, GLAST. This profile of marker expression is characteristic of neurogenic RG cells. Note that the expression of these markers is evenly distributed throughout the aggregates. RA-treated aggregates contain very few cells (<1%) expressing the markers Lim1/2, Hb9, and Isl1, and no cells expressing Pax7, Nkx2.2, or Olig2. A 3 hour pulse with BrdU shows the presence of proliferative cells in RA-treated aggregates. At least 5 aggregates from different experiments were analyzed for each marker. Bar represents 100 µm.

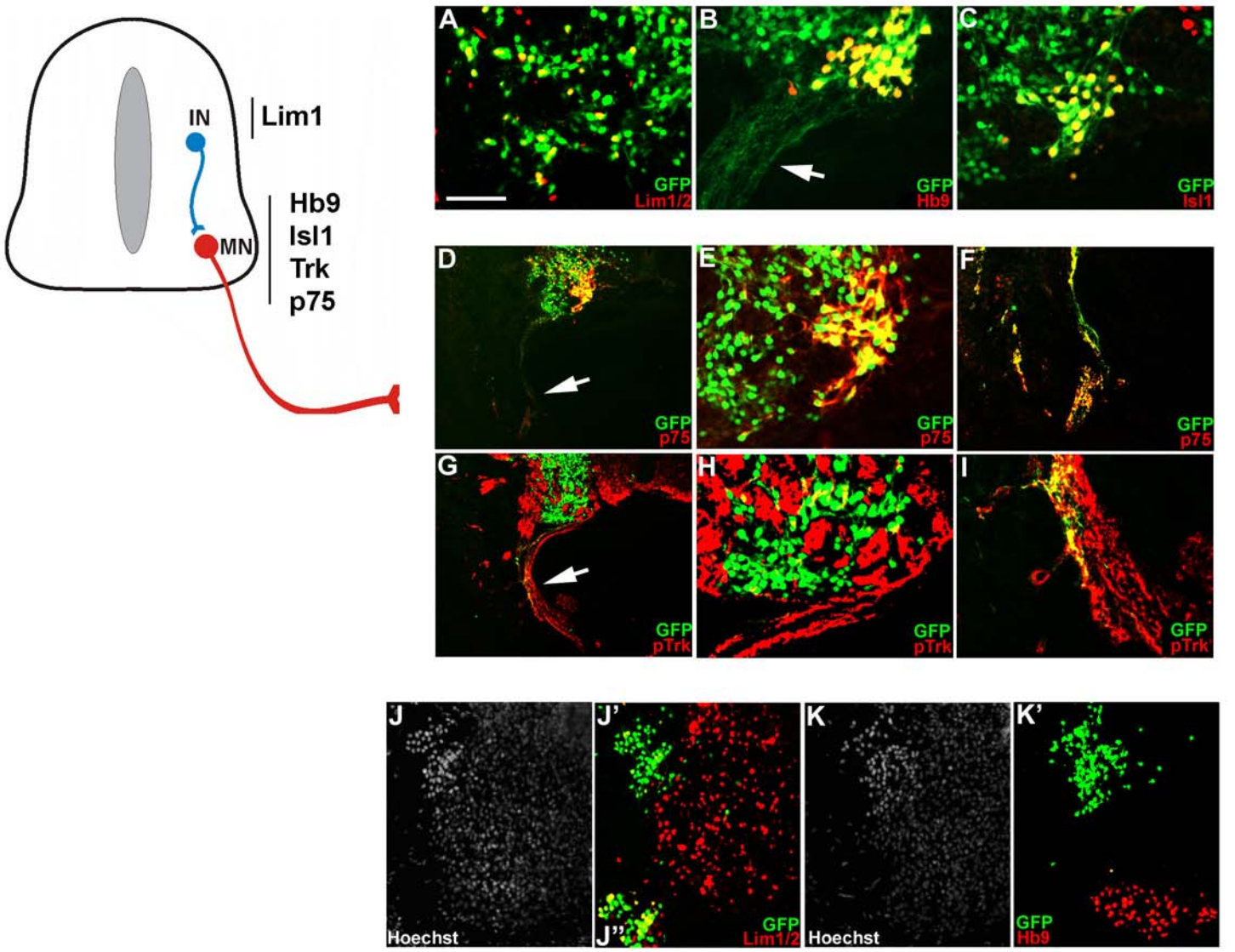
Fig.3



Integration of RA-treated ES cells in the chick neural tube

(A) A segment of one neural fold (~400 µm long) was removed and discarded at the level of the forelimb bud of E2 chick embryos (21 somites stage). The aggregates were gently dissociated with trypsin and implanted in the gap, and embryos allowed to develop until E8. (B) Identification of donor cells by nuclear morphology. Nuclear staining with Hoechst of 16 µm thick cryosections reveals differences in morphology between chick and mouse nuclei. (C,D) Transverse sections (16 µm thick) through chick embryos operated as described in (A). (C) Donor cells survive in large numbers and differentiate into GFP+ neurons throughout the operated side of the spinal cord. Left image shows nuclear staining revealing spinal cord morphology. Note that the operated side resembles the non-operated side in size and morphology. (D) GFP+ neurons populate the ventral spinal cord and extend axons towards the periphery. Axons can be observed projecting dorsally (arrowhead) and ventrally (arrow). GFP signal co-localizes with neurofilament (NF-M) staining (merged yellow). Bar represents 10 µm in B and 100 µm in C, D.

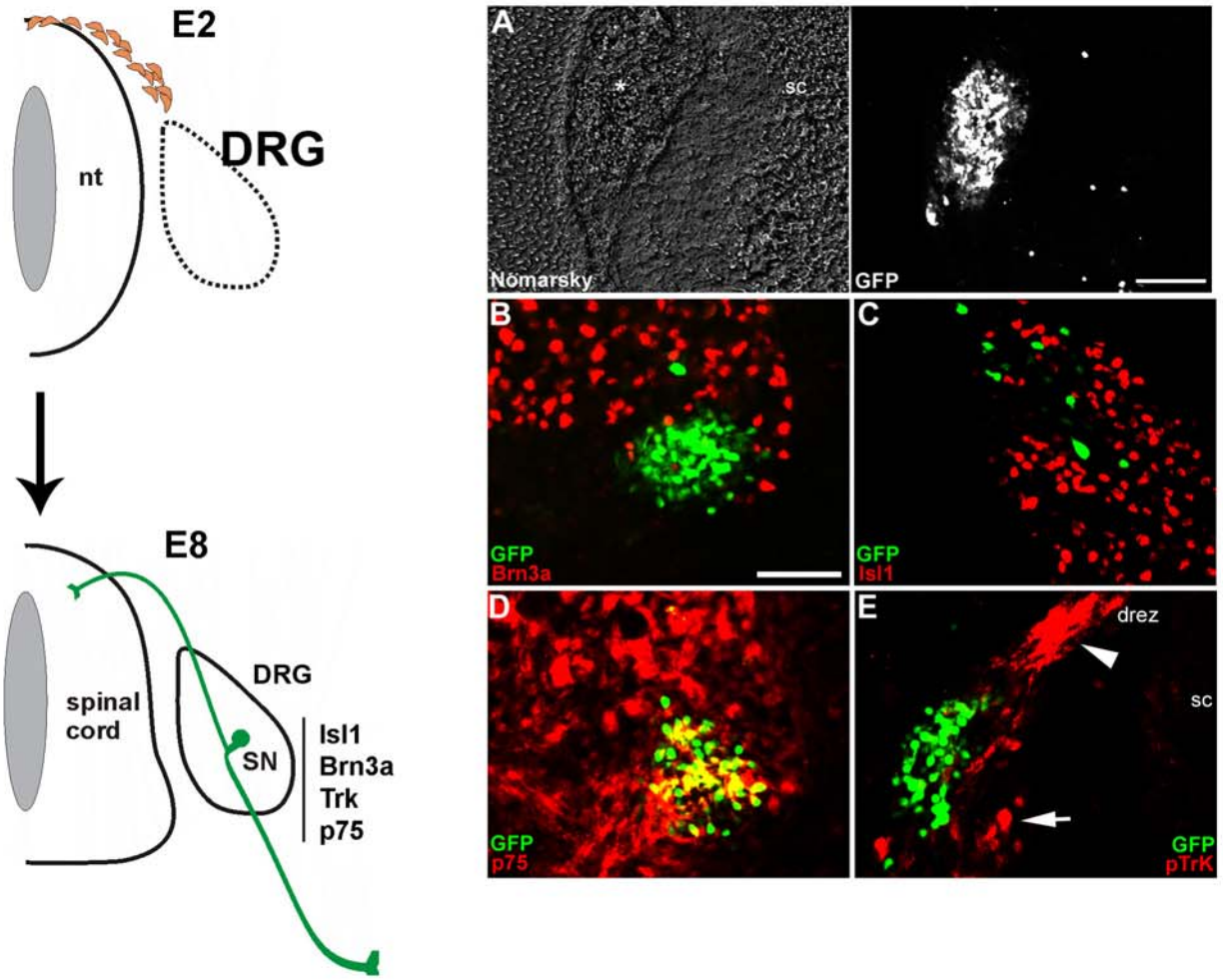
Fig.4



Progenitors from RA-treated Es cells differentiate into defined neuronal subtypes

The scheme represents the approximate location of interneurons (IN) and motoneurons (MN) in the chick spinal cord, and some of the characteristic markers expressed by these cells. (A-K) Transverse sections through E8 chick embryos operated at E2, showing endogenous GFP signal and labelled for the indicated markers. (A) GFP+ neurons located at mid levels in the spinal cord express Lim1/2 (merged yellow). Ventrally located GFP+ neurons express the motoneuron markers Hb9 (B) and Isl1 (C). Note in (B) the GFP+ axons (arrow) projecting from the Hb9+/GFP+ cells towards the periphery. (D-I) Expression of neurotrophin receptors by donor cells. (D) p75 expression is detected in ventrally located GFP+ neurons and their axons (arrow). (E) and (F) show at higher magnification the GFP+ somata and the axons indicated by arrow in (D). (G) Expression of Trk receptors is detected in ventrally located GFP+ neurons using pan-Trk antibodies. (H) and (I) show at higher magnification the GFP+ somata and the axons indicated by arrow in (G). (J-K) Consecutive sections of the same embryo double labelled by Hoechst (J, K) and by Lim1/2 (J') or Hb9 (K'). GFP+ cells located at the middle spinal cord express Lim1/2 (J' is a higher magnification of the same section), but do not express Hb9 (K'). Bar represents 50 µm in A-C, E, F, H, I, 100 µm in J-K' and 200 µm in D, G.

Fig.5



RA-treated ES cells fail to acquire sensory neuron characteristics

Scheme depicts neural crest cells migrating away from the neural tube (nt) at E2. By E8, these cells populate the DRG and differentiate into sensory neurons. These extend axons as depicted and express the indicated markers. (A) Nomarsky image (left) shows host DRG (*) colonized by GFP+ cells (right). Donor cells colonized much of the host DRG and the majority expressed GFP, indicating their neuronal differentiation. Note the lack of GFP+ processes. In this section mouse donor cells have migrated longitudinally, away from the site of implantation, and the spinal cord segment at this level is occupied mostly by chick cells. Expression of Brn3a (B) and Isl1 (C) in DRGs colonized by GFP+ cells. Note the absence of Brn3a and Isl1 expression by GFP+ cells (no merged yellow). (D) GFP+ cells express high levels of the p75 neurotrophin receptor in the DRG (merged yellow). (E) Expression of Trk receptors is detected in chick cells in the DRG (arrow) and in their axons (arrowhead) projecting to the spinal cord, but not in GFP+ donor cells. Cells expressing Brn3a or Isl1 always exhibited chick nuclear morphology (not shown). drez, dorsal root entry zone, sc, spinal cord. Bars represent 100 μm in A and 50 μm in B-E.

Table 1. Embryos operated in this analysis and integration of donor cells

Cell type implanted	Number of embryos operated	Number of embryos surviving operation	Number of embryos with GFP+ cells	% embryos containing GFP+ cells in VSC with axons	% embryos containing GFP+ cells in DRG
RA-treated cells	113	101	63	65.5 ± 27.3 (*n*=24)	59.2 ± 34.5 (*n*=27)
RA-untreated cells	86	71	56	74.9 ± 27.6 (*n*=16)	86.1 ± 16.4 (*n*=19)

Table 2. Neuronal differentiation of donor cells

Cell type implanted	% of mouse neurons in spinal cord	% of mouse neurons in DRG
RA-treated cells	78.5 ± 15.3	81.3 ± 5.5
RA-untreated cells	72.8 ± 19.2	65.4 ± 17.1

Table 3. Expression of neural markers by donor cells

Cell type implanted	Lim1/2 in SC	Hb9 in VSC	Isl1 in VSC	Brn3a in DRG	Isl1 in DRG
RA-treated cells	39.3 ± 10.2	73.6 ± 23.5	80 ± 26.4	0	0
RA-untreated cells	42.8 ± 10.9	15.4 ± 6.4	43.9 ± 36.8	36.7 ± 18.6	21.1 ± 4.7

Legend to Table 1

Analyses were performed on embryos from 16 independent experiments (9 with RA-treated ES cells and 7 with RA-untreated ES cells). Operated embryos were initially observed as whole mount and those showing a GFP signal at the implant region (third column) were sectioned. All these embryos contained numerous GFP+ cells in several continuous sections, but the number of these varied. GFP+ cells were frequently detected in the ventral spinal cord (VSC) extending axons towards the periphery (fourth column), and in the DRG (fifth column). In few sections, donor cells were detected only in the DRG, suggesting that they also migrated along the anterior-posterior axis. We failed to detect mouse nuclei in embryos that did not exhibit a GFP signal (~40%), suggesting that the donor cells did not survive in these animals. Results are (± s.e.m.) and n represents the number of embryos analyzed.

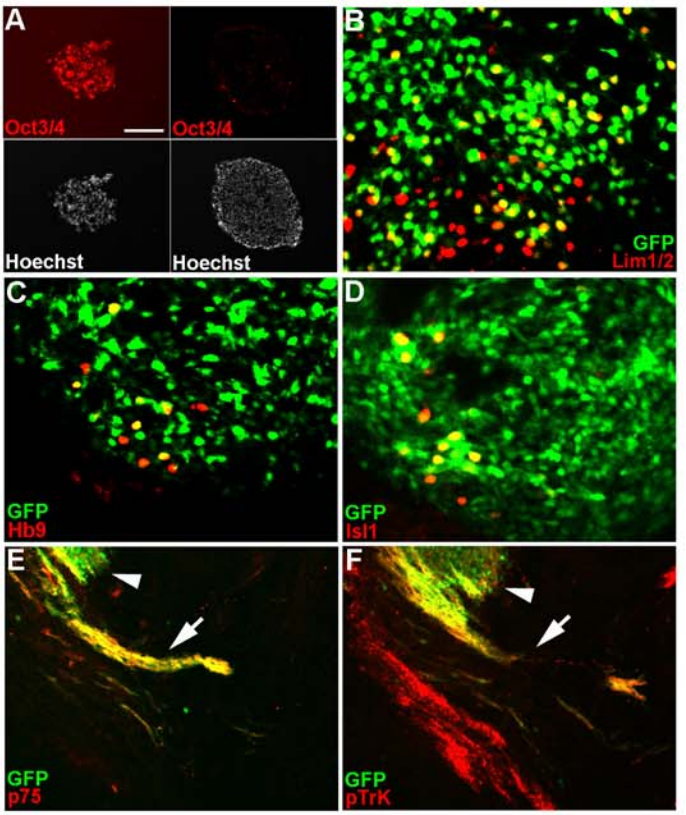
Legend to Table 2

The number of mouse nuclei was quantified in the spinal cord and in the DRG of embryos collected from 8 independent experiments. Results show the percentage (± s.e.m.) of Hoechst-stained mouse nuclei that expressed GFP, indicating their neural identity.

Legend to Table 3

The analyses were performed on sections from embryos implanted with RA-treated or with RA-untreated ES cells (5 independent experiments). Results show percentage (± s.e.m.) of GFP+ cells that expressed Lim1/2 throughout the spinal cord (SC), Hb9 or Isl1 in the ventral spinal cord (VSC), and Brn3a or Isl1 in the DRG.

Fig.6



Developmental potential of RA-untreated ES cells

ES cells were allowed to form aggregates for 36 hours and implanted into the chick neural tube. (A) Cryosections (12 µm thick) through aggregates double-labelled by Oct3/4 antibodies (upper row) and the nuclear staining Hoechst (lower row). After 36 hours (left column) the majority of the cells within the aggregates express Oct3/4. After 8 days (4-/4+ RA) very few cells within the aggregates maintain Oct3/4 expression (right column). (B-F) Transverse sections through E8 chick embryos operated as described previously showing endogenous GFP signal and labelled for the indicated markers. (B) ES cell-derived GFP+ cells located at the middle spinal cord express the interneuron marker Lim1/2 (merged yellow). Ventrally located GFP+ cells express the motoneuron markers Hb9 (C) and Isl1 (D). GFP+ cells in the ventral spinal cord elongate axons towards the periphery; both their somata (arrowheads) and axons (arrows) are labelled by p75 (E) and pan-Trk (F) antibodies. Bar represents 100 µm in A, 25 µm in B-D and 100 µm in E,F.

Fig.7

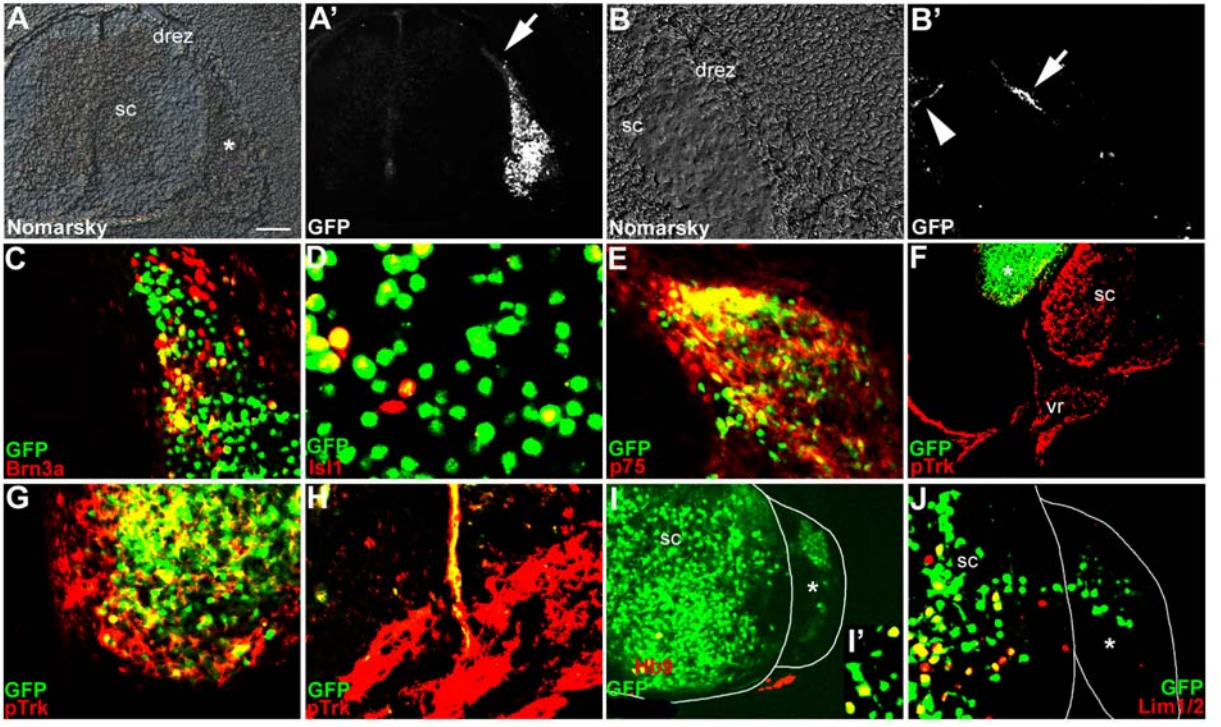


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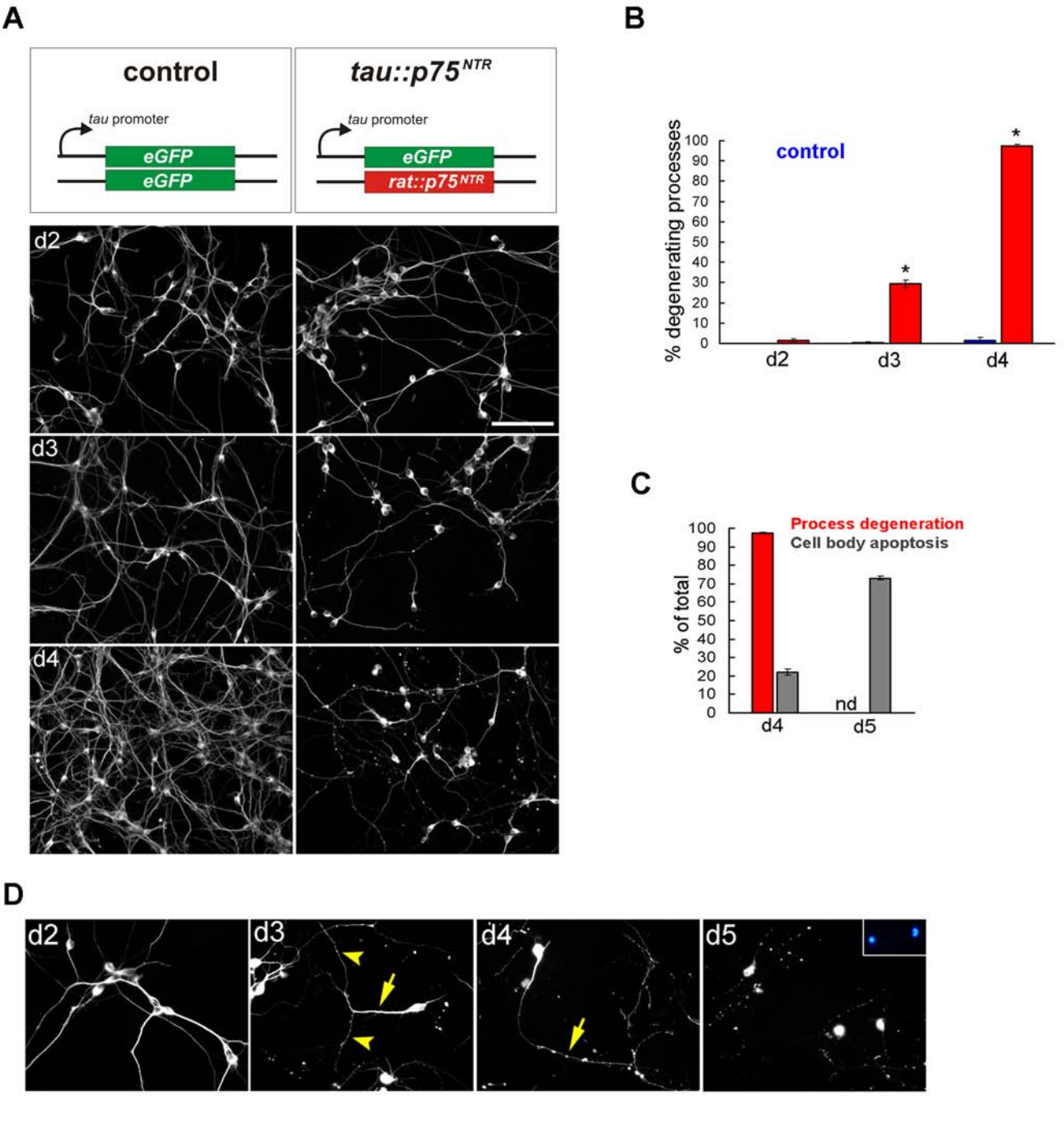
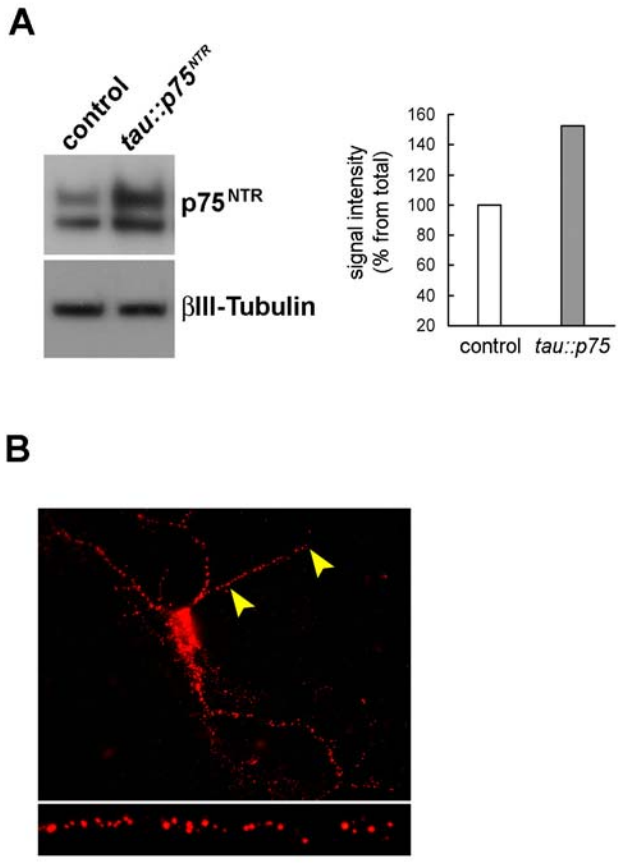
Fig.8



Expression of *p75^{NTR}* from the *tau* locus causes the degeneration of neuronal processes before the death of cell bodies.

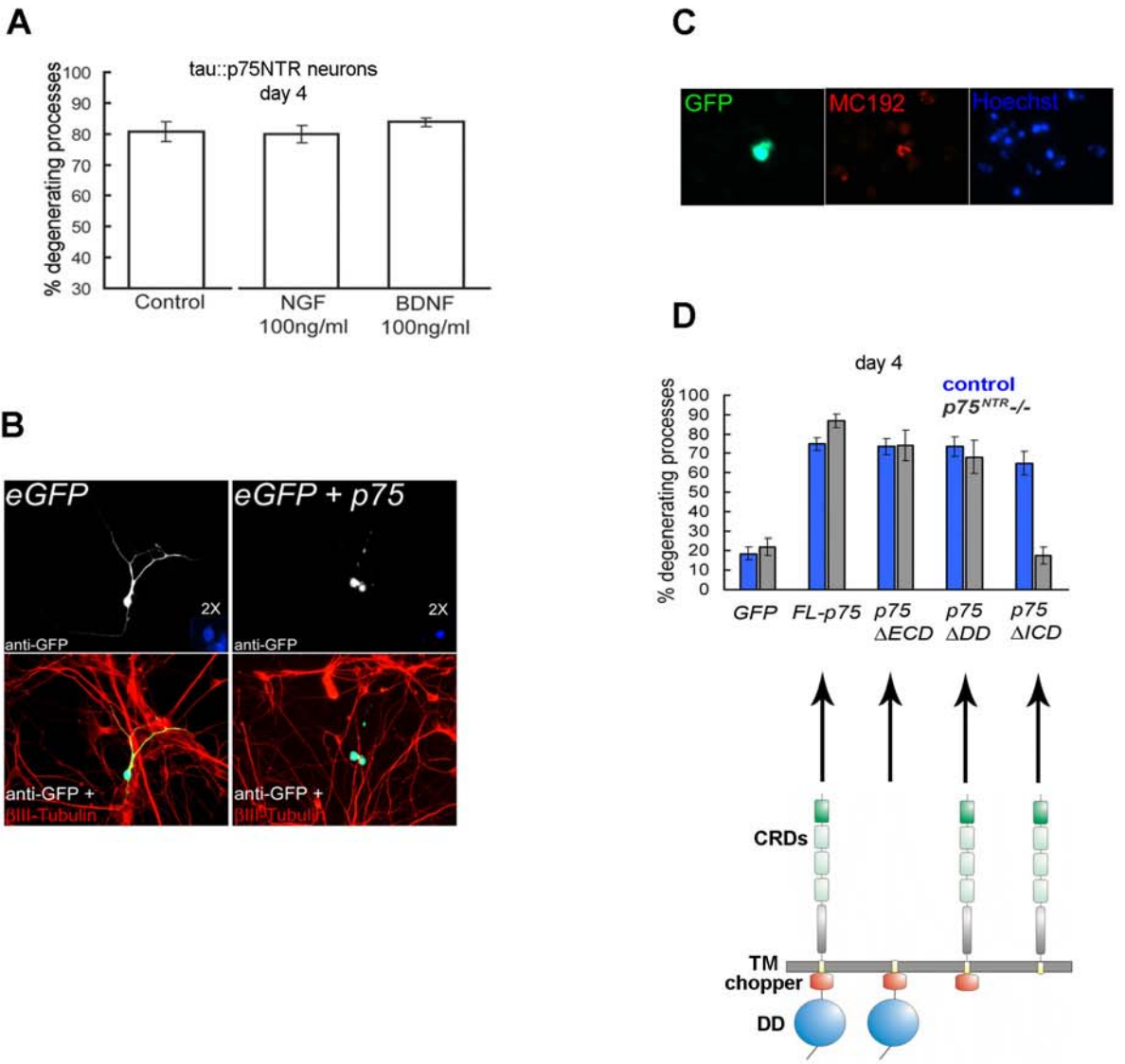
(A) ES cells were differentiated into neurons and stained for βIII-Tubulin 2-4 days after plating of progenitors. Process degeneration in *tau::p75^{NTR}* neurons starts at d3 and by d4, most processes are degenerating (A,B). (D) Representative examples of *tau::p75^{NTR}* neurons stained for βIII-Tubulin. Secondary branches (arrowheads) start degenerating at d3, while the principal processes originating from the cell body (arrow) degenerate at d4. Cell body apoptosis as evidenced by nuclear pyknotic morphology (inset, Hoechst staining) occurs by d5, when all processes had degenerated. (C) Quantitative comparison between process degeneration and cell body death in *tau::p75^{NTR}* neurons. Bar, 100 μm in A, 70 μm in D.

Fig.9



Expression levels and distribution of p75^{NTR}
(A) Western blot for p75^{NTR} at d2. The intensity of the p75^{NTR} band in *tau::p75^{NTR}* lysates is 57.1% higher than in control (see graph). (B) Non-permeabilized *tau::p75^{NTR}* d2 neurons stained for the extracellular domain of p75^{NTR}. Note that p75^{NTR} appears as clusters at the surface of processes and cell bodies. High magnification image shows p75^{NTR} clusters in the segment indicated between arrowheads. (C) The transferin receptor also appears to form clusters but these are distributed more evenly than p75^{NTR} clusters. GRIP-1 does not reveal the presence of clusters and exhibits an even staining. (D) Higher density of p75^{NTR} clusters is detected at regions where processes branch, indicated by the arrows.

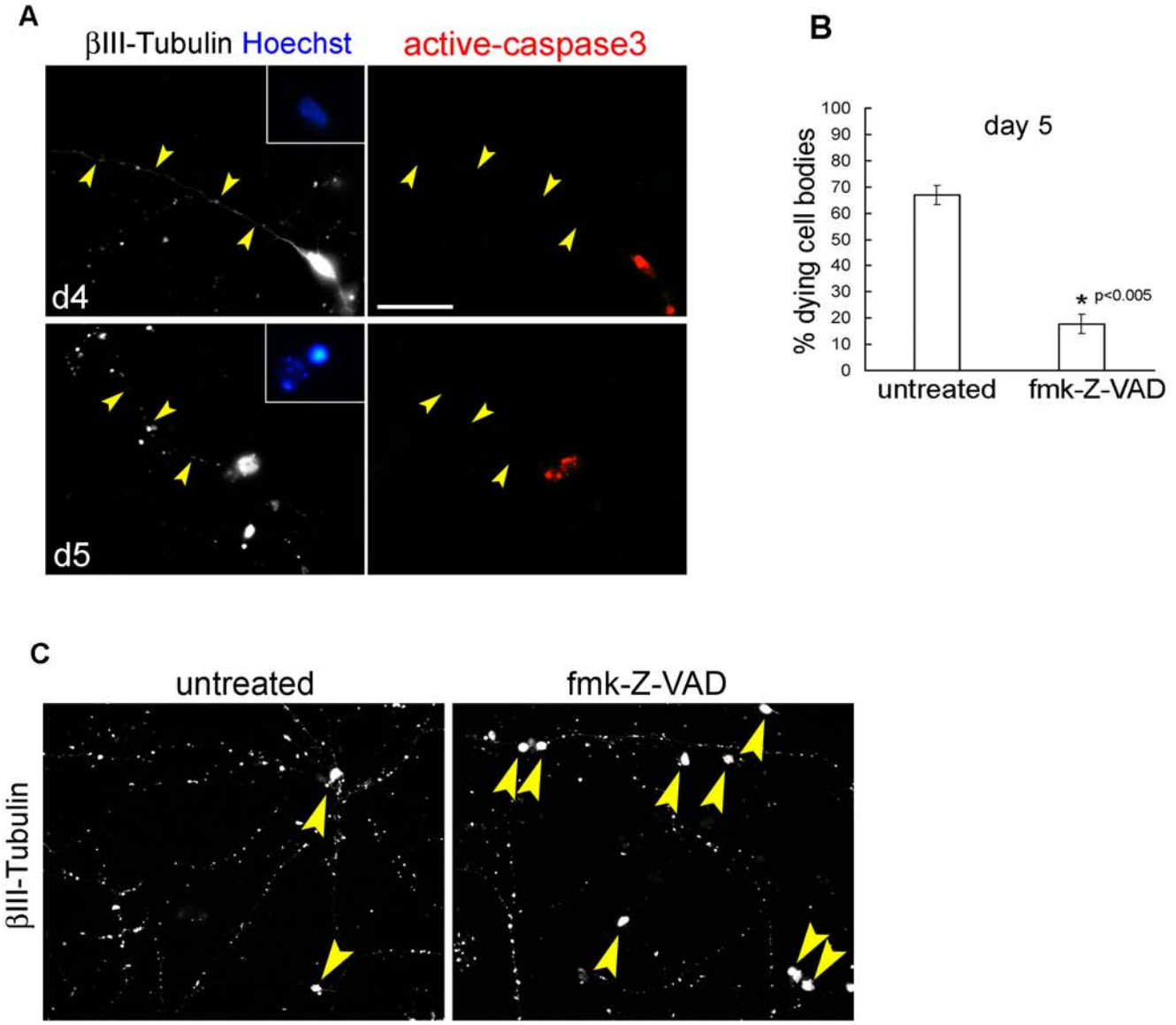
Fig.10



Treatment with neurotrophins and expression of deletion constructs

(A) Treatment of *tau::p75^{NTR}* neurons with NGF or BDNF (100 ng/ml) at day 2 does not prevent nor accelerate process degeneration at day 4. (B) ES cell-derived neurons were co-transfected with *GFP* and different *p75^{NTR}* deletion constructs. Transfection with GFP alone does not compromise neuronal survival (left panels). βIII-Tubulin labeling shows all neurons in the culture. Right panels show representative example of a neuron co-transfected with *GFP* and full length (FL)-*p75^{NTR}*. Note the almost complete degeneration of processes and the pyknotic morphology of the nucleus revealed by Hoechst staining (inset). (C) Staining with a rat-specific p75^{NTR} antibody (MC-192) shows expression only in co-transfected neurons that express GFP. (D) Quantitative analysis of process degeneration after transfection of control or *p75^{NTR}-/-* neurons with the following p75^{NTR} deletion constructs: FL- p75^{NTR}, p75^{NTR} ΔECD (lacking the entire extracellular domain), p75^{NTR} ΔDD (lacking the intracellular death domain) and p75^{NTR} ΔICD (lacking the entire intracellular domain). The scheme illustrates the deletion constructs used. CRDs, cystein-rich domains; DD, death domain; TM, transmembrane domain.

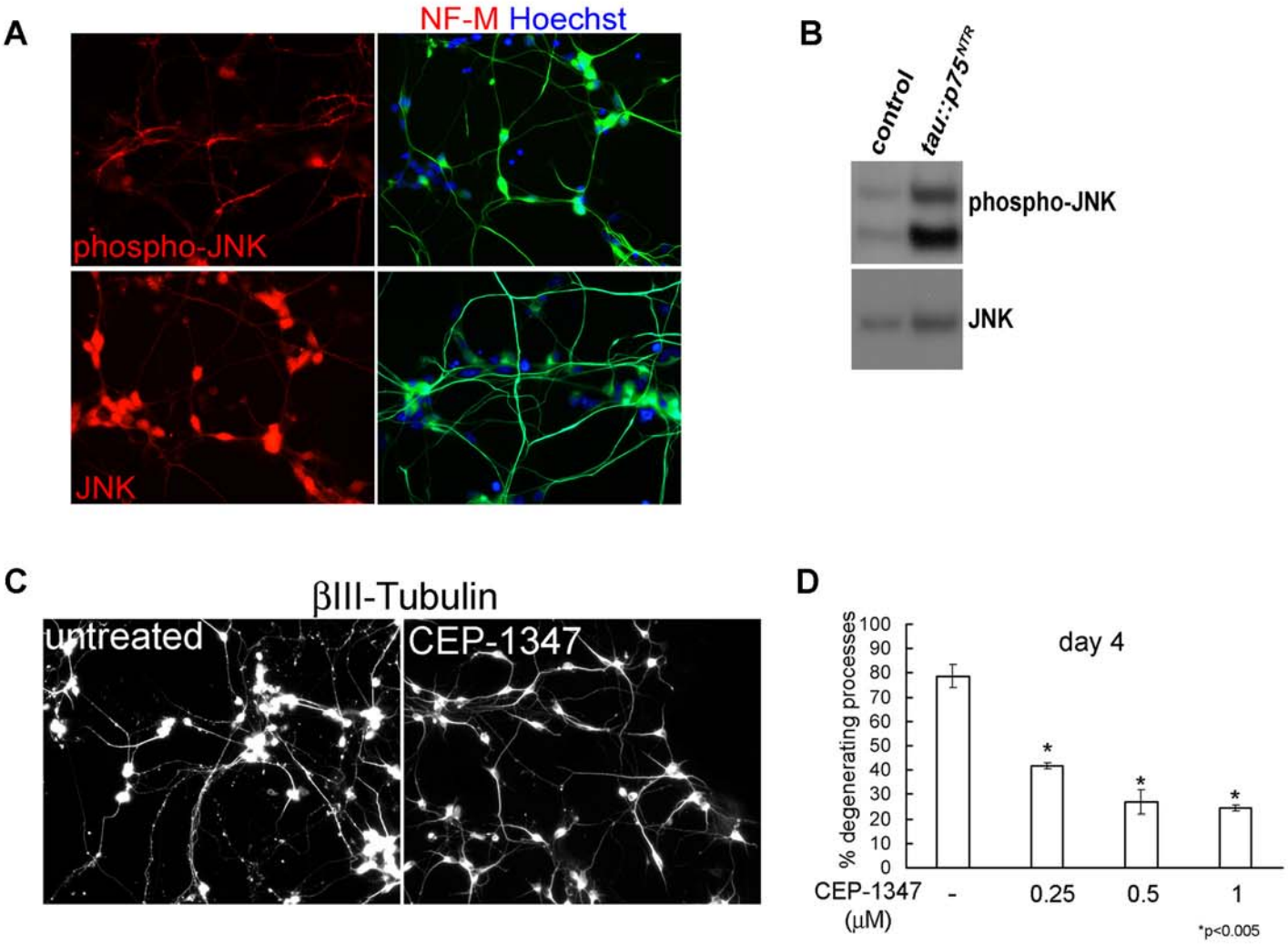
Fig.11



Process degeneration is independent of caspase activity

(A) *tau::p75*^{NTR} neurons double-labeled for βIII-Tubulin and an antibody against the activated form of caspase3. Caspase3 activity is detected neurons at d4 in the cell body, but not in the processes (arrowheads). By d5, caspase3 activity co-localizes with pyknotic nuclei in dying cell bodies. (B,C) Treatment of *tau::p75*^{NTR} neurons at d2 with the pan-caspase inhibitor fmk-Z-VAD (100 μM) rescues cell body apoptosis, but not process degeneration. In (C), remaining cell bodies in untreated and fmk-Z-VAD-treated *tau::p75*^{NTR} neurons at day 5 are indicated by arrowheads. Note that at this point all processes have degenerated in both conditions. **p* shows significant difference vs. untreated. Bar, 100 μm.

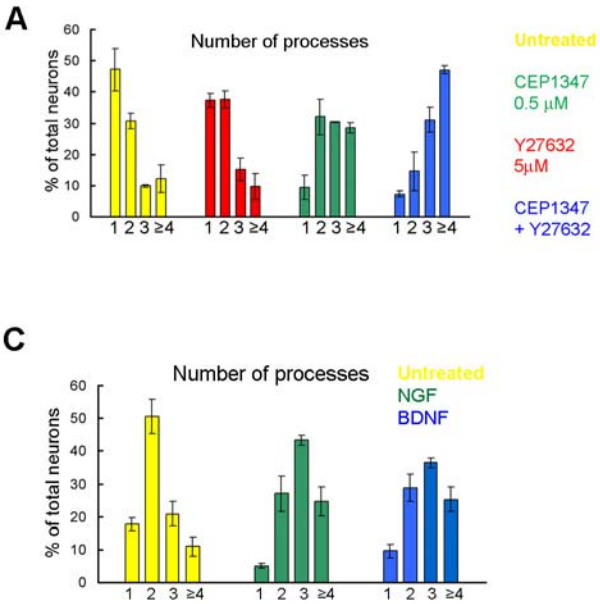
Fig.12



Process degeneration requires JNK activity

(A) Staining of *tau::p75^{NTR}* neurons at d2 shows phosphorylated JNK in processes, but not in cell bodies, while an antibody against total JNK shows reactivity in both neuronal compartments. (B) Western blot shows increased levels of phosphorylated JNK in *tau::p75^{NTR}* lysates at d2. Total JNK is detected in both lysates. (C,D) Process degeneration in day 4 *tau::p75^{NTR}* neurons is prevented by the JNK signaling inhibitor CEP-1347 (0.5 μM, applied at d2). (E) Western blot shows the levels of phosphorylated-BAD protein at day 1 and day 2 between control and *tau::p75^{NTR}* lysates. **p* shows significant difference vs. untreated. Bar, 50 μm in A and 100 μm in C.

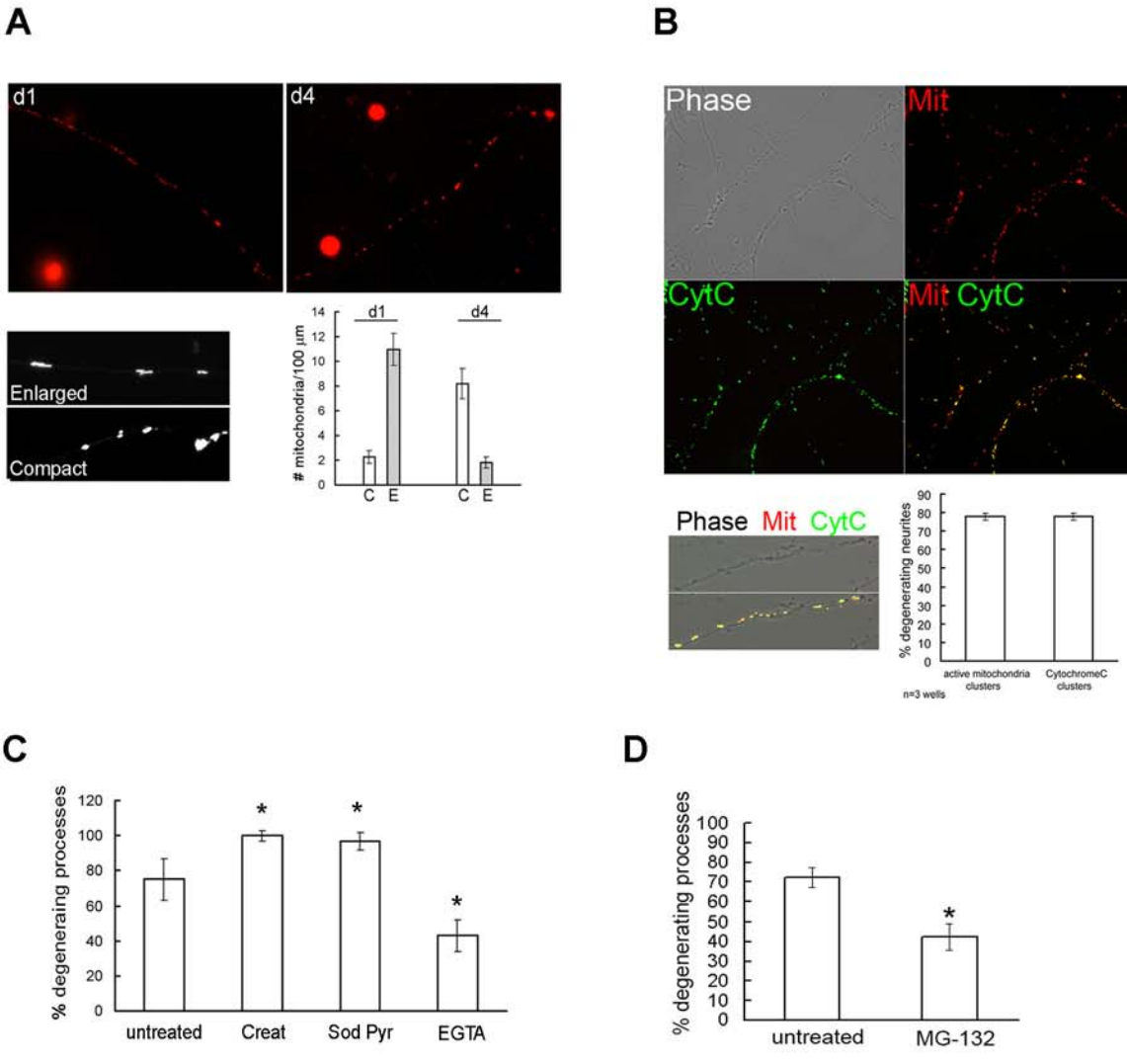
Fig.13



Negative regulation of process branching by p75^{NTR}

Quantitative analysis shows the number of processes growing directly from neuronal cell bodies in different ES cell-derived neurons and under different conditions at day 4. Each neuron was classified as having 1, 2, 3 or ≥4 processes. (A) Control neurons treated at day 2 with the JNK signaling inhibitor CEP-1347 (0.5 μM), the Rho kinase inhibitor Y27632 (5 μM) or both inhibitors together. (B) Comparison between untreated control and *p75^{NTR}-/-* neurons. (C) Control neurons treated at day 2 with NGF or BDNF (50 ng/ml).

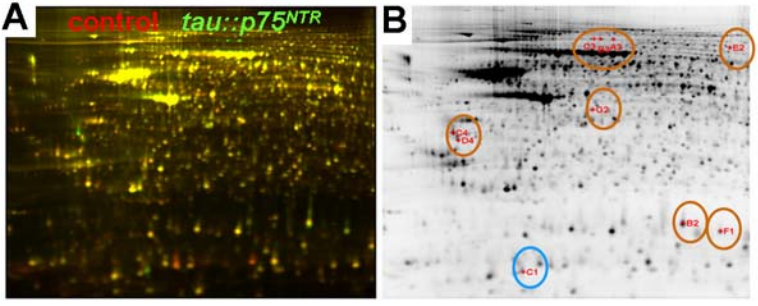
Fig.14



Role of mitochondria and of the ubiquitin-proteasome system in process degeneration

(A) Mitochondria in *tau::p75^{NTR}* neurons were labeled for 1 hour with Mitotracker-Orange at day 1 or day 4. Images show representative examples of mitochondria cluster along processes. The morphology of clusters was classified as either enlarged or compact (see black and white high magnification image). Quantitative analysis shows that most clusters exhibit an elongated morphology at day 1 and a compact morphology at day 4. (B) *tau::p75^{NTR}* neurons were double-labeled for Mitotracker-Orange and cytochrome C. High magnification image and graph show that ~80% of mitochondria clusters are labeled both for Mitotracker-Orange and cytochrome C. (C) Quantitative analysis shows percentage of process degeneration in *tau::p75^{NTR}* neurons at day 4 treated at day 2 with creatine, sodium pyruvate or EGTA. (D) Treatment at day 2 with MG-132 partially rescues process degeneration in *tau::p75^{NTR}* neurons at day 4.

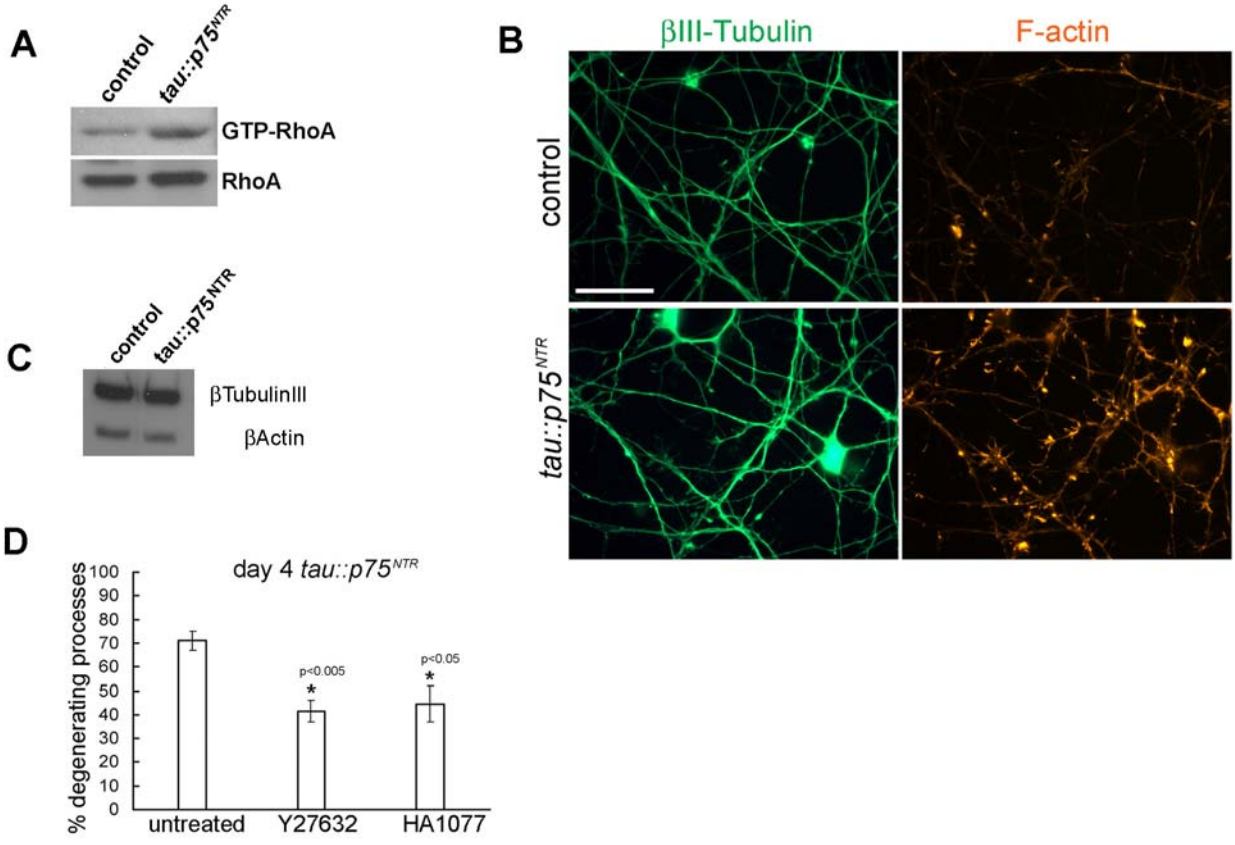
Fig.15



Proteomic analysis

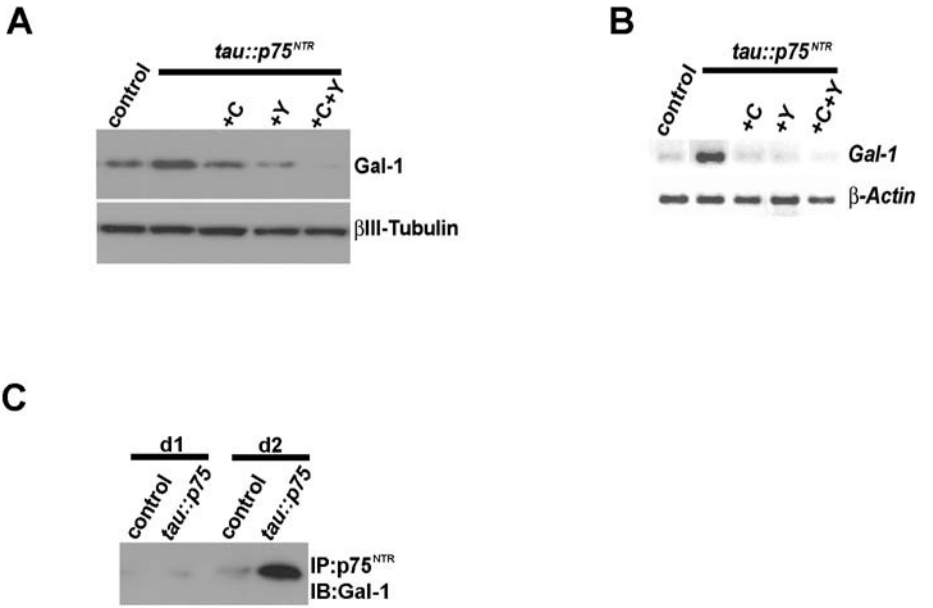
Control and *tau::p75*^{NTR} neurons were lysed at day 2 and the proteins in each lysate were labeled with a different fluorescent marker. (A) 2-dymensional-polacrylamide gel electrophoresis (2D-PAGE) shows fluorescently-labeled protein spots of control (red) or *tau::p75*^{NTR} (green) lysates. Note that the majority of the proteins are detected in both lysates (co-localization appears yellow). (B) The spots indicated were differentially expressed and were identified by mass spectrometry as: A3 B3 C3, gelsolin; B2, cofilin; F1, destrin; G2, calponin-3; C4 D4, tropomyosin; E2, caldesmon (brown circles) and C1, Galectin-1 (blue circle).

Fig.16



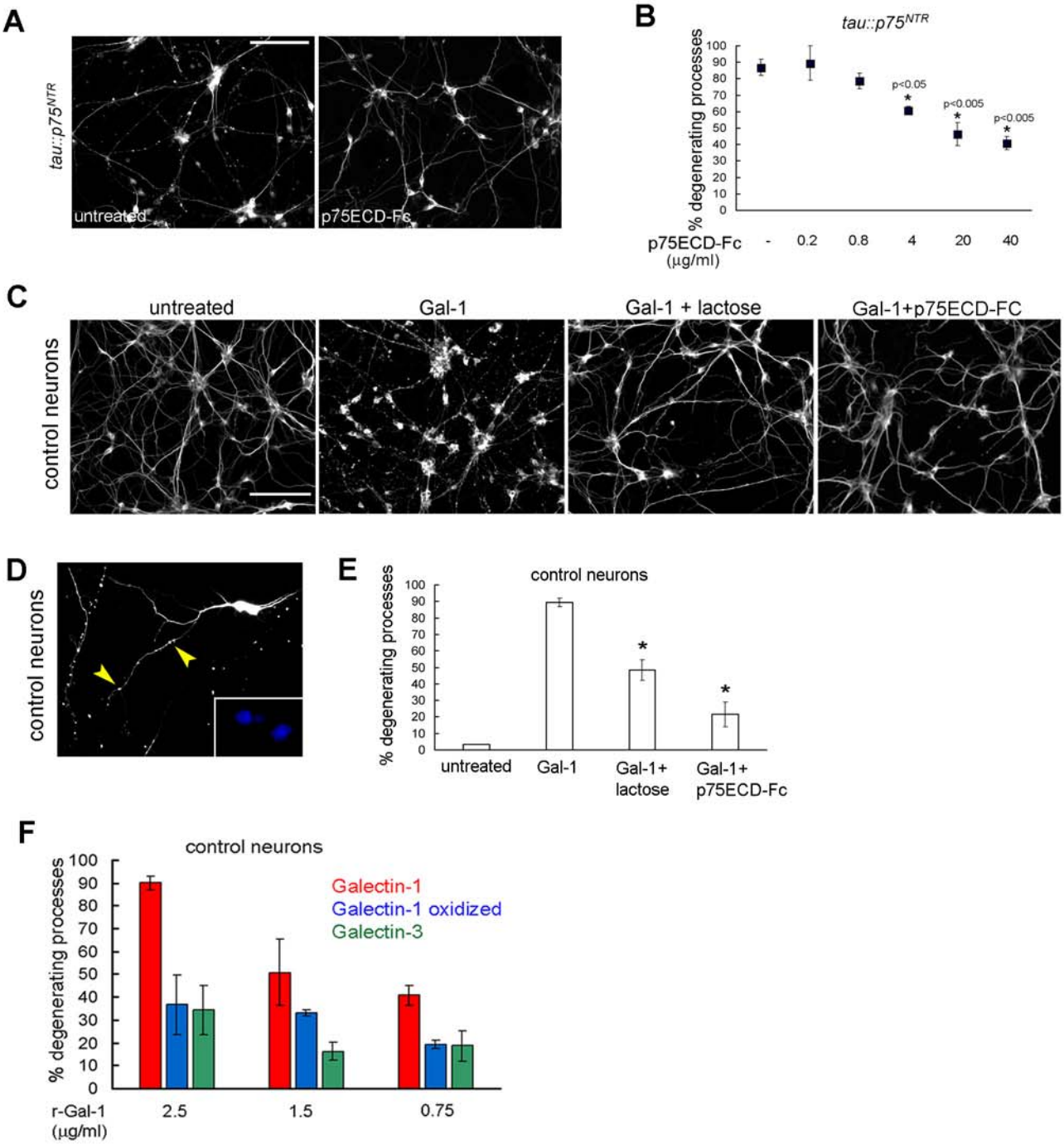
Involvement of RhoA signaling on p75^{NTR}-mediated process degeneration
(A) Increased levels of GTP-bound RhoA are detected in *tau::p75^{NTR}* lysates at d2. The levels of total RhoA are similar between lysates. (B) Staining of processes at d2 with Phalloidin-Rhodamine reveals abnormal beading of actin filaments in *tau::p75^{NTR}* neurons. At this point the microtubules in *tau::p75^{NTR}* cells do not exhibit signs of beading nor fragmentation and are indistinguishable from control. (C) The levels of total actin and tubulin are similar between control and *tau::p75^{NTR}* neurons at day 2. (D) Treatment of *tau::p75^{NTR}* neurons at d2 with the Rho kinase inhibitors Y27632 (2.5 μM) or HA1077 (5μM) prevents process degeneration at day 4. **p* shows significant difference vs. untreated. Bar, 30 μm.

Fig.17



Galectin-1 levels in ES cell-derived neurons and interaction with p75^{NTR}
Western blot (A) and RT-PCR analyses (B) show increased Galectin-1 protein and mRNA levels in *tau::p75^{NTR}* as compared with control cells at d2. Treatment of *tau::p75^{NTR}* neurons at d1 with 0.5 μM CEP-1347 (+C) and/or 2.5 μM Y27632 (+Y) prevent Galectin-1 up-regulation. βIII-Tubulin (A) and *β-Actin* (B) were used as control. (C) Co-immunoprecipitation experiment shows that Galectin-1 can be detected by Western blot after pull down with p75^{NTR} antibodies. Note that higher levels of Galectin-1 are pulled from *tau::p75^{NTR}* than from control lysates at day 2.

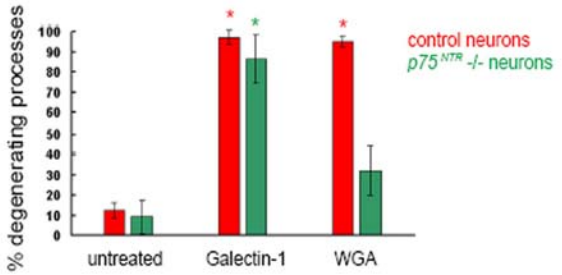
Fig.18



Killing of neurons by Galectin-1

(A,B) *tau::p75*^{NTR} neurons were treated at day 2 with a recombinant protein consisting of the extracellular domain of p75^{NTR} fused to the Fc region of a Human antibody. By day 4, process degeneration is prevented in a concentration-dependent manner. (C,E) Recombinant Galectin-1 (2 μg/ml) applied at d3 causes neuronal process degeneration in control neurons at d5. Co-treatment with lactose (30 mM) prevents the killing effect of Galectin-1 (βIII-Tubulin staining). (D) High magnification image shows a Galectin-1-treated control neuron. Arrowheads point at a degenerating process while the cell bodies are still healthy (inset, Hoechst staining). (F) Quantitative comparison between the effects of reduced Galectin-1, oxidized Galectin-1, and reduced Galectin-3. Note that at the concentrations at which reduced Galectin-1 kills, oxidized Galectin-1 and reduced Galectin-3 do not kill. **p* shows significant difference vs. Galectin-1 treatment alone. Bar, 100 μm in A and C and 50 μm in D.

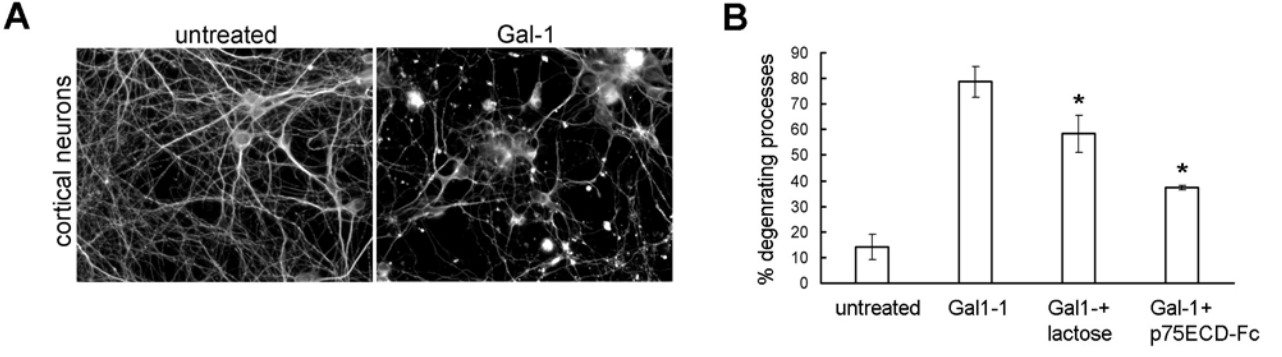
Fig.19



Comparison between the killing effects of Galectin-1 and WGA

Quantitative analysis shows the extent of process degeneration at day 5 caused by treatment of control or $p75^{NTR}$ -/- neurons with recombinant Galectin-1 or WGA (applied on day 4). While treatment with Galectin-1 kills both control and $p75^{NTR}$ -/- neurons, WGA kills control neurons, but not $p75^{NTR}$ -/- neurons.

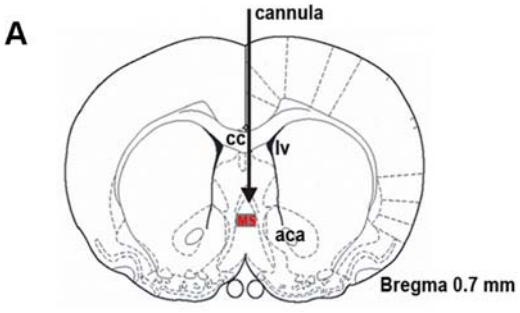
Fig.20



Vulnerability of cortical neurons towards Galectin-1

(A,B) Treatment with Galectin-1 (3 μg/ml applied at d5) of neurons isolated from E16.5 mouse cortex causes process degeneration at d7. Note that processes are affected before the cell bodies. **p* shows significant difference vs. Galectin-1 treatment alone. Bar, 50 μm.

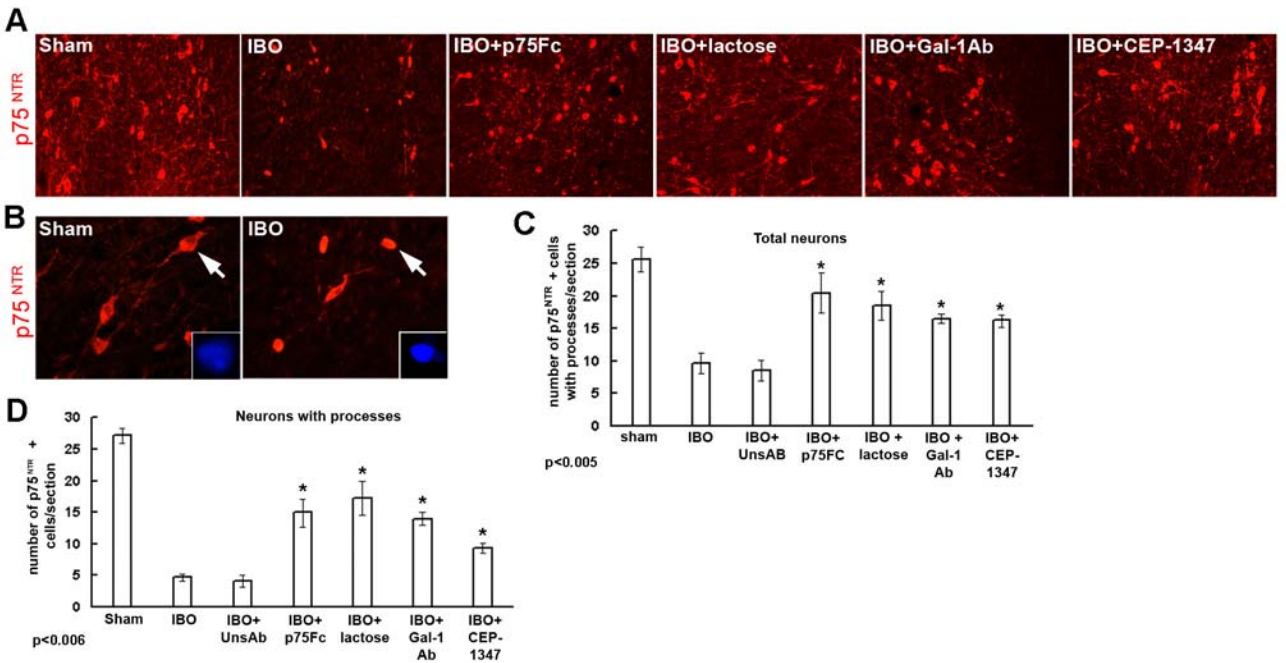
Fig.21



Galectin-1 is up-regulated by excitotoxicity in vivo

(A) Ibotenic acid (0.3 mg/ml) was stereotaxically injected into the medial septum as depicted in the scheme. The shaded box indicates the area of the medial septum where cholinergic neurons are normally concentrated and where the analysis was performed. (B) Ibotenic acid increases Galectin-1 immunoreactivity in the MS after 26 hours. Co-injection of CEP-1347 prevents Galectin-1 increased immunoreactivity. Bar, 100 μm.

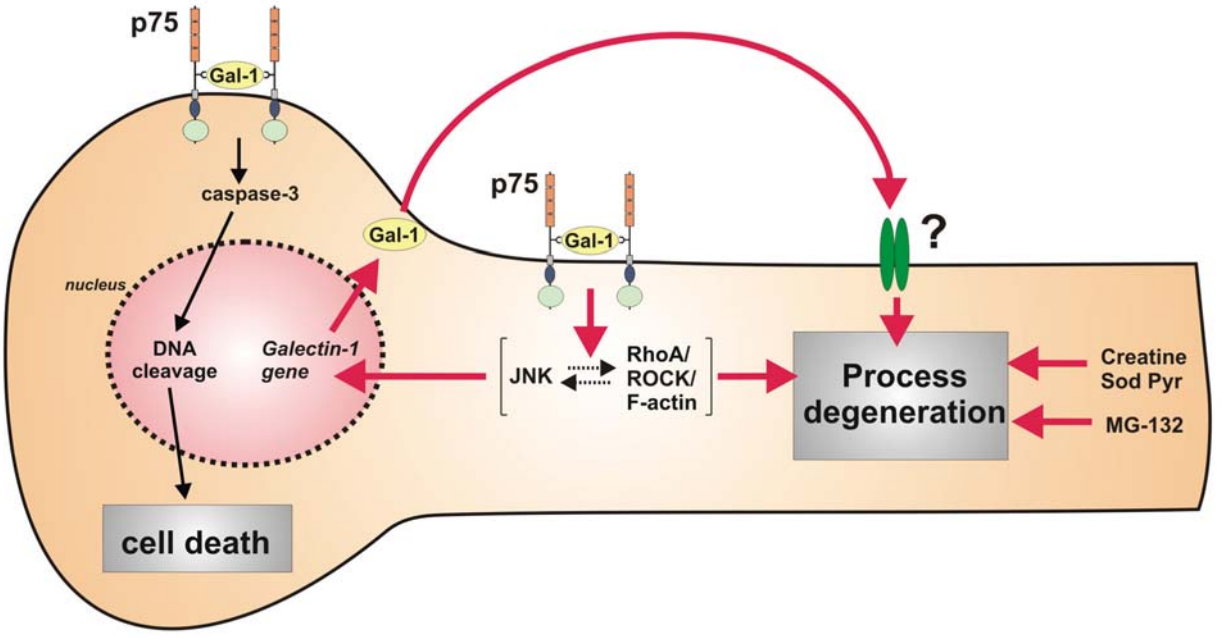
Fig.22



Extracellular Galectin-1 decoys prevent Galectin-1 excitotoxicity

(A) Large numbers of $p75^{NTR}$-positive cholinergic neurons are normally detected in the rat medial septum. Injection of IBO leads to the degeneration of these cells 26 hours after injection. (B) High magnification image shows that after 26 hours of IBO injection, most neurons exhibit increased $p75^{NTR}$ immunoreactivity, have lost their processes and start undergoing programmed cell death as evidenced by their pyknotic nuclear morphology (inset shows the Hoechst-stained nucleus of the neuron indicated by arrow). Co-injection of ibotenic acid and a soluble $p75^{NTR}$ fusion protein, lactose (250 mM), an anti-Galectin-1 antibody (0.5 mg/ml), or CEP-1347 (10 μM) prevents the loss of $p75^{NTR}$-positive neurons (C) and of their processes (D). Bar, 100 μm in B,C and 50 μm in D. aca, anterior commisure; lv, lateral ventricle; cc, corpus callosum; ms, medial septum.

Fig.23



Working model

During development or after injury, neurons express $p75^{NTR}$, a receptor of the TNF receptor superfamily that can activate the JNK and RhoA/Rho kinase signaling pathways. The work presented in this Thesis indicates that these pathways regulate the expression of the *Galectin-1* gene. Galectin-1 is somehow externalized and causes the degeneration of neuronal processes and their cell bodies, presumably by binding to yet unidentified receptors. $p75^{NTR}$ is not necessary for this to occur, as Galectin-1 also causes the degeneration of neurons lacking $p75^{NTR}$. Galectin-1 also binds directly to $p75^{NTR}$, which is heavily glycosylated, and this interaction might be important for the transport of $p75^{NTR}$ to the cell surface or for its clustering. In the cell body, $p75^{NTR}$ signaling induces caspase-3 activity leading to classical programmed cell body death.

CURRICULUM VITAE

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B.Sc./Masters Degree in Life Sciences (2000-2002), *magna cum laude*.
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PUBLICATIONS

- **Plachta N**, Annaheim C, Bissiere S, Hoving S, Voshol V, Bibel M, Barde Y-A (2006). Identification of Galectin-1 as a killer of neuronal processes. *Submitted*.
- Nikoletopoulou V, **Plachta N**, Allen ND, Haubst N, Götz M, Barde Y-A (2006). Mis-specification of progenitors and neuronal death revealed by Pax6-deficient embryonic stem sells. *Submitted*.
- **Plachta N**, Bibel M, Tucker KL, Barde Y-A (2004).Developmental potential of defined neural progenitors derived from mouse embryonic stem cells. ***Development* (21) 5449-56**
- **Plachta N**, Traister A, Weil M (2003). Nitric oxide is involved in establishing the balance between cell cycle progression and cell death in the developing neural tube. ***Experimental Cell Research* (2) 354-62**
- Traister A, Abashidze S, Gold V, Yairi R, Michael E, **Plachta N**, McKinnell I, Patel K, Fainsod A, Weil M (2004). BMP controls nitric oxide-mediated regulation of cell numbers in the developing neural tube. ***Cell Death and Differentiation* (8) 832-41**
- Traister A, Abashidze S, Gold V, **Plachta N**, Karchovsky E, Patel K, Weil M (2002). Evidence that nitric oxide regulates cell cycle progression in the developing chick neuroepithelium. ***Developmental Dynamics* (3) 271-6**

PROFESIONAL EXPERIENCE

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2001-2002	**Student researcher** at Dr. Miguel Weil's laboratory of Cell Biology, Department of Cell Research and Immunology, Tel Aviv University, Israel
1999-2000	**Student researcher** at Prof. Claudio Lazzari's laboratory of Insect Physiology, Faculty of Biology, University of Buenos Aires, Argentina

TALKS

From Bench to Bedside 2004, Neurex Programme, Basel, Switzerland, 2004, *"Testing the differentiation potential of defined neural progenitors derived from mouse embryonic stem cells"*

PUBLISHED ABSTRACTS

- Swiss Stem Cell Society Meeting, Basel, Switzerland, 2005

Plachta N, Annaheim C, Hoving S, Voshol V, Bibel M, Barde Y-A (2006). *"Identification of Galectin-1 as a killer of neuronal processes"*

- FENS Forum, Vienna, Austria, 2006

Plachta N, Annaheim C, Hoving S, Voshol V, Bibel M, Barde Y-A (2006). *"Identification of Galectin-1 as a killer of neuronal processes"*

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