

**Functions of TGF- β 2 and GDNF in the Development of the Mouse Nervous
System: Evidence from Double Mutant Mice**

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Dissertation

Submitted for the acquisition of Doctorate degree in Biology
School of Natural Sciences and Mathematics
George August University
Göttingen, Germany

by

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Göttingen, 2006

Dedication

**TO MY PARENTS,
MY SISTERS,
AND MY BROTHERS**

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Abbreviations

A β : amyloid β
AD: Alzheimer's disease
Alk: activin-receptor like kinase
ALS: amyotrophic lateral sclerosis
AP: anteroposterior
ART: artemin
Aq: aqueduct
BDNF: brain-derived neurotrophic factor
BMP: bone morphogenetic protein
ca: carotid artery
c-Jun: NH(2)-terminal kinase
CNS: central nervous system
CNTF: ciliary neurotrophic factor
CVG: cochleovestibular ganglion
DA: dopamine
DAB: diaminobenzidine
DRG: dorsal root ganglion
DV: and the dorsoventral
E: embryonic day
ECM: extracellular matrix
EGF: epidermal growth factor
ENS: enteric nervous system
FG: facial ganglion
FGF: fibroblast growth factor
g: gut
GDF: growth/differentiation factor
GDNF: glial cell line-derived neurotrophic factor
GFAP: glial fibrillary acidic protein
GPI: glycosylphosphatidylinositol
H&E: Hematoxylin-eosin-staining
hr: hour
HSCR: Hirschsprung disease
IEG: immediate early gene
IL: interleukin
INL: inner nuclear layer
ir: immunoreactivity/immunoreactive
ir: inner retina
LAP: latency associated protein
LC: locus coeruleus
LLC: large latent protein complex
LTBP: latent-TGF- β -binding protein
MAPK: mitogen-activated protein kinase
MEKK: MAPK/ERK kinase kinase
MGC: Mueller glial cells
MIS: Müllerian inhibiting substance
MMP: matrix metalloprotease
MN: motoneurons
NA: noradrenaline
NF: neurofilament
NGF: nerve growth factor

NPG: Nodose-Petrosal gnaglia
NRK: normal rat kidney
NT: neurotrophin
NTN: neurturin
ou: outer retina
OV: optic vesicle
PAI: plaminogen activator inhibitor
PBS: phosphate buffered saline solution
PCD: programmed cell death
PCNA: proliferative cell nuclear antigen
PCR: Polymerase chain reaction
PD: Parkinson's disease
PDGF: platelet-derived growth factor
PFA: Paraformaldehyde solution
PI3K: phosphatidylinositol-3-kinase
PMR: paramedian raphe
PNMT: phenylethanolamine-N-methyltransferase-positive cells
PNS: peripheral nervous system
PSP: persephin
RGC: retinal ganglion cells
s: stomach
SCG: superior cervical ganglion
Shh: sonic hedgehog
SNpc: substantia nigra pars compacta
sp: spinal cord
TGF- β : transforming growth factor-beta
TH: Tyrosine Hydroxylase
T β R: TGF- β receptor
t-PA: tissue plasminogen activator
TpOH: tryptophan hydroxylase
ub: urinary bladder
VTA: vental tegmental area
4V: 4th ventricle
5-HT: 5-hydroxytryptamine (serotonin)

1. Abstract:

A major area of investigations in neuroscience is directed at understanding factors that participate in neural survival versus death. A number of neurotrophic growth factors have been identified that promote neuronal survival and differentiation. The transforming growth factors-beta (TGF- β) constitute a family of multifunctional cytokines. Their functions include control of cell proliferation, differentiation and regulation of cell survival and death. Glial cell line-derived neurotrophic factor (GDNF) itself is distantly related to TGF- β . It maintains survival of various neuronal populations such as midbrain dopaminergic neurons and motoneurons. Many recent advancements have revealed that growth factors acting in synergy can regulate neuronal survival much more potently than any individual factor alone. Much evidence suggests that GDNF may require cofactors to act as a neurotrophic factor. The present work aims at elucidation of TGF- β 2 and GDNF synergism *in vivo* through generation of TGF- β 2/GDNF double mutant mice.

As mutant mice lacking TGF- β 2 or GDNF die during birth, double mutant mice have to be generated by first breeding heterozygous mice to generate double heterozygous mice and finally double mutant mice. The expected ratio was 1:16 embryos. A total of 270 embryos was obtained of which 11 instead of the expected 17 were double mutant mice. The embryos were obtained from embryonic days 12 to 18. To test the hypothesis of whether TGF- β 2/GDNF synergistically promote neuron survival, a wide spectrum of neuron populations was analysed at E18, the latest stage accessible before birth and consequently death of the mutant mice. At E18.5 there was a significantly decreased number of neurons detectable in the motoneuron population, the sympathetic ganglionic neurons as well as some parasympathetic neuron populations. The loss of parasympathetic neurons was comparable to the phenotype of GDNF mutant mice, whereas lumbar motoneurons and neurons of the SCG resulted in a clear unique phenotype due to the double null condition. These data suggest that TGF- β and GDNF synergise to promote neuron survival during development of lumbar motoneurons as well as SCG neurons.

Both TGF- β as well as GDNF are well known for their survival-promoting effect of midbrain dopaminergic neurons. Therefore, special attention was given to the analysis of their development. At embryonic stage 12.5 (E12.5), the total number of midbrain dopaminergic neurons was significantly decreased in mice deficient for TGF- β 2 ($Tgfb2^{-/-}$) compared to wild-type ($Tgfb2^{+/+}$) mouse embryos. This may give an indication that TGF- β 2 plays a role in the early induction of the dopaminergic neurons. Surprisingly, at E14.5 and E18.5, our analysis failed to reveal significant differences in the total number of TH-positive cells in the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) in $Tgfb2^{-/-}Gdnf^{+/+}$, $Tgfb2^{+/+}Gdnf^{-/-}$, $Tgfb2^{-/-}Gdnf^{-/-}$ mutant mouse embryos compared to the controls ($Tgfb2^{+/+}Gdnf^{+/+}$). This may indicate that these genes seem to have a marginal effect on the development of the midbrain dopaminergic neurons at these stages *in vivo*, but may be more important in postnatal maturation of the system. Moreover, the one year old $Tgfb2^{+/+}$ and double heterozygous ($Tgfb2^{+/+}Gdnf^{+/+}$) mice showed a marginal decrease (10% and 13%, respectively) in the dopaminergic neurons compared to the controls ($Tgfb2^{+/+}Gdnf^{+/+}$).

The locus coeruleus (LC) is the noradrenergic nucleus that is severely affected in neurodegenerative disorders. In this study, at E14.5 there were no significant differences observed in the total number of neurons within the LC between $Tgfb2^{-/-}$, $Gdnf^{-/-}$, $Tgfb2^{-/-}Gdnf^{+/+}$, $Tgfb2^{+/+}Gdnf^{-/-}$ mouse embryos and the controls ($Tgfb2^{+/+}Gdnf^{+/+}$).

The total number of serotonergic neurons at E12.5 was significantly decreased in mice deficient for TGF- β 2 ($Tgfb2^{-/-}$) compared with wild-type mouse embryos ($Tgfb2^{+/+}$). This may indicate that TGF- β 2 plays a role in the early induction of the serotonergic neurons. Moreover, there was a significant decrease in the total number of serotonergic neurons in $Tgfb2^{-/-}$ and $Tgfb2^{-/-}Gdnf^{+/-}$ in the paramedian raphe (PMR) at E18.5 compared with wild-type mouse embryos. On the other hand, quantification of rostral 5-HT-positive cells showed a decrease at E14.5 in the double mutant mice ($Tgfb2^{-/-}Gdnf^{-/-}$) compared with wild-type mouse embryos, but differences did not reach statistical significance. One out of three double knockout mice at E14.5 and E18.5 showed a severe defect (reduction) in the number of the rostral 5-HT-positive neurons which may be due to genetic penetrance.

Chromaffin cells are thought to develop from the same progenitors as sympathetic neurons. In the present study, effects of TGF- β 2 and GDNF on proliferation and differentiation of chromaffin cells in mouse adrenal chromaffin cells were investigated in a genetic mouse model. We observed a significant increase in the total number of tyrosine hydroxylase-positive cells (TH⁺) in $Tgfb2^{-/-}$ and $Tgfb2^{-/-}Gdnf^{-/-}$ double knockout mouse embryos at E14.5 and E14.5 compared to wild-type animals ($Tgfb2^{+/+}$), but no changes in the number of TH-immunoreactive cells were observed in GDNF mouse mutants. At E15.5 but not at E18.5, there was a marked increase in the number of proliferative cell nuclear antigen (PCNA⁺) positive chromaffin cells in $Tgfb2^{-/-}$ knockout embryos compared to the wild type group. On the other hand, there was a clear decrease in the ratio of total number of phenylethanolamine-N-methyltransferase-positive cells (PNMT⁺) to the total TH⁺ in $Tgfb2^{-/-}$ mouse embryos at E18.5 compared to wild type animals. This is the first documentation of the physiological significance of TGF- β 2, an isoform that has been suggested to play a role in the regulation of chromaffin cell proliferation and differentiation based on *in vitro* experiments.

$Tgfb2^{+/+}Gdnf^{-/-}$ and $Tgfb2^{-/-}Gdnf^{-/-}$ double mutant mouse embryos lack most of the enteric neurons using neurofilament (NF) antibody as a neuronal marker. This result is consistent with the results from GDNF knockout mice, $Gdnf^{-/-}$ mice showed an absence of the enteric nervous system (ENS) neurons.

In addition to the hypothesis-based analysis and results there were some obvious additional phenotypes detectable in TGF- β 2/GDNF double mutant mice. At E14.5 and E18.5 the entire neural retina of $Tgfb2^{-/-}Gdnf^{-/-}$ double mutant mouse embryos and $Tgfb2^{-/-}Gdnf^{+/-}$ littermates was significantly thicker than wild-type retina. Interestingly, the double mutant mice ($Tgfb2^{-/-}Gdnf^{-/-}$) showed a complete detachment of the retina from the underlying pigment epithelium at E18.5 and the retina was folded (coloboma formation) at E18.5. Furthermore, $Tgfb2^{-/-}Gdnf^{-/-}$ double mutant mice showed some phenotypes outside the central and peripheral nervous system. $Tgfb2^{-/-}Gdnf^{-/-}$ double mutant embryos showed a reduction in the thickness of the ventral body wall and muscle development, defects in extracellular matrix formation (ECM), and acceleration in molar tooth development.

In summary, TGF- β 2/GDNF mutant mice show a unique pattern of phenotypes that partly may be due to a synergism in regulating neuronal survival e.g. MN and SCG. Furthermore, they may also cooperate in other places by regulating proliferation, differentiation as well as production and composition of extracellular matrix. The data obtained also suggest a wide array of potential clinical implications, ranging from the understanding to the treatment of motoneuron to eye disease.

2. Introduction

The nervous system is a complex network of huge numbers of neurons that build appropriate connections and transmitting the required information. Although the nervous system has a lifelong synaptic plasticity, it is essentially built just once with little regenerative capacity, this means that neurons have to survive and function for a lifetime. Loss of neurons will eventually lead to functional impairments. Neuronal survival is a central issue both in nervous system development and regeneration. A major area of investigations in neuroscience is directed at understanding factors that participate in neuronal survival and death. Therefore they are good candidates to be responsible for different neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's, and Parkinson's diseases. Neurotrophic factors are a broad set of peptide growth factors that tightly regulate development and survival of neurons of the central and peripheral nervous system (Huang and Reichardt, 2001). Some neurotrophic factors also may be involved in the modification of neuronal connections in the developing brain. Though some neurotrophic factors act specifically on neurons, others affect both neuronal and non-neuronal cells (for review see Yuen et al., 1996). The neurotrophic factors divided into different families according to their structures and functions (Table 1) (Korsching, 1993; Arumäe et al., 1997). A prototypic neurotrophic factor is a secreted, target-derived protein that binds to a transmembrane receptor on the cell surface (Barbacid 1995; Segel and Greenberg, 1996). The receptor then dimerizes and is activated by transphosphorylation of the catalytic intracellular domain, which starts an intracellular signaling pathway leading to early and late transcriptional changes for specific genes in the nucleus. On the other hand, other neurotrophic factors such as the ciliary neurotrophic factor (CNTF) are secreted, but not derived from a distant target tissue (Sariola et al., 1994). Moreover, some molecules that are not secreted and have initially been known to

Table 1: Neurotrophic factor families (Korsching, 1993; Arumäe et al., 1997).

<p><u>Neurotrophins:</u> Nerve Growth Factor (NGF) Brain Derived Neurotrophic Factor (BDNF) Neurotrophin 3 (NT3) Neurotrophin 4/5 (NT4/5)</p>
<p><u>Neuropoietins:</u> Ciliary Neurotrophic Factor (CNTF) Leukemia Inhibitory Factor (LIF)</p>
<p><u>Insulin-like Growth Factors:</u> 1-2 (IGF-1, IGF-2)</p>
<p><u>Transforming Growth Factors-beta (superfamily more than 40 members):</u> Transforming Growth Factor β1-3 (TGF-β1, TGF-β2, TGF- β3) Glial Cell Line-Derived Neurotrophic Factor (GDNF) Neurturin (NTN) Persephin (PSP)</p>
<p><u>Fibroblast Growth Factors (23 members):</u> Acidic Fibroblast Growth Factor (FGF-1) Basic Fibroblast Growth Factor (FGF-2) Fibroblast Growth Factor-5 (FGF-5)</p>
<p><u>Other factors:</u> Platelet-Derived Growth Factor (PDGF) Stem Cell Factor (SCF) Transforming Growth Factor α (TGFα) Epidermal Growth Factor</p>

act as adhesion molecules have later been found to support neuronal cell differentiation. For example, the integrins and Eph family of tyrosine kinases are activated by membrane bound ligands and repulsive axon guidance signal (Segal and Greenberg, 1996).

2.1 TGF- β s superfamily

The TGF- β superfamily is a large group of extracellular growth factors controlling many aspects during development (for review see Kingsley, 1994; Massagué, 1998). The TGF- β superfamily consists of more than 35 members in vertebrates (Table 2; Chang et al., 2002), including TGF- β s, BMPs (bone morphogenetic proteins), GDFs (growth differentiation factors), activins, inhibins, Mullerian inhibiting substance (MIS) and Nodal (Miyazono et al. 2002; Chang et al., 2002; Dube et al., 1998 and Hogan, 1996). TGF- β /BMP-like proteins are found in vertebrate and invertebrates, including *C. elegans* and *D. melanogaster* (Newfeld et al., 1999; Patterson and Padgett, 2000). Many of these signalling proteins have important functions during early embryogenesis, organogenesis, after birth and in the adult, and for tissue homeostasis and repair (Kingsley, 1994; Hogan, 1996; Chang et al., 2002; Reddi, 2005). Alterations in the functions of TGF- β superfamily members have been attributed to the pathogenesis of several diseases such as cancer, skeletal dysplasias, immune tolerance and inflammation and infertility (Siegel and Massague, 2003; Thomas et al., 1997; Schmidt-Weber and Blaser, 2004; Dong et al., 1996).

2.1.1 TGF- β s family

2.1.2 TGF- β s isoforms

TGF- β s family are multifunctional cytokines with widespread distribution. The three mammalian TGF- β -isoforms are TGF- β 1, TGF- β 2 and TGF- β 3, each encoded by different genes and located on different chromosomes (Roberts and Sporn, 1990; Massague, 1990). TGF- β 1 was purified from human platelets (Assoian et al., 1983). The specific assay used

to monitor these purifications, which provided the original name for the peptide, was the ability of TGF- β to induce normal rat kidney (NRK) fibroblasts to grow and form colonies of cells in soft agar in the presence of epidermal growth factor (EGF; Roberts et al., 1981). Few years later, TGF- β 2 has been purified from tissues including porcine platelets (Cheifetz et al., 1985), bovine bone (Seyedin et al., 1985). The cloning of these proteins revealed that the precursors are encoded as preproteins consisting of 390 aa for TGF- β 1 and 412 aa for TGF- β 2 each carrying a 20-30 aa signal peptide on its N-terminus and resulting in a 112 aa processed mature form (Derynck et al., 1985; De Martin et al., 1987; Madisen et al., 1988). Doopin et al., (1992) were the first to solve the TGF- β 2 protein structure among TGF- β superfamily upon crystallographic determination. Moreover, TGF- β 3 has been identified from cDNA libraries derived from human ovary, placenta and umbilical cord (Ten Dijke et al., 1988; Derynck et al., 1988). TGF- β 4 have been cloned from chicken chondrocyte library (Jakowlew et al., 1988).

2.1.3 TGF- β activation

TGF- β s are secreted as large latent protein complexes (LLC) and directed to the extracellular matrix (reviewed by Annes et al., 2003). The three mammalian TGF- β isoforms; TGF- β 1, - β 2, and - β 3 are synthesized as 75kDa homodimeric proproteins (pro TGF- β). The TGF- β propeptide, which is termed latency associated protein (LAP), remains bound to TGF- β after secretion by non-covalent bond. However, within this complex TGF- β cannot bind its extracellular receptor. LAP forms a specific disulfide bridge with protein, the latent-TGF- β -binding protein (LTBP/Fibrillin protein family; for reviews see Hyytiäinen et al., 2004). Therefore, LLC includes mature TGF- β non-covalently bound to an LAP-LTBP complex. Therefore, TGF- β activity not be regulated by its synthesis or release from the cell but by release from the LLC. Several mechanisms known to activate TGF- β such as proteolytic activation of LAP by plasmin, matrix metalloproteinase-2

(MPP-2), reactive oxygen species, and by pH (reviewed by Annes et al., 2003; Massagué, 1998 and Krieglstein, 2006). Therefore, TGF- β availability is considered as an important step in TGF- β activation (Rifkin, 2005).

2.1.4 TGF- β receptor

TGF- β family ligands signal through a family of transmembrane serine/threonine kinases. On the basis of their structures and functions, the TGF- β receptors are divided into two types: type I and type II receptors. Type I and type II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively, which interact together upon ligand binding (for review see Chang et al., 2002). The extracellular regions of these receptors contain about 150 amino acids with 10 or more cysteines that determine the folding of this region. The type I receptors is a highly conserved 30 amino acid intracellular region immediately preceding the kinase domain; this 30-amino acid stretch is called the GS domain because of the SGSGSG sequence it contains (Wrana et al., 1994). Ligand-induced phosphorylation of the GS domain in the type I receptor by the type II receptor is required for the activation of the downstream signaling pathway (for review see Chang et al., 2002). Signal transduction from the receptor to the nucleus is mediated via members of effector molecules named SMADs (Baker and Harland, 1997; Heldin et al., 1997).

2.1.5 TGF- β and SMAD proteins

SMAD family proteins are the first identified substrates of type I receptor kinases and have important roles in the transduction of signals from the receptor to target genes in the nucleus. The *Drosophila* gene "mothers against decapentaplegic" (Mad), which constitutes a central component in Dpp signal transduction (Rafferty et al., 1995; Sekelsky et al., 1995), has been instrumental in the discovery of three homologous proteins in *C. elegans*, named Sma-2, -3, and -4 (Savage et al., 1996). After the founder members Sma and Mad, vertebrate homologues were named SMAD. About 10 vertebrate SMAD proteins have

been identified (Massague and Chen, 2000). Some members of the SMAD family play different roles in TGF- β family signaling. SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8 can be phosphorylated directly by type I receptors after ligand-induced dimerization of type I and type II receptors, and they are called receptor-regulated SMADs (R-SMADs; Massagué, 1998; Heldin et al., 1997; Massague and Chen, 2000). Phosphorylation of R-SMADs stimulates their interaction with the common SMAD followed by localization from the cytoplasm to the nucleus where they function as regulators of transcription of specific genes.

2.1.6 Expression and Biological activity of TGF- β s

TGF- β expression begins early in development. Using immunohistochemical methods, TGF- β expression is found in cartilage, teeth, muscle, bone, heart, blood vessels, haematopoietic cells, lung, kidney, eye, ear, gut, liver, skin and the nervous system (Heine et al., 1987). Marked TGF- β expression is observed in areas undergoing morphogenetic events, for example those involving epithelial-mesenchymal interactions or differentiation (Heine et al., 1987; Lehnert et al., 1988; Krieglstein et al., 1995a). In many locations TGF- β mRNA and protein expression is maintained postnatally (Thompson et al., 1989; Unsicker et al., 1991). The spectrum of functions of TGF- β s ranges from control of cell proliferation and differentiation, chemotaxis, production of extracellular matrix (ECM) components and immunosuppression, to regulation of cell death (Roberts and Sporn, 1990). Effects of specific isoforms largely depend on type and differentiation state of target cells and on the presence of other factors (Nathan and Sporn, 1991; Krieglstein et al., 1995a).

2.1.7 TGF- β and the nervous system

Several immunohistochemical and in situ hybridization studies provided evidence for the widespread distribution of TGF- β 2 and - β 3 and sites of their synthesis in the developing

and adult central and peripheral NS of mouse, rat, and chick (Flanders et al., 1991; Pelton et al., 1991; Unsicker et al., 1991,1996). During mouse development, TGF- β 2 and - β 3 proteins become first detectable immunohistochemically in radial glial cells, along peripheral nerves, and along CNS axon tracts, while TGF- β 1 is most prominent within the meninges at E12 (Flanders et al., 1991). At this stage, nerve cell bodies were still devoid of immunoreactivity, TGF- β 2 and - β 3 were not detectable in brain, spinal cord, and peripheral ganglionic nerve cell bodies of mouse embryos until E15. On the contrary, cells in the subventricular zone, subplate, and lamina I of the E16 mouse cortex stain positively for TGF- β (Flanders et al., 1991). As in the developing nervous system, TGF- β 2 and - β 3 are present in the adult nervous system. Unsicker et al., (1991) have been shown that both neurons and astroglial cells express TGF- β 2 and - β 3 with different levels in the rat CNS, depending on the brain regions and neuron type. Using Northern blotting and isoform specific antibodies for immunocytochemistry, TGF- β 2 mRNA were present in all brain areas including cerebral cortex, striatum, hippocampus, cerebellum and brainstem (Unsicker et al., 1991). In situ hybridization has revealed synthesis of TGF- β 2 synthesis in postnatal and adult mouse hippocampal, dentate gyrus, and Purkinje neurons (Constam et al., 1994). Moreover, TGF- β 3 mRNA is also widely expressed in the adult CNS, but at lower levels than TGF- β 2 mRNA. Recently, Farkas et al. (2003) showed that TGF β 2/3 were expressed in the notochord and floor plate and that neutralization of TGF β 2/3 abolished induction of dopaminergic neurons in rat primary cultures and E2 chick embryos. TGF- β has been shown to promote neuron survival of several neuron populations in vitro (Krieglstein et al., 1995b; Poulsen et al., 1994). Ishihara et al., (1994) have been reported that TGF- β 1 and - β 2 cause neurite sprouting and elongation of hippocampal neurons *in vitro*.

2.1.8 Phenotypes of TGF- β ligand, TGF- β receptor and SMAD deficient mice

To study the biological roles of TGF- β *in vivo*, mutations of individual genes within the TGF- β signalling molecules were generated. Analysis of the phenotype revealed significant unique functions of individual TGF- β isoforms as well as some overlapping functions.

2.1.8.1 TGF- β 1 mutant mice

Targeted disruption of the TGF- β 1 gene in mice results in diffuse and lethal inflammation. TGF- β 1 mutant mice appear normal and indistinguishable from their heterozygotes and wild-type controls for the first 2 weeks after birth. At about 3 weeks of age, all TGF- β 1 mutant mice develop a progressive wasting syndrome, resulting in death within a few days (Shull et al., 1992; Kulkarni et al., 1993). On the other hand, an embryonic phenotype has been described in TGF- β 1 mutant mice in only 50% of the mice reach parturition (Shull et al., 1992; Kulkarni *et al.*, 1993 ; Dickson *et al.*, 1995) . Dickson et al., (1995) have been shown that 50% of the TGF- β 1 null mutant and 25% of TGF- β 1 heterozygotes mice died by E10.5 due to defective haematopoiesis and endothelial differentiation of extraembryonic tissue. Many studies indicating that TGF- β 1 also has a role in cancer. Tang et al., (1998) showed that treatment of TGF- β 1 heterozygotes mice with carcinogens have an enhanced development of lung and liver cancer

2.1.8.2 TGF- β 2 mutant mice

TGF- β 2 knockout mice display many developmental defects. Homozygous TGF- β 2 mutant mice embryos die probably from congenital cyanosis at birth. Several malformations include cardiac, lung, spinal column, eye, limb, craniofacial, inner ear and urogenital defects (Sanford et al., 1997). Furthermore, defects in eye development include an ocular hypercellularity with respect to the posterior chamber as well as the inner and outer neuroblastic layers. Moreover, the corneal stroma is reduced and only one-third as thick as in wild-type animals. The developmental defects in TGF- β 2 mutant tissues involve

developmental processes as epithelial-mesenchymal interactions, cell growth, palatogenesis, and extracellular matrix production. Several affected tissues contain neural crest derived components and simulate neural crest deficiencies (Sanford et al., 1997).

2.1.8.3 TGF- β 3 mutant mice

Proetzel et al., (1995) showed that targeted disruption of the TGF- β 3 gene resulted in defective palatogenesis and delayed pulmonary development. Homozygous TGF- β 3 mutants mice die within 24 h of birth. TGF- β 3 mutant mice exhibit a cleft palate and thus, are unable to suckle, gasp for air and become cyanotic just before death (Proetzel et al., 1995; Kaartinen et al., 1995). The palatal shelves in homozygous TGF- β 3 mutants develop normally and they are in contact and adherent but fail to fuse together (Taya et al., 1999). No craniofacial abnormalities or malformations of any other organs were observed, except for the lung maturation (Proetzel et al., 1995; Kaartinen et al., 1995).

2.1.8.4 TGF- β 2/ β 3 double mutant mice

Recently, TGF- β 2/ β 3 double knockout mice were generated in our laboratory. Duenker and Krieglstein, (2002a) have been reported that TGF- β 2/ β 3 double knockout mice and their three allelic *Tgfb2*^{-/-}*Tgfb3*^{+/-} littermates display a lack of distal parts of the rib, a lack of sternal primordia, and failure in ventral body wall closure. Moreover, abnormalities in connective tissue composition and an early embryonic lethality (around embryonic day 15.5) are seen (Duenker and Krieglstein, 2002a). The eyes of TGF- β 2/TGF- β 3 double mutant mice display severe alterations in the morphology of the retina, lens, and cornea. In *Tgfb2*^{-/-}*Tgfb3*^{-/-} and *Tgfb2*^{-/-}*Tgfb3*^{+/-} littermates the retinas were consistently detached from the underlying pigment epithelium. Moreover, cornea, corneal stroma, and lens epithelium were significantly thinner in these mutants (Dünker and Krieglstein, 2003).

2.1.8.5 TGF- β receptor mutant mice

TGF- β -receptor type II (TBR-II) homozygous knockout mice die around E10.5 due to defective yolk sac hematopoiesis and vasculogenesis (Masanobu et al., 1996), whereas heterozygous mice are indistinguishable from their wild-type littermates. Therefore, TBR-II may play a role in signalling, haematopoiesis as well as in endothelial differentiation.

2.1.8.6 SMADs gene targeting

Targeting the SMAD2 gene results in embryonic lethality (before E8.5). At E6.5, Smad2 null mutant embryos are smaller than their wild-type littermates, lacking the extraembryonic portion of the egg cylinder, and have mesodermal defects. Waldrip and co-workers (1998) reported that Smad2 null mutant embryos do not form a head fold or primitive streak, suggesting that SMAD2 signalling determines the anterior-posterior polarity of the early mouse embryo. On the contrary to SMAD2 mutant, Smad3 mutant mice are viable and fertile (Datto et al., 1999; Zhu et al., 1998). SMAD3 mutant mice develop colorectal adenocarcinomas at the age of 4-6 months (Zhu et al., 1998). Recently, many studies showed that Smad4 mutant mice embryos die before E7.5, fail to gastrulate or to form mesoderm, have a reduced size and growth retardation, and show abnormal visceral endoderm development (Sirard et al., 1998; Yang et al., 1999). Phenotype analyses of genes ablation within the TGF- β signalling pathway support the notion that TGF- β plays a significant role in mouse development.

2.2 GDNF family

Glial cell line-derived neurotrophic factor (GDNF) family consists of small secreted proteins that are responsible for many functions inside as well as outside the nervous system during development (reviewed by Airaksinen et al. 1999; Unsicker et al., 1998). Lin et al., (1993) purified the first member of this family from the rat glial cell line B49 supernatant and called GDNF, it was shown to promote the survival and morphological differentiation of embryonic midbrain dopaminergic neurons. GDNF is a distant member

of the transforming growth factor β (TGF β) family (Lin et al. 1993). Few years later, new members of the GDNF family including neurturin (NRTN), persephin (PSPN) and Artemin (ARTN) were purified and characterised (Kotzbauer et al. 1996; Baloh et al. 1998; Milbrandt et al. 1998).

2.2.1 GDNF molecule

Lin and associates (1993) have been demonstrated that the coding region of the GDNF gene is 633 bp, which encoding for a 211 amino acid precursor polypeptide from which the mature GDNF of 134 amino acids is produced. By using RT-PCR, an alternatively spliced GDNF mRNA lacking 78 bp in the preproregion has also been found, but because it gives rise to the same mature GDNF, the function of the different splicing is still unknown (Suter-Crazzolaro and Unsicker, 1994). GDNF is heterogeneously glycosylated molecule and behaves like a disulfide-bonded homodimer. Surprisingly, the identified disulfide structure of GDNF is highly homologous to that of TGF- β 2 (Haniu et al., 1996). GDNF contains the seven conserved cysteine residues which found in all members of the TGF- β superfamily, but shares only about 20% amino acid sequence identity with any family member of the TGF- β superfamily (Lin et al., 1993). Data on the crystal structure of GDNF showed a similarity in the structure of GDNF protein to other members of the TGF- β family, GDNF became a member of the cysteine knot growth factor family (Eigenbrot and Gerber, 1997).

2.2.2 GDNF receptors and signal transduction

GDNF, ART, NTN, and PSP require two types of receptors on the plasma membrane of the target cell to induce the activation of intracellular signaling cascade (Airaksinen and Saarma, 2002). First, GFR α -1/4 surface receptors, which are the ligand-binding components. Second, RET; the transmembrane tyrosine kinase receptor; signals through the autophosphorylation of its multiple intracellular tyrosine residues. For signaling,

ligands of the GDNF family binds preferentially to the corresponding surface receptor GFR α -(1 to 4), leading to the subsequent interaction of the GFL/GFR α -1 to 4 complex with two molecules of RET, inducing its homodimerization, autophosphorylation and activation of signalling cascades to the nucleus to regulate specific genes (Treanor et al., 1996; Trupp et al., 1999). All four ligands use the same transmembrane receptor RET, but each ligand binds to a preferred GFR α surface receptor: GDNF binds preferentially to GFR α -1 receptor; NTN to GFR α -2; ART to GFR α -3 and PSP to GFR α -4 (Airaksinen and Saarma, 2002).

2.2.3 GDNF expression

GDNF exhibits an extensive patterns of expression during development inside as well as outside the nervous system. Stroemberg and coworkers (1993) demonstrated that the expression of GDNF mRNA in striatum peaked at birth in the rat, but was undetectable in the adult striatum, this data suggest a retrograde messenger role for GDNF in the nigrostriatal system during development. Using western blot technique, the distribution of GDNF protein was also demonstrated in neuronal somata, dendrites and axons (Kawamoto et al., 2000). Furthermore, GDNF mRNA was also found in human astrocytes *in vitro* (Moretto et al., 1996). In CNS, GDNF mRNA was detected in cotext cerebri, corpus pineale, hippocampus, thalamic nucleus, pons, dorsal horn of spinal cord (Nosrat et al., 1996). Using RT-PCR, the expression of GDNF transcripts were widespread in many organs in the newborn rat including kidney, lung, liver, spleen, skin, stomach, bone, heart, blood (Suter-Crazzolaro and Unsicker, 1994). GDNF mRNA is expressed also in the precursors of Sertoli cells in the testis, but not in the ovaries (Hellmich et al., 1996; Suvanto et al., 1996).

2.2.4 GDNF function

Many studies show that GDNF play a significant role inside as well as outside the nervous system. Lin et al., (1993) demonstrated that GDNF promote the survival and increased the high-affinity DA uptake of SN dopaminergic neurons *in vitro* and *in vivo* (Hudson et al., 1995). Using adult rat models, retrograde axonal transport of GDNF molecule from the DA terminal region to the SN demonstrated its trophic effects on DA neurons (Tomac et al., 1995). The pharmacological and biological effects of GDNF on midbrain dopaminergic neurones have been studied in several animal models (for review see Lapchak et al., 1997; Grondin et al., 1998). Therefore, GDNF is considered a potential drug candidate for the treatment of Parkinson's disease. Moreover, GDNF also has a trophic effect on noradrenergic neurons in the locus coeruleus which are severely affected in several neurodegenerative diseases of CNS (Arenas et al., 1995). Mount et al., (1995) demonstrated that GDNF enhance the development and morphologic differentiation of Purkinje cells, which are the efferent neurons of cerebellar cortex. In neonatal mice, overexpression of GDNF by muscle led to an increase in the number of motor axons innervating neuromuscular junctions (Nguyen et al., 1998). About half of the spinal cord motoneurons (MN) die at a time when they are establishing synaptic connections with their target muscles, their survival depends mainly on the access to several trophic factor (Oppenheim et al., 1995;1996). Several studies showed that GDNF has survival effect on MN obtained from E14 rat cultures (Henderson et al., 1994). Furthermore, GDNF rescues avian motor neurons from natural programmed cell death and also promotes the survival of enriched populations of motoneurons in culture (Oppenheim et al., 1995). In the presence of GDNF, growth of mouse neural-crest cultures has been shown to result in a significant increase in the number of tyrosine hydroxylase-positive cells with neuronal morphologies (Maxwell et al., 1996). Outside the nervous system, GDNF stimulated branching morphogenesis in the kidney and induced ectopic uretheric buds from the nephric duct

(Sainio et al., 1997). Recently, it was shown that GDNF regulate spermatogonia renewal and differentiation during spermatogenesis (Meng et al., 2000). During development, the expression of GDNF is also seen in a variety of other mesenchymal tissues such as limb buds and cartilage, although its function in these sites is still unknown (Hellmich et al., 1996; Suvanto et al., 1996).

2.2.5 GDNF mutant mice

GDNF knockout mice showed defects in the developing kidneys and lacked the enteric nervous system (ENS) (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). These mice showed complete renal agenesis due to the lack of induction of the uretheric bud, an early step in nephrogenesis. GDNF mutant mice die shortly after birth due to renal failure, and the absence of the enteric neurons. In the central nervous system, the total number of the midbrain dopaminergic neurons is normal. Only a small deficit in sensory, sympathetic and lumbar motoneurons has been detected. The phenotype of GDNF mutant mice is similar to both, $GFR\alpha^{-1/-}$ and $RET^{-/-}$ mice (Enomoto et al., 1998; Cacalano et al., 1998; Schuchardt et al., 1994), suggesting that they may constitute a common signaling pathways.

2.3 TGF- β and neurotrophic factor: synergism and cooperation

Several lines of evidence had suggested that GDNF may require co-factors for acting as a neurotrophic factor. Depending on *in vitro* experiments in which GDNF was shown to promote the survival of enriched sympathetic, parasympathetic and sensory embryonic chick neurons (Buj-Bello et al., 1995; Trupp et al., 1995), all cultures were done using serum either initially or throughout the experiment. In contrast, GDNF does not support the survival of most peripheral neurons in low-density dissociated cultures and defined media (Henderson et al., 1994). TGF- β and fibroblast growth factor-2 (FGF-2) synergistically promote early bovine embryonic development in the fourth cell cycle (Larson et al., 1992). Kriegstein and co-workers (1998b) have identified TGF- β as an essential component in

GDNF-mediated neurotrophic actions *in vitro* on peripheral and central NS neurons. Recently, Peterziel et al., (2002) demonstrated that TGF β -induced recruitment of the glycosyl-phosphatidylinositol-anchored GDNF receptor- α -1 (GFR- α -1) to the plasma membrane. This result is supported by the fact that GDNF in the presence of a soluble GFR- α -1 can promote survival in the absence of TGF- β (Peterziel et al., 2002). A major question regarding GDNF/TGF- β cooperativity and synergism is to understand the molecular mechanisms. An important component of GDNF/TGF- β signaling has been identified as an activation of phosphatidylinositol-3 (PI-3) kinase (Krieglstein et al., 1998b). The biological significance of the synergism between GDNF and TGF- β is supported by colocalization of the receptors for TGF- β and GDNF on several investigated GDNF responsive neuron populations *in vivo*. Figure 1 shows some possibilities of intracellular signalling cross talk between TGF- β and GDNF pathways.

2.4 Chromaffin cells

Chromaffin cells are neuroendocrine cells of neural crest origin that were termed '*chromaffin*' by Alfred Kohn (1902) because of their characteristic staining property by chrome salts. Unlike the closely related sympathetic neurons, which also originate from the neural crest and probably from a common progenitor cell, chromaffin cells are able to proliferate throughout their life span (Anderson, 1989; Unsicker, 1993; Unsicker et al., 1978). Even in adult animals, a small fraction of cells is undergoing cell division. Several studies showed that the capacity of the chromaffin cells to divide declines rapidly from birth to adulthood (Malvaldi et al., 1968; Tischler et al., 1989). Tischler et al., (1989) demonstrated that proliferation of chromaffin cells is thought to be regulated by interaction of neurogenic and hormonal signals *in vivo*. *In vitro* studies suggested that adrenal cortex-derived glucocorticoid hormones are important inhibitors of chromaffin cell mitosis (Lillien and Claude, 1985; Frödin et al., 1995). Recent studies have shown that TGF- β -

synthesized and secreted from developing chromaffin cells - inhibits DNA synthesis of chromaffin cells (Kriegstein and Unsicker, 1995c; Wolf et al., 1999). Reduction of endogenous TGF- β increases proliferation of developing chick adrenal chromaffin cells *in vivo* (Combs et al., 2002).

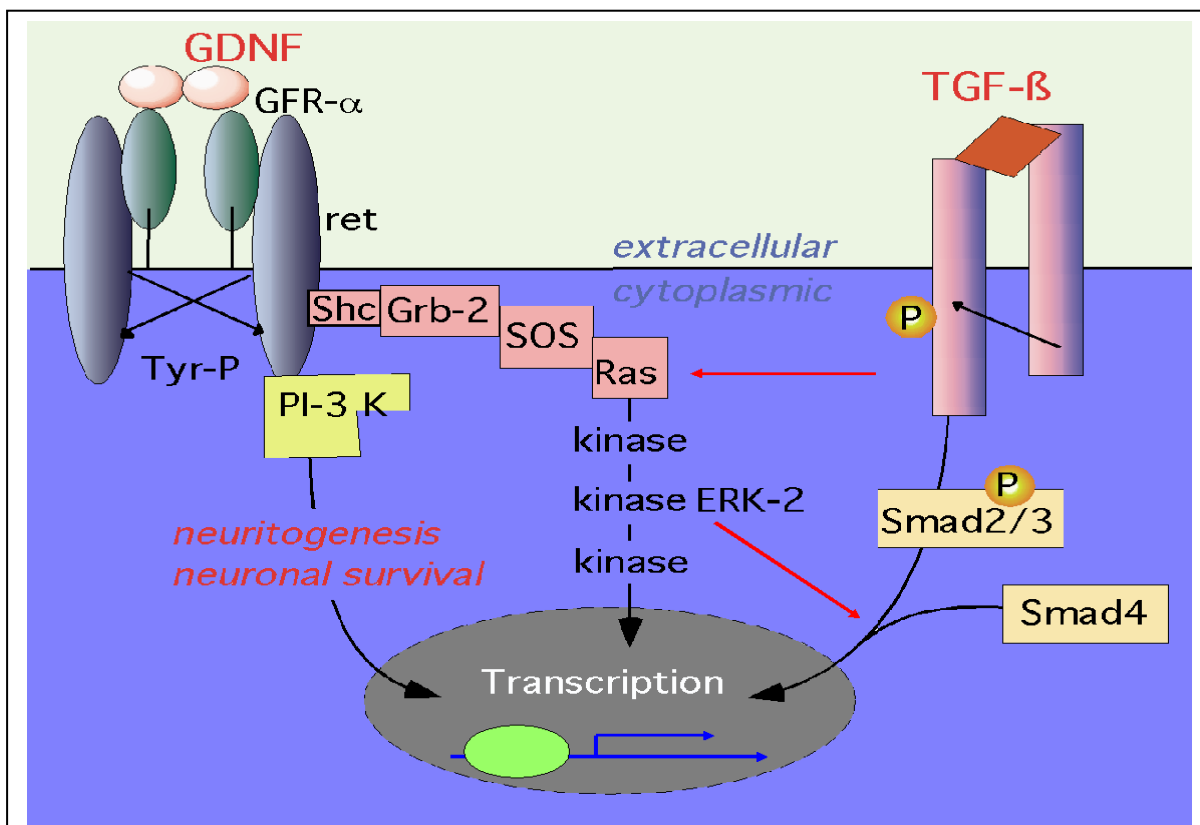


Figure 1: Scheme showing the downstream signalling pathways of GDNF (left) and TGF- β (right). Red arrows mark possible points of cross talk between the pathways.

2.5 Catecholaminergic and Serotonergic Neurons

Catecholaminergic and serotonergic neurons, known as monoaminergic neurons, produce their neurotransmitters through a different series of enzymatic modifications of the amino acid precursors tyrosine and tryptophan. Catecholaminergic neurons produce noradrenaline (NA), adrenaline or dopamine (DA). They all share the first two steps of the neurotransmitter-synthesis that leads to DA, adrenergic and noradrenergic (NA) neurons share a third step that leads to NA synthesis, and only adrenergic neurons express the enzyme for adrenaline synthesis (for review see Goriadis and Rohrer, 2002).

2.5.1. Dopaminergic neurons

Mammalian adult dopaminergic neurons have been divided into 18 different groups (A1-A18), with the most important groups populating in the ventral midbrain (A8, A9, and A10), and in the diencephalon (A11-A15), the telencephalon contains two groups of DA neurons, and these are restricted to the olfactory bulb (A16) and the retina (A17; Fig. 2-3). Midbrain dopaminergic neurons, also known as the neurons of the substantia nigra pars compacta (SNc; A9), and the ventral tegmental area (VTA; A10), innervate the striatum and neocortex, respectively (Björklund and Hökfelt 1984). Loss of substantia nigral neurons results in motor disorders which are the characteristic features of Parkinson's disease, whereas overstimulation of ventral tegmental DA neurons has been associated with several neurological disorders such as schizophrenia and drug addiction (Hirsch et al., 1998; Greenberg et al., 1974). Midbrain dopaminergic neurons can be identified by the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (reviewed by Hynes and Rosenthal, 1999). Di Porzio et al., (1990) showed that the first mouse dopaminergic neurons are detected at around E10, when TH-expressing cells appear just rostral to the isthmus. During the last two decades many studies using animal and cellular models have improved our knowledge about the development and specification of

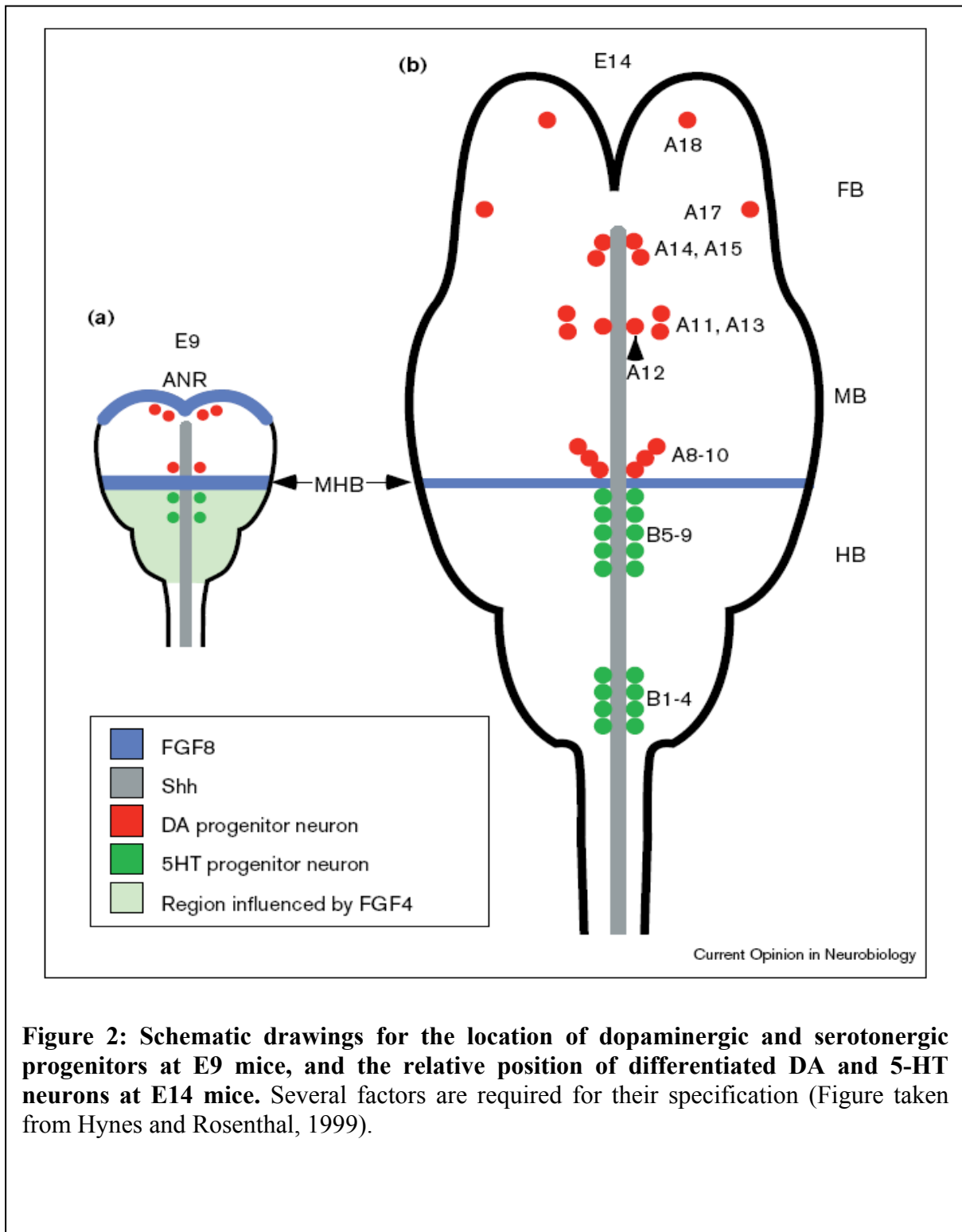


Figure 2: Schematic drawings for the location of dopaminergic and serotonergic progenitors at E9 mice, and the relative position of differentiated DA and 5-HT neurons at E14 mice. Several factors are required for their specification (Figure taken from Hynes and Rosenthal, 1999).

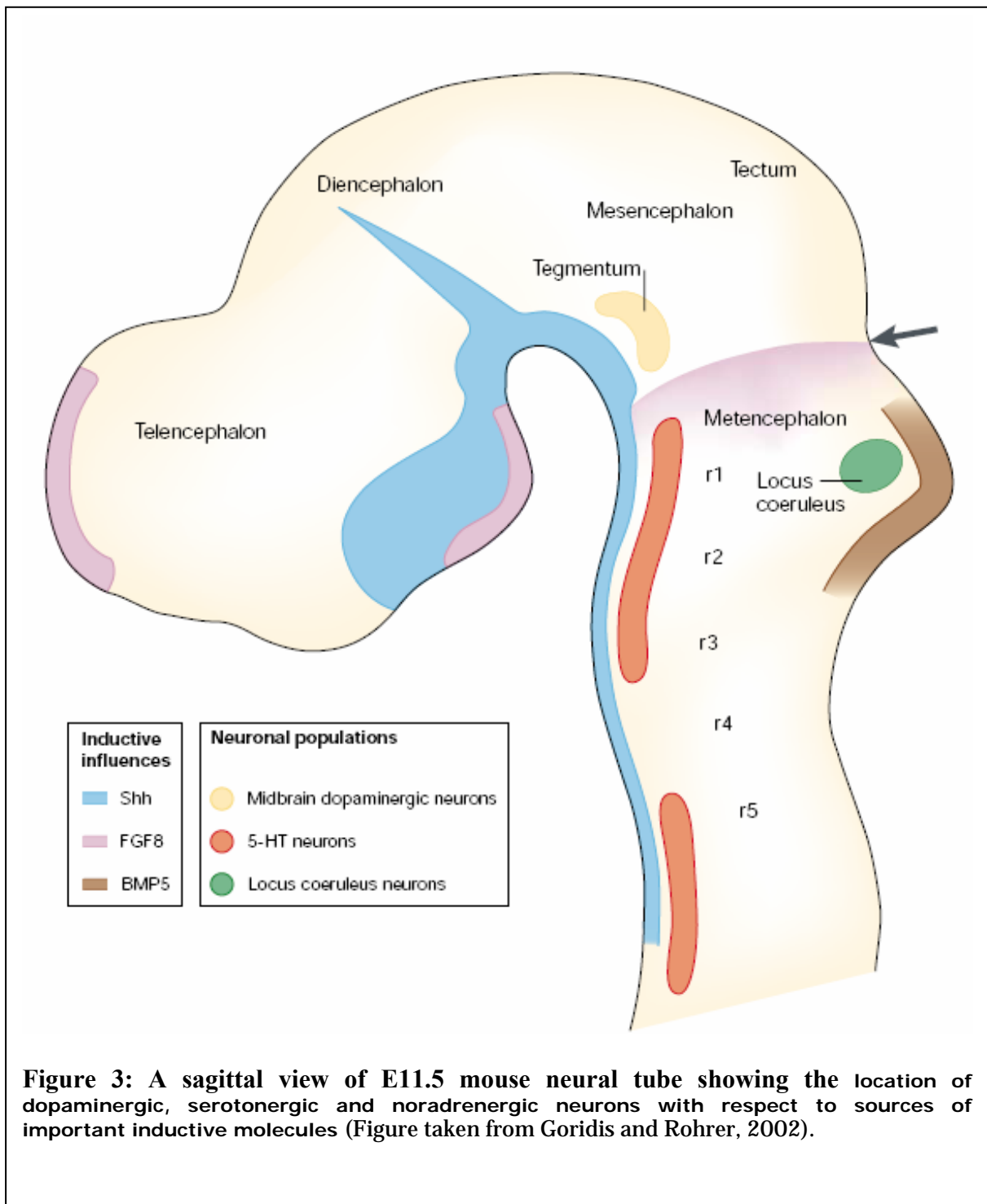


Figure 3: A sagittal view of E11.5 mouse neural tube showing the location of dopaminergic, serotonergic and noradrenergic neurons with respect to sources of important inductive molecules (Figure taken from Goridis and Rohrer, 2002).

midbrain dopaminergic neurons. Several intrinsic and extrinsic determinants have been identified that control neuronal fate and the establishment of the dopaminergic phenotype. The initial specification of dopaminergic neurons requires the patterning information that is provided by sonic hedgehog (Shh) and fibroblast growth factor-8 (FGF8). Both Shh and FGF8 belong to molecules whose expression patterns persist during embryonic development into the adult stage (reviewed by Goridis and Rohrer, 2002). Tanaka et al., (2001) showed that FGF8 immunoreactivity in the substantia nigra is reduced in cases of Parkinson's disease, compared with age-matched controls. Furthermore, FGF-8 mutant mice do not gastrulate and have no mesodermal derivatives (Sun et al. 1999). Shh is considered as an important factor for the induction of the floor plate at the ventral midline of the neural tube and contributes to the establishment of a polarity at the DV axis of the vertebrate nervous system (Wurst et al., 2001; for review see Roussa and Krieglstein, 2004). Recently, many studies dealing with the effects of TGF- β relating to dopaminergic neurons have focused on the regulation of the survival and death of mesencephalic dopaminergic neurons. These studies have identified members of the TGF- β superfamily as potent survival factors for midbrain dopaminergic neurons (see also Krieglstein, 2004). Poulsen et al. (1994) and Krieglstein et al. (1995) have been reported that TGF- β 2 and TGF- β 3 exert survival-promoting effects in cultured rat midbrain dopaminergic neurons. Treatment of cells dissociated from the ventral midbrain floor of E12 rats, with TGF- β , increases the number of TH-positive cells within 24 hr, whereas neutralization of endogenous TGF- β *in vitro* completely abolishes the induction of dopaminergic neurons. On the other hand, data suggest that TGF- β may cooperate with Sonic hedgehog (Shh) in dopaminergic neuron development (Farkas et al., 2003). It is shown that GDNF promoted the survival of SN dopaminergic neurons *in vitro* (Lin et al., 1993). The neurotrophic effect of GDNF is thought to be due to the synergistic and cooperative action of GDNF and TGF-

β whose underlying molecular mechanisms are becoming more clear (Krieglstein et al., 1998b; Peterziel et al., 2002). At transcriptional level, the specification and maintenance of midbrain DA neurons has been found to depend on the activity of several transcription factors. Several studies showed that *Nurr1* and *Lmx1b* transcription factor play a significant role during DA differentiation. Moreover, the *Pitx3* transcription factor and the retinoid-synthesizing enzyme *Aldh1* are specific markers of developing dopaminergic neurons (reviewed by Golidis and Rohrer, 2002). *Nurr1*^{-/-} mutant mice showed no midbrain dopaminergic neurons using a several markers (Zetterström et al., 1997). Few years later, further analysis showed that the neurons of *Nurr1*^{-/-} mutants that normally become DA neurons are born and express several of their specific markers, including *Pitx3* and *Lmx1b*, but fail to activate the *TH* gene (Wallen et al., 1999; Saucedo-Cardenas et al., 1998). In *Pitx3* mutant mice, no changes in the status of midbrain DA neurons or any other neuronal populations were reported (Semina et al., 2000; Rieger et al., 2001). Recently, the specification of midbrain DA neurons has been shown to depend on the *Lmx1b* gene. In *Lmx1b*^{-/-} mutant mice embryos, TH-positive cells are still born on schedule in the midbrain, and they express *Nurr1*, but they fail to activate the *Pitx3* gene and they die later on (Smidt et al., 2000). On the other hand, in *Lmx1b* mutant mice embryos, defects in dorsal midbrain structures have been detected (Smidt et al., 2000). Consistence with its expression patterns, *Aldh1* factor could have a role in DA specification. *Aldh1* codes for an aldehyde dehydrogenase which is involved in the synthesis of retinoic acid from vitamin A. It is expressed specifically in TH-positive mesencephalic neurons in the brain (McCaffery et al., 1994; Haselbeck et al., 1999).

2.5.2 Serotonergic neurons

Serotonergic neurons are involved in different autonomic and behavioral functions such as respiration, aggression, sleep-wake cycle, and anxiety (Feldman, 1997). Impairment in the

functions of brain serotonergic neurons is linked to neurological disorders, including depression, schizophrenia, and migraine (Feldman, 1997). Similar to dopaminergic neurons, serotonergic neurons arise from ventral neuroepithelial progenitors near to the floor plate (Reviewed by Goridis and Rohrer, 2002). 5-HT neurons have been divided into nine cell groups (B1-B9) in mammals (Fig. 2-3). The rostral 5-HT groups (B5-B9) populate the midbrain and rostral hindbrain, whereas the groups B1-B4 are located caudally (Paxinos, 1995). The rostral group have projections mainly to the forebrain, while the caudal group projections that reach into the cerebellum and the spinal cord. The serotonergic system develops very early in fetal life. The rostral group of serotonergic neurons starts to develop at E12–E13 in the rat, while the development of caudal 5-HT neurons in the medulla oblongata begins at E14–E15 (Wallace and Lauder, 1983; Aitken and Törk, 1988). The first 5-HT neurons have been detected at E11.5 using anti-5-HT antibodies in the mouse (Briscoe et al., 1999), although other data indicated that the first 5-HT neurons become postmitotic one day earlier (Taber-Pierce, 1973). The initial specification of 5-HT neurons depends on the combined effects of Shh, FGF-4, and FGF-8 (Ye et al., 1998). Moreover, at least three transcription factors (Nkx2.2, Pet1, and Gata3) were shown to have an important role in the early differentiation and specification of the rostral and caudal serotonergic neurons (reviewed by Goridis and Rohrer, 2002). Nkx2.2 transcription factor is expected to relay Shh signalling (Briscoe et al., 1999), while Pet1 seems to have an important role in the differentiation of both rostral and caudal neurons (Hendricks et al., 1999). Moreover, van Doorninck et al., (1999) reported that *Gata3* factor is required for the development of caudal group of 5-HT neurons. Consequently, the caudal raphe neurons appeared disorganized in *Gata3*^{-/-} mutant mice with only few cells express serotonin. In contrast, the rostral groups of 5-HT cells developed normally in *Gata3*^{-/-} deficient mice, although they also express *Gata3* (van Doorninck et al., 1999). Another

protein that appears to play a role in the differentiation and maintenance of 5-HT neurons is S-100b (Azmitia et al., 1990). Using RT-PCR and in situ hybridization, Galter et al., (1999) reported that TGF- β 2, - β 3 and the TGF- β type II receptor are expressed in the embryonic rat raphe at E14. Using cultures from the caudal rat E14 raphe, TGF- β 2 and - β 3 increased numbers of tryptophan hydroxylase (TpOH) -positive neurons (Galter et al., 1999). Furthermore, several factors including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) increased the numbers of tryptophan hydroxylase and serotonergic neurons over a four-day culture period at E14 (Galter and Unsicker, 1999).

2.5.3 Noradrenergic neurons

In the vertebrate peripheral nervous system, NA is found mainly in sympathetic neurons. In the vertebrate CNS, noradrenergic (NA) neurons form a distinct metencephalic nucleus consisting of the locus coeruleus (LC; groups A4 and A6) and a series cell groups that are distributed throughout the hindbrain (A1–A3, A5 and A7), (Fig. 2-3). The LC axons irrigate all brain regions, forming one of the widely distributed projection systems of the CNS (Niewenhuys, 1985; Cooper et al., 1977; Paxinos, 1995). The locus coeruleus is the most important noradrenergic nucleus in the brain and provides several innervation to many areas of the CNS (Loughlin and Fallon, 1985). This organization makes the LC a regulator of many essential functions, including motor output, attention, and memory (Barnes and Pompeiano, 1991). Furthermore, a notable loss of LC neurons and a severe impairment of some of these functions have been reported in the most important neurodegenerative disorders of the brain, such as Alzheimer's (Tomlison et al., 1981) and Parkinson's (Hassler, 1938). Many extrinsic and intrinsic factors are involved in the specification and differentiation of noradrenergic neurons. The specification of noradrenergic sympathetic neurons is initiated by bone morphogenetic proteins (BMPs)

and the transcription factors Mash1, Phox2b, dHand and Gata3, which, in turn, control the expression of noradrenergic properties (reviewd by Goridis and Rohrer, 2002). Many studies were done in order to elucidate factors responsible for maintaining the survival and phenotype of central noradrenergic neurons. Friedman et al., (1993) shown the neurotrophin 3 (NT-3), a member of the nerve growth factor family, is considered as a survival factor for LC noradrenergic neurons *in vitro*. Moreover, NT-3 has been shown to prevent the 6-hydroxydopamine (6-OHDA)-induced degeneration of LC noradrenergic neurons in the adult brain *in vivo* (Arenas and Persson, 1994). Surprisingly, NT-3 mutant mice showed no reduction in the number of neurons or in the level of tyrosine hydroxylase (TH) staining in the LC (Ernfors et al., 1994; Farifias et al., 1994) suggesting that other neurotrophic factors may be responsible for maintaining the survival of central noradrenergic neurons during development. Arenas et al., (1995) demonstrated that grafting of genetically engineered fibroblasts which express high levels of GDNF prevented the 6-hydroxydopamine-induced degeneration of rat noradrenergic neurons in the LC *in vivo*. These data revealed that GDNF may have a neurotrophic activity and therapeutic applications in neurodegenerative disorders affecting central noradrenergic neurons, such as Alzheimer and Parkinson's diseases.

2.6 Vertebrate eye development

Eye development is an intricate process requiring a series of specific inductive signals and precise morphogenetic movements. In mouse, eye development starts on embryonic day 8.5 when the optic vesicle (OV) grows out from the secondary prosencephalon. One day later, both the OV and the surface ectoderm invaginate. This invaginations lead to the formtion the two layered optic cup from the OV and the lens pit and subsequently the lens vesicle from the surface ectoderm (reviewed by Chow and Lang, 2001). One of the important events in eye development is represented by the establishment of asymmetries,

both in terms of cell type distribution and gene expression along the anteroposterior (AP) and the dorsoventral (DV) axes of the retina (Flanagan and Vanderhaeghen, 1998; Huh et al., 1999; Szel et al., 1996). The vertebrate neural retina is a well organized sensory organ that receives, integrates and transmits visual information. Light is received by the photoreceptor cells and converted into chemical signals that are sent through a series of different neurons (the bipolar cells, the amacrine cells, and the horizontal cells) to the retinal projections (reviewed by Peters, 2002). During development, progenitors proliferation is regulated by both extrinsic and intrinsic factors. In vertebrates, the cells that form the early optic vesicle are not distinguishable from other cell types, but all they express specific number of transcription factors, including Pax6, Otx2, Rx1, Lx2, and Six3, which are required to initiate eye development (Zuber et al., 2003). *Pax-6* is a patterning gene, expressed in the head, and it has been reported to regulate the patterning of eye and brain (Harris, et al., 1997). Mice and humans carrying mutations in the *Pax6* gene are born without eyes (Hill et al., 1992; Hanson et al., 1993). During vertebrate eye development, programmed cell death (PCD) is a key phenomenon in regulating cell numbers and tissue homeostasis. More than half of the developing cells are removed by PCD, so it makes a balance between cell proliferation and cell death during development (reviewed by Duenker, 2005). Several lines of evidence demonstrated that transforming growth factors-beta (TGF- β s) play a role in the regulation of PCD, for example, during digit formation (Dünker et al. 2002b), in the gastrointestinal epithelium (Dünker et al., 2002c), and in the peripheral and central nervous system (Krieglstein et al., 2000) including the developing retina (Dünker et al. 2001). Recently, Close et al., (2005) demonstrated that TGF- β receptors I and II are expressed in the rat retina and are located in nestin-positive progenitors early in development and in Mueller glia later in development. Furthermore, by using RT-PCR and immunohistochemistry, Close et al., (2005) reported that TGF- β 2 is the

most highly expressed TGF- β protein in postnatal inner retina. At E12.5 and E13.5, positive immunosignals for TGF- β 2, TGF- β 3, TGF β receptors I and II were found in the inner murine retina as well as in the future optic fiber layer (Duenker and Krieglstein, 2003). Retinae of anti-TGF- β -treated chick embryos, TGF- β 2 single and TGF- β 2/TGF- β 3 double mutant mice are much thicker compared to the control retinae (Duenker et al., 2001; Sanford et al., 1997; Duenker and Krieglstein, 2003).

GDNF is a widely distributed protein in the central, peripheral nervous system as well as outside the nervous system (Nosrat et al., 1996). GDNF mRNA is highly detectable in mouse retina during the early embryonic development (Nosrat et al., 1996). Furthermore, the two GDNF receptor compartments, GFR α -1 and c-Ret, are expressed on porcine retinal Mueller glial cells but not on photoreceptors (Hauck et al., 2006). Recently, using RT-PCR, GFR α -and c-Ret, are expressed by the degenerating photoreceptor cells but mainly by Mueller glial cells (MGC) in mouse (Delyfer et al., 2005). A growing number of evidence supported the concept that GDNF has neuroprotective effect during retinal degeneration. For example, GDNF have an indirect neuroprotective effect on the retinal photoreceptors in rd1 mice (Delyfer et al., 2005). Because of the close proximity of TGF- β 2 and GDNF expression in the retina and their wide range of actions, the analysis of mice that lacking both TGF- β 2 and GDNF is of prime interest.

3. Objectives:

Many recent advancements in our laboratory have revealed that growth factors acting in synergy can regulate neuronal survival much more effectively than individual factors alone. Impairment of neuronal survival is the cause of a considerable number of neurodegenerative diseases, such as Parkinson's disease, Morbus Alzheimer, and amyotrophic lateral sclerosis (ALS). TGF- β has been shown to act in synergy with neurotrophic factors, most strikingly in combination with GDNF. Since no information was available about the overall significance of TGF- β /GDNF cooperation and/or synergism for nervous system development, the present study was undertaken to test the paradigm of the developmental requirement of growth factor synergism and cooperation. Double knockout mice that lack both TGF- β 2 and GDNF were generated. Neuron populations analyzed included all those in which biological effects of either growth factor had been documented before: midbrain dopaminergic neurons, hindbrain serotonergic neurons, noradrenergic neurons in the locus coeruleus, lumbar spinal motoneurons, enteric neurons, dorsal root ganglionic neurons, superior cervical sympathetic neurons, cranial ganglionic neurons, neural retina and neuroendocrine chromaffin cells. Furthermore, this study also focused on tissues outside the nervous system following the expression of TGF- β 2 and GDNF. Since the single mice for either TGF- β 2 or GDNF die at birth, the nigrostriatal system of old mice which are heterozygous for both TGF- β 2 and GDNF was also analyzed. Finally, an understanding of the role of TGF- β /GDNF synergism *in vivo* through the analysis of the nervous system development of the corresponding double mutant mice can provide significant basic and clinical insight into mechanisms that may lead to overcoming neuron loss and verify potential targets for neurological diseases.

4. Materials and Methods

4.1 Animals

Tgfb2^{+/-} heterozygous mice were offspring from breeding pairs kindly provided by T. Doetschman, University of Cincinnati (Cincinnati, OH). The generation of these strain has been described elsewhere (see: Sanford et al., 1997). *Gdnf*^{+/-} heterozygous mice were offspring from breeding pairs kindly provided by Mart Saarma, Institute of Biotechnology, University of Helsinki, Viikki Biocenter, Finland. The generation of these strain has been described elsewhere (see: Pichel et al., 1996). Mice homozygous for TGF-β2 null mutants die from congenital cyanosis at birth (Sanford et al., 1997), while GDNF mutant mice die shortly after birth due to renal failure, and the absence of the enteric neurons, causing pyloric stenosis and dilation of duodenum (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996).

4.2 Generation of the double mutant mice

Tgfb2^{-/-}*Gdnf*^{-/-} double knockouts were generated by: (1) cross-breeding the two heterozygous strains (*Tgfb2*^{+/-} and *Gdnf*^{+/-}) resulting in *Tgfb2*^{+/-}*Gdnf*^{+/-} offsprings according to the Mendelian ratio and (2) setting up matings between these double-heterozygous mice (*Tgfb2*^{+/-}*Gdnf*^{+/-} X *Tgfb2*^{+/-}*Gdnf*^{+/-}). For morphological comparison in each case *Tgfb2*^{+/+}*Gdnf*^{+/+} littermates of the respective mutants were used as wild-type controls. The morning of the day on which a vaginal plug was detected in females mating was designated gestation day 0.5. Mice heterozygous for TGF-β2 and/or GDNF alleles have no apparent defects, and no significant embryonic lethality was observed (Sanford et al., 1997; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Experiments were performed according to the National Health and ethical regulations. Animals were kept in a regulated environment (23°C ± 1°C, 50 % ± 5 % humidity) on a 12-hr light:12-hr dark cycle, with food and water ad libitum.

4.3 DNA extraction

Total genomic DNA was isolated from the limbs and the tails of the embryos and the old animals using QIAamp DNA Mini Kit (QIAGEN; cat. no: 51306) following the manufacturer's instructions. In brief, tails or limbs were cut and incubated with ATL Buffer and Proteinase K overnight, then ethanol (96–100%) was added to the samples. The whole mixture applied to the QIAamp Spin Column. After that, the mixture was washed with AW1 and AW2 solutions. Finally the genomic DNA was extracted using AE Buffer and kept at $-20\text{ }^{\circ}\text{C}$.

4.4 Genotyping

Polymerase chain reaction (PCR); a common method of creating copies of specific fragments of DNA, was used to identify the genotypes of the animals (Mullis and Faloona, 1987). The reaction started with the denaturation of two strands of a DNA template. After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands (annealing). Finally, the DNA-Polymerase enzyme has to fill in the missing strands. It starts at the annealed primer (the free 3'-OH group) and works its way along the DNA strand (elongation). Repeating the previous steps (denaturation, annealing and elongation) for 35 cycles will exponentially enrich the reaction with the primer-flanked DNA sequence. The PCR reaction was carried out in a 50 μl reaction volume with the following constituents: gDNA, primers (Invitrogen), dNTPs (Cat. No:1969046; Roche), 10X PCR Buffer (-MgCl₂) (Invitrogen, part no:Y02028), *Taq* DNA polymerase recombinant (Invitrogen, cat. No:10342-020), 50 mM MgCl₂ (Invitrogen, part no:Y02016).

4.5 Mastermix preparation

To prepare a final reaction volume of 50 μl , the following volumes and concentrations was used:

5 µl 10x PCR Buffer (- MgCl₂)
 1.5 µl of 50 mM MgCl₂
 1 µl of 10 mM dNTP mix
 2 µl forward primer (20 µM) and 2 µl primer reverse (20 µM)
 0.5 µl Taq DNA polymerase (5 U/µl)
 38 µl distilled water

Then 5 µl of the gDNA was added and mixed by pulse-vortexing for 10 seconds

4.6 Primer sequences

TGF-β₂ +/+ :

Forward primer: b2-5': AAT GTG CAG GAT AAT TGC TGC (5'-primer at 5'-end of exon 6)

Reverse primer: b2-3': AAC TCC ATA GAT ATG GGC ATG C (3'-primer at 3'-end of exon 6)

PCR product: 132 base pairs

TGF-β₂ -/- :

Forward primer: b2-neo: GCC GAG AAA GTA TCC ATC AT (5'-primer at 3'-end of the neo-cassette)

Reverse primer: b2-3': AAC TCC ATA GAT ATG GGC ATG C (3'-primer at 3'-end of exon 6)

PCR product: 600 base pairs

GDNF +/+:

Forward primer: PGK: GAC TAC GGG AGG AGT AGA AG

Reverse primer: GDNF: TAT CGT CTC TGC CTT TGT CC

PCR product: 338 base pairs

GDNF -/-:

Forward primer: p1: CCA GAG AAT TCC AGA GGG AAA GGT C (5' – 3')

Reverse primer: p2 : CAG ATA CAT CCA CAC CGT TTA GCG G (5' – 3')

PCR product: 480 base pairs

4.7 PCR programmes

The amplification reaction was done in a PCR thermocycler (Mastercycler, Eppendorf)

using the following programmes:

For TGF-β₂ +/+ and TGF-β₂ -/- PCR:

Initial denaturation	3 minutes 93 °C
Denaturation	30 seconds 93 °C
Annealing	1 minutes 56 °C 35 x Cycle
Elongation	1 minute 72 °C
Final elongation	10 minutes 72 °C
Hold	4 °C

For GDNF +/+ and GDNF -/- PCR:

Initial denaturation	5 minutes 95 °C
Denaturation	1 minutes 94 °C
Annealing	1 minutes 58 °C 35 x Cycle
Elongation	1 minute 72 °C
Final elongation	10 minutes 72 °C
Hold	4 °C

4.8 Agarose gel electrophoresis

PCR products were analyzed on 2 % agarose gels (Cat. No:2267.3, Roth, Germany). Agarose was boiled in TAE buffer for about 6 minutes. The solidified gel was inside the running chamber that was filled with 1x TAE. PCR products were mixed with 5X loading buffer and loaded into the wells. Then the gels were run at 125 V for about 60 min. The gels were put in ethidium bromide bath with shaking for 30 minutes. Finally, the amplified PCR products were visualized by UV light and photographs were taken (Table 2).

4.9 Embryos preparation

Pregnant mothers were killed by curvical dislocation, then embryos were taken from the mother, washed with saline (0.09 % NaCl) and photographed to document any obvious phenotypic changes. At embryonic day (E) 18.5, embryos were transcidentally perfused with 4% paraformaldehyde (PFA). Brains, bodies, skulls and adrenal glands were fixed in Bouin's fixative (75% picric acid, 25% formaldehyde, and 5% glacial acetic acid) for several hours, dehydrated in a graded series of ethanol concentrations and embedded in paraffin wax. At E12.5 and E14.5, embryos were fixed directly with Bouin's fixative without perfusion with PFA, dehydrated in a graded series of ethanol concentrations and embedded in paraffin wax. Serial sections (10µm) were obtained using microtome (Reichert-Jung-2050), mounted on glycerin-coated slides dried at 38 °C and kept in oven for 2 days at 37 °C.

4.10 Morphological studies

4.10.1 Nissl-staining

Paraffin sections of transgenic and wild-type mice were deparaffinized, hydrated to distilled water and afterward transferred to a 1.5% cresylviolet solution for 5-10 minutes. After this, sections were rinsed in distilled water, incubated in a solution contained distilled water and drops of glacial acetic acid. Sections were then transferred to an ascending alcohol-series (50,70, 90, 96, 100 % ethanol), and finally to xylol, before they were mounted using entellan (Merch, Germany) and coverslipped.

4.10.2 Hematoxylin-eosin-staining (H&E)

Paraffin sections (10 μm) were deparaffinized, hydrated to distilled water and transferred to Mayers hemalaum solution (cat no: T865.2; Roth, Germany) for 10 minutes. After this, sections were rinsed in distilled water and washed in running tap water for 10 minutes. Then sections were incubated with 0.1 % eosin solution (cat. No:45380; Merch, Germany) for 10-15 minutes and rinsed with distilled water. Afterward, sections were transferred to an ascending alcohol-series (50,70, 90, 96, 100 % ethanol) and finally to xylol, before they were mounted using entellan (Merch, Germany) and coverslipped (Roth, Germany).

4.10.3 Trichrome staining

Connective tissue as well as hyaline cartilage was stained with trichrome staining according to Masson-Goldner, as described by Romeis (1989). In brief, sections were deparaffinized, hydrated to distilled water. Then sections were incubated with Weigert's iron hematoxylin working solution for 10 minutes and rinsed in running tap water for 10 minutes. Afterward sections were incubated with acid fuchsin solution, washed with 1% glacial acetic acid in distilled water, differentiated in phosphomolybdic acid-Orange G, and stained with light green. Finally, sections were dehydrated quickly through ascending

alcohol-series (50,70, 90, 96, 100 % ethanol) and finally to xylol, before they were mounted.

4.10.4 Morphology of the retina

Retinal morphology was studied on hematoxylin-eosin-stained histological paraffin sections of transgenic and wild-type mice. Measurements of thickness of retina was performed on digital images of matching sections in the optic nerve region using the Zeiss Axiovision image analysis software, version 3.0.6. Three random areas of each section were measured for each animal analyzed.

4.11 Immunohistochemistry

4.11.1 Tyrosine Hydroxylase (TH) stain

Tyrosine hydroxylase (TH) catalyzes the rate-determining initial step in the biosynthesis of catecholamines such as dopamine, noradrenaline and adrenaline. Therefore the antibody is used as a marker for dopaminergic and adrenergic neurons. Paraffin sections were deparaffinized, hydrated to distilled water and heated for 5 min in citrate buffer (0.1M, pH=6) in a microwave oven at 550 W to improve antigen retrieval. Endogenous peroxidase activity was quenched by pre-incubation in 3% H₂O₂ (Merck) for 10 minutes. Sections were preincubated with 10% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 for 1 hour. Then sections were incubated in a solution containing 5% NGS, 0.1% Triton-X in PBS, and monoclonal mouse TH anti-serum (MAB5280, Chemicon) at 4 C° overnight. After rinsing with PBS, sections were incubated with goat anti mouse (GaM; 1:50; Nordic) for one hour at room temperature, rinsed in PBS and incubated with mouse peroxidase anti peroxidase (PaP-M; 1:800; Nordic) for one hour at room temperature. Visualization of the immune complex was achieved by incubation with diaminobenzidine (DAB; Sigma) according to Adam's method (1977). The sections were dehydrated in ethanol and mounted using entellan. For the controls, in all cases PBS was substituted for

the primary antisera in order to test for nonspecific labeling. No specific cellular staining was observed when the primary antiserum was omitted.

4.11.2 Serotonin (5-hydroxytryptamine, 5-HT) stain

Serotonin (5-hydroxytryptamine, 5-HT) was used as a marker for hindbrain serotonergic neurons. Paraffin sections were deparaffinized, hydrated to distilled water and heated for 5 min in 0.1M citrate buffer in a microwave oven at 550 W to improve antigen retrieval. Sections were treated with 3% H₂O₂ (Merck) for 10 minutes to block endogenous peroxidase activity. Sections were preincubated with 10% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 for 3 hour. Then sections were incubated in a solution containing 5% NGS, 0.1% Triton-X in PBS, and rabbit 5-HT antiserum (S-5545, Sigma) at 4 C° overnight at a concentration of 1:1000. After rinsing with PBS, sections were incubated with goat anti rabbit (GaR; 1:100; Nordic) for one hour at room temperature, rinsed in PBS and incubated with rabbit peroxidase anti peroxidase (PaP-R; 1:800; Nordic) for one hour at room temperature. The immune complex was visualized by incubation with diaminobenzidine (DAB; Sigma) according to Adam's method (1977). Finally, Sections were then transferred to an ascending alcohol-series and finally to xylol, before they were mounted.

4.11.3 PCNA stain

To detect cells which are still proliferating, a mouse monoclonal antibody directed against the proliferative cell nuclear antigen protein (PCNA) was used (Novokastra). In brief, sections were deparaffinized, hydrated to distilled water and heated in citrate buffer in a microwave oven to improve antigen retrieval as described above. Sections were treated with 3% H₂O₂ in PBS to block endogenous peroxidase activity. Non-specific binding was blocked by pre-incubation in 10% normal goat serum (NGS; Sigma) containing 0.1% Triton for two hours. Sections were incubated with anti-PCNA antibody (1:100) at 4 °C

overnight. After rinsing with PBS, sections were incubated goat anti mouse (GaM), rinsed in PBS and incubated with mouse peroxidase anti peroxidase (PaP-M) as described above. The reaction was visualized with Ni-intensified DAB. Cell proliferation was quantified by counting PCNA-positive cells at 60 X magnification in two random visual fields in the central retina on either side of the optic nerve. Regionally matching sections were used for each animal counted (n=3).

4.11.4 Neurofilament stain

Neurofilaments are a type of intermediate filament that serve as major elements of the cytoskeleton supporting the axon cytoplasm. Rabbit polyclonal anti-neurofilament (NF) antibody (AB1981, Chemicon) was used as neuronal marker; it stains neurons of the central and peripheral nervous system. Sections were incubated with anti-NF antibody (1:1500) at 4 °C overnight. After that GaR and PaP-R was used as described above. The reaction was visualized with Ni-intensified DAB.

4.11.5 Alpha-smooth muscle actin stain

Mouse anti-smooth muscle actin (1:2000; A5228, Sigma) was performed to study the phenotype in the smooth muscle at embryonic stages. Immunohistochemistry was done following the manufacturer's instructions.

4.11.6 Chromaffin cells staining

For specific staining of chromaffin cells, paraffin sections of adrenal gland were immunostained using a rabbit anti-tyrosine hydroxylase (anti-TH) antibody (Chemicon). Immunostaining for PNMT (Phenylethanolamine-N-methyltransferase) was performed using a polyclonal rabbit anti-PNMT antibody (Chemicon) specifically staining differentiated chromaffin cells. To detect cells which are still proliferating, a mouse monoclonal antibody directed against the PCNA was used (Novokastr). Paraffin sections were deparaffinized and heated in citrate buffer (0.1M, pH=6) in a microwave oven at 600W for 5 min to

improve antigen retrieval. Endogenous peroxidase activity was quenched by pre-incubation in 3% H₂O₂ (Merck). Non-specific binding was blocked by pre-incubation in 10% normal goat serum (NGS; Sigma) containing 0.1% Triton for two hours. Sections were incubated with an anti-TH antibody (1:200) at 4 °C overnight and rinsed in phosphate buffer saline (PBS). The antibody was visualised by incubation with a goat anti-rabbit-Cy3 labelled (GaR-Cy3) secondary antibody (Molecular Probes) for one hour at room temperature. The section were mounted with prolong anti-fade kit (Molecular probes). For PNMT and PCNA staining, sections were incubated with anti-PNMT antibody (1:500) or anti-PCNA antibody (1:100), respectively, at 4 °C overnight. After rinsing with PBS, sections were incubated with GaR (1:100), goat anti mouse (GaM; 1:100; Nordic), respectively, for one hour at room temperature, rinsed in PBS and incubated with rabbit peroxidase anti peroxidase (PaP-R; 1:1000; Nordic) or mouse peroxidase anti peroxidase (PaP-M; 1:800; Nordic), respectively, for one hour at room temperature. Visualization of the immune complex was achieved by incubation with diaminobenzidine (DAB; Sigma) according to Adam's method (1977). The sections were dehydrated in ethanol and mounted. In order to evaluate the total number of TH⁺ and PNMT⁺ chromaffin cells, every third serial section of adrenal gland was counted to avoid double counting. For PCNA⁺ cells, the ratio for PCNA⁺/TH⁺ cells in a total of 300 TH⁺ cells was calculated. Data represent means of n=3 ± SEM. The significance of inter-group differences was detected by applying student *t*-test for unpaired samples. Differences were considered significance at P< 0.05 .

4.12 Adult animals and tissue processing

Tgfb2^{+/-}, *Gdnf*^{+/-} and double heterozygous (*Tgfb2*^{+/-}*Gdnf*^{+/-}) adult (12 months) mice and wild-type control littermates (*Tgfb2*^{+/+}*Gdnf*^{+/+}) were used and maintained in accordance with the institutional guidelines for animal rights. Animals were deeply anesthetized with xylazine (2%) and hostaket (100mg/ml) at a ratio of (0.1 ml/10gm body weight) then

transcardially perfused with 4% PFA. Brains were removed and immersed in the same fixative (4 % PFA) for one day then incubated in 30 % sucrose solution overnight. Serial sagittal sections (60 μ M) were made using a vibratome (Reichert-Jung Mod. 1206, Germany), kept in chambers filled with phosphate buffered saline (PBS) and treated with 3% H₂O₂ for 10 minutes to block endogenous peroxidase activity. Then sections were incubated with 10% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 overnight. Finally, sections were stained with TH antibody as a marker for dopaminergic neurons as mentioned before. The total number of the TH positive cells in the midbrain was estimated using the optical fractionator method (West et al., 1991; von Bohlen und Halbach et al., 2005). The thickness of the sections after staining were measured using the commercially available stereological system StereoInvestigator version 6 (MicroBrightField) and a computer-driven motorized stage. The instrumental thickness was 60 μ m and the postprocessing thickness was $25 \pm 0.5 \mu$ m. A systematic random series of sections throughout the SNpc-VTA was collected. The first sampled section was selected randomly by using the random number generator of StereoInvestigator. On each section selected, the outlines of the borders of the SNpc-VTA were drawn with $\times 5$ lens and a grid was placed randomly over the area of interest. At predetermined positions of the grid, cells were counted within three-dimensional optical disectors using an oil objective with a magnification of $\times 63$. The total number of neurons was estimated using the equation:

$$N = Q- \times t/h \times 1/asf \times 1/ssf$$

where N is the total neuronal number, Q- is the number of objects counted, t is the section height, h is the height of the sampling fraction, asf is the area sampling fraction, sf is the section sampling fraction.

4.13 Counting procedure and statistical analysis

The total number of cells of interest was evaluated by counting every fifth serial section (10 μm) of different tissues to avoid double counting. Cells of clear cytoplasm and clear nuclei were considered as positive cells. Data represent means of minimum $n=3 \pm \text{SEM}$. The significance of inter-group differences was detected by applying student *t*-test for unpaired samples. Differences were considered significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

4.14 Solutions

4.14.1 Phosphate buffer solution

27.6 gm $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (A) was dissolved in 1000 ml distilled water

35.6 gm $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (B) was dissolved in 1000 ml distilled water

190 ml of A was mixed with 810 ml of B to prepare 1L PB (0.2M; pH=7.4)

4.14.2 Phosphate buffered saline solution (PBS)

9.0 gm NaCl and 100 ml PB 0.1M were added to 900 ml distilled water to prepare 1L PBS (pH =7.2).

4.14.3 Paraformaldehyde solution (4%PFA)

40 gm of PFA (Merck, Germany) was dissolved in 500 ml distilled water, heated until 60 $^{\circ}\text{C}$. Aldehyde formation was occurred upon addition of drops of NaOH (1M). Then the solution was cooled, filtered and mixed with 500 ml of 0.2M PB to prepare 1L 4% PFA (pH=7.4).

4.14.4 Citrate buffer

21.01 gm $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ (A) was dissolved in 1000 ml distilled water

29.40 gm $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ (B) was dissolved in 1000 ml distilled water

190 ml of A was mixed with 810 ml of B to prepare 1L citrate buffer (0.1M; pH=6.0)

4.14.5 Tris acetate buffer (TAE, 50X, pH=7,9, 1L)

242 gm Tris

57.1 ml glacial acetic acid

18,61 gm EDTA (0.5M, pH=8.0)

5. Results

5.1 Mating and genotyping results

Tgfb2^{+/-}*Gdnf*^{+/-} double heterozygous mice presented no apparent defects. No embryonic lethality was observed, and the offsprings were viable and partially fertile. As a result of matings between TGF-β2/GDNF double-heterozygous mice (*Tgfb2*^{+/-}*Gdnf*^{+/-}), several double mutant mice embryos (*Tgfb2*^{-/-}*Gdnf*^{-/-}) and the other expected genotypes were obtained at developmental stages E12.5 to E18.5. As all embryos lacking both alleles of GDNF also lack both kidneys it is expected that double mutant mice would also die after birth as single mutant mice do. Table 2 summarizes the PCR results for the genotyping of the four alleles investigated. During this study 270 embryos were obtained, which included 11 double mutant mice (Table 3), i.e. 60% of the expected embryos according to Mendelian genetics. Gene-targeted mice with one GDNF-null allele show depletion of sperms, whereas mice overexpressing GDNF show accumulation of undifferentiated spermatogonia (Meng et al., 2000). Thus, heterozygous GDNF males have a reduced numbers of sperms resulting in infertility of some of the animals.

Table 3: Numbers of the double mutant mice obtained at different developmental stages.

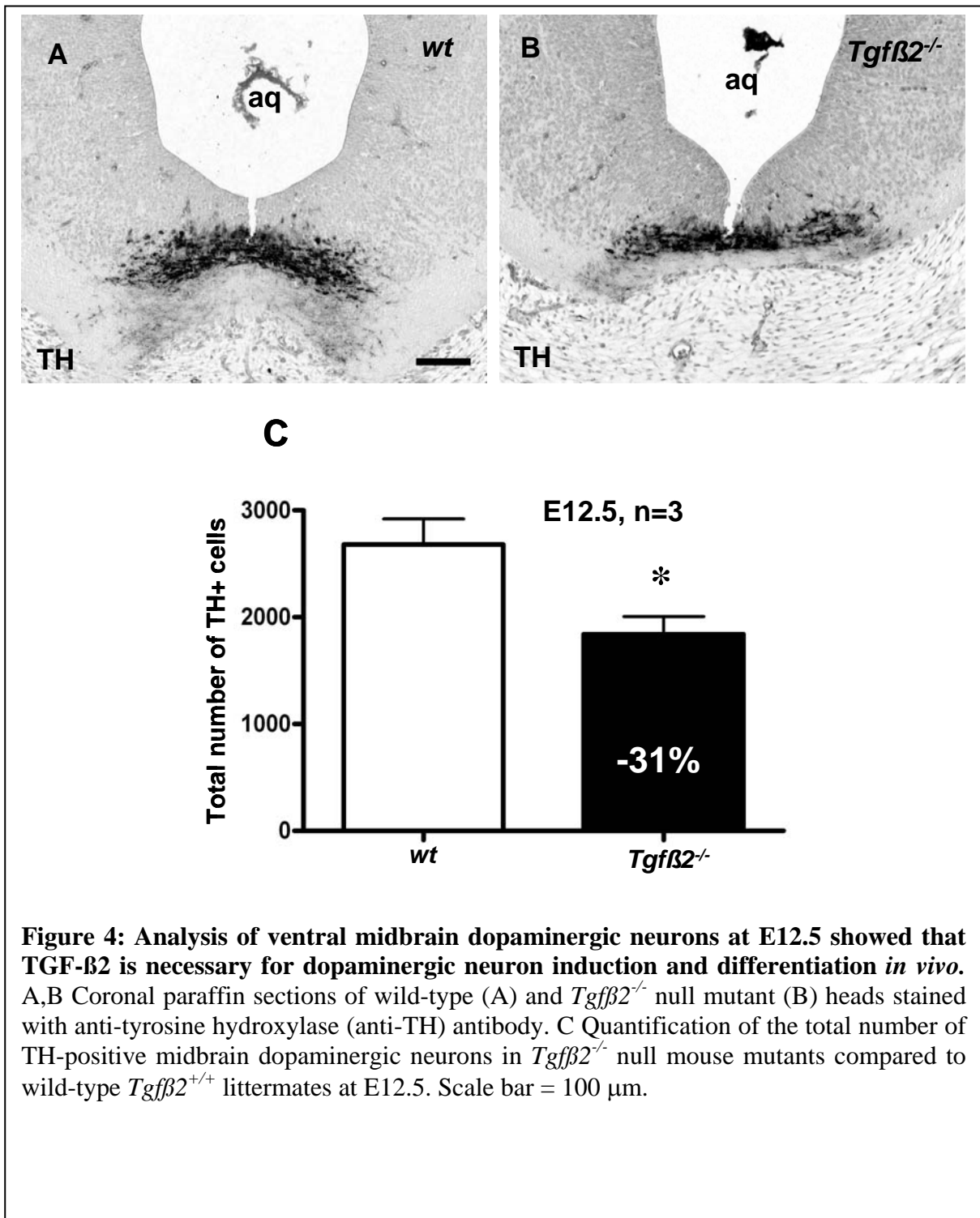
Age	# of <i>Tgfb2</i> ^{-/-} <i>Gdnf</i> ^{-/-} embryos
E12.5	2
E14.5	3
E16.5	2
E18.5	4
Total	11

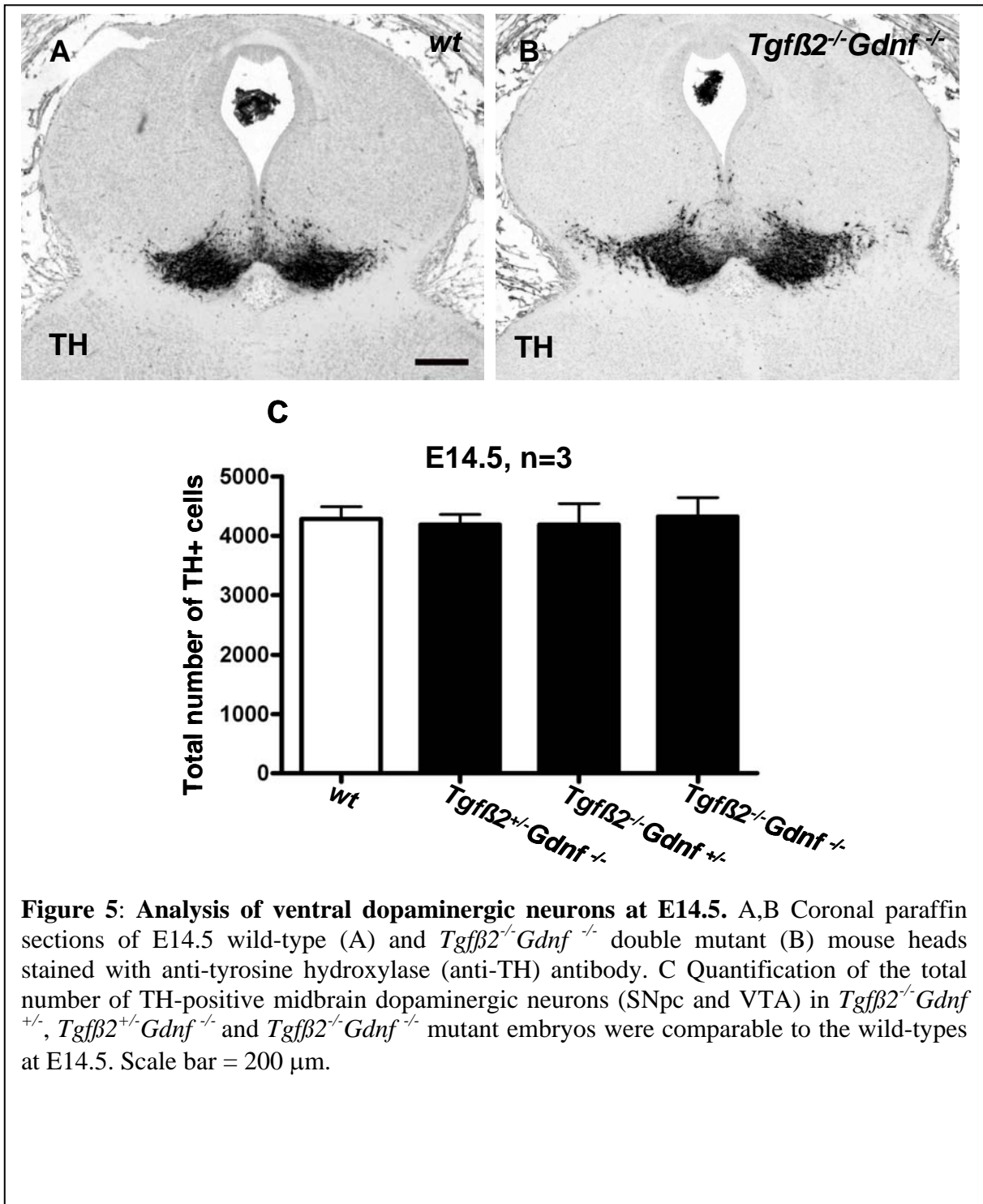
Table 2: Summary for the genotypes of offsprings obtained from matings between double heterozygote mice ($Tgfb2^{+/-}Gdnf^{+/-}$).

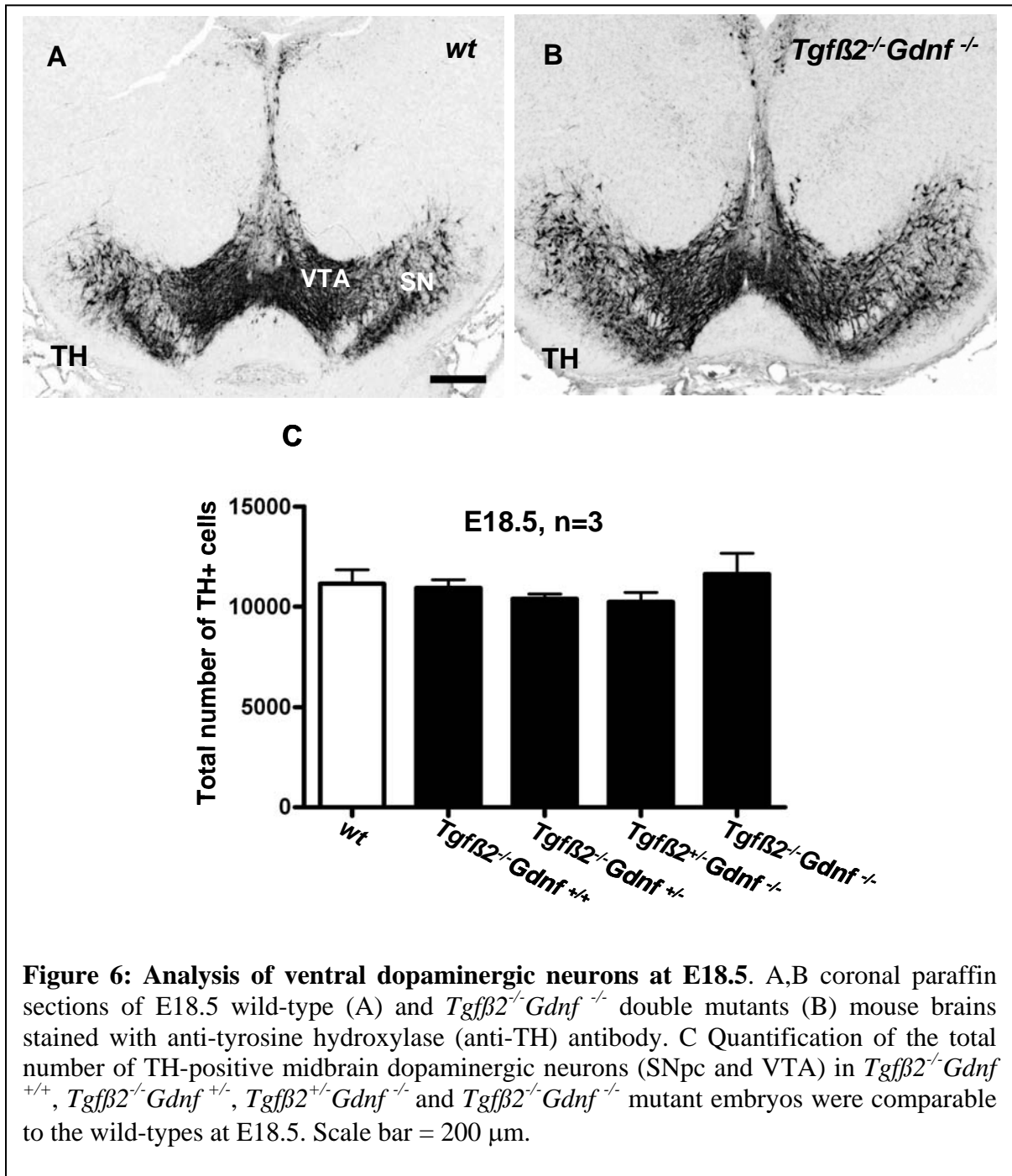
Genotypes	Number of Embryos (E12,E14,E16,E18)	Mendel's Genetics (Theoretically)
$Tgfb2^{+/+}Gdnf^{+/+}$	19	17
$Tgfb2^{+/-}Gdnf^{+/+}$	38	34
$Tgfb2^{+/+}Gdnf^{+/-}$	28	34
$Tgfb2^{+/+}Gdnf^{-/-}$	25	17
$Tgfb2^{-/-}Gdnf^{+/+}$	17	17
$Tgfb2^{-/-}Gdnf^{+/-}$	26	34
$Tgfb2^{-/-}Gdnf^{-/-}$	34	34
$Tgfb2^{+/-}Gdnf^{+/-}$	72	68
$Tgfb2^{-/-}Gdnf^{-/-}$	11	17
Total	270 Embryos	

5.2 Dopaminergic neurons

To determine the role of TGF- β 2 and GDNF in the development, induction and survival of midbrain dopaminergic neurons, single and double mutant mice for TGF- β 2 and GDNF were generated at different embryonic stages. At embryonic stage 12.5 (E12.5), the total number of midbrain dopaminergic neurons stained for tyrosine hydroxylase (TH) were significantly decreased in mice deficient for TGF- β 2 ($Tgfb2^{-/-}$) compared to wildtype ($Tgfb2^{+/+}$) mice embryos (Fig. 4). Surprisingly, at E18.5 (Fig. 5), the number of the dopaminergic neurons in SVpc and VTA of $Tgfb2^{-/-}$ mutant mice embryos were comparable to wildtype littermates. Furthermore, our analyses failed to reveal significant differences in the total number of TH-positive cells in substantia nigra pars compacta (SVpc) and ventral tegmental area (VTA) in $Tgfb2^{-/-}Gdnf^{+/+}$, $Tgfb2^{+/-}Gdnf^{-/-}$, $Tgfb2^{-/-}Gdnf^{-/-}$ mutant mice embryos compared to the controls ($Tgfb2^{+/+}Gdnf^{+/+}$) at E14.5 (Fig. 5) and E18.5 (Fig. 6). These data may indicate that lack of TGF- β 2 at E12.5 and earlier, delayed dopaminergic neuron development a little bit which could be compensated by E18.5. Otherwise TGF- β 2 and GDNF seemed to have only marginal effects on the development of the midbrain dopaminergic neurons at these stages *in vivo*. To test whether reduced levels of TGF- β 2 and GDNF may result in degeneration of nigrostriatal dopaminergic neurons with age, the total number of midbrain dopaminergic neurons in one year old $Tgfb2^{+/-}$ and double heterozygous ($Tgfb2^{+/-}Gdnf^{+/-}$) mice were analyzed. The $Tgfb2^{+/-}$ and double heterozygous ($Tgfb2^{+/-}Gdnf^{+/-}$) mice showed a marginal decrease (10% and 13% respectively) in the neuronal number compared to the controls ($Tgfb2^{+/+}Gdnf^{+/+}$). Differences did not reach statistical significance (Fig. 7). Because double mutant mice die at birth, we are unable to assess a possible role for GDNF and TGF- β 2 in the maintenance of these neurons during postnatal maintenance.







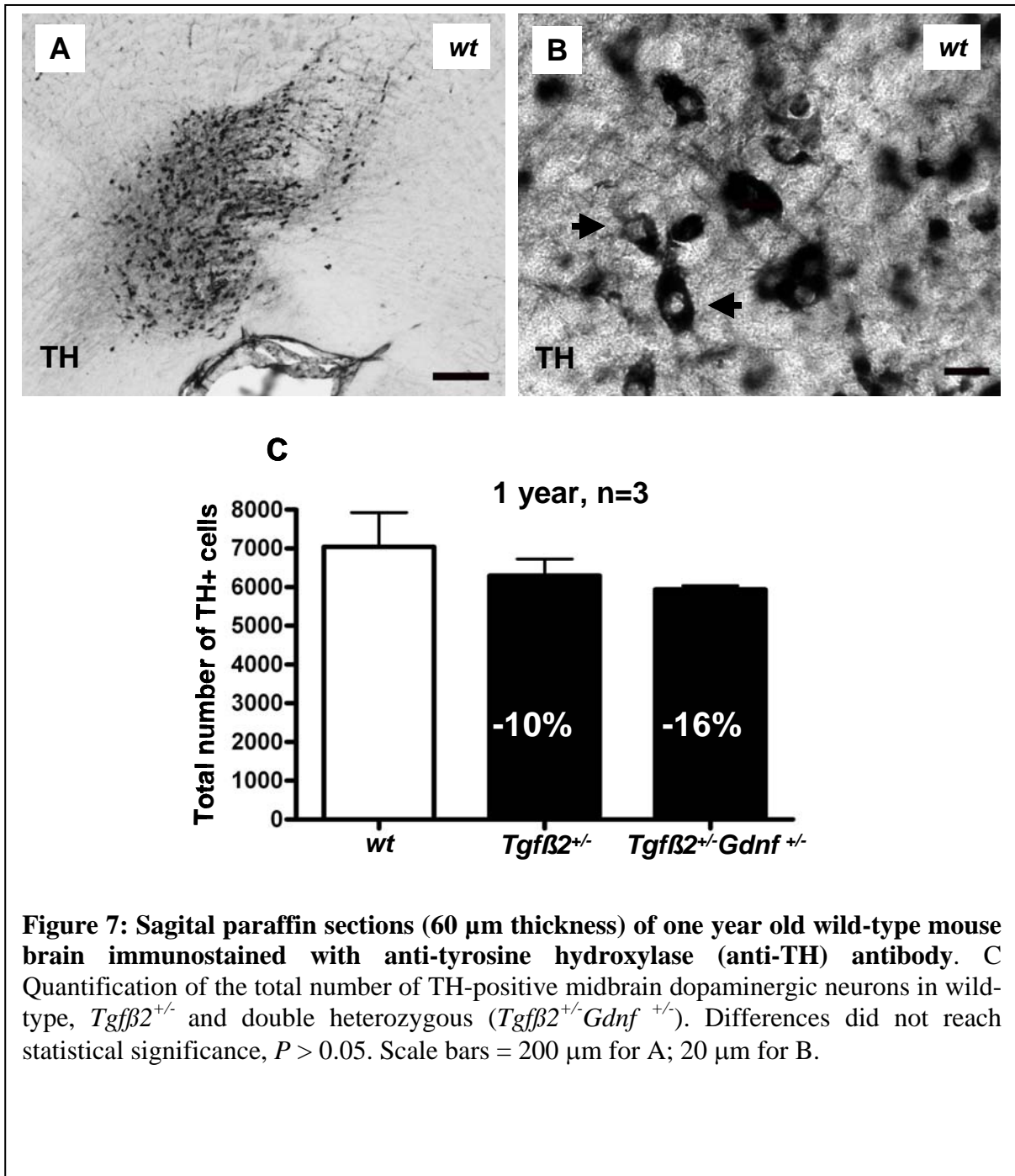
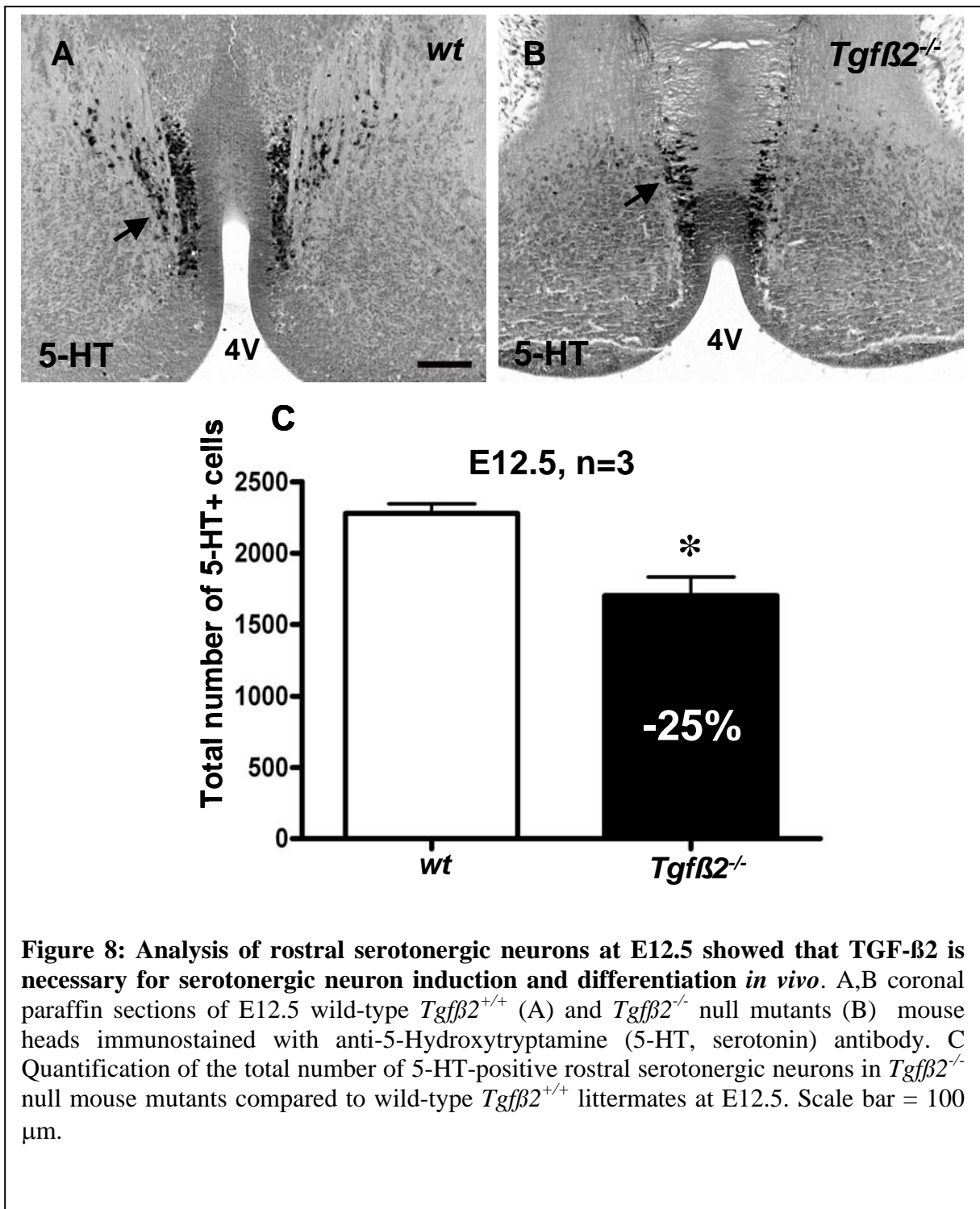


Figure 7: Sagittal paraffin sections (60 μm thickness) of one year old wild-type mouse brain immunostained with anti-tyrosine hydroxylase (anti-TH) antibody. C Quantification of the total number of TH-positive midbrain dopaminergic neurons in wild-type, $Tgfb2^{+/-}$ and double heterozygous ($Tgfb2^{+/-}Gdnf^{+/-}$). Differences did not reach statistical significance, $P > 0.05$. Scale bars = 200 μm for A; 20 μm for B.

5.3 Serotonergic neurons

To investigate a putative role of TGF- β 2 and GDNF in the induction and survival of hindbrain serotonergic neurons, single and double mutant mice for TGF- β 2 and GDNF were generated and analyzed at different embryonic stages. Figure 8 shows that at E12.5 the total number of 5-HT-immunoreactive neurons was significantly decreased (1702 ± 130 ; $P < 0.05$) in mice deficient for TGF- β 2 ($Tgfb\beta 2^{-/-}$), compared with wild-type mice embryos (2278 ± 68). Moreover, there was a significant decrease in the total number of serotonergic neurons in $Tgfb\beta 2^{-/-}$ (630 ± 71 ; $P < 0.05$) and $Tgfb\beta 2^{-/-}Gdnf^{+/-}$ (458 ± 79 ; $P < 0.01$) at the paramedian raphe (PMR) at E18.5 compared with wild-type (853 ± 49) (Fig. 9). On the other hand, although quantification of the rostral 5-HT-positive cells showed decrease at E14.5 in the double mutant mice ($Tgfb\beta 2^{-/-}Gdnf^{-/-}$), compared with wild-type mice embryos, these differences did not reach statistical significance ($P > 0.05$; Fig. 10). One of these three double knockout mice at E14.5 showed a severe defect (decrease) in the number of the rostral 5-HT-positive neurons (Fig. 11), probably because of genetic penetrance, whereas the caudal 5-HT-positive neurons in this embryo were comparable to the controls. Interestingly, at E18.5 too, one out of three of these double mutant mice showed a severe decrease in the rostral 5-HT-positive neurons (Fig. 12). In contrast, the number of the caudal 5-HT-positive neurons was found comparable to the controls. Furthermore, the number of rostral hindbrain serotonergic neurons in $Tgfb\beta 2^{-/-}Gdnf^{+/-}$, $Tgfb\beta 2^{+/-}Gdnf^{-/-}$ at E14.5 and $Gdnf^{-/-}, Tgfb\beta 2^{+/-}Gdnf^{-/-}$ at E18.5 showed no differences, compared to the wild-type embryos (Fig. 10 and 13). Since TGF- β 2 and GDNF single deficient mice die at birth, it was impossible to analyse these mice postnatally.



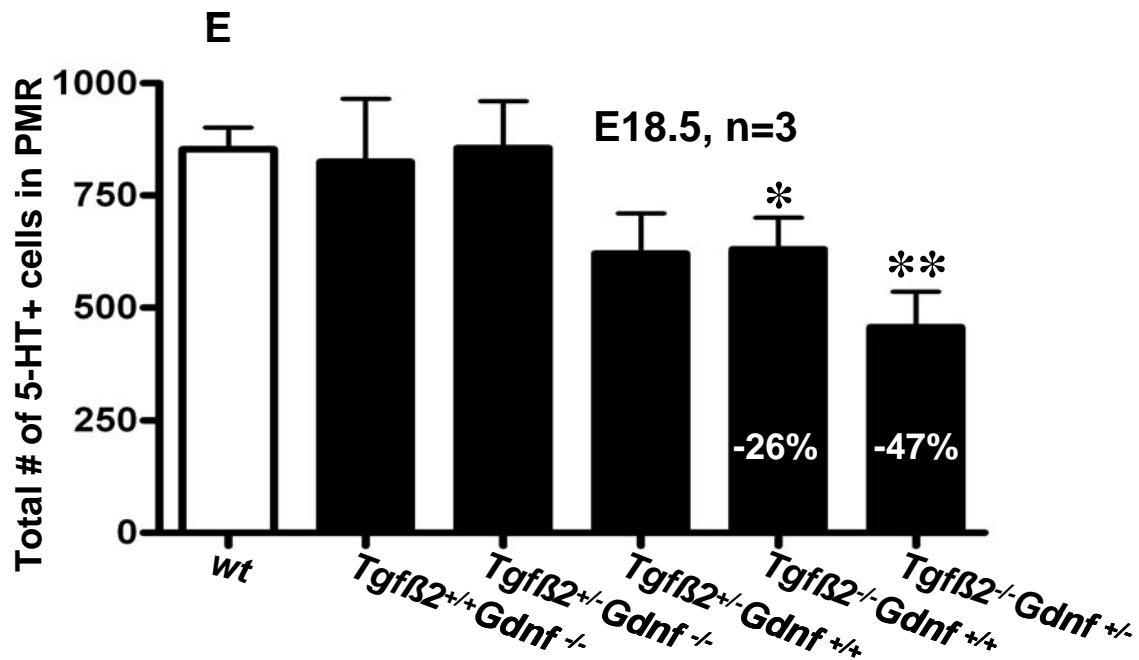
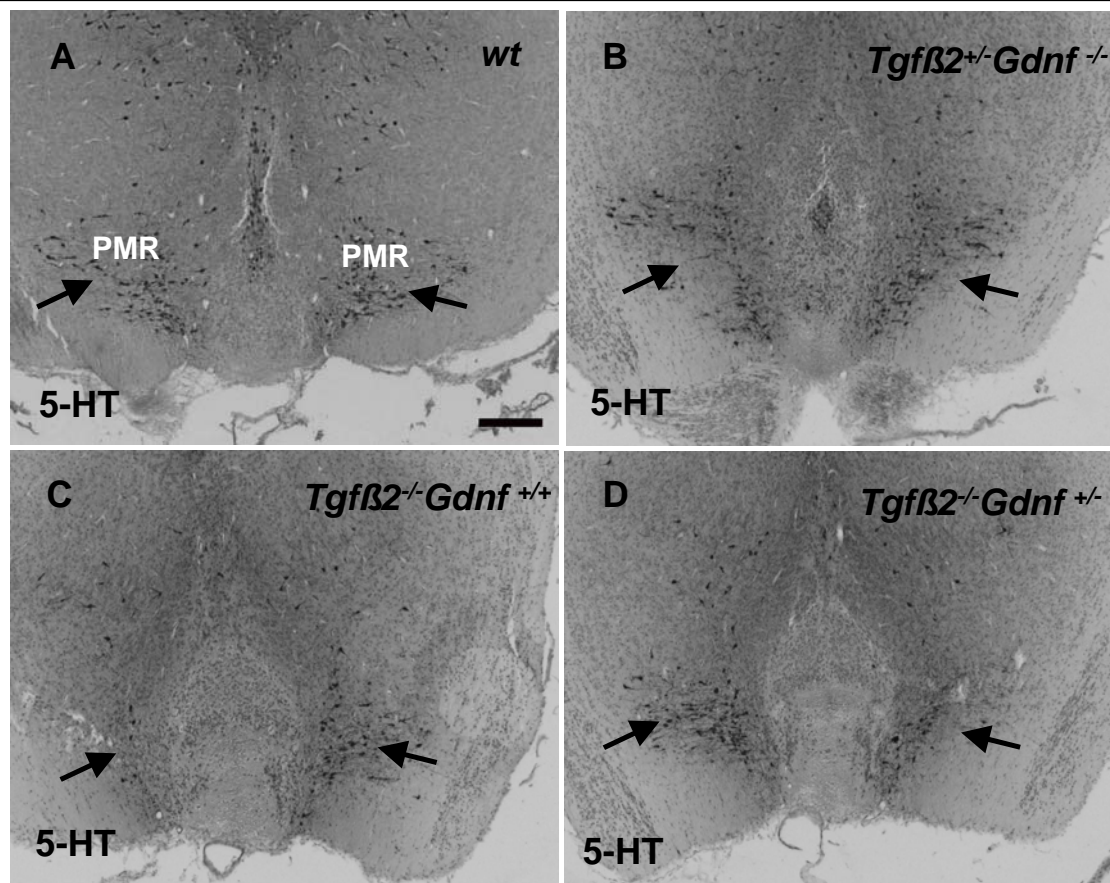


Figure 9: Analysis of serotonergic neurons in the paramedian raphe (PMR) at E18.5. A-D coronal sections of E18.5 mouse brains immunostained with anti-serotonin (anti-5-HT) antibody. C Quantification of the total number of 5-HT-positive neurons in the PMR of $Tgf\beta 2^{-/-}Gdnf^{+/+}$ and $Tgf\beta 2^{-/-}Gdnf^{+/-}$ mouse embryos were significantly decreased compared to wild-type controls at E18.5, while $Tgf\beta 2^{+/-}Gdnf^{-/-}$, $Tgf\beta 2^{+/-}Gdnf^{+/-}$ and $Tgf\beta 2^{+/-}Gdnf^{+/+}$ were developed normally. Scale bar = 100 μ m.

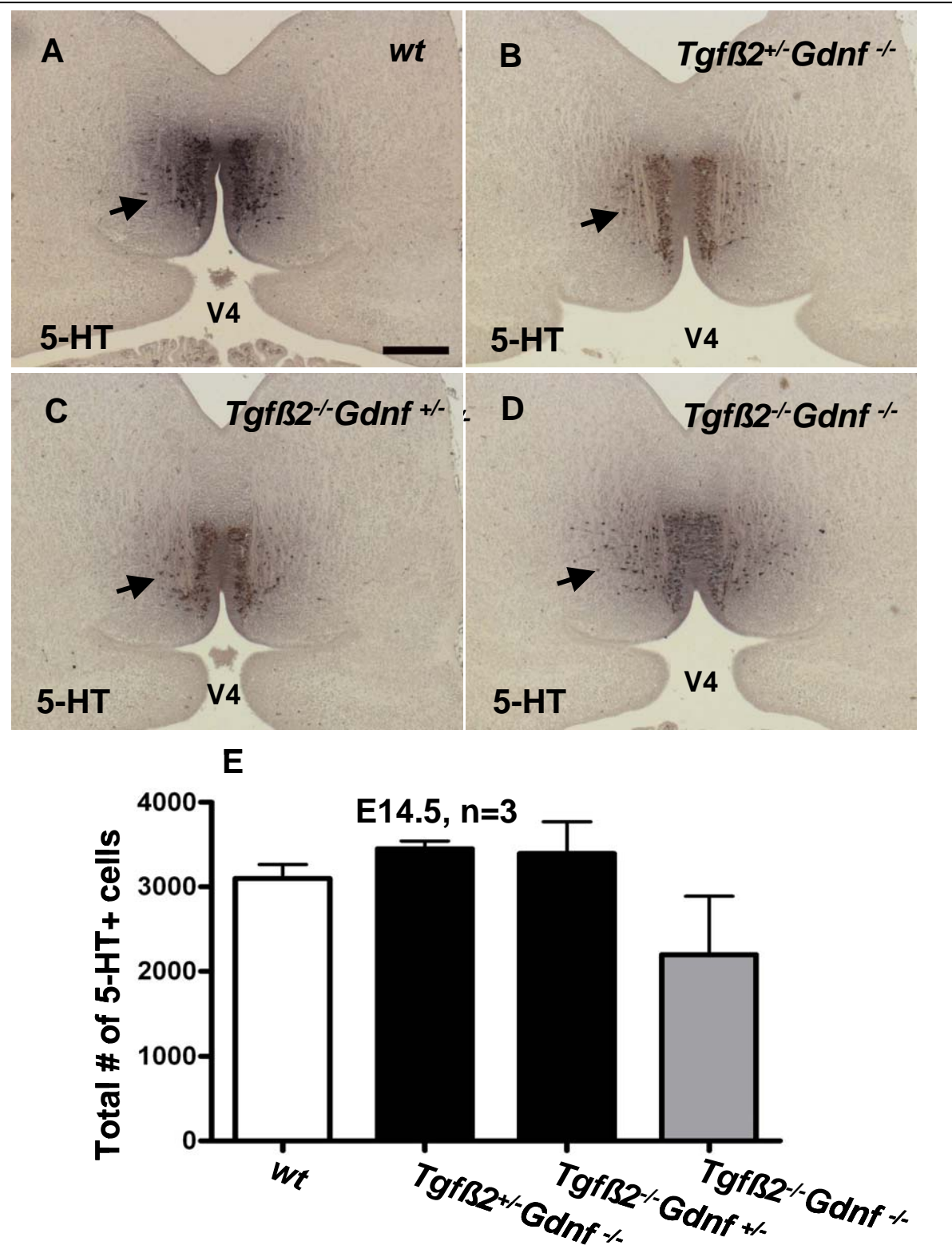
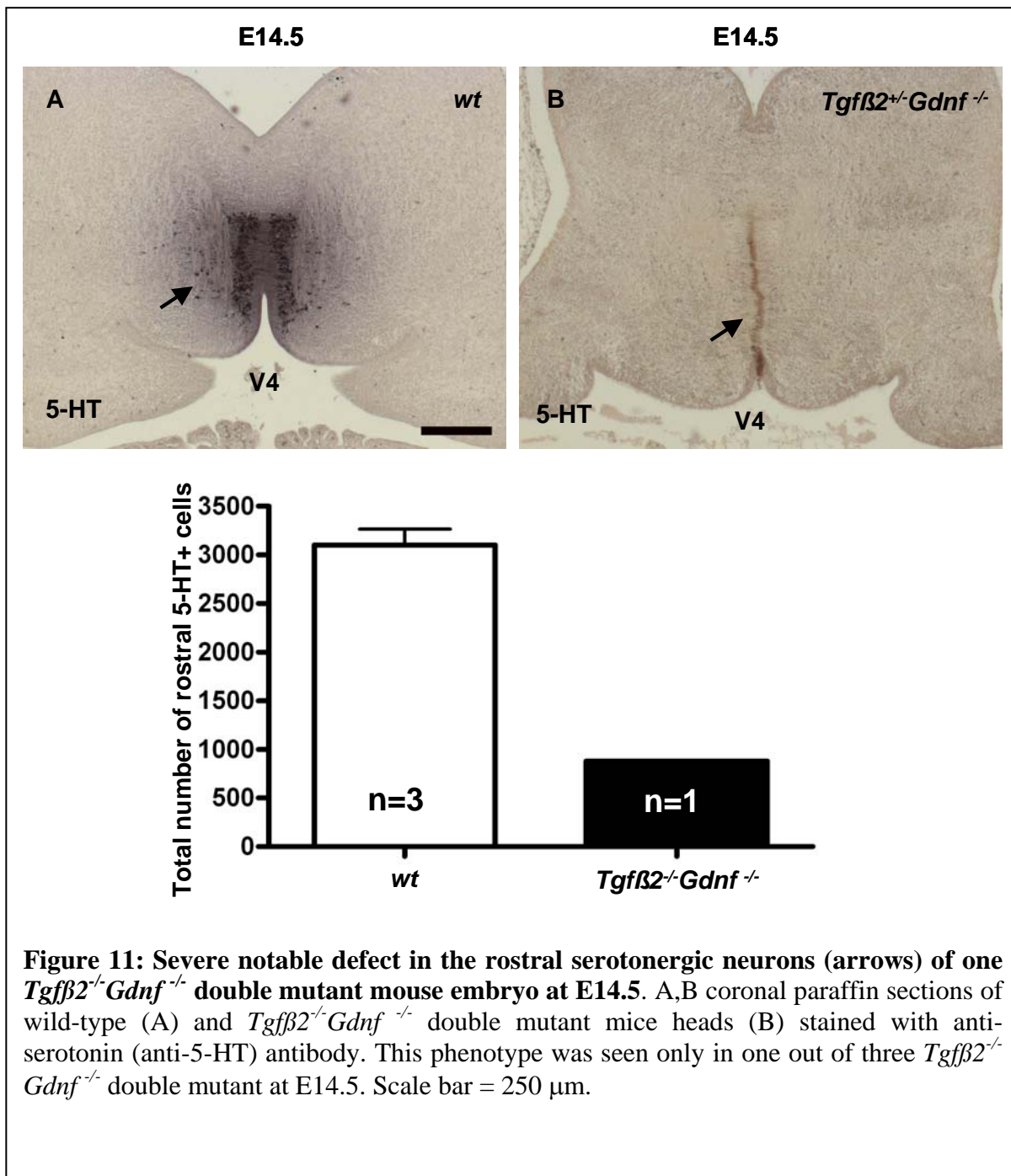


Figure 10: Analysis of rostral serotonergic neurons at E14.5. A-D coronal sections of E14.5 mouse heads immunostained with anti-serotonin (anti-5-HT) antibody. The total number of rostral serotonergic neurons (arrows) of $Tgf\beta 2^{+/-}Gdnf^{-/-}$ (B), $Tgf\beta 2^{-/-}Gdnf^{+/-}$ (C) and $Tgf\beta 2^{-/-}Gdnf^{-/-}$ mutant embryos were identical to the wild-type mice embryos (A). E Quantification of the total number of rostral 5-HT-positive neurons in $Tgf\beta 2^{+/-}Gdnf^{-/-}$, $Tgf\beta 2^{-/-}Gdnf^{+/-}$ and $Tgf\beta 2^{-/-}Gdnf^{-/-}$ mouse mutants compared to wild-type ($Tgf\beta 2^{+/+}Gdnf^{+/+}$) littermates at E14.5. Scale bar = 250 μ m.



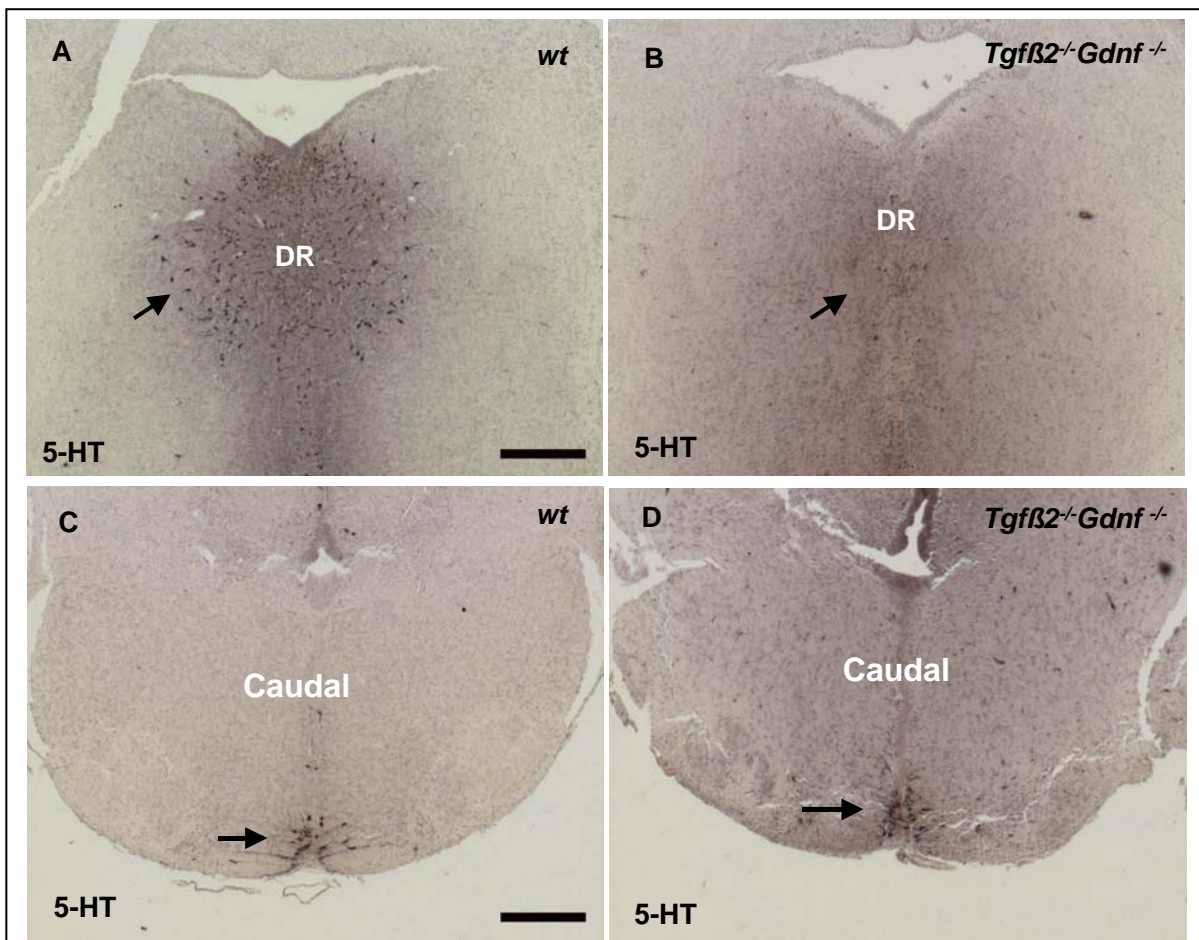


Figure 12: Severe notable defect in the rostral dorsal raphe (DR) serotonergic neurons (arrows) of one *Tgfβ2*^{-/-}*Gdnf*^{-/-} double mutant mouse embryo at E18.5. A-D coronal paraffin sections of wild-type (A, C) and *Tgfβ2*^{-/-}*Gdnf*^{-/-} double mutant mice heads (B, D) stained with anti-serotonin (anti-5-HT) antibody. The caudal serotonergic neurons was normal in the double mutant embryos. This phenotype was seen only in one out of three *Tgfβ2*^{-/-}*Gdnf*^{-/-} double mutant at E14.5. Scale bar = 250 μm.

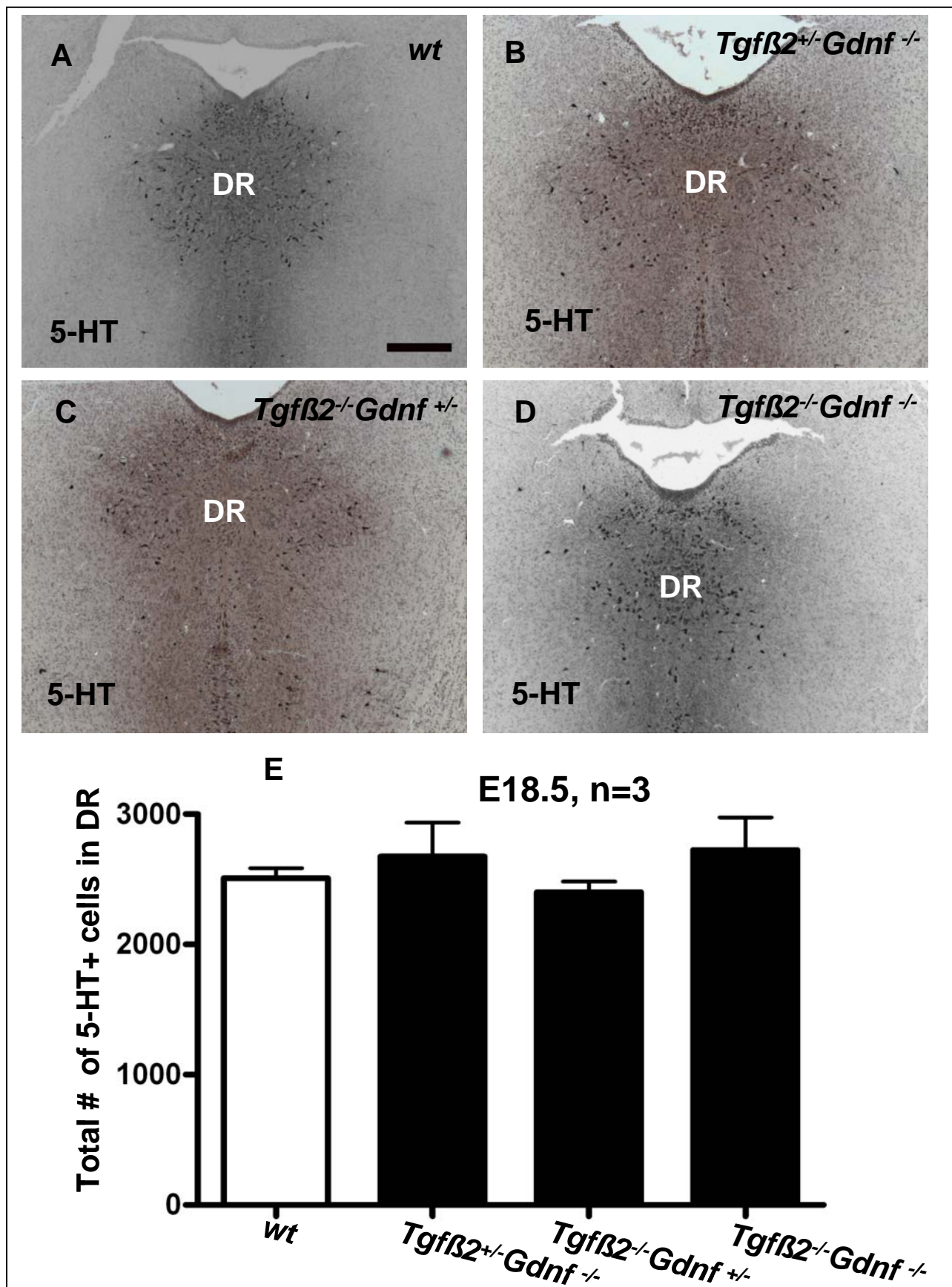


Figure 13: Analysis of dorsal raphe (DR) serotonergic neurons at E18.5. A-D Coronal sections of E18.5 mouse brains stained with anti-serotonin (anti-5-HT) antibody. E Quantification of the total number of 5-HT-positive dorsal raphe neurons (B7 serotonergic cell bodies) of $Tgf\beta 2^{+/-}Gdnf^{-/-}$ (B), $Tgf\beta 2^{-/-}Gdnf^{+/-}$ (C) and $Tgf\beta 2^{-/-}Gdnf^{-/-}$ mutant embryos were comparable to the wild-types (A). Scale bar = 250 μ m.

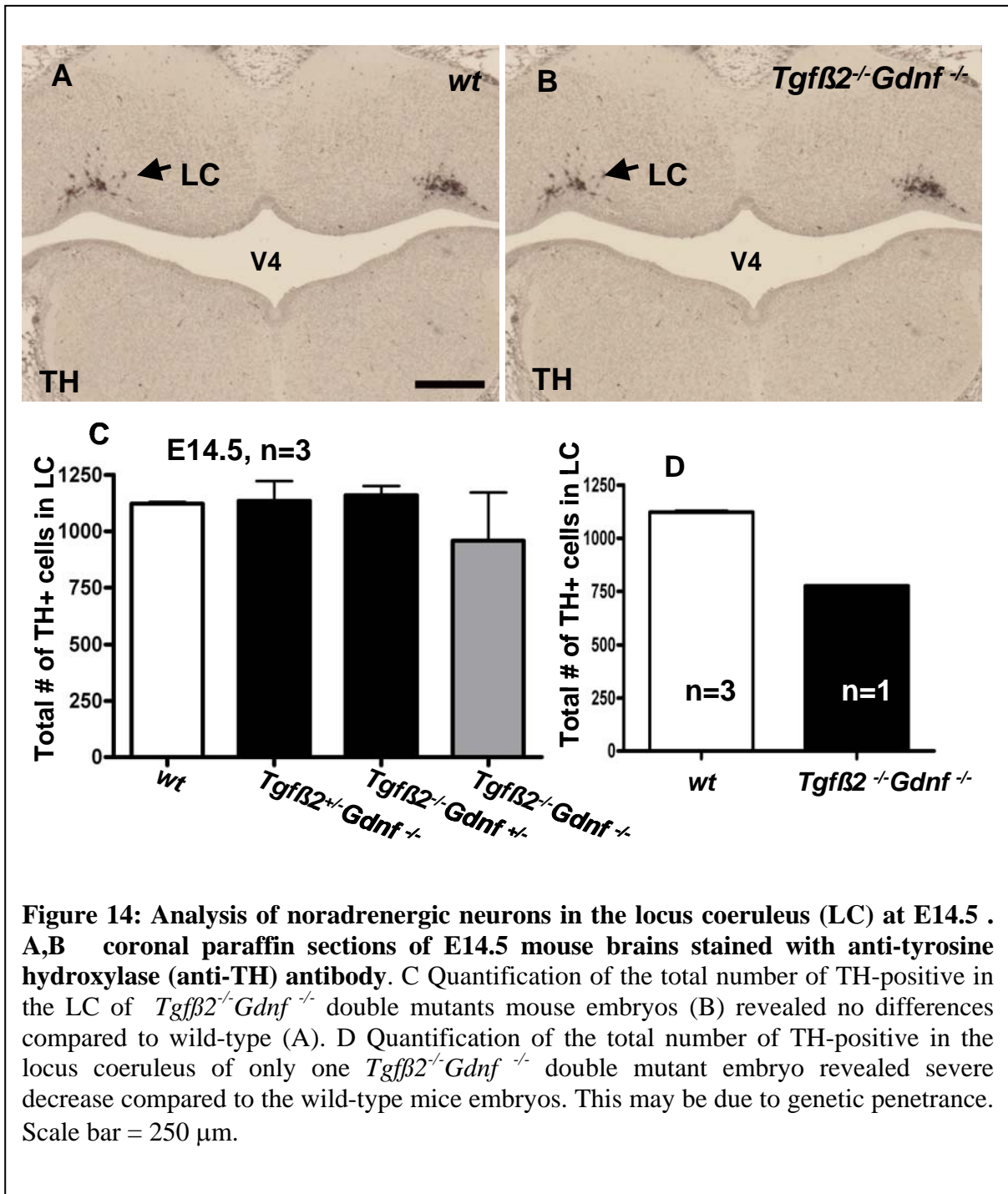
5.4 Noradrenergic neurons in the LC

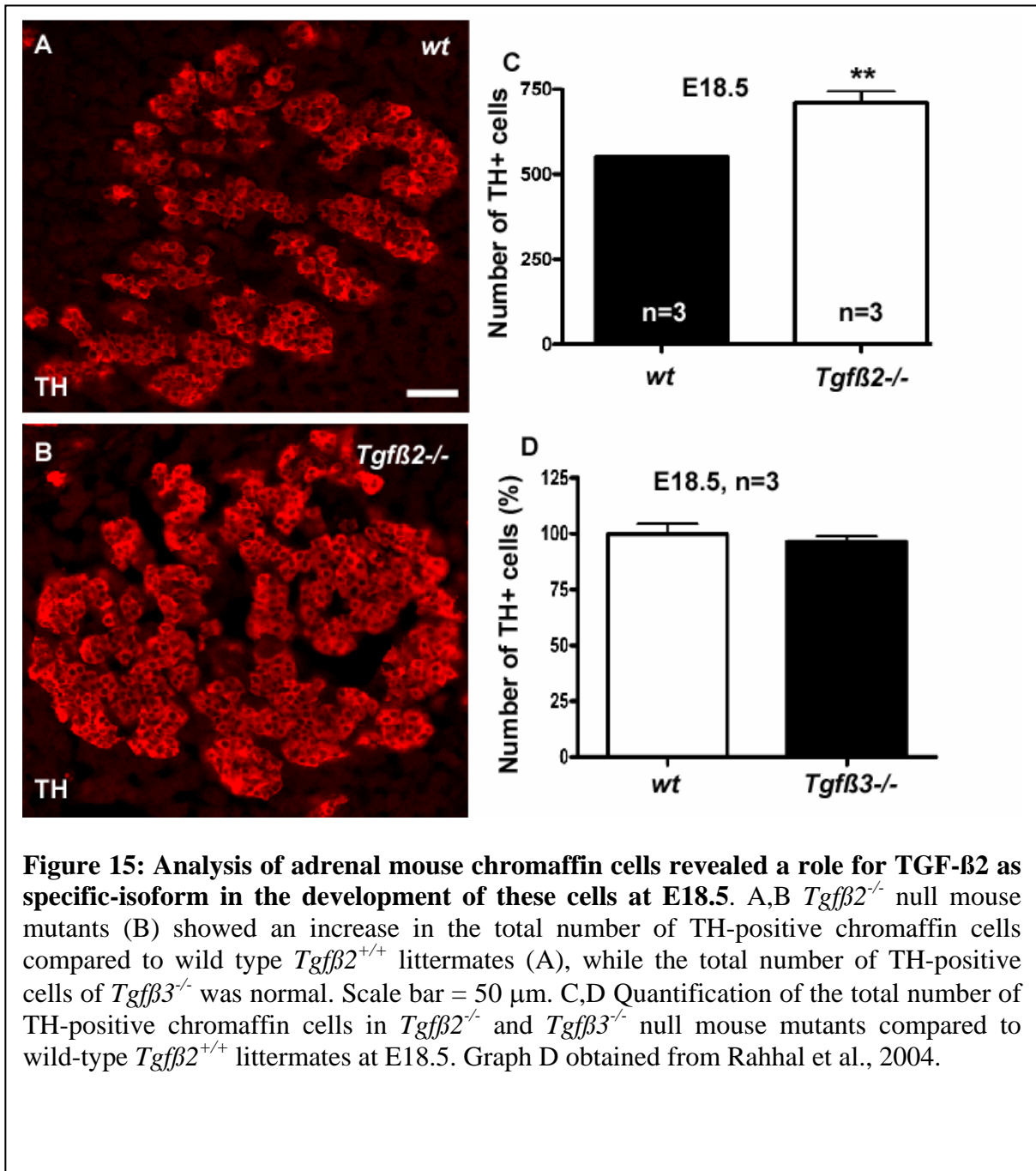
The locus coeruleus (LC) is the noradrenergic nucleus that is severely impaired in neurodegenerative disorders. Figure 14 shows that at E14.5 there were no differences observed in the number of neurons within the LC between $Tgfb2^{-/-}$, $Gdnf^{-/-}$, $Tgfb2^{-/-}Gdnf^{+/-}$, $Tgfb2^{+/-}Gdnf^{-/-}$ mice embryos and the controls ($Tgfb2^{+/+}Gdnf^{+/+}$). On the other hand, quantification of TH-positive cells in the LC showed a decrease at E14.5 in the double mutant mice ($Tgfb2^{-/-}Gdnf^{-/-}$) compared with wild-type mice embryos, but differences did not reach statistical significance ($p > 0.05$). One of these three double knockout mice (the same embryo which has severe rostral 5-HT phenotype) showed a severe decrease (about 40%) in the number of the TH-positive neurons in the LC (Fig. 14), this may be due to limited genetic penetrance.

5.5 Chromaffin cells

A previous study of our group showed that *in ovo* neutralization of all TGF- β isoforms results in a significant increase in the number of chromaffin cells in the developing quail adrenal gland (Combs et al., 2000). In the present study we investigated whether this increase in the number of chromaffin cells is mediated by TGF- β and/or GDNF by analyzing $Tgfb2^{-/-}$, $Tgfb3^{-/-}$, $Gdnf^{-/-}$ single and double mutant mice ($Tgfb2^{-/-}Gdnf^{-/-}$). Figures 15 and 16 showed that total numbers of TH⁺ chromaffin cells in mice deficient for TGF- β 2 ($Tgfb2^{-/-}$) and double mutant mice ($Tgfb2^{-/-}Gdnf^{-/-}$) were significantly increased compared to wild type mice embryos ($Tgfb2^{+/+}$), whereas the data for $Tgfb3^{-/-}$ at E18.5 and $Gdnf^{-/-}$ null mouse mutants at E14.5 reflect wild-type numbers, indicating that the deletion of TGF- β 3 or GDNF genes seemed to have no or only a marginal effect on the development of chromaffin cells. These data suggest that the effect of TGF- β on total numbers of chromaffin cells is regulated by TGF- β 2 rather than by TGF- β 3 or GDNF. Chromaffin cells retain the capacity to proliferate throughout their whole lifespan. In order

to elucidate the mechanisms leading to increased numbers of chromaffin cells we tested whether the increase in numbers of chromaffin cells may be a consequence of increased proliferation. Proliferating cells were identified by PCNA staining and chromaffin cells by co-staining for TH. Analyses at E15.5 (Fig. 17), an earlier time point in adrenal development, revealed a significant increase in the ratio of PCNA-positive chromaffin cells, suggesting that an increase in proliferation of chromaffin cells leads to an overall increase in chromaffin cell numbers when development proceeds. On the contrary, at E18.5, the ratio of the PCNA-positive cells were identical in the TGF- β 2 mutant mice compared to the controls (Fig. 18). During organogenesis proliferating cells may be delayed in their state of differentiation. Chromaffin cells differentiate from noradrenaline to an adrenaline producing cells. To test for their status of differentiation, tissue sections were stained for PNMT, a differentiation marker and counterstained for TH. At E18.5 about half of the cells express PNMT. As shown in Figure 19 fewer PNMT positive cells were detectable in adrenals from *Tgf β 2*^{-/-} mice than in organs from wild type litter mates. Quantification of this morphological data showed that the ratio of PNMT positive chromaffin cells is reduced from about 60% to about 35%. Together, these data provide clear evidence that TGF- β 2 has an isoform-specific role in the regulation of proliferation as well as differentiation of chromaffin cell in the developing murine adrenal gland.





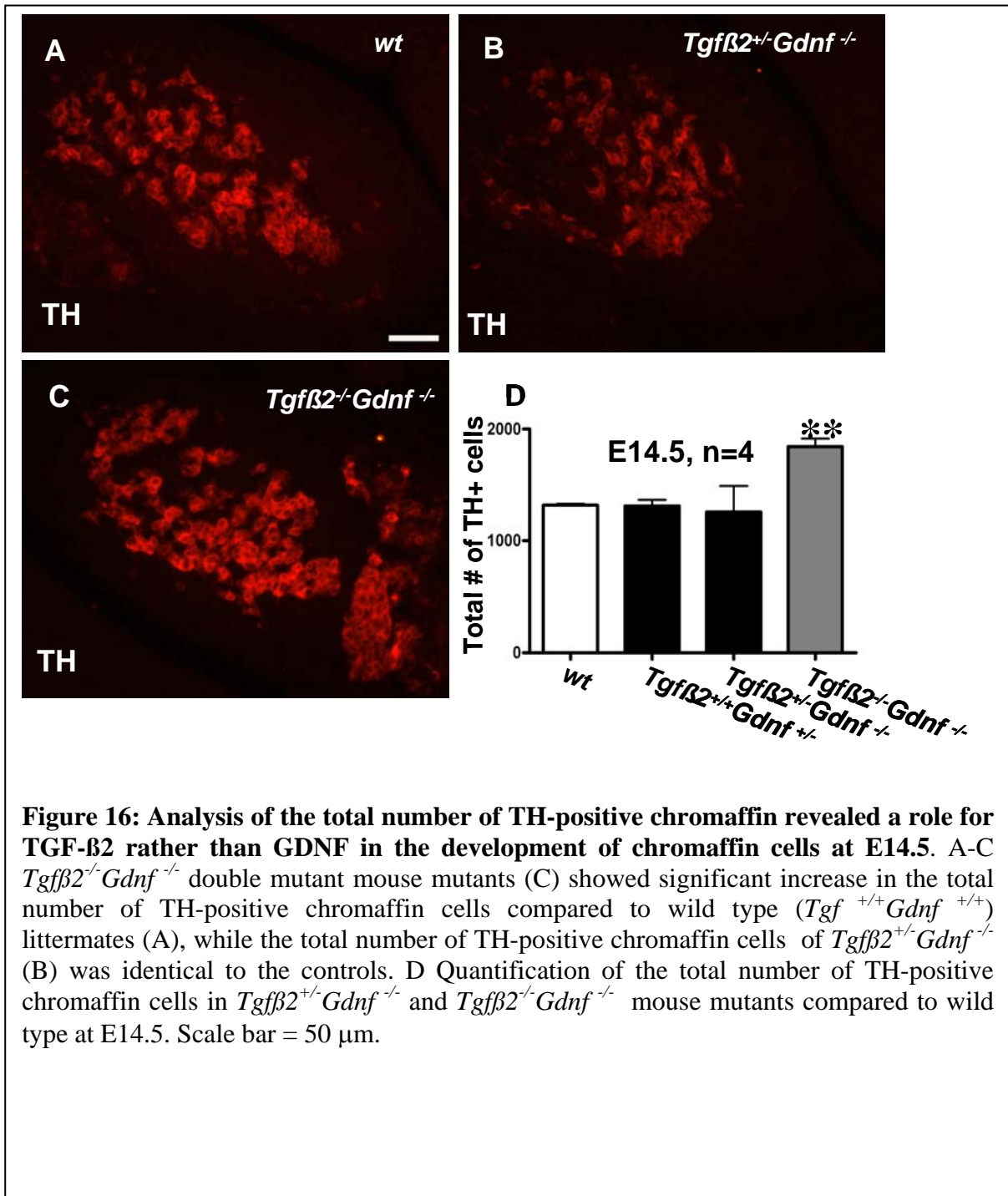


Figure 16: Analysis of the total number of TH-positive chromaffin revealed a role for TGF- β 2 rather than GDNF in the development of chromaffin cells at E14.5. A-C $Tgf\beta 2^{-/-}Gdnf^{-/-}$ double mutant mouse mutants (C) showed significant increase in the total number of TH-positive chromaffin cells compared to wild type ($Tgf^{+/+}Gdnf^{+/+}$) littermates (A), while the total number of TH-positive chromaffin cells of $Tgf\beta 2^{+/-}Gdnf^{-/-}$ (B) was identical to the controls. D Quantification of the total number of TH-positive chromaffin cells in $Tgf\beta 2^{+/-}Gdnf^{-/-}$ and $Tgf\beta 2^{-/-}Gdnf^{-/-}$ mouse mutants compared to wild type at E14.5. Scale bar = 50 μ m.

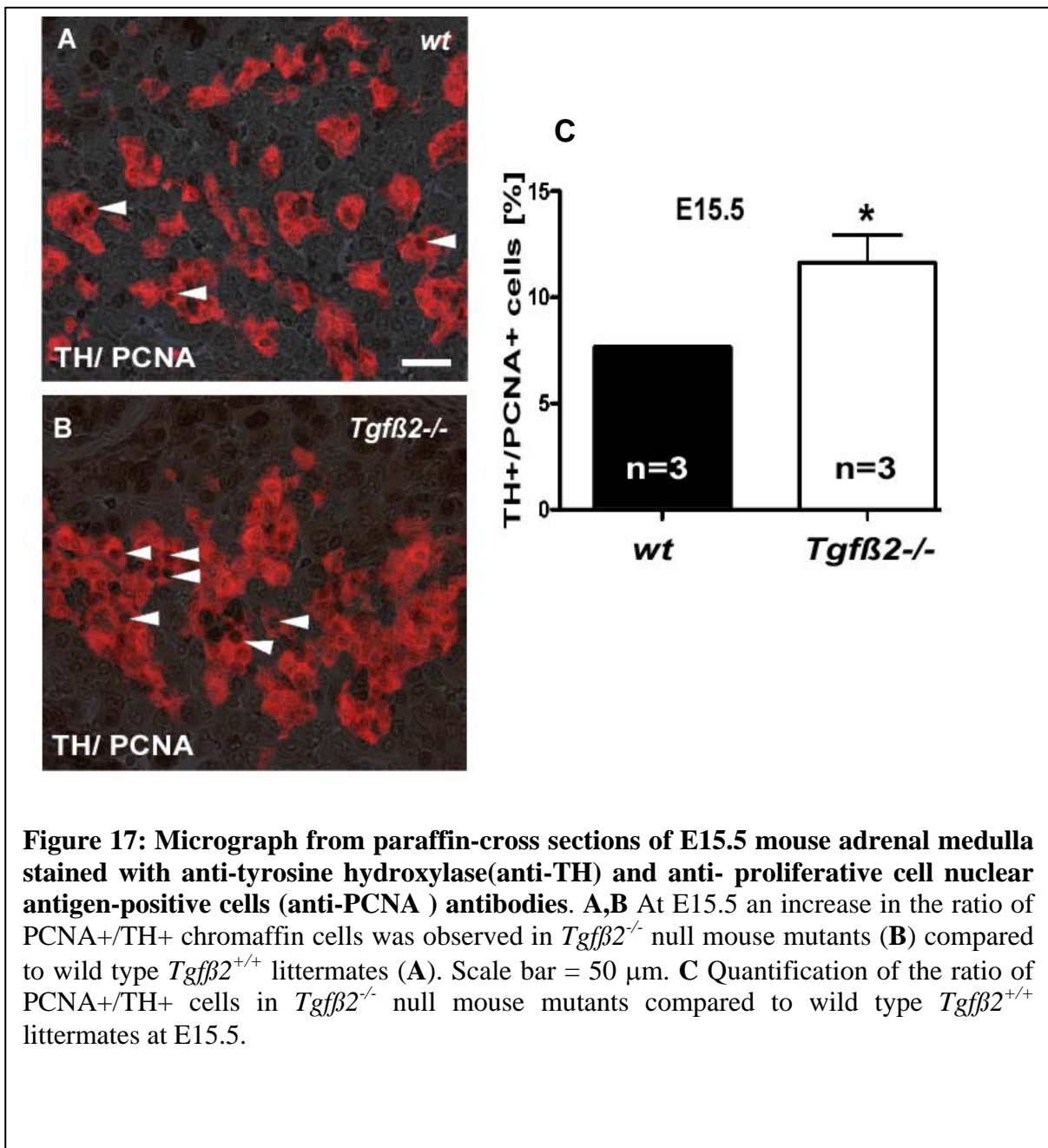


Figure 17: Micrograph from paraffin-cross sections of E15.5 mouse adrenal medulla stained with anti-tyrosine hydroxylase(anti-TH) and anti-proliferative cell nuclear antigen-positive cells (anti-PCNA) antibodies. A,B At E15.5 an increase in the ratio of PCNA+/TH+ chromaffin cells was observed in *Tgfβ2^{-/-}* null mouse mutants (**B**) compared to wild type *Tgfβ2^{+/+}* littermates (**A**). Scale bar = 50 μm. **C** Quantification of the ratio of PCNA+/TH+ cells in *Tgfβ2^{-/-}* null mouse mutants compared to wild type *Tgfβ2^{+/+}* littermates at E15.5.

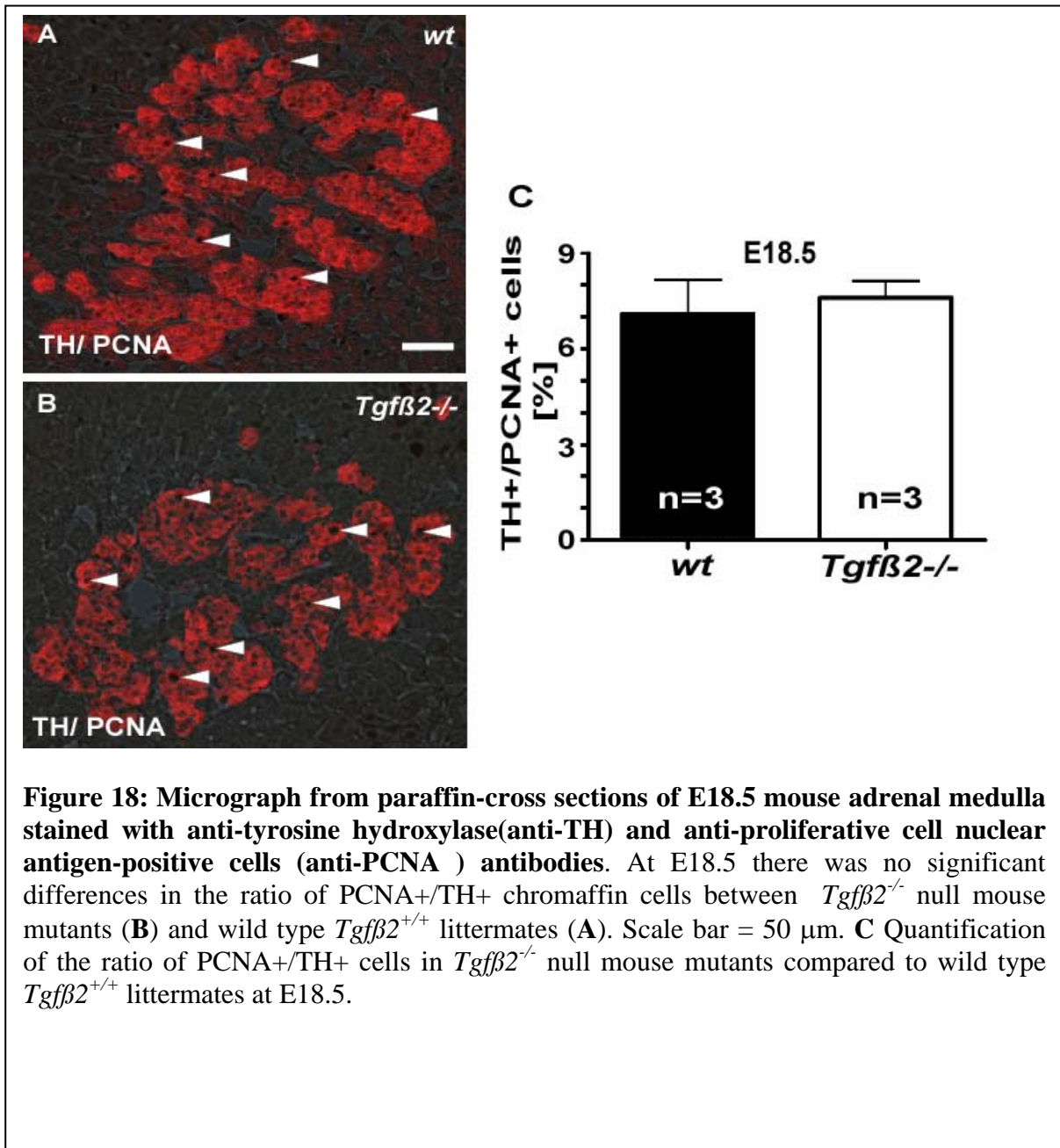


Figure 18: Micrograph from paraffin-cross sections of E18.5 mouse adrenal medulla stained with anti-tyrosine hydroxylase(anti-TH) and anti-proliferative cell nuclear antigen-positive cells (anti-PCNA) antibodies. At E18.5 there was no significant differences in the ratio of PCNA+/TH+ chromaffin cells between *Tgfβ2^{-/-}* null mouse mutants (**B**) and wild type *Tgfβ2^{+/+}* littermates (**A**). Scale bar = 50 μm. **C** Quantification of the ratio of PCNA+/TH+ cells in *Tgfβ2^{-/-}* null mouse mutants compared to wild type *Tgfβ2^{+/+}* littermates at E18.5.

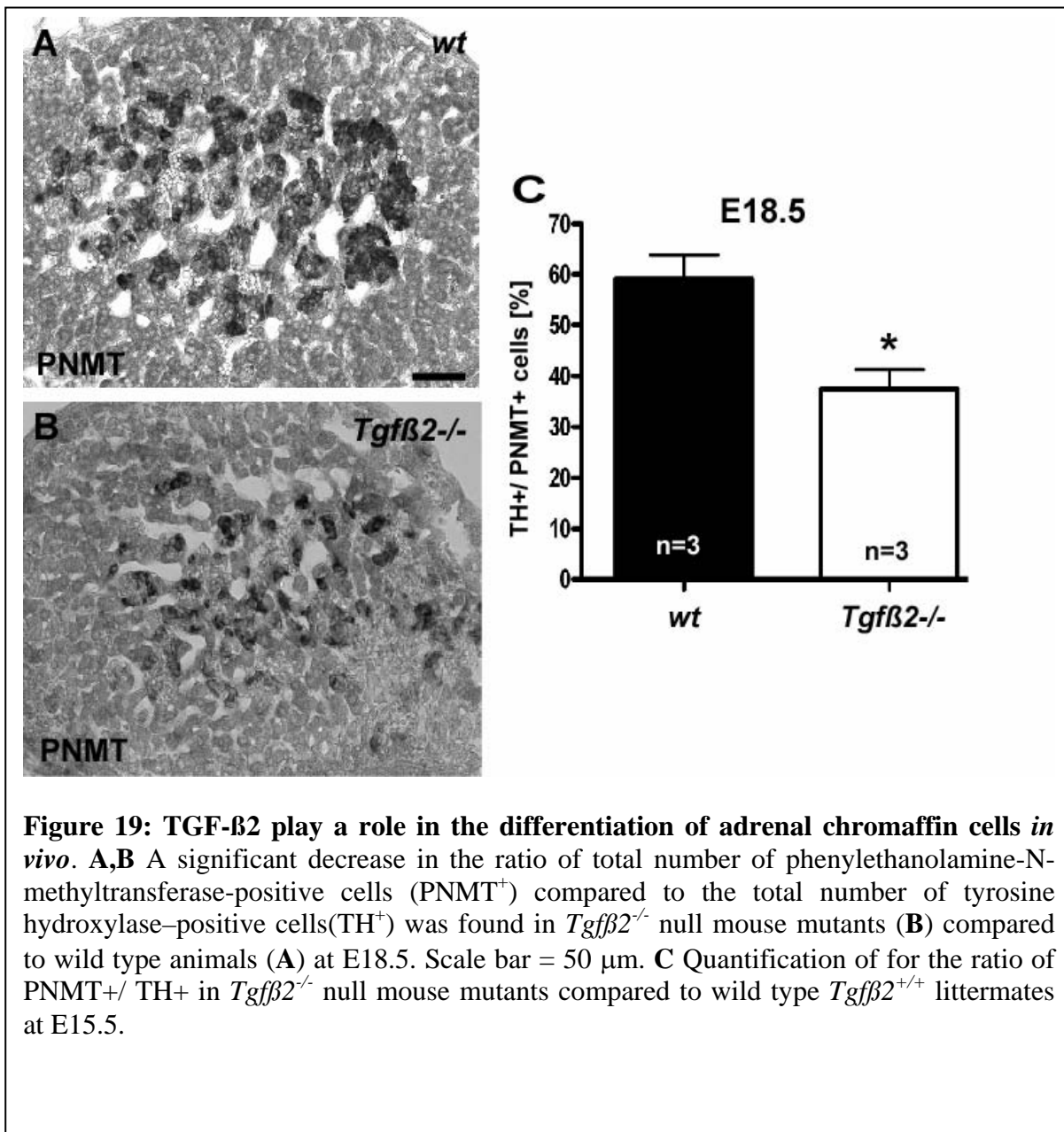


Figure 19: TGF- β 2 play a role in the differentiation of adrenal chromaffin cells *in vivo*. A,B A significant decrease in the ratio of total number of phenylethanolamine-N-methyltransferase-positive cells (PNMT⁺) compared to the total number of tyrosine hydroxylase-positive cells (TH⁺) was found in *Tgfβ2*^{-/-} null mouse mutants (B) compared to wild type animals (A) at E18.5. Scale bar = 50 μ m. C Quantification of for the ratio of PNMT⁺/ TH⁺ in *Tgfβ2*^{-/-} null mouse mutants compared to wild type *Tgfβ2*^{+/+} littermates at E15.5.

5.6 Eye development

The eye morphology of several mutant mice embryos for TGF- β 2 and GDNF including the double mutant embryos ($Tgfb\beta 2^{-/-}Gdnf^{-/-}$) were investigated using hematoxylin and eosin (H&E) stained sections. At E14.5 and E18.5 the entire neural retina of $Tgfb\beta 2^{-/-}Gdnf^{+/+}$, $Tgfb\beta 2^{-/-}Gdnf^{+/-}$ and $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ double mutant mice embryos were significantly thicker compared to wild-type retinæ (Figs. 20-21). Surprisingly, the retinal morphology of $Tgfb\beta 2^{+/-}Gdnf^{-/-}$ mutant mice was comparable to the controls (Figs. 20-21). This gives an indication that TGF- β 2 may play a significant role in the development of the vertebrate retina rather than GDNF. On the other hand, quantification of proliferative cell nuclear antigen (PCNA) positive cells in the central part of the retina at E14.5 revealed a significant increase in cell proliferation in $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ double mutant mice compared with wild-type retinæ (Fig. 22). Eyes of $Tgfb\beta 2^{-/-}Gdnf^{+/+}$, $Tgfb\beta 2^{-/-}Gdnf^{+/-}$ and $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ mutant mice displayed a vascularized accumulation of cells in the posterior chamber of the eye (primary vitreous) never seen in this amount in wild-type animals (Figs. 20-21). Interestingly, $Tgfb\beta 2^{-/-}Gdnf^{+/-}$ and $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ double mutant mice showed a complete detachment of the retina from the underlying pigment epithelium at E18.5 and the retina was folded (coloboma formation) (Fig. 21). The detachment of the retina and folding of the retina in $Tgfb\beta 2^{-/-}Gdnf^{+/-}$ and double mutant mice embryos support the notion of synergism and cooperation between TGF- β 2 and GDNF *in vivo*. Moreover, immunostaining at E18.5 for the transcription factor Pax-6, revealed the expected patterning of the retina in control eyes and a diffuse distribution in $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ mutant embryos (Fig. 23).

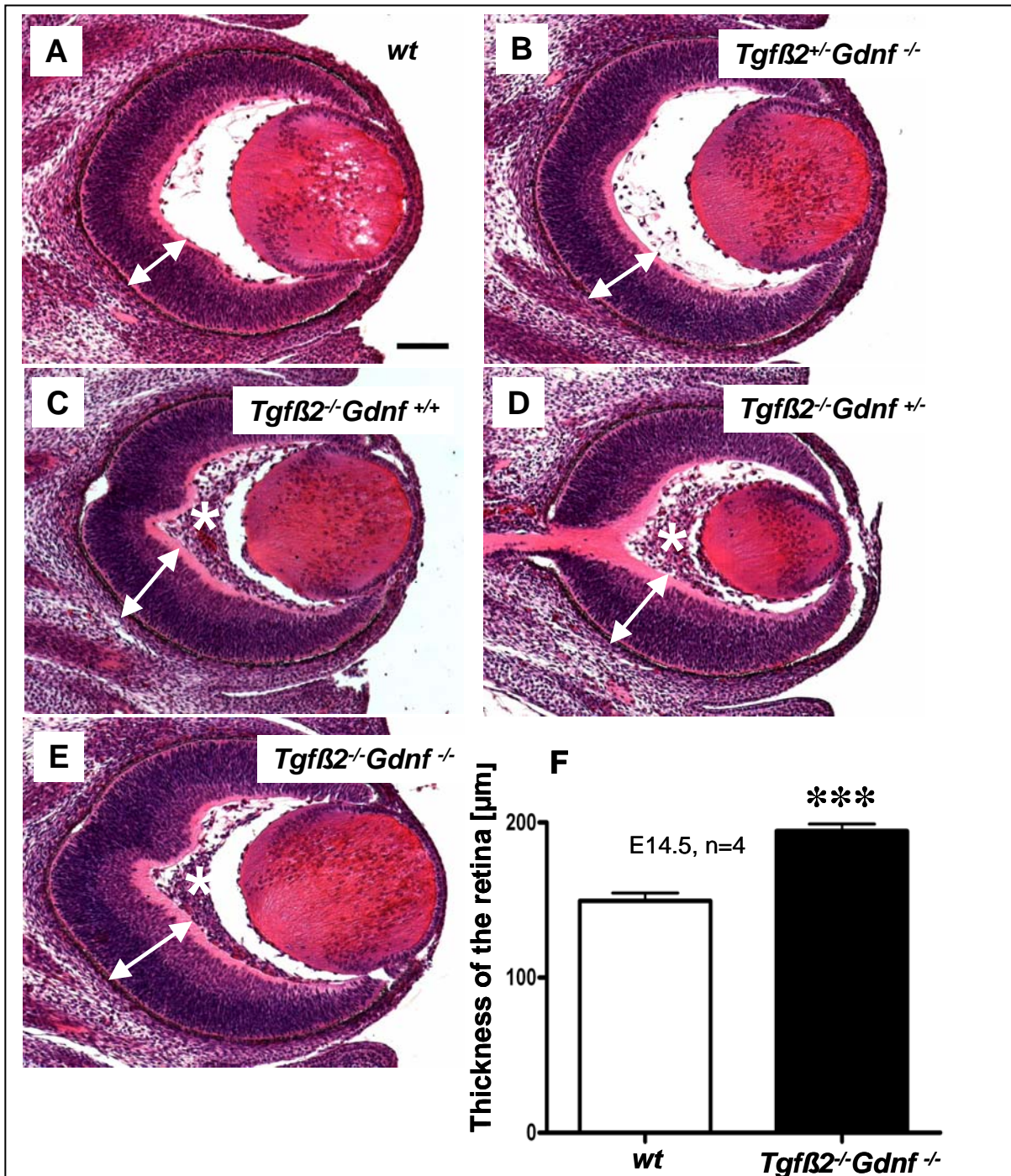


Figure 20: All the neural retinae of *Tgfb2*^{-/-}*Gdnf*^{+/+}, *Tgfb2*^{-/-}*Gdnf*^{+/-} and *Tgfb2*^{-/-}*Gdnf*^{-/-} mouse mutants embryos are notably thicker compared to wild-type retinae at E14.5. A-E Hematoxylin-eosin stained transverse sections of E14.5 wild-type (A), *Tgfb2*^{+/-}*Gdnf*^{-/-} (B), *Tgfb2*^{-/-}*Gdnf*^{+/+} (C), *Tgfb2*^{-/-}*Gdnf*^{+/-} (D) and *Tgfb2*^{-/-}*Gdnf*^{-/-} (E) mouse mutants heads. Eyes of *Tgfb2*^{-/-}*Gdnf*^{+/+}, *Tgfb2*^{-/-}*Gdnf*^{+/-} and *Tgfb2*^{-/-}*Gdnf*^{-/-} mutant mice display a vascularized accumulation of cells in the posterior chamber of the eye (white asterisks in C,D and E) never seen in wild-type animals. Eyes of *Tgfb2*^{+/-}*Gdnf*^{-/-} mutant mice (B) embryos were identical to the controls. F Quantification of central retinal layer thickness of the retina of *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant and wild-type mice. Scale bars: 250 μm.

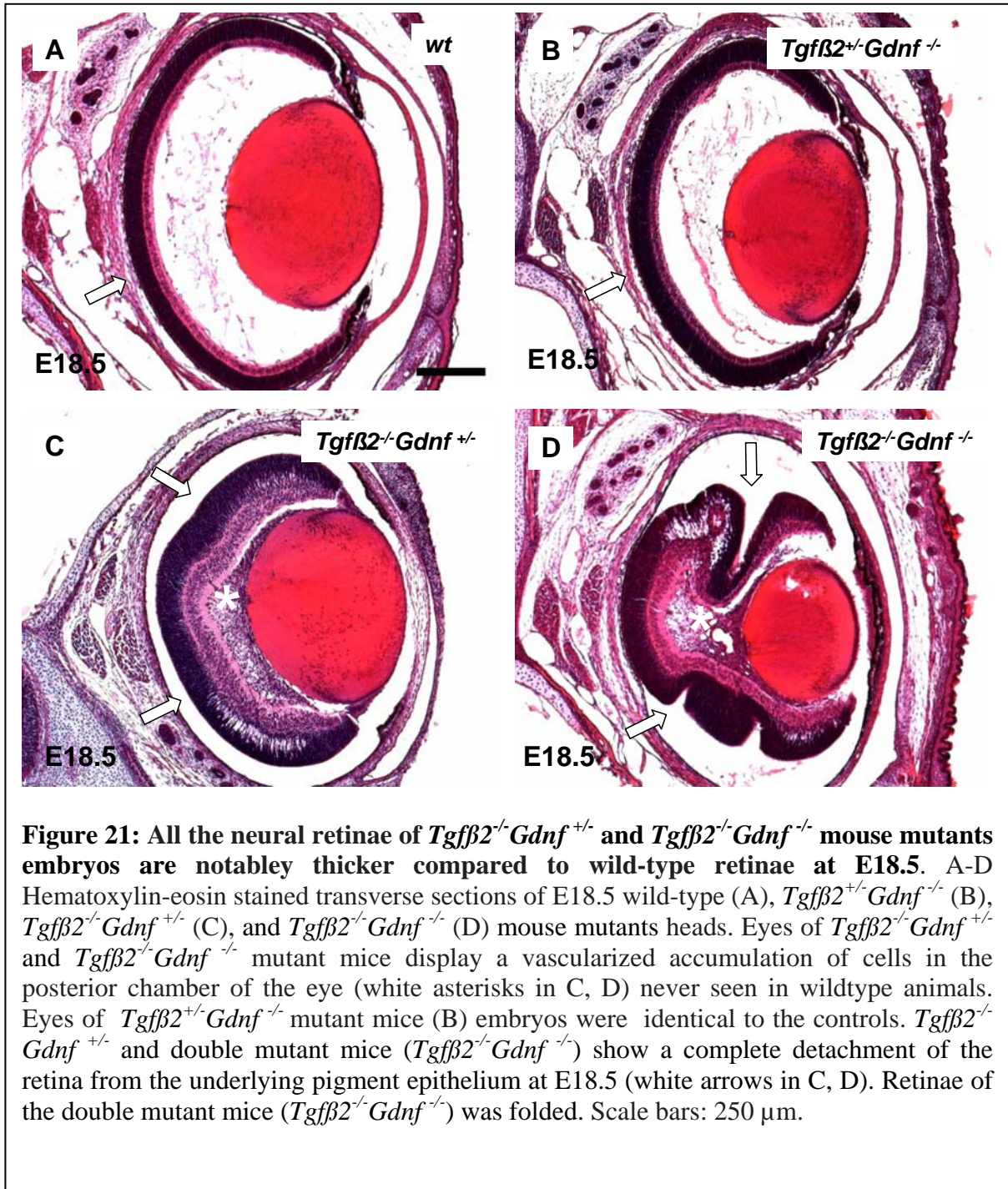


Figure 21: All the neural retinae of $Tgfb2^{-/-}Gdnf^{+/-}$ and $Tgfb2^{-/-}Gdnf^{-/-}$ mouse mutants embryos are notably thicker compared to wild-type retinae at E18.5. A-D Hematoxylin-eosin stained transverse sections of E18.5 wild-type (A), $Tgfb2^{+/-}Gdnf^{-/-}$ (B), $Tgfb2^{-/-}Gdnf^{+/-}$ (C), and $Tgfb2^{-/-}Gdnf^{-/-}$ (D) mouse mutants heads. Eyes of $Tgfb2^{-/-}Gdnf^{+/-}$ and $Tgfb2^{-/-}Gdnf^{-/-}$ mutant mice display a vascularized accumulation of cells in the posterior chamber of the eye (white asterisks in C, D) never seen in wildtype animals. Eyes of $Tgfb2^{+/-}Gdnf^{-/-}$ mutant mice (B) embryos were identical to the controls. $Tgfb2^{-/-}Gdnf^{+/-}$ and double mutant mice ($Tgfb2^{-/-}Gdnf^{-/-}$) show a complete detachment of the retina from the underlying pigment epithelium at E18.5 (white arrows in C, D). Retinae of the double mutant mice ($Tgfb2^{-/-}Gdnf^{-/-}$) was folded. Scale bars: 250 μ m.

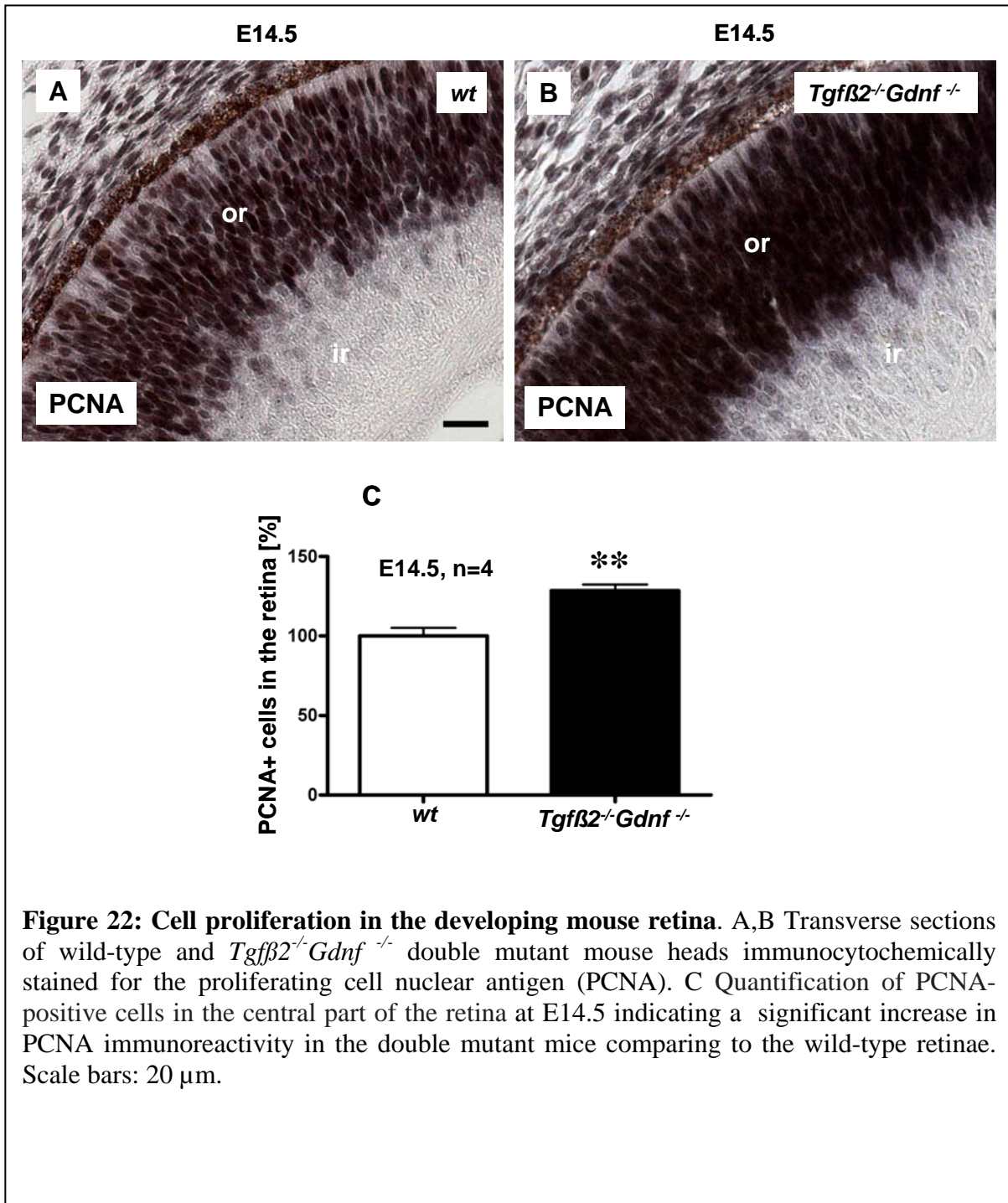
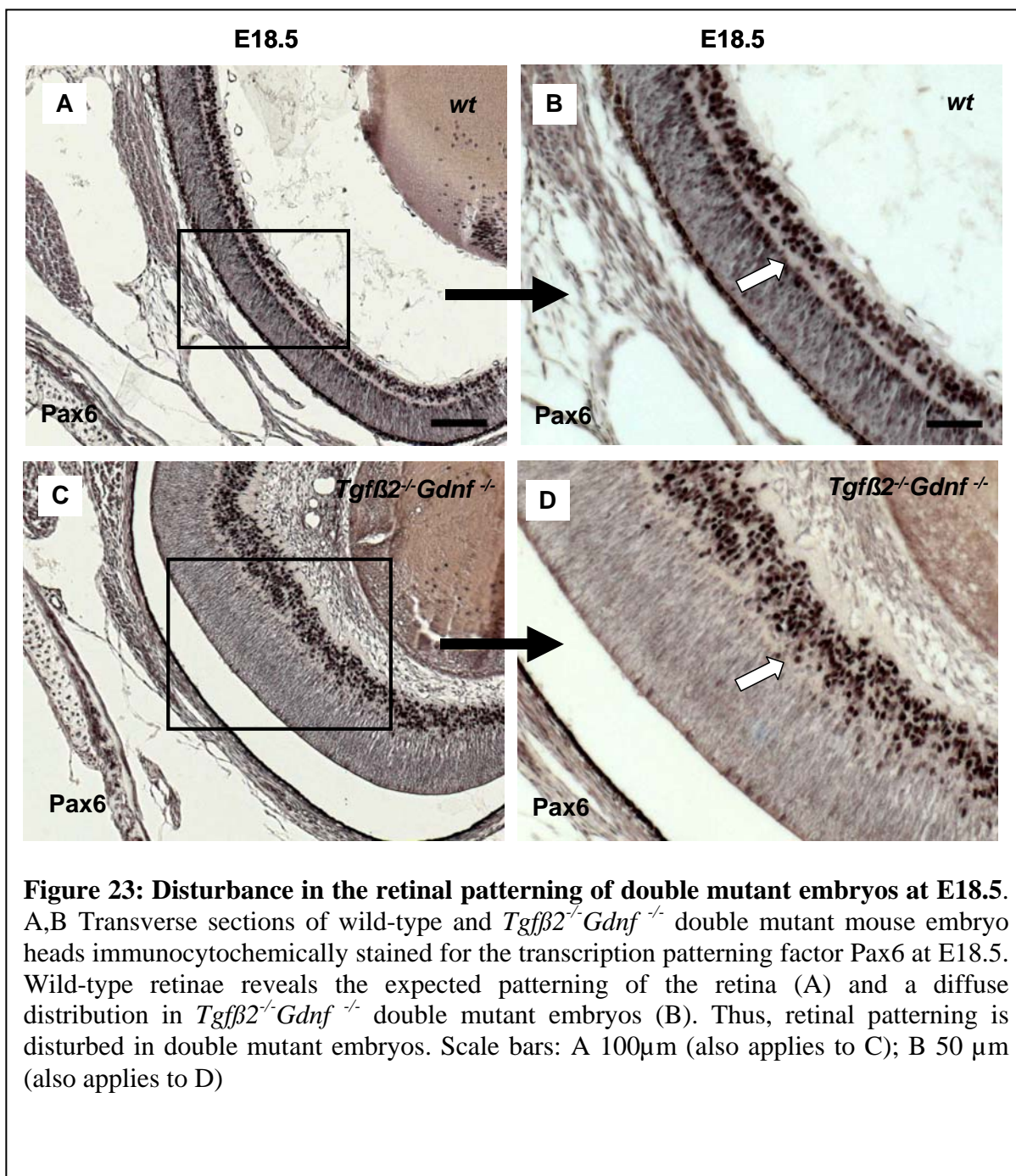


Figure 22: Cell proliferation in the developing mouse retina. A,B Transverse sections of wild-type and *Tgfβ2^{-/-}Gdnf^{-/-}* double mutant mouse heads immunocytochemically stained for the proliferating cell nuclear antigen (PCNA). C Quantification of PCNA-positive cells in the central part of the retina at E14.5 indicating a significant increase in PCNA immunoreactivity in the double mutant mice comparing to the wild-type retinae. Scale bars: 20 μm.



5.7 Enteric Nervous System

To examine the physiological role of TGF- β 2 and GDNF in the development of the mammalian enteric neurons *in vivo*, single and double mutant mice for TGF- β 2 and GDNF were generated. Figures 24 and 25 show that, at E14.5 and at E16.5, *Tgfb2*^{+/+}*Gdnf*^{-/-} and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos lack most of the enteric neurons using neurofilament (NF) antibody as a neuronal marker. This result is consistent with the results

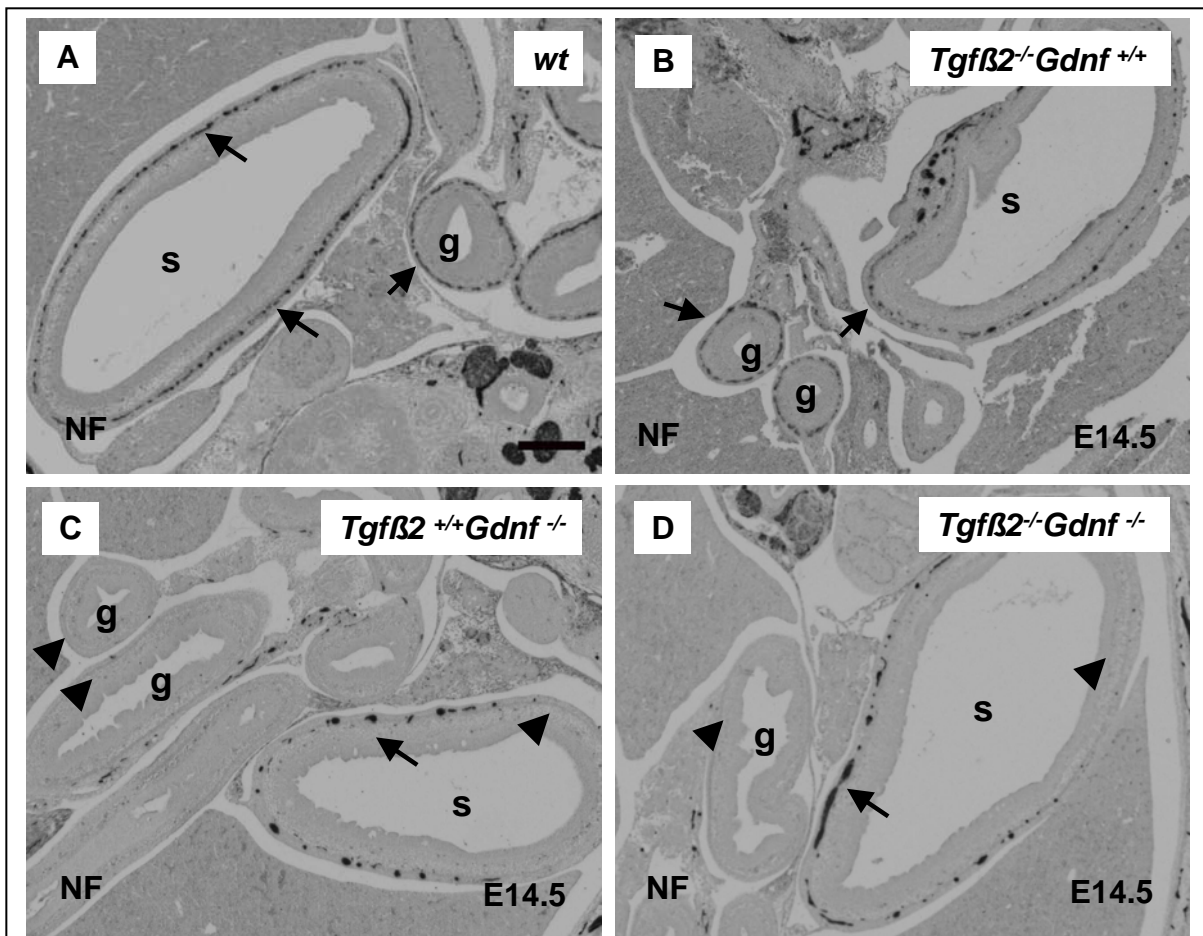


Figure 24: ENS neurons do not survive in single GDNF and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mouse embryos at E14.5. Transverse paraffin-sections (10 μ m) from bodies of wild-type (A), *Tgfb2*^{-/-}*Gdnf*^{+/+} (B), *Tgfb2*^{+/+}*Gdnf*^{-/-} (C), and *Tgfb2*^{-/-}*Gdnf*^{-/-} (D) were stained with anti-neurofilament (anti-NF). NF-staining revealed neurons in the stomach wall (s) and gut (g) in the wild-type (A) and *Tgfb2*^{-/-}*Gdnf*^{+/+} (B) mice embryos (arrows). However, this antibody labelled a few fibers in the stomach wall (arrows) and there was no neurons (arrowheads) in the midgut (C, D). This give an indication the the defect in the ENS in the *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mouse embryo is due to the absence of GDNF but not TGF- β 2. Scale bars: 100 μ m.

from GDNF knockout mice, *Gdnf*^{-/-} mice showed an absence of the enteric nervous system (ENS) due to failed migration of neural crest cells (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). ENS of *Tgfb2*^{-/-}*Gdnf*^{+/+} mutant mice embryos were comparable to the controls (*Tgfb2*^{+/+}*Gdnf*^{+/+}). This may indicate that TGF-β seems to have no or a marginal effect on the development of the enteric nervous system and the loss of the ENS is due to the absence of GDNF gene rather than TGF-β2.

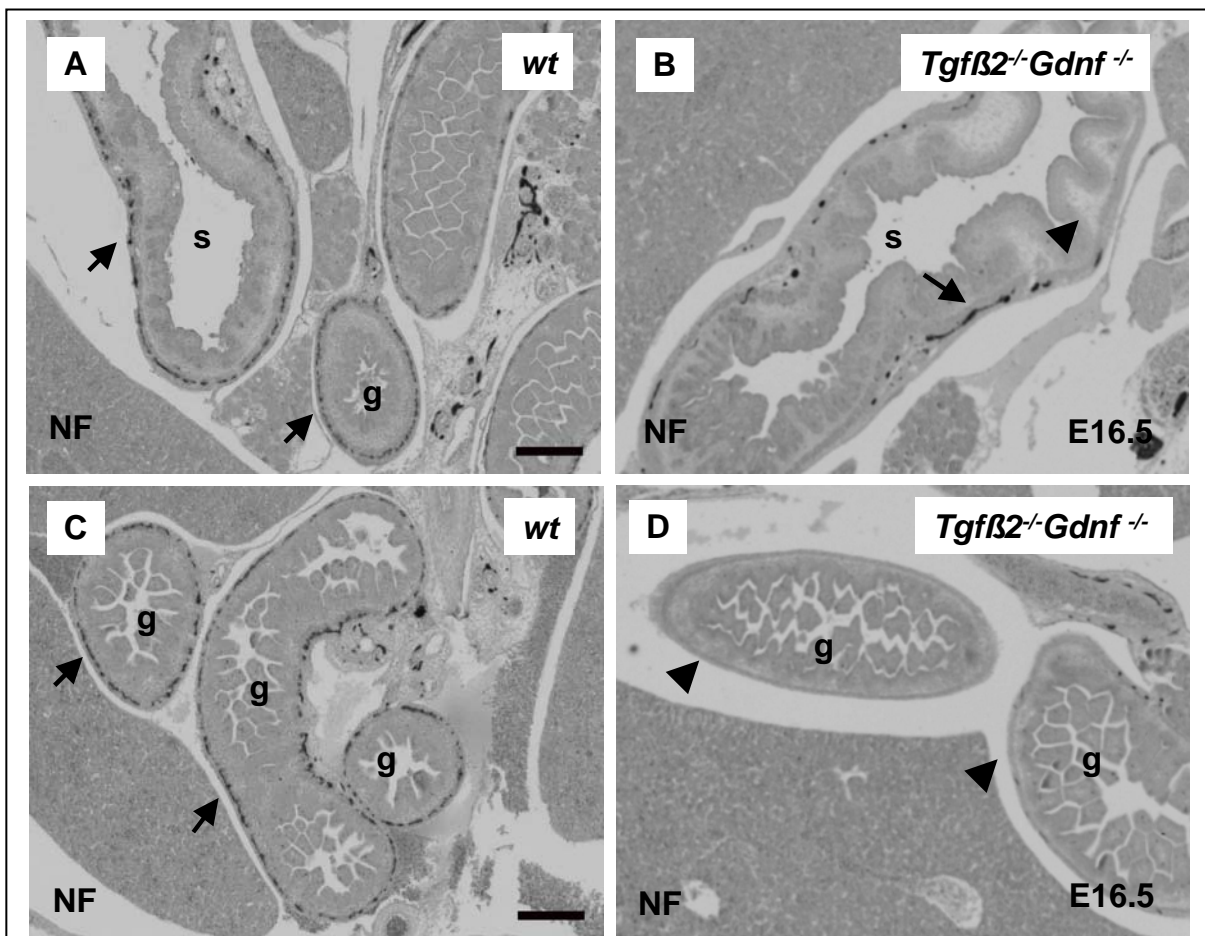


Figure 25: ENS neurons do not survive in *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mouse embryos at E16.5. Transverse paraffin-sections (10 μm) from bodies of wild-type (A, C) and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice (B, D) were stained with anti-neurofilament (anti-NF). NF-staining revealed neurons in the stomach wall (s) and gut (g) in the wild-type (A,C) mice embryos (arrows). However, this antibody labelled a few fibers in the stomach wall (arrows in B) and there was no neurons (arrowheads) in the midgut (D). This give an indication the the defect in the ENS in the *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mouse embryo is due to the absence of GDNF but not TGF-β2. Scale bars: 200 μm (also applies to B,C, D)

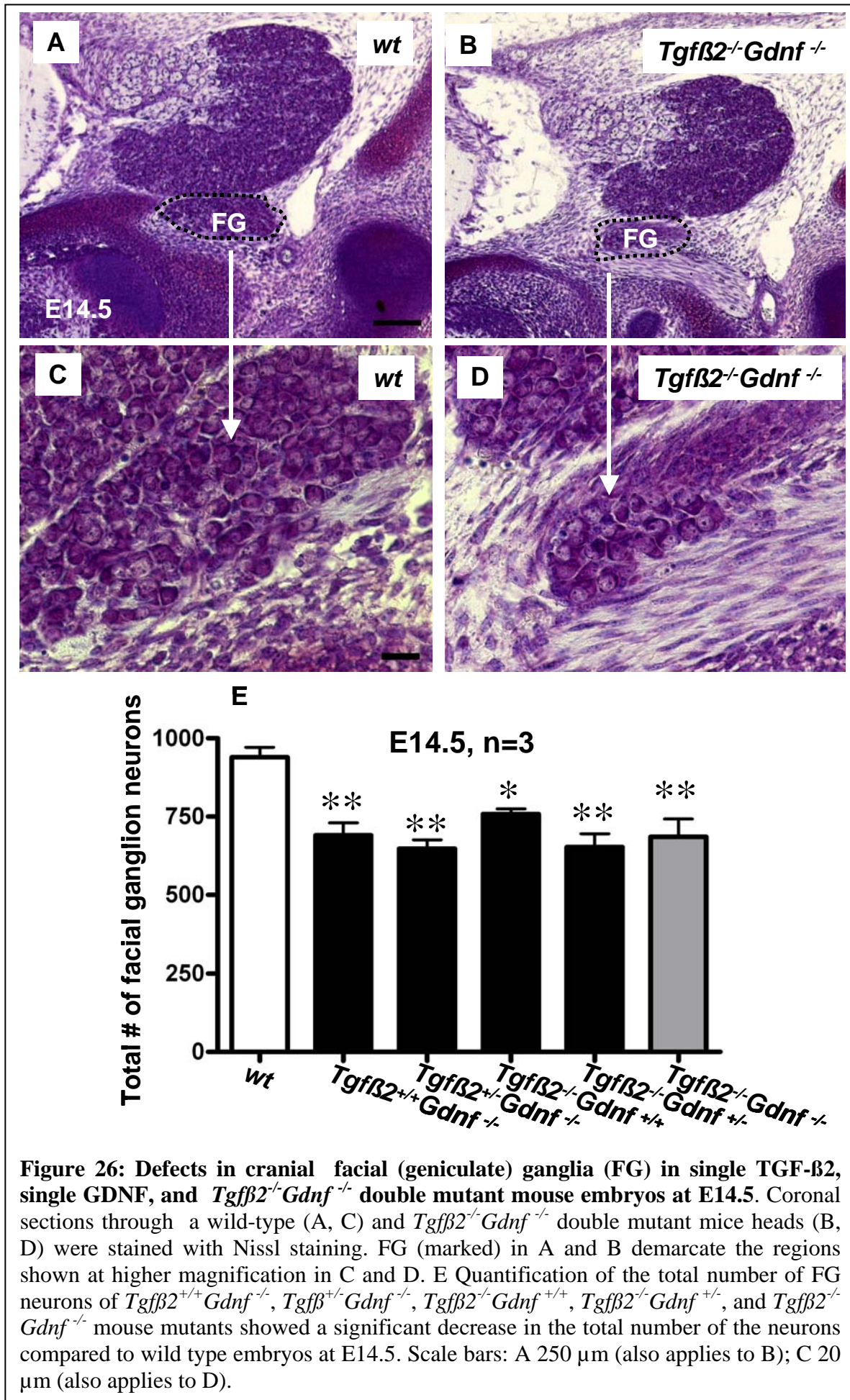
5.8 Peripheral ganglia

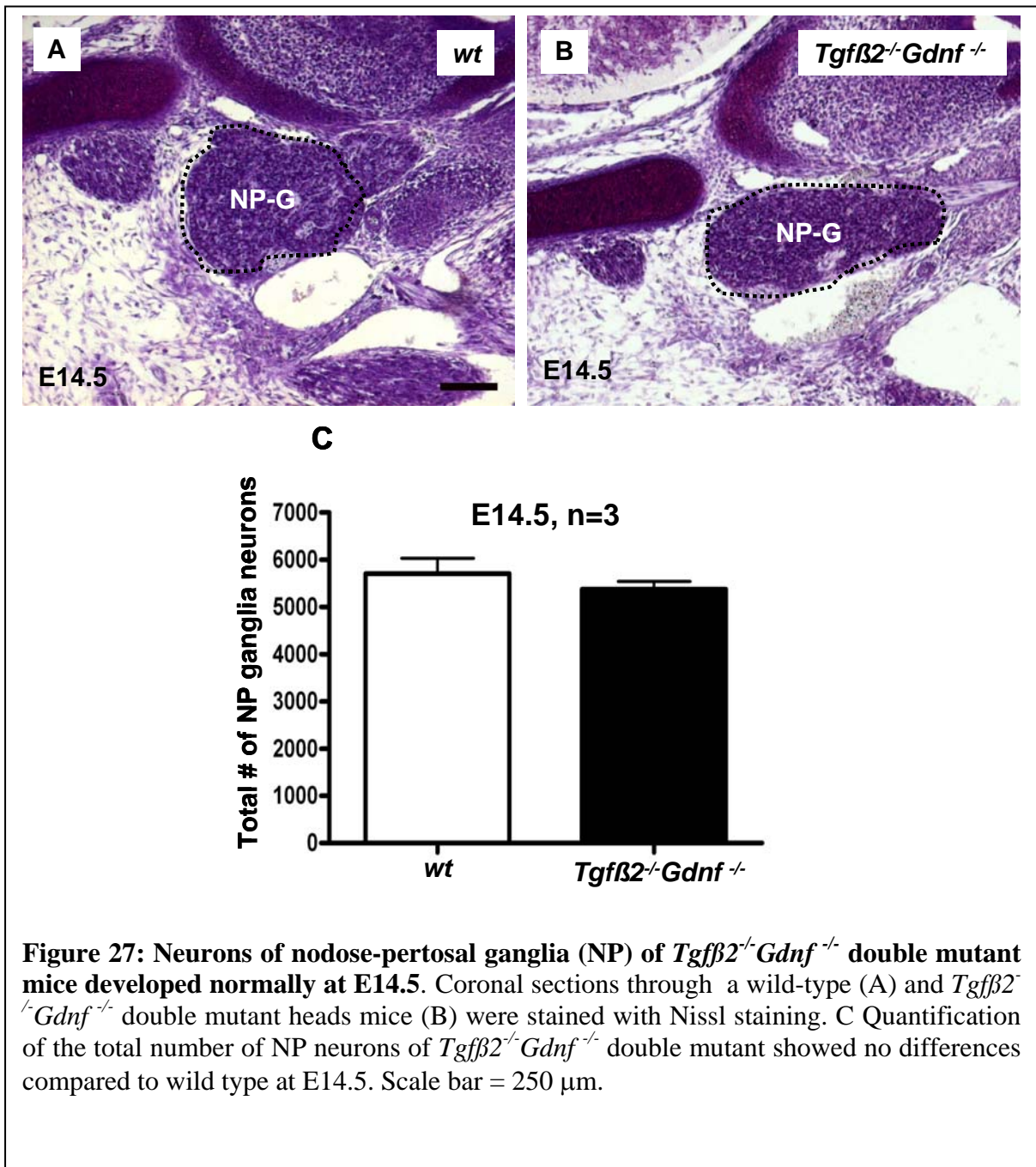
To assess the role of TGF- β 2 and GDNF in the development of the cranial (facial, nodose, petrosal), sensory dorsal root (DRG) and sympathetic superior cervical (SCG) ganglia, neurons of the ganglia of *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos were examined at E14.5 as well as at E18.5. To quantify the differences (reduction) of neurons in ganglia of mutant mice embryos, neurons in these ganglia were counted. Setting the cell numbers from wt mice embryos as 100 %, there were approximately 81 % of the neurons in facial (geniculate) ganglia detectable in the *Tgfb2*^{-/-}*Gdnf*^{+/-}, *Tgfb2*^{+/-}*Gdnf*^{-/-} and *Tgfb2*^{-/-}*Gdnf*^{-/-} mutant mice embryos at E14.5 compared to the wt mice (Fig. 26). In contrast to the facial ganglia, the other ganglia (nodose-petrosal ganglia, dorsal root ganglion) did not show any difference at E14.5 (Figs. 27-28 respectively). On the other hand, at E18.5, there was approximately 46 % reduction of the nodose-petrosal single GDNF mutant and 47 % of the double mutant mice (Fig. 29), a loss of 17 % of L5-DRG neurons in single GDNF mutant and 24 % of double mutant mice while the L5-DRG neurons in single TGF- β 2 mutant embryos was normal (Fig.30), 21 % neuronal cell loss in the SCG of single GDNF mutant and 46 % loss in the double knockout mice embryos compared to the controls mice (Fig. 31). As there is no loss of SCG neurons in TGF- β 2 single mutants (Fig. 31), the increased loss of neurons in double mutants versus GDNF single mutants represents a clear example of a synergistic effect. To test whether the TGF- β 2/GDNF-dependent loss of SCG neurons reflects a degeneration of neurons due to lack of trophic support or a developmental phenotype, earlier stages of SCG development were analyzed. At E16.5, there was a significant decrease in the total number of the total SCG neurons in the *Gdnf*^{-/-} mice (26 %) and a severe reduction (47 %) in these neurons in the double mutant mice (Fig. 32). This result is well consistent with the SCG counting results at E18.5. Surprisingly, the volume of the SCGs at E14.5, an earlier developmental stage, were identical in the double mutant

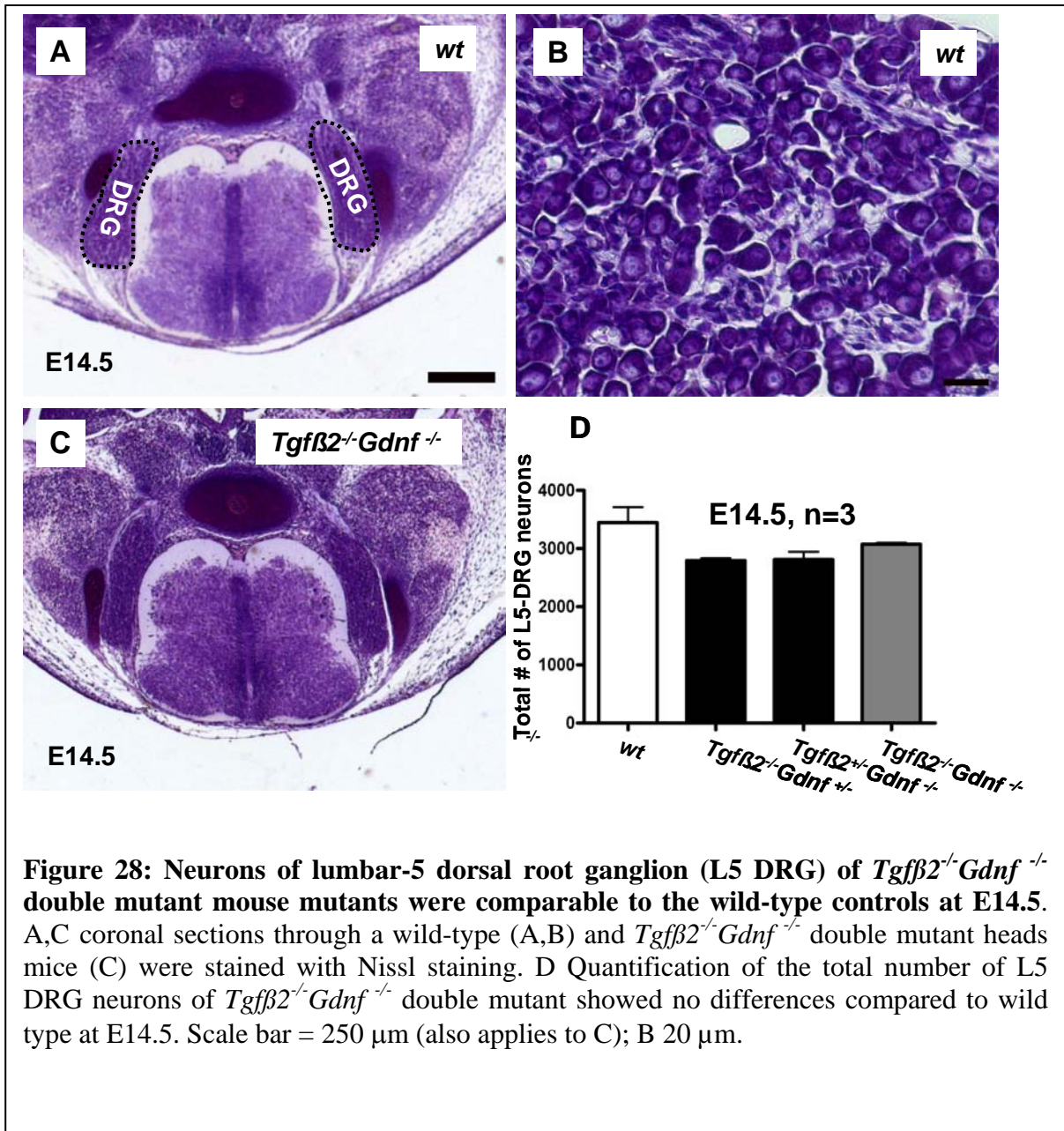
mice compared the wild-type controls (Fig. 33). Together, these data suggest that TGF- β 2 and GDNF cooperate in the regulation of SCG neuron survival and constitute a synergistic neurotrophic activity. Table 4 summarized the percentage in the reduction of the different mutant ganglia at different developmental stages compared to the wild-type controls.

Table 4: Summary for the reduction in the total number of different neuronal populations compared to the wildtype (100%). FG: Facial ganglia, NP-G: Nodpse-petrosal ganglia, L5-DRG: Lumber dorsal root ganglia, SCG: Superior cervical ganglion, MN: Motoneurons, NS: not significant. TGF- β 2 single mutant data in this table kindly provided by Anika Ferdinand. Differences were considered significance at * $P < 0.05$, ** $P < 0.01$.

	<i>Tgfb2</i> ^{-/-}	<i>Gdnf</i> ^{-/-}	<i>Tgfb2</i> ^{-/-} / <i>Gdnf</i> ^{-/-}
FG	E14.5 : -19% **	E14.5 : -19% **	E14.5 : -19% **
NP-G	----	----	E14.5 : NS
L5-DRG	E18.5 : NS	E18.5 : -17% *	E18.5 : -24% **
SCG	E18.5 : NS	E16.5 : -26% ** E18.5 : -21% , NS	E16.5 : -47% ** E18.5 : -46% *
MN (L1-L5)	E18.5 : NS	E18.5 : -18% *	E18.5 : -31% *







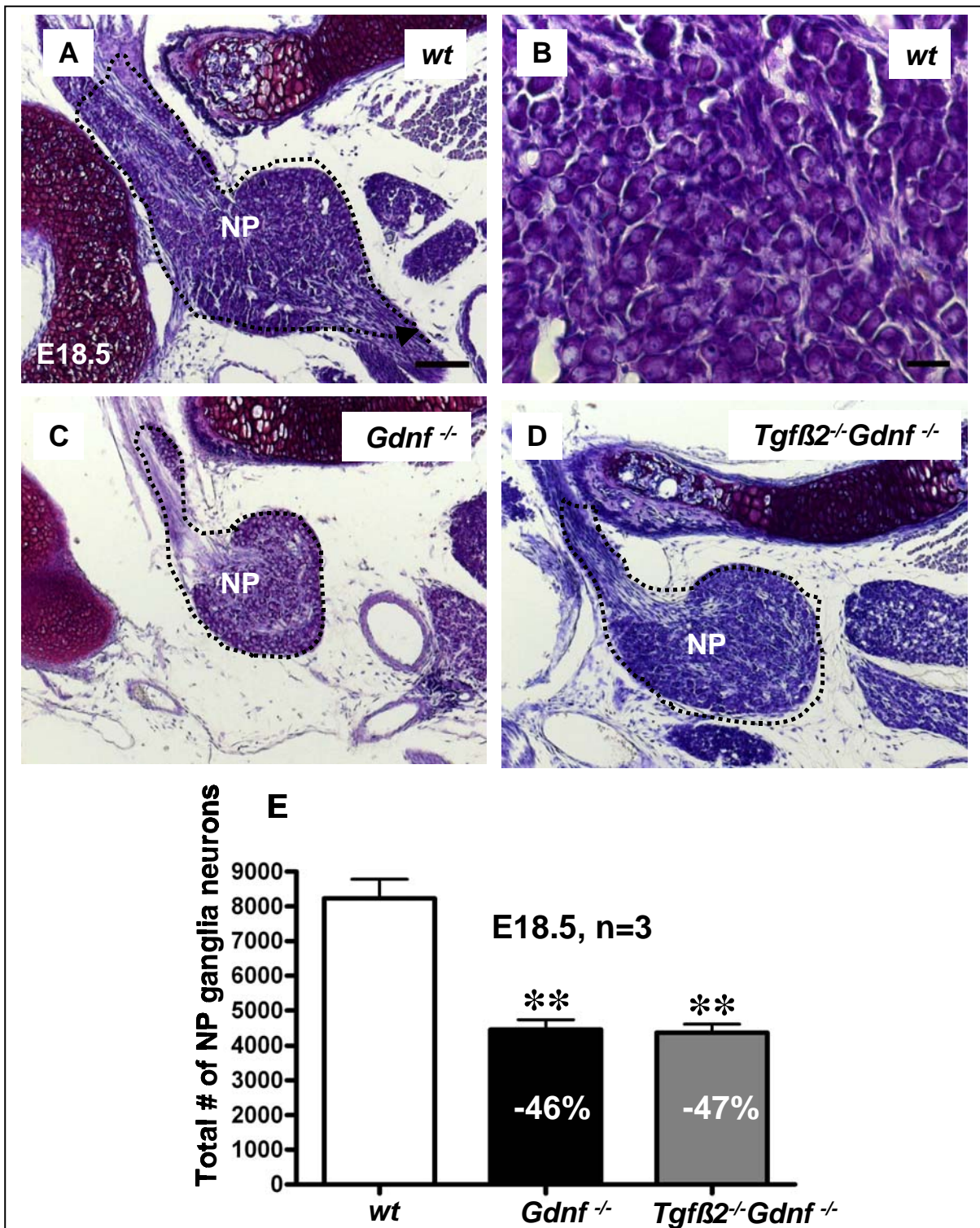
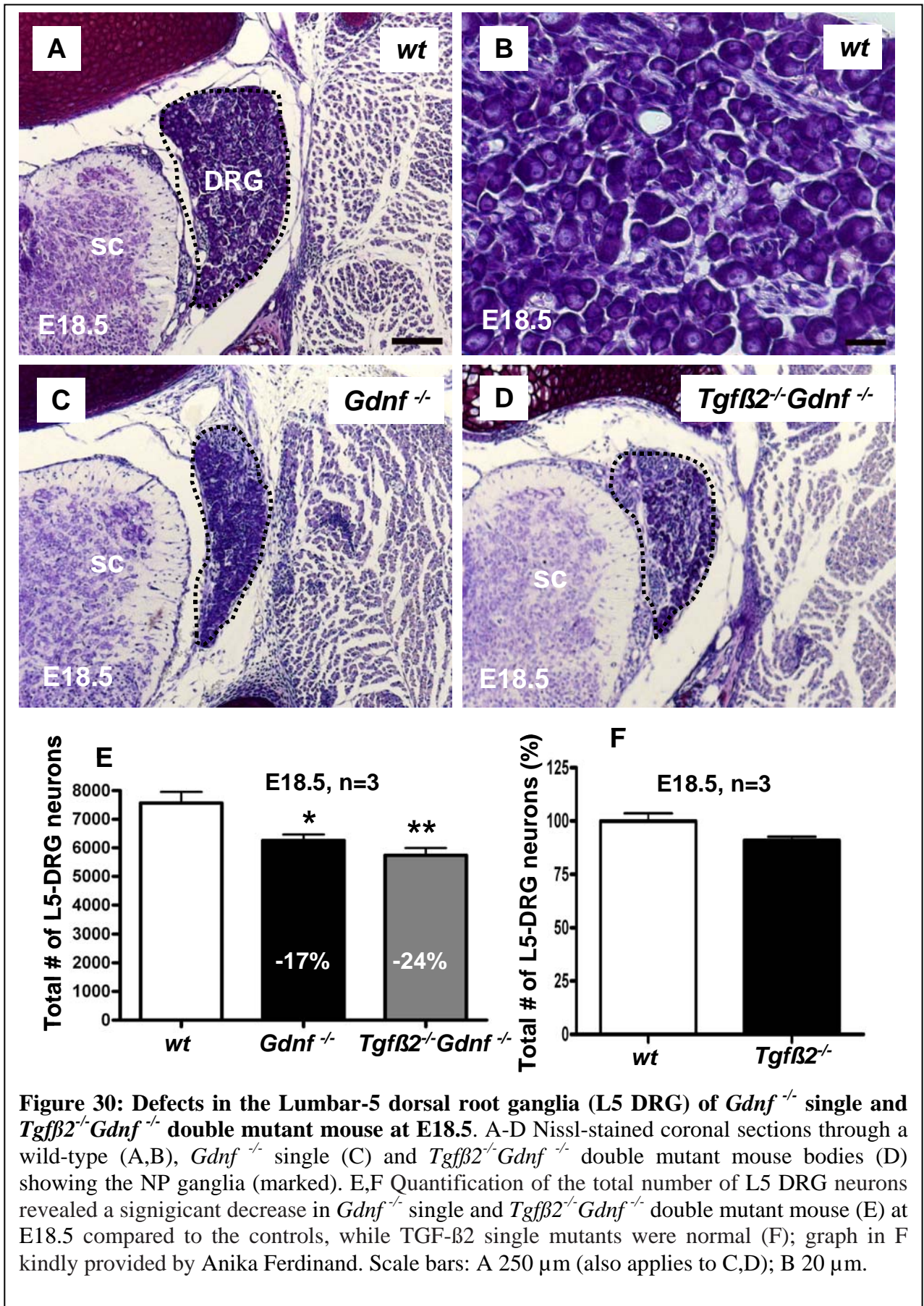
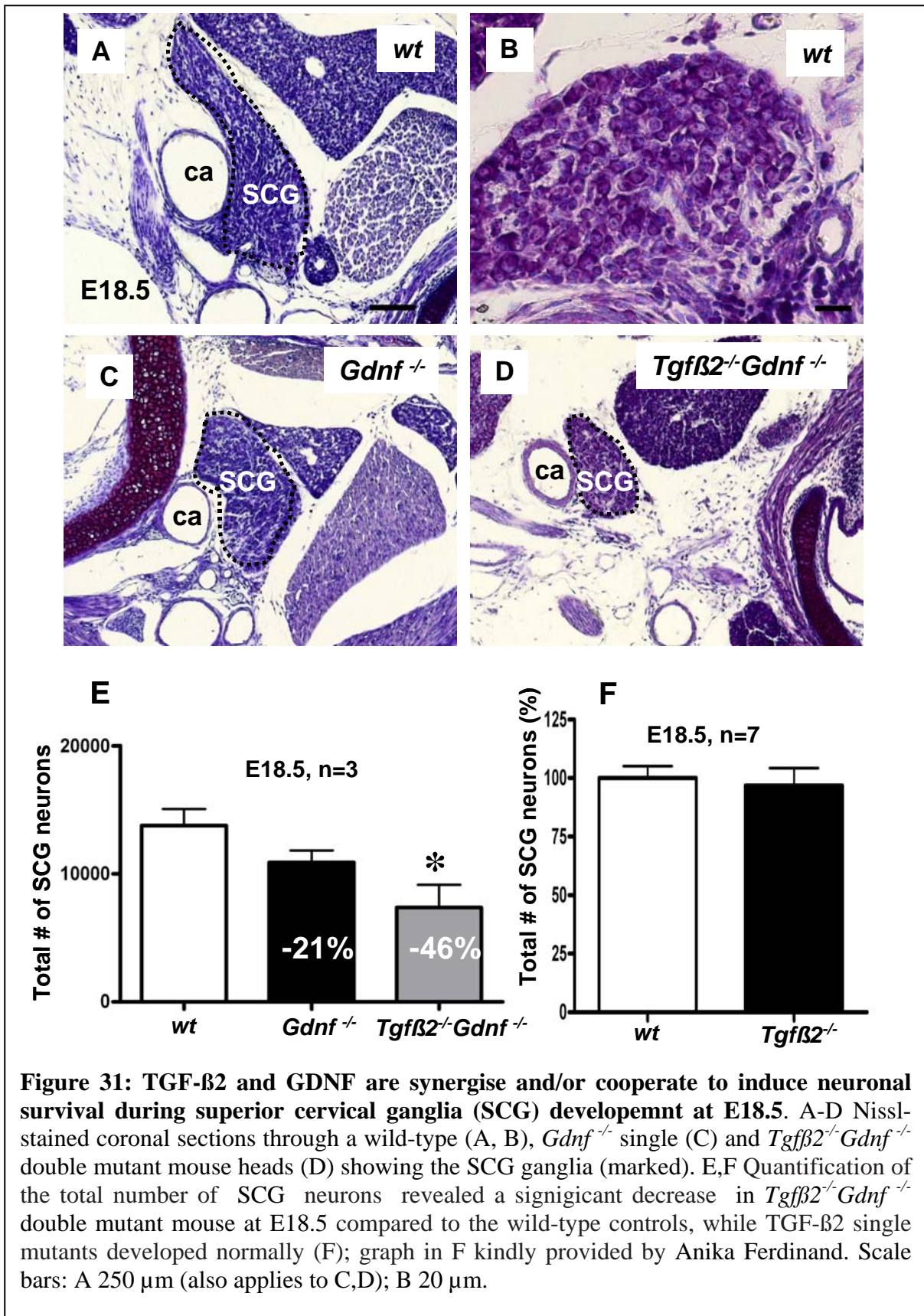
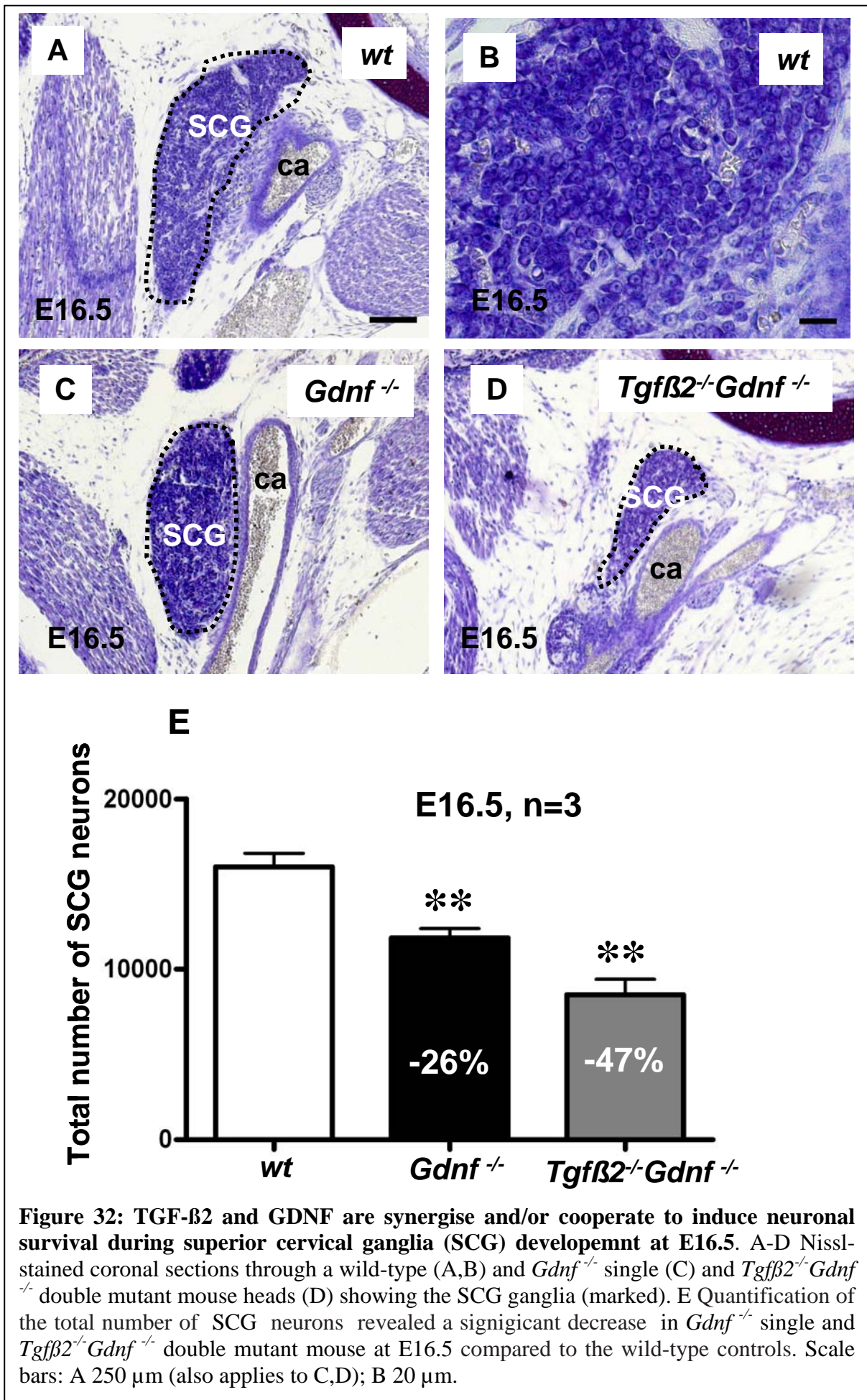
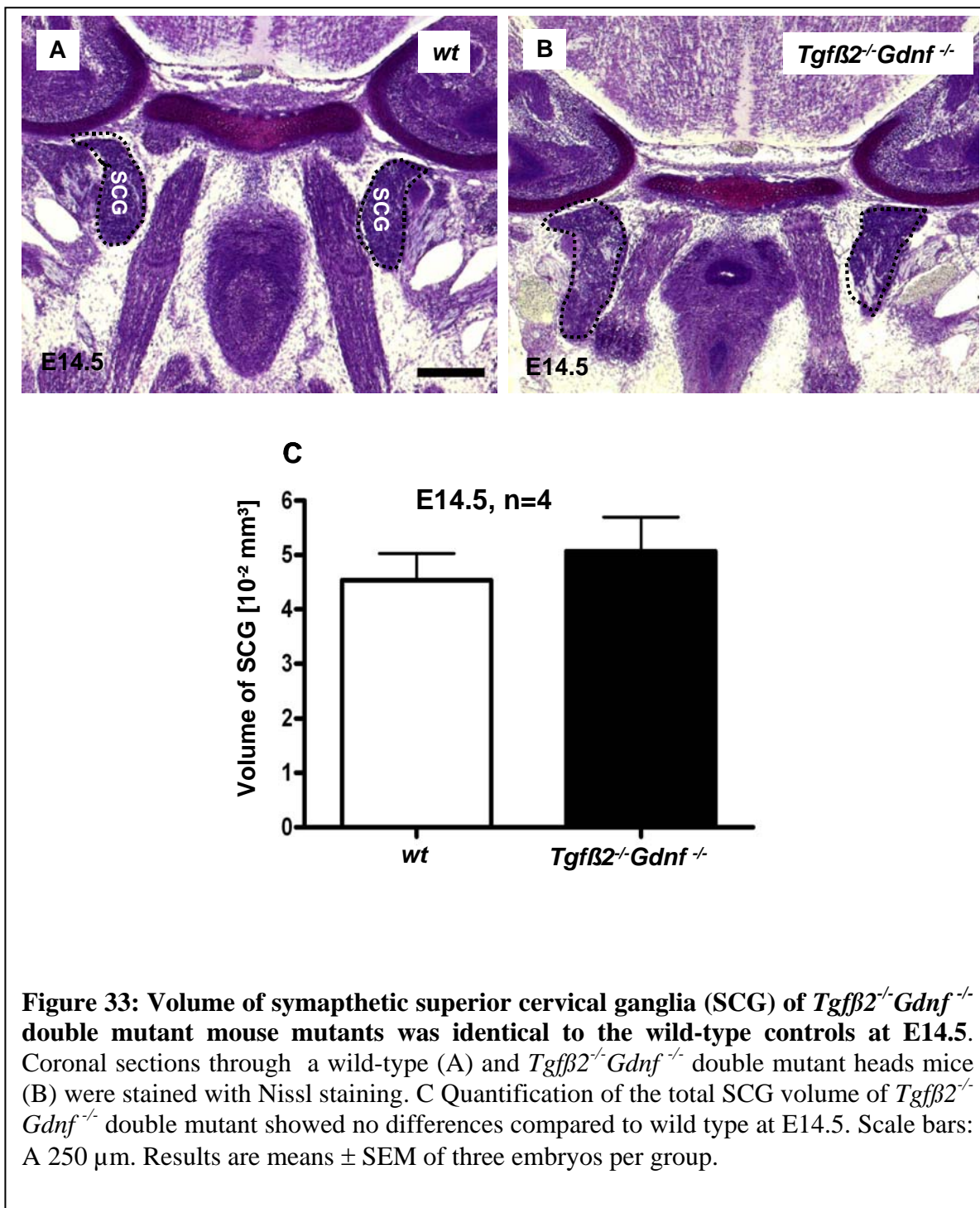


Figure 29: Defects in the nodose-petrosal ganglia (NP) of *Gdnf*^{-/-} single and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mouse at E18.5. A-D Nissl-stained coronal sections through a wild-type (A, B) and *Gdnf*^{-/-} single (C) and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mouse heads (D) showing the NP ganglia (marked). E Quantification of the total number of the NP neurons revealed a significant decrease in *Gdnf*^{-/-} single and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mouse at E18.5 compared to the wild-type controls. Scale bars: A 250 μ m (also applies to C,D); B 20 μ m.









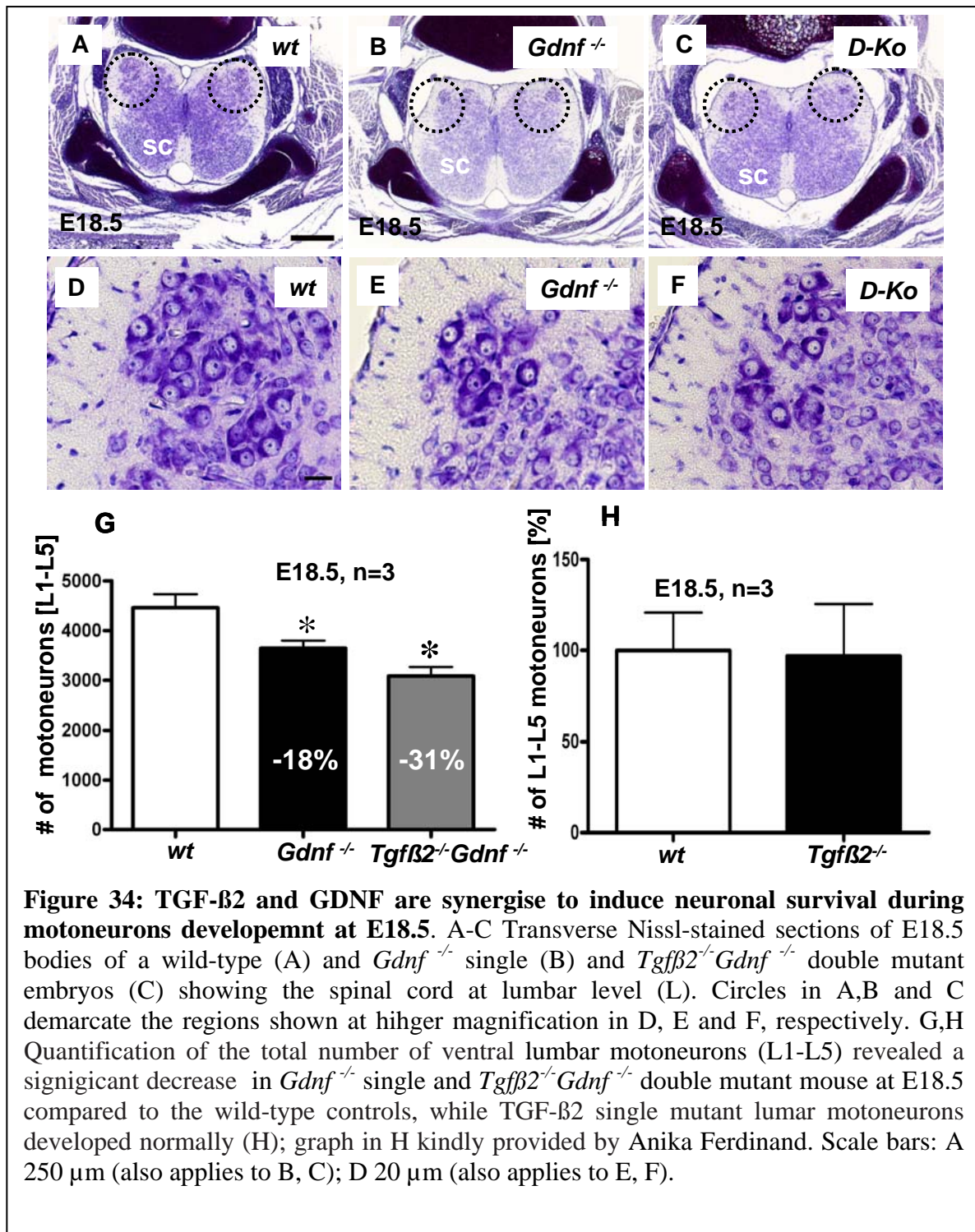
5.9 Motor neurons

Many neurotrophic factors with survival activity for motoneurons were first identified using cultures of purified embryonic motoneurons *in vivo*. It is still far from clear how they act *in vivo*, and whether other significant factors remain to be identified. To quantify the reduction in the lumbar motoneurons of mutant mice embryos, the large multipolar ventral MNs were counted using Nissl-stained sections. Figure 34 showed that, at E18.5, there was about 31 % decrease in the number of lumbar motoneurons (L1-L5) in the $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ double mutant mice embryos compared to the controls ($Tgfb\beta 2^{+/+}Gdnf^{+/+}$), while the GDNF single mutant mice showed only 18% reduction and TGF- β 2 single mutant showed no significant difference in the lumbar motoneurons (Table 4). Again, the increased motoneuron loss in $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ double mutant mice versus single mutant may be a result of cooperation and/or synergy between TGF- β 2 and GDNF. Pathological motoneuron death can occur as the result of the neurodegenerative disease ALS (amyotrophic lateral sclerosis) and SMA (spinal muscular atrophy).

5.10 Phenotypes outside the nervous system

5.10.1 Ventral body wall and muscle development

Histological Nissl-stained sections of an E14.5 $Tgfb\beta 2^{-/-}Gdnf^{+/+}$ and $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ mutant mice embryos demonstrated that the thickness of the ventral body wall at abdominal (Fig. 35) and urinary bladder (Fig. 36) levels was notably reduced compared to the wild-type situation, while the ventral body wall of $Tgfb\beta 2^{+/+}Gdnf^{-/-}$ embryos was normal (Fig. 35). For further investigation, immunohistochemistry for alpha-smooth muscle actin was performed. At embryonic stages, alpha-smooth muscle actin does not stain specifically for smooth muscles, but also considered to be a striated muscle cell feto-embryonic actin marker. It is transiently expressed in cardiomyoblasts and skeletal myoblasts and reappearing during neoplastic transformation of skeletal muscle (Babai et al., 1990). Figure



36 shows that the ventral body wall muscles of wild-type embryos continue close to the hernia, while the body wall muscles of *Tgfb2*^{-/-}*Gdnf*^{+/-} and *Tgfb2*^{-/-}*Gdnf*^{-/-} mutants do not reach the midline.

5.10.2 Extracellular matrix formation (ECM)

TGF-βs are known to have an effects on matrix formation. In E14.5 wild-type embryos, Trichrome stained sections showed that the connective tissue underlying the epidermis was stratified with collagen fibers in parallel layers (Fig. 37). On the contrary, the *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos exhibited loosely organized connective tissue, disturbed collagen fiber bundles which were notably thicker than the wild-type embryos (Fig. 37).

5.10.3 Molar tooth development

Growth factors regulate communication between cells in all developing organs. During tooth development, molecules in several conserved signal families mediate interactions both between and within the epithelial and mesenchymal tissue layers (reviewed by Thesleff and Mikkola, 2002). HE-stained paraffin sections of E14.5 heads of *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos showed a defect (acceleration in the tooth development) in the molar tooth morphogenesis compared to the wild-type embryos (Fig. 38). The *Tgfb2*^{-/-}*Gdnf*^{+/-} and *Tgfb2*^{+/-}*Gdnf*^{-/-} molar teeth at E14.5 were identical to the controls group (*Tgfb2*^{+/+}*Gdnf*^{+/+}). Surprisingly, at E18.5, the molar teeth of the *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos were not distinguishable from the wild-types molar teeth (Fig. 39). This give an indication that these factors (TGF-β2 and GDNF) may have an inhibitory affects during the molar teeth developemt at certin developmental stages.

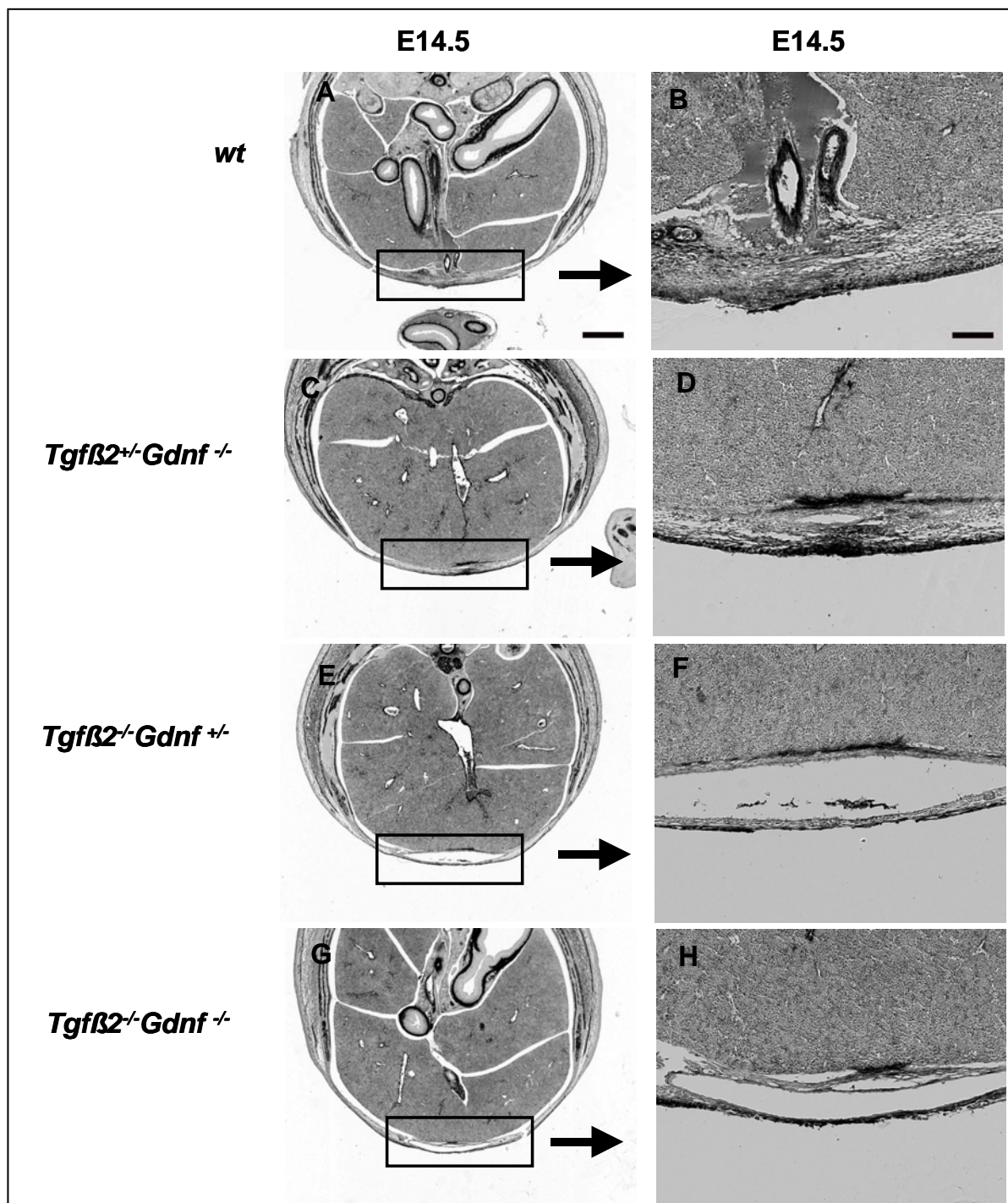


Figure 35: A-H Transverse sections of an E14.5 wild-type (A, B), *Tgfb2*^{+/-}*Gdnf*^{-/-} (C, D), *Tgfb2*^{-/-}*Gdnf*^{+/-} (E, F), and *Tgfb2*^{-/-}*Gdnf*^{-/-} double knockout mice bodies embryos (G, H) at level level immunocytochemically stained for alpha-smooth muscle actin, an embryonic striated muscle cell marker also staining smooth muscle cells. *Tgfb2*^{-/-}*Gdnf*^{+/-} (E, F), and *Tgfb2*^{-/-}*Gdnf*^{-/-} double knockout mice bodies revealed a decrease in the thickness of the ventral body wall compared to the wild-type controls. *Tgfb2*^{+/-}*Gdnf*^{-/-} (C, D) ventral body wall was identical to the controls. Black boxes in A, C, E, and G demarcate the regions shown at higher magnification in B, D, F and H, respectively. Scale bars: A 500 μ m (also applies to C, E, G); B 100 μ m (also applies to D, F, H).

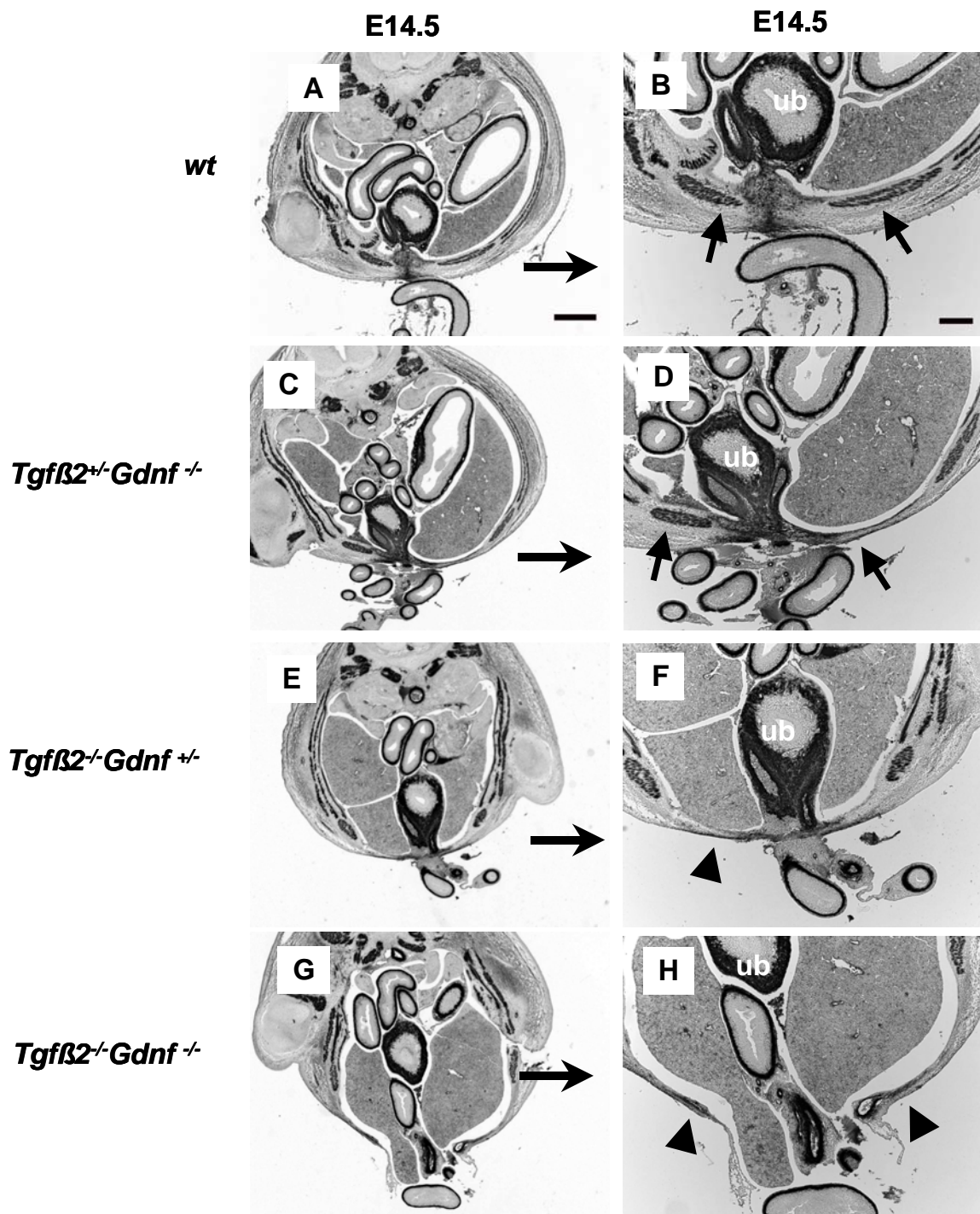
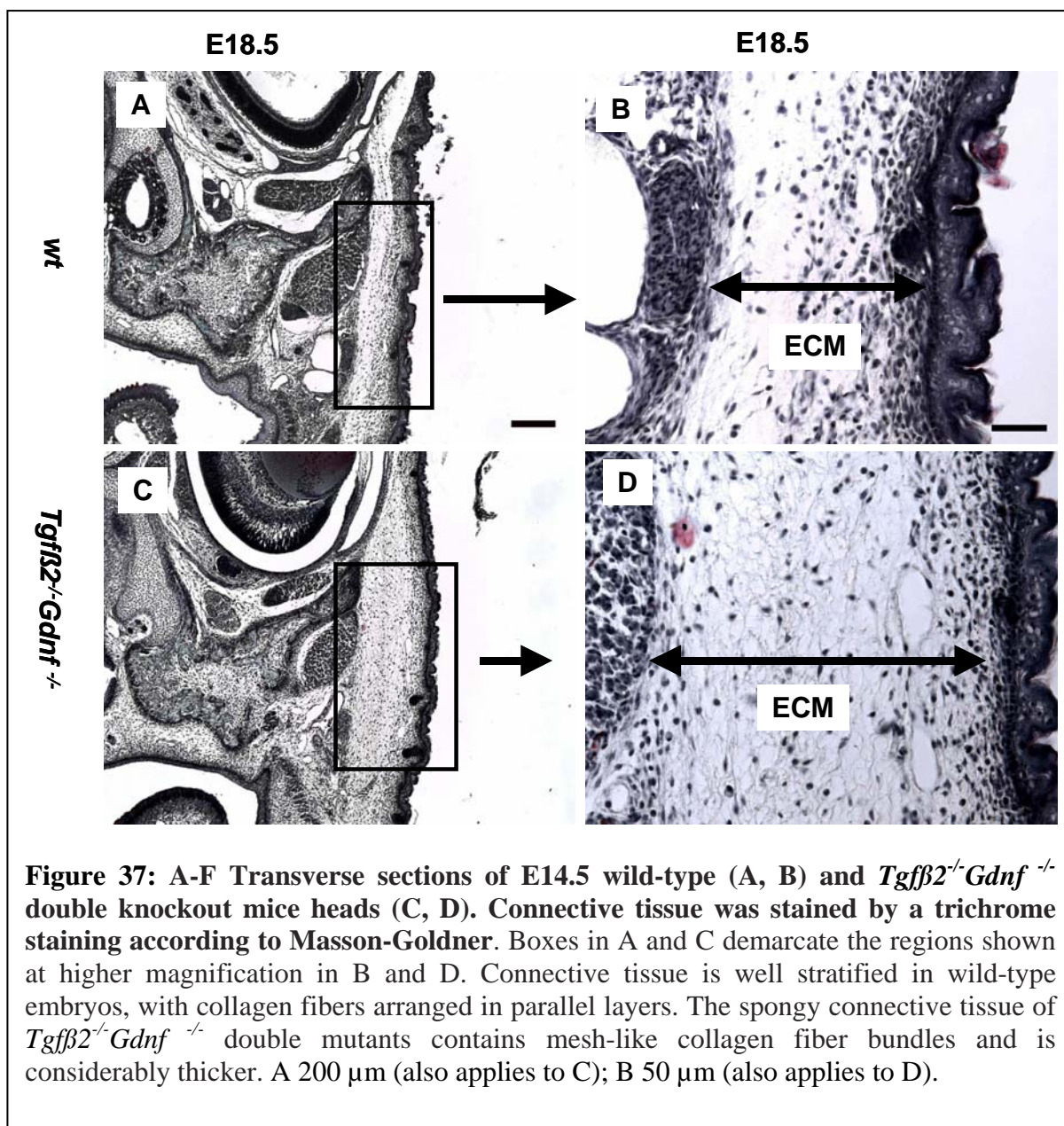


Figure 36: A-H Transverse sections of an E14.5 wild-type (A, B), $Tgfb2^{+/-}Gdnf^{-/-}$ (C, D), $Tgfb2^{-/-}Gdnf^{+/-}$ (E, F), and $Tgfb2^{-/-}Gdnf^{-/-}$ double knockout mice bodies embryos (G, H) at urinary bladder level immunocytochemically stained for alpha-smooth muscle actin, an embryonic striated muscle cell marker also staining smooth muscle cells. Body wall muscles of $Tgfb2^{-/-}Gdnf^{+/-}$ and $Tgfb2^{-/-}Gdnf^{-/-}$ double knockout mice do not reach the midline (arrowheads in F; arrowheads in H as compared to arrows in B). Black boxes in A, C, E, and G demarcate the regions shown at higher magnification in B, D, F and H, respectively. Scale bars: A 500 μ m (also applies to C, E, G); B 100 μ m (also applies to D, F, H).



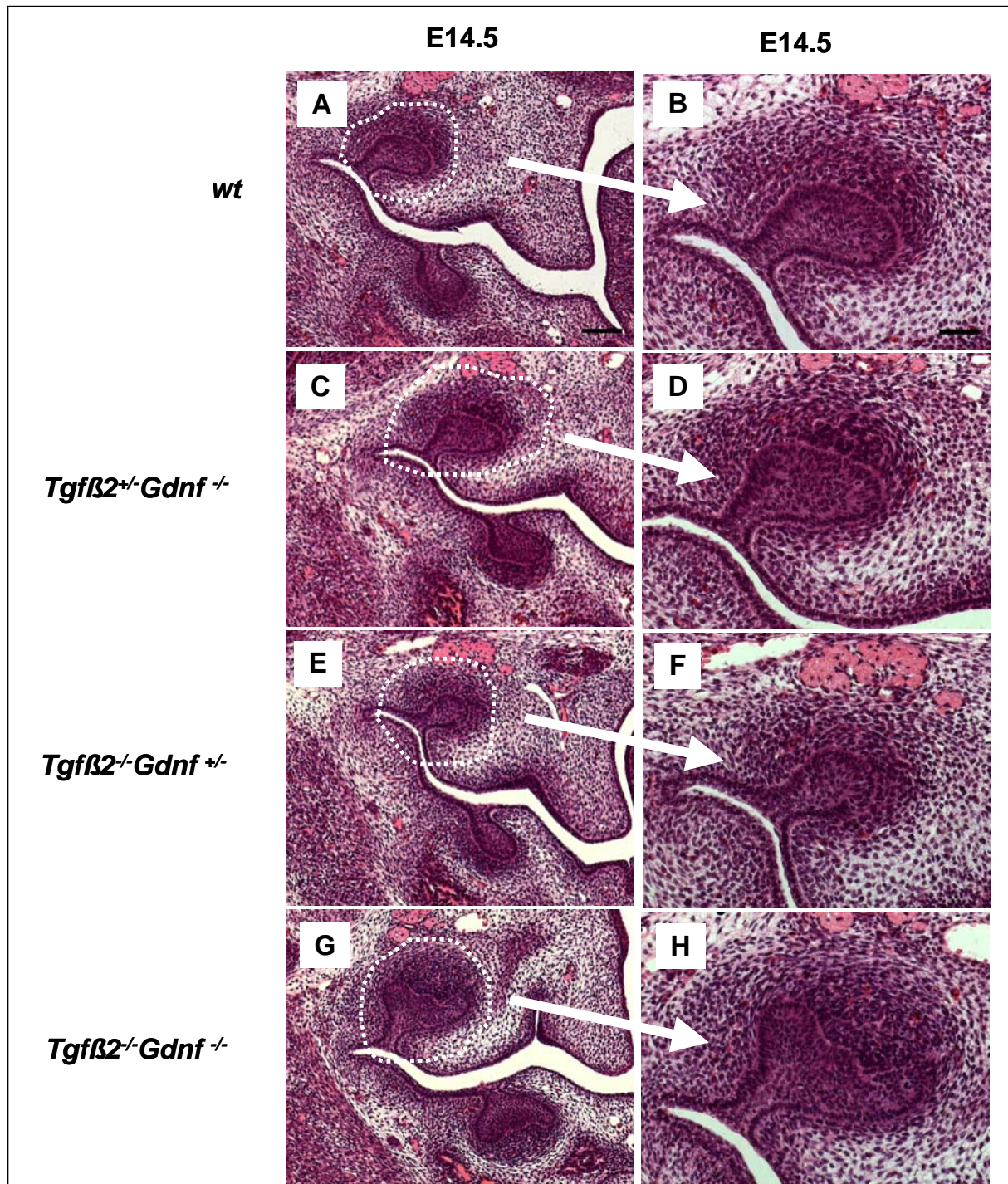
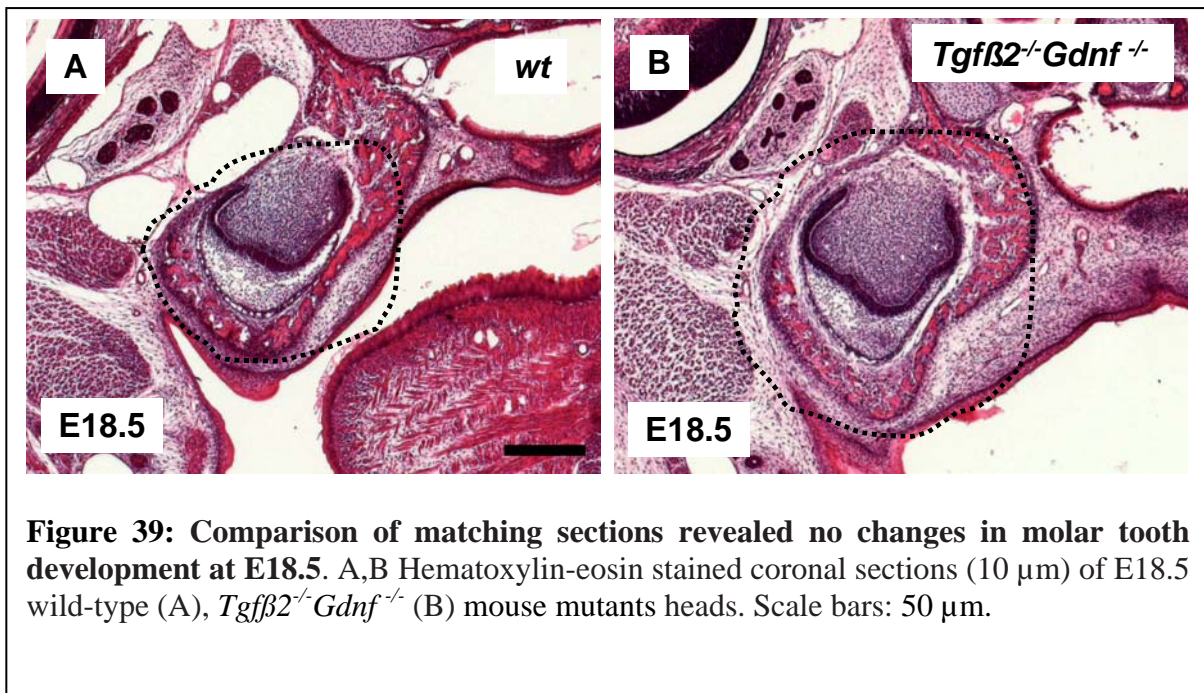


Figure 38: *Tgfb2^{-/-}Gdnf^{-/-}* double mutants mice (G,H) molar teeth revealed an acceleration in the development comparing to the wild-type controls at E14.5, while *Tgfb2^{+/-}Gdnf^{-/-}* (C, D) and *Tgfb2^{-/-}Gdnf^{+/-}* (E, F) were comparable to the controls. A-H Hematoxylin-eosin stained coronal sections (10 μ m) of E14.5 wild-type (A,B), *Tgfb2^{+/-}Gdnf^{-/-}* (C,D), *Tgfb2^{-/-}Gdnf^{+/-}* (E,F), and *Tgfb2^{-/-}Gdnf^{-/-}* (G,H) mouse mutants heads. This data may give an indication that TGF- β 2 and GDNF synergise together to act as inhibitory factors. Marked areas (upper molar tooth) in A, C, E, and G demarcate the regions shown at higher magnification in B, D, F and H, respectively. Scale bars: A 250 μ m (also applies to C, E, G); B 50 μ m (also applies to D, F, H).



6. Discussion

6.1 Mating and genotyping results

Mice homozygous for TGF- β 2 null mutants die from congenital cyanosis shortly before or during birth (Sanford et al., 1997), while GDNF mutant mice die shortly after birth due to renal failure, and the absence of the enteric neurons, causing pyloric stenosis and dilation of duodenum (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Since single TGF- β 2 or GDNF mutant mice die at birth, it is likely that the double mutant mice (*Tgfb2*^{-/-} *Gdnf*^{-/-}) die at or before birth. In the present study, we described the phenotypic changes observed in *Tgfb2*^{-/-} *Gdnf*^{-/-} double knockouts and their *Tgfb2*^{-/-} *Gdnf*^{+/-} and *Tgfb2*^{+/-} *Gdnf*^{-/-} littermates compared to the wild-type controls (*Tgfb2*^{+/+} *Gdnf*^{+/+}). Recently, it was shown that GDNF possess the ability to regulate spermatogonia renewal and differentiation during male spermatogenesis (Meng et al., 2000). In the present study, we also observed limited fertility of the *Tgfb2*^{+/-} *Gdnf*^{+/-} double heterozygous mice males. Many matings were not successful, and the successful matings was limited to specific males.

6.2 Choice of TGF- β 2 isoform

TGF- β s family are multifunctional cytokines with widespread distribution. There are three different mammalian TGF- β -isoforms; TGF- β 1, TGF- β 2 and TGF- β 3, each encoded by different genes and located on different chromosomes (Roberts and Sporn, 1990). TGF- β 2 was used in this study for several reasons. Analysis of *Tgfb2*^{-/-} *Tgfb3*^{-/-} double-deficient mice embryos revealed a more important role for TGF- β 2 mediating processes in the development of the eye (Duenker and Krieglstein, 2003) as well as in the development of the ribs and midline fusion (Duenker and Krieglstein, 2002a). Furthermore, TGF- β 2 has an isoform-specific role in the regulation of proliferation as well as differentiation of chromaffin cell in the developing murine adrenal gland (Rahhal et al., 2004).

6.3 Summary for some of the general phenotypes for TGF- β 2 single, GDNF single and TGF- β 2/GDNF double mutant mice embryos

Table 5 summarized some of the phenotypes which have been seen in TGF- β 2 single, GDNF single and TGF- β 2/GDNF double mutant mice embryos. It is clear that some of the phenotypes was due to absence of TGF- β 2 only, others due to the loss of GDNF. Interestingly, specific phenotypes in the eye, ECM, ventral body wall, and teeth developemnt were seen only in the double mutant mice (*Tgfb2^{-/-}Gdnf^{-/-}*). This finding support the notion of synergism and cooperation between these two factors outside the nervous system *in vivo*.

Table 5: Summary for some of the phenptypes which have been seen in TGF- β 2 single, GDNF single, and TGF- β 2/GDNF double mutant mice embryos.

	<i>Tgfb2^{-/-}</i>	<i>Gdnf^{-/-}</i>	<i>Tgfb2^{-/-}Gdnf^{-/-}</i>
Eye	<ul style="list-style-type: none"> • Increase in the thickness • Accumulation of cells in the posterior chamber of the eye 	Normal	<ul style="list-style-type: none"> • Increase in the thickness • Accumulation of cells in the posterior chamber of the eye • Folding of the retina (coloboma-like structure) • Detachment of the retina from the underlying pigment epithelium
ENS	Normal	Defect in the ENS	Defect in the ENS
Teeth	Normal	Normal	Acceleratuion in molar teeth development at E14.5
ECM	Normal	Normal	Increase in thickness of the ECM and disturbed collagen fiber.
Ventral body wall	NA	NA	Decrease in the thickness and absence of muscles in the medline
Kidney	Have kidney	No kidney	No kidney

6.4 TGF- β 2 and GDNF: synergism and cooperation

Many recent studies have revealed that growth factors acting in synergy can regulate neuronal survival much more effectively than individual factors alone. A good example of TGF- β /neurotrophic factor synergisms is the cooperativity of TGF- β and GDNF. In the present study, TGF- β 2 single, GDNF single and TGF- β 2/GDNF double mutant mice embryos were generated and analyzed. As there is no loss of SCG neurons in TGF- β 2 single mutants and only 21% loss of GDNF single mutants, the significantly increased loss of neurons at E18.5 in double mutants versus TGF- β 2, GDNF single mutants presents a clear example of a synergistic effect between these two factors *in vivo* (Fig. 31). To test whether the TGF- β 2/GDNF-dependent loss of SCG neurons at E18.5 reflects a degeneration of neurons due to lack of trophic support or a developmental phenotype, earlier stages of SCG development have to be analyzed. At E16.5, there was a significant decrease in the total number of the total SCG neurons in the *Gdnf*^{-/-} mice (26 %) and more severe reduction (47 %) in these neurons in the double mutant mice (Fig. 32). Surprisingly, the volume of the SCGs at E14.5, an earlier developmental stage, were normal in the double mutant mice compared to the wild-type controls (Fig. 33). Together, these data suggest for the first time that TGF- β 2 and GDNF cooperate and synergize in the regulation of SCG neuron survival during development and constitute a synergistic neurotrophic activity *in vivo*. On the other hand, the severe defect (reduction) in the number of the lumbar motoneurons of TGF- β 2/GDNF double mutant mice embryos at E18.5 support the notion of synergism and/or cooperation between these two factors *in vivo*. TGF- β 2 single mutant mice have marginal effects on the motoneurons development while GDNF single mutant embryos have only 18% loss in these neurons *in vivo* (Fig. 34). Furthermore, the folding (coloboma formation) of the neuronal retina of *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos was never seen neither in GDNF nor in TGF- β 2 single mutant mice embryos.

This is well consistent with the expression patterns of these factors in the retina (Nosrat et al., 1996; Duenker and Kriegstein, 2003). Interestingly, our data support the notion of synergism between TGF- β 2 and GDNF outside the nervous system. *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos showed an acceleration in the molar teeth development compared to the wildtype controls (Fig. 38). These factors seemed to synergize together to work as inhibitory factor during teeth development.

Our results are well consistent with other *in vitro* studies. When grown in serum-free media and in the absence of exogenous TGF- β , GDNF failed to promote survival of a large number of PNS and CNS neuron populations (Kriegstein et al., 1998b). Mechanisms, by which TGF- β generates neurotrophic effects and synergizes with other cytokines are begin to become more clear. Kriegstein and coworkers (1998a) have been showed that E8 chick ciliary neurons lose responsiveness to CNTF and GDNF when treated with phosphatidylinositol phospholipase C, which liberates GPI-linked α -receptors within the receptor complexes. In the presence of TGF- β , however, both CNTF and GDNF promote neuronal survival, suggesting that TGF- β may protect or restore the respective α receptors. The biological mechanism of the TGF- β /GDNF synergy is explained by several types of observations. First, both TGF- β and GDNF molecules are co-stored in and co-liberated from secretory granules of chromaffin cells in the adrenal gland (Kriegstein et al., 1998b). Second, neutralization of endogenous TGF- β in preganglionic sympathetic neurons abolishes the rescuing effect of GDNF on this neuron population (Schober et al., 1999a). Moreover, it was shown recently that TGF β -induced recruitment of the glycosyl-phosphatidylinositol-anchored GDNF receptor- α -1 (GFR- α -1) to the plasma membrane. This is supported by the fact that GDNF in the presence of a soluble GFR- α -1 can promote survival in the absence of TGF- β (Peterziel et al., 2002). An important component of GDNF/TGF- β signaling has been identified as an activation of

phosphatidylinositol-3 (PI-3) kinase (Kriegstein et al., 1998b), also, the biological significance of the trophic synergism of GDNF and TGF- β is also supported by colocalization of the receptors for both TGF- β and GDNF in all investigated GDNF responsive neuron populations *in vivo*. Figure 1 shows some of the expected pathways of interaction during the downstream signaling cascades between TGF- β and GDNF. Finally, an understanding of the role of TGF- β /neurotrophic factor synergism is considered an important issue to identify common factors that may act as new targets for the development of new therapeutic strategies for neurodegenerative and neurological diseases.

6.5 Dopaminergic neurons

Dopaminergic neurons of the ventral midbrain play important roles in the regulation of motor performances, cognition, and behavior. Parkinson's disease is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), with a marked loss of dopaminergic function in the striatum (Dauer and Przedborski, 2003). It is one of the most common neurological disorders of the elderly. Neurotrophic factors capable of sustaining the survival of otherwise degenerating dopamine neurons are thus of considerable interest (Weiss, 1993). The search for such neurotrophic factors has focused on dissociated cultures of embryonic midbrain (*in vitro*) and many mutant animal models (*in vivo*). Using *in vivo* mice experimental model, we found that, at embryonic day 12.5 (E12.5), the total number of midbrain dopaminergic neurons stained for tyrosine hydroxylase (TH), were significantly decreased in mice deficient for TGF- β 2 (*Tgfb2*^{-/-}) compared to wild-type (*Tgfb2*^{+/+}) mice embryonic littermates (Fig. 4). This observation is well consistent with the reported effects of TGF- β s *in vitro*; Farkas et al. (2003) showed that using E12.5 rat ventral mesencephalic cultures, both TGF- β 2 and TGF- β 3 are required for induction of midbrain dopaminergic neurons. They

demonstrated that TGF- β 2 and TGF β -3 proteins were expressed in mesencephalic cells. Furthermore, administration of neutralizing antibodies against all three mammalian TGF- β isoforms almost abolished the TH-positive phenotype in cultures of E12 rat ventral mesencephalon and E7 chick embryos (Farkas et al., 2003; Kriegstein et al., 2000). These data suggest that TGF- β is essentially involved in the early induction of the TH-positive phenotype. Recently, Roussa et al., (2006) showed that TGF- β 2/TGF- β 3 double mutant mice embryos revealed significantly reduced numbers of TH labelled cells in ventral mesencephalon. Several growth factors and cytokines have previously been shown to upregulate the catecholaminergic phenotype of neural crest-derived or midbrain neuronal cells. The transforming growth factor betas (TGF- β s) are a small family of growth factors with three mammalian isoforms, TGF- β 1, - β 2 and - β 3. Most cells are responsive to TGF- β s, and the TGF- β s have been implicated in a broad range of physiological and pathological processes (Roberts et al., 1990). TGF- β 1 synthesized and localized in the notochord and floor plate (Farkas et al. 2003; Unsicker et al., 1996). TGF- β 2 and TGF- β 3, which are distantly related to the GDNF, prevent the death of cultured dopaminergic neurons at picomolar concentrations *in vitro*. Moreover, they found that TGF- β 2, TGF- β 3, and GDNF are expressed sequentially as local and target-derived trophic factors and that subpopulations of dopaminergic neurons projecting to distant targets have access to only one of these factors (Poulsen et al., 1994; Kriegstein et al., 1995). These findings are consistent with the idea that GDNF, TGF- β 2, and TGF- β 3 are survival factors for embryonic midbrain dopaminergic neurons and may have applications as therapeutics for neurodegenerative diseases such as Parkinson's disease. By using *in situ* hybridization, both TGF- β 2 and GDNF mRNAs were detected in close proximity to the cell bodies of the TH-positive neurons in the rat ventral midbrain (Poulsen et al., 1994). At this stage, RT-PCR data revealed low levels of mRNAs for TGF- β 2, TGF- β 3, and GDNF in the

innervation targets of dopaminergic neurons. On the other hand, one day after birth, when the connections of dopaminergic neurons mature, mRNAs for TGF- β 2, TGF- β 3, and GDNF are no longer present in the ventral midbrain but are up-regulated in distinct innervation targets of the midbrain dopaminergic neurons (Poulsen et al., 1994). Krieglstein and Unsicker (1994) found that TGF- β has trophic and protective effects on the dopaminergic neurons isolated from the embryonic rat mesencephalic floor *in vitro*. Andrews et al., (2006) have been shown that all of the neurons in the striatum, substantia nigra, and globus pallidus in adult mice were strongly stained by the anti-TGF- β 2 antibody. The TGF- β 2 immunoreactivity was most intense in the cell bodies and axons. The SNpc and SN neurons were immunostained with a similar intensity. The different parts of the brain stained very differently with the anti-T β RII antibody. Only low levels of immunoreactivity were present in the SNpc. All parts of the SNpc lacked significant T β RII positive immunoreactivity (Andrews et al., 2006).

On the contrary, at E14.5 and E18.5 (Figs. 5-6), our analysis failed to reveal significant differences in the total number of midbrain dopaminergic neurons in SVpc and VTA in *Tgfb2*^{-/-}, *Gdnf*^{-/-}, *Tgfb2*^{-/-}*Gdnf*^{+/-}, *Tgfb2*^{+/-}*Gdnf*^{-/-}, *Tgfb2*^{-/-}*Gdnf*^{-/-} mutant mice embryos compared to the controls littermates (*Tgfb2*^{+/+}*Gdnf*^{+/+}). This may indicate that these genes seemed to have a marginal effect on the development and survival of these neurons at these stages (prenatally). Because our mutants die shortly after birth, we were unable to access a possible role for GDNF and TGF- β 2 in the maintenance of midbrain dopaminergic neurons after birth. These results obtained from knockout gene manipulation experiments do not rule out the possibility that GDNF and TGF- β 2 are important for the survival of the midbrain dopaminergic neurons, because the brain may be able to compensate for loss of any one factor during development. This compensatory function of CNS neurons has been shown in at least some of the knockout mice that lack other neurotrophic factors, such as

nerve growth factor (NGF) (Crowley et al., 1994). For example, it has been demonstrated that early development of the forebrain cholinergic neurons is not compromised in animals that are lacking NGF (Crowley et al., 1994), despite the fact that these cholinergic neurons have been shown to be dependent on NGF for their development (Honegger and Lenoir, 1982). Burke and coworkers have collected several evidence suggesting that dopaminergic neurons in the SN undergo ontogenetic cell death in a biphasic manner at postnatal day 2 and 14 (Oo and Burke, 1997). Kholodilov and coworkers (2004) have been demonstrated that GDNF suppresses apoptosis of nigral dopaminergic neurons both *in vitro* and during the biphasic period of ontogenetic cell death *in vivo*. Furthermore, neutralizing endogenous GDNF by the application of anti-GDNF antibodies to the striatum increases cell death, suggesting that endogenous GDNF act as a target-derived factor (Kholodilov et al., 2004). GDNF was first isolated from culture of supernatants of B49 glial cell line using its ability to support the survival and physiological differentiation of embryonic midbrain dopaminergic neurons and increased their high-affinity dopamine uptake *in vitro* (Lin et al., 1993; Lin et al., 1994) as the other related TGF- β 2 and TGF- β 3 molecules (Poulsen et al., 1994, Krieglstein and Unsicker, 1994). GDNF is expressed throughout the CNS, with the highest levels occurring in the P0 and P10 rat, in the human striatum, and at lower levels in the substantia nigra, suggesting that GDNF may act both as a local and a target-derived neurotrophic factor for nigrostriatal dopaminergic neurons which are severely affected in Parkinson's disease (reviewed by Krieglstein, 2004).

Because TGF- β 2 and GDNF die at birth, TGF- β 2 haploinsufficient ($Tgfb2^{+/-}$) and double heterozygous ($Tgfb2^{+/-}Gdnf^{+/-}$) mice was analyzed. At one year of age, $Tgfb2^{+/-}$ and $Tgfb2^{+/-}Gdnf^{+/-}$ showed a marginal decrease (about 10-12%) in the total number of dopaminergic neurons. Differences did not reach statistical significance (Fig. 7). Recently, Andrews and coworkers (2006) found that TGF- β 2 haploinsufficiency ($Tgfb2^{+/-}$) have

subclinical defects in the dopaminergic neurons of their substantia nigra pars compacta. At 6 weeks of age, the *Tgfb2*^{+/-} mice had 12% fewer dopaminergic neurons than wild-type littermates, which was marginally significant. In contrast to the young mice, the concentration of dopamine in the striatum of *Tgfb2*^{+/-} mice was only 70% of their wild-type littermates. The 50% reduction in TGF-β2 only produced a 10% loss of neurons. This is consistent with the known patterns of expression of the TGF-βs during development (Andrews et al., 2006). The SNpc dopaminergic neurons produce TGF-β2, and the neurons and glia of the striatum contain TBR1. Consequently, the loss of dopamine in the *Tgfb2*^{+/-} heterozygous adult mice may be as a result of disrupting communication between the dopaminergic neurons and the cells of the striatum (Andrews et al., 2006).

6.6 Serotonergic neurons

Several evidence suggest that numerous members of the TGF-β superfamily are expressed and have several functions in the developing nervous system. TGF-β2 and -β3 are consistently coexpressed in many neuron populations and astroglial cells within the adult CNS in mammals (Unsicker et al., 1996). The present study provides evidence for the regulation of the serotonergic cell phenotype by members of TGF-β superfamily at specific stages and regions. By using a genetic mouse model, we found that at E12.5 the total number of serotonergic neurons was significantly decreased in mice deficient for TGF-β2 (*Tgfb2*^{-/-}) compared with wild-type mice embryos (Fig. 8). Moreover, there was a significant decrease in the total number of serotonergic neurons in *Tgfb2*^{-/-} and *Tgfb2*^{-/-} *Gdnf*^{+/-} in the paramedian raphe (PMR) at E18.5 compared with wild-type mice embryos (Fig. 9). This data suggest that TGF-β2 is essentially involved in the induction of the 5-HT-positive phenotype at certain stages. These observations are well consistent with the reported effects of TGF-βs *in vitro*; in primary cell cultures from rat hindbrain raphe tissue, the two investigated TGF-β isoforms, TGF-β2 and -β3, similarly promoted the

serotonergic phenotype by inducing two major serotonergic markers, tryptophan hydroxylase (TpOH), and the plasma membrane transporter for 5-HT (Galter et al., 1999). This indicated that TGF- β s induce the serotonergic phenotype rather than promote survival. The TGF- β isoforms - β 2 and - β 3 can be detected by RT-PCR in the embryonic mouse hindbrain floor around E12.5 (Flanders et al., 1991), suggesting that they may have roles in events such as migration and differentiation of neurons. In rat embryonic hindbrain raphe, the mRNAs for the TGF- β isoforms 2 and 3 are present in the developing hindbrain raphe and may therefore be regarded as candidates for proteins with a regulatory ability for neurons that develop within the raphe including the serotonergic neurons (Galter et al., 1999). These data are consistent with the previous observation that T β R-II mRNA and protein are detectable using competitive RT-PCR in the developing rat brainstem at several developmental stages (E18, P6, adult) (Böttner et al., 1996; Vivien et al., 1998). Furthermore, several other factors including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were shown to play a role in the differentiation and maintenance of serotonergic neurons (Altar et al., 1994; Mamounas et al., 1995). Additionally, Galter and Unsicker (1999) reported that GDNF, NT-3, and NT-4 promote the serotonergic phenotype of neurons cultured from embryonic rat raphe region in terms of tryptophan hydroxylase (TpOH) expression.

On the contrary, the number of rostral hindbrain serotonergic neurons in *Tgfb2^{-/-}Gdnf^{+/-}*, *Tgfb2^{+/-}Gdnf^{-/-}* at E14.5 and *Gdnf^{-/-},Tgfb2^{+/-}Gdnf^{-/-}* at E18.5 were identical with wild-type embryos (Figs. 10 and 13). Since TGF- β 2 and GDNF single knockout mice die at birth, it was impossible to analyse these mice postnatally. It is possible that other molecules can compensate for the lack of GDNF and TGF- β 2 in the mutants at these stages. Interestingly, there was also in one double mutant embryo both at E14.5 and E18.5 a severe defect (decrease) in the rostral 5-HT-positive neurons (Figs. 11-12), the caudal 5-HT-positive

neurons were comparable to the controls. This observation may give an indication about limited synergism between TGF- β 2 and GDNF *in vivo*. The severe defect (decrease) in the rostral 5-HT-positive neurons which was seen only in one double mutant embryo at E14.5 and E18.5 may be because of genetic penetrance. In order to understand the exact mechanisms for the action of TGF- β s on these neuronal population, further detailed investigations are required.

6.7 Noradrenergic neurons in the LC

The noradrenergic neurons in the locus coeruleus (LC), the main noradrenergic center in the brain, were analyzed in the $Tgf\beta 2^{-/-}Gdnf^{-/-}$, $Tgf\beta 2^{-/-}Gdnf^{+/-}$, $Tgf\beta 2^{+/-}Gdnf^{-/-}$ and the controls ($Tgf\beta 2^{+/+}Gdnf^{+/+}$) mice embryos at E14.5. In contrast to the previous studies showing the effects of some of TGF- β superfamily on the induction and survival of the embryonic noradrenergic neurons, no obvious defects were seen in these neurons in the single or double mutants mice embryos for TGF- β 2 and GDNF (Fig. 14). These results suggest that *in vitro* results or manipulations in prenatal or postnatal animals may not always reflect the physiological role of the protein *in vivo*, or that the function of these factors is compensated in the mutant mice. GDNF is expressed in the noradrenergic neurons, suggesting that it may also be involved in the regulating survival and differentiation of non-dopaminergic neurons (Arenas et al., 1995). Narsat and coworkers (1996) found GDNF mRNA expression in the developing locus coeruleus with high peak shortly before birth (E19-E21) and after birth. This patterns of expression may give an indication about roles for GDNF in the postmaturation of these neurons. Grafting of genetically Engineered fibroblasts which express high levels of GDNF prevented the 8-hydroxydopamine-induced degeneration of noradrenergic neurons in the LC *in vivo* (Arenas et al., 1995). On the contrary, neutralization of TGF- β *in vivo* in chick (E2-7) had no effect on the number of the TH-positive noradrenergic neurons in the locus coeruleus

(Farkas et al., 2003). Huang et al., (2005) found that GDNF protein is required for differentiation of pontine A5 noradrenergic neurons and patterning of central respiratory output, also, there was a marked decrease in the number of tyrosine hydroxylase (TH) positive A5 noradrenergic neurons. Moreover, in dissociate cultures of the A5 noradrenergic cell group from rat embryos, GDNF increases TH cell number and neurite growth without affecting total neuronal survival or proliferation of TH neurons. These effects of GDNF are inhibited by function blocking antibodies against endogenous brain derived neurotrophic factor (BDNF), indicating that GDNF requires BDNF as a cofactor to stimulate differentiation of A5 noradrenergic neurons (Huang et al., 2005).

6.8 Chromaffin cells

The present study suggests a specific role for the TGF- β 2 isoform rather than TGF- β 3 and GDNF in the regulation and control of chromaffin cell proliferation and differentiation. TGF- β , IGF-II (insulin like growth factor-II), and NGF (nerve growth factor) are potent promoters of chromaffin cell proliferation *in vitro* (Lillien and Claude, 1985; Tischler et al., 1989; Frödin and Gammeltoft, 1994, Wolf et al., 1999). PACAP has been identified as a negative regulator of chromaffin cell proliferation *in vitro* (Frödin et al., 1995). TGF- β s play a role in stimulation and inhibition of astriogial cell proliferation and regulation of their phenotypic expressions (Flanders et al., 1993). *In vivo*, proliferation of chromaffin cells is thought to be regulated by interaction of neurogenic and hormonal signals (Tischler et al., 1989). Wolf et al. (1999) have shown that TGF- β did not interfere with the spontaneous proliferation of chromaffin cells dissociated from postnatal day 6 rat adrenal gland in culture. However, when DNA synthesis and proliferation of chromaffin cells were stimulated by FGF-2 and insuline-like growth factor II , TGF- β had a pronouced inhibitory effect.

TGF- β immunoreactivity occurs in developing mouse chromaffin cells (Flanders et al., 1990). TGF- β is also synthesized and secreted by chromaffin cells (Wolf et al., 1999) via exocytosis (Krieglstein and Unsicker, 1995c). Chromaffin cells express TGF- β receptors which may be activated by upon release of chromaffin cell-derived TGF- β (Wolf et al., 1999). Culturing of mouse neural-crest cells in the present of glial cell line-derived neurotrophic factor (GDNF) has been shown to result in a significant increase in the number of tyrosine hydroxylase-positive cells with neuronal morphologies (Maxwell et al., 1996). Mice deficient for the NGF receptor *trkA* show clear losses in chromaffin cells numbers until day 12 postnatally (Schober et al., 1999b). Mice lacking FGF-2 and PACAP and TGF- β 2 have not been analyzed with regard to their adrenal phenotype. Thus, TGF- β 2 is the first identified peptide growth factor, shown to be a negative regulator of chromaffin cell proliferation and a stimulator for differentiation of chromaffin cells from norepinephrine producing cells to epinephrine producing cells. TGF- β is known as a contextually acting molecule, as its action depend on environmental cues such as the cell type, their state of differentiation and the presence or absence of other growth factors, to stimulate or inhibit proliferation (Nathan and Sporn 1991; Roberts and Sporn, 1990). Several mechanisms have been suggested by which TGF- β suppresses cell growth (Wolf et al., 1999). TGF- β inhibits mitosis by blocking the transition from G1 to S phase in epithelial cells (Alexandrow et al., 1995). TGF- β prevents the hyperphosphorylation of the retinoblastoma gene product (pRB), which is necessary for the progression from G1 to S phase, via interaction with cyclins and cyclin-dependent kinases (Alexandrow et al., 1995; Howe et al., 1991; Koff et al., 1993; Laiho et al., 1990). Previous studies showed that a reduction in the endogenous TGF- β increased proliferation of developing adrenal chromaffin cells *in vivo* in quail embryos, and these result are consistant with our result in on mice embryos (Combs et al., 2000).

The present study adds the murine neuroendocrine cells (chromaffin cells) to the list of documented cell types responding to TGF- β by inhibition of proliferation and stimulation of differentiation. These data also extends the list of neural crest derived cells whose development is specifically impaired by the lack of TGF- β 2 (Sanford et al., 1997). *In vitro* TGF- β isoform treatment may results in reductive biological effects, *in vivo*, however, TGF- β isoforms seem to serve unique roles. Future studies will reveal if TGF- β 2 acts alone or in cooperative synergism with other growth factors.

In conclusion, these data provide clear evidence that TGF- β 2 has an isoform-specific role in the regulation of proliferation as well as differentiation of chromaffin cell in the developing murine adrenal gland.

6.9 Eye developmental defects

In order to determine the putative functions of TGF- β 2 and GDNF in the development of mouse retina, several mutant mice embryos for TGF- β 2 and GDNF including the double mutant embryos ($Tgfb2^{-/-}Gdnf^{-/-}$) were generated and analyzed. At E14.5 and E18.5 the entire neural retinae of $Tgfb2^{-/-}Gdnf^{+/+}$, $Tgfb2^{-/-}Gdnf^{+/-}$ and $Tgfb2^{-/-}Gdnf^{-/-}$ double mutant mice embryos were notably thicker than the wild-type retinae (Figs. 20-21). Moreover, eyes of $Tgfb2^{-/-}Gdnf^{+/+}$, $Tgfb2^{-/-}Gdnf^{+/-}$ and $Tgfb2^{-/-}Gdnf^{-/-}$ double mutant mice displayed a vascularized accumulation of mesenchymal cells in the posterior chamber of the eye never seen in wild-type control animals (Fig. 20-21). Surprisingly, the retinal morphology of $Tgfb2^{+/-}Gdnf^{-/-}$ mutant mice were identical to the controls (Fig. 20-21). Sanford and coworkers (1997) were the first to report the hyperplasia of the retina in $Tgfb2^{-/-}$ single mutant mice. $Tgfb2^{-/-}Gdnf^{-/-}$ double mutant mice exhibit a significant increase in the number of proliferating cells as shown by PCNA immunostaining, this may give an explanation that the increase in the thickness of the retina is in part due to an increase in cell proliferation (Fig. 22). Furthermore, our results may be explained by the other reported

effect of TGF- β in mouse keratinocyte cell line; Alexandrow et al., (1995) reported that TGF- β inhibits mitosis by blocking the transition from G1 to S phase. Moreover, TGF- β 2 has an inhibitory effect during murine chromaffin cell development *in vivo* (Rahhal et al., 2004). It is most likely that the increase in the thickness of the retinae and the accumulation of the mesenchymal cells in the posterior chamber of the eye is may be due to the absence of the inhibitory effects of TGF- β on cell proliferation and/or disturbance in ontogenic cell death. Interestingly, the double mutant mice (*Tgfb2^{-/-}Gdnf^{-/-}*) and *Tgfb2^{-/-}Gdnf^{+/-}* mutant showed a complete detachment of the retina from the underlying pigment epithelium at E18.5 and the retina of the double mutant mice was folded (coloboma formation) at E18.5 (Fig. 21). These phenotypes have never been seen in other mutants for TGF- β 2 or GDNF and support the notion that TGF- β 2 and GDNF act synergistically during the eye development. TGF- β s are well known regulators during extracellular matrix formation (Roberts et al. 1992). Therefore the detachment of the retina from the underlying pigment epithelium at E18.5 may reflect a requirement of TGF- β for matrix formation and expression of specific cell adhesion molecules that may be lost and/or affected in the mutant mice (Grotenhorst 1997; Hocevar and Howe 2000). These results are well consistent with the expression patterns of TGF- β 2 and GDNF in mice, rat, and porcine retinae during development (Duenker and Krieglstein, 2003; Hauck et al., 2006; Nosrat et al., 1996). Many investigators have shown the potential therapeutic value of GDNF as neuroprotective factor in retinal degeneration. GDNF has survival effect on retinal ganglion cells (RGC) after axotomy and also has been proven to be very effective in retarding retinal photoreceptor (PR) degeneration in the rd1 mouse (Koeberle et al., 1998; Frasson et al., 1999). Recently, Nishikiori et al., (2005) reported that the intravitreal GDNF level increased in proliferative diabetic retinopathy (PDR) in human patients, suggesting that GDNF play a role in the pathogenesis of PDR. The phenotypes that seen in

Tgfb2^{-/-}Gdnf^{-/-} double mutant mice are well consistent in part with other observations in chick embryos treated with a neutralizing antibody against TGF- β (Duenker et al., 2001); retinae of anti-TGF- β -treated chick embryos were notably thicker than the control retinae, folded, and detached from the adjacent cell layers. These phenotypes supported the wide range of actions of TGF- β s during development including proliferation, cell death, and extracellular matrix formation. Moreover, transgenic animals carrying deletions of the apoptosis execution genes *Apaf1* and *Bax* have some comparable retinal phenotypes as in *Tgfb2^{-/-}Gdnf^{-/-}* mutant mice (Cecconi et al. 1998; Mosinger Ogilvie et al. 1998). Consistence with our observations in E14.5 and E18.5 *Tgfb2^{-/-}Gdnf^{-/-}* double null mutants, eyes of E12.5 *Apaf1^{-/-}* mutant mice showed a thickened folded retina and small lens as compared to the wild-type embryos (Cecconi et al. 1998). Furthermore, the inner nuclear layer (INL) of *Bax* mutant mice retina was thicker than normal retinae (Mosinger Ogilvie et al. 1998). In our study, immunostaining at E18.5 for the transcription factor Pax-6, reveals the expected patterning of the retina in control eyes and a diffuse distribution in *Tgfb2^{-/-}Gdnf^{-/-}* mutant embryos. Retinal patterning is disturbed in *Tgfb2^{-/-}Gdnf^{-/-}* double mutant embryos (Fig 23), but there was no notable changes in the Pax6 expression in the double mutants notable compared to wild-type embryos. This is well consistent with the results obtained from the TGF β receptor II mutant mice (Ittner et al., 2005). In conclusion, the present study showed that TGF- β 2 play more important role than GDNF in some parameters during eye development, and also, TGF- β 2 and GDNF act synergistically in other specific biological aspects during retinal development *in vivo*. In order to understand the exact mechanisms of action for both TGF- β 2 and GDNF during eye development, further detailed investigations are required.

6.10 Enteric Nervous System

The enteric nervous system (ENS) is a complex network of neurons and glia that innervate the wall of the intestine that controls intestinal motility, regulates mucosal secretion and also modulates sensation from the gut (Costa and Brookes, 1994, Kunze and Furness, 1999). During development, ENS precursors actively divide within the intestine to produce enough neurons to populate the gut. In this study, the enteric nervous system in single mutant mice for TGF- β 2 (*Tgfb2*^{-/-}), GDNF (*Gdnf*^{-/-}), and double knockout mice embryos (*Tgfb2*^{-/-}*Gdnf*^{-/-}) were generated and analyzed at E14.5 and E16.5 (Figs. 24-25). Neurons at the levels of the small and large intestine, that are colonized by vagal neural-crest-derived cells were completely absent in single GDNF mutant embryos as well as in double mutant mice embryos. The ENS of the TGF- β 2 mutant mice developed normally. This give an indication that the phenotype in the *Tgfb2*^{-/-}*Gdnf*^{-/-} mice is due to the absence of GDNF rather than TGF- β 2. This result is well consistant with other previous studies on the GDNF mutant mice (Moore et al., 1996; Pichel et al., 1996). Sánchez et al., (1996) analyzed the GDNF mutant mice at different developmental stages (E9.5-E13.5) in which ENS precursors populate the gastrointestinal tract wall. At E12.5, p75 neurotrophin receptor-positive neurons were detected only in the oesophagus and in the stomach wall, but not in the more distal midgut intestinal loops. In newborn animals, no ENS neurons were detected in the lower oesophagus, stomach, samll intestine, and colon. The effects of GDNF on the ENS neurons is well consistant with the expression patterns of GDNF in the gastrointestinal tract. By using in situ hybridization, GDNF mRNA was expressed extensively in the gastrointestinal tract of the rat during the development with high peak shortly before and after birth (Nosrat et al., 1996). Moreover, GDNF mRNA transcripts have been detected at high levels in the outer mesenchymal layer throughtout the length of the developing gastrointestinal tract (Hellmich et al., 1996). At E14, GFR α -1 is detected within the gut wall in a region broader than that occupied by the neural crest. This data

suggest that GFR α -1 protein is present on both neural crest and mesenchymal components of the gut wall (Gianino et al., 2003). Schuchardt and coworkers (1994) demonstrated the absence of the ENS in mice lacking the orphan tyrosine kinase receptor Ret. Thus, GDNF involved in c-Ret-mediated signaling during ENS development. Some human genetic disorders, e.g. Hirschsprung disease (HSCR) which causes megacolon formation, are characterized by defects in gastrointestinal innervation. Mutations in the human RET locus have been demonstrated in some familial forms of HSCR (Pasini et al., 1996). Thus, the loss of ENS neurons in GDNF mutant mice embryos suggests that mutations in the GDNF gene may also cause HSCR.

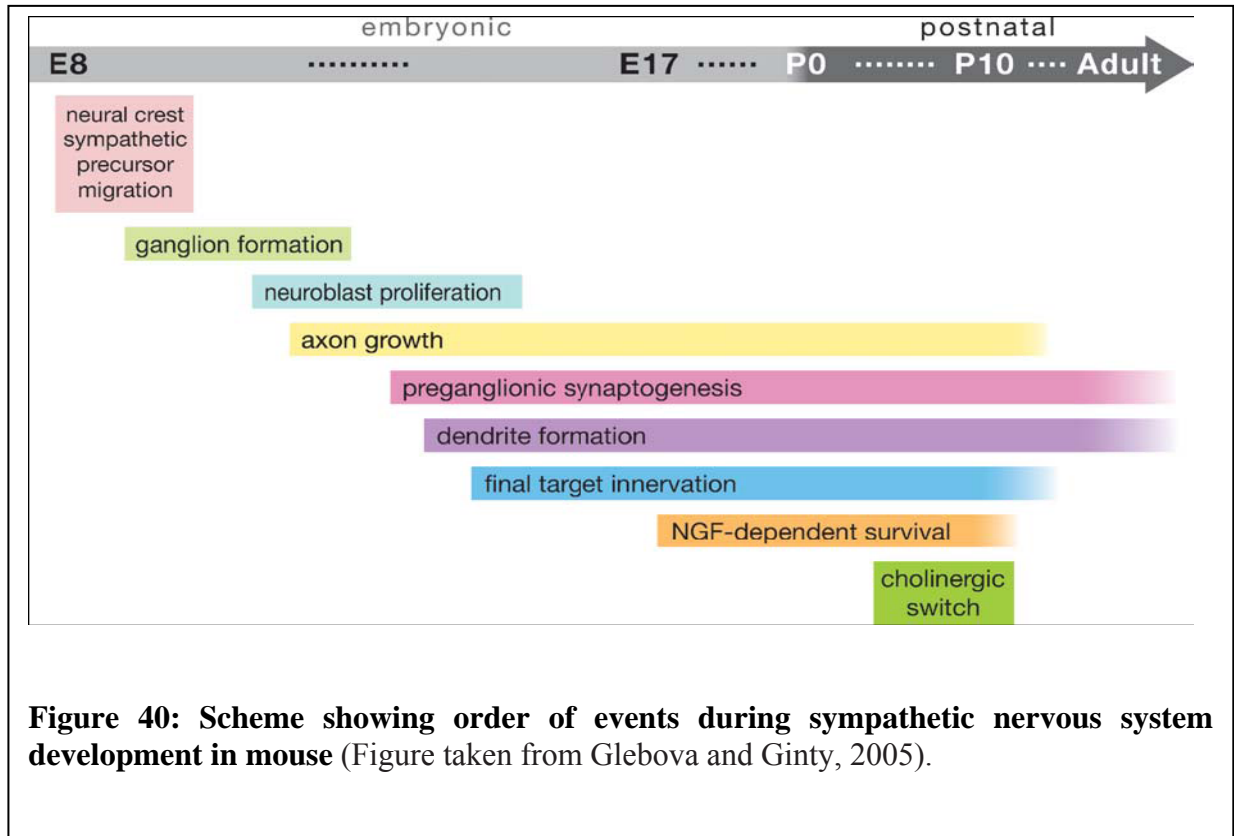
6.11 Peripheral ganglia

The development and maintenance of the vertebrate nervous system requires the activity of a wide range of polypeptides known as neurotrophic factors. To examine the physiological role of neurotrophic factors in the developing mammalian peripheral ganglia, genetically modified null-mutated animals that lack GDNF and TGF- β 2 factors were generated and analyzed. In the present study, we found that at E18.5, *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos show a significant reduction in the number of these ganglia neurons (Figs. 29-33). On the contrary, at E14.5, DRG and nodose-petrosal ganglia and the volume of SCGs were comparable to the wild-type controls. These data support the notion of degeneration due to the absence of GDNF. Buj-Bello et al., (1995) proved that GDNF is an age-specific survival factor for sensory and autonomic neurons, its mRNA is expressed in the tissues innervated by these neurons. Moreover, GDNF promotes the survival of sympathetic, enteroceptive, and small and large cutaneous sensory neurons in chicken culture (Buj-Bello et al., 1995). These results show that GDNF promotes the survival of several PNS neurons and suggest that GDNF may be important for regulating the survival of different populations of neurons at different stages of their development.

Previous studies have shown that several populations of peripheral neurons, including the trigeminal, dorsal root, and superior cervical ganglion, as well as the NPG, require more than one trophic factor for their survival during development (Reichardt and Farinas, 1997).

6.11.1 Superior cervical ganglion (SCG)

The SCG of GDNF mutant mice only display a limited reduction (20%) in volume (Sánchez et al., 1996). Consistent with this study, we found in this study that GDNF single mutant mice display about 21% reduction (E18.5) in the number of the neurons in this ganglia, while the SCG neurons of single TGF- β 2 mutant mice was normal (Figs.31). Surprisingly, *Tgfb2^{-/-}Gdnf^{-/-}* double mutant mice embryos display a severe defect in the total number of SCG neurons (-46%) at E16.5 as well as at E18.5 (Figs.31-32). Since the volume of E14.5 SCGs in the *Tgfb2^{-/-}Gdnf^{-/-}* double mutant mice embryos were comparable to the controls groups, this gives an indication that the sympathetic precursors in the *Tgfb2^{-/-}Gdnf^{-/-}* double mutant mice embryos migrate from the neural crest and proliferate to form the SCG ganglion (Fig. 33). The severe defect (reduction) in the total number of SCG neurons at E16.5 and E18.5 may support the notion of synergism between GDNF and TGF- β 2 to induce neuronal survival in the SCG neurons directly and/or indirectly and constitute a synergistic neurotrophic activity through helping the SCG neurons to reach their final targets innervation at later stages during axonal growth and synaptogenesis (Fig. 34, 40). These data are in line with the findings that the immunohistochemical labeling of tyrosine hydroxylase is reduced in the SCG cells in the GDNF mutant mice and also that the sympathetic innervation is decreased in blood vessels and glands of the nasal and oral mucosa (Granhölm et al., 1997). Superior cervical ganglion (SCG) expressed low levels of GDNF mRNA (Trupp et al., 1995). Schuchardt and coworkers (1994) showed that there is a complete loss of superior cervical ganglion



(SCG) neurons in mice lacking Ret (Schuchardt et al., 1994), whereas no changes was reported in the neuronal number in GFR α 1-deficient mice (Cacalano et al., 1998; Enomoto et al., 1998). This give an indication that GDNF may mediate signals through other GFR α family members. In the mouse, primary formation of the sympathetic ganglia depends on proper ventral migration of the sympathoadrenal lineage of the neural crest cells (Britsch et al., 1998). After migration, these neural crest cells coalesce adjacent to the dorsal aorta to form the primary sympathetic chain. Recently, Enomoto and coworkers (2001) demonstrated that newborn Ret mutant mice display complex deficits in the sympathetic ganglion that include impaired migration, reduced axonal projection and increased neuronal cell death. During migration and axonal projection of sympathetic precursors, ARTN, but not other GFLs, is expressed in various blood vessels. Moreover, ARTN stimulates neurite outgrowth from sympathetic ganglion explants and also has an ability to direct axonal growth. This data identifies RET and artemin as central regulators of early sympathetic innervation (Enomoto et al., 2001). In our study, the SCG phenotype in *Tgfb2*^{-/-}/*Gdnf*^{-/-} double mutant mice was seen at E16.5 and E18.5 (after migration), but the SCG volume in *Tgfb2*^{-/-}/*Gdnf*^{-/-} double mutant mice was comparable to the controls at E14.5. Therefore, the SCG defect in double mutant embryo at E16.5 and E18.5 may be explained by the inability of SCG neurons to reach their final targets and/or direct survival effects for TGF- β 2 and GDNF rather than migration defect.

6.11.2 Dorsal root ganglion (DRG)

Dorsal root ganglion (DRG) sensory neurons undergo naturally occurring cell death during development, and axotomy-induced cell death. L5-DRG neurons of the GDNF single mutant embryos display about 17 % reduction, while *Tgfb2*^{-/-}/*Gdnf*^{-/-} double mutant mice embryos display about 27% reduction (Fig. 30). On the contrary, L5-DRG neurons of the TGF- β 2 single mutant embryos developed normally (Fig. 30). These data are well

consistent with the other studies on the DRG of the single GDNF mutant mice (Moore et al., 1996). Dorsal root ganglion expressed low levels of GDNF mRNA during rat development (Trupp et al., 1995).

6.11.3 Nodose-Petrosal ganglia (NPG)

One of the most severely affected neuronal populations in GDNF knockout mice is the nodose-petrosal ganglion complex (NPG) of primary cranial sensory neurons, in which 40% of cells die by birth (Moore et al., 1996). In this study, single GDNF and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos display about 47% in the number of neurons in NPG (Fig. 29). This gives an indication that the phenotype in the double mutant mice is due to the loss of GDNF rather than TGF-β2. The absence of the phenotype at E14.5 (Fig. 27) may be explained by the survival effect of GDNF at later stages (E18.5). Some NPG targets, including the carotid body, have been reported to express GDNF mRNA (Nosrat et al., 1996), raising the possibility that survival of some NPG neurons is supported by target-derived factors as GDNF. Moreover, GDNF also promoted survival of about half of the neurons in embryonic chick nodose ganglion and a small population of embryonic sensory neurons in chick dorsal root and rat trigeminal ganglia (Trupp et al., 1995). Okano et al. (2005) found that TGF-β2 promotes the formation of the mouse cochleovestibular ganglion in organ culture. Furthermore, they showed, by in situ hybridization, that TGF-β2, TGF-β type I and type II were expressed in the otic epithelium at the OV stages between embryonic days (E) 9.5 and 11.5. This pattern of expression may suggest a role for TGF-β2 in the development of CVG (Okano et al., 2005).

Finally, GDNF seems to have more survival effects on the peripheral ganglia than TGF-β2. On the other hand, GDNF and TGF-β2 may synergize and/or cooperate to induce neuronal survival during SCG development. Thus, future studies using conditional gene knock-outs

for the TGF- β receptors will hopefully help further understanding the fundamental significance of TGF- β in the signaling of GDNF and other neurotrophic factors.

6.12 Motor neurons

Motoneurons are irreplaceable cells, that in humans can survive for about 100 years. Their death causes disability, leading to paralysis and death. One of the criteria for a motoneuron survival factor is that it should be present in the appropriate place and at suitable time (Oppenheim RW, 1996). TGF- β 2 and GDNF satisfy this criterion, with apparent redundancy. In this study, spinal motoneurons of single *Gdnf*^{-/-}, single TGF- β 2 and *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos were analyzed at E18.5. *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant embryos showed 31% reduction (Fig. 34) in the total number of the lumbar motoneurons (L1-L5). On the other hand, as reported in this and other studies from GDNF-deficient mice, only about 18% of the spinal motoneurons are lost in the absence of GDNF (Moore et al., 1996). The additional reduction in the double knockout mice may be explained by cooperation and/or synergism between TGF- β 2 and GDNF in the development of the motoneurons. This synergism and cooperation may be supported by the finding that single TGF- β 2 mutant mice embryos failed to have a phenotype in the lumbar motoneurons. These data are well consistent with the expression patterns of both TGF- β 2 and GDNF in the spinal cord as well as with many other studies supporting the survival effects of these two factors *in vitro*. Studies by Zurn et al., (1996) suggest that GDNF, because of its synergistic effects with BDNF and CNTF, might be profitably combined with other trophic factors to prevent losses of motoneurons. By using in situ hybridization, GDNF was detected in the dorsal horn of spinal cord around birth (Nosrat et al., 1996). Motoneurons purified from E14 rat spinal cord expressed mRNA signals for TGF- β 2, TGF- β 3 and T β R-II along with the GDNF receptors GFR α -1 and c-Ret (Krieglstein et al., 1998b). The survival of developing motoneurons depends on muscle- and CNS-derived

trophic factors (Oppenheim, 1989; Henderson et al., 1993). Henderson and co-workers (1994) were the first to find that GDNF supported the survival of purified embryonic rat motoneurons in culture, also, GDNF rescues and prevents the atrophy of facial motoneurons that have been deprived of target-derived survival factors by axotomy in newborn rats (Henderson et al., 1994). Moreover, GDNF could also rescue developing avian motoneurons from naturally occurring cell death *in vivo* (Oppenheim et al., 1995; Houenou et al., 1996). Together with its synthesis by striated muscles, Schwann cells and cells at the tips of motor axons (Henderson et al., 1994; Wright and Spnider, 1996), GDNF may have survival and physiological effects during the development of motoneurons. The GFR α -1 mutant mice exhibit small losses of lumbar spinal and trigeminal nucleus motor neurons, while the facial motor neurons developed normally (Cacalano et al., 1998). TGF- β s prevent the death of isolated immature motoneurons *in vitro*, in cooperation with glial cell line-derived neurotrophic factor (Krieglstein et al., 1998b). Using E14 rat embryo motoneurons, subnanomolar concentrations of TGF- β 1 (40-500 pM) promoted survival effect on motoneurons after 9-11 days in culture (Martinou et al., 1990). Moreover, TGF- β 2, GDNF, and BDNF were shown to have survival effect on lesioned adult rat motoneurons (Sakamoto et al., 2003). Motoneurons were shown to synthesize the three proteins involved in transforming growth factor-beta 2 signalling (types I, II TGF- β receptor and betaglycan) and to transport them anterogradely (Jiang et al., 2000a). Transforming growth factor-beta 2 was detected in motoneurons and injured nerves, indicating that motor neurons may be exposed to several sources of TGF- β 2 (Jiang et al., 2000a). Adding of low level of TGF- β 2 (6 ng) near to the avulsed motor nucleus caused a significant attenuation of this death (Jiang et al., 2000b). This dose of TGF- β 2 is low compared to that used with GDNF or BDNF in previous studies of avulsed motoneurons (Li et al., 1995; Novikova et al., 1997). TGF-beta rescues target-deprived preganglionic

sympathetic neurons in the spinal cord of adult rat adrenal medulla *in vivo* (Blottner et al., 1996). Finally, GDNF and TGF- β 2 may be useful in the treatment of motoneurons disorders like amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy. The therapeutic usefulness may be restricted to cotreatment with other factor(s) to achieve more neurotrophic activity.

6.13 Phenotypes outside the nervous system

6.13.1 Ventral body wall and muscle development

E14.5 $Tgfb\beta 2^{-/-}Gdnf^{+/+}$ and $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ mutant mice embryos demonstrated that the thickness of the ventral body wall at abdominal (Fig. 35) and urinary bladder (Fig. 36) levels was notably reduced compared to the wild-type situation. On the other hand, the ventral body wall muscles of wild-type embryos continue close to the hernia, while the body wall muscles of $Tgfb\beta 2^{-/-}Gdnf^{+/+}$ and $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ mutants do not reach the midline. This gives an indication that the phenotypes in the ventral body wall is because of lack of TGF- β 2 rather than GDNF. At early developmental stages, alpha-smooth muscle actin does not stain specifically for smooth muscles at embryonic stages, but also considered to be a striated muscle cell feto-embryonic actin marker. It is transiently expressed in cardiomyoblasts and skeletal myoblasts and reappearing during neoplastic transformation of skeletal muscle in rat and mouse embryos (Babai et al., 1990). Recently, Li et al., (2006) demonstrated that TGF- β may have an important role in chicken skeletal muscle formation through modulation of the expression of both extracellular matrix proteins and cellular receptors important in the control of cell migration and growth regulation (Li et al., 2006).

6.10.2 Extracellular matrix formation (ECM)

Layering of the connective tissues were severely disturbed in $Tgfb\beta 2^{-/-}Gdnf^{-/-}$ double knockouts which display disorganized connective tissue and disturbed collagen fiber bundles (Fig. 37). Coordinate regulation of production of extracellular matrix components

(ECM) is important for tissue homeostasis. TGF- β s are known to have effects on matrix deposition by increasing the expression of certain proteins such as fibronectin and collagens, upregulating inhibitors of matrix proteases and suppressing the synthesis of proteases, which degrade matrix components (Grotendorst 1997, Hocevar and Howe 2000). Therefore, the phenotype that have been seen in *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutants may be explained in terms of lack of ECM molecules, such as fibronectin, which in turn results in disturbed appearance of fibers normally arranged in parallel bundles. Because these areas of decreased attachment include many ECM-free spaces, the connective tissues appear much thicker.

6.10.3 Molar tooth development

During tooth development, molecules in several conserved signal families mediate interactions both between and within the epithelial and mesenchymal tissue layers. The same molecules are used during development repeatedly, and several growth factors are expressed in epithelial signaling centers (reviewed by Thesleff and Mikkola, 2002). *Tgfb2*^{-/-}*Gdnf*^{-/-} double mutant mice embryos showed an acceleration in the molar tooth development compared to the wild-type embryos (Fig. 38). The *Tgfb2*^{-/-}*Gdnf*^{+/-} and *Tgfb2*^{+/-}*Gdnf*^{-/-} molar teeth at E14.5 were identical to the controls group (*Tgfb2*^{+/+}*Gdnf*^{+/+}). This give an indication for a synergism between TGF- β 2 and GDNF in molar tooth development at specific stages of development, the effects of these two factors were inhibitory. This data are well consistent with the expression patterns of GDNF and TGF- β during teeth development. Nosrat and coworkers (1996) detected the GDNF mRNA in mice teeth with high peak of expression around birth (E17-P3). TGF- β 1 expression was shown to shift between epithelium and mesenchyme and to be associated with known inductive tissue interactions. During budding, TGF- β 1 is expressed in epithelium, then shifts to the condensing mesenchyme; and during cap stage it is strongly expressed in the

cervical loop epithelium (Vaahtokari et al., 1991). The three mammalian TGF- β isoforms; TGF- β 1, -2, and -3 are intensely expressed during odontoblast and ameloblast differentiation (Pelton et al., 1990; Vaahtokari et al., 1991).

7. Referances

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8. Appendix

8.1 List of Figures

Figure 1: Scheme showing the downstream signalling of GDNF (left) and TGF- β (right).

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8.3 Curriculum Vitae

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- Roussa, E, Oehlke, B, **Rahhal, B**, Wiehle, M, and Krieglstein, K (2006). Differential effects of the TGF- β superfamily members on dopaminergic phenotype induction. (Submitted to *J. Neurobiology of disease*)
- **Rahhal B**, Heermann S, Roussa E, Arumä U, Sariola H, Saarma M, Krieglstein K (2007). TGF-b and GDNF synergistically regulate development of the sympathetic nervous system *in vivo*. (In Preparation)

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