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Laminar Organization of Cerebral Cortex in Transforming Growth Factor Beta Mutant Mice

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Abstract

Transforming growth factor betas (TGFβs) are one of the most widespread and versatile cytokines. The three mammalian TGFβ isoforms, β1, β2, and β3, and their receptors regulate proliferation of neuronal precursors as well as survival and differentiation in neurons of developing and adult nervous system. Functions of TGFβs has a wide spectrum ranging from regulating cell proliferation and differentiation, production of extracellular matrix components, chemotaxis, and immunosuppression, to the regulation of cell death. While there is a complete lack or low levels of TGFβ1 in the unlesioned nervous system, a widespread expression of TGF β2 and TGF β3 can be seen in different areas of the CNS and PNS. Consistent co-expression of TGFβ2 and β3 in neurons, astroglial, and Schwann cells is indicative of their multiple effects on neurons and glial cells. Based on overlapping expression of TGF β2 and TGF β3 and therefore their functions, our study was designed to differentiate between the functions of the two isoforms during development. Because mice lacking both isoforms die around embryonic day 15 (E15), we decided to use single homozygous and double heterozygote null mutant embryos who survive and lack any abnormal nervous system phenotype after birth. The expression pattern of markers for extracellular matrix protein such as reelin and chondroitin sulfate proteoglycan (CSPG) as well as those for cytoskeletal proteins such as microtubule associated protein (MAP), a marker for differentiated neurons, was studied in E14.5 and E16.5 mice embryos both by immunohistochemistry and *in situ* hybridization. Despite a reduced expression of CSPG and MAP proteins that was seen only in TGF beta 3 null mutants, $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$, no abnormal localization of these markers and no change in the expression at mRNA level was noticed. The normal localization of the markers as well as their unchanged mRNA expression suggest that TGF β2 compensates for the lack of TGF β3 and therefore the two isoforms act in parallel to ensure the stability of brain cytoarchitecture during development.

Keywords: Transforming growth factor beta (TGF*β*); Mutant; Cerebral cortex; Embryo

Introduction

Neurotrophic factors, including the family of transforming growth factors β, regulate neuronal growth and differentiation [1]. Like other neurotrophic factors, TGF β is known to trigger both neuronal survival as well as apoptosis. Using antibodies against TGF β in E6 chicks, both CNS and peripheral neurons are rescued from natural occurring cell death to a great extent [2]. Conversely, TGF β has also been suggested as a potent modulator of neurotrophin-mediated survival, both *in vivo* and *in vitro* (*e.g.* a cofactor for neurotrophic actions

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of GDNF; [3]), while there is no evidence for independent function of TGF β on survival. Moreover, TGF β is known to regulate differentiation i.e., regulation of neurite growth, transmitter synthesis and synapse formation [4]. TGF β is also known for orchestrating the axis formation [5] possibly by positioning and concentrating growth factors in the extracellular matrix [6] which is also of a great importance in cell migration and adhesion [7]. Cell positioning and laminar organization of developing brain might therefore be regulated by TGF β indirectly.

The laminar organization of mouse cerebral cortex begins with the appearance of the preplate, located above the ventricular zone, from embryonic day E10-12. When the cortical plate neurons exit the cell cycle near the ventricular surface, they migrate radially and split the preplate into marginal zone and subplate at E16.5 [8,9].

To investigate laminar organization of brain, the expression pattern of different related markers was studied. Markers included those of extracellular matrix such as reelin and chondroitin sulfate proteoglycan (CSPG) as well as those of cytoskeletal proteins such as microtubule associated protein (MAP) considered to be a marker for differentiated neurons. These proteins develop early in development, prior to the appearance of histological abnormalities, and therefore could be used as valuable traits [10]. Reelin protein that functions similar to the components of the extracellular matrix proteins is expressed early in development in marginal zone of the cortex, cerebellum and hippocampus [10, 11]. CSPG is associated with the preplate and its derivatives, subplate and marginal zone [12]. MAP is expressed in cortical plate and subplate, located beneath the cortical plate [10].

To elucidate whether cell migration and laminar organization of the developing brain is controlled by TGF β, we have therefore used TGF β knockout mice to study the expression pattern of different markers such as reelin, MAP and CSPG both by immunohistochemistry and *in situ* hybridization. Due to wide and overlapping expression of the two TGF β isoforms, null mutation of either of these isoforms alone may not produce any nervous system phenotype. Also due to lethality of double homozygous mutant TGF β mice ($Tgf\beta2^{-/-}Tgf\beta3^{-/-}$, [13]), single homozygote and double heterozygote null mutant mice for TGF β2 and TGF β3 with different genotypes were investigated.

Our results from immunohistochemical studies in E16 mutants show that although there is no defect in localization of extracellular matrix and cytoskeletal markers in general, there is a reduced cortex level of MAP and CSPG only in *Tgfβ3* mutants. Reduced but not eliminated or dislocated expression in TGF $\beta3^{-/-}$ null mutant suggest that TGF β2 compensates for the lack of TGF β3 to regulate extracellular and cytoskeletal proteins required for cell positioning of the cerebral cortex.

Materials and Methods

Mice and Tissue Preparation

Male and female mice (obtained from breeding colonies generated in University of Heidelberg, Germany) were considered for mating. Mutant mice were obtained by mating heterozygous with genotypes of $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$ males to heterozygous or homozygous females. For morphological comparison in each case $Tgf\beta2^{+/+}Tgf\beta3^{+/+}$ littermates of the respective mutants were used as wild-type controls. Females were examined each morning and if a plug was observed, the stage of development was designated as embryonic day 0.5 (E0.5). Embryos were taken at E14.5, E16.5 and E18.5 with a piece of tail cut for genotyping and the rest fixed in Bouin's fixative (Perfusion was performed for E16.5 and E18.5 embryos). Following fixation, the embryos were paraffin embedded and sectioned by using microtome (Leica) both horizontally and sagitally. For genotyping analysis, tail DNA was extracted by using mini quick spin columns (Roch), amplified by PCR, using specific sets of primers of *Tgfβ2* and *Tgfβ3* and gel electrophoresed. The results of genotyping showed that different genotypes were available at different stages: The available genotypes were $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$ and $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$ at E14.5 and $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$, $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$, $Tgf\beta2^{-/-}Tgf\beta3^{+/+}$, $Tgf\beta2^{+/-}Tgf\beta3^{-/-}$ at E16.5 and $Tgf\beta3^{+/+}Tgf\beta3^{-/-}$ at E18.5. For each genotypes n=5 embryos were considered for immunohistochemical and in situ hybridization studies.

Immunohistochemistry

Using the antigen unmasking procedure, immuno-histochemistry for reelin, MAP and CSPG were performed. Briefly, following dewaxing in xylene and series of isopropanol, slides were microwave heated in a sodium citrate buffer (PH 6) for 5 min. They were then incubated in 3% hydrogen peroxide (Merck, 10 min), followed by two washes in PBS. Using avidin-biotin blocking kit (Vector Laboratories), nonspecific binding to avidin and biotin was blocked. The slides were then incubated with mouse IgG blocking agent (Vector laboratories, 1 h), followed by incubating with primary antibodies (1:1000 for reelin; a gift from Faissner, A.., 1:500 for MAP and 1:200 for CSPG; overnight at 4°C).

After several washes with PBST, the slides were then incubated with secondary biotinylated anti-mouse IgG (1:250, 1 h at RT), followed by incubating in horseradish peroxidase kit (Vector laboratories, 1 h at RT) and developing in DAB (diaminobenzidine tetrahydrochloride). After extra washes with PBS and distilled H2O, slides were air dried, dehydrated using series of alcohol, cleared by xylene and mounted by DEPEX.

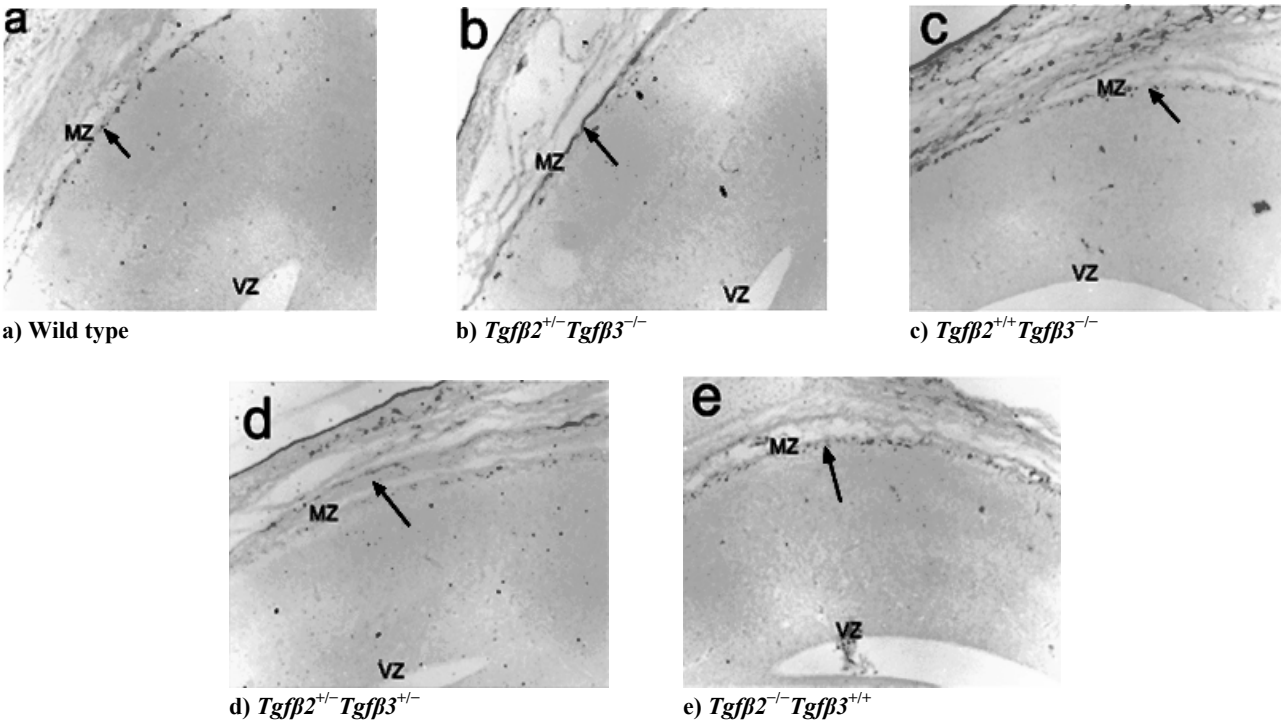
In situ *Hybridization*

A non radioactive *in situ* hybridization was performed using a DIG labeled MAP cDNA probe (designed in Heidelberg University and tested for its specificity). Briefly, cryostat frozen sections were cut, mounted on coated slides and reached to the room temperature before use. Incubating with a cDNA probe, the slides were coverslipped in a humid chamber for overnight. The next day, slides were washed in a wash buffer at 68C (containing 50% formamide, 620 mM NaCl, 10 mM EDTA, 20 mM PIPES-Na, 0.2% SDS, 50 mM DTT, 250 µg/ml denatured salmon sperm DNA and yeast tRNA), followed by washes in MABT buffer (maleic acid and NaCl) at room temperature. Slides were then incubated with anti-DIG antibody for overnight. After 24 h, the slides were washed in MABT buffer and developed by NBT/BCIP in dark.

Results

Immunostaining of Reelin, MAP and CSPG in Brain Cortex at E14.5, E16.5 and E18.5

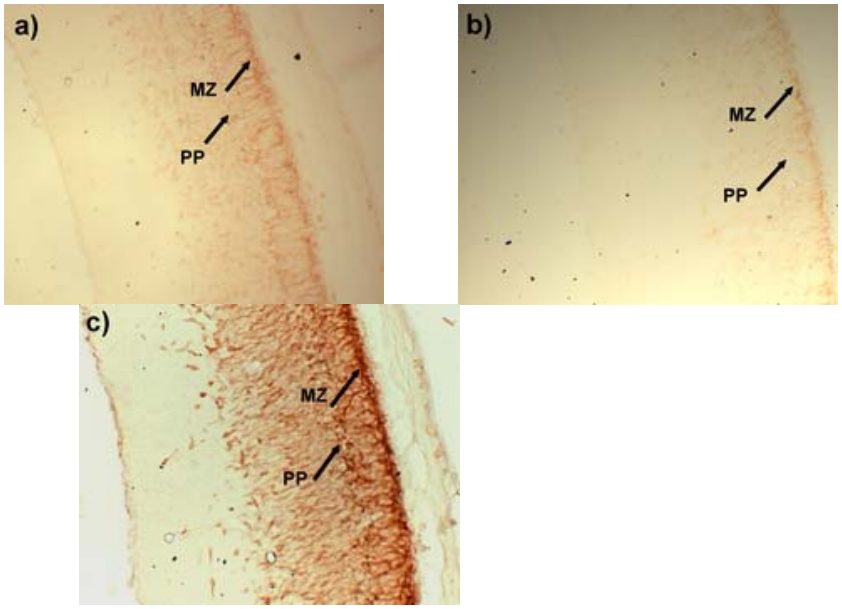
Starting from E14.5, the expression level of the neuronal markers associated with cortical layers, such as reelin, MAP and CSPG were studied and the architecture of the cortical layers was therefore investigated. Using anti-reelin antibodies in E14.5 mutants, we found that reelin is not detectable at this stage. Our results in either in mutants or the wild type E16.5 embryos show that even at this stage there is not any strong staining of the reelin positive neurons in marginal zone, (Fig. 1a, b, c, d, and e). No significant difference can therefore be distinguished between the wild type (Fig. 1a) and the mutants (Fig. 1b; $Tgf\beta2^{+/-}Tgf\beta3^{-/-}$), (Fig 1c; $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$) (Fig. 1d; $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$), (Fig. 1e; $Tgf\beta2^{-/-}Tgf\beta3^{+/+}$).



a) Wild type **b)** $Tgf\beta2^{+/-}Tgf\beta3^{-/-}$ **c)** $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$

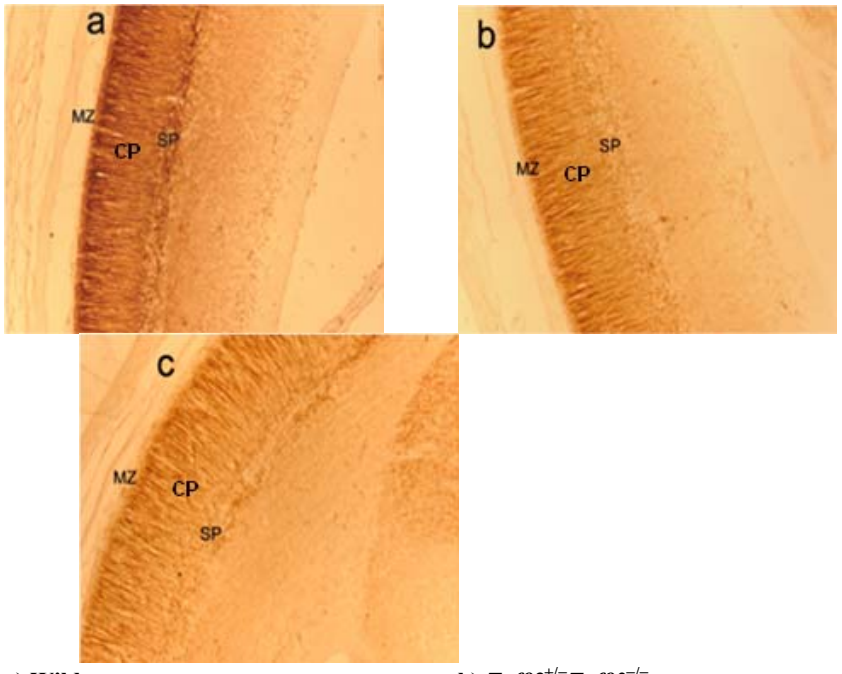
d) $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$ **e)** $Tgf\beta2^{-/-}Tgf\beta3^{+/+}$

Figure 1. Photomicrographs of reelin immunostaining in marginal zones of brain cortex in E16.5 mutants and the wild type. There is a general weak reelin immunostaining at this stage and therefore no visible difference could be detected in the intensity of reelin in marginal zones of brain cortex in different groups. Wild type (a), $Tgf\beta2^{+/-}Tgf\beta3^{-/-}$ (b), $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$ (c), $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$ (d), $Tgf\beta2^{-/-}Tgf\beta3^{+/+}$ (e).

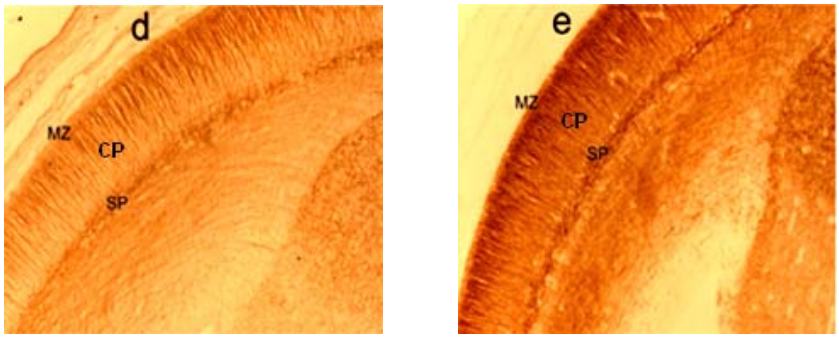


a) ***Tgfβ2*$^{+/-}$*Tgfβ3*$^{+/-}$ **b)** ***Tgfβ2*$^{+/+}$*Tgfβ3*$^{-/-}$ **c) Wild type**

Figure 2. Photomicrographs of MAP immunostaining in marginal zone and preplate region of brain cortex in E14.5 mutants and the wild type. Marginal zone and preplate region of homo- and hetero-zygote null mutants for TGF β3 (a: *Tgfβ2*$^{+/-}$*Tgfβ3*$^{+/-}$; b: *Tgfβ2*$^{+/+}$*Tgfβ3*$^{-/-}$) show reduced intensities of MAP immunostaining compared to these regions in the wild type (c).



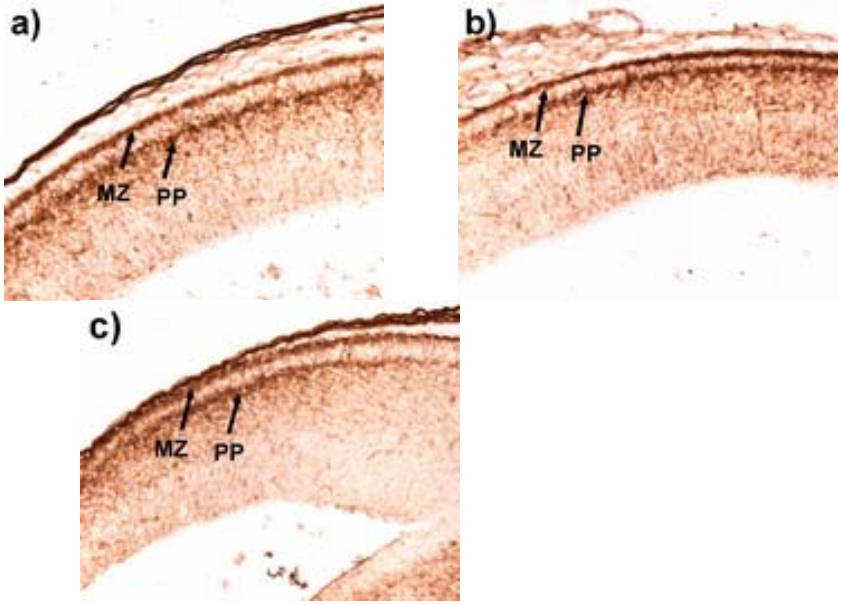
a) Wild type **b)** ***Tgfβ2*$^{+/-}$*Tgfβ3*$^{-/-}$ **c)** ***Tgfβ2*$^{+/+}$*Tgfβ3*$^{-/-}$



d) $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$ **e)** $Tgf\beta2^{-/-}Tgf\beta3^{+/+}$

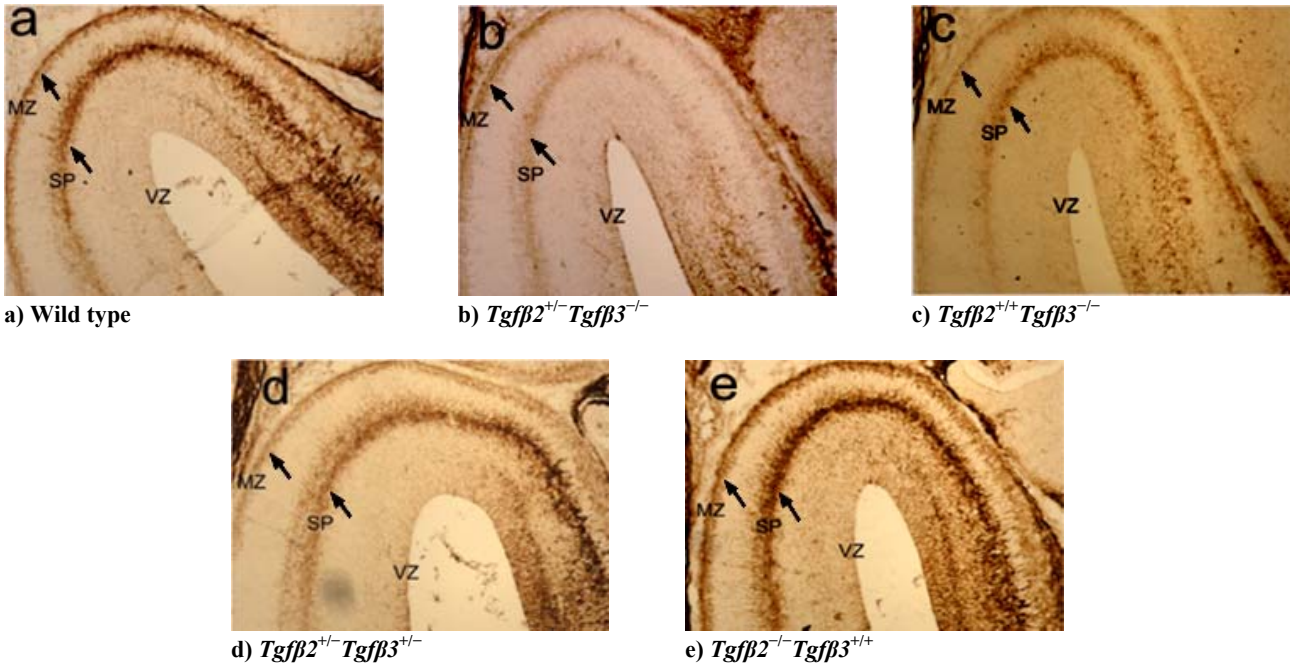
Figure 3. Photomicrographs of MAP immunostaining in cortical plate and subplate region of brain cortex in E16.5 mutants and the wild type. In homo- and hetero-zygote null mutants for TGF β3 (b: $Tgf\beta2^{+/-}Tgf\beta3^{-/-}$, c: $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$, d: $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$), there are reduced intensities of MAP immunostaining in cortical plate and subplate regions compared to these regions in the wild type (a). Cortical plate and subplate regions in homozygote TGF β2 mutant ($Tgf\beta2^{-/-}$) mice (e) had similar intensities of MAP immunostaining compared to those in the wild type (a).

The results of MAP immunostaining in cortical plate and subplate regions of E14.5 embryos (Fig. 2) show that in mutants with a genotype of either $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$ (Fig. 2a) or $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$ (Fig. 2b), neurons and their processes stain significantly weaker than those in the wild type mice (Fig. 2c). Consistently, the results of MAP staining in cortical plate and subplate regions of E16.5 embryos show that homo- and hetero-zygote null mutants for TGF β3 (Fig. 3b; $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$, Fig. 3c; $Tgf\beta2^{+/-}Tgf\beta3^{-/-}$, Fig 3d; $Tgf\beta3^{+/-}Tgf\beta3^{+/-}$), have weakly been stained in comparison with the wild type (Fig. 3a). In contrast the



a) $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$ **b)** $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$ **c) Wild type**

Figure 4. Photomicrographs of CSPG immunostaining in marginal zone and preplate region of brain cortex in E14.5 mutants and the wild type. There is no visible difference in intensities of CSPG immunostaining in marginal zone and preplate regions of homo- and hetero-zygote null mutants for TGF β3 (a: $Tgf\beta2^{+/-}Tgf\beta3^{+/-}$; b: $Tgf\beta2^{+/+}Tgf\beta3^{-/-}$) compared to these regions in the wild type (c).



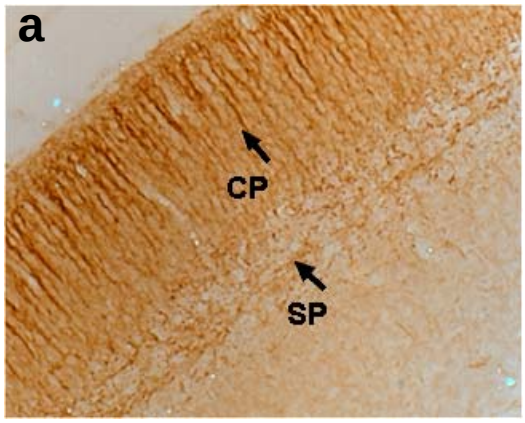
a) Wild type **b) *Tgfβ2*$^{+/-}$*Tgfβ3*$^{-/-}$** **c) *Tgfβ2*$^{+/+}$*Tgfβ3*$^{-/-}$**

d) *Tgfβ2*$^{+/-}$*Tgfβ3*$^{+/-}$ **e) *Tgfβ2*$^{-/-}$*Tgfβ3*$^{+/+}$**

Figure 5. Photomicrographs of CSPG immunostaining in marginal zone and subplate region of brain cortex in E16.5 mutants and the wild type. In homo- and hetero-zygote null mutants for TGF β3 (b: *Tgfβ2*$^{+/-}$*Tgfβ3*$^{-/-}$, c: *Tgfβ2*$^{+/+}$*Tgfβ3*$^{-/-}$, d: *Tgfβ2*$^{+/-}$*Tgfβ3*$^{+/-}$), there are reduced intensities of CSPG immunostaining in marginal zone and subplate region compared to these regions in the wild type (a). Marginal zone and subplate region in homozygote TGF β2 mutant (*Tgfβ2*$^{-/-}$) mice (e) had similar staining intensities compared to those in the wild type.

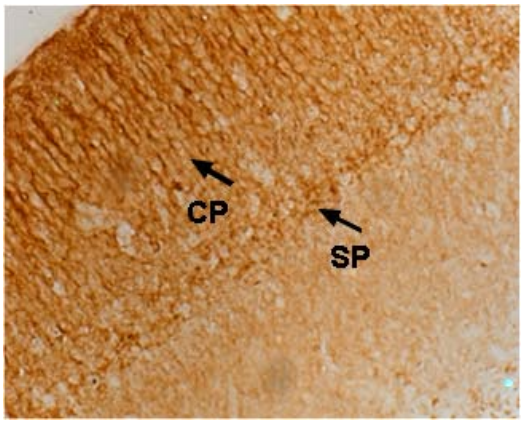
staining intensity in cortical plate and subplate regions of embryos with a genotype of *Tgfβ2*$^{-/-}$*Tgfβ3*$^{+/+}$ (Fig. 3e) was similar to that in the wild type.

Using an anti-CSPG, a strong immunostaining of CSPG can be seen in marginal zone and preplate region at E14.5 (Fig. 4) and in marginal zone and subplate region at E16.5 (Fig. 5) both in mutants and the wild type. While there is no difference in the staining intensity of CSPG in marginal zone and preplate region of the E14.5 wild type and the mutants, there is a reduced staining intensity in marginal zone and subplate region in homo- and hetero-zygote null mutants for TGF β3 E16.5 mutants compared to those in the wild type (Fig. 5b; *Tgfβ2*$^{+/+}$*Tgfβ3*$^{-/-}$, Fig. 5c; *Tgfβ2*$^{+/-}$ *Tgfβ3*$^{-/-}$, Fig. 5d; *Tgfβ2*$^{+/-}$*Tgfβ3*$^{+/-}$). Marginal zone and subplate region in homo- and hetero-zygote null mutants for TGF β2 (Fig. 5e) had similar staining intensities compared to those in the wild type.



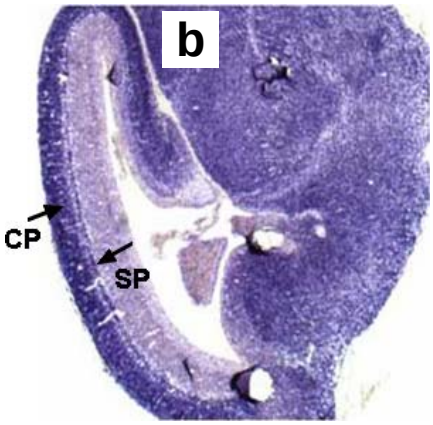
a) *Tgfβ2*$^{+/+}$*Tgfβ3*$^{-/-}$

b



b) Wild type

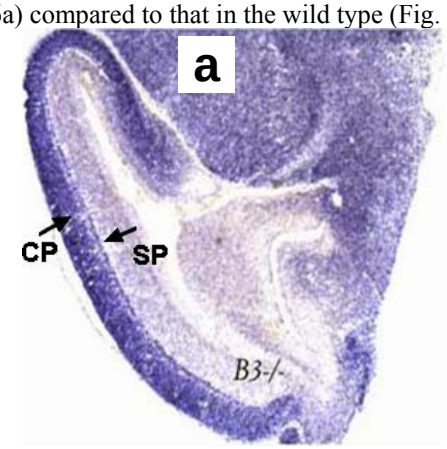
Figure 6. Photomicrographs of MAP immunostaining in cortical plate and subplate region of brain cortex in E18.5 TGF $\beta3^{-/-}$ mutant and the wild type. There is a minor reduced intensity of MAP immunostaining in cortical plate and subplate regions of E18.5 TGF $\beta3^{-/-}$ mice (a) compared to that in the wild type (b). Also there are some minor disturbances in cytoarchitecture of subplate region (arrow headed) in TGF $\beta3^{-/-}$ brain cortex (a) compared to that in the wild type (b).



b) Wild type

Figure 7. Photomicrographs of MAP mRNA level in marginal zone and subplate region of brain cortex in E16.5 TGF $\beta3^{-/-}$ mutant and the wild type. There is no significantly reduced level of MAP mRNA in cortical plate and subplate regions of E16.5 TGF $\beta3^{-/-}$ mutant (a) compared to those in the wild type (b).

Altogether the results of immunostaining at E16.5 show that cortex in homo- and hetero-zygote null mutants for TGF β3 should be carefully looked at to confirm the observed changes. The first approach was therefore looking at the cortex in E18.5 homozygote null mutants for TGF β3 to see if the intensity of MAP is also defected at this stage. As shown in Figure 6, there is a minor reduction of the MAP intensity in cortical plate and subplate region in E18.5 TGF $\beta3^{-/-}$ mice (Fig. 6a) compared to that in the wild type (Fig. 6b). Also there are some minor disturbances in cyto-architecture of subplate region in TGF $\beta3^{-/-}$ brain cortex (Fig. 6a) compared to that in the wild type (Fig. 6b).



a) ***Tgfβ2*** $^{+/+}$ ***Tgfβ3*** $^{-/-}$

In situ *Hybridization for MAP mRNA*

Our second approach was to investigate if the reduction at protein level also exists at mRNA level. Using a MAP cDNA probe, we performed *in situ* hybridization at E16.5. As shown in Figure 7, there is no significant reduction of MAP mRNA level in cortical plate and subplate region in E16.5 TGF β $3^{-/-}$ mutant (Fig. 7a) compared to that in wild type (Fig. 7b). Therefore despite significant reduction at protein level in cortical plate and subplate region in homo- and hetero-zygote null mutants for TGF β3, no changes at mRNA level can be detected in these areas.

Discussion

TGF β is known for positioning and concentrating growth factors in the extracellular matrix [6], which is of a great importance in cell adhesion and migration [7]. Also Unsicker and colleagues [14] showed that TGF βs induce neurite outgrowth in chick DRGs, therefore are potent regulators of neuron differentiation. A study by Flanders and colleagues [15] on distribution of TGFβ2 and β3 immunoreactivities in mouse embryo revealed that peripheral nerves, radial glial cells, and CNS axon tracts express TGFβ2 and β3 as early as embryonic day 12.5 (E12.5) to E13. Nerve cell bodies however are still devoid of immunoreactivity at this stage, suggesting that

cells accompanying axon bundles are the sites of TGFβ storage. TGFβ2/β3 does not become detectable in brain, spinal cord, and peripheral ganglionic nerve cell bodies in mouse embryos until E15. Expression of TGF β2 and β3 on radial glial cells and axons early in development [1] as well as their regulation of extracellular matrix [6,16] and cytoskeletal proteins provide more evidence on the role of these two isoforms in cell adhesion, migration and differentiation. Due to overlapping expression of TGF β2 and β3 throughout CNS, studying mutants of either isoforms would be of a great advantage for classification of their functions.

Localization of extracellular matrix proteins, CSPG and reelin as well as cytoskeletal protein, MAP, is not impaired in developing brains of TGF β 2/3 heterozygote mutants

Our results from null mutants for either isoforms showed that there was no apparent abnormality in localization of CSPG, reelin and MAP in developing brains of the mutants at E14.5 and E16.5. In other words, CSPG, reelin and MAP were still present in cortical plate and marginal zones with no dislocation or deletion. Despite lack of impairment in cortex layering of our mutants, reduced immunostaining intensity for CSPG and MAP was seen in the cortex of E16.5 TGF β3 but not in that of TGF β2 mutants, indicating that TGF β3 contributes more than TGF β2 to regulate cell migration and differentiation. The reduction but not elimination of the expression however points at compensatory function of TGF β2 isoforms, suggesting that both isoforms control cell migration and differentiation. The role of both TGF β2 and β3 in regulating the expression of cell adhesion molecules has also been addressed elsewhere [16]. While compensation of the two isoforms secures the expression of extracellular matrix proteins and therefore cytoarchitecture of the cortex, there are evidence elsewhere indicating that cortex development is severely impaired if reelin itself is knocked out (*e.g.* reeler mice; ref. 10).

In contrast to the immunohistochemical findings which is indicative of the reduced level of MAP protein in TGF β3 null mutants, our results of *in situ* hybridization does not show any changes in the level of MAP mRNA in the cortex of these mutants in comparison with that of the wild type. The discrepancy between the results of *in situ* and immunohistochemistry has also been reported elsewhere. For example Zendman and colleagues [17] have shown that using anti-tumor marker provides lower and varying percentages of positivity compared with mRNA-based frequencies, suggesting that mRNA-based studies do not always represent protein expression.

Altogether, our results in this study show that TGF β2 and TGF β3 act in parallel to secure the development of cytoarchitecture of the brain. A better understanding of TGF β function during brain development will be accomplished from future studies on mice deficient for TGF β receptor.

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