Cell Transplantation

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Journal:	*Cell Transplantation*
Manuscript ID	CT-1913.R2
Manuscript Type:	Special Issue: ASNTR
Date Submitted by the Author:	n/a

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Keywords:	Parkinson’s disease, Cell transplantation, Ventral mesencephalon, Neurotrophic Factors, Dopamine

Abstract:	Idiopathic Parkinson’s disease is a progressive neurodegenerative disorder clinically manifested by cardinal motor symptoms including tremor at rest, bradykinesia and muscle rigidity. Transplantation of dopaminergic neurons is an experimental therapy for Parkinson’s disease, however, limited by suboptimal integration and low survival of grafts. Pretreatment of donor tissue may offer a strategy to improve properties of transplanted dopaminergic neurons and thereby clinical outcome. We have previously shown that a combination of neurotrophin-4/5 (NT-4/5) and glial cell line-derived neurotrophic factor (GDNF) demonstrated additive effects on rat ventral mesencephalic tissue. The present study investigated the effects of NT-4/5 and GDNF as single factors or in combination on dopaminergic neurons in organotypic explant cultures of fetal human ventral mesencephalon. For that purpose free-floating roller-tube cultures were prepared from ventral mesencephali and the equally sized pieces grown for one week in presence or absence of neurotrophic factors. Both neurotrophic factors increased dopamine content in the culture medium and number of tyrosine hydroxylase immunoreactive (ir) neurons, most prominently after combined GDNF+NT-4/5 treatment. Culture volumes did

	not differ between groups, while content of lactate dehydrogenase in the culture medium was moderately reduced in all treated groups. In conclusion, we identified that a combination of GDNF and NT-4/5 robustly promoted differentiation and survival of human fetal ventral mesencephalic dopaminergic neurons, an observation with potential promising impact for cell replacement approaches in Parkinson's disease.

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Running title: GDNF and NT-4/5 promote human nigral neurons

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Abstract

Idiopathic Parkinson's disease is a progressive neurodegenerative disorder clinically manifested by cardinal motor symptoms including tremor at rest, bradykinesia and muscle rigidity. Transplantation of dopaminergic neurons is an experimental therapy for Parkinson's disease, however, limited by suboptimal integration and low survival of grafts. Pretreatment of donor tissue may offer a strategy to improve properties of transplanted dopaminergic neurons and thereby clinical outcome. We have previously shown that a combination of neurotrophin-4/5 (NT-4/5) and glial cell line-derived neurotrophic factor (GDNF) demonstrated additive effects on rat ventral mesencephalic tissue. The present study investigated the effects of NT-4/5 and GDNF as single factors or in combination on dopaminergic neurons in organotypic explant cultures of fetal human ventral mesencephalon. For that purpose free-floating roller-tube cultures were prepared from ventral mesencephali and the equally sized pieces grown for one week in presence or absence of neurotrophic factors. Both neurotrophic factors increased dopamine content in the culture medium and number of tyrosine hydroxylase immunoreactive (ir) neurons, most prominently after combined GDNF+NT-4/5 treatment. Culture volumes did not differ between groups, while content of lactate dehydrogenase in the culture medium was moderately reduced in all treated groups. In conclusion, we identified that a combination of GDNF and NT-4/5 robustly promoted differentiation and survival of human fetal ventral mesencephalic dopaminergic neurons, an observation with potential promising impact for cell replacement approaches in Parkinson's disease.

Key Words: Parkinson's disease, Cell transplantation, Ventral mesencephalon, Neurotrophic factors, Dopamine

Introduction

Progressive loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta is critically implicated for the motor symptoms observed in patients suffering from Parkinson's disease (PD). While drug therapies provide an effective means to substitute for the missing intrastriatal dopamine long-term treatment is often going along with severe side-effects. In this context, cell replacement strategies in PD have emerged as an experimental therapeutic concept. Thereby, the transplanted cells serve as a biological pump releasing dopamine. Human fetal nigral tissue can be transplanted safely and it has been shown that grafts survived and re-innervated the host striatum of PD patients[1-4]. This approach, however, is limited by suboptimal survival and differentiation of grafted DAergic neurons[1].

Hence, alternative sources of transplantable DAergic neurons have been investigated including induced pluripotent stem cells (iPSC) and embryonic stem cells (ESC) but also their capacity to re-innervate the diseased striatum in rodents was found to be critical[5]. A number of neurotrophic factors have been described to increase the survival and growth of DAergic neurons including the glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-4/5 (NT-4/5)[6]. GDNF belongs to the transforming growth factor-β superfamily and mediates its cellular responses by a multicomponent receptor complex composed of the RET and GDNF family receptor α (GFR-α). In cultures of fetal midbrain, GDNF promotes survival and differentiation of DAergic neurons and increase high-affinity dopamine uptake[7,8]. NT-4/5 is a member of the neurotrophin family. Upon TrkB receptor binding NT-4/5 initiates intracellular signaling cascades including the Ras-PI3K-Akt and the phospholipase-C gamma 1 pathways[9,10]. In mesencephalic primary cultures, NT-4/5 has been shown to enhance the morphological complexity and to stimulate the survival of DAergic neurons[11,12]. In line with these reports, we have shown that GDNF and NT-4/5 administration resulted in a significant higher survival rate of rat VM tyrosine hydroxylase immunoreactive (TH-ir) neurons grown for one week as free-floating roller-tube (FFRT) cultures and also increased their DA release[6].

Transient storage of donor tissue in FFRT culture offers the possibility of exposing cells with growth factors prior to grafting. We and others have previously shown that pretreated donor tissue resulted in a better outcome[13]. Support for growth factor pre-treatment prior to transplantation is furthermore given by a recent study which demonstrated that FGF2-mediated pre-grafting expansion of primary VM precursor cells considerably improved dopaminergic cell survival and functional restoration in a rat model of PD[14].

To the best of our knowledge so far no studies have been performed analyzing the effects of a combined GDNF and NT4/5 administration on VM tissue of human origin. Hence, in the present study we aimed at investigating whether treatment with combined exposure to GDNF and NT-4/5 improves DAergic neurons survival and augments dopamine release as compared to single factor treatment.

Methods

Human tissue

All experiments were carried out in accordance with the Ethics Committee of the Medical Faculty of the University of Bern and the Ethics Committee of the State Bern, Switzerland (study No. 181/07). Written consent was given by the women seeking abortion.

Tissue culture

Free-floating roller-tube (FFRT) cultures were prepared from ventral mesencephali (VM) derived from aborted human fetuses as described in detail by Spenger and co-workers[15,16]. The embryonic age of the specimen was 8 to 9 weeks post-conception for analysis of cell numbers and dopamine content and 7 to 10 weeks post-conception for Western blot analysis and lactate dehydrogenase (LDH) measurements as determined by the method of Evtouchenko and coworkers[17]. In brief, the ventral mesencephalic tissue were dissected out and divided into two equal sized portions corresponding to the left and right side and then further divided into equally sized small pieces. Each piece was transferred into labeled conical plastic tubes (Falcon) containing 1 ml of culture medium and placed in a roller-drum in an incubator for one week[16]. The cultures were grown in medium containing 55% Dulbecco´s modified Eagle medium (DMEM, Gibco Reinach, Switzerland), 32,5% Hanks balanced salt solution (HBSS, Gibco), 1,5% glucose, 10% fetal calf serum (FCS, Gibco No. 013-06290) and 1% 0.01 M Hepes (Merck KGaA, Darmstadt, Germany). Antibiotics (Gibco) were present during the entire culture period. The culture tubes were randomly assigned to the GDNF (10 ng/ml; Promega AG, Dübendorf, Switzerland), NT-4/5 (10 ng/ml; Promega), the GDNF plus NT-4/5 group and to the control group (n= 4 - 12 cultures per group for each of the three independent experiments). The medium was changed every other day. Neurotrophic factors were added at day 0 and then at each medium change. Control

cultures were grown in medium with no trophic factors added.

Analysis of dopamine content in the culture medium by HPLC

At the end of the culture period the medium was collected and a dopamine stabilizing solution (0.22 mg metabisulfite per 50 μl in 7.5% orthophosphoric acid) was added. Dopamine was determined using reversed phase HPLC with electrochemical detection as described in detail elsewhere[6]. Mean dopamine content in control cultures was 24.1 ± 4.4 pg/ml (mean ± SEM).

Immunohistochemistry and morphometric analyses

Cultures were washed two times with ice cold Hank's balanced salt solution, pooled and fixed in 4 % paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4) for 45 min. at room temperature followed by cryoprotection in 20% sucrose in 0.1M PBS containing 0.01% NaN_3 for 24 hrs. Cultures were sectioned at 20 *μ*m on a freezing microtome (Frigocut 2800 N, Reichert-Jung) and mounted on gelatin chrome-alum precoated glass slides. Sections were washed in PBS preincubated for 60 min. in PBS containing 0.3% Triton X-100 and 10% horse serum and incubated for 48 h at 4°C with primary antibodies against rabbit anti-TH (1:1000, Pel Freez Bio, Rogers, AR, USA) diluted in PBS containing 0.1% Triton X-100 and 2.5% horse serum. Following washes, sections were incubated with biotinylated anti-rabbit antibodies (1:200; Vector, Burlingame, California, USA). Bound antibodies were visualized with the avidin-peroxidase complex method and the metal enhanced 3, 3'-diaminobenzidine-based kit (Pierce, Rockford, Illinois, USA). Slides were then washed, dehydrated and covered with Eukitt (O. Kindler GmbH, Freiburg, Germany). Quantification of TH-ir neurons was performed by bright field microscopy by means of a neuron tracing system using a x20 objective[6]. Cell numbers were corrected for double counting according to Abercrombie's formula [18] and were normalized as TH-ir cell densities (number of TH-ir cells / mm^2) and shown as percent of controls. Mean TH-ir cell densities in control

cultures were 71.3 ± 8.3 cells / mm^2 (mean ± SEM). Culture volumes were assessed by computer-assisted image analysis as described elsewhere[6]. Mean culture volumes in control cultures were 0.47 ± 0.07 mm^3 (mean ± SEM).

Analysis of lactate dehydrogenase content in the culture medium

At the end of the culture period the medium was collected and used for Lactate dehydrogenase (LDH) level measurements as described in detail earlier[6]. Values are expressed as percent of controls of four independent experiments (n=17 - 20 per group).

Western blotting

Proteins were analyzed by SDS-polyacrylamide minigel electrophoresis as described previously[6]. Briefly, cultures were washed two times with ice cold Hank's balanced salt solution and then pooled for each group. The prepared lysates were subjected to a 12% SDS-polyacrylamide separating gel and the proteins blotted to a polyvinylidene difluoride membrane (BioRad, Basel, Switzerland). After electrophoretic transfer the blots were decorated with mouse anti-TH (1:500, Millipore, Billerica, Massachusetts, USA) or mouse anti-GFAP (1:500, Millipore) antibodies. Visualization of bound antibody was performed using anti-mouse antibody-peroxidase conjugates (1:10000, Nordic Immunological Laboratories, USA) and the ECL method (Amersham, Glattbrug, Switzerland). For loading control and densitometric analyses membranes were re-probed with mouse monoclonal alpha tubulin (1:20000, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Experiments were done in triplicate.

Statistics

For the statistical analysis a commercially available software package was used (GraphPad Prism 6, La Jolla, California, USA). The experimental groups were compared using the Kruskal-Wallis nonparametric ANOVA test in combination with the

Dunn's multiple comparison post hoc analysis. Differences were considered statistically significant at $p < 0.05$. Values are presented as mean ± SEM.

Results

Immunohistochemical analysis of the cultures disclosed higher TH-ir neurons cell densities in the GDNF, NT-4/5 and GDNF plus NT-4/5 treated groups (3 fold, 5 fold and 9 fold after GDNF, NT-4/5 and the combination treatment, respectively) as compared to untreated controls (Fig. 1A). Moreover, the combination treatment resulted in increased surviving TH-ir cell numbers as compared to all other groups (Fig. 1A). In contrast, culture volume did not differ significantly between controls and experimental groups with slightly increased volumes found for the GDNF plus NT-4/5 group (by 22%) as compared to controls (Fig. 1B). Similar to the outcome seen on cell densities of TH-ir neurons, significantly augmented TH protein levels in tissue lysate were observed in the growth factor treated groups as compared to controls (Fig. 2A,B). We next reasoned whether the release of dopamine might have paralleled these effects on the TH-ir neurons. Indeed the levels dopamine in the culture medium were increased in all neurotrophic factor treated groups (4.5 fold, 5.5 fold and 6.5 fold after GDNF, NT-4/5 and the combination treatment, respectively) as compared to controls. Accordingly, the treatment with NT-4/5 and GDNF+ NT-4/5 exerted the same increase in dopamine release in the culture medium (Fig. 1C).

Western blot analysis for GFAP did not show increased levels in the neurotrophic factor treated cultures as compared to controls (Fig. 2C,D). The level of lactate dehydrogenase (LDH), released into the culture medium from dead or degenerating cells was lower after growth factor treatment as compared to untreated control cultures (100.0 ± 5.2%, 90.5 ± 1.3%, 91.4 ± 0.9%, and 90.2 ± 1.6% for control, GDNF, NT-4/5 and the combination treatment, respectively).

Discussion

In the present work we demonstrate that the combination of NT-4/5 and GDNF has an additive effect on TH-ir cell numbers in organotypic explant cultures of fetal human ventral mesencephalon. Moreover, we have found that the combined treatment is superior to each of the single factors in improving the survival and the expression of structural and biochemical functional markers of cultured fetal DAergic neurons. Overall these observations substantiate and extend our earlier report on VM-FFRT cultures of rat origin[6].

Optimal growth, differentiation, and survival of DAergic neurons during development require a concerted action of different neurotrophic factors. In translational settings, several studies have addressed whether the neuronal supporting actions of different neurotrophic factors including GDNF and neurturin can be exploited to improve the efficacy of cell transplantation approaches for PD. Indeed, GDNF has already been reported to reduce apoptosis in DAergic neurons[19] and to promote DAergic fiber growth in fetal nigral grafts[20]. Application of NT4/5 and GDNF furthermore, protected DAergic neurons from toxin-induced cell death[21] and GDNF was found to exert anti-apoptotic effects on the DAergic neurons[19,22]. These observations suggest that the reduction of apoptosis events might be one of the mechanisms involved in the higher number of TH-ir neurons in the NT4/5 and GDNF treated samples. Indeed, this notion seems to be supported by the lower LDH levels measured after growth factor treatment. Notably and conversely to the additive actions observed on TH-ir cell densities, however, levels of released LDH did not differ amongst the growth factors treated groups. Thus, this outcome does not support the idea that the higher TH-ir cell densities are the result of a substantial reduction in cell death; rather these findings might be better explained by an enhanced specific maturation and / or differentiation of the TH-ir cells sub-population. In agreement with this hypothesis we have observed no significant differences in culture volume between groups. Moreover, in agreement to our previous study using cultures of rat origin[6], GFAP

protein levels were not affected by the treatments. Nevertheless, the mechanisms underlying the increased TH-ir cell densities were not investigated in the present study and we cannot exclude that other neuronal phenotypes were also promoted by the treatments. In fact, it has been reported that GDNF and neurotrophins stimulate the survival and growth of a wider range of neurons including striatal and cortical GABAergic neurons[9,23-25].

From a functional standpoint in the cell transplantation paradigm for PD, the capacity to release substantial amount of dopamine is a crucial disease changing pattern. We previously demonstrated that BDNF treatment increased the number of cultured DAergic neurons and elevated dopamine levels in the culture medium[26]. Moreover, we reported a moderate but beneficial survival promoting effect of combined GDNF and BDNF treatment on rat fetal nigral tissue[20]. In the present study, HPLC measurements of dopamine in the culture medium disclosed pronounced effects after single factor treatment as compared to controls. Interestingly, only a tendency for additivity was detected. Although this outcome appears in contrast to the findings of Studer and co-workers, i.e. that TH-ir cell numbers correlate with dopamine content in the culture medium[27], it should be noted that in the mentioned study only cultures not exposed to growth factors were investigated. The mechanisms accounting for our observation need to be further explored and might involve differences in the degree of DAergic neurons maturation induced by NT4/5 and GDNF possibly due to differences in the expression of the respective receptors. Importantly to note, even though Mendez and co-workers reported a substantial increased fluorodopa uptake in two parkinsonian patients receiving grafts exposed to GDNF[28] it is clearly necessary to verify the effectiveness of the combined NT4/5 and GDNF pre-treatment regimen of human mesencephalic tissue in animal models of PD.

Conclusions

Recent evidence supports the view that the intrastriatal delivery of dopaminergic neurons might develop into a safe and effective therapeutic option for PD[29]. Although the human iPSC-derived DAergic cells hold a considerable potential due to the unrestricted availability, high purity and minor ethical concerns, they appear to be less effective compared to the fetal-derived neurons. On the other hand the limited availability of fetal donor tissue and the generally poor survival of the grafted DAergic neurons are still major issues to be solved for successful cell replacement interventions[29]. The present study demonstrates that exposure of human DAergic nigral neurons grown as FFRT cultures to a combination of GDNF and NT-4/5 significantly improved quality of donor tissue and that this combination is overall superior compared to the single factor treatments. Further studies in PD will be needed to verify the significance of the pre-engraftment treatment described here. Nevertheless, it can be assumed that our findings likely also have impact for cell replacement approaches using iPSC- and / or ESC-derived donor cells.

Acknowledgments

We are grateful to Dr. Matthias Bischof, Tanja Bosnjak, Sandra Krebs, Inge Nielsen, and Dr. Alberto Perez-Bouza, Dr. Jürgen Sautter and Dr. Christian Spenger for excellent technical assistance and their interest in the work. We are indebted to the Department of Gynecology, University of Bern, Switzerland for their contribution. This research was supported by the HANELA Foundation and the Swiss National Science Foundation (No. 3100A0-112529). MM was supported by the Danish Parkinson Association, IMK Almene Fond, Kirsten and Freddy Johansens Fond and Hørslev Fonden.

Conflicts of interest

All authors state to have no conflict of interest (financial or otherwise) with the submitted work.

Figure legends

Fig. 1. Effects of GDNF (gray bars), NT-4/5 (dark gray bars) and combined neurotrophic factor treatment (black bars) on tyrosine hydroxylase (TH) positive cell densities (A), culture volume (B) and dopamine content in the culture medium (C). Ventral mesencephalic FFRT cultures were grown for one week in absence (control, open bars) or presence of neurotrophic factors. Data in the bar graphs are given as a percentage of control values and are expressed as mean + SEM. *: $p < 0.05$ vs corresponding control. [a]: $p < 0.05$ vs all groups.

Fig. 2. Representative microphotographs TH-ir cells from human ventral mesencephalic free-floating roller-tube cultures grown for 7 days without (control; A) or with addition of NT-4/5 and GDNF (comb; B). Scale bars: 100µm and 50µm (inserts). Representative Western blot analyses for TH (C) and GFAP (D) protein levels from human ventral mesencephalic free-floating roller-tube cultures grown for 7 days without (control) or with addition of NT-4/5 and GDNF (comb). Note that the combined treatment resulted in a significantly increased TH signal intensity as compared to the control group (C) whereas no difference in GFAP signal intensities between groups was observed (D). Membranes were re-probed with α-tubulin as a loading control. *: $p < 0.05$ vs corresponding control.

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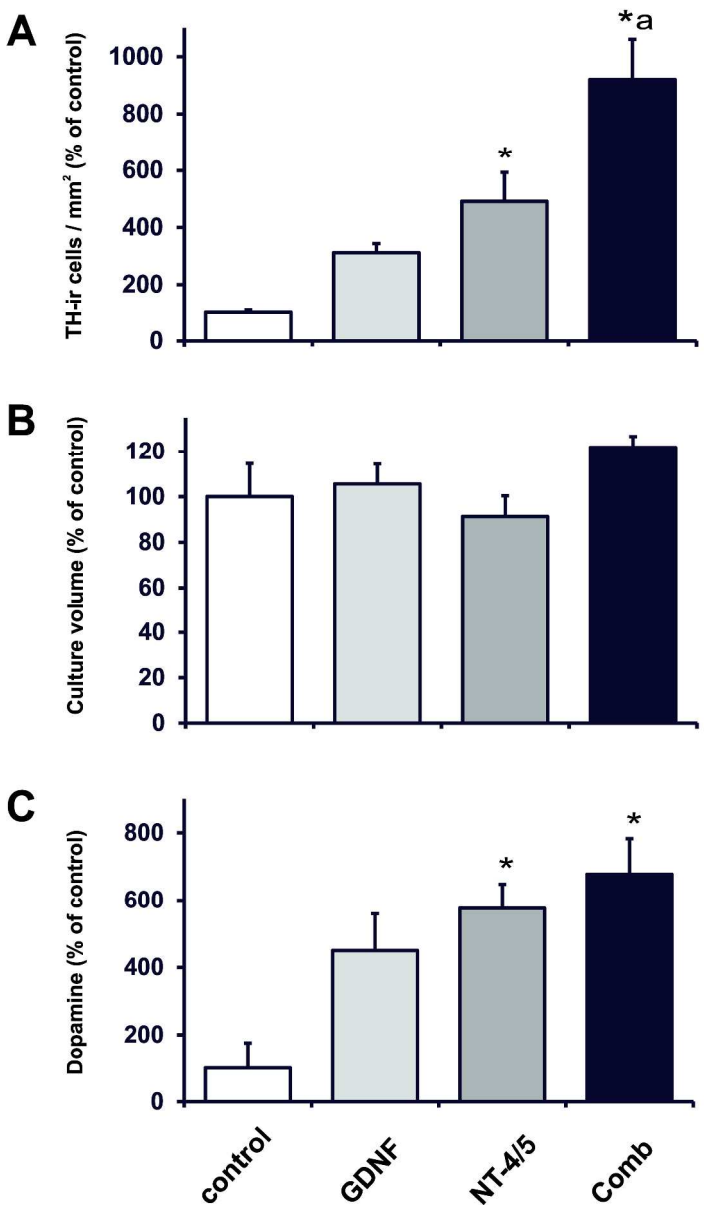

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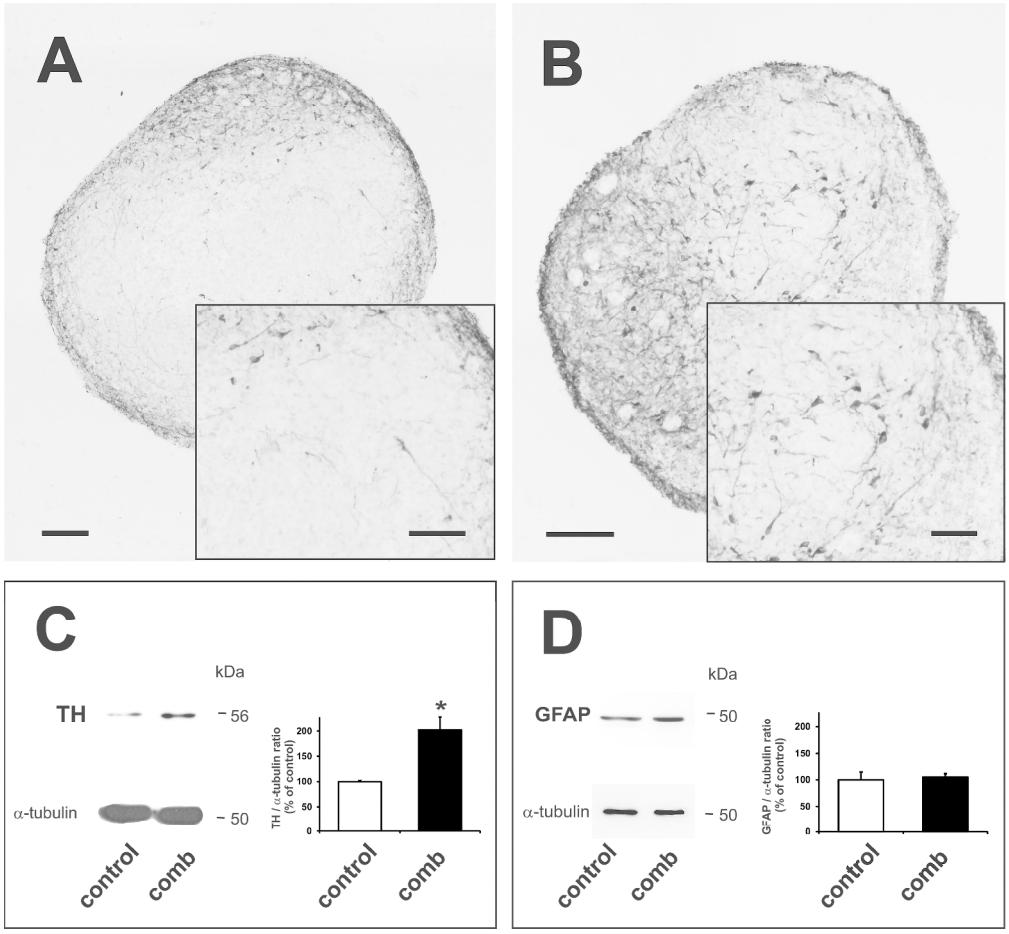

Fig. 2

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