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EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) ON MOUSE FETAL VENTRAL MESENCEPHALIC TISSUE

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Abstract

The symptoms of Parkinson's disease occur due to degeneration of dopamine neurons in substantia nigra. It has been demonstrated that glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor when it comes to protect and enhance survival of dopamine neurons in animal models of Parkinson's disease. The aim of this study was to evaluate short- and long-term effects of GDNF on survival and nerve fiber outgrowth of dopamine cells and astrocytic migration in mouse fetal ventral mesencephalic (VM) tissue. Primary tissue cultures were made of mouse fetal VM tissue and evaluated at 7 and 21 days in vitro (DIV) in terms of dopaminergic nerve fiber outgrowth and astrocytic migration when developed with GDNF present, partially, or completely absent. The results revealed that VM tissue cultured in the absence of GDNF did not exhibit any significant differences in migration of astrocytes or dopaminergic nerve fiber outgrowth neither after 7 DIV nor after 21 DIV, when compared with tissue cultured with GDNF present. Migration of astrocytes and dopaminergic nerve fiber outgrowth reached longer distances when tissue was left to develop for 21 DIV in comparison with 7 DIV. In order to study the long-term effects of GDNF, mouse fetal dopaminergic tissue was transplanted into the ventricles of adult mice and evaluated after 6 months. No surviving dopamine neurons were present in the absence of GDNF. In contrast dopamine neurons developed with GDNF did survive, indicating that GDNF is an essential neurotrophic factor when it comes to long-term dopamine cell survival. More cases have to be assessed in the future in order to strengthen the findings. Thus, transplanted dopamine neurons will be assessed after 3 and 12 months in order to map out when dopamine neurons deprived of GDNF undergo degeneration.

Keywords: Parkinson's disease, dopamine, glial cell line-derived neurotrophic factor (GDNF), ventral mesencephalon, astrocytes.

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1 Introduction

1.1 Background

1.1.1 Basal ganglia

The basal ganglia consist of subcortical nuclei located in the prosencephalon (forebrain), mesencephalon (midbrain) and diencephalon. Nuclei of basal ganglia are forming a circuit and project to motor cortex, thereby participating in control of movement. Basal ganglia consist of the subthalamic nucleus, the globus pallidus, the striatum, and the substantia nigra. Substantia nigra is divided into two segments, substantia nigra pars reticulata (SNpr) and substantia nigra pars compacta (SNpc). Whilst SNpr is involved in forwarding signals from the striatum to the cortex via thalamus, SNpc contains dopamine producing cells that project to the striatum. In the striatum, dopamine is released and bound to dopamine receptors that are located on the striatal neurons, which utilizes GABA as a neurotransmitter. The response depends on the receptors involved and the striatum can regulate the dopamine release in SNpc with GABA. The striatum then forwards the signal to the motor cortex, via SNpr and thalamus, where movement is controlled. The striatum receives excitatory input from almost the entire cerebral cortex but also from thalamus [1]. Degeneration of the dopamine cells in SNpc causes Parkinson's disease.

1.1.2 Dopamine

Dopamine is formed in the synthesis pathway of adrenaline and noradrenalin, though it is a distinct neurotransmitter and not only an intermediate molecule [2][3]. Dopamine is formed when the enzyme tyrosine hydroxylase (TH) converts tyrosine into dopa followed by the reaction of aromatic amino acid dopa-decarboxylase (AADC) that converts dopa into dopamine. Later in this reaction noradrenalin is synthesized with dopamine-β-hydroxylase (DBH) and adrenaline with phenylethanolamine-N-methyltransferase (PNMT) (figure 1) [4]. Dopamine producing neurons are found in several clusters in the brain, each of them labeled with an A and a following number [5]. Substantia nigra (labeled A9) and ventral tegmental area (labeled A10) consist of a large proportion of the dopamine cells in the brain.

Tyrosine $\Rightarrow$ Dopa $\Rightarrow$ Dopamine $\Rightarrow$ Noradrenalin $\Rightarrow$ Adrenaline
TH AADC DBH PNMT

Figure 1. The synthesis of the neurotransmitters dopamine, noradrenalin, and adrenaline from the amino acid tyrosine.

1.1.3 Parkinson's disease

Parkinson's disease is named after the English physician James Parkinson who first described the disease in 1817. The disease has an onset at late middle age and the symptoms occur due to degeneration of dopamine neurons in SNpc. It is a progressive disease in the sense that the patient's conditions worsen as the loss of dopamine neurons continues. The definite cause for the degeneration of these dopamine neurons has yet not been determined, although it has been shown that genetic, inflammatory, and environmental factors are decisive. Due to excessive muscle contraction Parkinson's disease patients suffer from symptoms such as tremors, rigidity of muscles, difficulties with posture and balance and slowness or

absence of movements. These symptoms occur as a result when approximately 50% of the dopamine neurons have degenerated and 80% of the dopamine levels in the striatum are lost. Other malfunctions that often occur concurrently with Parkinson's disease are depression, dementia, cognitive dysfunction, anxiety, and sleeping difficulties [1].

1.1.4 Treatment

A cure for Parkinson's disease has yet not been found, though treatments to relieve the symptoms are available. To compensate for the lack of dopamine, patients with Parkinson's disease are treated with orally administrated 3,4-dihydroxyphenyl-L-alanine (L-dopa). L-dopa is used because of its ability to cross the blood-brain barrier, while dopamine is unable to do so due to its water solubility. After entering the central nervous system L-dopa converts into dopamine by the enzyme AADC. Unfortunately, this treatment loses its effectivity with the progressive loss of dopamine neurons. Common side effects of L-dopa treatment are dyskinesias (involuntary movements) that are thought to occur when serotonin neurons instead of dopamine neurons convert L-dopa into dopamine. Serotonin neurons can store and release exogenously administrated L-dopa but lack the regulatory mechanisms needed [6]. Other treatments involve dopamine agonists that are used to stimulate the dopamine-receptors and antimuscarinics that are used to reduce the excessive muscle contraction. Deep brain stimulation has been performed on patients with severe symptoms and far progressed Parkinson's disease. In the absence of a permanent cure, patients eventually become rather disabled as the disease progresses [1]. In order to gain full recovery, regeneration of dopamine cells that innervate the striatum has to be achieved. Attempts have been done in this area, namely by transplanting fetal dopamine cells into the striatum of patients.

1.1.5 Transplantations

Attempts to transplant fetal ventral mesencephalic (VM) dopamine producing neurons into the striatum in patients with advanced Parkinson's disease have given variable results. Some patients have experienced an improvement in motor function whilst others have had none or very little improvement. The same variable results have been seen for the L-dopa induced dyskinesias, where in some cases the dyskinesias has gotten worse after transplantation [7] [8] [9]. VM tissue, as dissected for grafting, contains other neuronal types apart from dopamine neurons. A study where a rat model for Parkinson's disease was used demonstrated that successful transplantation in terms of reduced dyskinesias and improved motor function are due to transplantation of grafts with many dopamine neurons and none or few serotonin neurons, whilst grafts with few dopamine neurons and many serotonin neurons showed a worsening of L-dopa induced dyskinesias and no motor function improvement [6]. It is also known that functional recovery after transplantation is partly dependent on the number of surviving dopamine neurons and the degree of dopaminergic innervation of the host striatum [10]. Furthermore, it has been observed in animal models of Parkinson's disease that grafted dopamine neurons innervate the striatum in a sparse and widespread pattern, instead of dense and patchy [11].

Dopamine nerve fiber outgrowth has been observed in tissue cultures from rat VM area. These studies show that the dopamine nerve fiber outgrowth comes in two

temporally separate waves [12]. The first wave is seen approximately after 3 days in vitro (DIV) and is not guided by astrocytes, called non-glia-mediated nerve fiber outgrowth. This outgrowth reaches long distances, but degenerates with time. The second wave follows the first but is guided by astrocytes, glia-mediated nerve fiber outgrowth, and is long-term lasting. This outgrowth is shorter and forms dopamine rich patches [13] [14]. Knowledge of what guides the dopamine nerve fiber outgrowth is of great importance and studies have been done in this area. A great number of nerve growth factors have been utilized to affect nerve fiber formation. One such growth factor that has been studied for its effects on dopamine neurons is glial cell line-derived neurotrophic factor (GDNF).

1.1.6 Glial cell line-derived neurotrophic factor

Glial cell line-derived neurotrophic factor (GDNF) is produced and secreted by glial cells (for example astrocytes) in the brain and was first cloned in 1993 by Lin et al. Lin and colleagues also demonstrated that GDNF enhances the survival of cultured dopamine neurons [15]. The levels of GDNF are known to be higher during development than in adulthood, therefore indicating that it is an important factor during development [16] [17] [18]. It has been demonstrated that GDNF is a potent neurotrophic factor when it comes to protecting dopamine neurons in rat models of Parkinson's disease [19] [20] [21] [22]. Due to its ability to promote survival in dopamine neurons, GDNF administration in combination with grafting in animal models of Parkinson's disease has been performed as an attempt to enhance innervation of host brain from transplanted tissue. The outcome showed enhanced survival of transplanted dopamine neurons, although GDNF did not change the sparse pattern of dopamine nerve fiber outgrowth into a patchy pattern [23].

Because of the successful experiments with GDNF in animal models of Parkinson's disease, clinical trials have been performed where patients were treated with GDNF. Patients that received intra-ventricular injections of GDNF showed no motor improvement and experienced many side effects such as nausea, anorexia, vomiting, psychotic manifestations etc. On the other hand, patients receiving intra-putaminal infusions improved their motor functions, experienced no side effects and reduced L-dopa induced dyskinesias with 64% [24] [25]. Although more research needs to be done regarding dosage and administration before treatment with GDNF can be performed safely and without the risk of severe side effects.

Due to its ability to protect dopamine neurons against degeneration and to promote cell survival, the effects of GDNF were evaluated in the present study. Mouse fetal VM tissue developed with GDNF present and absent was compared. Mice lacking the gene for GDNF die shortly after birth due to their inability to develop kidneys and major parts of their enteric nervous system. They also exhibit damage on their motor, sensory and sympathetic neurons [26] [27] [28]. Therefore were studies limited to fetal GDNF knockout tissue. Heterozygous mice survive with one allele but their striatal levels of GDNF are lower than in the wild type mouse [29]. In this study, tissues from fetuses with different GDNF genotypes were compared when it comes to number of surviving dopamine cells, formation of dopamine nerve fibers, and migration of astrocytes.

1.2 Aims

To study short- and long-term effects of GDNF on survival and nerve fiber outgrowth of dopamine cells and astrocytic migration in tissue from fetal VM area in mice.

The specific aims were:

- To evaluate dopaminergic nerve fiber outgrowth and astrocytic migration during early development by culturing mouse fetal VM tissue with GDNF absent and present.
- To evaluate long-term survival of dopamine neurons in tissue developed in the presence and absence of GDNF by transplantation of mouse fetal VM tissue and lateral ganglionic eminence (LGE) into the lateral ventricles of mice.

2 Materials and methods

2.1 Procedures

2.1.1 Primary ventral mesencephalic tissue cultures

All experiments on animals were approved by the local ethical committee. Cell cultures were made of tissue containing dopamine neurons from the VM area in mice, which is the area where substantia nigra will develop. The tissue was taken from mouse fetuses at embryonic day 14 (E14). Fetuses had different genotypes for the gene GDNF; thus wild types (gdnf $^{+/+}$) having both alleles, heterozygous (gdnf $^{+/-}$) having one allele, and knockouts (gdnf $^{-/-}$) none. Glia-mediated as well as non-glia-mediated nerve fiber outgrowth of the dopamine cells were determined and compared for tissue when developed in the absence of GDNF (gdnf $^{-/-}$) or with GDNF present (gdnf $^{+/+}$ or gdnf $^{+/-}$). In addition, astrocytic migration was calculated. Tissue was cultured for 7 days and 21 days, including time as a factor.

Heterozygous mice for the gene *gdnf* were mated which resulted in fetuses with different genotypes, that is gdnf $^{+/+}$, gdnf $^{+/-}$, and gdnf $^{-/-}$. The pregnant mice were deeply anesthetized with 4 % isofluran (Baxter Medical AB, Kista, Sweden) using Univentor 400 anesthesia unit (AgnThos, Stockholm, Sweden), thereafter were their neck dislocated. The fetuses were cut out at E14 with a crownrump-length (CRL) of 12-13 mm. The VM area was dissected in Dulbecco's modified Eagle's medium (DMEM; Gibco) under a dissection microscope and sterile conditions. The VM tissue was cut in a total of six pieces, approximately 350 µm thick, three from each side of the midline and put in two drops of chicken plasma (Sigma) on coverslips pretreated with poly-D-lysine (5 mg/ 100 ml distilled H_2O; Sigma). The drops was stirred together with one drop of thrombin (Sigma) and left to dry for 20 minutes. The coverslips were then put in 15 ml falcon tubes containing 0.9 ml medium with 10 µl antibiotics (10 000 units/ ml penicillin, 10 000 µg/ml streptomycin, 25 µg/ml amphotericin; Gibco) per ml medium and incubated in a roller-drum device at 37ºC and 5 % carbon dioxide (CO_2). The medium consisted of 55% DMEM, 32.5% Hanks' balanced salt solution (HBSS, Gibco), 10% fetal bovine serum (FCS; Gibco), 1.5% glucose, and 1% Hepes (Gibco) and was changed every 3-4 days. Antibiotics were excluded after the first medium change.

2.1.2 Transplantation of fetal dopaminergic tissue

In order to further study the effects of GDNF, fetal mouse dopaminergic tissue was transplanted into the lateral ventricles of adult mice and left to develop in vivo for 6 months. After this time the tissue was evaluated for the number of surviving dopamine neurons and compared when developed with GDNF present or absent.

Heterozygous mice for the gene *gdnf* were mated, which resulted in fetuses with different genotypes for GDNF i.e. gdnf $^{+/-}$, gdnf $^{+/+}$ or gdnf $^{-/-}$. The fetuses were cut out at E14 according to the procedure described above. VM and lateral ganglionic eminence (LGE) were dissected from the fetuses and put in drops of DMEM, LGE being the area from where the striatum will develop. Meanwhile, gdnf $^{+/+}$ mice were deeply anesthetized with 4 % isofluran. The VM and LGE tissues were thereafter injected into the left and right ventricle of the gdnf $^{+/+}$ mice. The coordinates were: anterior/posterior at bregma level, +/- 0.8 mm lateral to the bregma, and 3.5 mm below the dura. One gdnf $^{+/+}$ mouse received one unilateral VM/LGE transplant into each lateral ventricle, where each VM/LGE co-grafts were collected from different fetuses. The next host received tissue from the same fetuses, thus creating pairs of hosts carrying identical genotypes of transplants. The transplanted tissues were left to grow in the ventricles for 6 months and after this time evaluated for differences in survival of tissue with different genotype, i.e. when developed with GDNF absent or present.

2.1.3 Fixation and preparation of transplanted tissue

After 6 months, the tissue was fixed and prepared for evaluation. The transplanted gdnf$^{+/+}$ mice were deeply anesthetized by an injection of 0.3 ml pentobarbital (60 mg/ml) into the abdomen. When deeply anesthetized, a needle was inserted into the left ventricle of the heart and the right atrium was punctured. The system was washed with 20 ml Tyrode solution followed by fixation with 100 ml 4% paraformaldehyde (diluted in 0.1 M phosphate buffer, pH 7.4). The brains were dissected and portfixed in 4% paraformaldehyde (in 0 .1 M phosphate buffer) for 60-90 minutes and kept in the refrigerator. The brains were then rinsed in 10% sucrose (with 0.01% sodium acetate). The sucrose solution was changed several times during the first and second day, where after the brains were kept in sucrose solution in the refrigerator until further processed. When evaluated, the area of the brain containing the ventricles with the transplanted tissue was cut into 14 µm thin sections using a cryostat (Microm, HM 500 M) and attached to slides.

2.1.4 Genotyping

In order to determine the genotype (gdnf $^{+/+}$, gdnf $^{+/-}$, or gdnf $^{-/-}$) for fetuses that tissue was obtained from, DNA was extracted and analyzed with polymerase chain reaction (PCR). Intestines from the fetuses were collected in eppendorf tubes and kept on ice during the dissection. DNA was extracted by adding 200µl lysis-buffer [25 mM sodium hydroxide (NaOH) and 0.2 mM disodium ethylene-dimine-tetra-acetic-acid (EDTA; pH 12)] to the tubes and left to incubate for 1 h at 95 ºC in a Thermomixer compact (Eppendorf). Tubes were cooled to 4ºC and thereafter 200µl neutralizing-buffer [40mM Tris-Hydrochloric acid (HCl), pH 5] was added and the tubes were kept in the fridge. A total volume of 20µl was added to each tube containing 5 units of TAQ polymerase (Fermentas), 10 mM dNTP (Fermentas), 25 mM $MgCl_2$ (Fermentas), 2µl 10X Buffer (Fermentas), 3µl of sense and antisense primer (Promega), and 1.5 µl DNA. A negative control free

from DNA was also included. Two tubes was set up for each DNA sample containing primers to detect gdnf$^{+/+}$ and gdnf $^{-/-}$.
Primers sense -5' CGG AGC CGG TTG GCG CTA CCG G 3'- and antisense -5' ACG ACT CGG ACC GCC ATC GGT G 3' to detect gdnf $^{-/-}$ was added to one tube, and primers sense -5' CCA GAG AAT TCC AGA GGG AAA GGT-3'and antisense -5' CAG ATA CAT CCA CAC CGT TTA GCG G-3' for detection of gdnf$^{+/+}$ was added to the other tube. PCR was performed in a thermal cycler (PTC-200, MJ Research, Inc.) and initiated with a denaturation step for 4 minutes at 92ºC. 40 cycles followed where each cycle consisted of denaturation for 1 minute at 92ºC followed by annealing for 1 minute at 56ºC and finally elongation for 2 minutes at 72ºC.The PCR products were analyzed with gel electrophoresis using a 2% agarose (Fermentas) gel. A 344 basepair (bp) long fragment was seen for gdnf $^{+/+}$ and a 255 bp long fragment indicated gdnf $^{-/-}$. A fragment was received from both reactions for gdnf $^{+/-}$. A 100 bp DNA ladder (Fermentas) was used as a reference.

2.1.5 Immunohistochemistry

After 7 and 21 days in vitro, cultured tissue was fixed on coverslips with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and then rinsed 3 times with 0.1 M phosphate buffer saline (PBS; pH 7.4). The tissue was incubated for 48 h at 4ºC with primary antibodies, diluted to 1:1500, against tyrosine hydroxylase (TH; mouse anti-TH, Immunostar Inc.). Blocking with 5% goat serum (diluted in 0.1 M PBS) followed in 15 minutes at room temperature. To visualize TH-positive (TH+) cells Alexa Fluor® 594 goat-antimouse IgG (Molecular Probes, Inc.) secondary antibodies were added (concentration 1:500) with an incubation period of 1 h in the dark, at room temperature. Tissue was also incubated with rabbit anti-vimentin primary antibodies (Abcam; concentration 1:100) for 48 h at 4ºC. Vimentin is an astrocytic marker, thus enabling visualization of astrocytic migration. Alexa Fluor® 488 goat-antirabbit IgG (Molecular Probes, Inc.) secondary antibodies were added at a concentration of 1:500 for 1h at room temperature and kept dark. Finally, cell nuclei were visualized with 4', 6-diamidino-2-phenylindole (DAPI) for 15 minutes (concentration 1:50 diluted in 0.1 M PBS). Cells were rinsed 3 times with 0.1 M PBS (pH 7.4) between every incubation step and all antibodies were diluted with 1% Triton-X in 0.1 M PBS. The coverslips were mounted with 90% glycerol in 0.1 M PBS.

Brain sections with transplanted VM and LGE tissues were incubated with antibodies against TH. The slides were rinsed in 0.1 M PBS (pH 7.4) for 15 minutes and then incubated for 48 h in the refrigerator with mouse anti-TH (Immunostar, Inc) primary antibodies at a concentration of 1:1500. Blocking with 5 % goat sera in 0.1 M PBS was performed for 15 minutes and Alexa Fluor® 594 goat anti-mouse IgG secondary antibodies were added to the slides and incubated for 1 h at room temperature kept in the dark. The slides were washed three times in PBS after every incubation period and all antibodies were diluted with 0.3 % Triton-X in 0.1 M PBS. Slides were mounted with 90% glycerol in 0.1 M PBS.

2.2 Evaluation

Evaluation of cell cultures and transplanted VM and LGE tissues were performed using fluorescence microscopy. The evaluation was blinded in the sense that during measurements it was not known whether the tissue had developed with GDNF absent or present. After measurements and calculations, statistical analysis was performed using one-way analysis of variance (ANOVA), Fisher's post-hoc test and independent sample t-test. ANOVA reveals differences between the groups being compared whilst Fisher's post-hoc test determines between which groups there is a difference and whether the difference is significant. Independent sample t-test was done when fewer than 3 groups were compared. A significant difference was revealed by a significance value (p) less than 0.05.

2.2.1 Evaluation of primary ventral mesencephalic tissue cultures

Measurements were made on tissue cultures containing more than 20 TH+ neurons. Vimentin-positive astrocytic migration and TH+ nerve fiber outgrowth were measured using a scale that was mounted in one ocular in the microscope. The astrocytic migration as well as the TH+ nerve fiber outgrowth was measured in four directions from the periphery of the tissue slice. The distances were converted into millimeters and a mean value was calculated for the migrations. The TH+ nerve fiber outgrowth comes in two waves. The first wave grows in the absence of astrocytes and is referred to as the TH+ non-glia-mediated outgrowth. The second wave of fibers, the TH+ glia-mediated outgrowth, follows the astrocytes and grows onto them. These two different types of TH+ nerve fiber outgrowths were distinguished and measured separately. The values for the astrocytic migration and the TH+ non-glia- and glia-mediated nerve fiber outgrowths were compared for tissue obtained from gdnf $^{+/+}$, gdnf $^{+/-}$, or gdnf $^{-/-}$ fetuses by using ANOVA and Fisher's post-hoc test. In addition, an independent sample t-test was used to analyze possible differences in astrocytic migration and TH+ nerve fiber (non-glia as well as glia-mediated) outgrowth when tissue was cultured at 7 and 21 days in vitro (DIV).

2.2.2 Evaluation of transplanted fetal dopaminergic tissue

Transplanted tissue in the lateral ventricles was observed and TH+ cells with a visible entire nucleus were counted. Every fourth section was counted, giving an indication of the amount surviving dopamine neurons that are present in the transplant. It also indicates whether there are differences in cell survival in tissue obtained from gdnf $^{+/+}$, gdnf $^{+/-}$, and gdnf $^{-/-}$ fetuses. A dopamine neuron is approximately 20μm in diameter and since every brain section is 14μm thick, dopamine cells are only counted once. The results were statistically analyzed using an ANOVA and post-hoc test.

3 Results

3.1 Primary ventral mesencephalic tissue cultures

3.1.1 Ventral mesencephalic tissue cultures at 7 DIV

Descriptive data received from the statistical analysis such as the number of cases (N), mean values, and standard error of means (SEM) revealed that there were small differences in astrocytic migration and non-glia-mediated nerve fiber outgrowth. The glia-mediated outgrowth was very even (see table 1) when

compared for tissue with different GDNF genotypes. The differences observed were not significant ($p > 0.05$) (see table 2). It was not possible to compare TH+ non-glia-mediated nerve fiber outgrowth due to lack of presence in gdnf$^{-/-}$ cultures. Furthermore, the TH+ non-glia-mediated nerve fiber outgrowth was absent in 33.3% of cases for gdnf $^{+/+}$ tissue, 54.5% for gdnf $^{+/-}$ tissue, and 66.7% for gdnf $^{-/-}$ tissue. TH+ glia-mediated nerve fiber outgrowth was missing in 33.3% of cases for gdnf $^{+/+}$ tissue, 9.1% for gdnf $^{+/-}$ tissue, and 33.3% for gdnf $^{-/-}$ tissue.

Table 1: Descriptive data such as number of cases (N), mean value, and standard error of means (SEM) for astrocytic migration and TH-positive nerve fiber outgrowth at 7 days in vitro.

Outgrowth	Genotype	N	Mean (mm)	SEM
astrocytic	gdnf $^{+/+}$	3	0.383	0.058
	gdnf $^{+/-}$	11	0.507	0.076
	gdnf $^{-/-}$	3	0.704	0.157
Non-glia-mediated	gdnf $^{+/+}$	3	1.363	0.200
	gdnf $^{+/-}$	11	1.071	0.125
Glia-mediated	gdnf $^{+/+}$	3	0.341	0.034
	gdnf $^{+/-}$	11	0.313	0.039
	gdnf $^{-/-}$	3	0.313	0.150

Table 2: Significance values (p) received when tissue with different GDNF genotypes were compared at 7 days in vitro, where a significant difference is seen when $p < 0.05$.

Outgrowth	Genotype	Genotype	p
astrocytic	gdnf $^{+/+}$	gdnf $^{+/-}$	0.439
	gdnf $^{+/-}$	gdnf $^{-/-}$	0.227
	gdnf $^{-/-}$	gdnf $^{+/+}$	0.122
Glia mediated	gdnf $^{+/+}$	gdnf $^{+/-}$	0.783
	gdnf $^{+/-}$	gdnf $^{-/-}$	1.000
	gdnf $^{-/-}$	gdnf $^{+/+}$	0.831

3.1.2 Ventral mesencephalic tissue cultures at 21 DIV

Descriptive data (N, mean values, and SEM) concerning migration revealed that there were small differences in astrocytic migration and TH+ non-glia-mediated and glia-mediated nerve fiber outgrowth (see table 3) when compared for tissue with different GDNF genotypes, but the differences were not significant ($p > 0.05$) (see table 4). At this time point the TH+ non-glia-mediated nerve fiber outgrowth was missing in 46.2% of cases for gdnf $^{+/-}$ tissue and 50.0% for gdnf $^{-/-}$ tissue, whilst it was present in every case for gdnf $^{+/+}$ tissue. TH+ glia-mediated nerve fiber outgrowth was present in every case regardless of genotype.

Table 3: Descriptive data such as number of cases (N), mean values, and standard error of means (SEM) for astrocytic migration and TH-positive nerve fiber outgrowth at 21 days in vitro.

Outgrowth	Genotype	N	Mean (mm)	SEM
Astrocytic	gdnf $^{+/+}$	13	1.113	0.229
	gdnf $^{+/-}$	3	1.345	0.118
	gdnf $^{-/-}$	6	1.557	0.142
Non-glia mediated	gdnf $^{+/+}$	13	3.383	0.063
	gdnf $^{+/-}$	3	3.254	0.315
	gdnf $^{-/-}$	6	3.465	0.337
Glia mediated	gdnf $^{+/+}$	13	0.792	0.177
	gdnf $^{+/-}$	3	1.000	0.089
	gdnf $^{-/-}$	6	0.879	0.209

Table 4. Significance values (p) received when tissue with different GDNF genotypes were compared at 21 days in vitro, where a significant difference is seen when $p < 0.05$.

Outgrowth	Genotype	Genotype	p
astrocytic	gdnf $^{+/+}$	gdnf $^{+/-}$	0.380
	gdnf $^{+/-}$	gdnf $^{-/-}$	0.301
	gdnf $^{-/-}$	gdnf $^{+/+}$	0.136
Non-glia mediated	gdnf $^{+/+}$	gdnf $^{+/-}$	0.793
	gdnf $^{+/-}$	gdnf $^{-/-}$	0.670
	gdnf $^{-/-}$	gdnf $^{+/+}$	0.889
Glia mediated	gdnf $^{+/+}$	gdnf $^{+/-}$	0.401
	gdnf $^{+/-}$	gdnf $^{-/-}$	0.525
	gdnf $^{-/-}$	gdnf $^{+/+}$	0.748

3.1.3 Ventral mesencephalic tissue cultures at 7 versus 21 DIV

Migration of astrocytes and TH+ nerve fiber outgrowth were evaluated for tissue with the same genotype but when cultured 7 versus 21 days, thereby including time as a factor.

It was observed that there were a significant difference in distance reached in gdnf $^{+/+}$ ($p < 0.05$), gdnf $^{+/-}$ ($p < 0.001$), as well as in gdnf $^{-/-}$ ($p < 0.01$) tissue, when migration of astrocytes were compared in tissue cultured 7 versus 21 days (see table 5 and figure 2).

A significant difference was also seen over time in the outgrowth of TH+ non-glia-mediated nerve fibers; $p < 0.05$ for gdnf $^{+/+}$ and $p < 0.001$ for gdnf $^{+/-}$ (see table 5 and figure 3). A comparison for gdnf$^{-/-}$ was not possible to perform due to lack of data.

When it comes to the outgrowth of TH+ glia-mediated nerve fibers, there was a difference in nerve fiber lengths when compared between time points, although the difference was only significant for gdnf $^{+/-}$ ($p < 0.001$) tissue (see table 5 and figure 4).

Table 5: Differences in astrocytic migration and TH-positive nerve fiber outgrowth when compared at 7 and 21 days in vitro for tissue with the same GDNF genotype.

Outgrowth	**Genotype**	**Mean value 7 DIV** (mm)	**Mean value 21 DIV** (mm)	**p**
Astrocytic	gdnf $^{+/+}$	0.383	1.113	0.037
	gdnf $^{+/-}$	0.507	1.345	0.000
	gdnf $^{-/-}$	0.704	1.557	0.008
Non-glia-mediated	gdnf $^{+/+}$	1.363	3.383	0.044
	gdnf $^{+/-}$	1.071	3.254	0.000
Glia-mediated	gdnf $^{+/+}$	0.341	0.792	0.145
	gdnf $^{+/-}$	0.313	1.000	0.000
	gdnf $^{-/-}$	0.313	0.879	0.194

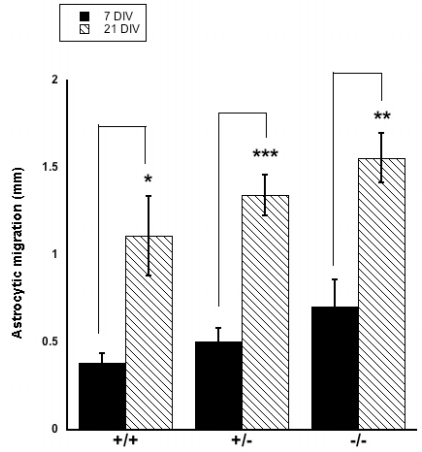

Figure 2: Astrocytic migration from tissue slice at 7 versus 21 days in vitro. Astrocytes have reached significantly longer distances at 21 days in vitro regardless of genotype. Mean values, standard error of means, and the significance level are illustrated. The level of significance is marked with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

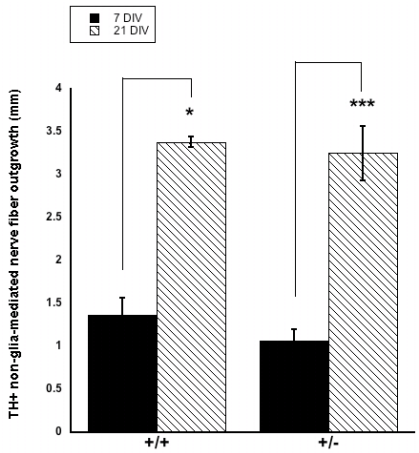

Figure 3: TH-positive non-glia-mediated nerve fiber outgrowth at 7 versus 21 days in vitro. TH-positive nerve fibers have reached significantly longer distances at 21 days in vitro in gdnf $^{+/+}$ and gdnf $^{+/-}$ tissue. Mean values, standard error of means, and the significance level are illustrated. The level of significance is marked with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

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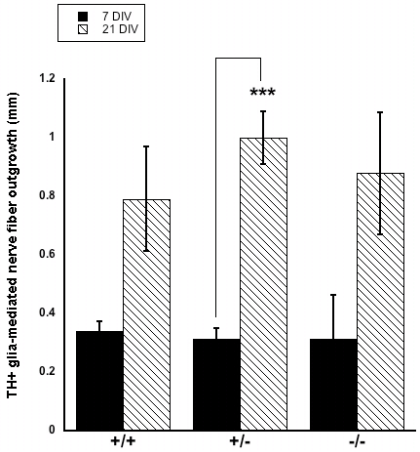

Figure 4: TH-positive glia-mediated nerve fiber outgrowth at 7 versus 21 days in vitro. Nerve fibers have reached significantly longer distances in gdnf $^{+/-}$ tissue at 21 days in vitro, but not in gdnf $^{+/+}$ or gdnf $^{-/-}$ tissue. Mean values, standard error of means, and the significance level are illustrated. The level of significance is marked with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

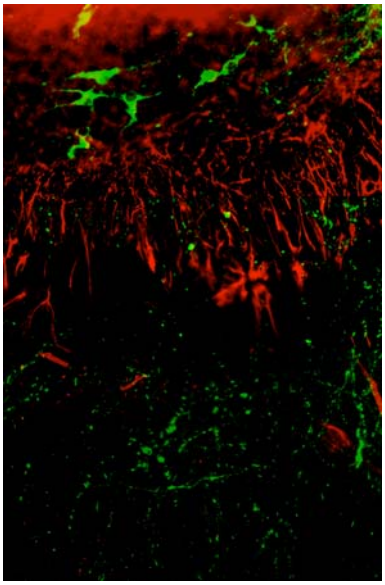

Figure 5: Migration of astrocytes (red) and TH-positive nerve fibers (green) at 7 days in vitro.

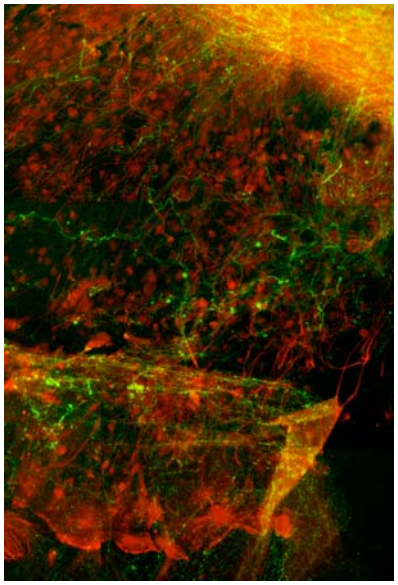

Figure 6: Migration of astrocytes (red) and TH-positive nerve fibers (green) at 21 days in vitro.

3.2 Transplanted fetal dopaminergic tissue

After being developed in the lateral ventricles of mice for 6 months, it was observed that transplanted fetal gdnf$^{+/+}$ (see figure 7 and 8) and gdnf $^{+/-}$ tissue contained varying numbers of surviving dopamine neurons, whilst tissue from gdnf $^{-/-}$ (see figure 9) tissue had no surviving dopamine neurons. The mean value of surviving dopamine neurons was highest in gdnf $^{+/+}$ tissue, gdnf $^{+/-}$ having a lower number (see table 6 and figure 10). The statistical analysis revealed that there was a significant difference ($p < 0.01$) in dopamine cell survival when comparing gdnf $^{+/+}$ and gdnf $^{-/-}$ tissue. Despite the fact that a difference was seen when comparing gdnf $^{+/+}$ with gdnf $^{+/-}$ tissue and gdnf $^{-/-}$ with gdnf $^{+/-}$ tissue, the difference seen was not significant ($p > 0.05$) (see table 7 and figure 10).

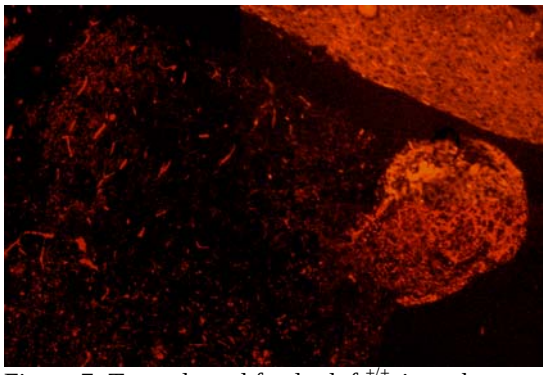

Figure 7: Transplanted fetal gdnf $^{+/+}$ tissue have surviving dopamine neurons at 6 months after the transplantation to the lateral ventricles of adult mice (red = TH+).

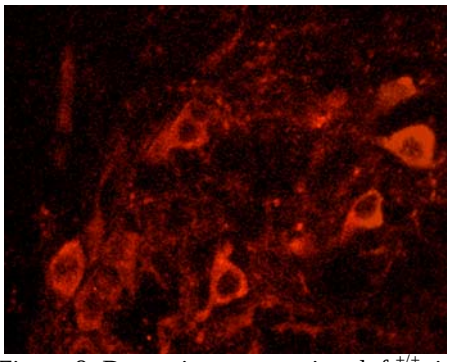

Figure 8: Dopamine neurons in gdnf $^{+/+}$ tissue (red = TH+).

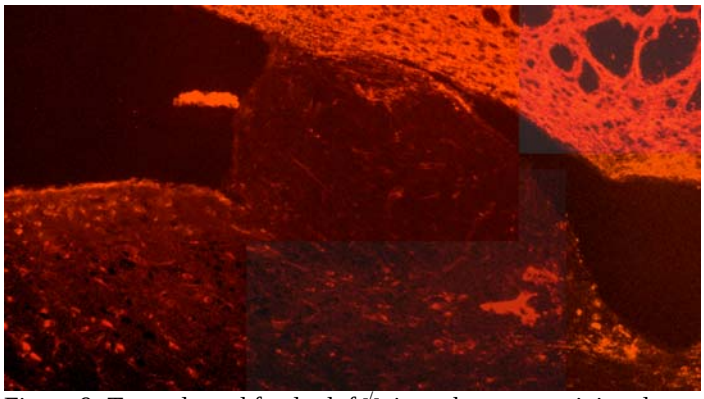

Figure 9: Transplanted fetal gdnf $^{-/-}$ tissue has no surviving dopamine neurons at 6 months after grafting to the lateral ventricles of adult mice (red = TH+).

Table 6: Descriptive data such as number of cases (N), mean values of surviving dopamine neurons, and standard error of means (SEM) for transplanted tissue with different GDNF genotype.

Genotype	**N**	**Mean value**	**SEM**
gdnf $^{+/+}$	4	137.50	36.55
gdnf $^{+/-}$	9	65.33	20.12
gdnf $^{-/-}$	3	0.00	0.001

Table 7: Significance values (p) received when the mean values of surviving dopamine neurons in tissue with different GDNF genotypes were compared.

Genotype	**Genotype**	**p**
gdnf $^{+/+}$	gdnf $^{+/-}$	0.063
gdnf $^{+/-}$	gdnf $^{-/-}$	0.120
gdnf $^{-/-}$	gdnf $^{+/+}$	0.009

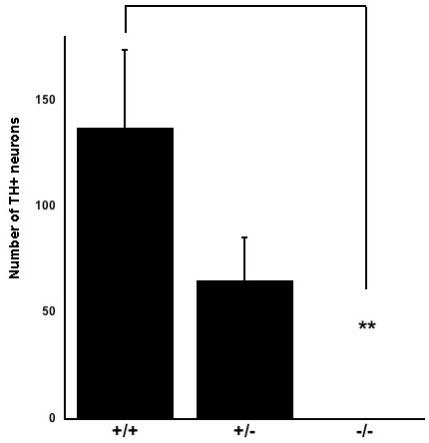

Figure 10: A significant difference was seen in dopamine cell survival when comparing gdnf $^{+/+}$ and gdnf$^{-/-}$ tissue. Mean value, standard error of means, and the level of significance (** for $p < 0.01$) are illustrated for the number of TH-positive neurons in gdnf $^{+/+}$, gdnf $^{+/-}$, and gdnf$^{-/-}$ transplants at 6 months.

4 Discussion

4.1 Primary ventral mesencephalic tissue cultures

4.1.1 Ventral mesencephalic tissue cultures at 7 DIV

At this time point no significant difference was found in astrocytic migration or TH+ glia-mediated nerve fiber outgrowth for tissues with different GDNF genotypes. The TH+ non-glia-mediated nerve fiber outgrowth was not possible to evaluate due to shortage of data for one group (gdnf $^{-/-}$). When analyzing tissue cultures at 7 DIV, a large variety in migration of astrocytes and TH+ nerve fibers was observed in tissue with the same GDNF genotype. This can be explained by dopamine cells not being fully developed and activated at this early time point. An example of this could be the fact that the TH+ non-glia-mediated nerve fiber outgrowth was missing in 33.3% of cases for gdnf $^{+/+}$ tissue, 54.5% for gdnf $^{+/-}$ tissue, and 66.7% for gdnf $^{-/-}$ tissue. Additionally, absence of TH+ glia-mediated nerve fiber outgrowth was seen in 33.3% of cases for gdnf $^{+/+}$ tissue, 9.1% for gdnf $^{+/-}$ tissue, and 33.3% for gdnf $^{-/-}$ tissue. The absence of TH+ nerve fiber outgrowth indicates that tissue cultured for 7 days has not developed all cellular mechanisms needed and therefore not exhibiting differences for tissue with different GDNF genotype. In a prior study VM tissue from gdnf $^{+/+}$, gdnf $^{+/-}$, and gdnf $^{-/-}$ mice were cultured for 12 DIV. It was seen that while TH+ non-glia-guided nerve fiber outgrowth did not differ for tissue with different GDNF genotype, the glia-guided did. TH+ glia-guided nerve fibers along with astrocytes reached significantly shorter distances in gdnf $^{-/-}$ tissue than in gdnf$^{+/+}$ tissue [14]. In other words, VM tissue developing for a longer period of time does exhibit differences in tissue when cultured with GDNF present or absent.

4.1.2 Ventral mesencephalic tissue cultures at 21 DIV

Tissue with different GDNF genotypes cultured for 21 days did not exhibit any significant differences when it comes to astrocytic migration or TH+ non-glia- and glia-mediated outgrowth. A possible explanation is that VM tissue lacking GDNF partly or completely develops mechanisms to compensate the lack of GDNF as it

is left to incubate for a longer period of time. These compensatory mechanisms might be other neurotrophic factors counterweighing for the shortage of GDNF. Actually, it has been demonstrated that gdnf $^{+/+}$ and gdnf $^{-/-}$ animals evaluated during postnatal day 1 do not differ in their dopamine systems [30]. In the present study, TH+ glia-mediated nerve fiber outgrowth was found in every case regardless of genotype, supporting the theory of compensatory mechanisms developed to counteract the deficiency of GDNF. The fact that the TH+ non-glia-mediated nerve fiber outgrowth was missing in 46.2% of cases for gdnf $^{+/-}$ tissue and 50.0% for gdnf $^{-/-}$ tissue, whilst it was present in every case for gdnf $^{+/+}$ tissue is normal at this time point, since this type of outgrowth degenerates with time.

A comparison was made in terms of astrocytic migration and TH+ nerve fiber outgrowth (non-glia- and glia-mediated) in tissue with the same GDNF genotype over the two time points 7 and 21 DIV. The distances reached were significantly longer at 21 DIV for all cases, except for glia-mediated nerve fiber outgrowth for gdnf $^{+/+}$ and gdnf $^{-/-}$ tissue where a non-significant difference was observed. The fact that the length of glia-mediated nerve fiber outgrowth was longer at 21 DIV than after 7 DIV is normal since the dopamine neurons need time to develop.

In order to empower the findings of this study, more cases have to be evaluated in the future. Differences between groups examined are not possible to discover when the number of cases is too small. By gathering more material and results, differences not yet found can be revealed.

4.2 Transplanted fetal dopaminergic tissue

Transplanted dopaminergic tissue was left to develop in the ventricles of adult gdnf $^{+/+}$ mice for 6 months. When evaluated at this time point it was found that tissue from gdnf $^{+/+}$ and gdnf $^{+/-}$ fetuses contained variable numbers of surviving dopamine neurons, whilst tissue from gdnf $^{-/-}$ fetuses had no surviving dopamine neurons. The mean value of surviving dopamine neurons was 137.5 for gdnf $^{+/+}$ tissue, 65.33 for gdnf $^{+/-}$, tissue and 0 for gdnf $^{-/-}$ tissue, although the difference was only significant when comparing gdnf $^{+/+}$ and gdnf $^{-/-}$ tissue ($p = 0.009$). The degeneration of dopamine neurons in gdnf $^{-/-}$ tissue occurs probably due to a defect in their dopamine system as a consequence to the absence of GDNF because preliminary data show survival of transplanted gdnf $^{-/-}$ tissue at 6 weeks. It seems as if GDNF is a crucial neurotrophic factor in terms of long-term dopamine cell survival. The compensatory systems mentioned above for dopaminergic tissue cultured 21 DIV seems to not be long-term lasting. These mechanisms, thought to counteract the insufficient amounts of GDNF in gdnf $^{+/-}$ and gdnf $^{-/-}$ tissue, seem to be inadequate leading to degeneration of the dopamine neurons. In a previous study, gdnf $^{+/+}$ and gdnf $^{+/-}$ mice were compared when it comes to motor and dopamine neuronal function. The results indicated that motor activity declined earlier in gdnf $^{+/-}$ mice (at 12 months of age) than in gdnf $^{+/+}$ mice. Moreover, a decrease in substantia nigra TH immunostaining was seen in all mice although it was accelerated in gdnf $^{+/-}$ mice [31].

In order to enhance the findings in this study, more cases have to be evaluated in the future. Also, transplanted dopaminergic tissue will be assessed after 3 and 12 months. The results will give important information regarding dopamine cell survival when developed with GDNF present or absent, and at what time point

differences start to show. In an un-published study, transplanted dopaminergic tissue was evaluated after 6 weeks. No differences were seen in cell survival when comparing gdnf $^{+/+}$, gdnf $^{+/-}$, and gdnf $^{-/-}$ tissue at that time point.

5 Conclusions and recommendations

Mouse fetal VM tissue cultured in the absence of GDNF did not exhibit significant differences in migration of astrocytes or TH+ non-glia- and glia-mediated nerve fiber outgrowths neither at 7 DIV nor at 21 DIV, when compared with tissue cultured with GDNF present. Migration of astrocytes and TH+ non-glia- and glia-mediated nerve fiber outgrowths reached longer distances when tissue was left to develop for 21 DIV in comparison with 7 DIV. More cases have to be assessed in the future in order to strengthen the findings.

Mouse fetal VM dopamine neurons developed in the absence of GDNF did not survive the time period of 6 months, meanwhile dopamine neurons developed with GDNF present survived. This indicated that GDNF is an essential neurotrophic factor when it comes to long-term survival of dopamine neurons. In the future, dopaminergic tissue will be further evaluated at 6 months but also at 3 and 12 months. By investigating dopaminergic tissue at different time points it is possible to map when dopamine neurons deprived of GDNF undergo degeneration.

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