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Effects of glial cell line-derived neurotrophic factor deletion on ventral mesencephalic organotypic tissue cultures

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) is potent for survival and promotion of nerve fibers from midbrain dopamine neurons. It is also known to exert different effects on specific subpopulations of dopamine neurons. In organotypic tissue cultures, dopamine neurons form two diverse nerve fiber growth patterns, targeting the striatum differently. The aim of this study was to investigate the effect of GDNF on the formation of dopamine nerve fibers. Organotypic tissue cultures of ventral mesencephalon of *gdnf* gene-deleted mice were studied. The results revealed that dopamine neurons survive in the absence of GDNF. Tyrosine hydroxylase immunoreactivity demonstrated, in *gdnf* knockout and wildtype cultures, nerve fiber formation with two separate morphologies occurring either in the absence or the presence of astrocytes. The outgrowth that occurred in the absence of astrocytes was unaffected by *gdnf* deletion, whereas nerve fibers guided by the presence of astrocytes were affected in that they reached significantly shorter distances from the *gdnf* gene-deleted tissue slice, compared to those measured in wildtype cultures. Treatment with GDNF reversed this effect and increased nerve fiber density independent of genotype. Furthermore, migration of astrocytes reached significantly shorter distances from the tissue slice in GDNF knockout compared to wildtype cultures. Exogenous GDNF increased astrocytic migration in *gdnf* gene-deleted tissue cultures, comparable to lengths observed in wildtype tissue cultures. In conclusion, cultured midbrain dopamine neurons survive in the absence of GDNF, and the addition of GDNF improved dopamine nerve fiber formation — possibly as an indirect effect of astrocytic stimulation.

Keywords

GDNF; Knockout; Organotypic culture; Ventral mesencephalon; Dopamine; Neural plasticity; Astroglia; Parkinson's disease

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) was cloned in 1993 (Lin et al., 1993) and has been shown to be a potent factor for survival of embryonic midbrain dopamine neurons *in vitro* and *in vivo* in normal rat brain (Hudson et al., 1995; Lin et al., 1993; Strömberg et al., 1993). GDNF has also demonstrated neuroprotective properties in dopamine-injured rats (Beck

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et al., 1995; Granholm et al., 1997a; Kearns and Gash, 1995; Sauer et al., 1995). Furthermore, GDNF mRNA has been detected in the striatum at higher levels during development than in adult animals, suggesting that it is an important factor during development (Choi-Lundberg and Bohn, 1995; Schaar et al., 1993; Strömberg et al., 1993). In addition, GDNF can be retrogradely transported from the striatum to dopamine neurons located in the ventral mesencephalon (Barroso-Chinea et al., 2005; Tomac et al., 1995b), suggesting a trophic role of GDNF for the nigrostriatal system in the adult brain. Indeed, studies on dopamine-lesioned adult animals have shown that intrastrially administered GDNF can stimulate axonal sprouting of the remaining dopamine neurons (Beck et al., 1995; Grondin et al., 2002; Rosenblad et al., 1998; Tomac et al., 1995a).

The effects of GDNF on ventral mesencephalic dopamine neurons made this neurotrophic factor attractive for use in combination with grafting of dopamine neurons in animal models of Parkinson's disease (Apostolides et al., 1998; Granholm et al., 1997a; Rosenblad et al., 1996; Sautter et al., 1998; Strömberg et al., 1993; Sullivan et al., 1998; Yurek, 1998). The results revealed, as expected, enhanced survival of grafted dopamine neurons. Interestingly, when transplanted dopamine neurons are allowed to reinnervate adult striatum, the dopaminergic nerve fiber outgrowth was diffuse, sparse, and widespread and not as dense as the islands of dopamine terminals formed during *in situ* development (Strömberg et al., 1997). GDNF also improved the graft-derived reinnervated volume of adult host striatum, but did not change the pattern of dopaminergic reinnervation, i.e. from diffuse and sparse to dense and patchy (Törnqvist et al., 2000). However, GDNF treatment of mature dopamine grafts did not affect survival and outgrowth from grafted dopamine neurons (Johansson et al., 1995; Winkler et al., 2006), but enhanced nerve fiber formation within the grafts, a phenomenon also found using other factors (Björklund et al., 1998). This phenomenon may cause reduced graft function since high dopamine activity surrounding the grafted dopamine neurons should, theoretically, shut down neuronal activity for the dopamine neurons that target the host striatum, thus reducing dopamine release in the host target. Knowledge about what may guide neurons to target the adult striatum, when forming their nerve fibers, is therefore important.

To study what may guide dopamine nerve fiber growth, organotypic tissue cultures have been utilized. Using this technique, it has been shown that dopamine nerve fiber outgrowth is formed in two morphologically separate waves in rat (Johansson and Strömberg, 2002). The first wave of dopamine outgrowth is seen in the absence of astrocytes and occurs as a robust growth as early as 3 days *in vitro* (DIV). These nerve fibers degenerate at later time points and are followed by a second wave of dopamine outgrowth. This second wave of dopamine nerve fiber formation is, in contrast to the first wave, guided by astrocytes, forms striatal dopamine-rich patches, and survives long-term (Johansson and Strömberg, 2003). GDNF was, as the name implies, first purified from a glioma cell line (rat B49) and found to be produced by glial cells in the brain (Lin et al., 1993; Schaar et al., 1993). It is still not known, however, whether GDNF may affect the different growth patterns formed in organotypic tissue cultures.

The GDNF knockout (*gdnf*^{-/-}) mouse can be used as a model to study effects of GDNF on the dopamine system. These mice display deficits in sensory, sympathetic, and motor neurons and fail to develop kidneys, ureters, and most of the enteric nervous system (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). *Gdnf*^{-/-} mice die shortly after birth, as a consequence of these deficiencies, limiting experiments on *gdnf*^{-/-} to fetal tissue. Mice heterozygous for GDNF (*gdnf*^{+/-}) survive the shortcoming of one allele, but their striatal levels of GDNF are reduced (Airavaara et al., 2004). Although the nigrostriatal dopamine system appears to undergo normal development prenatally in GDNF gene-deleted animals, isolated midbrain dopamine neurons seem to be dependent on the presence of GDNF in order to mature in postnatal environment (Granholm et al., 2000). Moreover, a recent study has shown that dopamine neurons in aged mice heterozygous for *gdnf* gene deletion undergo an increased rate

of degeneration (Boger et al., 2006). Since GDNF levels are higher during development than during adulthood, the loss of dopamine in aged GDNF heterozygous mice might be a consequence of GDNF deficiency during early development. Furthermore, it is not known how GDNF affects the astroglial cells during nerve fiber formation. One way to study developmental effects of GDNF on dopamine nerve fiber formation is to isolate tissue from *gdnf* gene-deleted fetuses and cultivate as organotypic tissue cultures. The aim of this study was to investigate whether developmental deficits in the nigrostriatal nerve fiber production occur in *gdnf*^{-/-} tissue, and if so, whether this is accompanied by astrocytic development.

2. Results

Organotypic slice cultures from fetal ventral mesencephalon (VM) were used to study the effect of *gdnf* gene deletion on tyrosine hydroxylase (TH)-positive neurons and their nerve fiber outgrowth. Embryonic day (E) 14 mice were phenotyped for the mutant allele and fetal VM from *gdnf*^{-/-}, *gdnf*^{+/-}, and WT mice were cultivated for 12 days *in vitro* (DIV). Surviving TH-positive cells were found in *gdnf*^{-/-}, *gdnf*^{+/-}, and wildtype (WT) cultures, and no apparent differences in cell morphology or cell number were found between the three genotypic cultures (Fig. 1). TH-positive nerve fiber outgrowth was found both in *gdnf*^{-/-}, *gdnf*^{+/-}, and WT cultures. The TH-positive nerve fiber formation occurred in two morphologically different growth patterns where one outgrowth was radiating from the periphery of the tissue slice without changing direction and in the absence of astroglial cell bodies, as revealed by the absence of DAPI-positive nuclei (Fig. 2a). The other growth pattern of TH-positive nerve fibers appeared glia-mediated and grew onto a single layer of migrating S100-positive astrocytes (Figs. 2b, c). These TH-positive nerve fibers branched and formed a network overlying distally located astrocytes. Both types of TH-positive outgrowth were present in cultures from *gdnf*^{-/-}, *gdnf*^{+/-}, and WT tissues. The two morphologically different growth patterns were displayed over different areas of the periphery of the tissue slice.

The two different growth patterns of TH-positive outgrowth were evaluated separately. Measurements showed that the glia-guided TH-positive outgrowth reached significantly shorter distances from the periphery of the tissue slice in cultures from *gdnf*^{-/-} mice compared to that measured in WT cultures ($p < 0.05$, $n = 12$ *gdnf*^{-/-}, $n = 26$ WT; Figs. 3a and 4). The effect of a partial GDNF deletion demonstrated that TH-positive outgrowth in cultures from *gdnf*^{+/-} tissue was found to be shorter than WT but longer than measured in *gdnf*^{-/-} tissue cultures, although not significant. Thus, one allele of the *gdnf* gene may promote TH-positive outgrowth more than the absence of both alleles, as in the case of *gdnf*^{-/-} tissue. The reduced distance of glia-guided TH-positive outgrowth seen in cultures derived from *gdnf*^{-/-} tissue was counteracted by the addition of GDNF in the medium ($p < 0.05$, $n = 12$ *gdnf*^{-/-}, $n = 13$ *gdnf*^{-/-} + GDNF; Figs. 3a and 4). No significant effect of additional GDNF was seen on the length of TH-positive nerve fibers in cultured tissue from *gdnf*^{+/-} and WT mice. TH-positive nerve fiber density revealed neither differences in density within the tissue slice nor in the outgrowth in *gdnf*^{-/-}, *gdnf*^{+/-}, or WT cultures (Fig. 3b). The addition of GDNF resulted, however, in significantly increased nerve fiber density of TH-positive outgrowth in slice cultures from all genotypes ($p < 0.001$, $n = 7$ *gdnf*^{-/-}, $n = 7$ *gdnf*^{-/-} + GDNF; $p < 0.05$, $n = 10$ *gdnf*^{+/-}, $n = 9$ *gdnf*^{+/-} + GDNF; $p < 0.01$, $n = 8$ WT, $n = 9$ WT + GDNF; Fig. 3b).

TH-positive outgrowth seen in the absence of S100-positive astroglia did not differ in length of nerve fibers between cultures derived from any genotype. Treatment with GDNF did not affect the length or density of these nerve fibers. The TH-positive nerve fibers (in controls) formed without astroglia association measured approximately 3000 μ m from the tissue slice, while the glia-associated outgrowth measured approximately 600 μ m (Fig. 5). Thus, the glia-guided TH-positive outgrowth was significantly shorter, for all genotypes, than the TH-positive outgrowth formed in the absence of glia ($p < 0.001$).

S100-positive astrocytes migrated from the tissue slice forming a single cell layer. Measurements showed that S100-positive astrocytes in the *gdnf*^{-/-} cultures migrated and reached significantly shorter distances from the tissue slice compared to that measured in WT and *gdnf*^{+/-} cultures ($p < 0.01$, $n = 7$ *gdnf*^{-/-}, $n = 22$ *gdnf*^{+/-}, $n = 23$ WT; Figs. 6 and 7). The addition of GDNF to the medium reversed the shorter distance seen in *gdnf*^{-/-} cultures (Figs. 6 and 7). Thus, the astrocytes reached distances of the same magnitude as that seen in WT cultures without GDNF treatment (Fig. 7). Administration of GDNF through the medium exerted no significant effect on S100-positive astrocytic migration in WT and *gdnf*^{+/-} cultures. DAPI staining revealed the presence of cells other than S100-positive peripheral to, and between, the migrating S100-positive astrocytes (Fig. 2d). This phenomenon was found in all types of cultures.

GDNF levels measured, using ELISA, in medium from control cultures without the addition of GDNF were below detection limit. The addition of GDNF showed no differences in GDNF concentration in medium used for *gdnf*^{-/-}, *gdnf*^{+/-}, or WT cultures. The concentration of GDNF ranged from 784 ± 111 , 763 ± 114 , to 665 ± 45 pg/ml for medium from *gdnf*^{-/-}, *gdnf*^{+/-}, and WT cultures, respectively. GDNF levels in E14 fetal brains were 1.67 ± 0.25 pg/ml in WT brains ($n = 9$), 0.61 ± 0.09 pg/ml in *gdnf*^{+/-} brains ($n = 14$), and no GDNF protein was found in *gdnf*^{-/-} brains ($n = 5$).

3. Discussion

The results from this study revealed that surviving TH-positive neurons were found in organotypic slice cultures from both WT and *gdnf* gene-deleted tissue. Interestingly, the previously documented two morphologically different types of dopaminergic outgrowth from slice cultures, i.e. glia-guided vs. non-glia-guided, were present in WT and *gdnf*^{-/-} tissue cultures (Johansson and Strömberg, 2002, 2003). While the non-glia-guided TH-positive outgrowth appeared unaffected in terms of distribution and length of outgrowth by the absence of GDNF, the glia-guided TH-positive outgrowth as well as the migration of S100-positive astroglia were reduced in cultures derived from knockout tissue. The addition of exogenous GDNF to the knockout cultures counteracted these effects. Furthermore, no difference was observed regarding viability of the TH-positive neurons.

In a previous grafting study, little or no TH-positive neuronal survival was shown after grafting of tissue from *gdnf* knockout fetuses (Granhölm et al., 2000). The extremely poor survival of the grafted VM *gdnf*^{-/-} tissue is probably not an effect solely caused by the lack of GDNF since in the present study TH-positive neurons survived in VM cultures of *gdnf*^{-/-} tissue with no difference compared to WT cultures. This is strengthened by the fact that the grafts were implanted to dopamine-depleted striata, where dopamine depletion upregulates endogenous striatal GDNF protein levels (Nakajima et al., 2001; Yurek and Fletcher-Turner, 2001), while measurements in the present paper showed no detectable GDNF protein in control medium. Thus, grafted dopamine neurons may have access to GDNF from the striatum while our cultured dopamine neurons did not. Furthermore, with respect to dopamine development *in situ*, in newborn *gdnf* gene-deleted mice the dopamine system appears to be unaffected (Granhölm et al., 1997b). It may be argued that GDNF can reach the *gdnf*^{-/-} fetus via circulation from the mother, and therefore a normal development of the dopamine system is seen. This paper shows, however, that dopamine neurons survive in the absence of GDNF from day E14 and that GDNF levels are below detection limit in *gdnf*^{-/-} fetuses at that time point. It may still be possible that the time differences of 12 DIV and 8 weeks grafting may explain the difference in cell survival. It is well documented though that cell death is an early event, which should be bypassed in both the cultures and grafting experiments at the time of evaluation (Barker et al., 1996; Branton and Clarke, 1999; Clarkson et al., 1995; Mahalik et al., 1994). Thus, GDNF

may have a greater effect on neurite formation than on cell viability of TH-positive neurons, a hypothesis also supported by others (Jacobsen et al., 2005).

The results from this study showed that the glia-guided outgrowth reached shorter distances when the cultures were derived from *gdnf*^{-/-} tissue. It is not known whether this outgrowth really is mediated by astroglia. However, these nerve fibers have always been shown to be associated with the presence of astrocytes and long-term survival (Johansson and Strömberg, 2002, 2003). Thus, when proliferation of astroglia is stimulated resulting in enhanced glia migration, the glia-guided outgrowth is enhanced to grow for longer distances, and when astroglia proliferation is inhibited the outgrowth is decreased (Johansson and Strömberg, 2002). Furthermore, migration of astroglial cells is preceding the glia-guided outgrowth (Johansson and Strömberg, 2003). It was noted that other cells than TH- and S100-immunoreactive cells were present among the migrating astrocytes as revealed from the DAPI staining. It has been shown that these cells may be either oligodendrocyte precursors or microglia, and they do not appear to have any influence on TH-positive nerve fiber formation (Berglöv et al., in press). Since the distance of S100-positive astrocytes had migrated was reduced in *gdnf*^{-/-} cultures, the reduced outgrowth might be an indirect effect of inhibition of proliferation and/or migration of astrocytes. It cannot be determined that the effect was specific directly to GDNF, although addition of GDNF enhanced the distance of astrocytic migration. Indeed, it has been shown that astrocytes under certain conditions express the GDNF receptor GFR α 1, but also that GDNF has no effect on cultured astrocytes (Bresnahan and Antaur, 2000; Wood et al., 2005).

Interestingly, the more transient non-glia-mediated TH-positive outgrowth was not affected by *gdnf* gene deletion when comparing distances that nerve fibers reached. Thus, lack of GDNF appeared to affect only neuritic growth supported by astroglia. It has been suggested in other studies that GDNF affects calbindin-positive dopamine neurons, neurons located in A10 rather than A9 (Gerfen et al., 1987; Meyer et al., 1999). Dissections from E14 fetuses include both A9 and A10 dopamine neurons. Furthermore, it has been shown that GDNF may alter the target of innervation in tissue cultures such that dopamine neurons from A9, which normally avoid growing into cortical co-cultures, is triggered to do so by exogenous GDNF (Jaumotte and Zigmond, 2005). Moreover, it is known that dopamine neurons in A10 are less vulnerable than neurons in A9, which has been suggested to be partly due to the fact that GDNF expression is higher in ventral than dorsal striatum. Consequently, A10 neurons are exposed to higher concentrations of GDNF than A9 through the retrograde transport from target to neurons (Barroso-Chinea et al., 2005). Thus, there are strong indications that A10 neurons are more affected by GDNF than A9 dopamine neurons. However, we have not been able to characterize the glia-guided versus non-glia-guided types of outgrowth to be produced by dopamine neurons derived from A9 vs. A10. Using aldehyde dehydrogenase (ALDH1A1) as a marker for A9 neurons (Chung et al., 2005), both glia- and non-glia-guided outgrowth are ALDH1A1-positive and thus formed by A9 dopamine neurons (Berglöv et al., in press). It was, however, clear from this study that dopamine neurons producing the non-glia-guided outgrowth were not affected by the absence of GDNF.

The length of the TH-positive nerve fiber outgrowth was not affected by the addition of GDNF to the medium to WT and *gdnf*^{+/-} cultures, but the TH-positive nerve fiber density increased. Cultures treated with GDNF exhibited, independent of genotype, an increased glia-mediated nerve fiber outgrowth density compared to that measured in cultures without GDNF treatment. This suggests that GDNF may exert moderate effects on nerve fiber extension but is more important for nerve fiber density. This increased density may reflect increased number of TH-positive neurons producing nerve fibers or increased branching of nerve fibers. Indeed, recent data have shown that GDNF increases the number of branching points among TH-positive fibers (Schaller et al., 2005).

In conclusion, isolated TH-positive neurons survived in the absence of GDNF, and GDNF exerted effects on TH-positive neurons that formed nerve fibers onto astroglia. The absence of GDNF in *gdnf*^{-/-} cultures reduced migration of astrocytes, which was probably the reason why reduced nerve fiber outgrowth was observed. Interestingly, exogenous GDNF did not affect astrocytic migration in WT cultures.

4. Experimental procedures

4.1. Organotypic tissue cultures from *gdnf*^{-/-}, *gdnf*^{+/-}, and WT tissues

All animal experiments were approved by the local animal ethical committee. The animals were kept under a 12:12 h day–night cycle and were given free access to water and pellets. Adult mice, heterozygote for GDNF null mutation, were mated. Mice carrying embryonic day (E) 14 fetuses (counting the day of vaginal plug as E0) were deeply anaesthetized with 4% isoflurane (Baxter Medical AB, Kista, Sweden) using Univentor 400 anesthesia unit (AgnThos, Stockholm, Sweden) before neck dislocation. The fetuses were cut out and VM tissue was dissected under sterile conditions in Dulbecco's modified Eagle's medium (DMEM; Gibco) under a dissection microscope. The VM tissue from each fetus was divided in 6 pieces (3 pieces from each side of the midline, approximately 350 µm thick) using a scalpel and placed on sterile coverslips pretreated with poly-D-lysine (5 mg/100 ml distilled H₂O; Sigma) in a drop of chicken plasma (Sigma) and stirred together with a drop of thrombin (Sigma). The plasma/thrombin clots were left to dry in 15–20 min before insertion of the coverslips into 15 ml Falcon tubes with 0.9 ml medium. The medium was composed of DMEM, Hanks' balanced salt solution (HBSS, Gibco), and fetal bovine serum (Gibco) to a final volume of 10%, glucose, and HEPES (Gibco). Additionally, antibiotics were added to a volume of 0.1% (10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin; Gibco). Cultures were treated with GDNF (Promega) through the medium (10 ng/ml medium; *n* = 25 for WT, *n* = 16 for *gdnf*^{+/-}, *n* = 13 for *gdnf*^{-/-}), and medium without GDNF served as control cultures (*n* = 26 for WT, *n* = 22 for *gdnf*^{+/-}, *n* = 14 for *gdnf*^{-/-}). The tubes were thereafter placed in an incubator at 37 °C and in 5% CO₂ in a “roller-drum” rotating at a speed of one turn every 2 min and cultivated for 12 days (Gähwiler et al., 1997; Stoppini et al., 1991). The cultures were supplied with fresh medium every 3–4 days. Antibiotics were excluded starting with the first medium change.

4.2. Genotyping

The fetuses, from which the VM tissues were dissected, were genotyped to confirm their identity as *gdnf*^{-/-}, *gdnf*^{+/-}, or WT. The genotyping was performed using polymerase chain reaction (PCR). Genomic DNA was prepared from tissue not used for culture. The tissue was lysed in 0.5 ml lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl, 200 mg/ml Proteinase K; Promega) at 56 °C over night in a thermomixer (Thermomixer Compact, Eppendorf). After homogenization 1 µl/ml Ribonuclease A (RNaseA; Sigma) was added and the samples were incubated at 37 °C for 30 min. Thereafter 200 µl of phenol/chloroform/isoamylalcohol 25:24:1 (Sigma) was added to each sample and the suspensions were centrifuged at 14,000 rpm for 5 min. The supernatants were transferred into new tubes, inverted together with 100 µl chloroform and centrifuged for 14,000 rpm in 5 min. The supernatants were once more transferred to new tubes and the DNA was precipitated with 1 ml cold isopropanol. The DNA was stabilized at –20 °C for 30 min and thereafter pelleted by centrifugation for 5 min at 14,000 rpm at 4 °C. The pellets were washed once with 200 µl cold 70% ethanol and centrifuged as above. The purified DNA pellets were dissolved in 200 µl 1× TE buffer (Promega) and incubated at 37 °C for 2 h. The PCR reactions were accomplished in a thermal cycler (PTC-200, MJ Research, Inc.) and the DNA was assayed for the presence of WT and knockout alleles. Hence, there were two separate PCR reactions using either WT or knockout primers. The PCR was performed in a total reaction volume of 20 µl, which contained

1.5 µl genomic DNA, 30 µM sense primer, WT — 5'-CCA GAG AAT TCC AGA GGG AAA GGT C-3' or knockout — 5'-CGG AGC CGG TTG GCG CTA CCG G-3' (TAG Copenhagen, Copenhagen), 30 µM antisense primer, WT—5'-CAG ATA CAT CCA CAC CGT TTA GCG G-3' or knockout—5'-ACG ACT CGG ACC GCC ATC GGT G-3' (TAG Copenhagen, Copenhagen), 250 µM dNTP (Promega), 2 µl 10× PCR buffer (Promega), 2.5 mM MgCl₂ and 2 units of Taq Polymerase (Promega). Negative controls, without DNA, were also included. The PCR reaction was run for 40 cycles, each cycle consisting of denaturation at 92 °C for 1 min, annealing at 56 °C for 1 min and elongation at 72 °C for 2 min. The cycles were preceded by a denaturation step, 92 °C for 4 min. The PCR products were analyzed by gel electrophoresis on a 2% agarose gel (Agarose, LMP preparative grade for small fragments, Promega) stained with ethidium bromide. Amplification of the WT allele gave a band of 344 bp, while the mutant allele gave a band of 255 bp (Granhölm et al., 2000). A 100 bp DNA ladder (Promega) was used as reference.

4.3. Immunohistochemistry

The cultures were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature at 12 DIV. They were thereafter rinsed in phosphate buffered saline (PBS; pH 7.4) before incubation with antibodies, raised against TH (mouse anti-rat diluted 1:1 500; Interassay) and for the astrocytic marker S100 (rabbit anti-cow diluted 1:400; Dako Patts). The cultures were incubated in primary antibodies for 48–72 h in a humid chamber at 4 °C. Alexa Fluor® conjugates (Molecular Probes, Inc.) were used as secondary antibodies and applied at room temperature for 1 h. TH was visualized with Alexa Fluor 594 (goat anti-mouse, diluted 1:500), and visualization of S100 was performed with Alexa Fluor 488 (goat anti-rabbit diluted 1:500). All antibody dilutions were made in 0.1 M PBS containing 1% Triton X-100 (Merck). Rinsing was performed in PBS between each of the incubations. Cell nuclei were visualized using staining with DAPI diluted 1:50. After rinsing, the cultures were mounted in 90% glycerin diluted in PBS.

4.4. ELISA

Enzyme-linked immunosorbent assay (ELISA) was utilized to determine GDNF protein levels in E14 brain tissue from *gdnf*^{-/-} (*n* = 5), *gdnf*^{+/-} (*n* = 14), and WT (*n* = 9) mice and in culture medium collected at medium change after 3–4 days of incubation (*gdnf*^{-/-} *n* = 4, *gdnf*^{+/-} *n* = 6, WT *n* = 6). The tissue was homogenized with lysis buffer and protease inhibitor. GDNF protein levels were determined in tissue homogenates and media using a commercially available assay kit from R&D Systems. Briefly, 96-well flat bottom plates were coated overnight with the corresponding capture antibody. Duplicate samples of either brains or media were added to the wells and incubated at room temperature for 2 h. The plates were then incubated in the detection antibody for 2 h followed by incubation with the Streptavidin–HRP antibody. All unbound conjugates were removed by subsequent wash steps according to the R&D Systems protocol. After an incubation with chromogenic substrate (Turbo TMB), color change was measured in an ELISA plate reader at 450 nm. A standard curve using known amounts of GDNF protein was generated to determine sample concentrations. Using these kits, GDNF can be quantified in the range of 7.8–500 pg/ml. GDNF was expressed as pg/mg tissue.

4.5. Image analysis and statistics

The cultures were analyzed using a fluorescence microscope with a digital camera (Hamamatsu) connected to a computer. Images were analyzed using Openlab software (Improvion). Images of identical areas were captured at different wavelengths and merged to achieve pictures of double and triple staining.

S100-positive astrocytic migration and TH-positive nerve fiber outgrowth were measured using a scale mounted in one ocular of the microscope. Astrocytic migration was measured in

four perpendicularly placed directions from the periphery of the tissue slices to the distal end that the S100-positive astrocytes had migrated. The two different types of TH-positive nerve fiber formation were measured separately. The TH-positive outgrowth growing in the absence of astrocytic cell bodies was determined by measuring the distance from the most distally located nerve fibers to the periphery of the tissue slice. The average length of the second type of TH-positive outgrowth was calculated from measurements of nerve fibers performed in areas with TH-positive fibers growing onto astroglia using the same parameter as that for measuring the non-glia-associated nerve fibers. Nerve fiber density was measured on images captured with a CCD camera (ProgRes C14, Jenaoptic) using the NIH Image software. Optical density of TH immunoreactivity was estimated on binary images in a set frame and expressed as gray values set from 0 to 255 (Strömberg et al., 2005). Measurements were performed within the tissue slice and over the glia-guided and non-glia-guided nerve fiber outgrowth. In measurements performed within the tissue slice, areas where TH-positive neurons were present were avoided. One to four measurements were performed per culture, depending if four different areas of outgrowth were available for measurement.

All measurements were performed on blind coded cultures. Statistical analysis was performed on mean values calculated from each culture using one-way analysis of variance (ANOVA) followed by Fisher's post-hoc test. Values are expressed as means $\pm$ SEM.

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Fig. 1.

Viable TH-positive neurons in *gdnf* gene-deleted cultures. Representative views of TH-positive neurons in WT (a, $n = 26$), *gdnf*^{+/−} (b, $n = 22$), and *gdnf*^{−/−} (c, $n = 14$) fetal ventral mesencephalic tissue, cultured for 12 DIV, showed no apparent morphological differences. Scale bar: a–c = 50 μ m.

Fig. 2.

Nerve fiber formation from midbrain WT organotypic cultures. Fetal ventral mesencephalic tissue cultures showing TH (red) and S100 (green) immunoreactivity at 12 DIV. DAPI staining (blue) revealed the presence or absence of cell nuclei. The TH-positive fibers occurred in two different morphological growth patterns, one in the absence of other cells as revealed by a lack of DAPI labeling (a) and the other in the presence of S100-positive astrocytes (b, c). TH-positive nerve fibers growing in the absence of astrocytes and other cell types were radiating from the tissue slice, without changing direction, as observed at the distal end of the fiber outgrowth (a). The glia-mediated TH-positive nerve fibers had branched and formed a network (c), and no TH-positive fibers extended outside the astrocytic layer in the most distal regions from the tissue slice (b). DAPI staining showed the presence of cells other than S100-positive astrocytes between the migrating S100-positive cells (c, see arrows). TH (red), S100 (green), and DAPI (blue) are represented in panels a–c and S100 (green), and DAPI (blue) are represented in panel d. Scale bar: a–c = 50 μ m.

Fig. 3.

GDNF effects on length and density of TH-positive outgrowth from ventral mesencephalic cultures. Graphs showing TH-positive glia-guided outgrowth at 12 DIV; (a) distance from tissue slice and (b) nerve fiber density. The glia-guided TH-positive outgrowth reached significantly shorter distances from the tissue slice in *gdnf*^{-/-} compared to WT (a), and measurements were performed from the periphery of the tissue slice to the distal end nerve fibers reached. Treatment with GDNF (10 ng/ml medium) reversed the effect of the deletion (a). No significant effect of GDNF treatment was seen on the length of glia-guided TH-positive fibers in *gdnf*^{+/-} and WT cultures (a). The addition of GDNF resulted in significantly increased nerve fiber density of glia-guided TH-positive outgrowth in cultures from all genotypes. In

figure b, significance comparison of nerve fiber density was performed between GDNF treatment within each genotype. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; a: WT $n = 26$, WT + GDNF $n = 25$; $gdnf^{+/-}$ $n = 17$; $gdnf^{+/-}$ + GDNF $n = 15$; $gdnf^{-/-}$ $n = 12$; $gdnf^{-/-}$ + GDNF $n = 13$; b: WT $n = 8$, WT + GDNF $n = 9$; $gdnf^{+/-}$ $n = 10$; $gdnf^{+/-}$ + GDNF $n = 9$; $gdnf^{-/-}$ $n = 7$; $gdnf^{-/-}$ + GDNF $n = 7$.

Fig. 4. GDNF effects on nerve fiber formation. Fetal ventral mesencephalic organotypic tissue cultures (ts) at 12 DIV showing TH-positive nerve fibers growing from WT (a, representative of $n = 26$), WT treated with GDNF (10 ng/ml medium) (b, representative of $n = 25$), $gdnf^{-/-}$ (c, representative of $n = 14$), and $gdnf^{-/-}$ treated with GDNF (10 ng/ml medium) (d, representative of $n = 13$). The TH-positive nerve fibers reached shorter distances in $gdnf^{-/-}$ cultures (c) compared to WT cultures (a). The addition of GDNF (10 ng/ml medium) counteracted the effect of the knockout (d). Scale bar: a–d = 100 μ m.

Fig. 5.

Quantification of distances for the non-glia-guided and glia-guided TH-positive nerve fiber outgrowth. Measurements were performed from the periphery of the tissue slice to the distal end the nerve fibers had reached. Ventral mesencephalic tissue slices were cultured for 12 DIV. The non-glia-guided TH-positive outgrowth reached significantly longer distances from the tissue slice compared to the glia-guided TH-positive outgrowth in all genotypes. The glia-guided TH-positive outgrowth reached significantly shorter distances from the tissue slice in *gdnf*^{-/-} compared to WT (**p* < 0.01). Significance comparison of non-glia-guided with the glia-guided TH-positive outgrowth of each genotype. ****p* < 0.001, non-glia-guided: WT *n* = 24, *gdnf*^{+/-} *n* = 14, *gdnf*^{-/-} *n* = 14; glia-guided: WT *n* = 26, *gdnf*^{+/-} *n* = 22, *gdnf*^{-/-} *n* = 12.

Fig. 6.

Astrocytic migration in *gdnf*^{-/-} tissue cultures. Fetal ventral mesencephalic tissue was cultured for 12 DIV. S100-immunoreactivity showed astrocytic migration from tissue slice (left in pictures) from WT (a), *gdnf*^{-/-} (b), WT treated with GDNF (10 ng/ml medium) (c), and *gdnf*^{-/-} treated with GDNF (10 ng/ml medium) (d) cultures. The S100-positive astrocytes migrated over shorter distances in *gdnf*^{-/-} cultures (b) compared to WT cultures (a). The addition of GDNF (10 ng/ml medium) counteracted the effect of the deletion (d). Additional GDNF did not exert any effect on the S100-positive migration in WT (c). Panels a–d are representing *n*'s of 26 = WT, 25 = WT + GDNF, 14 = *gdnf*^{-/-}, 13 = *gdnf*^{-/-} + GDNF. Scale bar: a–d = 200 μ m.

Fig. 7.

S100-positive astrocytic migration from ventral mesencephalic tissue slice at 12 DIV. The distance was calculated from the periphery of the tissue slice to the distal end the astrocytes had migrated. The migrating S100-positive astrocytes reached significantly shorter distances from the tissue slice in *gdnf*^{-/-} compared to *gdnf*^{+/-} and WT. The shorter distance in *gdnf*^{-/-} was counteracted by the addition of GDNF (10 ng/ml medium). Additional GDNF did not exert any significant effect on the S100-positive migration in *gdnf*^{+/-} and WT cultures. ** $p < 0.01$, WT $n = 23$, WT + GDNF $n = 21$, *gdnf*^{+/-} $n = 22$, *gdnf*^{+/-} + GDNF $n = 16$, *gdnf*^{-/-} $n = 7$, *gdnf*^{-/-} + GDNF $n = 8$.