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DUAL EFFECTS OF TNF α ON NERVE FIBER FORMATION FROM VENTRAL MESENCEPHALIC ORGANOTYPIC TISSUE CULTURES

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Abstract

Tumor necrosis factor alpha (TNF α) is toxic to dopamine neurons and increased levels of TNF α are observed in Parkinson's disease. Dopamine nerve fiber outgrowth in organotypic cultures of fetal ventral mesencephalon occurs in two waves. The early appearing nerve fibers are formed in the absence of astroglia, while migrating astrocytes guide the late appearing dopamine nerve fibers. TNF α (40 ng/ml) was added to the medium of organotypic ventral mesencephalic tissue cultures between days 4–7 or 11–14. The cultures were evaluated at days 7 or 19 to study effects of TNF α on both types of nerve fiber formation. Tyrosine hydroxylase (TH) -immunohistochemistry demonstrated that the number of cultures showing non-glial-guided TH-positive outgrowth was reduced compared to controls, when TNF α was added at day 4. By contrast, the glial-guided TH-positive nerve fiber outgrowth and the astrocytic migration reached significantly longer distances by early TNF α treatment. Ki67-immunohistochemistry revealed that TNF α did not affect proliferation of astrocytes. Treatment with TNF α and antibodies against TNF α receptor 1 between days 4–7 revealed that the non-glial-guided TH-positive outgrowth reappeared. TNF α treatment between days 11–14 triggered neither the TH-positive glial-guided outgrowth, nor promoted the astrocytic migration to reach longer distances. The number of microglia was significantly increased after the late but not early TNF α treatment. In conclusion, TNF α is toxic for the non-glial dopaminergic nerve fiber outgrowth but stimulates the glial-guided outgrowth and the migration of astrocytes at an early time point. TNF α increased the number of microglia in VM tissue cultures after late but not after early treatment.

Keywords

ventral mesencephalon; TNF α ; dopamine; nerve fiber formation

1. INTRODUCTION

In neurodegenerative diseases, such as in Parkinson's disease, neuroinflammation plays a critical role. During neuroinflammation as well as in Parkinson's disease, the levels of proinflammatory cytokines are elevated (Boka et al. 1994; Mogi et al. 1994; Sawada et al. 2006). Tumor necrosis factor alpha (TNF α) belongs to the family of proinflammatory cytokines. The levels of TNF α are elevated in Parkinson's disease, and TNF α is known to induce apoptotic cell death (Kolesnick and Golde 1994; Mogi et al. 1994). TNF α is produced by activated microglia and the number of activated microglial cells are increased in the brains

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of parkinsonian patients (McGeer and McGeer 1997). Microglia are already activated during the early phase of Parkinson's disease, and the presence of activated microglia is correlated with decrease in dopamine nerve fiber density (Ouchi et al. 2005).

It has been shown that dopamine neurons are vulnerable to TNF α in tissue culture (McGuire et al. 2001). Furthermore, it has been documented that the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is less effective in TNF α deficient mice (Ferber et al. 2004), suggesting that TNF α takes part in the degenerative process. However, TNF α can also protect injured dopamine neurons in tissue culture (Shinpo et al. 1990). This neuroprotective effect is further confirmed in an in vivo study where the presence of TNF α at an early, transient event after injury of the dopamine system is beneficial for dopamine cell survival and regeneration (Gemma et al. 2007). Thus, there are indications that TNF α exerts a dual effect.

TNF α exerts its effect via two receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (Lewis et al. 1991; Tartaglia et al. 1993; Tartaglia et al. 1991). These receptors are localized on neurons, including the nigral neurons, as well as on glial cells (Boka et al. 1994; Dziewulska and Mossakowski 2003; McGuire et al. 2001). TNFR1 induces cell death via a cascade of death proteins while TNFR2 instead stimulates survival and migration. Moreover, double mutant mice for TNFR1 and TNFR2, but not single mutants for either one of the two receptors, are protected against mechanical or toxic insults to the dopamine neurons (Rousselet et al. 2002; Sriram et al. 2002; Wessig et al. 2005). Thus, although TNFR2 is supposed to exert neuroprotective properties, both receptors need to be blocked to prevent degenerative effects of TNF α .

Astrocytes and microglia are mobilized after injury, infection, or in neurodegenerative diseases. Especially, the loss of dopaminergic neurons in Parkinson's disease is associated with an increased glial reaction. Astrocytes respond particularly to proinflammatory cytokines and it is believed that cytokines participate in astrocyte activation (McNaught and Jenner 2000). In organotypic slices cultures of fetal ventral mesencephalon (VM), dopamine neurons form their nerve fibers either in the absence of astroglial cell bodies or onto a monolayer of astrocytes (Johansson and Strömberg 2002). The two sequences of outgrowth are temporally separated, and the first formed nerve fibers appear prior to proliferation and migration of astroglia. When the astrocytes proliferate, they also migrate away from the tissue slice and form a monolayer to finally surround the tissue slice. As the astrocytes start to migrate, the initially formed nerve fibers are retracted and the secondary formed nerve fibers grow onto the astrocytes. Both types of outgrowth are often visible in the same culture over different areas of the periphery of the tissue slice, but over time, nerve fibers representing the initial wave of growth disappear and the newly formed nerve fibers are attached to astrocytes (Berglöf et al. 2007; Johansson and Strömberg 2003). Since proinflammatory cytokines are toxic to dopamine neurons, but leave a subset of dopamine neurons unaffected (McGuire et al. 2001), and affect astrocytes, which appear to exert important guiding cues on dopamine nerve fiber formation, the aim of this investigation was to study effects of TNF α on astrocytic migration and how this affected dopamine nerve fiber formation. Therefore, effects of TNF α were investigated during early or late treatment to determine possible diverging effects on nerve fiber formation with regard to their relationship to astrocytes.

2. RESULTS

In E14 ventral mesencephalic (VM) organotypic slice cultures, tyrosine hydroxylase (TH) - positive nerve fibers appeared in two morphologically different growth patterns, either in the presence or the absence of migrating astrocytes (Fig. 1). TH-positive nerve fibers were found in the absence of glial cells, as revealed by negativity to S100-immunoreactivity and DAPI-

positive nuclei, while the TH-positive growth formed onto migrating astrocytes became obvious first when astrocytes had started to migrate to form a monolayer surrounding the tissue slice. The two growth patterns were often displayed over different areas of the periphery of the same tissue slice.

Effects of early TNF α treatment

VM cultures were treated with TNF α (40 ng/ml) and effects were examined at 7 or 19 days in vitro (DIV) using TH- and S100-immunohistochemistry. Treatment with TNF α during days 4–7 in vitro reduced the number of cultures with the non-glial-associated TH-positive growth. At 7 DIV, 16.0% of the cultures treated with TNF α displayed the non-glial-associated TH-positive growth while it was found in 36.8% of control cultures (Table 1). At 19 DIV, the number of cultures where the non-glial-associated growth was present was reduced to 31.6% in control cultures and remained at 16.7% after the early TNF α treatment (Table 1). The non-glial-associated TH-positive outgrowth often had a dotted appearance as if nerve fibers were degenerating at the longer time point.

The early TNF α treatment (days 4–7) promoted the presence of glial-associated TH-positive outgrowth to an extent of 88.0% at 7 DIV compared to 78.9% in the control cultures (Table 1). The length of the glial-associated TH-positive outgrowth treated with TNF α between days 4–7 was significantly enhanced ($p < 0.001$; $F_{6,115} = 12.64$; control $n = 30$, TNF α days 4–7 $n = 27$) compared to control cultures at 7 DIV (Fig. 1a, b and Fig. 2a). Nerve fiber outgrowth in treated cultures reached nearly twice the length compared to controls. At 19 DIV, there was no difference in the length of glial-associated TH-positive outgrowth after the early TNF α treatment (Fig. 2a). TH-positive neurons had no apparent change in morphology after TNF α treatment (Fig. 3a–d).

Astrocytes migrated significantly longer distances ($p < 0.001$; $F_{6,157} = 39.88$; control $n = 34$, TNF α days 4–7 $n = 28$) from the periphery of the tissue slice after the early TNF α treatment when compared to control cultures at 7 DIV (Fig. 1a, b and Fig. 2b). At 19 DIV, no difference in astrocytic migration was observed between TNF α treated and control cultures.

Effects of late TNF α treatment

At 19 DIV, the non-glial-guided TH-positive outgrowth was absent in cultures treated with TNF α between days 11–14, while still present in 31.6% of control cultures (Table 1). There was no difference in the length of glial-associated TH-positive outgrowth after the late TNF α treatment when compared to control cultures at 19 DIV (Fig. 2a). Furthermore, TNF α treatment did not affect the distance that astrocytes migrated (Fig. 1c, d and Fig. 2b).

TNF α effects on the presence of microglia

The presence of Iba-1-immunoreactive microglia was studied after treatment with TNF α . The early TNF α treatment (days 4–7) did not affect the number of microglia that was present in the tissue slice at 7 DIV (Fig. 4a). At 19 DIV, the number of Iba-1-immunoreactive microglia was moderately reduced, but no significant difference compared to cell counts performed at 7 DIV or to control cultures at 19 DIV. Counting microglial profiles after the late TNF α treatment (11–14 DIV) demonstrated a significant augmentation in the number of microglia at 19 DIV, when compared to control cultures or cultures treated at the earlier time point ($p < 0.05$; $F_{4,42} = 1.94$; control $n = 6$; TNF α days 4–7 $n = 8$; TNF α days 11–14 $n = 9$; Fig. 3e–j and Fig. 4a).

Effects of antibodies against TNFR1 or TNFR2

The effects of TNF α were investigated by adding antibodies against TNFR1 or TNFR2 to the medium to block the effects mediated through TNFR1 or TNFR2, respectively. VM slice

cultures were treated between days 4–7 and TH- and S100-immunohistochemistry were performed at 7 DIV. First, single treatment with antibodies against TNFR1 and TNFR2 was investigated. Since the antibody solutions contained sodium azide, control cultures were given the same concentrations as in the antibody solutions, i.e. 12.5 µg/ml and 25.0 µg/ml sodium azide, and compared to untreated controls. The results revealed no differences in astrocytic migration, TH-positive outgrowth or distribution of non-glial vs. glial-associated outgrowths. Therefore control cultures were pooled to one group.

VM cultures treated with antibodies against TNFR1 or TNFR2 was performed in the concentrations of 2.5 and 5.0 µg/ml during 4–7 DIV. Both doses and antibodies affected TH-positive outgrowth such that the non-glial-mediated nerve fibers were present and measured similar lengths, around 1 mm, as compared to controls (1127 ± 114 µm for controls, 994 ± 135 µm for TNFR1 antibody, 1117 ± 86 µm for TNFR2 antibody using 5.0 µg/ml antibody). The glial-associated outgrowth could not be found after antibody treatment in any dose or treatment. The overall impression of TH-immunoreactivity in cultures treated with low (2.5 µg/ml) or high (5.0 µg/ml) dose of antibodies against TNFR1 or TNFR2 revealed that the neurons did not appear healthy, and the tissue contained many TH-immunoreactive dots as if neurites were degenerating. Most of the TNFR2 antibody-treated cultures in the high dose (5.0 µg/ml) came loose during the incubation. Therefore, no measurements were possible to be performed on those cultures. The high dose (5.0 µg/ml) of antibodies against TNFR1 reduced astrocytic migration from 459.6 ± 30.9 µm in control cultures to 310.7 ± 72.8 µm after treatment and the length became significantly shorter compared to TNFα treatment alone ($p < 0.05$, $F_{9,115} = 10.19$, control $n=30$, TNFR1 antibody $n=7$, TNFα $n=28$). The low dose of antibody treatment did not affect the attachment of the cultures to the substrate and both TNFR1 and TNFR2 antibody treatments reduced the lengths of astrocytic migration compared to TNFα treatment but did not differ from controls (Fig. 4b).

Effects of antibodies against TNFR1 or TNFR2 on TNFα treatment

At 7 DIV, cultures treated with TNFα in combination with antibodies against either TNFR1 or TNFR2 in two doses, a low (2.5 µg/ml) or a high (5.0 µg/ml) dose. TNFα treatment with antibodies against TNFR1 enhanced the number of cultures displaying the non-glial-associated growth to 50.0% (2.5 µg/ml) and 63.6% (5.0 µg/ml) from 16.0% of cultures treated with TNFα only. The addition of the high dose of antibodies against TNFR2 (5.0 µg/ml) did not affect the presence of non-glial-associated TH-positive growth compared to TNFα treatment only, 14.0% and 16.0%, respectively. However, when adding the low dose of TNFR2 antibodies (2.5 µg/ml) to TNFα treatment, the non-glial-associated growth was observed in 50.0% of the cultures (Fig. 5).

Blocking TNFR1 resulted in reduced number of cultures displaying the glial-associated TH-positive growth to 45.0% of the cultures after the early TNFα treatment. The distance these nerve fibers reached was significantly shorter (264.8 ± 44.48 µm) than that measured for TNFα treated cultures (627.4 ± 93.1 µm; $p < 0.01$; $F_{8,131} = 14.029$; TNFα days 4–7 $n=27$; TNFα + TNFR1 antibodies $n=9$), but within the same range as for control cultures at 7 DIV. In only 18.2% of the VM cultures treated with TNFα and antibodies against TNFR2 the glial-associated TH-positive growth was displayed. The distance these nerve fibers reached from the tissue slice was comparable to control cultures at 7 DIV. However, these TH-positive nerve fibers were visualized as dots rather than undisrupted nerve fibers and had no healthy appearance and were therefore never used for the statistics. The two doses used gave similar results.

By blocking either TNFR1 or TNFR2 (both doses) in VM cultures treated with TNFα between days 4–7, astrocytes migrated significantly shorter distances from the tissue slice compared to cultures treated with TNFα at 7 DIV ($p < 0.001$; $F_{9,115} = 10.19$; control $n=30$; TNFα days 4–7

n=28; TNF α + TNFR1 antibodies n=7; TNF α + TNFR2 antibodies n=6; Fig. 4b). There was no difference when compared to control cultures at the same time point.

Blocking TNF α function

Since the results from VM cultures treated with either antibodies against TNFR1 or TNFR2 alone or together with TNF α were similar, the next experiment was performed to neutralize any possible endogenous production of TNF α . Thus, adding TNF α antibodies to the medium resulted in no TH-positive outgrowth, although TH-positive neurons were present within the tissue slice. Furthermore, the TH-positive neurons were located to one cluster of neurons and no viable TH-immunoreactivity was found outside the area of TH-positive neurons (Fig. 5b, c). The S100-positive astrocytes migrated significantly shorter distances when compared to TNF α treated astrocytes ($p < 0.001$; $F_{9,115} = 10.19$; TNF α antibody n=7; TNF α n=28), but did not differ from controls (Fig. 4b). In addition, the migrated S100-positive astrocytes appeared shrunken in size compared to astrocytes in any other treatment (Fig. 5d, e).

Effects of TNF α treatment on astrocyte proliferation

The distance that astrocytes migrated from the periphery of the VM tissue slice was significantly longer when treated with TNF α between days 4–7 compared to control cultures, an effect that was blocked by adding antibodies against TNFR1, TNFR2, or TNF α . Cultures were therefore processed for immunohistochemistry using the astrocytic marker S100 and for the antibody against Ki67. Ki67 is expressed in all proliferating cells during G1, S, M and G2 phases of the cell cycle. Cell counts of S100/Ki67-positive astrocytes revealed no difference in the number of cells in cultures exposed to TNF α or by adding TNF α along with TNFR1 antibodies as compared to control cultures 7 DIV (Fig. 1f–h and Fig. 6). Thus, TNF α stimulated the migration but not the proliferation of astrocytes.

3. DISCUSSION

The main findings from this study demonstrate that TNF α exerts dual actions i.e. toxic and growth promoting. Thus, TNF α is toxic to the non-glial-associated nerve fiber production and the effect is mediated via TNFR1, while TNF α enhances the glial-guided TH-positive outgrowth during an early, but not late, event, which requires both TNFR1 and TNFR2. The triggered glial-guided nerve fiber formation might be an indirect effect caused by improved astroglia migration. Late TNF α treatment, between days 11–14, did not affect the glial-associated outgrowth but increased the number of microglia present in the cultures, while the early treatment did not affect the presence of microglia.

In previous studies, TNF α has shown to act detrimentally on dopamine neurons. For instance, intraparenchymal injection of TNF α into the medium forebrain bundle of the rat leads to dopamine neuron cell loss in ventral mesencephalon (Carvey et al. 2005). The toxic action has been suggested to be mediated via TNFR1 and data presented by Clarke et al. indicate improved survival of transplanted fetal dopaminergic neurons by including TNFR1 antibody in the cell suspension to block TNF α (Clarke and Branton 2002). The action of TNF α appears more complicated than regarding the effects either to be toxic strictly through TNFR1 or to act growth promoting via TNFR2 only, as previously suggested (Fontaine et al. 2002). For instance, TNF α levels are increased after MPTP treatment (Sriram et al. 2002). However, both TNFR1 and TNFR2 need to be deleted to protect against MPTP lesions (Sriram et al. 2002). Thus, one of the two receptors is not enough to block the toxic effect. In the present study, TNF α improved the glial-guided TH-positive outgrowth and migration of astrocytes. Separate blocking the exogenously added TNF α by each of the two receptors, equally affected the distances that migrating astrocytes and glial-guided outgrowth reached. It is known that TNFR1 is expressed in astrocytes (Figiel and Dzwonek 2007; McGuire et al. 2001). Furthermore, TNF α upregulates

the expression of TNFR2, which normally is low in astrocytes (Choi et al. 2005). TNF α induces also astrogliosis (Sriram et al. 2002), which leads to enhanced production of astrocytic glial cell line-derived neurotrophic factor (GDNF) (Kuno et al. 2006). Interestingly, in VM cultures from GDNF knockout tissue, the astrocytic migration and glial-mediated nerve fiber outgrowth is impaired (af Bjerkén et al. 2006). Thus, blocking one of the two TNF α receptors might impair production of growth promoting factors. Furthermore, treating the VM cultures with antibodies against either of the two TNF α receptors alone abolished the glial-associated nerve fiber formation. Moreover, adding antibodies against TNF α to the cultures totally blocked all nerve fiber production. This indicates that the shortened glial-associated outgrowth seen after TNF α plus receptor antibody treatment was probably due to a dose effect. The results indicate that although adding TNF α to the cultures improved the glial-associated outgrowth there is an endogenous production of TNF α in the tissue, or the medium, which is important for nerve fiber formation during development, since no nerves were present after TNF α antibody incubation. Therefore, both TNFR1 and TNFR2 appear to be needed for TNF α to stimulate the migration of the astrocytes and the glial-guided TH-positive outgrowth at the early time point. Thus, TNFR1 appears in this situation to mediate growth-promoting effects.

The toxic effect of TNF α was demonstrated on the non-glial-associated outgrowth, which was reduced by half of the control cultures during the early treatment and absent after the late treatment. Blocking TNFR1 counteracted this effect. Thus, it appears that TNF α , when acting through TNFR2, promoted the presence of non-glial-associated nerve growth, especially since blocking TNFR1 actually enhanced the growth compared to control cultures. This was further demonstrated when blocking TNFR2 using the high dose, leaving TNFR1 active, which did not change the presence of non-glial-associated growth compared to treatment with TNF α alone. Thus, it seems that the subpopulation of TH-positive neurons producing the non-glial-associated nerve fibers are sensitive to TNF α toxicity via TNFR1. Furthermore, TNF α could promote the presence of the non-glial-associated growth when acting through TNFR2. Thus, in this case it appears as if TNFR1 and TNFR2 have direct opposite effects.

Although the presence of the non-glial-associated growth was affected by TNF α , it seemed that at 19 DIV, the TH-positive neurons producing the glial-associated nerve fibers were still robust, showing no difference from control cultures, when treated at the early or late time points. Other studies have shown a low, but still surviving subpopulation of cultured TH-positive neurons after treatment with TNF α (McGuire et al. 2001). Thus, subpopulations of dopamine neurons are affected differently by TNF α . Interestingly, it has been documented that TNF α treatment of cultures from E12.5 fetuses can enhance the number of dopamine neurons while the same treatment reduces the number of dopamine neurons in cultures from E14 and E16 fetuses (Doherty 2007). This might be due to the fact that the survival time was much shorter in those experiments allowing no comparison for long-term culturing as in the present study. Thus, if TH-positive neurons producing the glial-mediated dopamine nerve fibers were lost during TNF α treatment, it did not affect the nerve fiber production at long-term survival.

When plating TH-positive neurons from fetal ventral mesencephalon, subpopulations of dopamine neurons from A8, A9, and A10 are included (Dahlström and Fuxe 1964). It is well known that A9 dopamine neurons are more vulnerable to toxic insults than A10 neurons (Barroso-Chinea et al. 2005; Gibb 1992; Gonzalo-Hernandez et al. 2004; Kish et al. 1988). It cannot be determined from this study whether TNF α treatment affected specifically A9 or A10 dopamine neurons. However, the late TNF α treatment killed 100% of the non-glial-mediated nerve fibers. A9 and A10 TH-positive neurons can form both the non-glial- and glial-guided TH-positive outgrowths (Berglöf et al. 2007), which suggests that the toxic effect of TNF α affected both A9 and A10 neurons and was not correlated to either subtype of dopamine neurons, but rather to the type of morphological nerve fiber growth. Indeed, the TNF α receptors are differently expressed over subgroups of lateral and medial substantia nigra dopamine

neurons (Duke et al. 2007), which suggests that subgroups of A9 neurons need to be considered for differences in vulnerability. In conclusion, TNF α is toxic for the non-glial-associated dopaminergic nerve fiber outgrowth when mediated through the TNFR1, but not the TNFR2. Furthermore, TNF α stimulates the glial-guided outgrowth as well as the migration of astrocytes at an early time point, and this function requires both TNFR1 and TNFR2. Thus, TNF α exerts dual effects on VM dopamine neurons.

4. EXPERIMENTAL PROCEDURE

Dissection and tissue preparation

Fetal VM organotypic tissue cultures were prepared from tissue obtained from Sprague-Dawley pregnant rats. The animals were kept in a 12/12 h light/dark cycle and with access to food and water ad libitum. Pregnant rats were deeply anesthetized using isoflurane prior to neck dislocation, and the fetuses were obtained. The VM tissue was dissected from embryonic day (E) 14 fetuses under a dissection microscope and sterile conditions. Dulbecco's modified Eagle's medium (DMEM; Gibco) was used for dissection. The dissected tissue was sliced with a tissue chopper in 300 μ m coronal sections and transferred into DMEM, and each tissue slice was cut in the midline. One such piece was used for each culture. The experiments were approved by the local ethics committee.

Tissue cultures

The tissue slices were plated on washed, autoclaved, and poly-D-lysine coated (5 mg/100 ml dH₂O; Sigma-Aldrich) 12 $\times$ 24 mm coverslips using the "roller-drum" culture technique (Gähwiler et al. 1997). Each tissue piece was placed in two drops of chicken plasma (Sigma-Aldrich). One drop of thrombin (1,000 units/ml; Sigma-Aldrich) was mixed with the chicken plasma and the tissue piece was placed in the center of the coverslip. The plasma/thrombin clot was dried for 15–20 minutes before the coverslips were placed in 15 ml Falcon tubes containing 0.9 ml of medium. The tubes were inserted into a "roller-drum" placed in an incubator. The cultures were continuously rotating at a speed of 0.5 turns per minute.

The culture medium contained 55% DMEM, 32.5% Hanks' balanced salt solution (Gibco), 10% fetal bovine serum (Gibco), 1.5% glucose (Gibco) and 1% Hepes (Gibco). All ingredients were mixed and filtered through a sterile filter with pore size of 0.22 μ m (Sterivex, Millipore). The medium was stored at –20°C and thawed to 37°C before use. Antibiotics were added to the medium to a final concentration of 1% (10,000 units/ml penicillin, 10 mg/ml streptomycin, 25 μ g/ml amphotericin; Gibco) and used at plating. At the first medium change and thereafter, antibiotic was excluded. The medium was changed twice a week.

Rat tumor necrosis factor alpha (TNF α ; 40 ng/ml; R&D Systems; see McGuire et al. 2001) was added to the medium between days 4–7 (n=65) or 11–14 (n=26) and the cultures evaluated at 7 (control n=102; TNF α days 4–7 n=47) or 19 (control n=36; TNF α days 4–7 n=18; TNF α days 11–14 n=26) days in vitro (DIV). Antibodies against TNF α receptor 1 (TNFR1; 2.5 μ g/ml; n=7 and 5.0 μ g/ml; n=16; goat polyclonal antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or TNF α receptor 2 (TNFR2; 2.5 μ g/ml; n=6 and 5.0 μ g/ml; n=15; rabbit polyclonal antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, (Clarke and Branton 2002; Tacheuchi et al. 2006)) were added together with TNF α to the medium between 4–7 DIV and evaluated at 7 DIV. In addition, antibodies against TNFR1 and TNFR2 (2.5 μ g/ml; TNFR1 n=4, TNFR2 n=5, or 5.0 μ g/ml; TNFR1 n=7, TNFR2 n=6) were added without TNF α to test for specific effects of the antibodies per se. Since sodium azide was included in the antibody solutions, control cultures (n=14) also received the same concentration of sodium azide as given to the cultures including antibodies (12.5 μ g/ml n=4; 25 μ g/ml n=6). TNF α and

TNF α + antibodies against TNFR1 or TNFR2 were added once, when medium change was made at day 4.

Finally, neutralizing antibodies against TNF α (0.15 μ g/ml; n=7; Serotec Oxford, UK) was added to the medium during days 4–7 in vitro. The dose was recommended by the manufacturer. The time points chosen for this study was based on when the two sequences of outgrowth appears: the non-glial-associated growth is pronounced already at day 3–5 and the glial-mediated outgrowth at day 10–14 (Johansson and Strömberg 2003).

Immunohistochemistry

The VM cultures were fixed at 7 or 19 DIV with 2% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4) for 1 h. After fixation, the cultures were rinsed 3 times for 10 minutes with phosphate buffered saline (PBS; pH=7.4) before incubation in primary antibodies raised against the indirect marker for dopaminergic cells i.e. tyrosine hydroxylase (TH; diluted 1/300; mouse monoclonal; DiaSorin, MN, USA) followed by either S100 (diluted 1/400; polyclonal rabbit; Dako Patts, Denmark) to visualize astrocytes, Iba-1 (diluted 1/1000; rabbit polyclonal; Wako Chemicals, Germany) to visualize microglia, or Ki67 (diluted 1/150; mouse monoclonal; Novocastra Laboratories Ltd. UK), which is expressed in all proliferating cells during G1, S, M and G2 phases of the cell cycle (Schluter et al. 1993). Antibodies against Ki67 were applied after preincubation in PBS containing 0.3% Triton-x-100 for 1h before antibody application. Antibody incubations were performed in 48–72 h at 4°C. After rinsing in PBS, cultures were incubated with the secondary antibodies Alexa 488 (1/500; goat-anti-mouse or 1/200; goat-anti-rabbit; Molecular Probes, USA) and Alexa 594 (1/500; goat-anti-mouse or 1/500; goat-anti-rabbit; Molecular Probes, USA) for 1h at room temperature. Additionally, DAPI (1/50, Molecular Probes, USA) was used to stain the cell nuclei. Cultures were incubated in DAPI for 10 min at room temperature. All antibodies (except Ki67) and DAPI were diluted in 1% Triton X-100 in PBS. Incubations were performed in a humidified atmosphere. All cultures were triple labelled, which was performed in sequence with one antibody at the time. After additional rinsing the cultures were mounted in 90% glycerin in PBS.

Image analysis and statistics

Image analysis using Openlab software (Improvision) was utilized to analyze the cultures. The distances that the TH-positive nerve fibers had reached and the astrocytes had migrated were analyzed. The distances were measured from the periphery of the tissue slice to the distal end that the TH-positive nerve fibers or astrocytes had reached. The periphery of the tissue slice was defined based on the density of DAPI-positive cell nuclei. As the astrocytes started to migrate from the tissue piece, the density of DAPI-positive nuclei was drastically reduced. All cultures, where TH-positive nerve fiber outgrowth was determined, were processed for S100-immunohistochemistry. The TH-positive nerve fibers were defined as glial-associated when growing on astrocytes and as non-glial-associated when no astrocytic cells were present among the nerve fibers. Four measurements of glial-associated TH-positive growth as well as for astrocytic migration were performed for each culture. Cultures with less than 50 TH-positive neurons were excluded. The number of Iba-1-positive cells present in the tissue slices was counted using a standardized frame and a 20x objective in 2 areas per culture. Ki67-positive astrocytes were calculated in the area of migrating single layer of astrocytes using the same procedure as for the Iba-1-positive microglia. All measurements were performed randomly on a blind basis. Statistical analysis were performed on means per slice culture using one-way analysis of variance followed by Fisher post hoc analysis and expressed as means $\pm$ SEM.

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Fig. 1.

Dopamine nerve fiber outgrowth after treatment with $\text{TNF}\alpha$. Dopamine neurons were visualized using tyrosine hydroxylase (TH) and astrocytes were visualized using S100, for cell nuclei staining, DAPI was used in VM organotypic tissue cultures. The VM cultures were treated with $\text{TNF}\alpha$ between days 4–7 (b) or between days 11–19 (d) and fixed either day 7 (a, b) or day 19 (c, d). The glial-guided TH-positive outgrowth is enhanced by the early treatment (b), and the astrocytic migration reached longer distances from the tissue slice compared to that measured in the control cultures at 7 DIV (a). $\text{TNF}\alpha$ treatment of VM cultures evaluated at 19 DIV (d) did neither demonstrate a difference for the glial-guided outgrowth nor for the astrocytic migration compared to control at day 19 (c). The non-glial-associated TH-positive outgrowth was determined by the absence of glial cells, seeing by the negativity to DAPI (e). In cultures processed for the astrocytic marker S100 and the proliferation marker Ki67 (f–h), the number of Ki67-positive cells did not differ between the control (f), cultures treated with $\text{TNF}\alpha$ (g), or $\text{TNF}\alpha$ plus antibodies against TNFR1 (h). Scale bar: a–d = 100 μm , e–h = 150 μm .

Fig. 2.

The distances for glial-guided TH-positive outgrowth and astrocytic migration from the periphery of the tissue slice. The distance of glial-guided TH-positive outgrowth in cultures treated with TNF α between days 4–7 (n=27) was significantly longer than that measured in control cultures at 7 DIV (n=30) (a). This difference seen at 7 DIV was no longer present at 19 DIV (control n=25, TNF α days 4–7 n=7, TNF α days 11–14 n=16). Thus, the enhanced outgrowth seen at the early time point in TNF α treated cultures, reached its maximal length before control cultures did. The migration of S100-positive astrocytes was significantly enhanced after TNF α treatment between days 4–7 at 7 DIV (n=28) (b). TNF α treatment

between days 11–14 did not induce this effect when studying the cultures at 19 DIV (control n=30, TNF α days 4–7 n=10, TNF α days 11–14 n=17). ***p<0.001

Fig. 3.

Effects of TNF α on TH-positive cell morphology and on the presence of Iba-1-positive microglia. The appearance of TH-positive neurons (a–d) was not affected by the early (b) or late (d) TNF α treatment as compared to controls at 7 (a) or 19 (c) DIV. Microglia cells were more frequently found in cultures treated between days 11–14 (g, j) compared to the early treatment (f, i) and control cultures (g, h) at 19 DIV. A change in morphology of the microglia from a flat and rounded in control cultures and after the early treatment (e, f) to process-bearing microglia could be observed after TNF α treatment during days 11–14 (g). Scale bar: a–d = 50 μ m, e–g = 75 μ m, h–j = 230 μ m.

Fig. 4.

Cell counts of microglia and astrocytic migration after blocking the effects of TNF α with antibodies against TNFR1, TNFR2, or TNF α . When counting the number of microglial cells using the pan-microglia marker Iba-1, no difference was found at 7 DIV (control n=11, TNF α days 4–7 n=13) (a). At 19 DIV the number of microglia was significantly increased after TNF α treatment between days 11–14 (n=9), compared to control (n=6) and to cultures treated between days 4–7 (n=8) (a). Astrocytic migration was determined in VM cultures treated with TNF α (n=28) and antibodies against either TNFR1 (n=4, 2.5 $\mu\text{g}/\text{ml}$) or TNFR2 (n=5, 2.5 $\mu\text{g}/\text{ml}$) alone, or TNF α together with the receptor antibodies between days 4–7 (b). The distance that astrocytes migrated from the tissue slice did not differ from control level (n=30) when

blocking TNF α with antibodies against either TNFR1 (n=7) or TNFR2 (n=6) (b), while TNF α (n=28) treatment alone significantly enhanced astrocytic migration. Neutralizing TNF α by adding TNF α antibodies (n=7) resulted in reduced astrocytic migration compared to TNF α treated cultures. None of the treatments, except for TNF α , were changed from controls. *p<0.05, ***p<0.001

Fig. 5. Blocking the effects of $\text{TNF}\alpha$ with antibodies against TNFR1 , TNFR2 or $\text{TNF}\alpha$. TH- and S100-immunoreactivity in control (a, d), and antibody treatment of $\text{TNF}\alpha$ (b, c, e), TNFR1 (f; 2.5 $\mu\text{g/ml}$), TNFR2 (h; 2.5 $\mu\text{g/ml}$) and $\text{TNF}\alpha$ plus antibodies against TNFR1 (g) and $\text{TNF}\alpha$ plus antibodies against TNFR2 (i) in VM cultures at 7 DIV. Blocking $\text{TNF}\alpha$ receptors promoted the presence of non-glial-associated TH-positive outgrowth. Neutralizing endogenous $\text{TNF}\alpha$ production by adding antibodies against $\text{TNF}\alpha$ abolished the presence of TH-positive nerve fibers (b). TH-positive neurons were found in cultures treated with antibodies against $\text{TNF}\alpha$, but degenerating areas in the tissue slice was apparent by the areas of TH-immunoreactive dots (c) and S100-positive astrocytes was smaller in size (e) compared to controls (d). Scale bar: a, b, c = 100 μm , d, e = 50 μm , f-i = 135 μm .

Fig. 6. Effects of TNF α on astrocytic cell division. In VM cultures treated with TNF α (n=6) or TNF α +antibodies against TNFR1 (n=9) between days 4–7 and studied at day 7 for S100 and the proliferation marker Ki67, there was no difference in the proliferation in the treated cultures compared to controls (n=13).

Table 1
The number of cultures expressing the non-glial- and glial-mediated TH-positive outgrowth in % of total number of cultures.

	7 DIV		19 DIV	
	Control	TNF α days 4-7	Control	TNF α days 4-7
% of the cultures with the non-glial guided TH-positive outgrowth	36.8%	16.0%	31.6%	16.7%
% of the cultures with glial-guided TH-positive outgrowth	78.9%	88.0%	94.7%	76.7%
				96.2%