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RESEARCH ARTICLE

Hypothermia-Induced Neurite Outgrowth is Mediated by Tumor Necrosis Factor-Alpha

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Keywords
brain slices, hypothermia, TNF-alpha, neurite outgrowth, traumatic brain injury, regeneration.

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Abstract

Systemic or brain-selective hypothermia is a well-established method for neuroprotection after brain trauma. There is increasing evidence that hypothermia exerts beneficial effects on the brain and may also support regenerative responses after brain damage. Here, we have investigated whether hypothermia influences neurite outgrowth in vitro via modulation of the post-injury cytokine milieu.

Organotypic brain slices were incubated: deep hypothermia (2 h at 17°C), rewarming (2 h up to 37°C), normothermia (20 h at 37°C). Neurite density and cytokine release (IL 1beta, IL-6, IL-10, and TNF-alpha) were investigated after 24 h. For functional analysis mice deficient in NT-3/NT-4 and TNF-alpha as well as the TNF-alpha inhibitor etanercept were used. Hypothermia led to a significant increase of neurite outgrowth, which was independent of neurotrophin signaling. In contrast to other cytokines investigated, TNF-alpha secretion by organotypic brain slices was significantly increased after deep hypothermia. Moreover, hypothermia-induced neurite extension was abolished after administration of the TNF-alpha inhibitor and in TNF-alpha knockout mice. We demonstrate that TNF-alpha is responsible for inducing neurite outgrowth in the context of deep hypothermia and rewarming. These data suggest that hypothermia not only exerts protective effects in the CNS but may also support neurite outgrowth as a potential mechanism of regeneration.

INTRODUCTION

Traumatic brain injury (TBI) remains a significant cause of death and severe disability worldwide. High morbidity and long-term problems in performing the activities of daily life are a result of a TBI (34). Systemic or brain-selective hypothermia has been established as an effective neuroprotective treatment in multiple studies; however, management of unwanted side effects, such as hypotension or hypovolemia, is of key importance (33). Several mechanisms of neuronal protection induced by hypothermia have been proposed, including reduction of the cerebral metabolic rate (9, 21), suppression of epileptic activity and seizures (36) and the reduction of excessive free radical production (27, 34).

Moreover, hypothermia can prevent secondary damage, which is initiated through inflammatory responses following TBI (2). Cytokines and activated microglia extend neuronal injury and sensitize the injured brain to a second insult. We have previously demonstrated that hypothermia suppresses inflammation in stimulated microglial cells via the ERK signaling pathway (38) and downregulates MCP-1 and interleukin (IL)-8 release in activated endothelial cells (7, 8).

Finally, there are some indications that hypothermia may not only influence neuronal cell survival but also promote regenerative responses after brain damage (37). Because hypothermia influences the inflammatory response after brain trauma and as inflammatory cytokines play a major role in modulating neurite outgrowth and regeneration (17, 18), we investigated in organotypic brain slice cultures whether hypothermia and rewarming influence neurite outgrowth after injury via modulation of the post-injury cytokine milieu. We analyzed the secretion of four inflammation-associated cytokines that have been shown to influence neurite extension. We demonstrated here that tumor necrosis factor (TNF)-alpha levels were significantly upregulated after hypothermia and rewarming in contrast to IL-1beta, IL-6 and IL-10. In functional assays we provide for the first time evidence that TNF-alpha is responsible for hypothermia-induced neurite extension.

These data indicate that TNF-alpha may play a key role in hypothermia-associated axon growth as a potential mechanism of

hypothermia-induced regeneration after central nervous system (CNS) trauma.

MATERIALS AND METHODS

Animals and factors

C57BL/6 wild-type mice, NT-3 and NT-4 knockout mice [NT-3$^{+/+}$/NT-4$^{-/-}$ and NT-3 ± /NT-4$^{-/-}$] as well as TNF-alpha-deficient mice (1) were used for the experiments on postnatal day two. It was necessary to use NT-3 ± /NT-4$^{-/-}$ mice, as those with a complete knockout for both NT-3 and NT-4 die within hours after birth. All mice were housed in a conventional animal facility (Center for Anatomy, Charité-Universitätsmedizin) and the experiments were performed in accordance with German guidelines on the use of laboratory animals.

K252a (Calbiochem, Schwalbach, Germany), which is a potent inhibitor of TrkA, TrkB and TrkC (but not of p75NTR) in nanomolar concentrations and does not block other tyrosine kinases (14, 23, 32), was used in concentrations of 100 nM or 1 μM in dimethyl sulfoxide (DMSO; Calbiochem, Bad Soden, Germany) compared with DMSO alone at the same concentration. Etanercept (Enbrel® Whyeth, Munster, Germany) was used in a concentration of 80 μg/mL to block TNF-alpha based on an effective *in vivo* concentration (12).

Acute organotypic slice cultures

Collagen type I from rat tail was dissolved in 0.1 M acetic acid at a final concentration of 2 mg/mL. Organotypic slice cultures were prepared on postnatal day 2 from mouse entorhinal cortex as previously described (20, 39). The collagen cultures were incubated in a humidified atmosphere with 5% CO_2 for 24 h according to the time-temperature protocol.

Time-temperature protocol

Acute organotypic brain slices were prepared and directly cooled down from 37°C to 17°C. Deep hypothermia (17°C) was maintained for 2 h followed by rewarming up to 37°C for a period of 2 h. After a follow-up phase of 20 h at 37°C cytokine release and neurite outgrowth were analyzed. Normothermic control slices were incubated at 37°C throughout the experiments.

Enzyme-linked immunosorbent assay (ELISA)

Conditioned medium from treated slice cultures was tested for IL-6, TNF-alpha, IL-1beta and IL-10 using commercially available sandwich ELISAs according to the manufacturer's instructions (BD Bioscience, Heidelberg, Germany). Absorbance was measured at 450 nm in the microplate reader (Multiskan Ascent; Thermo Electron Corporation, Vantaa, Finland).

Immunohistochemistry

The entorhinal cortices (ECs) were washed three times with phosphate-buffered saline (PBS) and fixed for 20 minutes in 4% paraformaldehyde. The ECs were then incubated for 1 h in PBS with 10% normal goat serum (NGS; Sigma, St. Louis, MO, USA) and 0.2% Triton X-100 (Sigma) in PBS. The first antibody, Tau-1 (mouse monoclonal, MAB3420, Millipore, Billerica, MA, USA) or glial fibrillary acidic protein (GFAP; mouse monoclonal, Sigma, G3893), was added (1:500) for 4 h (Tau1) or overnight (GFAP) with 10% NGS in PBS at room temperature (RT). The ECs were then washed three times in PBS (the last time for 20 minutes) and then incubated for 2 h at RT with the secondary antibody goat anti-mouse (1:1000), AL488 (A11029, Invitrogen, Carlsbad, CA, USA), in PBS with 10% NGS. Immunofluorescence was photodocumented with a fluorescent microscope (BX50; Olympus, Hamburg, Germany) and a CoolSNAPTIMES camera (Photometrics, Tucson, AZ, USA).

Measurement of neurite density

To evaluate neurite density we used a standard protocol for the evaluation of neurite outgrowth as described previously (20, 39) (Figures 1 and 3A,B), which was improved using image analysis software (Image J, Wayne Rasband, NIH) to quantify neurite density (Figures 2 and 4). The concave part of the entorhinal cortex explants was photo-documented using a 10× objective (Olympus IX70). To determine neurite density, image processing was based on the Sobel algorithm, which measures a two-dimensional spatial gradient emphasizing regions of high spatial density that correspond to neurites. The mean intensity was then calculated in a standardized area parallel to the brain slice edge in a micrograph of every organotypic brain slice.

Statistical analysis

The Mann–Whitney *U* one-way analysis of variance by ranks was used to determine whether significant differences were present. *P* values were considered significant when below 0.05.

RESULTS

We investigated the effects of deep hypothermia and rewarming on neurite outgrowth from acute organotypic brain slices using a dynamic time–temperature protocol over 24 h. Organotypic brain slices were embedded in a three-dimensional collagen matrix (Figure 1A). The concave part of the entorhinal cortex explants was photo-documented (Figure 1B). To confirm that the observed extensions from the brain slice are neurites, immunofluorescence was performed using a specific antibody against Tau-1 (Figure 1C). A specific antibody against GFAP as a marker for astrocytes did not mark any extension (Figure 1D) similar to the secondary antibody alone as negative control (Figure 1E). Higher magnification of Tau-1-labeled neurites showed characteristic growth cones (Figure 1F, arrows) suggesting that these neurites are axons and not dendrites. To precisely quantify neurite outgrowth we improved a standard protocol (20, 39) by using an image analysis software (Figure 2). The concave part of the entorhinal cortex explants was photo-documented using a 10× objective. Figure 2A illustrates a brain slice with intermediate outgrowth, and Figure 2B a slice with strong outgrowth; the white box in Figure 2A indicates the area shown at higher magnification in Figure 2E. To quantify the density of the outgrowing neurites (Figure 2A,B; arrowheads) image processing based on the Sobel algorithm was performed

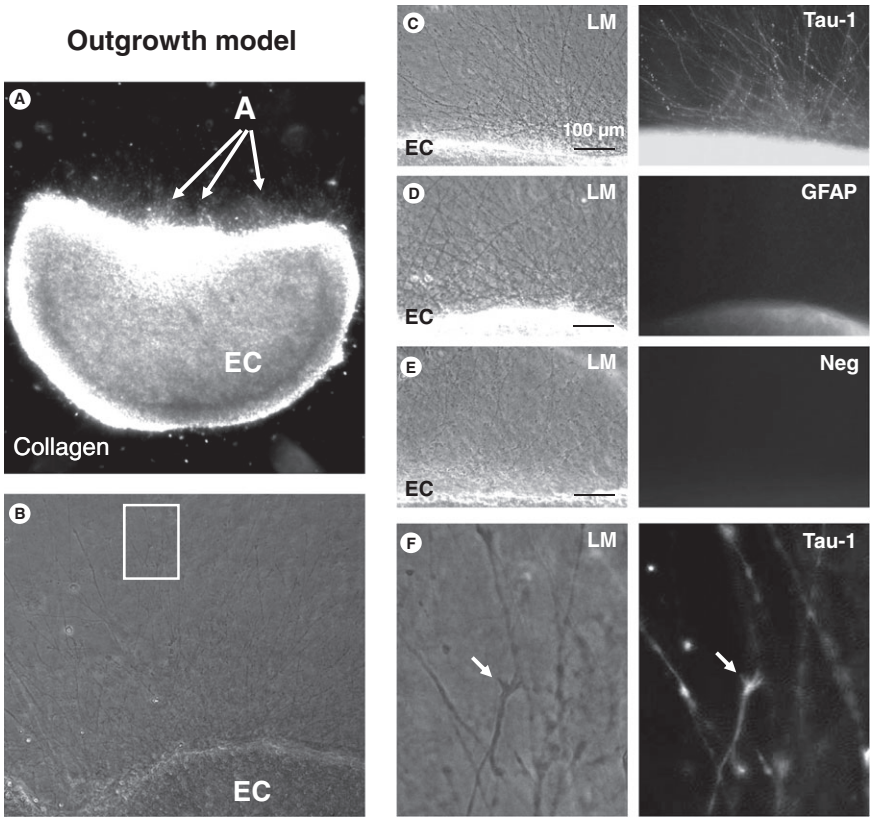

Figure 1. *Outgrowth model.* **A.** Outgrowth assay (see Material and Methods section for details); the entorhinal cortex (EC) explant was embedded in a three-dimensional collagen I gel matrix; the density of the outgrowing neurites (arrows) was photo-documented at higher magnification and quantified by two blinded investigators. **B.** Neurite outgrowth in control organotypic brain slices. The white box indicates the area shown at higher magnification in 1F. **C.** Multiple neurites are visible by light-microscopy (LM) growing out of the concave part of the EC explant. Immunofluorescence staining using specific antibodies reveals immunreactivity against Tau-1, which is found on neurites but not on glial cells. **D.** No immunoreactivity of the extensions is detectable using a specific antibody against glial fibrillary acidic protein (GFAP) as a marker for astrocytes and their extensions. **E.** No immunoreactivity of the extensions is found using the secondary antibody alone as a negative control. **F.** Higher magnification of the white box in 1B: Tau-1-labeled neurites at light microscopy (LM) show characteristic growth cones that are also immunoreactive for Tau-1 as a marker for neurites.

(Figure 2C,D). The mean intensity was then calculated in a standardized area parallel to the brain slice edge (indicated as white boxes in Figure 2C,D). At higher magnification characteristic growth cones are visible (Figure 2E, arrows). Compared with control brain slices (Figure 2F) that were kept at 37°C during the experiment, applying the dynamic time-temperature protocol over 24 h lead to significantly increased neurite density in brain slices after deep hypothermia and rewarming (Figure 2G,H).

As NT-3 and NT-4 are the major neurotrophins responsible for neurite growth from organotypic brain slices (Hechler & Hendrix, under review) we investigated brain slices derived from mice either homozygous for NT-4 deficiency (NT-4$^{-/-}$) or with a combined homozygous NT-4 deficiency and heterozygous NT-3 deficiency (NT-3 ± /NT-4$^{-/-}$) (a full NT-3 knockout is lethal). Neurite growth was still increased by hypothermia in brain slices derived from NT-4 KO mice (Figure 3A). Furthermore, a combination of NT-4 deficiency and a substantial reduction of NT-3, in mice homozygous for NT-4 deficiency and heterozygous for NT-3 deficiency, did not abolish the growth-stimulatory effect of hypothermia on neurites (Figure 3A).

To further investigate whether hypothermia-induced neurite outgrowth is independent of neurotrophin signaling, we applied K252a, which is a potent inhibitor of the neurotrophin receptors TrkA, TrkB and TrkC in nanomolar concentrations (14, 23, 32). The application of 100 nM to the culture medium reduced neurite growth from control slices but did not abolish the significant stimulation of neurite growth by deep hypothermia and rewarming (Figure 3B). A higher concentration of K252a (1 M), which may also block other tyrosine kinases, reduced neurite outgrowth in controls; however, there was still a strong and statistically significant stimulating effect of deep hypothermia and rewarming (Figure 3B).

In a next step we investigated whether the levels of selected inflammation-associated cytokines like IL-1beta, IL-6, IL-10 and TNF-alpha secreted by organotypic brain slices are modulated 24 h after experimental start by deep hypothermia and rewarming. The secretion of IL-1beta (37°C: 2200 fg/mL ± 110; hypothermia and rewarming: 1200 fg/mL ± 970), IL-6 (37°C: 166 pg/mL ± 9.19; hypothermia and rewarming: 168 pg/mL ± 9.90) and IL-10 (37°C: 5700 fg/mL ± 3970; hypothermia and rewarming: 6400 fg/mL ± 3360) by organotypic brain slices was not substantially modulated (Figs 3C–E) in contrast to TNF-alpha secretion, which was significantly increased nearly fourfold (37°C: 1200 fg/mL ± 230; hypothermia and rewarming: 4300 fg/mL ± 1140) after deep hypothermia and rewarming (Figure 3F).

Based on these finding we further explored whether the TNF-alpha upregulation plays a causal role in stimulating neurite extension after hypothermic treatment. As a first step, we demonstrated that TNF-alpha was sufficient to increase neurite extension from brain slices (Figure 4A,B,G). TNF-alpha increased neurite density by nearly 45%, a similar effect to that of deep hypothermia/rewarming (Figure 4G). Next we used the TNF-alpha inhibitor etanercept (Figure 4C,D,G), which fully abolished the stimulatory effect of hypothermia and rewarming. Furthermore, the absence of endogenous TNF-alpha in slices derived from TNF-alpha-deficient mice (Figure 4E–G) fully eliminated the effect of deep hypothermia and rewarming.

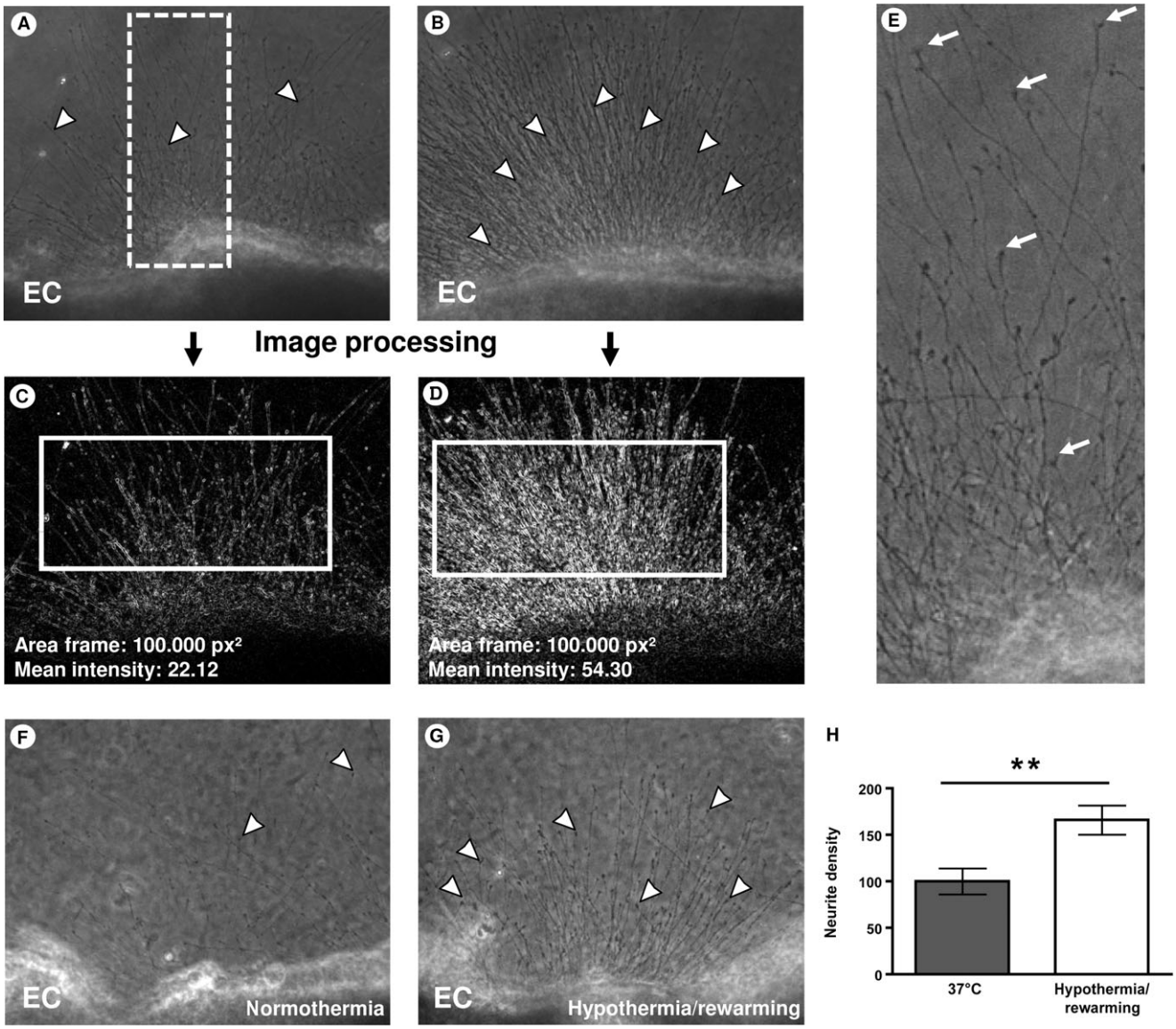

Figure 2. *Quantification by image analysis reveals stimulation of neurite outgrowth by hypothermia and rewarming.* **A, B.** Representative photomicrographs of a brain slice with intermediate outgrowth (A) and a brain slice with strong outgrowth (B); the white dotted box in Figure 2A indicates the area shown at higher magnification in 1E. **C, D.** To quantify the density of the outgrowing neurites (arrowheads) image processing based on the Sobel algorithm was performed to determine the mean intensity in a standardized area parallel to the brain slice edge (indicated as white boxes). **E.** Higher magnification of the white dotted box in 1A; characteristic growth cones (arrows) are visible at the end of the neurites. **F.** Control brain slices kept at 37°C during the experiment display average growth of neurites (arrowheads). **G.** Applying the dynamic time–temperature protocol over 24 h leads to significantly increased neurite density after deep hypothermia and rewarming. **H.** Analysis of neurite density reveals a significant increase of more than 60% in organotypic brain slice cultures after deep hypothermia. $**P < 0.01$; arrow heads: single neurites. Abbreviation: EC = entorhinal cortex.

DISCUSSION

TBI is a major source of death and disability. Estimates run as high as 10 million cases of TBI per year worldwide. Around 40% of the patients are left with permanent neurological disabilities (34). In addition to the emotional effects the financial burden is also enormous.

The neurological outcome after a period of ischemia is established to a substantial degree by mechanisms during the post-injury period. Therefore ischemia plays a key role in all forms of brain injury and preventing ischemic injury is central to all neuroprotective strategies. One well-established method for neuroprotection in the context of TBI is the direct application of systemic or brain-restricted hypothermia to prevent this secondary injury (5).

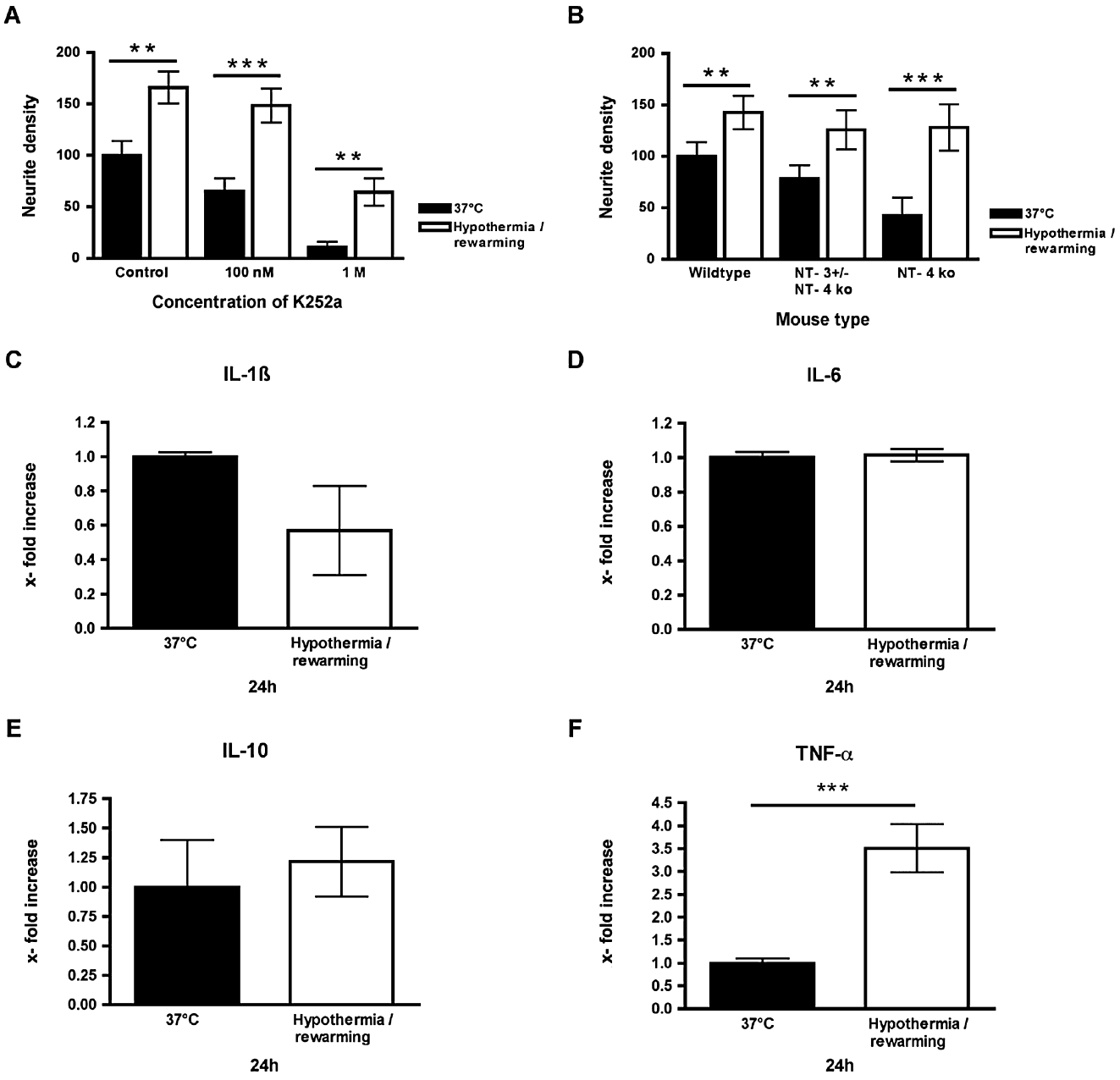

Figure 3. Hypothermia-induced neurite growth is independent of neurotrophins and deep hypothermia upregulates the secretion of TNF-alpha by organotypic brain slices. **A.** Neurite growth is increased by deep hypothermia and rewarming in wildtype mice as well as in NT-4 or NT-3 ± /NT-4 mice. **B.** Hypothermia-induced increase in neurite growth remains present after application of the pan-neurotrophin receptor antagonist K252a, which is a potent inhibitor of TrkA, TrkB and TrkC in nanomolar concentrations compared with the solvent DMSO alone at the same concentration. **C–E.** The levels of IL-1beta, IL-6 and IL-10 do not significantly change after deep hypothermia and rewarming. **F.** Deep hypothermia and rewarming significantly upregulate (nearly fourfold) the secretion of TNF-alpha by organotypic brain slices $***P < 0.001$.

In a meta-analysis looking at the effects of hypothermia on clinical neurological outcome in patients with TBI, most investigations showed that cooling can be effective if the treatment is initiated early and continued for long enough and if patients are rewarmed slowly (33). Therefore, in our cell culture model, acute organotypic brain slices were directly cooled down to 17°C and after 2 h the slices were rewarmed over a period of 2 h to 37°C. Deep hypothermia and rewarming hereby led to increased neurite outgrowth.

The multifaceted benefits of hypothermic treatment include a reduced rate of cerebral metabolism and epileptic discharges as well as the production of reactive oxygen species (9, 21, 27, 36).

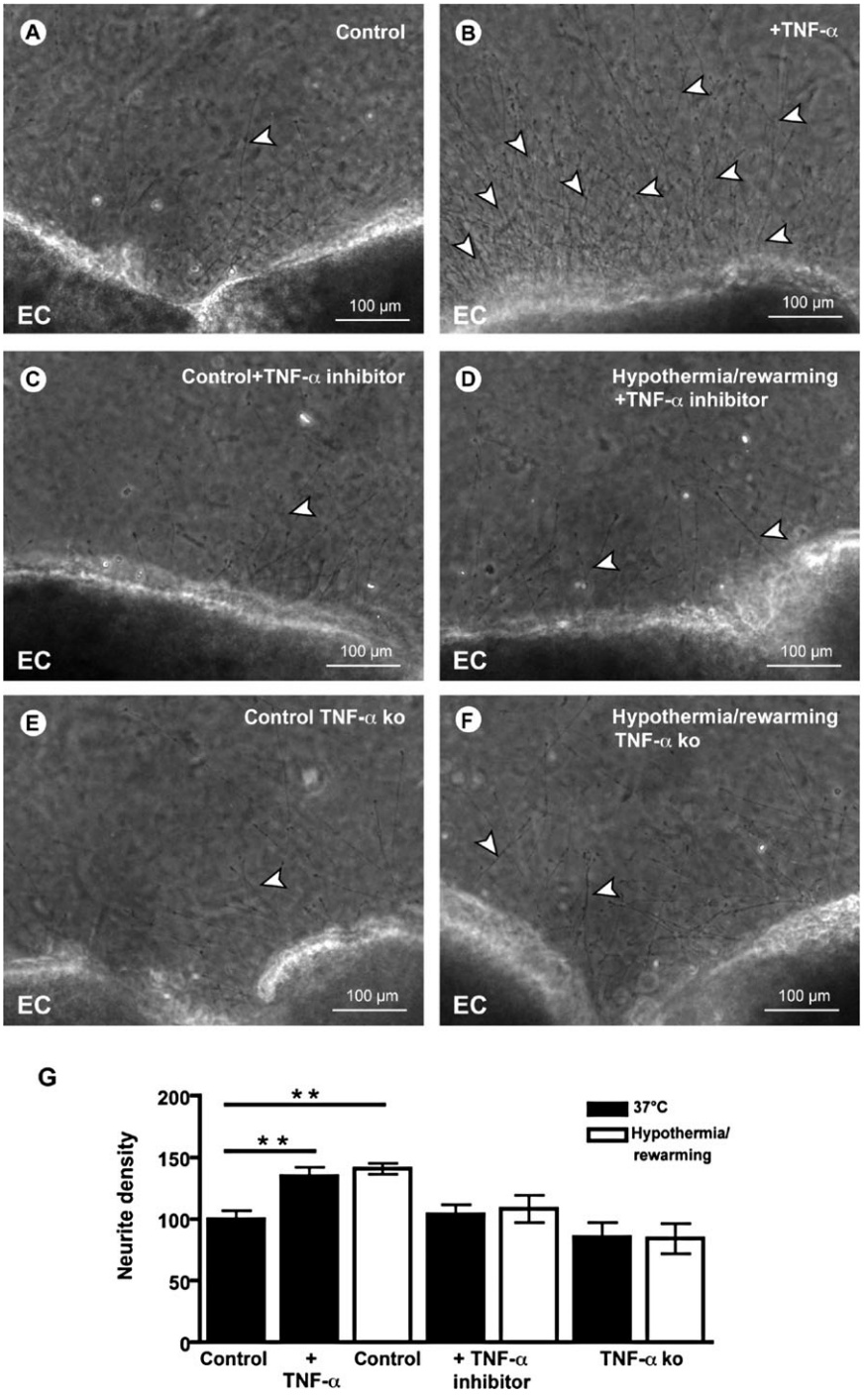

Figure 4. *TNF-alpha is necessary and sufficient for hypothermia-induced neurite growth.* **A.** Representative photomicrograph of a normothermic organotypic brain slice showing moderate outgrowth of neurites (arrowhead). **B.** Hypothermia and rewarming induce a substantial increase of the outgrowth of neurites (arrowheads). **C.** The TNF-apha inhibitor etanercept does not influence neurite outgrowth of normothermic brain slices compared with controls. **D.** Etanercept inhibits hypothermia-induced neurite growth. **E.** TNF-alpha absence in brain slices derived from TNF-alpha-deficient mice blocks hypothermia-induced neurite growth. **F.** TNF-alpha-deficient mice fully eliminated the effect of deep hypothermia and rewarming. **G.** TNF-alpha significantly increases neurite density similar to hypothermia and rewarming. In the presence of the TNF-alpha inhibitor etanercept and in the absence of TNF-alpha in TNF-alpha-deficient mice the hypothermia-induced increase in neurite growth is abolished. $^{**}P < 0.01$. Abbreviation: EC = entorhinal cortex.

We and others have previously demonstrated that hypothermia also modulates the brain immune system (37–39, 42).

Based on these findings we addressed the question of whether hypothermia may support not only neuronal cell survival (37) but also regenerative responses after brain damage (38). In contrast to previous studies focused mainly on neuroprotection we investigated whether hypothermia induces neurite growth via neurotrophins and/or inflammation-associated cytokines—namely IL-1beta, IL-6, IL-10 and TNF-alpha—that have been shown to modulate neurite outgrowth and regeneration (17, 18). Organotypic brain slice cultures underwent a dynamic time-temperature protocol of deep hypothermia and rewarming over 24 h resulting in a significant increase of neurite growth.

Organotypic brain slices are a well-established *in vitro* model to study, e.g., electrophysiological processes and cell survival as well as neurite plasticity, growth and regeneration (16). As brain slices are acutely cut out of the living brain they are in fact a trauma model because most neurites are dissected, the blood–brain barrier

is heavily damaged, many cells die, and astrocytes and immune cells become highly activated (10, 19, 43).

The extensions growing out of the organotypic brain slices show immunoreactivity against Tau-1, which is primarily found on axons but also on dendrites. In contrast, these extensions do not show imunoreactivity against GFAP, a standard marker for astrocytes and their extensions. Morphological criteria used in single cell cultures (e.g., the presence of an axon hillock, the angle of branches) are not applicable to fasciculated distal ends of neurites exiting from brain slices because the cell bodies are embedded in the slices. The Tau-1 immunoreactivity and the morphological appearance of the neurites, in particular the presence of multiple growth cones, are good arguments in favor of considering these extensions to be axons.

As NT-3 and NT-4 are the major neurotrophins responsible for neurite growth in organotypic brain slices (16), we investigated whether these neurotrophins are also responsible for the hypothermia-induced increase in neurite growth.

Surprisingly, hypothermia still increases neurite growth even in the absence of NT-4 in brain slices derived from NT-4 KO mice. Furthermore, the additional reduction in NT-3 secretion, in mice homozygous for NT-4 deficiency and heterozygous for NT-3 deficiency, does not abolish the growth-stimulatory effect of hypothermia on neurites.

As mice with a full knockout of NT-3 are not viable, we cannot determine with these experiments whether the remaining NT-3 still plays a role in hypothermia-induced neurite extension. To address the question of whether hypothermia-induced neurite outgrowth is independent of neurotrophin signaling, we applied K252a, which is a potent inhibitor of the neurotrophin receptors TrkA, TrkB and TrkC in nanomolar concentrations (14, 23, 32). Surprisingly, neurotrophin receptor inhibition by K252a application did not abolish hypothermia-induced increase in neurite extension. These data indicate that the increase of neurite extension by hypothermia is independent of neurotrophin signaling.

There is increasing evidence in the literature and from our own laboratory (17, 18) that inflammation-associated cytokines play a key role in the stimulation of axonal regeneration. Therefore, we further investigated whether hypothermia influences neurite outgrowth *in vitro* via modulation of the post-injury cytokine milieu. The analysis of the levels of IL-1beta, IL-6, IL-10 and TNF-alpha, which are known to influence neurite extension, revealed that only TNF-alpha secretion is significantly increased (nearly four fold) after deep hypothermia and rewarming of organotypic brain slices.

TNF-alpha is a pleiotropic cytokine that induces neuroprotective and neurotoxic effects after CNS injury (25, 26, 29). It is upregulated within 1 h after CNS injuries in neurons, astrocytes, macrophages and microglia. TNF-alpha exerts pro- or anti-apoptotic effects on CNS neurons depending on cell type and assay (4, 6, 30). Similarly, TNF-alpha has differential effects on neurite extension (17, 18). Previously, we and others have demonstrated in organotypic dorsal root ganglia cultures that TNF-alpha reduces neurite outgrowth in the peripheral nervous system (15, 24). In organotypic mesencephalic brain slices TNF-alpha has been shown to support glia-dependent neurite growth (28). In the present study we have demonstrated that TNF-alpha increases neurite outgrowth in brain slices by nearly 45%, thus, at a level comparable with the stimulatory effects of hypothermia.

The use of the TNF-alpha inhibitor etanercept as well as the absence of endogenous TNF-alpha in slices derived from TNF-alpha-deficient mice fully abolished the effect of deep hypothermia and rewarming. Interestingly, etanercept did not substantially influence cell survival (data not shown), suggesting that hypothermia-induced neurite extension is mediated by TNF-alpha whereas hypothermia-induced cell survival is an independently regulated process.

There are good indications that TNF-alpha may have protective and maybe even proregenerative functions in the damaged CNS *in vivo*. In two models of hippocampal excitotoxic and ischemic injury, mice deficient in both TNF receptors were found to be more susceptible (3, 11). Preconditioning with TNF-alpha appears to be neuroprotective in ischemic cerebral injury (13, 31). TNF receptor-deficient mice also show exacerbated brain damage after traumatic injury (41). Finally, TNF-alpha appears to facilitate the regeneration of the injured adult rabbit optic nerve.

In line with this discussion, it has been shown that chilling and rewarming of murine brain slices lead to a substantial proliferation of dendritic spines of mature hippocampal neurons (22). Similar structural changes have been reported for dendrites of CA3 pyramidal cells in ground squirrels after arousal from hibernation (35), suggesting a physiological role of neuronal plasticity after hypothermia and rewarming. The role of TNF-alpha in these processes is not known.

However, as TNF-alpha is a pleiotropic cytokine exerting neuroprotective as well as neurotoxic effects after CNS injury (40), it will be important to analyze whether the TNF-alpha-induced neurite growth shown in the present study is truly beneficial (i.e., pro-regenerative) or whether it may even have detrimental effects such as contributing to posttraumatic epileptogenesis.

The data presented here clearly show that TNF-alpha is necessary and sufficient to increase neurite outgrowth in the context of deep hypothermia and rewarming. This study provides for the first time evidence that hypothermia not only exerts protective effects in the CNS but also supports neurite outgrowth via TNF-alpha upregulation as a possible mechanism of regeneration.

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