Aus dem Institute of Cell Biology and Neurobiology

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Hypothermia and C3 peptide promote neurite outgrowth and regeneration after traumatic CNS injury

zur Erlangung des akademischen Grades

Doctor of Philosophy in Medical Neurosciences

(PhD in Medical Neurosciences)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

von

Francesco Boato

Aus Venedig

Gutachter: 1. Prof. Dr. Sven Hendrix

2. Prof. Elena E. Pohl

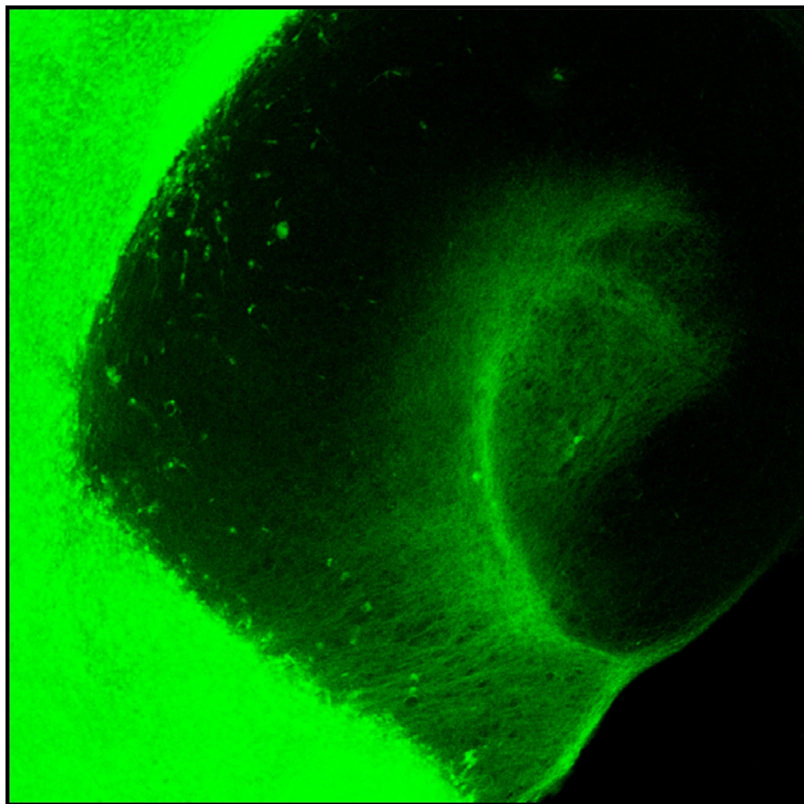
3. PD Dr. Kirsten Haastert

Datum der Promotion: 19/11/2010

Institute of Cell Biology and Neurobiology

Center for Anatomy
Charité – Universitätsmedizin Berlin

Hypothermia and C3 peptide promote neurite outgrowth and regeneration after traumatic CNS injury.



by Francesco Boato

Supporting Professor: Prof. Dr. Sven Hendrix

International Graduate Program Medical Neurosciences
Academic year: 2009-2010.

Table of Contents

1. Preface 5
2. Abstract 6
3. Introduction and Aims 7
4. Results 8
5. Discussion 12
6. Material and Methods 16
7. References 19
8. Declaration of own contribution 21
9. Complete list of publications 23
10. Selbstständigkeitserklärung 26
11. Acknowledgments 27

1. <u>Preface</u>

The present short dissertation has the aim to summarize three relevant and independent publications in which I participated during my PhD thesis (Höltje *et al.* 2009; Schmitt *et al.* 2009; Boato *et al.* 2010), with particular focus on my direct contribution. The dissertation follows the guidelines of the "Publication-Based Thesis" within the context of the "International Graduate Program Medical Neurosciences" at the Charité–Universitätsmedizin Berlin. Background information, methodological details as well as parts of results, figures and discussion had to be shortened due to space limitations, but can be found in the respective publications, which are inserted in their entire form in section 9 of this thesis.

2. Abstract

In response to injury and inflammation of the CNS, the expression of inflammatory mediators is often altered, and several of these factors contribute directly to the development of the neuronal injury. Hypothermia (systemic or brain-selective) influences the inflammatory response and is a well-established method for neuroprotection after brain trauma. Here we provide evidence that hypothermia led to a significant increase of neurite outgrowth from brain slices (independent of neurotrophin signalling), accompanied by an increased secretion of TNF-α. Moreover, hypothermia-induced neurite extension was abolished after administration of TNF-α inhibitor and in TNF-α knockout mice. We suggest then that hypothermia not only exerts protective effects in the CNS, but also support neurite outgrowth via TNF-α as a potential mechanism of regeneration.

Importantly TNF-α is known to exert its action trough the cellular pathway of the small GTPase RhoA, which plays an active and versatile role in the formation and development of axons and dendrites. Effects of RhoA are often studied by the Rho-inactivating C3 transferase (C3bot) from *Clostridium botulinum*. We previously reported that transferase-deficient C3bot also exerted axonotrophic activity. Using organotypical slice cultures and a hippocampal-entorhinal cortex lesion model, we detected trophic effects of a 29 amino acid transferase-deficient fragment from the C-terminus of C3bot ($C3bot^{154-182}$) on length and density of outgrowing fibers from the entorhinal cortex, that were comparable to the effects elicited by full-length C3bot. *In vivo*, functional recovery and regeneration of corticospinal tract (CST) fibers following spinal cord injury by compression or dorsal hemisection in mice was monitored after application of the transferase-deficient C3bot. $C3bot^{154-182}$ significantly improved locomotor restoration in both injury models as assessed by several behavioral paradigms. These data were supported by tracing studies showing an enhanced regenerative growth of CST fibers in treated animals. Additionally, $C3bot^{154-182}$ stimulated regenerative growth of raphespinal fibers and improved serotonergic input to lumbar α-motoneurons. The observed effects were probably due to a non-enzymatic down-regulation of active RhoA by the C3 peptide as indicated by pull-down experiments. In conclusion, $C3bot^{154-182}$ represents a novel, promising tool to foster axonal protection and/or repair, as well as functional recovery after traumatic CNS injury.

3. Introduction and Aims

Traumatic brain (TBI) and spinal cord (SCI) injuries are significant causes of death and severe disability worldwide; they result in high morbidity and long-term problems in performing the activities of daily life (1). Systemic or brain-selective hypothermia has been established as an effective neuroprotective treatment in multiple studies (2,3) and moreover can prevent secondary damage, which is initiated through inflammatory responses following injury (3). There are some indications that hypothermia may not only influence neuronal cell survival, but also promote regenerative responses after brain damage (4). For these reasons, and since inflammatory cytokines play a major role in modulating neurite outgrowth and regeneration (5, 6), our aim (7) was to investigate whether hypothermia and rewarming influence neurite outgrowth after injury via modulation of the post-injury cytokine milieu. We demonstrated that tumor necrosis factor-alpha (TNF-α) 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 for TNF-α involvement in hypothermia-induced neurite extension.

Importantly TNF-α is known to exert its action trough the cellular pathway of the small GTPase RhoA (8), which are key molecules in orchestrating cytoskeletal rearrangements linking surface signals to cytoskeleton-associated proteins (9-11). Bacterial C3 transferases have been used since their discovery (over 20 years ago) to study the function of Rho proteins in virtually all cellular systems of eukaryotic origin (12,13) and have been proven to foster neurite outgrowth and regeneration (14-18). Its mode of action, namely enzymatic inactivation of Rho proteins (especially RhoA) is well understood. Non-enzymatic interactions and cellular effects were also recently discovered. Using primary cultures of hippocampal neurons, it was demonstrated that C3bot possesses an additional axonotrophic function independent from its enzymatic activity (14). Our aim (15) was to identify the precise region of C3bot responsible for the neurotrophic effect, by using various C3bot-derived peptide fragments that lack enzymatic transferase activity. Application of a 29-amino acid fragment (C3bot$^{154-182}$) influenced also fiber outgrowth and reinnervation of target tissues in organotypical hippocampal/entorhinal slice cultures, more closely related to the *in vivo* situation. Furthermore, C3 proteins were successfully used to improve functional recovery after spinal cord injury (SCI, an established *in vivo* model for investigating the intrinsically limited neuronal regeneration of the CNS) (16,17). Since it was clear that C3bot also exerts its growth-promoting effects on neurons by an enzyme-independent activity, the aim of our most recent study (18) was to investigate the ability of C3bot$^{154-182}$ to stimulate central axonal repair and functional recovery after contusive SCI or dorsal hemisection. Additionally, the effect on the maintenance of neuromuscular junctions of tibial skeletal muscles and the putative C3bot$^{154-182}$-mediated effects on active RhoA levels of certain subsets of cultivated neurons were investigated.

4. 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 and the concave part of the entorhinal cortex explants was photo-documented. To confirm that the observed extensions from the brain slice are neurites, immunofluorescence was performed using a specific antibody against Tau-1. A specific antibody against GFAP as a marker for astrocytes did not mark any extension. Higher magnification of Tau-1-labeled neurites showed characteristic growth cones suggesting that these neurites are axons and not dendrites. To precisely quantify neurite outgrowth we improved a standard protocol (15,19) by using image analysis software. The concave part of the entorhinal cortex explants was photodocumented using a 10X objective (see the section material and methods Figure 3). To quantify the density of the outgrowing neurites image processing based on the Sobel algorithm was performed. The mean intensity was then calculated in a standardized area parallel to the brain slice edge. Compared with control brain slices 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.

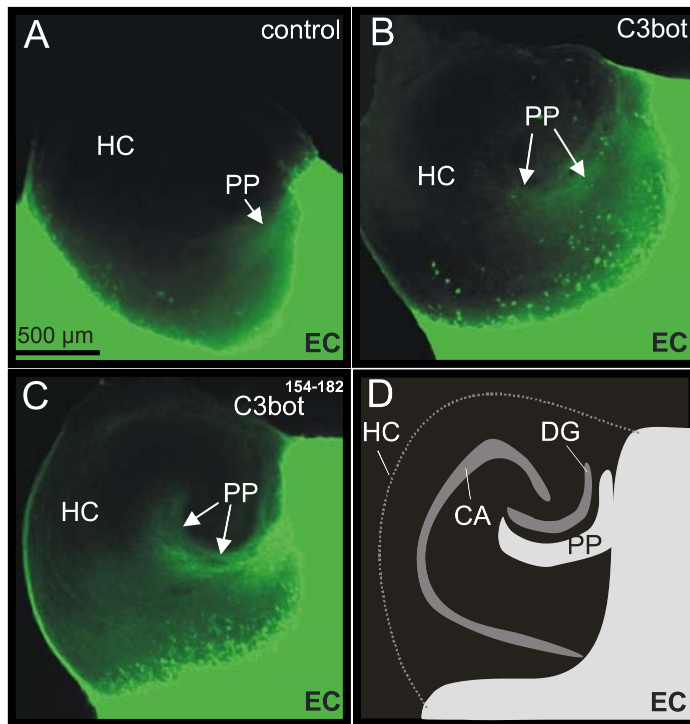
As NT-3 and NT-4 are the major neurotrophins responsible for neurite growth from organotypic brain slices we investigated brain slices derived from mice either homozygous for NT-4 deficiency (NT-4-/-) or with a combined homozygous NT-4 deficiency and heterozygous NT-3 deficiency (NT-3+/- /NT-4-/-) (a full NT-3 knockout is lethal). Neurite growth was still increased by hypothermia in brain slices derived from NT-4 KO mice. 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. 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 (20-22). 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. In a next step we investigated whether the levels of selected inflammation-associated cytokines like IL-1beta, IL-6, IL-10 and TNF-α 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, in contrast to TNF-α 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. Based on these finding we further explored whether the TNF-α upregulation plays a causal role in stimulating neurite extension after hypothermic treatment. As a first step, we demonstrated that TNF-α was sufficient to increase neurite extension from brain slices. TNF-α increased neurite density by nearly 45%, a similar effect like deep hypothermia/rewarming. Next we used the TNF-α inhibitor etanercept, which fully abolished the stimulatory effect of hypothermia and rewarming. Furthermore, the absence

of endogenous TNF-α in slices derived from TNF-α-deficient mice fully eliminated the effect of deep hypothermia and rewarming (7).

Since there are strong evidences that TNF-α influences nurite growth trough the cellular pathway of RhoA (8), which is a small GTPase very important in orchestrating cytoskeletal rearrangements (9-11), we studied the effect on neurite outgrowth, neuroprotection and regeneration mediated by bacterial C3 transferases or derived peptides, which are intensively used to study the function of Rho proteins. The C3 isoforms C3bot (from *Clostridium botulinum*) and C3lim (from *Clostridium limosum*) can perform an enzymatic inactivation of Rho by ADP-ribosylation. As previously shown, C3bot additionally harbors an axonotrophic activity, which is independent from its enzymatic activity and not shared by C3 proteins from other sources (14). Using overlapping peptides from the C3bot sequence, we identified (15) a small peptide of 29 amino acids (covering residues 154-182) from the C-terminal region of C3bot that promotes both axonal and dendritic growth, as well as branching of hippocampal neurons, at submicromolar concentrations. Several C3bot constructs, including the short peptide, enhanced the number of axonal segments from mid- to higher-order segments. C3bot$^{154-182}$ also increased the number of synaptophysin-expressing terminals, up-regulated various synaptic proteins, and functionally increased the glutamate uptake. Staining against the vesicular glutamate and GABA transporters further revealed that the effect was attributable to a higher number of glutamatergic and GABAergic inputs on proximal dendrites of enhanced green fluorescent protein (EGFP)-transfected neurons (results not discussed). Furthermore, we studied the influence of C3 proteins on axon outgrowth under conditions closely related to the *in vivo* situation, namely the organotypical brain slice culture. Dissected entorhinal cortex slices were incubated for 48 h with C3bot (a concentration of 300 nM was used to overcome a putative restricted diffusion of full-length protein into the collagen matrix used) and C3bot$^{154-182}$ (50 nM). Length and density of regrowing axons mainly belonging to the perforant path in the *in vivo* situation were evaluated. Both parameters were significantly increased by both C3bot and the peptide. C3bot$^{154-182}$ increased axonal length and density by 44 and 37%, respectively. Full-length C3bot was able to increase the length by 60% and density by 38%. We then used another organotypical culture system that allows investigating the ability of axons to reinnervate target tissues after lesion. The hippocampal-entorhinal cortex coculture is widely used to study axon growth and pathfinding (23,24). We used an EGFP/wild-type culture model that combines the entorhinal cortex of a β-actin-EGFP mouse with the hippocampus of a wild-type mouse. EGFP-expressing axons are clearly detectable in the nonfluorescent wild-type hippocampus. Special emphasis was taken on the perforant path that originates from the upper layers of the entorhinal cortex and terminates in the marginal zones of the hippocampus and the outer molecular layers of the dentate gyrus. Slice cocultures were incubated with C3bot or C3bot$^{154-182}$ for 48 h as in the outgrowth assay. In contrast to the control conditions, in which only a moderate reinnervation of wild-type hippocampus by EGFP expressing axons of the perforant path was observable (Figure.1A-D) both full-length C3bot and C3bot$^{154-182}$ enhanced the reinnervation significantly by 40% (Figure 1E).

Prompted by these results we investigated the *in vivo* effects of the 29-amino acid fragment C3bot$^{154-182}$ on functional recovery after contusion injury or dorsal hemisection of the spinal cord in mice. Gel foam patches soaked in C3bot$^{154-182}$ solution (40 μM, 610 ng per animal) were applied directly above

the injury site. We analyzed the locomotor function in these mice using the Basso Mouse Scale (25), an open-field test and Rotarod treadmill to analyze the performance under forced movement.



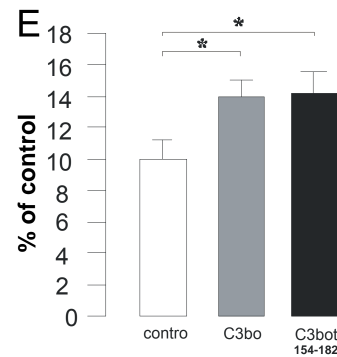


Figure 1. ***Reinnervation of hippocampus by fibers of perforant path is enhanced by C3bot$^{154-182}$ and C3bot.***
A, B, C. β-Actin-EGFP expressing entorhinal cortex (EC) was cocultured with a littermate wild-type hippocampus (HC). Reinnervation of hippocampus by green fluorescent fibers of perforant path (PP) was followed after application of C3bot$^{154-182}$ or C3bot for 48 h. **D.** Schematic illustration of the model. Explants were positioned rearranging correct anatomy. CA, cornu ammonis; DG, dentate gyrus. **E.** Measurements of fluorescence intensity of ingrowing fibers. Application of both C3bot$^{154-182}$ and C3bot enhanced perforant path formation by 40%.

In the BMS analysis, the locomotor function was significantly increased during the whole observation period in contusion-injured mice treated with C3bot$^{154-182}$ patches. The motor performance of both treated and untreated mice was more affected in the hemisection model than after contusion injury. The clear beneficial effect of the C3 peptide in the former model was particularly pronounced during the last two weeks of the observation period; in the case of hemisection, the observation period was extended to four weeks (compared to three weeks in the contusion model) as the lesion was more severe. Since correct foot placing is associated with proper CST function (26,27), we also analyzed stepping and correct paw positioning scores for the contusion model. Treated animals showed improved stepping and especially paw positioning from day 8 on, with substantial improvement over subsequent days, whereas control animals had minimal scores throughout the observation period. Furthermore, C3bot$^{154-182}$ application increased the latency for the mice to fall from the Rotarod in both models. Detailed photodocumentation revealed that treated mice displayed more efficient climbing behavior on the turning wheel, while control mice showed a tendency to lose grip early, at low rotation speed. After completing the behavioral examination we addressed whether the improved recovery of treated animals included enhanced axonal growth of descending spinal motor fibers. Analysis of the BDA-traced corticospinal tract showed a significantly increased percentage of nerve fibers between the end of the tract and the center of the contusion injury lesion. Moreover, the number of fibers passing through the lesion center was significantly increased at 0.5 mm (Figure 2A-C). In line with the latter, an increased number of BDA-positive fibers caudal to the lesion site was detected following hemisection and treatment with the C3 peptide (Figure 2D-F). We even detected an increased

percentage of regenerating nerve fibers as far as 5 mm caudal from the lesion center (Figure 2F). Taken together, the data at this stage provided strong evidence of a C3bot$^{154-182}$-mediated improvement of axonal sprouting and/or regeneration following damage to the spinal cord. Additionally, the early onset of improvement, especially in the BMS tests following contusion injury, indicated that the C3 peptide might also have neuroprotective effects.

To detect possible effects of C3bot$^{154-182}$ on the lesion size and reactive gliosis, spinal cord sections were double-stained for glial acidic fibrillary protein (GFAP) and myelin basic protein (MBP). Evaluation of contusion-induced lesion size revealed a reduction of tissue damage by 25% following administration of C3 peptide. On the other hand, gliosis as measured by perilesional GFAP expression by reactive astrocytes was not significantly altered by C3bot$^{154-182}$. In the hemisection model, neither lesion size nor astrogliosis were affected by the peptide. However, recovery of function after SCI might not exclusively rely on regenerative growth of CST fibers.

To test for beneficial effects of C3 peptide on other tracts beside the CST we visualized serotonergic raphespinal projections by an anti-serotonin (5HT) antiserum in mice injured by hemisection. In the ventral funiculus the total length of serotonergic fibers was assessed cranial and caudal to the lesion site. Whereas the total fiber length cranial to the lesion was unaltered after application of C3bot$^{154-182}$, it was significantly increased caudal to the lesion at more than 3-fold compared to control mice. Serotonergic fibers set up a network of projections to the grey matter in order to contact interneurons and motoneurons. Research has suggested that serotonergic fibers originating from the brainstem might form conventional synapses with theses neurons within the ventral horn (28). It is well established that serotonergic input to spinal motoneurons activates motor functions (29). Consequently, we addressed the question whether treatment with C3 peptide leads to an increased serotonergic input to lumbar motoneurons and whether this contributes to the improved motor outcome. At lumbar levels L1-L2 we counted 5HT-positive boutons on α-motoneurons of the ventral horn. We found that application of C3bot$^{154-182}$ considerably increased the average number of serotonergic contacts from 3.6 (per 100 µm cell perimeter) to 10.3. Notably, the increased serotonergic contacts corresponded very well to the increased density of serotonergic fibers within the ventral funiculus caudal to the lesion site. Taken together, these data provide strong evidence for a C3 peptide-mediated improved serotonergic input to lumbar α-motoneurons, thereby contributing to an enhanced hind limb motor performance.

In addition to the investigations described above, we studied the putative effects of C3bot$^{154-182}$ treatment on the neuromuscular junctions of tibial muscles of the hind limb (*M. tibialis cranialis*) following injury at the end of the observation period. The tibial muscles are crucially involved in lifting the paw during movement on both level surfaces and when clinging to a (rotating) rod. After longitudinal sectioning, we applied the established labeling technique using Alexa Fluor 488-coupled α-bungarotoxin (30) to visualize and quantify motor endplates. Following contusion, the number of motor endplates decreased by 17% (normalized to the number of muscle fibers) in the control/SCI group compared to intact mice. This reduction in the number of motor endplates was completely prevented by administration of C3bot$^{154-182}$. After hemisection, loss of endplates in general was more severe. The number of endplates in the PBS group was declined to 55%. Treatment with the C3

peptide restored the number of endplates to 85%. No obvious differences in the size or general morphology of endplates were detected between the groups.

To gain insight into the molecular mechanisms contributing to the observed morphological changes we determined active RhoA levels by Rhotekin pull-down analysis from hippocampal cell lysates. RhoA is known to be crucially involved in actin and microtubule dynamics. Application of C3bot$^{154-182}$ at final concentrations of 10 or 30 nM to hippocampus cultures resulted in a strong reduction of active RhoA levels, while total RhoA protein expression was unchanged. Thus, despite the lack of enzymatic activity, the C3 peptide is likely to exert its effects by means of a RhoA-dependent mechanism.

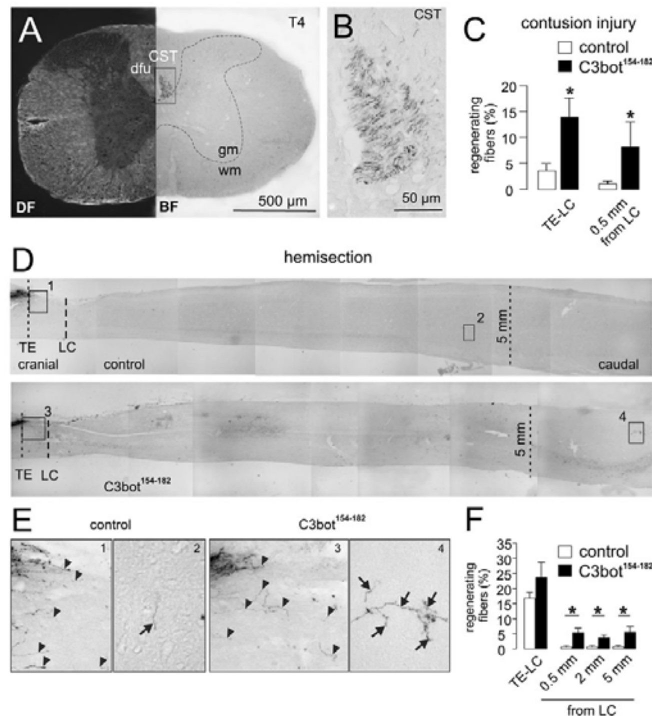


Figure 2. Quantification of nerve fibers between the tract end and the lesion center and caudal to the lesion in two models of spinal cord injury in mice. **A**. Cross section of spinal cord at thoracic level 4 (T4) illustrating tracing of the left CS. BF, brightfield illumination; DF, darkfield illumination; dfu, dorsal funiculus; gm, grey matter; wm, white matter. **B.** Enlargement of the boxed area in A depicting the dorsal corticospinal tract (CST). **C**. The percentage of labeled nerve fibers was significantly increased in contusion-injured mice treated with C3bot$^{154-182}$2 compared with controls. Bars represent the percentage of nerve fibers in the area between the end of the CST and the lesion center (TE-LC) and in the area 0.5 mm distal to the lesion center (0.5 mm from LC). PBS, *n*=6 animals; C3bot$^{154-182}$, *n*=5 animals **D**. Parasaggital spinal cord sections of mice that underwent SCI by dorsal hemisection. Images show CST tracing. TE, tract end; LC, lesion center. **E**. Higher magnification of the numbered and boxed areas in D. Arrowheads indicate nerve fibers between the tract end and the lesion site, arrows indicate fibers passing the lesion center. **F**. The percentage of labeled nerve fibers caudal to the lesion was significantly increased in hemisection-injured mice treated with C3bot$^{154-182}$ compared with control mice. Bars indicate the percentage of nerve fibers in the area TE-LC and in the area 0.5 mm , 2 mm and 5 mm distal to the lesion center. PBS, *n*=5 animals; C3bot$^{154-182}$, *n*=7 animals. *$P<0.05$.

5. Discussion

TBI and SCI are major sources of death and disability. Around 40% of the TBI patients are left with permanent neurological disabilities and the percentage is even higher when talking about SCI (1). In addition to the emotional effects the financial burden is also enormous. One well-established method for neuroprotection in the context of TBI is the direct application of systemic or brain-restricted hypothermia to prevent secondary injury (31). 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 (2). 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.

We and others have previously demonstrated that hypothermia also modulates the brain immune system (4,19,32,33). Based on these findings we addressed the question of whether hypothermia may support not only neuronal cell survival (4,32) but also regenerative responses after brain damage (31). In contrast to previous studies focused mainly on neuroprotection we investigated whether hypothermia induces neurite growth via neurotrophins and/or inflammation-associated cytokines that have been shown to modulate neurite outgrowth and regeneration (5,6). 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 neurite plasticity, growth and regeneration (24). 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 (34-36). As NT-3 and NT-4 are the major neurotrophins responsible for neurite growth in organotypic brain slices (24), 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. 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 (20-22). 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. The analysis of the levels of IL-1beta, IL-6, IL-10 and TNF-α, which are known to influence neurite extension, revealed that only TNF-α secretion is significantly increased (nearly four-fold) after deep hypothermia and rewarming of organotypic brain slices. TNF-α is a pleiotropic cytokine that induces neuroprotective and neurotoxic effects after CNS injury (37-39). In the present study we have demonstrated that TNF-α 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-α inhibitor as well as the absence of endogenous TNF-α in slices derived from TNF-α-deficient mice fully abolished the effect of deep hypothermia and rewarming. 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 (40). Similar structural changes have been reported for dendrites of CA3 pyramidal cells in ground squirrels after arousal from hibernation (41), suggesting a physiological role of neuronal plasticity after hypothermia and rewarming. The role of TNF-alpha in these processes is not known. However, in the context of our study it will be important to analyze whether the TNF-α-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 so far clearly show that TNF-α is necessary and sufficient to increase neurite outgrowth in the context of deep hypothermia and rewarming. This part of the study provides for the first time evidence that hypothermia not only exerts protective effects in the

CNS but also supports neurite outgrowth via TNF-α upregulation as a possible mechanism of regeneration.
Most probably, as previously shown (8), TNF-α influence neurite outgrowth trough the cellular pathway of a small GTPase, namely RhoA which has been intensively studied in virtually any CNS injury model *in vitro* and *in vivo* for his capacity of orchestrating cytoskeletal rearrangements (9-11). Interestingly the C3 isoforms C3bot and C3lim are able to enzymatic inactivation of Rho by ADP-ribosylation and are then extensively used in cell biology to analyze Rho-dependent effects on axonal and dendritic development. Since C3bot additionally harbors an axonotrophic activity, which is independent from its enzymatic activity and not shared by C3 proteins from other sources (14) we studied the effect of a 29 amino acids long enzyme-deficient peptide derived from the C-terminal part of C3bot (C3bot$^{154-182}$). C3bot$^{154-182}$ exhibits trophic effects on neuronal morphology, and enhances axonal outgrowth and regeneration (reinnervation) in organotypical brain slices. In contrast to full-length enzyme-deficient C3botE174Q (14), C3bot$^{154-182}$ also alters dendritic morphology. In hippocampal primary cultures, C3bot$^{154-182}$ resulted in significant enhancement of axonal and dendritic outgrowth. Whether or how C3bot peptides enter the neuron is unclear so far.
C3 proteins have proven to foster neuronal survival and regeneration in a variety of model systems, *e.g.*, the optic nerve (42). The present study (15) demonstrates the beneficial outcome of C3 protein treatment for neuronal outgrowth and even reinnervation of denervated target tissues such as the hippocampus formation by perforant path fibers of the entorhinal cortex. One might speculate whether the enhanced ingrowth of fibers is the mere result of a boosted growth program of entorhinal neurons alone or also results from alterations within the target tissue like the expression of attractive cues. So far, however, there is no evidence for C3 protein effects on such mechanisms. Above all, it undoubtedly shows that a short region of C3bot exhibiting no enzymatic activity is sufficient to trigger comparable promoting effects. Since C3bot-mediated effects described so far may not exclusively rely on ADP ribosylation of Rho proteins, but rather reflect the net outcome of differentially triggered pathways converging in putative common downstream cytoskeletal targets also *in vivo*, we tested the peptide in two models of spinal cord injury.
Our latest study clearly shows that treatment with C3bot$^{154-182}$ is also effective *in vivo* and results in improved recovery from spinal cord injury. We studied two spinal cord injury models. Although the most common type of SCI in humans involves compressive impact (43,44), cutting CST fibers by performing a hemisection up to at least the central canal creates a more defined lesion and allows distinguishing between newly formed, therefore regenerated, and spared fibers (43,45). Two experimental settings designed to observe functional recovery after spinal cord injury detected an improved motor performance in treated animals as early as within the first week following compression injury. This early onset of improved functional recovery might indicate not only beneficial effects on neurological repair mechanisms but also a neuroprotective effect of C3bot$^{154-182}$. This evidence was underlined by the fact that treatment with the peptide resulted in reduced tissue damage (lesion size) following contusive SCI.
The lesion size was unaltered after dorsal hemisection and the beneficial effects of the peptide appeared later than using the contusion injury model. In the latter, the BMS score demonstrated significant benefit only two days post-injury and this positive trend persisted throughout the almost 3-

week observation period. Upon completion, treated animals exhibited a higher degree of coordination between fore limbs and the affected hind limbs (25). Additionally, improved locomotor restoration, such as frequent plantar stepping and parallel paw position relative to the body axis, contributed to the improved outcome. Both plantar stepping and especially paw positioning showed the highest improvement following C3bot$^{154-182}$ treatment during the final 2 weeks of the observation period. Furthermore, the BMS open-field data were well supported by the Rotarod forced-movement experiments and emphasized the effectiveness of the treatment. Hemisection represented a more severe injury in general (both control and treated animals performed worse than after contusion injury, according to the BMS scores and the Rotarod experiments). Nevertheless, in both tests the effect of the C3 peptide could be observed, particularly in the final days of the observation period.

To determine whether the improved functional recovery after SCI was reflected by histological differences between the different treatment groups, we first quantified BDA+ axons of the corticospinal tract (CST). In animals treated with C3bot$^{154-182}$ a significantly enhanced number of BDA-labeled axons caudal to the lesion site were observed following both contusion injury and hemisection. Increased numbers of fibers cranial to the lesion were also observed in the case of contusion. Indicators such as distance or time course of growth can also be employed to characterize axonal response in terms of plasticity, sprouting or regeneration (46). Particularly in the hemisection model, we detected alterations in axonal growth over moderate distances (< 1 mm from the corticospinal tract end), which might indicate local regenerative sprouting, but also over a distance as far as 5 mm from the lesion center, which is likely to represent regeneration. We can exclude that the fibers detected caudal to the lesion originated from uninjured ventral CST fibers because in our hemisection model the ventral funiculus was severed by a median cut in addition to the transection of the dorsal half of the spinal cord. Based on morphological criteria the BDA+ fibers crossing the lesion site appear to represent newly established fibers, even if in the compression injury model we cannot exclude that they represent branching from spared fibers. Supporting our CST data, an enhanced number of serotonergic fibers caudal to the lesion were observed in mice treated with C3bot$^{154-182}$ following hemisection. Moreover, serotonergic input to lumbar α-motoneurons of the ventral horn was enhanced by the peptide to a similar degree. The primary role of 5HT in the mammalian spinal cord mainly originating from medullary raphe nuclei seems to be the facilitation of motor performance, as well as the coordination between motor, autonomic and sensory systems (47-50). Our findings of improved serotonergic input to lumbar motoneurons thus provide strong evidence for one likely mechanism that leads to enhanced functional recovery in mice treated with C3bot$^{154-182}$. However, a mixture of axonal responses, including those of local circuits, for example at lumbar spinal cord segments harboring the hind limb α-motoneurons, may have additionally contributed to the observed improved clinical outcome. Animals treated with C3bot$^{154-182}$ exhibited also a reduced motor endplate loss in a tibial muscle (*M. tibialis cranialis)* following both lesion types. Following hemisection, the number of motor endplates was more strongly reduced than after contusion of the spinal cord.

As mentioned above, numerous studies have demonstrated that clostridial C3 proteins foster axonal repair mechanisms after traumatic injury to the CNS inhibiting the small GTPase Rho. The pull-down studies presented here provide the first evidence of the molecular mechanisms of C3bot$^{154-182}$ action. Our data clearly show that also C3bot$^{154-182}$ can reduce active RhoA levels by an as yet unknown non-

enzymatic mechanism. Possibilities include that RhoA may be influenced directly by the internalized peptide or indirectly via GEF (guanine nucleotide exchange factors) inhibition or GAP (GTPase activating proteins) activation. In summary, our data indicate a direct, neuron-specific effect because no enzyme-independent effects were observed on glial cells in response to C3 preparations (51-53). The clear specificity of effects makes this peptide an excellent candidate for fostering neuronal-process growth without eliciting potentially unwanted glia-derived side effects (characteristic of the full length enzyme). Taken together, these data indicate that this short C3bot-derived amino acid fragment C3bot$^{154-182}$ is a novel and promising tool to specifically enhance process outgrowth of central neurons and promote recovery after traumatic injury to the CNS.

6. Material 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-α-deficient mice (54) were used for the experiments on postnatal day two. Balb/c mice for in vivo experiments were used at 9–11 weeks old. K252a (Calbiochem, Schwalbach, Germany), which is a potent inhibitor of TrkA, TrkB and TrkC (20-22), was used in concentrations of 100 nM or 1 mM in dimethyl sulfoxide (DMSO). Etanercept (Enbrel® Whyeth, Munster, Germany) was used in a concentration of 80 mg/mL (55). C3bot$^{154-182}$ was synthesized at IPF PharmaCeuticals GmbH (Hannover, Germany). C3bot was expressed as recombinant GST-fusion protein in *E. coli* TG1 harboring the respective DNA fragment in the plasmid pGEX-2T (15).

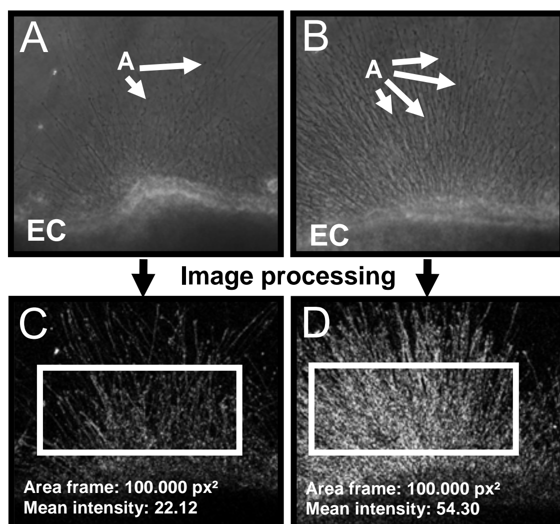


Figure 3. ***Quantification by image analysis reveals stimulation of neurite outgrowth by hypothermia and rewarming.***
A, B. Representative photomicrographs of a brain slice with intermediate outgrowth (A) and a brain slice with strong outgrowth (B). **C, D.** To quantify the density of the outgrowing 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).

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. All organotypic slice cultures were prepared on postnatal day 2 from mouse entorhinal cortex as previously described (19). The collagen cultures were incubated for 24 h or 48 h. To analyze reinnervation of the denervated hippocampus an entorhinal cortex of a β-actin-EGFP mouse was combined with a hippocampus of a wild-type (EC-HC). The cultures were prepared on membrane (Millipore) and incubated for 48h as described above (24). ***Time-temperature protocol and Enzyme-linked immunosorbent assay (ELISA):*** Acute organotypic brain slices were cooled

down from 37°C to 17°C 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. Conditioned medium from slice cultures was tested for IL-6, TNF-α, IL-1beta and IL-10 using ELISAs according to the manufacturer's instructions (BD Bioscience, Heidelberg, Germany). ***Spinal cord injury, corticospinal tract tracing and locomotion tests:*** Spinal cord compression injury, CST tracing and analysis was performed as described previously (56). Briefly, Balb/C mice underwent a partial laminectomy at thoracic level T8, and a contusion lesion was performed with a SPI Correx Tension/Compression Gage (Penn Tool) at 20 cN for 1 s. For the spinal cord bilateral dorsal hemisection, iridectomy scissors were used to transect left and right dorsal funiculus, the dorsal horns (57) and additionally the ventral funiculus. After positioning of the gelfoam patch on top of the perforated dura, the muscles were sutured. A small hole was then drilled into the skull and a Hamilton syringe was inserted into the motor cortex to apply 2 µl biotinylated dextran amine (10% BDA). BDA^+ CST nerve fibers cranial and caudal to the lesion were counted in serial sections (56) and normalized to the number of labeled fibers within a standardized 20 µm-wide area across the mid dorso-ventral diameter of the CST in cross sections at the T4 level cranial to the lesion. Mice were continuously tested for functional recovery within three or four weeks (contusion injury or hemisection, respectively) following SCI with the Basso Mouse Scale (25). Subscores for stepping performance and correct paw positioning were evaluated individually. Also Rotarod performance (58) was determined continuously up to the end of the observation periods. ***Histological staining and analysis:*** The entorhinal cortices (ECs) and EC-HC were washed and fixed for 20 minutes in 4% PFA. Spinal cord and *M. tibialis cranialis* cryosections (respectively 20 and 30 µm thick) from PFA perfused animals and ECs were then incubated with 10% normal goat serum (NGS; Sigma) and 0.2% Triton X-100 (Sigma). For ECs the first antibody, Tau-1 (mouse monoclonal, Millipore) or glial fibrillary acidic protein (GFAP; mouse monoclonal, Sigma), was added for 2h. The ECs were then incubated for 2 h at RT with the secondary antibody. To evaluate neurite density of the ECs we used a standard protocol for the evaluation of neurite outgrowth as described previously (15,24), which was improved using image analysis software based on the Sobel algorithm (Image J, NIH) to precisely quantify neurite density (Figure 3). To evaluate EC-HC reinnervation Photomicrographs were then analyzed with MetaMorph Image Software (VisitronSystems) to determine the average intensity of the GFP positive axons in a standardized area. For measurement of lesion size and gliosis in the injured spinal cord we performed a double-staining against GFAP and myelin basic protein (MBP, rabbit polyclonal). Quantification of GFAP expression was performed by intensity analysis as previously described (59). For analysis of serotonergic fibers and 5HT-positive boutons sections were incubated with a polyclonal anti 5-HT antiserum. 5-HT expressing fibers were documented and analysed using Image J. For camera lucida tracing, Adobe Photoshop software was used. The number of 5HT-positive boutons on motoneurons was calculated as previously described (60). For motor endplate staining on *M. tibialis cranialis*, sections were incubated for 2 hours at RT with 2 µg/ml Alexa Fluor 488-conjugated α-bungarotoxin (Molecular Probes). Every second section was analyzed and the total number of motor endplates per individual section was determined. As secondary antibody goat anti-mouse AL488 (A11029, Invitrogen) was used for ECs, Alexa Fluor 594 horse anti-mouse and Alexa fluor 488 goat anti-rabbit for EGFP and MBP staining, and Alexa 488 goat

anti-rabbit (Molecular Probes) for 5-HT. ***Primary cultures:*** For hippocampal neurons cells were prepared from hippocampi from mice at E16, dissociated mechanically centrifugated and plated on glass cover slips precoated with poly-L-lysine/collagen. One day after plating, C3bot peptide was added to the culture medium and at five days *in vitro* (DIV5) neurons were fixed and permeabilized. Hippocampal neurons were stained by antibody against neurofilament protein (NFP) and microtubule associated proteins 2 (MAP2) (Chemicon International) overnight and at 4°C and secondary antibodies were applied for 2h. Hippocampal neurons were documented using a DFC 490 digital camera and morphometrically analyzed using Neurolucida software (MicroBrightField). ***Rho GTPase pull-down assay:*** The Rho binding domain C21 was expressed as GST fusion protein in E. coli and purified by affinity chromatography using glutathione-sepharose. Hippocampal neurons were lysed and the obtained suspension was sonicated and centrifugated. Lysates were then added to glutathione-bound GST-C21 (rhotekin) for 1 h (4°C). Bound proteins were mobilized by incubation with Laemmli sample buffer at 95 °C for 10 min. Samples were subjected to SDS-PAGE and Western blot analysis. ***Statistical analysis:*** Data represent mean values ± SEM. Locomotion tests were analyzed using a two-way ANOVA. Statistical significance of cytochemical data was tested using the Mann Whitney U test. In general, p-values < 0.05, < 0.01, or < 0.001 were marked *, **, or ***, respectively.

7. References

[1] Polderman, K.H., Mechanisms of action, physiological effects, and complications of hypothermia. *Crit Care Med* 37 (7 Suppl), S186-202 (2009).

[2] Polderman, K.H., Induced hypothermia and fever control for prevention and treatment of neurological injuries. *Lancet* 371 (9628), 1955-1969 (2008).

[3] Atkins, C.M. *et al.*, Hypothermia treatment potentiates ERK1/2 activation after traumatic brain injury. *Eur J Neurosci* 26 (4), 810-819 (2007).

[4] Schmitt, K.R. *et al.*, Methylprednisolone attenuates hypothermia- and rewarming-induced cytotoxicity and IL-6 release in isolated primary astrocytes, neurons and BV-2 microglia cells. *Neurosci Lett* 404 (3), 309-314 (2006).

[5] Hendrix, S. & Nitsch, R., The role of T helper cells in neuroprotection and regeneration. *J Neuroimmunol* 184 (1-2), 100-112 (2007).

[6] Hendrix, S. & Peters, E.M., Neuronal plasticity and neuroregeneration in the skin -- the role of inflammation. *J Neuroimmunol* 184 (1-2), 113-126 (2007).

[7] Schmitt, K.R. *et al.*, Hypothermia-Induced Neurite Outgrowth is Mediated by Tumor Necrosis Factor-Alpha. *Brain Pathol* (2009).

[8] Neumann, H. *et al.*, Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by a rho-dependent mechanism. *J Neurosci* 22 (3), 854-862 (2002).

[9] Luo, L., Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annu Rev Cell Dev Biol* 18, 601-635 (2002).

[10] Raftopoulou, M. & Hall, A., Cell migration: Rho GTPases lead the way. *Dev Biol* 265 (1), 23-32 (2004).

[11] Govek, E.E., Newey, S.E., & Van Aelst, L., The role of the Rho GTPases in neuronal development. *Genes Dev* 19 (1), 1-49 (2005).

[12] Aktories, K. & Just, I., Clostridial Rho-inhibiting protein toxins. *Curr Top Microbiol Immunol* 291, 113-145 (2005).

[13] Vogelsgesang M, P.A., Aktories K., C3 exoenzymes, novel insights into structure and action of Rho-ADP-ribosylating toxins. *Naunyn Schmiedebergs Arch Pharmacol.* 5-6 347-360. (2007).

[14] Ahnert-Hilger, G. *et al.*, Differential effects of Rho GTPases on axonal and dendritic development in hippocampal neurones. *J Neurochem* 90 (1), 9-18 (2004).

[15] Höltje, M. *et al.*, A 29-amino acid fragment of Clostridium botulinum C3 protein enhances neuronal outgrowth, connectivity, and reinnervation. *FASEB J* 23 (4), 1115-1126 (2009).

[16] Dergham, P. *et al.*, Rho signaling pathway targeted to promote spinal cord repair. *J Neurosci* 22 (15), 6570-6577 (2002).

[17] Lord-Fontaine, S. *et al.*, Local inhibition of Rho signaling by cell-permeable recombinant protein BA-210 prevents secondary damage and promotes functional recovery following acute spinal cord injury. *J Neurotrauma* 25 (11), 1309-1322 (2008).

[18] Boato, F., C3 peptide treatment enhances functional recovery from spinal cord injury by improved axonal regenerative growth of descending fiber tracts. *Journal of Cell Science* 123 (2010).

[19] Schmitt, K.R. *et al.*, S100B modulates IL-6 release and cytotoxicity from hypothermic brain cells and inhibits hypothermia-induced axonal outgrowth. *Neurosci Res* 59 (1), 68-73 (2007).

[20] Goldberg, D.J. & Wu, D.Y., Tyrosine phosphorylation and protrusive structures of the growth cone. *Perspect Dev Neurobiol* 4 (2-3), 183-192 (1996).

[21] Koizumi, H., Morita, M., Mikami, S., Shibayama, E., & Uchikoshi, T., Immunohistochemical analysis of TrkA neurotrophin receptor expression in human non-neuronal carcinomas. *Pathol Int* 48 (2), 93-101 (1998).

[22] Ohmichi, M. *et al.*, The tyrosine kinase inhibitor tyrphostin blocks the cellular actions of nerve growth factor. *Biochemistry* 32 (17), 4650-4658 (1993).

[23] Frotscher, M. & Heimrich, B., Formation of layer-specific fiber projections to the hippocampus in vitro. *Proc Natl Acad Sci U S A* 90 (21), 10400-10403 (1993).

[24] Hechler, D., Nitsch, R., & Hendrix, S., Green-fluorescent-protein-expressing mice as models for the study of axonal growth and regeneration in vitro. *Brain Res Rev* 52 (1), 160-169 (2006).

[25] Basso, D.M. *et al.*, Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J Neurotrauma* 23 (5), 635-659 (2006).

[26] De Ryck, M., Van Reempts, J., Duytschaever, H., Van Deuren, B., & Clincke, G., Neocortical localization of tactile/proprioceptive limb placing reactions in the rat. *Brain Res* 573 (1), 44-60 (1992).

[27] Metz, G.A. & Whishaw, I.Q., Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. *J Neurosci Methods* 115 (2), 169-179 (2002).

[28] Rajaofetra, N. *et al.*, Transplantation of embryonic serotonin immunoreactive neurons into the transected spinal cord of adult monkey (Macaca fascicularis). *Brain Res* 572 (1-2), 329-334 (1992).

[29] Jacobs, B.L., Martin-Cora, F.J., & Fornal, C.A., Activity of medullary serotonergic neurons in freely moving animals. *Brain Res Brain Res Rev* 40 (1-3), 45-52 (2002).

[30] Guntinas-Lichius, O. *et al.*, Factors limiting motor recovery after facial nerve transection in the rat: combined structural and functional analyses. *Eur J Neurosci* 21 (2), 391-402 (2005).

[31] Christian, E., Zada, G., Sung, G., & Giannotta, S.L., A review of selective hypothermia in the management of traumatic brain injury. *Neurosurg Focus* 25 (4), E9 (2008).

[32] Schmitt, K.R. *et al.*, Hypothermia suppresses inflammation via ERK signaling pathway in stimulated microglial cells. *J Neuroimmunol* 189 (1-2), 7-16 (2007).

[33] Wang, Y. & Chambers, K.C., Cooling lesions of the lateral parabrachial nucleus during LiCl activation block acquisition of conditioned taste avoidance in male rats. *Brain Res* 934 (1), 7-22 (2002).

[34] Eyupoglu, I.Y., Savaskan, N.E., Brauer, A.U., Nitsch, R., & Heimrich, B., Identification of neuronal cell death in a model of degeneration in the hippocampus. *Brain Res Brain Res Protoc* 11 (1), 1-8 (2003).

[35] Heppner, F.L., Skutella, T., Hailer, N.P., Haas, D., & Nitsch, R., Activated microglial cells migrate towards sites of excitotoxic neuronal injury inside organotypic hippocampal slice cultures. *Eur J Neurosci* 10 (10), 3284-3290 (1998).

[36] Wolf, S.A. *et al.*, Neuroprotection by T-cells depends on their subtype and activation state. *J Neuroimmunol* 133 (1-2), 72-80 (2002).

[37] Lotocki, G., Alonso, O.F., Dietrich, W.D., & Keane, R.W., Tumor necrosis factor receptor 1 and its signaling intermediates are recruited to lipid rafts in the traumatized brain. *J Neurosci* 24 (49), 11010-11016 (2004).
[38] Lotocki, G. *et al.*, Therapeutic hypothermia modulates TNFR1 signaling in the traumatized brain via early transient activation of the JNK pathway and suppression of XIAP cleavage. *Eur J Neurosci* 24 (8), 2283-2290 (2006).
[39] Martin-Villalba, A. *et al.*, Therapeutic neutralization of CD95-ligand and TNF attenuates brain damage in stroke. *Cell Death Differ* 8 (7), 679-686 (2001).
[40] Kirov, S.A., Petrak, L.J., Fiala, J.C., & Harris, K.M., Dendritic spines disappear with chilling but proliferate excessively upon rewarming of mature hippocampus. *Neuroscience* 127 (1), 69-80 (2004).
[41] Popov, V.I., Bocharova, L.S., & Bragin, A.G., Repeated changes of dendritic morphology in the hippocampus of ground squirrels in the course of hibernation. *Neuroscience* 48 (1), 45-51 (1992).
[42] Hu, Y., Cui, Q., & Harvey, A.R., Interactive effects of C3, cyclic AMP and ciliary neurotrophic factor on adult retinal ganglion cell survival and axonal regeneration. *Mol Cell Neurosci* 34 (1), 88-98 (2007).
[43] Sekhon, L.H. & Fehlings, M.G., Epidemiology, demographics, and pathophysiology of acute spinal cord injury. *Spine (Phila Pa 1976)* 26 (24 Suppl), S2-12 (2001).
[44] Sicotte, M. *et al.*, Immunization with myelin or recombinant Nogo-66/MAG in alum promotes axon regeneration and sprouting after corticospinal tract lesions in the spinal cord. *Mol Cell Neurosci* 23 (2), 251-263 (2003).
[45] Steward, O., Zheng, B., & Tessier-Lavigne, M., False resurrections: distinguishing regenerated from spared axons in the injured central nervous system. *J Comp Neurol* 459 (1), 1-8 (2003).
[46] Cafferty, W.B., McGee, A.W., & Strittmatter, S.M., Axonal growth therapeutics: regeneration or sprouting or plasticity? *Trends Neurosci* 31 (5), 215-220 (2008).
[47] Skagerberg, G. & Bjorklund, A., Topographic principles in the spinal projections of serotonergic and non-serotonergic brainstem neurons in the rat. *Neuroscience* 15 (2), 445-480 (1985).
[48] Allen, G.V. & Cechetto, D.F., Serotoninergic and nonserotoninergic neurons in the medullary raphe system have axon collateral projections to autonomic and somatic cell groups in the medulla and spinal cord. *J Comp Neurol* 350 (3), 357-366 (1994).
[49] Kim, J.E., Liu, B.P., Park, J.H., & Strittmatter, S.M., Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. *Neuron* 44 (3), 439-451 (2004).
[50] Boido, M. *et al.*, Embryonic and adult stem cells promote raphespinal axon outgrowth and improve functional outcome following spinal hemisection in mice. *Eur J Neurosci* 30 (5), 833-846 (2009).
[51] Höltje, M. *et al.*, Role of Rho GTPase in astrocyte morphology and migratory response during in vitro wound healing. *J Neurochem* 95 (5), 1237-1248 (2005).
[52] Höltje, M. *et al.*, Glutamate uptake and release by astrocytes are enhanced by Clostridium botulinum C3 protein. *J Biol Chem* 283 (14), 9289-9299 (2008).
[53] Hoffmann, A. *et al.*, Inhibition of Rho-dependent pathways by Clostridium botulinum C3 protein induces a proinflammatory profile in microglia. *Glia* 56 (11), 1162-1175 (2008).
[54] Abe, K. *et al.*, Distinct contributions of TNF and LT cytokines to the development of dendritic cells in vitro and their recruitment in vivo. *Blood* 101 (4), 1477-1483 (2003).
[55] Genovese, T. *et al.*, Immunomodulatory effects of etanercept in an experimental model of spinal cord injury. *J Pharmacol Exp Ther* 316 (3), 1006-1016 (2006).
[56] Sieber-Blum, M. *et al.*, Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord. *Mol Cell Neurosci* 32 (1-2), 67-81 (2006).
[57] Simonen, M. *et al.*, Systemic deletion of the myelin-associated outgrowth inhibitor Nogo-A improves regenerative and plastic responses after spinal cord injury. *Neuron* 38 (2), 201-211 (2003).
[58] Sheng, H. *et al.*, A no-laminectomy spinal cord compression injury model in mice. *J Neurotrauma* 21 (5), 595-603 (2004).
[59] Wang, X. *et al.*, Ibuprofen enhances recovery from spinal cord injury by limiting tissue loss and stimulating axonal growth. *J Neurotrauma* 26 (1), 81-95 (2009).
[60] Müllner, A. *et al.*, Lamina-specific restoration of serotonergic projections after Nogo-A antibody treatment of spinal cord injury in rats. *Eur J Neurosci* 27 (2), 326-333 (2008).

8. Declaration of own contribution

Here follow the detailed contributions of the doctoral candidate Francesco Boato to the submitted publications.

- **Publication 1**

Höltje M, Djalali S, Hofmann F, Münster-Wandowski A, Hendrix S, **Boato F**, Dreger SC, Grosse G, Henneberger C, Grantyn R, Just I, Ahnert-Hilger G.

"A 29-amino acid fragment of Clostridium botulinum C3 protein enhances neuronal outgrowth, connectivity, and reinnervation."

FASEB Journal. 2009 Apr; 23(4):1115-26. Epub 2008 Dec 1.

Contribution in percent: 20%;

Detailed contribution: Conduction and analysis of key experiments (e.g. organotypic explants culture from enthorinal cortex -EC- in collagen and co-culture EC – Hippocampus on membrane); presentation of the results at international conferences and meetings (Society for Neuroscience 2009; CORTEX meeting in Oslo 2009; Berlin Brain Days 2010); participation in writing the manuscript; contribution for the revision of the manuscript.

- **Publication 2**

Schmitt K, **Boato F**, Diestel A, Hechler D, Kruglov A, Berger F, Hendrix S.

"Hypothermia-induced neurite outgrowth is mediated by TNF-alpha."

Brain Pathology. 2009 Dec 8. [Epub ahead of print].

Contribution in percent: 40%;

Detailed contribution: Active participation in planning the study and most of the experiments; conduction and analysis of key experiments (organotypic explants

culture in collagen under several condition, immunohistochemistry, intensity and morphological analysis, use of TNF-alpha inhibitor and TNF-alpha-KO mice experiments); improving of culturing and establishment of a novel analysis method; presentation of the results at international conferences and meetings (CORTEX meeting in Berlin 2008 and in Oslo 2009; Ecole Normale Superieur meeting in Paris 2010); participation in writing the manuscript; extensive contribution for the revision of the manuscript (including additional experiments, analysis and editing of the text).

- **Publication 3**

Boato F, Hendrix S, Huelsenbeck SC, Hofmann F, Große G, Djalali S, Klimaschewski L, Auer M, Just I, Ahnert-Hilger G, Höltje M.

"C3 peptide treatment enhances functional recovery from spinal cord injury by improved axonal regenerative growth of descending fiber tracts."

Journal of Cell Science. 2010 [*in Press*].

Contribution in percent: 70%;

Detailed contribution: Active participation in planning the study and all the experiments; conduction and analysis of the experiments (spinal cord contusion injury and hemisection, behavioural tests, immunocyto- and immunohistochemistry, morphological analysis, microscopy); improvement of injury model and establishment of new behavioural paradigms, presentation of the results at international conferences and meetings (CORTEX meeting in Berlin 2008 and in Oslo 2009; Society for Neuroscience 2009; Berlin Brain Days 2009; Ecole Normale Superieur meeting in Paris 2010); active participation in writing the manuscript; extensive contribution for the revision of the manuscript (including additional key experiments, analysis and editing of the text).

____

Francesco Boato

9. Complete publications list

Publications in peer-reviewed journals

- Höltje M, Djalali S, Hofmann F, Münster-Wandowski A, Hendrix S, **Boato F**, Dreger SC, Grosse G, Henneberger C, Grantyn R, Just I, Ahnert-Hilger G. *"A 29-amino acid fragment of Clostridium botulinum C3 protein enhances neuronal outgrowth, connectivity, and reinnervation."* **FASEB Journal**. 2009 Apr; 23(4):1115-26. Epub 2008 Dec 1.

- Schmitt K, **Boato F**, Diestel A, Hechler D, Kruglov A, Berger F, Hendrix S. *"Hypothermia-induced neurite outgrowth is mediated by TNF-alpha."* **Brain Pathology**. 2009 Dec 8. [Epub ahead of print]

- **Boato F**, Hendrix S, Huelsenbeck SC, Hofmann F, Große G, Djalali S, Klimaschewski L, Auer M, Just I, Ahnert-Hilger G, Höltje M. *"C3 peptide treatment enhances functional recovery from spinal cord injury by improved axonal regenerative growth of descending fiber tracts."* **Journal of Cell Science**. 2010 [*in press*].

- **Boato F***, Hechler D*, Nitsch R, Hendrix S. *"Differential regulation of axon outgrowth and reinnervation by neurotrophin-3 and -4 in the hippocampal formation."* **Experimental Brain Research**. 2010 [*under revision*]. * = equally contributing.

Talks in international conferences and meetings

- **Boato F**, Schmitt K, Diestel A, Hechler D, Nitsch R and Hendrix S. *"Hypothermia-induced neurite outgrowth is mediated by TNF-alpha."* University Pierre and Marie Curie (**UPMC**), Paris 2010

- **Boato F**, Höltje M, Schmitt K, Diestel A, Hechler D, Nitsch R and Hendrix S. *"RhoA pathways in axonal regeneration in vitro and in vivo."* **École Normale Supérieure**, Paris 2010

- Höltje M, **Boato F**, Hendrix S, Klimaschewski L, Just I and Ahnert-Hilger G. "*Clostridial C3 proteins: tools to foster regeneration from CNS injuries.*" **SNI Lectures**, Innsbruck 2010

- **Boato F**, Hechler D, Schwab M, Nitsch R and Hendrix S. *"T helper-2 calls mediate recovery from spinal cord injury via interleukin-4."* **Co.R.T.Ex** meeting, University of Oslo 2009

- Witzel C, **Boato F**, Koulaxouzidis G, Knie B, Infanger M and Hendrix S. "The effect of TRAIL on CNS and PNS trauma." Deutsche Arbeitsgemeinschaft für Mikrochirurgie (**DAM**), Erlagen 2009

- Höltje M, **Boato F**, Hendrix S, Klimaschewski L, Just I and Ahnert-Hilger G. *"Enhanced axonal sprouting and functional recovery after spinal cord injury by a peptidic fragment from Clostridium botulinum C3 protein."* Arbeitstagung der Anatomischen Gesellschaft, Würzburg 2009

- **Boato F**, Nitsch R and Hendrix S. *"Effect of IL-4 and IL-10 on outgrowth and reinnervation in vitro and in vivo."* Co.R.T.Ex meeting, Berlin 2008

- **Boato F**, Barber PR, Bauerschmidt C and Rothkamm K. *"Characterization and scoring of γH2AX AND 53BP1 foci: manual vs automatic."* Radiobiology Symposium, University of Oxford 2007.

Poster presentations in international conferences and meetings

- **Boato F**, Hendrix S, Hofmann F, Djalali S, Klimaschewski L, Auer M, Just I, Ahnert-Hilger G and Höltje M. *"A 29 aa long fragment from clorstridium botolinum C3 protein foster muscle reinnervation and functional recovery after spinal cord injury."* Berlin Brain Days (BBD) 2009

- **Boato F**, Hendrix S, Hofmann F, Djalali S, Klimaschewski L, Auer M, Just I, Ahnert-Hilger G and Höltje M. *"Enhanced Regeneration and functional recovery after spinal cord injury by a peptidic fragment from clorstridium botolinum."* Society for Neuroscience (SfN), Chicago 2009

- Höltje M, **Boato F**, Hendrix S, Huelsenbeck SC, Klimaschewski L, Auer M, Just I and Ahnert-Hilger G. *“C3 peptide treatment enhances functional recovery from spinal cord injury by improved regenerative growth of corticospinal and raphespinal fibers.”* 105th Annual Meeting der Anatomischen Gesellschaft, Hamburg 2010

- **Boato F**, Hendrix S and Nitsch R. *“T helper-2 cells mediate outgrowth and reinnervation via interleukin-4.”* BCRT meeting 2008.

- **Boato F**, Hechler D, Rosenberger K, Nitsch R and Hendrix S. *“Synergetic effects of IL-4 and IL-10 on axonal outgrowth and reinnervation.”* SfN, Washington 2008.

- **Boato F**, Rosenberger K, Nitsch R and Hendrix S. *“IL-10 induces axonal outgrowth and reinnervation via IL-4r pathways.”* Berlin Neuroscience Forum (BNF) 2008.

- Höltje M, Hofmann F, **Boato F**, Hendrix S, Just I and Ahnert-Hilger G. “*Neuronal Outgrowth and Re-innervation is Enhanced by Clostridial C3 Proteins.”* Arbeitstagung der Anatomischen Gesellschaft, Würzburg 2008

- **Boato F**, Barber PR, Locke R, Pierce GP, Vojnovic B, Bauerschmidt C and Rothkamm K. *“Automated scoring and characterisation of radiation-induced γH2AX and 53BP1 foci.”* **Posterprize**: Research Annual Meeting, Belfast 2007

10. Selbstständigkeitserklärung

„Ich, Francesco Boato, erkläre, dass ich die vorgelegte Dissertation mit dem Thema:

“Hypothermia and C3 peptide promote neurite outgrowth and regeneration after traumatic CNS injury.”

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin 12.05.2010 ____________________

Francesco Boato

11. Acknowledments

Once a bright mind told me that the acknowledgments is the most important part of a PhD thesis because it is the only one that everybody reads; and I agree, but not for the same reason. It is my deep-seated opinion that feeling good with the people around you is by far the most important thing to feel good with yourself and appreciate your own life and work; and the aim of these lines is not only to thank who helped me with my work, but mainly to thanks the people who contributed creating this incredibly welcoming environment.

These years at Charité had been for me an enormous surprise. I came to Berlin without almost any knowledge about neuroscience and without any friends; and now I'm leaving it with more friends that I can handle and with a big motivation to carry on my research in this field. A big help to make everything easier came from Pauline whom followed, loved and helped me so much throughout this entire experience making me feel at home; but also the link with my family and friends from Venice was never lost, thanks (or not) to easyJet, there have hardly been two weeks on a raw without some guest in our leaving room! I can't describe how fulfilling this experience was for me. I should spend pages thanking all the people which "gave me something" during these years… I'll try to keep it short.

These projects have been carried out under the flawless supervision of Professor Robert Nitsch and the superb guidance of Professor Sven Hendrix. Professor Nitsch provided a great working environment, and I am sincerely grateful for his availability and intellectual generosity and for allowing me to work on these challenging projects. Sven Hendrix introduced me into the scientific world and helped me for every (big or small) step of my scientific development, with many advices, suggestions and creative solutions. I want to thank him for his continued support as a supervisor and friend; and I am grateful for his enthusiasm, patience and extreme clarity in explaining difficult topics. I also would like to sincerely thank Markus Höltje for the many advises and for the helpful discussions and collaborations. He supported me in many difficult situations and solved many problems I didn't know how to handle.

Thank you to Karen for her patience in teaching me new techniques and for the friendship I received, and of course to Doreen, Julia and Mareike for their competence, their invaluable help and flexibility.

A warm thanks to the entire Cell Biology and Neurobiology department for the familiarity they offered me; and in particular to Sabrina, Tobias, Rike, Leslie, Lorenza, Eleonora, Debora, Peter, Eli, Thomas, Jens, Martin, Jan, Pawel and all the other PhD students, to make the work environment intellectually stimulating and the lab work a lot more fun that what I expected!
A very warm thanks to Anna, Gina, Gürlsel, Oscar, Ryan, and Steph for the countless discussions, exchange of thoughts, movies, dinners, parties and beers; here I would have A LOT more to say about everyone of them, but they already know that their friendship means a lot to me, also because it keeps reminding me that it is possible to have a great time out of the lab also with scientists! And thanks of course to the easy going Mark, to Ingo and to Hagen to make every moment we spend together even more agreeable. Thanks also to the fellows from CORTEX, to make this "excellence" program excellent, not only concerning science! And thanks to Lutz and Professor Dirnagl for making things a lot easier in many occasions.

The biggest thanks goes of course to my family, but I'm more comfortable to write this in Italian for obvious reasons.
Senza il supporto e il calore della mia famiglia in qualsiasi situazione, avrei potuto fare ben poco. Grazie mamma e grazie babbo per essere stati dalla mia parte per ogni scelta e in ogni situazione, e per aver avuto sempre un consiglio, una soluzione o un'improbabile alternativa da suggerirmi! Sono innumerevoli le cose che ancora avete da insegnarmi, tu mamma con l'Ariella e le tue molteplici amiche riesci a rendere tangibile il concetto stesso di società e tu papà con il tuo spirito curioso, stravagante ma comunque precisissimo riesci a far nascere in me l'interesse in cose che non avrei mai approcciato da solo. E un grazie a Skype per le molteplici telefonate che ci ha offerto. Grazie Chiara, il bene che ti voglio non é quantificabile, non so bene come renderlo comprensibile per iscritto, ogni volta che ci vediamo sembra che sia passato non più di un secondo dalla volta prima, il nostro legame non conosce ne tempo ne distanza, e so che sarà sempre così. E grazie Lorenzo per occuparti di lei ed essere diventato un amico in questi anni!
E tornando un secondo a Berlino, grazie ancora ed infinitamente Pao, per essere venuta con me ed aver quindi portato una parte della mia famiglia dentro la casa in cui vivo, è inestimabile poter condividere tutte le avventure e le emozioni con

qualcuno che ami, non riesco ad immaginare come farei senza. E grazie ad Alain e Jeannette per appoggiare tutte le difficili scelte che dobbiamo fare.
Grazie ai vicinissimi di casa in via Fusinato, che nella fattispecie sono l'indistruttibile nonna Luciana, le affettuose zie Leda e Monica che mi accolgono ogni volta che suono il campanello di casa loro nei momenti più impensabili, e soprattutto a Marco e Gianluca che ringrazio per le mille risate che ci facciamo ogni volta che ci vediamo!
Inoltre grazie agli zii Stefano, Marco, Sandro e Mimmo, nonché ai cugini Lalla, Vania, Giulia, David, Matteo, Riccardo e Nicolò ma soprattutto ai nuovi arrivati Matilda, Beatrice, Alessandro e Tommaso per ricordarmi fino a che punto sia unica la famiglia Boato, di cui vado ogni giorno fiero.
Grazie a Gian, al Vergo, a Luca, a Joseph e la Chiara e a Francesco e la Vero, anche in questo caso avrei pagine da scrivere, ma loro sanno quanto importanti sono per me e quanto bello sia trovarli sempre lì quando torno a Venezia, e grazie alla Giulietta romana, alla Madda, alla Giulia parigina e a Scarpa per ricordarmi che è bello anche quando bisogna fare 2000 km per vedere gli amici più cari e grazie anche per ospitarmi ogni volta che vengo a trovarvi.
E ovviamente non posso dimenticare tutti gli altri amici che ho fatto in questi anni a Berlino, in particolare grazie a Denis e l'Alessandra per la loro positività, esuberanza ed energia, e alla Cecilia, l'Alessandra Ch e la Sara per tutti i sorrisi che mi hanno regalato; and a big thanks to Soelly and Lukas, Audrey, Sandro, Morgane, Thomas and Katha not to allowed me to rest, even for only one weekend.
E poi alla gentilissima Sara, all'irrefrenabile Tyu, a Davide, alla Lalla, al Gaspa e Mitu, a Fabio, al Madda e a molti altri per farmi sentire in vacanza e farmi scoprire sempre cose nuove della cittá in cui vivo.
Di sicuro mi sto dimenticando qualcuno, forse proprio te che stai leggendo, ma credetemi, l'amicizia che ti ho dimostrato è sincera, e questa è solo una dimenticanza dovuta alla stanchezza.

Per finire c'è qualcun altro che non ho ancora nominato, ma in questo caso l'ho fatto per un motivo ben preciso, mi piacerebbe infatti dedicare questa tesi a Jacopo, mio nipotino, perché grazie a lui sento la mia famiglia ancora più vicina, e questo rende TUTTO molto più facile!!!