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Dissertation

Die Rolle des Parasympathikus in der Entstehung schlaganfallassoziierter Infektionen

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Abstract

Within the first three months after stroke onset up to 95% of stroke patients experience at least one relevant complication which has an important impact on survival. Infection is the most relevant complication with frequencies between 21 – 65%. Among infections, stroke-associated pneumonia has the highest attributable mortality in stroke patients and is considered as the most common serious medical complication in stroke care. Moreover, infection has a tremendous negative impact on the neurological outcome after stroke, and prevention of infection might improve functional recovery.

The first aim of this PhD thesis was to establish a suitable tool to assess long-term functional outcome after experimental stroke in mice. As patients might suffer from severe motor deficits after stroke, and as automated gait analysis is a reliable tool to measure motor function in small rodents, mice were tested before and after experimental stroke for gait deficits. Ten days after 60 min Middle Cerebral Artery occlusion, the use of hind limbs was impaired. Additionally, interlimb coordination was disturbed.

The second study aims to investigate whether preventive antibiotic treatment inhibits stroke-associated pneumonia and improves long-term outcome after stroke. Whereas preclinical research uses placebo as control, and clinical studies compare against standard antibiotic treatment after diagnosis of pneumonia, in this study current stroke care was modeled in mice, applying lung MRI and clinical scores to diagnose murine pneumonia. Standard antibiotic treatment reduced the post-stroke mortality as well as the preventive treatment approach, however at the cost of a worse neurological outcome in gait analysis compared to the preventive approach.

In the third part, the reason for the increased risk for infection after stroke was examined. In addition to the sympathetic nervous system and the HPA axis, the parasympathetic nervous system is a major contributor in the intense bidirectional brain-immune communication. Applying heart rate variability as an indicator for parasympathetic activity, an increased parasympathetic activity within the first days after stroke was observed. After experimental stroke, vagotomised mice and mice deficient for $\alpha 7$ nicotinic acetylcholine (ACh) receptor did not develop pneumonia as shown by a lower bacterial burden in the lung. Parasympathetic signaling impairs both macrophages and alveolar epithelium cells after stroke.

In summary, this thesis adds a small piece to the puzzle of disturbed brain-immune communication after stroke. I established a new method to assess long-term functional outcome in mice with stroke, tested the influnces of infection on functional outcome in a mouse model of standard stroke care and examined the influence of the parasympathetic nervous system on post-stroke infections.

Zusammenfassung

Innerhalb der ersten drei Monate nach einem Schlaganfall erleiden bis zu 95% der Patienten mindestens eine relevante Komplikation, die einen Einfluss auf die Überlebensrate hat. Infektionen sind die relevantesten Komplikationen mit einer Prävalenz zwischen 21 und 65%. Aus der Gruppe der Infektionen ist die Schlaganfall assoziierte Pneumonie die häufigste Todesursache von Schlaganfall-Patienten und wird als häufigste schwere medizinische Komplikation in der Schlaganfallversorgung angesehen. Darüber hinaus haben Infektionen einen großen Einfluss auf die neurologische Prognose nach Schlaganfall, und die Prävention von Infektionen könnte die funktionelle Genesung verbessern.

Das erste Ziel dieser Dissertation ist die Etablierung einer geeigneten Untersuchung des funktionellen Defizits nach experimentellem Schlaganfall in Mäusen. Da Schlaganfall-Patienten mitunter an schweren motorischen Störungen leiden, und da automatisierte Ganganalyse in kleinen Nagetieren schon erfolgreich eingesetzt wird, wurde das Gangbild von Mäusen vor und nach experimentellem Schlaganfall untersucht. Zehn Tage nach 60-minütigem Verschluss der Arteria cerebri media war die Nutzung der Hinterbeine eingeschränkt und die Koordination zwischen den Beinen gestört.

Die zweite Studie untersucht ob die präventive Gabe von Antibiotika durch die Verhinderung von Infektionen einen positiven Einfluss auf die Langzeit-Prognose nach Schlaganfall hat. Gegenwärtig gibt es nur präklinische Studien mit Modellen, die Placebo-kontrolliert sind, oder klinische Studien, in denen in der Kontrollgruppe nach der Diagnose einer Infektion antibiotisch behandelt wurde. Daher modellierten wir die Standard-Pflege für Schlaganfall in Mäusen, in dem wir Lungen-MRTs und klinische Scores zur Diagnose der murinen Pneumonie nutzten. Die Standard-Therapie mit Antibiotika nach Diagnose reduzierte die Mortalität im gleichen Maße wie die präventive Gabe, allerdings auf Kosten eines schlechteren neurologischen Ergebnisses verglichen mit der präventiven Gabe.

Im dritten Teil soll der Grund für das erhöhte Infektionsrisiko nach Schlaganfall näher untersucht werden. Zusätzlich zum Sympathischen Nervensystem und der Hypothalamus-Hypophysen-Nebennieren-Achse spielt das parasympathische Nervensystem eine wichtige Rolle in der intensiven wechselseitigen Kommunikation zwischen Gehirn und Immunsystem. Mittels Herzfrequenzvariabilitätsmessungen als Indikator für parasympathische Aktivität konnte eine erhöhte parasympathische Aktivität in den ersten Tagen nach experimentellem Schlaganfall beobachtet werden. Mäuse mit Vagotomie und transgene Mäuse ohne $\alpha 7$ Acetylcholin (ACh) Rezeptor entwickeltem nach experimentellem Schlaganfall keine Pneumonie, wie sich an der geringeren Keimlast in der Lunge zeigte. Es zeigte sich, dass der Parasympatikus nach Schlaganfall sowohl Makrophagen als auch alveoläre Epithelzellen in ihrer Funktion hemmt.

Zusammenfassend fügt diese Dissertation ein kleines Puzzleteil zu der nach Schlaganfall gestörten Kommunikation zwischen Gehirn und Immunsystem hinzu. Ich habe eine Methode etabliert, die in längeren Zeiträumen die Messung der neurologischen Funktion von Mäusen mit Schlaganfall erlaubt, habe den Einfluss von Infektionen auf die neurologische Funktion von Mäusen mit Schlaganfall gestestet und den Einfluss des parasympathischen Nervensystems auf Infektionen nach Schlaganfall charakterisiert.

Contents

List of abbreviations

α7 nAChR	α7 nicotinic acetylcholine receptor
ACh	Acetylcholine
AChE	Acetylcholinesterase
AM	alveolar macrophages
ARRIVE	Animal Research: Reporting of In Vivo Experiments
BAL	broncho-alveolar lavage
BM	bone marrow
BP	blood pressure
CDC	Centers for Disease Control and Prevention (USA)
cfu	colony-forming units
CNS	Central Nervous System
DBP	diastolic blood pressure
ECG	electro-cardiogram
G	Gauge
HPA	hypothalamo-pituitary-adrenal axis
HR	heart rate
ko	knock out
LB	lysogeny broth medium
LPS	Lipopolysaccharide
MAP	mean arterial pressure
MCAo	Middle Cerebral Artery occlusion (an experimental model for stroke)
mL	mililiter
MRI	magnet resonance imaging
PAT	preventive antibiotic treatment
SAP	stroke-associated pneumonia
SPF	specific pathogen free
SBP	systolic blood pressure
UTI	urinary tract infections
TE	echo time (MRI)
TLR	Toll like receptor
TR	repetition time (MRI)
WT	wildtype

Introduction

Neurological outcome of stroke and its implication for research models

Stroke is one of the most important diseases in industrialised countries, associated with high morbidity and mortality [1]. In Germany, with 250 000 cases and a mortality of about 30%, stroke is the third leading cause of death. Additionally stroke is the leading cause for adult disability[2]. Despite the medical need, therapeutic options are limited to thrombolysis with tissue plasminogen activator, which can only be used in 5-10% of patients due to the narrow time window of 4.5 hours [3], and specialized care in dedicated stroke units [4]. In addition, a small proportion of patients could also benefit from endovascular thrombectomy [5]. .

Three months after stroke, half of the surviving stroke patients have still not gained full recovery and 25 % of survivors are unable to master their daily life without professional care [2]. To allow translation of experimental findings to the clinics, effective functional tests for experimental stroke outcome are desperately needed [6]. While proper tests in patients for evaluating motor as well as cortical impairment are widely available, animal behaviour after cerebral ischemia remains to be difficult to assess in particular in mouse models of stroke. One of the most obvious reasons is the ecological niche as prey animal, implying compensatory mechanisms for hiding sickness symptoms as a key factor for survival [7]. Different behavioural tests have been proposed in relation to experimental stroke in mice, among them many for locomotion such as rope walk [8], grid walk [9], ladder rung test [10] and foot print analysis [11]. As stroke patients show impaired swing initiation [12], inadequate leg propulsion and subsequent compensatory mechanisms [13, 14], gait analysis might be also a promising tool to examine functional deficits in mice. With the development of suitable equipment, gait analysis has become a promising option in research.

Stroke associated infections

In addition to the neurological consequences, patient suffering from stroke regularly develop fever and subsequently have a high risk to die, as being described for the first time by Christoph Wilhelm Hufeland, the first dean of the Charité [15]. In spite of this early observation, medical complications after stroke remained a neglected field in stroke research. According to recent data between 60 and 85 % of stroke patients experience medical complications [16-18]. In line with Dr. Hufeland's description, fever is one of the most common symptoms [19] occurring in up to 61% of stroke patients [20] and worsening neurological outcome [16, 21, 22]. One of the most important reasons for developing fever is infection, and stroke associated infection is a common

complication in stroke care. With frequencies between 21 and 65% [19, 22-24], the incidence of infectious complications is significantly higher than the general prevalence of hospital acquired infections, ranging from 6 to 9 % [25]. Among those, bacterial pneumonia with an incidence of 5 – 22 % and urinary tract infections (UTI) with an incidence of 6 – 27% are the most common complications after stroke [19]. By comparison, non-stroke patients in a geriatric hospital have an average incidence of pneumonia of 3.5% [26]. Being the reason for about one third of the attributable mortality [27], pneumonia is the most common cause of death both in the acute phase after stroke [28-30] and after hospital discharge [31].

Alongside the medical relevance of stroke associated infection, an American study estimated the mean adjusted costs for hospitalisation of patients with stroke-associated pneumonia more than three times higher than for patients without pneumonia ($21,043 vs. $ 6209) [32]. Hence the authors calculated the annual costs of stroke-associated pneumonia in the US at up to US$ 459 million.

Stroke associated infection is not only a medical problem affecting mortality, it has also a tremendous impact on neurological outcome. [33-35] Stroke patients suffering from pneumonia were over 70% more likely to require extended care after discharge and hence to have a lower quality in daily life [32]. Patients experiencing infections after stroke have a much higher risk for unfavourable outcome compared to patients with infections present at hospital admission [36].

Even in specialized stroke units pneumonia remains a common and severe clinical challenge [34, 37] and thus alternative treatment strategies are of urgent need. Despite successful prevention of infection by preventive antibiotic treatment (PAT) in an experimental stroke model [38], randomized controlled phase IIb trials on PAT after stroke did not reveal a clear result [39-41]. A meta-analysis on these trials suggests that PAT reduces the occurrence of post-stroke infections, but remains ineffective in terms of outcome [42].

Stroke models have to reflect the clinical situation and care aspects [43]. In terms of neurological outcome, the discrepancy between preclinical success [38] and failure in clinical studies [42] might be explained by the fact that mice were treated either with preventive antibiotics or placebo in study. In the clinical studies, preventive antibiotic treatment was compared to the current guidelines, recommending antibiotic treatment directly after diagnosis of pneumonia. The challenge in modelling the clinical situation is to reliably diagnose pneumonia in living mice to start antibiotic treatment. In patients diagnosis of chest infection is based on the CDC criteria

[44] including radiological, clinical and laboratory findings, whereas in the mouse established criteria do not exist.

Stroke induced immune-depression syndrome

Cerebral ischemia leads to a number of predisposing factors for infections, among them aspiration, bedridden state, impairment of protective reflexes, decreased level of consciousness and mechanical ventilation. [45-49] However, they cannot explain the increased risk for infection alone. Several distinct small brain areas account for impairment of swallowing, but infectious complications are correlated with larger insular location of the insult. [50, 51] And with respect to the observation, that approximately 50% of healthy subjects aspirate pharyngeal fluid during sleep in similar amounts than stroke patients [52-54], there is strong evidence for additional mechanisms beside aspiration.

To react appropriately to challenges from the environment, the nervous and immune systems are closely interconnected in an intense bidirectional communication. [55-58] Receptors in the peripheral nervous system register the status of the immune system to higher centers of the CNS. After processing these signals the immune system is influenced by homeostatic signals mainly via three important pathways: the hypothalamo-pituitary-adrenal axis (HPA), the sympathetic nervous system and the parasympathetic nervous system [55, 57, 59]. With a plethora of studies reporting impaired immune responses after stroke and other diseases of the CNS [60-65], a secondary CNS-injury induced immunodepression is currently regarded as the cause for the increased risk for infections [23, 66-68]. In light of experimental and clinical observations CNS injury likely disturbs the normally well-balanced interplay between these two supersystems resulting in a profound and long-lasting immunodepression [23], as described in stroke [69], traumatic brain injury [70] and spinal cord injury [71, 72]

After stroke, well known communication pathways such as sympathetic nervous system and hypothalamus-pituitary-adrenal axis have been shown to play an important role in stroke induced immunodepression [69], acting mainly on the adaptive immune system [73, 74] and especially on iNK-T cells [75].

On the other hand the innate immune system is regulated by the parasympathetic nervous system acting via the vagus nerve and the neurotransmitter acetylcholine [76, 77]. In models of sepsis, experimental activation of the vagus nerve, either by electrical stimulation [78, 79] or by pharmacological activation via nicotine [80], led to a significant reduction in production of pro-inflammatory cytokine TNF-α, the most important cytokine of macrophages, and other pro-

inflammatory cytokines. The molecular basis is a nicotinic, α-Bungarotoxin sensitive acetylcholine receptor, the α7 nAChR, as demonstrated in knockout animals [81, 82]. Although the α7 nAChR being expressed on a variety of cells including macrophages, T-cells, neurons and glial cells, only α7nAChR expression on bone marrow-derived non–T cells is required for the integrity of the inflammatory reflex [83].

Recent research revealed at least for sepsis a much more elaborate way of communication, passing from the brain stem to the celiac ganglion as origin of the splenic nerve. From there signals via the adrenergic splenic nerve terminate at specialized T cells in the spleen, that deliberate Acetylcholine to influence macrophages [84].

Importantly, the vagus nerve also provides information on the peripheral immune status to the CNS. Peripheral receptors detect inflammatory mediators and relay this information via the vagus nerve to higher centers of the brain, among those the dorsal vagal complex and the nucleus tractus solitarius [85]. Under physiological conditions, this so-called "cholinergic anti-inflammatory pathway" acts as a feedback loop preventing a potentially noxious overreaction of the immune system [86-88]. The hypothesis of this work is that after stroke this feedback mechanism is activated without prior stimulation, hence leaving the patient without proper immune response after stroke.

Aims

The first aim of the first study was to investigate the influence of a common model of severe stroke, 60 min Middle Cerebral Artery occlusion, on mid- to long-term gait impairments. Further, the second aim was to investigate the applicability of gait analysis for experimental stroke studies with respect to effect sizes and required sample size.

The aim of the second study was to investigate the impact of preventive antibiotic treatment on long-term functional outcome in comparison to the current "gold standard" for treating post-stroke infections and to placebo. Current guidelines recommend an immediate treatment after diagnosis, hence we developed diagnostic criteria for murine pneumonia, similar to the CDC criteria for human [44].

Finally, the third study will focused on the underlying mechanisms of stroke-induced immunodepression and the subsequent high risk for infectious complications. I investigated to what extent the parasympathetic nervous system plays a role in the pathophysiology of post-

stroke infections. Special emphasis will be put on the question of the target cells of parasympathetic signalling in the lung.

Materials and Methods

Due to the variety of methods, only key methods are described here in brief. All details are described in the three publications listed in the appendix.

Animals and Housing

Experiments were performed in accordance with the European directive on the protection of animals used for scientific purposes and the respective German legislation after approval by the relevant authority, Landesamt für Gesundheit und Soziales, Berlin, Germany (registration numbers G0253/08, G0467/09, G0107/12). Experimental design followed the recommendations of the ARRIVE guidelines [89].

Male SPF C57Bl6/J mice (Charles River Laboratories, Sulzfeld, Germany), gender mixed α7 nicotinic acetylcholine receptor (α7 nAChR) knockout (ko) mice [90] and wild type (WT) littermates respectively (B6.129S7-Chrna7^{tm1Bay}/J; JAX-stock 003232; The Jackson Laboratory, Bar Harbour, USA) were housed in groups of 5 - 8 in cages lined with chip bedding and environmental enrichment (mouse tunnel and igloo, running wheel until experimental stroke; Plexx B.V., Elst, The Netherlands) on a 12 h light/dark cycle (change 7 o' clock) with ad libitum access to food (standard chow) and water. At the time of the experiment, mice were 11 – 14 weeks old.

Bone-marrow chimeric mice were generated as described previously [91]. We reconstituted 6 to 8 week old α7 nAChR ko or WT recipient mice with bone marrow derived from the tibias and femurs of adult WT or α7 nAChR ko mice, in all four feasible combinations. Reconstitution was assessed 8 weeks after transplantation by PCR analysis of peripheral blood.

Experimental stroke

The surgical procedure of middle cerebral artery occlusion (MCAo) was performed as described elsewhere in detail [92] according to the standard operating procedures of our lab [93]. In brief, after a ventral cervical midline incision a small silicon-coated filament was introduced over the common carotid artery and the internal carotid artery into the circle of Willis blocking the origin of the Middle Cerebral Artery. The filament was left in place for 60 min. Body temperature was

controlled throughout the whole procedure and isoflurane (Abott, Wiesbaden, Germany) in a 1:2 mixture oxygen/nitrous oxide was used for anaesthesia.

Gait Analysis

Gait analysis in mice was performed with an automated computer assisted method (CatWalk™, Noldus Information Technology, Wageningen, The Netherlands) according to manufactures instructions and published procedures [94]. In brief, in a dark and silent room mice passed an elevated 1.3 meter long glass plate with the home cage used as bait at the end of the walkway. They were trained three times before the first measurement to get familiar with the setup.

The glass plate is illuminated from the side, and contact of animal paws with the glass plate lead to a changed refractive index of the internally reflected fluorescent light, which then leaves the glass plate and is reflected downwards. A high-speed camera underneath the glass plate captures the images which are subsequently analyzed by the connected computer program.

Diagnosis of infection and quantitative analysis

Infection was either measured by MRI of inflammatory sequelae, or by broncho-alveolar lavage after intubation. MRI was performed 3 days after MCAo in Isoflurane anaesthesia using a 7 Tesla rodent scanner (Pharmascan 70/16, Bruker BioSpin, Ettlingen, Germany) and a 1H-RF volume resonator. Triggered on ECG and respiration to avoid motion artefacts, I examined the lung using a T1 weighted FLASH sequence. I considered areas with a signal-to-noise ratio larger than 3.5 as signals of lung inflammation [95] and expressed this as a fraction of whole lung area.

Broncho-alveolar lavage (BAL) was performed after intubating mice under medetomidin/ midazolam anaesthesia with a 22G peripheral venous catheter as described elsewhere [96]. Subsequently, 0.4 ml of saline plus 0.2 ml air were applied over the tubus and immediately withdrawn. [97] For quantitative analysis (colony forming units – cfu) samples were serially diluted and grown on LB plates for 18 hours.

Vagotomy

Cervical vagotomy was performed 5 days before experimental stroke at the same side of the neck as the MCAo surgery. In brief, under isoflurane anaesthesia the left cervical vagus nerve was carefully and bluntly dissected from the common carotid artery and transected. In sham-operated mice, the left vagus nerve was exposed and isolated from the surrounding tissue but was not transected.

Telemetry

Blood pressure (BP) and heart rate (HR) were measured by telemetry combined with fast Fourier transform analysis of BP and HR as described elsewhere. [98, 99] In brief, telemetric devices were implanted in a subcutaneous pocket along the right flank in mice. Blood pressure was measured in the abdominal aorta with catheter access through the right femoral artery.

Statistics

Data were analyzed with SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). Normal distribution of variables was verified with Kolmogorov-Smirnov test. Parametric and non-parametric test were applied as indicated in the papers. In all cases, a type I error (α) of 0.05 and type II error (β) of 0.2 was accepted. *P*-values of less than 0.05 were considered statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Data are expressed as mean ± standard deviation (SD) and represented as Box plots with whiskers Minimum to Maximum, unless otherwise stated. For calculating effect sizes and a priori sample sizes the program G*Power 3.1.2 [100] was applied. To analyze gait data in study 1 and 2, I used the comparison for effect sizes described by Matthews and Altman [101] and standard procedures such as the standard error of differences [102].

Results

Study 1: Gait analysis as a measure for outcome in experimental stroke

We hypothesized that experimental stroke in mice affects the complex coordination of gait and that these gait abnormalities can be measured even after longer periods of time.

Automated gait analysis provides a sensitive tool to examine locomotion and limb coordination in small rodents. We compared gait parameters from mice measured before and 10 days after experimental stroke (60 min MCAo). The approximately 250 parameters can be grouped into four larger categories: spatial and kinetic characteristics of individual paws, comparative paw measures and interlimb coordination.

The spatial parameters maximum contact area and maximum intensity were significantly decreased in both hind paws after stroke (Figure 1), indicating a less powerful use of the hindlimbs. Again in the hind limbs, individual paw kinetics were altered in terms of a significant reduction in paw's swing speed. Comparative paw measures describe measurements that are related to the whole step cycle. The altered parameters indicated an impaired subtask "swing" in

the hindlimbs and a compensatory use of frontlimbs. Furthermore, mice showed a disturbed interlimb coordination represented by changes in regularity index and phase dispersion.

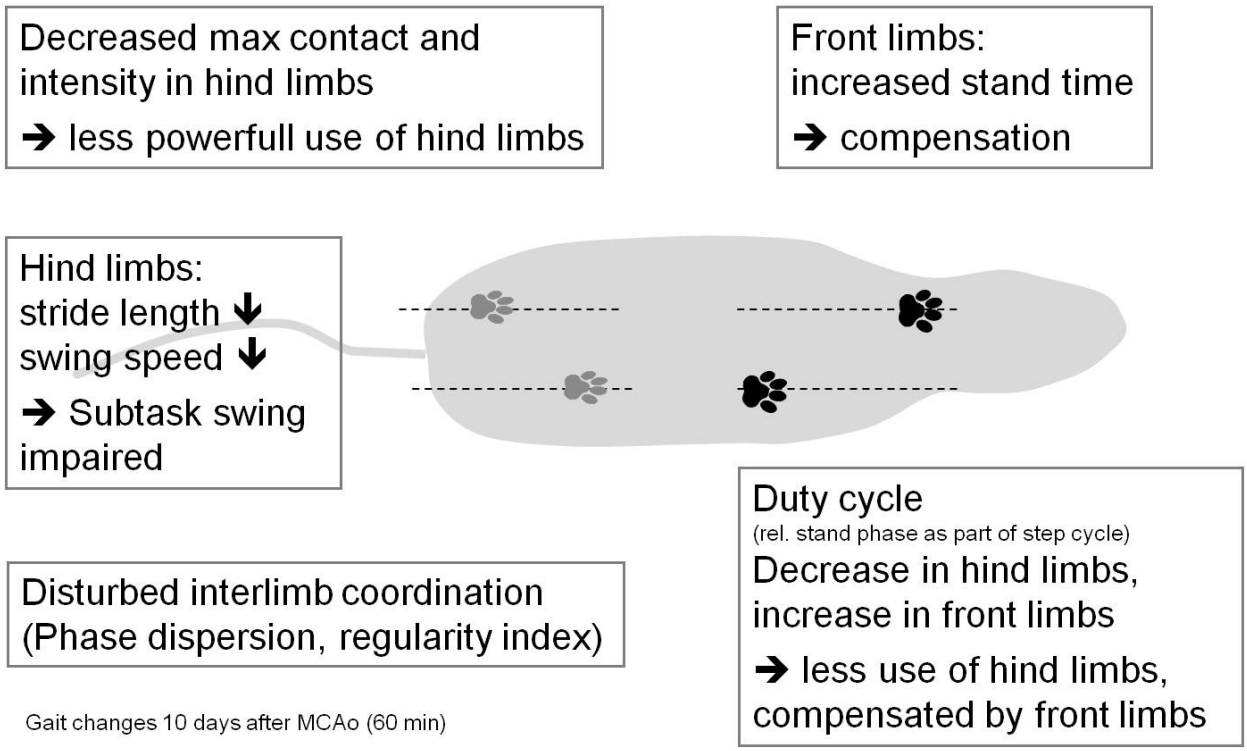

Figure 1 Summary of gait parameters that change 10 days after experimental stroke.

To assess whether gait analysis is applicable to assess improvements by neuroprotective compounds, I applied a model calculation. Here the use of effect size statistics can reduce the sample size to 16 – 21 animals, if we assume an improvement by 50 – 60 % as a biologically relevant improvement.

Study 2: Comparison of preventive antibiotic treatment and standard treatment of post-stroke pneumonia

The hypothesis of the second study was that prevention of post-stroke infection improves functional outcome compared to placebo and gold standard, i.e. therapeutic antibiotics after diagnosis of infection.

Our experimental setup included three groups. One group received preventive antibiotic treatment immediately after experimental stroke, a second group got placebo treatment. The third group got antibiotic treatment after diagnosis of pneumonia, modelling the current standard therapy in stroke-associated pneumonia care.

To test this hypothesis, I established a protocol to reliably diagnose pneumonia in mice, consisting of the Mouse General Health Score and radiological confirmation by MRI. The MRI protocol was verified against post-mortem bacteriological analysis.

Despite manifest infections in both placebo and standard treatment group, similar survival rates between 80 and 90 % were observed both after standard and preventive antibiotic treatment. In contrast, only 55% of placebo treated animals experienced the end of study at day 14.

However, preventively treated mice showed an improved functional outcome in gait analysis 10 days after experimental stroke, both compared to placebo and standard treatment. Interestingly, worse functional outcome correlated with an increased infiltration of leukocytes into the brain.

Study 3: Stroke induced parasympathetic activation mediates immunodepression in lung
We hypothesized that the parasympathetic nervous system is activated after stroke, hampering a proper immune response especially of macrophages and alveolar epithelium cells.

A suitable approach to measure the status of the autonomic nervous system is the measurement of heart rate variability [103, 104], and I used the telemetry approach to obtain measurements in an undisturbed, quiet environment. To validate the parameters of parasympathetic activity, I used atropine and metropolol before start of the experiment. I observed an increase in heart rate variability (LF-HRV, RMSSD, SD-RRI) and an increase in baroreflex sensitivity on day 1, 3 and 5 after stroke, indicating an increased parasympathetic activity. Baroreflex sensitivity (BRS) is the ability to adopt the sinus frequency in response to blood pressure changes, also influenced by parasympathetic activity.

To unravel the effect of this increase parasympathetic activity on the immune system, mice underwent unilateral cervical vagotomy 5 days before MCAo or sham surgery. Vagotomized animals showed significant reduction in bacterial burden in the lung, and the effect could be reversed by nicotine treatment.

Further characterizing the target receptor, I used α7 knock-out mice and wildtype littermates. Again, a significant reduction in bacterial burden could be observed if no α7 receptor was present while no differences in heart rate or infarct volume were observed.

To disentangle the impact of bone marrow (BM)-derived pulmonary immune cells, e.g. alveolar macrophages (AM) and lung epithelial cells on α7 nAChR dependant post-stroke immunodepression, we generated chimeric mice by transplanting BM from α7nAChR KO mice

or WT littermates into lethally irradiated either α7nAChR KO or WT recipient mice. In accordance with the knock-out experiment, ko→ko chimeras had a significantly smaller bacterial burden compared to WT→WT animals. Both WT→ko and ko→WT animals had intermediate values between WT and ko phenotype. A similar picture was observed ex vivo in cytokine response assays with different stimuli.

Discussion

Study 1: Gait analysis as a measure for outcome in experimental stroke

In summary, we observed deficits in both hind paws 10 days after 60 min MCAo. The reduced maximum contact area and maximum intensity might be an indicator of reduced propulsion and weight bearing in both hind paws [105]. We could observe an impaired subtask swing in gait patterns, being in line with observations in patients [106]. The observed increase in stand and duty cycle in the front limbs might be explained by compensatory mechanisms taking over the lack of effective hind limb use. In addition, mice showed an impaired interlimb coordination shown by more irregular step patterns and altered phase dispersions.

Our results are in line with other publications in the rat [107-109] and could be reproduced in general by other groups [110-112]. Analyzing the applicability of gait analysis in testing novel stroke treatments, only a few parameters appear to be suitable to detect gait improvements due to the limited effect size. However the sample size would be still quite high, as estimated between 15 and 20 animals per group. Nevertheless gait analysis can be regarded as a suitable tool to analyze long-term functional outcome in experimental stroke models.

Study 2: Comparison of preventive antibiotic treatment and standard treatment of post-stroke pneumonia

Infection is also a major bias in experimental stroke studies [113]. Due to the clinical relevance, several mouse models of stroke associated infection have been established [114]. In experimental models, post-stroke infection worsens stroke outcome in terms of mortality, expression of pro-inflammatory cytokines, infarct volume and short-term behavioural testing [38, 69, 115]. Hence mouse models are suitable for investigations on stroke-associated pneumonia [114].

Whereas clinical studies demonstrated that antibacterial prophylaxis reduced the occurrence of post stroke infections, but has no tremendous impact on mortality and functional outcome [42], studies in mice revealed a clear positive effect on mortality and outcome [38]. However mice

were treated either with preventive treatment or with placebo, not reflecting the clinical situation of "treatment after diagnosis" properly.

To address this discrepancy, we established a protocol to diagnose pneumonia in living mice similar to the CDC criteria in human, including clinical and radiological signs. Both Mouse General Health Score and lung MRI were effective in detecting mice with pneumonia.

Applying this to our stroke model, we were able to model the clinical situation properly. Similar to the clinical studies, preventive antibiotic treatment reduced the incidence of infection significantly. Again resembling the results from clinical studies, treatment with antibiotics improved survival, independent of whether it was given preventively or immediately after diagnosis of pneumonia. On the other hand, even a timely treatment after the diagnosis of pneumonia could not reverse the negative effect on functional outcome, as shown in gait analysis. Hence we assume that antibiotic treatment after diagnosis might be too late for preventing negative effects on neurological outcome.

Two recent large randomized clinical trials [116, 117] examined the impact of preventive antibiotic treatment on stroke outcome. In both trials preventive antibiotics did not result in a better functional outcome measured by modified ranking scale, and was not able to significantly reduce the frequency of pneumonia. In contrast to our homogenous mouse population at high risk for post-MCAo infection, the patient population was much more divers and not all patients had a high risk for pneumonia. Additionally, while antibiotic treatment in mice started immediately after ischaemia, the treatment start for patients was 24 to 48 hours after the ischaemic event [118]. In light of the results of the clinical trials, the growing problem of bacterial resistance [119] and the immanent adverse events of antibiotics [120, 121] in my view the preventive use of antibiotics cannot be recommended as standard therapy for stroke patients.

While the results of this thesis show in an ideal and surely simplified model that prevention of infection could be of benefit regarding functional and medical outcome, the translation into the complex clinical routine is more challenging and requires probably a more targeted approach. A better understanding of the underlying mechanisms leading to post-stroke immunodepression could unravel new diagnostic and therapeutic targets. On the one hand, this may help to identify patients at risk to tailor a suitable preventive treatment. Currently there are different attempts to identify suitable biomarkers in large clinical trials [122, 123].

On the other hand, counteracting the underlying mechanisms might be a better strategy to prevent stroke-associated pneumonia, especially as preventive antibiotics were not as effective as it was hoped. A lot of basic research on the underlying mechanisms has been done in the past ten years [51, 69, 74, 75, 124-130], and the third study of this thesis will contribute to that field. However, translation into therapeutic approaches is still an open task.

Study 3: Stroke induced parasympathetic activation mediates immunodepression in the lungs

Previous work concerning stroke induced immunodepression focussed on adaptive immunity, such as CD4 T-cells [69, 131], invariate natural killer T-cells [75] or B-cells [69, 126], mainly mediated by sympathetic influences [132]. Recently, excessive release of High Mobility Group B1 (HMGB1) from the ischaemic brain was shown to contribute to the induction of this immunosuppressive state, and accordingly post-stroke immunodepression appears to be a more complex phenomenon [130].

In contrast to the adaptive immune system, innate immune system reacts immediately after exposure to pathogens, hence acting as a first line of defence [133, 134]. Whereas the adaptive immune system has been in the focus of immunological research and was regarded as the more important part of immune responses for a long time, the innate immunity is increasingly regarded as the central defence mechanism [135]. Consequently we hypothesized that stroke-induced immunodepression also inhibits innate immunity, leading to a breakdown of the immunological barriers. The parasympathetic nervous system was shown to play a crucial role in controlling innate immunity, mainly macrophages [77] and eventually also lung epithelium cells [136].

Using heart rate variability measurements, we could observe an increased parasympathetic activity in the first days after experimental stroke, which could not be explained by differences in activity. Importantly, species specific differences in the interpretation of the parameters have to be taken into account [99, 104, 137], as for example low frequency HR oscillations are an indicator for parasympathetic activity in mice and for both sympathetic and parasympathetic activity in humans [138]. Alterations in heart rate variability have been linked to changes of peripheral markers of immune function [139-142].

According to our hypothesis, a disruption of the parasympathetic signalling should restore the body's ability to fight infections after stroke. Both after vagotomy and knock-out of the target receptor α7 nAChR mice bacterial burden was significantly reduced and almost as low as in healthy animals, supporting this hypothesis.

A recent study reports that knockout of α7nAChR decreases P. aeruginosa induced lung injury and mortality after stroke [143]. However, this study focuses on the effects of an already occurring pneumonia, whereas we address the question of the onset of stroke-induced immunodepression. In contrast to our study they choose an infection model that also causes severe infection in otherwise healthy mice. [144]

However it remained unknown which are the main target cells of parasympathetic signalling after stroke. Lung epithelium cells are far more than just a physical barrier [145], as they express TLR receptors [146] and other receptors to detect pathogens, and as they produce cytokines and antimicrobial peptides [147-150]. Apart from its physical barrier function, pulmonary epithelium can kill pathogens directly [148, 151] and is able to trigger innate and adaptive immune responses [152]. Furthermore, lung epithelial cells play an important role in fighting viral infections [151]. Other groups showed that lung epithelium expresses α7nAChR [136, 153, 154].

Based on our observations in α7nAChR chimeric mice, both pulmonary macrophages' and alveolar epithelium cells' functions are impaired by cholinergic signaling after stroke and the function of either of these cell types is required for adequate immune responses. A similar picture is observed ex vivo, where epithelium cells and macrophages respond to cholinergic stimuli with a reduction in cytokine production.

Interestingly, especially α7nAChR knock out macrophages also react on nicotine, raising the question for other receptors involved. Different groups were even unable to detect α7nAChR on pulmonary macrophages [155-158]. Although α7nAChR play an important role in attenuation of autoreactive CNS inflammation, also other nAChR are critically involved [159]. A few studies also report on effects of other nAChRs like α9nAChR or β2nAChR [156, 160, 161] on innate immune cells, and in a very recent study β2 and α9 nAChRs were involved in nicotine dependant modulation of immune function in experimental autoimmune encephalitis (EAE) in mice [162]. Also our results in ex vivo stimulation with nicotine and the α7 nAChR-specific PNU282987 support the involvement of other nAChRs in cholinergic immune signaling. Hence a further characterization of other nicotinic receptors is of critical importance to completely understand the complex interaction in cholinergic brain immune signaling after stroke.

Considering the anti-inflammatory effect of ACh, it is plausible that Acetylcholinesterase (AChE) activity is an intrinsic regulator of inflammation [163]. Indeed, peritoneal injection of AChE inhibitors reduces serum pro-inflammatory cytokine levels and improves survival in a murine model of sepsis; intravenous AChE inhibitors reduce IL-1β in brain and blood in mice [164]; and

AChE activity in circulation is inversely related to serum IL-6 levels induced by endotoxin in humans [165]. Therefore not only increased signaling but also decreased clearance by change of AChE expression might contribute to the cholinergic effects on post-stroke immunodepression, and there are first pieces of evidence in terms of serum cholinesterase activity in patients' serum [166].

One might consider stroke-induced immunodepression as a protective mechanism to prevent auto-aggressive immune responses [167]. Moreover, stimulation of the vagus nerve has recently been suggested as a potential neuroprotective therapy [168-172]. With respect to the results of this thesis, we're facing two sides of a coin, as on the one hand the increased cholinergic status could be protective for the brain, but on the other hand it may expose the patients to an increased risk for infections and subsequent higher mortality.

Surgical manipulation and isoflurane anaethesia affect immunological responses after stroke [173]. To allow conclusions from the available models, appropriate controls that were exposed to the same surgery and the same anaethesia were used, implying that the observed differences are independent of the surgery.

In conclusion, based on my results presented here, the protective feedback mechanism of parasympathetic activation, preventing the body from potentially harmful over-activation of immune cells, appears to turn into a destructive event after stroke. Here, parasympathetic signals were shown to disable the innate immunity, namely epithelium cells and macrophages, leaving the body unprotected against bacterial invasion.

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Appendix

Publikation 1: Gait analysis as a method for assessing neurological outcome in a mouse model of stroke

Hetze S, Romer C, Teufelhart C, Meisel A, **Engel O** (2012) Gait analysis as a method for assessing neurological outcome in a mouse model of stroke. *Journal of Neuroscience Methods* 206:7-14

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Publikation 2: Superiority of preventive antibiotic treatment compared to standard treatment of post-stroke pneumonia in experimental stroke: A bed to bench approach

Hetze S*, **Engel O***, Romer C, Mueller S, Dirnagl U, Meisel C, Meisel A (2013) Superiority of preventive antibiotic treatment compared with standard treatment of poststroke pneumonia in experimental stroke: a bed to bench approach. *Journal of Cerebral Blood Flow and Metabolism* 33:846-54

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Publikation 3: Cholinergic pathway suppresses pulmonary innate immunity facilitating pneumonia after stroke

Engel O, Akyüz L, da Costa Gozalves A, Winek K, Dames C, Thielke M, Herold S, Böttcher C, Priller J, Volk H-D, Dirnagl U, Meisel C, Meisel A (2015) Cholinergic pathway suppresses pulmonary innate immunity facilitating pneumonia after stroke. *Stroke* first published online on October 8 2015 as doi:10.1161/STROKEAHA.115.008989

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„Ich, Odilo Randolf Engel, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema „Die Rolle des Parasympathikus in der Entstehung schlaganfallassoziierter Infektionen“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Berlin, den Odilo Engel

Anteilserklärung

Odilo Engel hatte folgenden Anteil an den vorgelegten Publikationen:

Publikation 1: Hetze S*, Römer C*, Teufelhart C, Meisel A, Engel O (2012) Gait analysis as a method for assessing neurological outcome in a mouse model of stroke. *Journal of Neuroscience Methods* 206:7-14

Idee und Konzeption der Studie gemeinsam mit Prof. Andreas Meisel, Projektmanagement, Durchführung und Auswertung der Experimente gemeinsam mit Co-Autoren, statistische Auswertung inklusive Entwicklung der statistischen Methodik, Interpretation der Ergebnisse in Zusammenarbeit mit Co-Autoren, Koordination und Beteiligung am Schreiben der Publikation Der maßgebliche wissenschaftliche Anteil lag in der selbstgestellten Aufgabenstellung, der Evaluierung verfügbarer neurologischer Tests für Mäuse und der Umsetzung zur Ganganalyse. Ferner war Odilo Engel alleinig für die Anwendbarkeitsanalyse (statistische Modellrechnung) verantwortlich.

Publikation 2: Hetze S*, Engel O*, Römer C, Mueller S, Dirnagl U, Meisel C, Meisel A (2013) Superiority of preventive antibiotic treatment compared with standard treatment of poststroke pneumonia in experimental stroke: a bed to bench approach. *JCBFM* 33:846-54

Idee der Studie gemeinsam mit Dr. Christian Meisel und Prof. Andreas Meisel, Konzeption der Studie gemeinsam mit Susann Hetze, Dr. Christian Meisel und Prof. Andreas Meisel, Durchführung und Auswertung der Experimente gemeinsam mit Co-Autoren (insbesondere Etablierung des Lungen-MRTs, MCAo Operation), statistische Auswertung, Interpretation der Ergebnisse in Zusammenarbeit mit Co-Autoren, Beteiligung am Schreiben der Publikation
Der maßgebliche wissenschaftliche Anteil lag in der Erarbeitung der Idee basierend auf eigenen Beobachtungen im Labor, sowie der Beteiligung an der Untersuchung der Hypothese und der Beteiligung an Auswertung und Interpretation der Ergebnisse.

Publikation 3: Engel O*, Akyüz L*, da Costa Goncalves AC, Winek K, Dames C, Thielke M, Herold S, Böttcher C, Priller J, Volk HD , Dirnagl U, Meisel C* , Meisel A* (2015) Cholinergic pathway suppresses pulmonary innate immunity facilitating pneumonia after stroke. *Stroke*

Konzeption der Studie gemeinsam mit Dr. Christian Meisel und Prof. Andreas Meisel, Projektmanagement, Durchführung und Auswertung der Experimente gemeinsam mit Co-Autoren (alle in vivo Experimente), statistische Auswertung, Interpretation der Ergebnisse in Zusammenarbeit mit Co-Autoren, Schreiben der Publikation (erster Entwurf & Koordination)
Der maßgebliche wissenschaftliche Anteil lag in der Konzeption der in vivo Experimente, der verantwortlichen Durchführung der Versuche in Mäusen und der verantwortlichen Auswertung und Interpretation der Ergebnisse. Die geteilte Erstautorenschaft mit Levent Akyüz basiert auf der Teilung der Verantwortlichkeiten. Während Odilo Engel für den tierexperimentellen Teil verantwortlich war, zeichnete sich Levent Akyüz für den immunologischen Teil verantwortlich.

Darüberhinaus war Odilo Engel in allen Studien als Projektleiter gem. Tierschutzgesetz verantwortlich für alle Aspekte der Versuchstiere, insbesondere für den Tierschutz und die Einhaltung der entsprechenden gesetzlichen Bestimmungen.
Rein vorsorglich sei angemerkt, dass die Durchführung von Tierversuchen nach den heutigen fachlichen und tierschutzrechtlichen Ansprüchen in der hier vorliegenden Komplexität nur im Team durchgeführt werden kann. Damit ergeben sich zwangsläufig längere Autorenlisten.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

Unterschrift des Doktoranden/der Doktorandin

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Shared first authorships are indicated with a star (*).

Impact factors for the journals of the three publications used for the cumulative thesis:

Abbreviated Journal Title	ISSN	JCR Data						*Eigenfactor*® Metrics	
		Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	*Eigenfactor* Score	*Article Influence* Score
J NEUROSCI METH	0165-0270	12656	2.025	2.245	0.529	306	8.5	0.01989	0.753
J CEREBR BLOOD F MET	0271-678X	15903	5.407	5.455	0.974	232	8.0	0.02926	1.785
STROKE	0039-2499	58619	5.761	6.578	1.311	572	8.7	0.10295	2.140

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