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DISSERTATION

# Stroke, stress, and depression – evidence from a brain ischemia  mouse model

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## SUMMARY


### ABSTRACT

Emerging evidence in the last decade points out an association between stress, depression and stroke. Clinical studies report that a considerable portion of stroke survivors develop depression after stroke. Also, an increasing number of clinical case-control studies indicate an association between stress levels and ischemic stroke. Experimental studies focusing on the bi-directional interaction between stroke and stress as well as depression are crucial to better understand the pathophysiology of stroke and depression. This PhD work focuses on three separate projects that investigate the nature and mechanisms of this relationship.

In study 1 the effects of 30 minutes left and right middle cerebral artery occlusion (MCAo) on mouse “anxiety”-like and “depression”-like behavior were evaluated. Left MCAo but not right MCAo led to chronic depression and anxiety-like behavior; citalopram treatment was shown to reverse these effects. 30 min MCAo caused significant and chronic reduction of striatal dopamine (DA) levels. Striatal DA loss was normalized to a large degree by citalopram treatment. Further histological analysis revealed a significant loss of dopaminergic neurons in ipsilateral ventral tegmental area (VTA) and Substantio Nigra (SN), as assessed by Tyrosine Hydroxylase (TH) immunostaining and NeuN-immunostaining. Cell loss in SN and VTA, and effects of MCAo were also significantly attenuated by citalopram treatment. Our study indicate that it is possible to model Post stroke depression in mouse models and provide interesting findings regarding the possible mechanisms that result in the observed changes, such as sustained depletion of dopamine.

Study 2 and 3 investigate the effects of chronic stress on stroke outcome, endothelial function and oxidative stress load. Over the course of four weeks animals were exposed to a chronic stress procedure. Animals of the treatment group were given the I(f)-channel inhibitor ivabradine or glucocorticoid antagonist mifepristone. In both studies stress

exposure increased adrenal gland weight, overall corticosterone levels and increased the lesion size after MCAo. Heart rate was both acutely and chronically elevated in the stressed mice. Both studies confirmed that chronic stress exposure impairs endothelium function and down regulates eNOS mRNA levels in the brain and aorta along with increasing the markers of oxidative stress. Both Ivabradine and mifepristone treatment reduced lesion size, normalized endothelial dysfunction and reduced oxidative burden. Both studies indicate that a significant reduction in endothelial function along with markedly increased oxidative stress may be a possible pathway by which stress can facilitate stroke

## INTRODUCTION

Emerging evidence in the last decade points out an association between stress, depression and stroke which seems to be bi-directional. On one side of this interaction stands the high frequency of depression in stroke survivors[1]. Clinical studies indicate that a considerable portion of stroke survivors develop depression after stroke which negatively impacts stroke outcome with increased morbidity, mortality and poorer functional recovery. Prevalence of post stoke depression (PSD) varies from 20% to 80%, and it is under diagnosed and undertreated[1, 2]. Several studies have shown that biological and psychosocial factors do play significant roles in the development of post stroke emotional disturbances[1-3]. There is a lower prevalence of depression and other mood symptoms in orthopedic patients with similar disabilities compared to stroke survivors. In addition, patients with anosognosia (ie, those who do not recognize that they suffer from stroke) also develop PSD. Those observations indicate a biological mechanism in PSD pathophysiology[1].

In order to explain the possible biological mechanisms leading to PSD, the “amine hypothesis” was proposed, according to which, ischemic lesions by damaging the projections ascending from midbrain and brainstem, decrease the bioavailability of biogenic amines such as dopamine (DA), serotonin (5HT), and norepinephrine (NE). This

in turn leads to mood disturbances[1, 3]. However, the overall pathopysiology of PSD is only poorly understood, and preclinical and translational research on PSD is largely lacking.

On the other side of the interaction between stroke and depression, stands the observation that psychosocial distress and depression increase stroke risk[4]. The relation of chronic stress to cardiovascular diseases is well documented, but its association to stroke is not sufficiently established[5]. An increasing number of clinical case-control studies indicate an association between stress levels and ischemic stroke[4, 6, 7]. Accumulating evidence suggests that chronic psychosocial stress in humans may represent an independent risk factor for stroke incidence[4, 6]. Experimental studies document that stress-elevated levels of stress hormones and exposure to chronic stress worsen stroke outcome in animals[8, 9]. While some of the adverse effects of stress can be partially explained, studies examining the underlying pathophysiological mechanisms linking stress to increased risk for stroke are scarce, so that the reasons behind this interaction remain elusive[10].

Further studies focusing on the bi-directional interaction between stroke and stress as well as depression are crucial to reach a better understanding of the pathophysiology of stroke and depression. During my PhD studies I worked on a number of projects in which we tried to unearth the nature and mechanisms of this relationship. Three of those studies have already been published and represent the core of my PhD work. A brief description of our aims in each study is below:

**Experimental aims:**

***Study 1:*** "Exofocal dopaminergic degeneration as antidepressant target in mouse model of poststroke depression."

The effects of 30 minutes left and right middle cerebral artery occlusion (MCAo) on mouse "anxiety"-like and "depression"-like behavior were evaluated. Antidepressant treatment (citalopram 13mg/kg body weight intraperitoneal injections) was used to demonstrate that any behavioral change observed could be reversed with treatment and is thus an indicator

of an “emotional” disturbance. MRI and various histological means were employed to examine the possible anatomical and functional reasons behind observed behavior. To identify biological correlates of post stroke depression, we quantified corticosterone levels in serum-derived and brain-derived neurotrophic factor levels in brain.

***Study 2:*** “Stress worsens endothelial function and ischemic stroke via glucocorticoids.” Several separate cohorts of “chronically stressed” and naïve animals were used in this study to examine the effects of stress on stroke incidence and outcome. A batch of stressed animals was subjected to 30 minute MCAo to evaluate the detrimental effects of stress on lesion size. In another group of animals, possible mechanisms behind this interaction were investigated by measuring the effects of stress on blood pressure, heart rate, endothelial relaxation along with eNOS and markers of oxidative damage in aorta and brain. A glucocorticoid antagonist (mifepristone 25mg/kg intraperitoneal injections) was used for the treatment group to investigate to what extent glucocorticoid receptor activation takes part in the adverse affects of stress.

***Study 3:*** “Heart rate contributes to the vascular effects of chronic mental stress: effects on endothelial function and ischemic brain injury in mice.”

In this study, we aimed to investigate the effects of chronic stress on stroke outcome, endothelial function and oxidative stress load. Over the course of six weeks, animals of the treatment group were given the I(f)-channel inhibitor (ivabradine 10mg/kg body weight) in their chow to evaluate the heart-rate reducing effects of ivabradine on our experimental endpoint. In the second week of the treatment regimen, 2 groups were assigned to 28-day stress procedure. We measured the effects of chronic stress on heart rate, stroke lesion size, endothelial relaxation, CD31 positive cell count in the brain and eNOS mRNA and markers of oxidative damage in aorta and brain.

## MATERIALS AND METHODS

All materials and methods used are listed and described in the publications listed in the appendix. In this section, only the main materials and methods related to key findings, models and experimental aims are summarized.

### Animals and housing

All experiments were performed according to national and international animal care guidelines and were approved by the local official institute, the 'Landesamt für Gesundheit und Soziales', Berlin, Germany. For all experiments, Wild type male 129/SV mice aged 6 to 8 weeks were used and housed in standard mouse cages, in groups of 5 – 6 mice per cage, at 22-23°C with a standard light dark cycle (7am – 7pm).

### Cerebral ischemia

Mice were anesthetized with 1.0% isofluorane in 69% $N_2O$ and 30% $O_2$ using a vaporizer and subjected to left or right filamentous 30-min MCA occlusion or sham operation with monitoring for regional cerebral blood flow and temperature as described (Endres et al 1998). For sham operation, the filament was introduced in the left or right internal carotid artery, without advancing it further, and withdrawn after 30 min MCAo model was employed in all 3 studies listed.

### Magnetic resonance imaging

T2-weighted images at 7 T (Pharmascan; Bruker Biospin, Ettlingen, Germany) were obtained using a fat-suppressed two-dimensional turbo spin-echo sequence (repetition time: 5109 msec; echo time: 65.2 msec). A 2x2 cm field of view, a 128x128 matrix and an in-plane resolution of 156 xm were used; slice thickness was 0.4 mm with no interslice distance. MRI measurements were used in study 3.

**Chronic stress procedure**

The chronic stress procedure was carried out as follows: briefly, the procedure consisted of 1) exposure to rat, 2) restraint stress, and 3) tail suspension, which were applied in the following order: days 1-7, exposure to a rat; days 8-10, restraint stress; days 11-14, tail suspension; days 15-21, exposure to rat; days 22-25, restraint stress; and days 26-28, tail suspension. This procedure was used in studies 2 and 3.

***Exposure to rat:*** At the beginning of the dark phase of the circadian cycle, two mice were placed inside a cage, with diameters of 16x14x22 cm, which was then placed inside a rat cage, with diameters of 33x19x55 cm. A rat was introduced into the rat cage and remained there for 15 hours (11:30pm – 2:30pm). To facilitate olfactory sensing, the mouse cage contained holes (diameter 0.7 cm) in the side walls. After termination of the procedure, animals were returned to their home cages for the rest of the day.

***Restraint stress:*** Plastic restrainers (50 ml syringes) were prepared with air holes. Animals were placed inside the restraining syringe (internal diameter 30 mm) for 2.5 h during the dark phase (7:30pm – 10:00pm).

***Tail suspension stress****:* Approximately, 1 cm from the end, each mouse's tail was taped (3M Durapore tape) to a piece of metal tubing fixed to a wall. Mice were suspended by the tail approximately, 80 cm above the ground for 6 min/day. The procedure started at 7:30pm.

**Telemetric continuous recordings of blood pressure and heart rate in mice**

For telemetric recordings, TA-PAC20 transmitters (DSI, St. Paul, MI, USA) were implanted into the left femoral artery (n=4-5 animals per group). The transponder was inserted into a subcutaneous pouch in the left flank of the animal. Following a recovery period of 10 days, heart rate and blood pressure were recorded every 5 minutes for 20 seconds on 32 consecutive days and averaged as presented in the figures. Data was stored and analyzed using the Dataquest A.R.T. software 3.0. This procedure was used in study 2 and study 3.

## Behavioral testing

Behavioral analyses were performed in the dark phase of a 12:12 hour light–dark cycle. Tests were ordered to proceed from less stress-producing to more invasive. Behavioral tests were performed as a part of study 3.

## Spontaneous locomotor activity

For the continuous monitoring of spontaneous locomotor activity, animals were individually placed in single cages (30 x 20 x 15 cm), evenly distributed across rows and columns for each group. Animals from different groups were placed in alternating order to control for a position effect. Time, speed, and distance of spontaneous locomotor activity was measured overnight over a period of 8 hours. Dim, indirect illumination in the neutral setting of a soundproof chamber provided uniform and sufficient light for automated detection of movements.

## Elevated plus maze test

The elevated plus maze is widely accepted as a method for determining anxiety and innate fear in rodents. The elevated plus maze was made of black Plexiglas and consisted of two opposite open arms (30 x 5 x 0.25 cm) and two enclosed arms (30 x 5 x 15 cm) with side and end walls. The arms extend from a central platform (5 x 5 cm) standing on a tripod 50 cm above the floor. The plus maze was placed inside a soundproof chamber with neutral environment and dim indirect light. At the beginning of the test, each mouse was placed in the center of the maze facing one of the closed arms. The cumulative time spent on the open arms, the number of open arm visits (i.e., entry with the center of the body), and locomotor activity (speed and distance) were measured during a 5-min observation period. The time spent on the open arms was used as an index of anxiety.

**Porsolt forced swim test**

The modified version of the Porsolt forced swim test, an animal model of depression sensitive to antidepressant treatment, was performed. Mice were placed individually in glass cylinders (15-21cm), filled with 22°C warm water to a depth of 8.5 cm, for a period of 300 sec. Behavior was monitored using a time sampling technique for subsequent analysis. Climbing behavior was defined as upward directed movements of the forepaws along the side of the swim chamber. Swimming behavior was defined as movement (usually horizontal) throughout the swim chamber. Floating (immobility) was assigned when no additional activity was observed other than that required for keeping the head above water. For each behavior, latency, total time and attempts (total number of events) were recorded. The rater was uninformed of the individual groups.

**Novelty suppressed feeding test**

The novelty-suppressed feeding paradigm is based on an approach–avoidance conflict between the fear of moving into the center of a brightly illuminated arena, and the drive to ingest food. The testing apparatus consisted of an open field. Mice were food-deprived for 48 hours before behavioral testing. At the time of testing, a food pellet was placed on a round white paper in the center of the box. An animal was placed in a corner of the box and the latency to eat the pellet was measured. If the animal had not begun feeding within 6 min, it was assigned a latency score of 6 min.

**Sucrose preference test**

Mice were simultaneously given a free choice between two bottles, one with 1% sucrose solution and another with tap water, for 12 h. The test started with the onset of the dark (active) phase of the animals' cycle. Animals were not deprived of food or water previous to the test. To prevent the possible effects of a side-preference in drinking behavior, the position of the bottles in the cage was switched after 6 h during the test. The intake of water and 1%-sucrose solution, and the total intake were estimated by weighing the bottles before and after access to liquids. The preference for sucrose was calculated as the percentage of the sucrose solution, consumed out of the total amount of liquid drunk.

## RESULTS

The results of the studies are described in detail in the publications listed in the appendix. In this section only the main findings are summarized and briefly discussed.

***Study 1:*** "Exofocal dopaminergic degeneration as antidepressant target in mouse model of poststroke depression." This study has the following major findings:

Left MCAo but not right MCAo led to chronic depression and anxiety-like behavior; citalopram treatment was shown to reverse these effects. In evaluation, mice, examined at 14 weeks of age, displayed depressive-like syndrome with anxiety and anhedonia when they had undergone 30 min lMCAo. RMCAo animals on the other hand, showed only signs of hyperactivity.

30 min MCAo caused significant and chronic reduction of striatal dopamine (DA) levels. Striatal DA loss was normalized to a large degree by citalopram treatment. In the Porsolt swim test it was observed that Striatal DA levels and despair-like behaviors (as measured by the parameters: total time floating and latency to float) correlated significantly. Additionally, there was a significant inverse correlation between striatal DA levels and the latency to feed in the novelty-suppressed feeding test.

Regardless of the lesion site (left or right), MCAo triggered a progressive secondary degeneration in the midbrain. Hyperintensity, which was absent in initial scans, in substantia nigra (SN) and ventral tegmental area (VTA) (day2), appeared in subsequent MRI measurements (7 days post MCAo), indicating that the secondary lesion evolved during the first week after the operation, presumably via retrograde valerian degeneration. Further histological analysis revealed a significant loss of dopaminergic neurons in ipsilateral VTA and SN, as assessed by Tyrosine Hydroxylase (TH) immunostaining and NeuN-immunostaining. Cell loss in SN and VTA, and effects of MCAo were also significantly attenuated by citalopram treatment.

To further evaluate possible downstream mechanisms by which MCAo may induce depression like behavior, mRNA levels of dynorphine – the k receptor-preferring opioid - were assesed in nucleus accumbens (NA). MCAo -induced an increase in dynorphine levels in ipsilateral NA, and citalopram treatment reduced the increase.

Striatal atrophy observed to result from MCAO was also reduced by citalopram treatment.

***Study 2:*** "Stress worsens endothelial function and ischemic stroke via glucocorticoids."

- As expected, chronic stress exposure increased adrenal gland weight and overall corticosterone levels. Mifepristone treatment in naïve animals conferred similar effects on adrenal weight and corticosterone levels.
- Acutely, all three types of stressors that constitute the chronic stress paradigm caused significant increases in heart rate (HR) without any major change in blood pressure (BP). mifepristone treatment did not alleviate the acute effects of stressors on HR or BP.
- When averaged over the course of 28 days, analysis of the physiological parameters revealed a significant increase in HR in the chronic stress group. This effect was only slightly reduced by mifepristone. Chronic stress did not alter systolic or diastolic BP over 28 days. Mifepristone treatment on the other hand conferred a minor reduction in systolic and diastolic BP.
- As a result of chronic stress exposure, endothelium-dependent vasodilation was impaired; reactive oxygen species and lipid hydroperoxide production in the vasculature and the brain were increased. Also, brain and vascular levels of nitric oxide synthase (eNOS) were reduced in the chronic stress group. Mifepristone treatment significantly attenuated the observed effects.
- After 30 minutes transient MCAo, mice that were subjected to chronic stress showed increased lesion volumes. Treatment with mifepristone decreased lesion volumes in these animals. Interestingly, mifepristone treatment in the naïve (unstressed) group also caused an increase in the lesion volumes compared to control group.

- During transient MCAo, absolute blood flow measurement via C-iodoantipyrine autoradiography showed that the tissue volume with severely reduced blood flow in chronically stressed animals tended to be larger than that in non-stressed animals.

***Study 3:*** "Heart rate contributes to the vascular effects of chronic mental stress: effects on endothelial function and ischemic brain injury in mice."

- Exposure to stress significantly increased heart rate in both acute and chronic conditions. In the ivabradine-treated stress group, a 15% reduction in HR was observed. Ivabradine treatment also reduced heart rate significantly during stress exposure and lowered the baseline heart rate by 100bpm.
- Endothelial function as assessed by carbachol-induced relaxation rates in aortic rings showed significant endothelial relaxation impairment in the chronic stress group. Chronic ivabradine treatment significantly improved endothelial function in the stress group but no marked change was observed in treated non-stressed animals.
- In aortic tissue, markers of oxidative stress such as NADPH oxidase activity and lipid hydroperoxidates were significantly increased by exposure to chronic stress. Ivabradine treatment significantly reduced those adverse changes in the aorta. In the brain tissue, an increase in lipid hydroperoxidases was observed with no apparent change in NADPH oxidase activity. Ivabradine treatment normalized the changes in lipid hydroperoxidase levels caused by stress in the brain.
- The expression of eNOS mRNA in brain and aorta was reduced by chronic stress. Only in aortic tissue did heart rate reduction by ivabradine treatment normalize this stress-induced eNOS down regulation.
- After 30 minutes transient MCAo, mice that were subjected to chronic stress showed increased lesion volumes. Chronic treatment with ivabradine radically reduced this increase in lesion size. Interestingly, ivabradine treatment in naïve animals did not confer a significant beneficial effect on lesion size.
- Exposure to chronic stress did not affect the density of CD31-positive cells in brain, whereas ivabradine treatment induced a significant increase in both stress and naïve groups.

## DISCUSSION

Three separate studies that investigate the interaction between stroke and stress as well as depression constitute this PhD thesis. In the first study, our data suggest that it is possible to model core features of depression after brain ischemia in mice. Main behavioral findings of “Study 1“ demonstrate that animals subjected to left MCAo have increased anxiety in the novelty suppressed feeding test and the elevated plus maze; depressive-like behavior manifested as despair in Porsolt’s forced swim test and anhedonia as assessed by sucrose consumption test. Interestingly, RMCAo animals did not exhibit any anxiety or depression-related features in any test, but rather displayed hyperactivity as assessed by spontaneous locomotor activity testing. Importantly, observed deficits in behavioral phenotype were reversed with citalopram treatment, which further accounts for the validity of our model as a rodent PSD model.

Translating human psychiatric conditions to animal models is a significant challenge given the huge differences between humans and rodents. Despite difficulties, various behavioral tests such as the Porsolts forced swim test, tail suspension test, learned helplessness test and sucrose consumption test are successfully used to evaluate some core symptoms of depression (anhedonia, despair) in rodents[11]. Up to date, efforts to model spontaneous PSD in rodent focal ischemia models however, yielded some inconsistent results. In our earlier studies, we documented increased anxiety in ischemic animals at 8 weeks post stroke[12]. Kilic et al confirmed increased anxiety after LMCAo using the open field testing at earlier time points [13]. A hedonic deficit as assessed by the sucrose consumption test was reported 2 weeks after MCAo intervention[14]. Conversely, other studies failed to detect anxiety at 1 week post-stroke [15]. Our study models and describes a robust depressive and anxious behavioral phenotype in mice with left striatal lesions. Our study also provides interesting findings regarding the possible mechanisms that result in the observed changes, such as sustained depletion of dopamine, secondary degeneration in SN and VTA, and increased mRNA levels of dynorphine in NA.

PSD does not only affect patients' overall quality of life, but also disturbs poststroke recovery and increases mortality[1, 2]. Understanding the complete pathophysiology of this condition is therefore of utmost importance. Despite several reports indicating a correlation between left hemisphere lesion with PSD and anxiety in human patients, the study results are inconclusive[16, 17]. Our study indicates a clear effect of the lesion location on behavioral outcome which favors both the biological (not purely psychological) explanation of PSD and lesion-specific behavioral outcomes reported by some studies. In addition, the significant depletion of biogenic amines in the striatum in our stroke model is in accordance with the amine hypothesis of PSD. In our model, we have documented that the observed depletion of DA is a result of secondary degeneration in SN and VTA, which evolves gradually over the course of a week. Similar findings in human patients were reported in a few case reports, but secondary degeneration in SN and VTA has not been a major focus of interest or a major therapeutic target. Inspired by our study in rodents, a clinical study that is currently being conducted by our group has been investigating the incidence of secondary degeneration in patients with striatal lesions. Preliminary data also suggests a very similar pattern and timeline of degeneration in human survivorsl (unpublished data). Another striking finding of our study was the observation that citalopram treatment initiated as late as 7 days poststroke reduced striatal atrophy, almost normalized DA levels and prevented secondary degeneration. It has previously been reported that SSRI treatment poststroke improves functional and cognitive recovery[18]. Our paper provides experimental data and gives important insight in to the possible therapeutic effects of SSRI treatment. Taken together, we believe that our experimental study is a good example of a bench to bedside translational research with significant clinical implications.

Study 2 and study 3 investigate the effect of chronic stress on stroke outcome with different pharmaceutical interventions. In both studies, exposure to 28 days of chronic stress increased the lesion volume after stroke, confirming the deleterious effect of stress on stroke outcome. Heart rate was both acutely and chronically elevated in the stressed mice. Long-term heart rate monitoring revealed that mice did not show marked habituation to stressors, and the effects of each individual stressor on sympathetic activation remained

effective throughout the experiment. In addition, circulating glucocorticoid levels and adrenal weight were increased in the stress group, indicating an increase in hypothalamic–pituitary–adrenal system activity (study 2).

Both studies confirmed that chronic stress exposure impairs endothelium function and downregulates eNOS mRNA levels in the brain and aorta. This observed reduction in eNOS mRNA levels is presumably due to GC action on a suppressive GC response element in the eNOS promotor region[19]. In study 2, chronic stress induced a marked increase in SO production in aortic tissue and a significant increase in brain lipid hydroperoxides. These data are in agreement with studies in rodents showing that chronic stress induces brain oxidative stress, which may exacerbate damage after ischemia.

A glucorticoid receptor antagonist – mifepristone – was used as our intervention in study 2. 28 days of chronic mifepristone treatment blocked the detrimental effects of chronic stress on endothelial function, reduced the stress-induced increases in lipid hydroperoxides and reduced increases in SO production. In addition, reduced eNOS levels and lesion size exacerbation caused through chronic stress were normalized by treatment. On the other hand, mifepristone treatment had statistically significant yet only very minor effects on heart rate. Over all, the detrimental effects of chronic stress on endothelium-dependent vasodilation and stroke outcome were normalized to great extent with mifepristone treatment, indicating that GC receptor-mediated cascades play a significant part in the observed negative effects.

In study 3, the I(*f*)-channel inhibitor ivabradine was used to counteract the effects of chronic stress. Six weeks of chronic treatment restored the detrimental effects of stress on endothelial function. There was a significant reduction of oxidative stress parameters and a marked decrease in stroke lesion size in the ivabradine-treated stress group. NADPH oxidase activity in the aorta and aortic lipid peroxidation were normalized to a great extent with ivabradine treatment, which may account for the improved endothelial function. Heart

rate reduction, induced by chronic ivabradine treatment, also normalized the stress-induced eNOS reduction.

The link between stress and cardiovascular diseases is well established. Despite the common notion that stress may precipitate stroke, the actual link is not clear. Several case studies and related literature reviews indicate an association between chronic stress and high risk of stroke[5-7]. It is not clear, however, whether it is stress or stress-related habits and behavior that potentiate stroke risk. Stroke can essentially be considered a disease of the arterial endothelial cell layers. Cumulative damage to endothelium may lead to stroke. The initial cascade that leads to atherosclerosis also starts with minor damage to endothelia, and atherosclerosis can occlude an artery or create a thromboembolism. It has been shown that endothelial dysfunction alone is an independent risk factor for stroke[20]. Our studies confirm the experimental finding that stress exacerbates stroke. In addition, as observed in both of our studies, a significant reduction in endothelial function along with markedly increased oxidative stress may be a possible pathway by which stress can facilitate stroke. Protecting endothelial integrity represents a therapeutic target, and stress-induced endothelial dysfunction described in our animal experiments can be used as an animal model to test novel drugs and interventions.

## REFERENCES


1. Loubinoux, I. et al. Post-stroke depression: mechanisms, translation and therapy. J Cell Mol Med 16, 1961-9 (2012).
2. Lenzi, G. L., Altieri, M. & Maestrini, I. Post-stroke depression. Rev Neurol (Paris) 164, 837-40 (2008).
3. Fang, J. & Cheng, Q. Etiological mechanisms of post-stroke depression: a review. Neurol Res 31, 904-9 (2009).
4. Guiraud, V., Amor, M. B., Mas, J. L. & Touze, E. Triggers of ischemic stroke: a systematic review. Stroke 41, 2669-77 (2010).
5. Backe, E. M., Seidler, A., Latza, U., Rossnagel, K. & Schumann, B. The role of psychosocial stress at work for the development of cardiovascular diseases: a systematic review. Int Arch Occup Environ Health 85, 67-79 (2012).
6. Egido, J. A. et al. Is psycho-physical stress a risk factor for stroke? A case-control study. J Neurol Neurosurg Psychiatry 83, 1104-10 (2012).
7. Suadicani, P., Andersen, L. L., Holtermann, A., Mortensen, O. S. & Gyntelberg, F. Perceived psychological pressure at work, social class, and risk of stroke: a 30-year follow-up in Copenhagen male study. J Occup Environ Med 53, 1388-95 (2011).
8. Stuller, K. A., Jarrett, B. & DeVries, A. C. Stress and social isolation increase vulnerability to stroke. Exp Neurol 233, 33-9 (2012).
9. Jin, Z., Wu, J., Oh, S. Y., Kim, K. W. & Shin, B. S. The effect of stress on stroke recovery in a photothrombotic stroke animal model. Brain Res 1363, 191-7 (2010).
10. Sapolsky, R. M. Stress, Glucocorticoids, and Damage to the Nervous System: The Current State of Confusion. Stress 1, 1-19 (1996).
11. Cryan, J. F. & Mombereau, C. In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. Mol Psychiatry 9, 326-57 (2004).
12. Winter, B. et al. Anxious and hyperactive phenotype following brief ischemic episodes in mice. Biol Psychiatry 57, 1166-75 (2005).

13. Kilic, E. et al. Delayed melatonin administration promotes neuronal survival, neurogenesis and motor recovery, and attenuates hyperactivity and anxiety after mild focal cerebral ischemia in mice. J Pineal Res 45, 142-8 (2008).
14. Craft, T. K. & DeVries, A. C. Role of IL-1 in poststroke depressive-like behavior in mice. Biol Psychiatry 60, 812-8 (2006).
15. Gaur, V. & Kumar, A. Behavioral, biochemical and cellular correlates in the protective effect of sertraline against transient global ischemia induced behavioral despair: possible involvement of nitric oxide-cyclic guanosine monophosphate study pathway. Brain Res Bull 82, 57-64 (2010).
16. Starkstein, S. E. et al. Depression after stroke: the importance of cerebral hemisphere asymmetries. J Neuropsychiatry Clin Neurosci 3, 276-85 (1991).
17. Wongwandee, M., Tangwongchai, S. & Phanthumchinda, K. Relationship between poststroke depression and ischemic lesion location. J Med Assoc Thai 95, 330-6 (2012).
18. Mead, G. E. et al. Selective serotonin reuptake inhibitors (SSRIs) for stroke recovery. Cochrane Database Syst Rev 11, CD009286 (2012).
19. Liu, Y., Mladinov, D., Pietrusz, J. L., Usa, K. & Liang, M. Glucocorticoid response elements and 11 beta-hydroxysteroid dehydrogenases in the regulation of endothelial nitric oxide synthase expression. Cardiovasc Res 81, 140-7 (2009).
20. Sunbul, M. et al. Endothelial dysfunction is an independent risk factor for stroke patients irrespective of the presence of patent foramen ovale. Herz (2013).

**ANTEILSERKLÄRUNG**

*Mustafa G. Balkaya contributed in the following percentage to the submitted publications:*

**Publication 1:**

Kronenberg G*, **Balkaya M***, Prinz V, Gertz K, Ji S, Kirste I, Heuser I, Kampmann B, Hellmann-Regen J, Gass P, Sohr R, Hellweg R, Waeber C, Juckel G, Hörtnagl H, Stumm R, Endres M. (2012) Exofocal dopaminergic degeneration as antidepressant target in mouse model of poststroke depression. Biol Psychiatry 72(4):273-81

**% 30**

Individual contribution: Conceived and designed the research, established and performed the behaviour tests, acquired, analyzed and interpreted behavior data, partially drafted the manuscript and made critical revision of the manuscript and correspondence to reviewer comments.

**Publication 2:**

**Balkaya M***, Prinz V*, Custodis F, Gertz K, Kronenberg G, Kroeber J, Fink K, Plehm R, Gass P, Laufs U, Endres M (2011) Stress Worsens Endothelial Function and Ischemic Stroke via Glucocorticoids. Stroke. 42(11):3258-64

**% 35**

Individual contribution: Conceived and designed the research, established and performed the stress paradigm, acquired heart rate and blood pressure data, analyzed and interpreted the data, performed statistical analysis, drafted the manuscript, corresponded to reviewer comments.

**Publication 3:**

Custodis F, Gertz K, **Balkaya M**, Prinz V, Mathar I, Stamm C, Kronenberg G, Kazakov A, Freichel M, Böhm M, Endres M, Laufs U (2011) Heart rate contributes to the vascular effects of chronic mental stress: effects on endothelial function and ischemic brain injury in mice. Stroke 42(6):1742-9

**% 20**

Individual contribution: Contributed to the design of the experiment, established and performed the stress paradigm, partially drafted the manuscript and made critical revision of the manuscript and correspondence to reviewer comments.

## SELECTED PUBLICATIONS / LITERATURHINWEIS

Kronenberg G*, Balkaya M*, Prinz V, Gertz K, Ji S, Kirste I, Heuser I, Kampmann B, Hellmann-Regen J, Gass P, Sohr R, Hellweg R, Waeber C, Juckel G, Hörtnagl H, Stumm R, Endres M. (2012) Exofocal dopaminergic degeneration as antidepressant target in mouse model of poststroke depression. Biol Psychiatry 72(4):273-81 Impact factor 2011: 8.283

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Custodis F, Gertz K, Balkaya M, Prinz V, Mathar I, Stamm C, Kronenberg G, Kazakov A, Freichel M, Böhm M, Endres M, Laufs U (2011) Heart rate contributes to the vascular effects of chronic mental stress: effects on endothelial function and ischemic brain injury in mice. Stroke 42(6):1742-9 Impact factor 2011: 5.729

### Links to the selected publications

Electronic versions of the dissertations do not contain the original publications due to copy rights. Publications that are mentioned above can be reached from the following links.

Biol Psychiatry 72(4)...
**DOI:** http://dx.doi.org/10.1016/j.biopsych.2012.02.026
Stroke. 42(11)…
**DOI:** http://dx.doi.org/10.1161/STROKEAHA.110.607705
Stroke 42(6)...
**DOI:** http://dx.doi.org/10.1161/STROKEAHA.110.598607

## CURRICULUM VITAE

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

## LIST OF PUBLICATIONS

Balkaya M*, Kröber J*, Gertz K, Peruzzaro S, Endres M. (2013) Characterization of long-term functional outcome in a murine model of mild brain ischemia. J Neurosci Methods. 2013 Mar 15;213(2):179-87

Balkaya M, Kröber JM, Rex A, Endres M. (2013) Assessing post-stroke behavior in mouse models of focal ischemia. J Cereb Blood Flow Metab. 2013 Mar;33(3):330-8

Kronenberg G*, Balkaya M*, Prinz V, Gertz K, Ji S, Kirste I, Heuser I, Kampmann B, Hellmann-Regen J, Gass P, Sohr R, Hellweg R, Waeber C, Juckel G, Hörtnagl H, Stumm R, Endres M. (2012) Exofocal dopaminergic degeneration as antidepressant target in mouse model of poststroke depression. Biol Psychiatry 72(4):273-81

Gertz K, Kronenberg G, Kälin RE, Baldinger T, Werner C, Balkaya M, Eom GD, Hellmann-Regen J, Kröber J, Miller KR, Lindauer U, Laufs U, Dirnagl U, Heppner FL, Endres M (2012) Essential role of interleukin-6 in post-stroke angiogenesis. Brain. 135(Pt 6):1964-80.

Balkaya M*, Prinz V*, Custodis F, Gertz K, Kronenberg G, Kroeber J, Fink K, Plehm R, Gass P, Laufs U, Endres M (2011) Stress Worsens Endothelial Function and Ischemic Stroke via Glucocorticoids. Stroke. 42(11):3258-64

Custodis F, Gertz K, Balkaya M, Prinz V, Mathar I, Stamm C, Kronenberg G, Kazakov A, Freichel M, Böhm M, Endres M, Laufs U (2011) Heart rate contributes to the vascular effects of chronic mental stress: effects on endothelial function and ischemic brain injury in mice. Stroke 42(6):1742-9

Balkaya M, Endres M . Behavioral Testing in Mouse Models of Stroke. Neuromethods Volume: 47, chapter 13, 179-197

Royl G, Balkaya M, Lehmann S, Lehnardt S, Stohlmann K, Lindauer U, Endres M, Dirnagl U, Meisel A (2009) Effects of the PDE5-inhibitor vardenafil in a mouse stroke model. Brain Res 1265:148-57.

Ji S, Kronenberg G, Balkaya M, Färber K, Gertz K, Kettenmann H, Endres M (2009) Acute neuroprotection by pioglitazone after mild brain ischemia without effect on long-term outcome. Exp Neurol 216(2):321-8

Kronenberg G, Harms C, Sobol RW, Cardozo-Pelaez F, Linhart H, Winter B, Balkaya M, Gertz K, Gay SB, Cox D, Eckart S, Ahmadi M, Juckel G, Kempermann G, Hellweg R, Sohr R, Hörtnagl H, Wilson SH, Jaenisch R, Endres M (2008) Folate deficiency induces neurodegeneration and brain dysfunction in mice lacking uracil DNA glycosylase. J Neurosci 28(28):7219-30.

Prinz V, Laufs U, Gertz K, Kronenberg G, Balkaya M, Leithner C, Lindauer U, Endres M (2008) Intravenous rosuvastatin for acute stroke treatment: an animal study. Stroke 39(2):433-8.

Kronenberg, G, Harms, C, Sobol, R.W, Cardozo-Pelaez, F, Linhart, H, Balkaya, M., Gertz, K., Eckart, S., Hellweg, R., Hoertnagl, H., Jaenisch, R., Endres, M (2007) Role of uracil misincorporation for neurogeneration and brain dysfunction incuded by chronic folate deficiency (Conference Paper) J Cereb Blood Flow Metab 27(Issue Supp. 1): BO08-04

Winter B, Juckel G, Viktorov I, Katchanov J, Gietz A, Sohr R, Balkaya M, Hörtnagl H, Endres M (2005) Anxious and hyperactive phenotype following brief ischemic episodes in mice. Biol Psychiatry 57(10):1166-75.

**ERKLÄRUNG**

„Ich, Mustafa G. Balkaya, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „ Stroke, stress ,and depression – evidence from a brain ischemia  mouse model“ 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.“

**Affidavit**

I, Mustafa G. Balkaya certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Stroke, stress ,and depression – evidence from a brain ischemia  mouse model“. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein

Istanbul, den 04.04.14

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