STRESS – INDUCED CHANGES IN GENE TRANSCRIPTIONS AND TRANSLATIONS RELATED TO ALZHEIMER'S DISEASE

Summary of the Ph.D. thesis

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1. INTRODUCTION

Although stress is an integral part of life (with mild stressors possibly being useful), intense or prolonged exposure to stress may lead to a variety of neurodegenerative disorders, such as Alzheimer's disease (AD). The relationships between stress, depression and AD have been examined both in epidemiological investigations in humans and under experimental conditions. Epidemiological evidence has indicated that individuals prone to experience psychological distress are 2.4 times more receptive to the development of AD than non-stressed individuals. Moreover, 25-40% of AD patients suffer from co-morbid depression, possibly making it a significant risk factor for AD.

In in vitro and in vivo experiments, stress reduced the spine density within hours in vulnerable dendritic domains of the hippocampal pyramidal cells. A decrease in dendritic spine density is a prominent phenomenon in cases of early AD, which correlates significantly with the progressive decline in mental functions. The major cytoskeletal component of dendritic spines is filamentous actin, which plays a key role in the morphogenesis, maintenance and plasticity of spines. The most important regulators of actin dynamics are members of the actin – depolymerization factor (ADF)/cofilin family. Exposure to neurodegenerative stimuli causes neurons to reorganize their actin cytoskeleton into rod – like inclusions.

AD is pathologically characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). Senile plaques observed in the brains of AD patients contain deposited Aβ – protein. Aβ is a protein consisting of 40 – 42 amino acids and cleaved from the APP by the secretase proteins. In physiological conditions, APP is cleaved by α – secretase, forming a non – toxic, soluble APP. In AD, β – and γ – sectretases cleave APP, leading to the non – soluble Aβ – protein and inducing the appearance of aggregated extracellular senile plaques.

Other abnormal accumulations in the brain of AD patients are abundant filamentous tau lesions. In the physiological lesions, tau protein binds to axonal microtubules and regulates their assembly and transport. In AD, tau is hyperphosphorylated by a number of kinases such as mitogen – activated protein kinase – 1 (MAPK – 1). Tau hyperphosphorylation is deleterious to neurons, leading to microtubule degeneration, impairment of axonal transport and synaptic function, and cell death.

Despite evidence that gender has a pivotal role in the different responses to stress, little is known about which factors are common or different in mediating the stress response in males or females. Women are twice more likely to suffer from stress – related psychiatric disorders, such as depression, than men. Furthermore, the presence of AD is higher in women than in men. This difference between genders might partly be biologically determined.

In addition to gender differences, stress is influenced by many factors such as its type or duration, individual sensitivity, and the brain region. An ideal experimental animal model should reproduce the response of stress – induced molecular aspects and should be able to represent the pathophysiological features of human disease. Different acute and chronic stress protocols in laboratory rodents have been developed and used in literature. The importance of various parameters can be established from separate experiments.

Although recent work has studied the biochemical relationships between stress, depression and AD, there are still many unanswered questions. In contrast to the wide methodological repertoire of available animal stress models, the effect of different physical and psychological stressors on the cytoskeletal and AD – related gene regulation in the rat brain have not yet been compared.

2. AIMS

The present study sought to determine whether experimental stress modulates the underlying mechanisms of the hallmark neuropathological features of AD.

The main aims of our studies were the following:

1. To compare the effects of four widely – used stress protocols investigating the transcription and translation of main cytoskeletal components, such as β – actin and cofilin.
2. To examine the pathognostic role of the MAPK – 1 gene or protein induced by the four different types of stress in AD.
3. To investigate the possible impact of APP mRNA and protein changes in the AD pathomechanism in different stress models.
4. To study the acute and chronic stress response of the transcripton and translation of the genes and proteins in question.
5. To establish which brain region is more sensitive to stress.
6. To evaluate the combinatorial effects of gender and stress on the actin cytoskeleton and its regulation.

3. MATERIALS AND METHODS

3.1. Animals

Adult Wistar rats (200 – 300 g; n=6 – 10/group) were housed in a temperature (22±1 °C) and humidity (55±5%) controlled room on a 12 h light – dark cycle (lights on from 8.30 a.m. to 8.30 p.m.) and allowed free access to tap water and rat chow. In each of the stress procedures (RS, EFSS, FSS and PSS) the animals were divided into 5 experimental groups. Group 1 comprised the controls, while groups 2, 3, 4 and 5

were subjected to the given stress for 3, 7, 14 or 21 days, respectively. The control animals were left completely undisturbed.

The day after the last stress procedures (at 8 a.m.), the rats were anaesthetized with 8% chloral – hydrate and, following the transcardial perfusion with cold saline solution, the cerebral hemispheres were separated and the hippocampus and frontal cortex were dissected on an ice – cold tile. The same animals were used to measure mRNA and protein levels, but they were selected randomly to eliminate the changes induced by laterality. The samples were frozen with dry ice powder and stored at -80 °C until further experimental processing.

3.2. Stress procedures

3.2.1. Restraint stress

As in the standard protocol of Pitman et al. (1988), RS was applied by placing the rats into plastic, light – depriving tubes (10 cm in diameter and 25 – 30 cm long, where they could not move in any direction) for 5 h daily.

3.2.2. Electric foot – shock stress

EFSS was applied as in the protocol described by Tsukada et al. (2003) and Robbins and Ness (2008) by exposing the rat's footpad to a constant current produced with a foot – shock generator. In the acute stress experiment a total of 6 random shocks, each with an intensity of 1 mA for 750 ms, were administered within a period of 2 min, daily, for 3 consecutive days. In the chronic stress experiment, 10 random shocks, 0.6 mA in intensity, lasting for 2 s were administered daily within a period of 5 min for 7, 14 or 21 consecutive days.

3.2.3. Forced swimming stress

The FSS protocol described by Porsolt et al. (1978) was used in our experiments. Each rat was placed in a vertical Plexiglas cylinder (height 45 cm, diameter 19.4 cm) containing 32 cm of water maintained at 23 °C for 10 min, then removed and allowed to dry before being returned to their cages.

3.2.4. Psychosocial stress

The protocol of Gerges et al. (2001) was used in our experiments. Rats were kept with the same cage mates for at least 1 week to allow the establishment of social hierarchy. At the end of that period, 2 rats from each cage, randomly chosen, were switched once a day at the same time of day from one cage to the other for a period of 3 days. Analogous procedures were carried out for periods of 7, 14 and 21 days.

3.3. Total RNA isolation and reverse transcription

Total cellular RNA was extracted from the hippocampus and frontal cortex by means of the NucleoSpin RNA II Total RNA isolation kit (Macherey – Nagel, Düren, Germany) according to the manufacturer's instructions. Reverse transcription reactions were carried out for each RNA sample, subsequently followed by first – strand cDNA synthesis from total RNA samples by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). 2 ng of total mRNA were transcribed into cDNA. The samples were then cooled down to 4 °C, and finally stored at -20 °C until qRT – PCR.

3.4. Real – time polymerase chain reaction

Reactions were performed with RotorGene 3000 (Corbett Research, Sydney, Australia). Gene – specific primers designed by using Primer Express software

(Applied Biosystems, Foster City, CA, USA) were used. The relative gene expression was normalized to glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH). The results were analyzed by the $2^{-\Delta\Delta CT}$ method.

3.5. Western blotting

The brain regions were homogenized in a lysis buffer Homogenates were centrifuged and the supernatants were used for protein assays. Proteins were measured with bicinchoninic acid. After denaturation, 20 μg of protein were separated on 12% and 8% SDS – polyacrylamide gel and electroblotted onto nitrocellulose membranes. The samples were blocked in a solution of 0.1 M Tris – buffered saline containing 0.02% Tween 20 (TBST) supplemented with 5% non – fat milk for 1 h. The membranes were then incubated overnight with mouse monoclonal anti – β – actin, rabbit polyclonal cofilin (D59) antibody, mouse monoclonal ERK 1/2 antibody, mouse monoclonal anti – APP and mouse monoclonal anti – GAPDH. The next day, after washes with TBST, horseradish – peroxidase – labelled anti – mouse IgG, horseradish – peroxidase – labelled anti – rabbit IgG secondary antibodies were applied for 1 h. The nitrocellulose membranes were subsequently washed with TBST, and then incubated with the Supersignal® West Pico Chemiluminescent Substrate and exposed to Kodak autography film. The optical densities of the immunoreactive bands were quantified by means of Image Software. The amounts of examined proteins were calculated by comparison with the optical density of internal control. For each blot of β – actin, cofilin, ERK 1/2 and APP, the relative protein level was calculated from the ratio of absorbance of β – actin/GAPDH, cofilin/GAPDH, ERK1/2 /GAPDH and APP/GAPDH. This was considered as 100 % in the control group, and the data of different time points were compared to this ratio.

3.6. Statistical Analysis

In the case of male rats and stress types, data were analyzed by two – way ANOVA with SPSS 15.0 Software: Stress types (RS, EFSS, FSS, PSS) x Exposure times (3, 7, 14 and 21 days). Significant main effects and interactions were followed by *post hoc* comparisons using the General Linear Model. The comparisons within the same group were assessed by Student's T – test and by one – way ANOVA followed by the Bonferroni and Games – Howell *post hoc* tests. In the case of female rats, all data were analyzed by one – way ANOVA with SPSS 15.0 Software, followed by the Bonferroni *post hoc* tests. The level of significance of comparisons was taken as $p < 0.05$. All data are reported as mean ± SEM.

4. SUMMARY OF THE RESULTS

4.1. Stress – induced alterations in male rats

4.1.1. Body, adrenal gland and thymus weights of stressed male rats

Stress signals are easily monitored during the experiment or at the end of the experimental period by a decrease in body weight (BW), weight of thymus and adrenal glands of stressed animals compared to control. In the RS experiment, the relative decrease in BW was significant by day 3; and the reduction remained significant at all examined time points. EFSS also caused a significant lack of gain in BW, but only in response to chronic stress. However, exposure to FSS and PSS did not provoke any appreciable difference in BW.

RS caused a significant increase after 14 and 21 days, EFSS induced a significant increase after 7 and 14 days in the weight of the adrenal glands. In contrast, FSS and PSS did not result in any significant change. The thymus weight relative to BW was significantly decreased only by day 21 after. RS.

4.1.2. Effects of different stress types on β – actin transcription and translation levels

In the hippocampus, RS, EFSS and FSS caused significant increases in β – actin mRNA expression by day 3. The RS – induced transcription of β – actin mRNA followed a U – shaped time course.

The RS and EFSS – induced transcription of β – actin mRNA followed a U – shaped time course. In the case of FSS, the time course was not U – shaped: significant elevations were observed on days 3 and 7. In contrast to the physical stressors, PSS did not influence the β – actin mRNA transcription neither in the hippocampus nor in the frontal cortex. Similarly to the changes induced by RS and EFSS in the transcription of β – actin mRNA, the protein level changes described a U – shaped time course. Neither FSS nor PSS modified the hippocampal β – actin levels significantly. In the frontal cortex, none of the applied stressors caused any significant changes in the levels of β – actin.

4.1.3. Effects of different stress types on cofilin transcription and translation levels

Changes in the expression of cofilin mRNA in the hippocampus were observed only in the case of RS. EFSS, FSS and PSS had no effect on the expression of cofilin mRNA at any tested time point. In the frontal cortex none of the applied stressors caused any significant changes in the levels of cofilin.

4.1.4. Effects of different stress types on MAPK – 1 transcription and of RS stress on the ERK 1/2 translation levels

Changes in the expression of MAPK – 1 mRNA in the hippocampus were observed only in the case of RS. As in the cases of β – actin and cofilin, the MAPK – 1 data described a U – shaped time course. Western blotting experiments

did not reveal significant changes after RS in the amount of MAPK – 1 (ERK 1/2) protein.

4.1.5. Effects of different stress – types on APP transcription and translation levels

Changes in the expression of APP mRNA in the hippocampus were observed only in the case of RS. The hippocampal APP mRNA level was increased significantly only on day 21. None of the applied stressors caused any significant changes in the expression of APP mRNA in the frontal cortex. There were significant changes in the hippocampal APP protein levels on days 3, 7 and 21, respectively.

4.2. Stress – induced alterations in female rats

4.2.1. Body, adrenal gland and thymus weights of the stressed group

The stressed animals gained BW at a significantly slower rate than the control group by day 21. The adrenal gland weight relative to the BW was not significantly elevated by RS. The weight of thymus in the stressed animals was significantly lower on days 14 and 21 compared to control.

4.2.2. Effects of RS on β – actin transcription and translation levels

Similarly to the male rats, RS induced a U – shaped time course in the hippocampal β – actin mRNA expression changes, but a significant increase was found only on day 21. Western blotting experiments revealed that exposure to RS did not elevate the amount of β – actin protein in any of the examined regions.

4.2.3. Effects of RS on cofilin transcription and translation levels

RS caused a significant elevation in the cofilin mRNA expression by day 3 and 7 in the frontal cortex only. RS did not cause any significant change in the

hippocampal levels of cofilin, but there were significant changes in the frontal cortex cofilin protein levels on day 7.

5. CONCLUSIONS AND REMARKS

Experimental data and clinical studies support that chronic and transient stress types contribute to development of AD. Many studies described stress protocols in laboratory rodents have been developed and used in literature, but our data are the first to demonstrate cytoskeletal effects of different physical and psychological stressors. These stress – types give rise to different quantitative and kinetic changes in the transcription and translation of the main components of cytoskeletal organization in hippocampal homogenates of male rats. Our results also have important implications regarding the need for the careful selection of different stress models and their methodological importance.

In addition to stress – induced alterations in the cytoskeletal components, RS also has an impact on the hippocampal transcription of MAPK – 1 and APP in male rats. The effects caused by stress in AD – related genes may contribute to the development of AD – induced reduction of synaptic plasticity.

The fact that these molecular alterations were detected mostly in the hippocampus, tends to suggest that this brain area may be the most stress – sensitive formation in the central nervous system. Effects of RS and EFSS in the hippocampal mRNA expression of β – actin, cofilin and MAPK – 1 show a U – shaped time course, from which we deduced that stress induced a time – dependent alteration and depletion of compensatory mechanisms.

Our data are the first to demonstrate that RS indicates a gender – dependent regulation of neuronal cytoskeletal components. They further suggest that a

difference in actin regulatory mechanisms in females may be associated with the rate of neurodegenerative disease in women as compared to men.

These changes additionally indicate a very delicate stress-, time- and gender – dependent neuronal cytoskeletal and AD – related gene regulation in the rat brain. Our results may contribute to the selection of appropriate stress models in connection with the development of certain stress – related human conditions. These changes may participate in the progression of cognitive dysfunction in AD.

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