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

Ph.D. Thesis

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PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. Sántha P, Pákáski M, Fazekas OC, Fodor EK, Kálmán S, Kálmán J Jr, Janka Z, Szabó G, Kálmán J. Restraint Stress in Rats Alters Gene Transcription and Protein Translation in the Hippocampus. (2012) Neurochemical Research 37:958 – 964. IF: 2.240

II. Sántha P, Pákáski M, Fazekas Ö, Szűcs Sz, Fodor EK, Kálmán J, Jr, Kálmán S, Szabó Gy, Janka Z, Kálmán J. Acute and chronic stress induce changes in gene trancriptions related to Alzheimer's disease. (2012) Clinical Neuroscience 65:195 – 200. IF: 0.348

III. Sántha P, Pákáski M, Fodor EK, Fazekas OC, Kálmán S, Kálmán J Jr, Janka Z, Szabó G, Kálmán J. Cytoskeletal protein translation and expression in the rat brain are stressor – dependent and region – specific. (2013) PLOS ONE 8: e73504. IF: 3.730

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ABBREVIATIONS

AD	Alzheimer's disease
ADDL	Aβ – derived diffusible ligands
ADP	adenosine diphosphate
Aip1	actin – interacting protein 1
ANOVA	analysis of variance
ApoE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid – β
BACE	β – secretase enzyme
BW	body weight
CRH	corticotropin – releasing hormone
CSF	cerebrospinal fluid
EFSS	electric foot – shock stress
ERK 1/2	extracellular signal – regulated kinase 1/2
F – actin	filamentous actin
FSS	forced swimming stress
G – actin	globular actin
GAPDH	glyceraldehydes – 3 – phosphate dehydrogenase
HPA	hypothalamo – pituitary – adrenal
LTP	long – term potentiation
MAPK – 1	mitogen – activated protein kinase – 1
NFTs	neurofibrillary tangles
PSS	psychosocial stress
qRT – PCR	quantitative real – time polymerase chain reaction
RS	restraint stress

1. INTRODUCTION

1.1. Stress

Stress is an integral part of life and although it is a commonly used word, it is not a useful term for science. Even though, at present, there is no universally accepted definition, we can find many theories in literature. The term ‘stress’, as it is currently used was coined by Hans Selye, who defined it as a difficult and complex response of the body to any negative challenge subjected to the organism, calling this reaction in the body ‘general adaptation syndrome’ (Kim and Diamond, 2002, Bao et al., 2008). He made it clear that not all states of stress were noxious when he coined the terms ‘eustress’ and ‘distress’ (Chrousos and Gold, 1992). Most of these episodes can be controlled and may even be necessary for survival, but intense or prolonged exposure to stress – as environmental factors – may lead to a variety of disorders, such as hypertension (Sparrenberger et al., 2009), diabetes mellitus (Marcovecchio and Chiarelli, 2012), and atherosclerosis (Gu et al., 2012). It may also lead to a variety of neuropsychiatric disorders: mood and anxiety disorders, schizophrenia and neurodegenerative diseases, e.g. Alzheimer’s disease (AD) (Chen et al., 2008).

1.2. Stress hypothesis in AD

In old age, major negative life events, somatic illness with chronic pain, emotional and psychological traumas may all lead to the existence of stress and increase the development of depression. Furthermore, depression itself may be a prodromal symptom of dementia syndromes. Retrospective epidemiological studies have been published that depression also plays a key role as a risk factor in the development of AD. These evidences have indicated that the development of cognitive dysfunction or AD is common in individuals who are often exposed to periods of stress throughout their whole lives (Wilson et al., 2005). The co – morbidity of depression and AD is not uncommon. 25 – 40% of AD patients suffer from co – morbid depression, possibly making it a significant risk factor for AD (Caballero et al., 2006).

The pathomechanism of stressful experiments on brain damage are as of yet unknown, but in recent decades, much work has focused on the stress – induced negative impact on brain function (Kim and Diamond, 2002, Kim et al., 2013, Schoenfeld and Gould, 2013).

Chronic stress has an activating effect on the hypothalamo – pituitary – adrenal (HPA) axis. In response to stressors, the hypothalamus releases corticotropin – releasing hormone (CRH), which then in turn triggers the pituitary gland which leads to increased release of ACTH and steroid hormones from the adrenal cortex (cortisol in humans, corticosterone in rodents) (Bao et al., 2008, Rothman and Mattson, 2010). Blood of AD patients indicates increased levels of corticosteroids, similar to patients suffering from depression or anxiety disorders (Csernansky et al., 2006).

In normal conditions, glucocorticoids play a key role in the metabolism of glucose. As a response to stress, this increased hormone level inhibits the glucose uptake by hippocampal neurons and glial cells (McEwen et al., 1992). Chronically elevated glucocorticoid levels are usually associated with memory dysfunction and cause hippocampal functional and morphological alterations (Coluccia et al., 2008). Data from literature indicate that elevated cortisol levels can cause reductions in hippocampus volume and changes in neuronal morphology such as a decrease in a number of dendritic branch points and synapses (Sapolsky et al., 1986, Tata and Anderson, 2010). The hippocampus is crucially involved in both memory and the neuroendocrine regulation of stress hormones (Kim and Diamond, 2002). The response to stress may lead to damage in this region, so that these alterations contribute to development of cognitive dysfunction and AD.

Previous genetic studies indicate that the ε4 allele of the apolipoprotein E (ApoE) gene is not only involved in lipid transport and metabolism, but that it is also associated with AD (Mayeux et al., 1998). The presence of ApoE ε4 allele increases the risk of the disease by 3 – fold in heterozygous carriers and by 15 – fold in homozygous carriers (Farrer et al., 1997). Peskind et al. (2001) demonstrated that presence of the ApoE ε4 allele was reflected in the cortisol levels of cerebrospinal fluid (CSF). They measured cortisol levels in CSF and determined ApoE genotypes for AD and control patients. The CSF cortisol concentration was significantly higher within the ε4 genotype than in the ε2 or ε3 allele (Peskind et al., 2001). Other observations supported that prolonged stress and ApoE ε4 interact to increase the declarative memory deficits and the risk of developing AD (Peavy et al., 2007).

1.3. Neuropathological changes in the AD brain

AD is pathologically characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) (Castellani et al., 2008). One of the central mechanisms hypothesized to contribute to AD is the amyloid – β (Aβ) cascade. 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 APP by 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 (Haass and Selkoe, 1993, Ferreira et al., 2007). More recent studies have demonstrated an alternative representation of the Aβ cascade hypothesis. According to this Aβ – derived diffusible ligands (ADDL) hypothesis, the fibrillar amyloid is not the only toxic form of Aβ. Aβ also assembles into soluble forms but this aggregated peptide is much smaller than the well – known amyloid fibrils. These globular oligomers, ADDLs, could affect neurons by impairing synaptic plasticity and inducing nerve cell death and memory loss (Klein et al., 2001, Krafft and Klein, 2010).

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) (Medeiros et al., 2011). Tau hyperphosphorylation is deleterious to neurons, leading to microtubule degeneration, impairment of axonal transport and synaptic function, and cell death (Huang and Jiang, 2009).

Clinical and research data support that a decrease of dendritic spine density is also a prominent phenomenon in early cases of AD, which correlates significantly with the progressive decline of mental functions (Davies et al., 1987, Kojima and Shirao, 2007, Scheff et al., 2007, Penzes and Vanleeuwen, 2011). These morphological changes are characterized by dendritic spine size and number (Penzes and Vanleeuwen, 2011). Spines are small protrusions which appear in excitatory synapses within the brain. The decrease in a number of dendritic spines in the hippocampus and prefrontal cortex has a stronger correlation with the progressive decline than with NFTs and neuronal loss (DeKosky and Scheff, 1990).

In addition to the previous neuropathological changes, another hallmark of AD are the Hirano bodies (Hirano, 1994). These interneuronal inclusions contain mainly actin and actin – binding proteins. All of these assume that Hirano bodies are an abnormal organization of the neuronal cytoskeleton (Galloway et al., 1987).

1.4. Stress – induced pathological signs related to AD

Examining the different pathophysiological routes in AD genesis, their stress – induced origin has been proven in many cases (Fig. 1.).

The levels of β – secretase enzyme (BACE) and of its substrate, βAPP, are selectively increased after glucocorticoid administration resulting in increased production of Aβ (Green et al., 2006). Remarkably, administering glucocorticoids to the triple – transgenic (3×Tg – AD) mice – which develop both Aβ and tau pathologies in an age – dependent manner (Oddo et al., 2003) – exacerbated the formation of both lesions (Green et al., 2006). AD mouse models exposed to restraints also showed increased levels of Aβ, plaque deposition and enhancements of tau hyperphosphorylation, and neuritic atrophy of cortical neurons (Lee et al., 2009). Shifting APP processing toward β – amyloidogenesis by upregulating BACE and its substrate βAPP by induction through stress conditions has been confirmed in another double transgenic APP/PS1 mouse model (Devi et al., 2010).

There are also literature data regarding that chronic stress exposure may contribute to the development and progression of tau pathology in AD. Repeated stress exerted cumulative increases in hyperphosphorylated tau and resulted in its sequestration into insoluble NFTs, a defining feature of AD (Rissman et al., 2007, Yan et al., 2010).

Additionally, chronic stress causes dendritic regression and loss of dendritic spines in neurons that is accompanied by deficits in synaptic plasticity. Previous studies have proven that elevated glucocorticoid levels induced by stress – cause the impairment of long – term potentiation (LTP) (Kim and Haller, 2007). LTP is a neuronal mechanism playing a major role in learning and memory (Rowan et al., 2007). The inhibition of LTP has been proposed to underlie the synaptic dysfunction and lead to cognitive decline in AD (Rowan et al., 2003). Stress – induced changes are observed mainly in the hippocampus and prefrontal cortex.

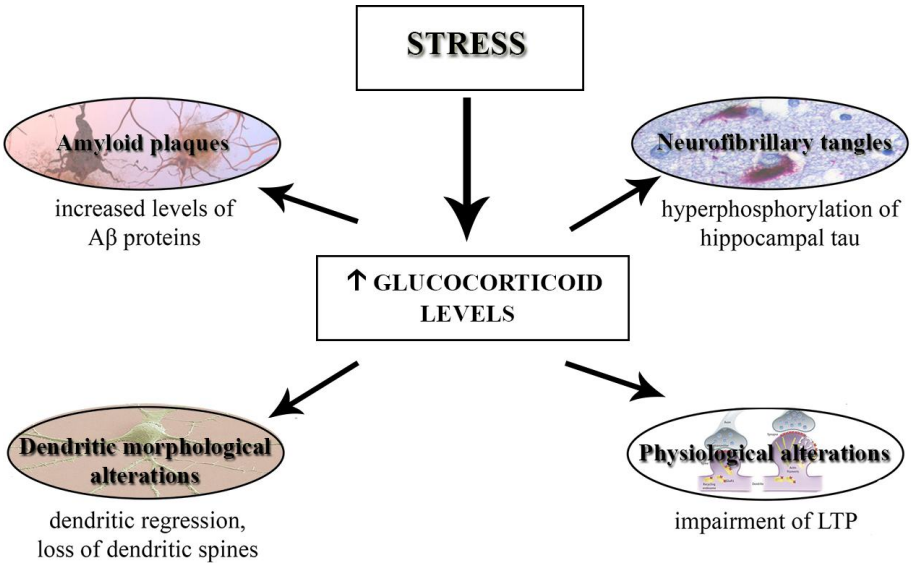

Fig. 1.: Stress – induced alterations in the development of AD.

1.5. Actin dynamics and regulation

The major cytoskeletal component of dendritic spines is filamentous actin (Sekino et al., 2007). Actin is a cytoskeletal protein expressed ubiquitously in eukaryotes. It is composed of three distinct isoforms: α – actin isoforms occur in the contractile apparatus and smooth muscle, while the two cytoplasmic actin isoforms, β – and γ – actin, are found in all tissues (Cheever and Ervasti, 2013). The ability of monomeric, globular actin (G – actin) to rapidly assemble and disassemble into polymer filaments (F – actin) is essential for cell motility, structure and integrity (Maloney and Bamburg, 2007, Bamburg and Bloom, 2009).

F – actin is the major cytoskeletal component of the dendritic spines and plays a key role in the morphogenesis, maintenance and plasticity of spines (Bamburg and Wiggan, 2002, Kojima and Shirao, 2007, Sekino et al., 2007). The most important regulators of actin dynamics are members of the actin – depolymerization factor ADF/cofilin family (Bamburg and Bloom, 2009). Three forms are expressed in mammals: one of them, cofilin – 1, appears in neurons.

This neuronal cofilin is regulated by the ratio of its concentration to those of actin and other actin – binding proteins (Schubert and Dotti, 2007, Bernstein and Bamburg, 2010). Data from literature indicate that low (less than 1%) concentrations of cofilin in a filament result in

persistent filament serving (Fig. 2A). In the case of a higher cofilin/actin ratio (1:10 to 1:2), the cofilin induces a rapid and transient disassembly by stabilizing F – actin in a twisted form. Other actin – binding proteins, such as actin – interacting protein 1 (Aip1), bind to cofilin – actin filaments and enhance the severing and depolymerizing activity of cofilin, potentiating the generation of actin monomers. In cells under stress, where ADP – actin levels are elevated, the cofilin – saturated F – actin assembles into rod – shaped bundles. Whether cofilin binds to the filaments depends on the release of inorganic phosphate (Pi) after actin ATP hydrolysis, because Pi binds antagonistically to cofilin. In addition, release of Pi is increased 10 – fold by cofilin binding (Fig. 2B) (Bamburg et al., 2010).

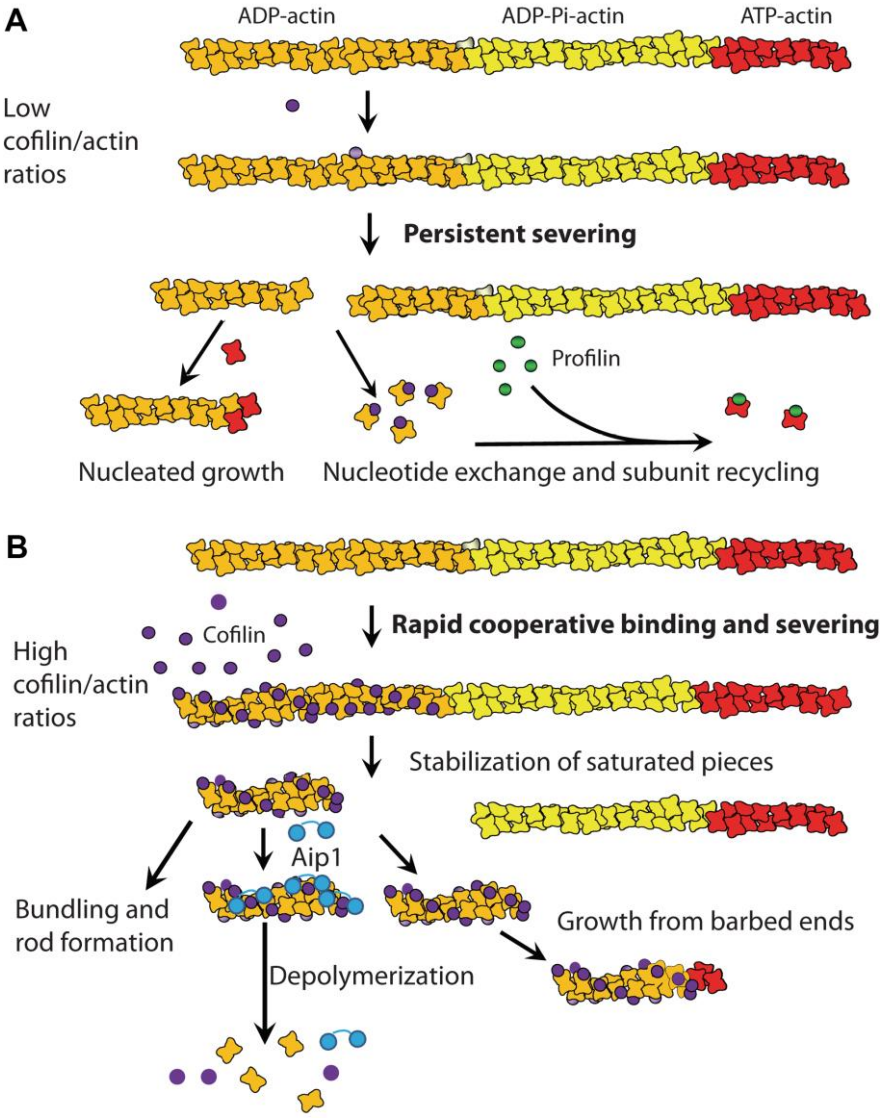

Fig. 2.: The effects of the ratio of cofilin/actin subunits on actin dynamics. ADP – Pi – actin: *ADP* – inorganic phosphate – actin; Aip1*:* actin – interacting protein 1 *(Bamburg et al., 2010).*

These intracellular cofilin – actin rod formations can occlude the neurites by disrupting microtubules, causing neuritic atrophy. They may also participate in amyloid production (Minamide et al., 2000) and may contribute to the formation of abnormal NFTs by the tau protein binding to rods and aiding in their assembly into paired helical filaments (Bamburg et al., 2010). In addition, cofilin over – expression causes a decrease in the number of presynaptic varicosities and impairs both basal synaptic transmission and LTP (Maloney and Bamburg, 2007).

Another regulatory factor is the MAPK – 1 (also called extracellular signal – regulated kinase 2 – ERK 1/2) which contributes to F – actin stabilization and arrangement (Jafari et al., 2012). MAPK – 1 is also responsible for the hyperphosphorylation of tau, leading to microtubule degeneration and cell death in AD.

1.6. Gender differences in stress and AD

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 (Vina and Lloret, 2010, Bangasser and Valentino, 2012, Chen et al., 2012). This difference between genders might partly be biologically determined. Clinical studies have found that stress has shown greater activity in the brains limbic system and there is a larger cortisol response in women compared to men (Peeters et al., 2003, Wang et al., 2007, Lebron – Milad et al., 2012).

Few comparative investigations have also been made concerning the influence of gender in animal models (Faraday, 2002, Trentani et al., 2003). Similarly to human data, one of the most important evidences supporting the differences between sexes are the levels of HPA – axis hormones: the basal levels of glucocorticoid and corticosterone are higher in female than male rats. Moreover, female rodents release more ACTH and corticosterone, and the levels of these hormones remain elevated for a longer period of time in response to stress (Bangasser and Valentino, 2012). Gonadal hormones are at least partly responsible in the mechanisms underlying the sex difference in stress HPA axis activity.

The HPA axis is not the only factor that influences stress susceptibility. The brain serotonin (5 – HT) system has previously been shown to be involved in the development of psychiatric disorders. Sach et al. (2014) have proven that gender and 5 – HT deficiency present a differential impact on hippocampal cell proliferation (Sachs et al., 2014).

1.7. Experimental stress models

Effects of stress are influenced by many factors such as gender, its type or duration, individual sensitivity, and the brain region (Chen et al., 2012). 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. Most experimental stressors indicated both physical and psychological effects at the same time. Restrain stress (RS) has been commonly used to study stress – induced biological, biochemical and physiological responses in animals. Animals are confined inside a plastic tube where they can not move in any direction; this situation induces an inescapable physical and mental stress (Pitman et al., 1988, Jaggi et al., 2011). Similarly to RS, electric foot – shock stress (EFSS) causes not only a physical but also a psychological stress response. The main advantage of this stress model is that the stressor factors, such as time, frequency, and intensity can be exactly controlled (Tsukada et al., 2003, Robbins and Ness, 2008). Forced swimming stress (FSS) predominantly reproduces physical stress. The response to FSS is induced by a stressful new situation from which the animal is not able to escape (Jaggi et al., 2011). In contrast to physical stressors, psychosocial stress (PSS) does not cause physical injury or discomfort, but reproduces psychological stress. It disrupts the social hierarchy and the rats involved must adjust to a new social situation. This model resembles work – related stress (Gerges et al., 2001).

Previous studies have proven that acute and chronic RS and EFSS can cause decreases in neurogenesis (Rosenbrock et al., 2005, Radley et al., 2006, Dagyte et al., 2009), while chronic PSS partially blocks the early LTP of the CA1 area of the hippocampus (Gerges et al., 2001). RS can change the status of the microtubular dynamics in the rat hippocampus, causing

an involution of structural neuronal plasticity, thereby playing a part in the pathophysiology of stress – related conditions (Bianchi et al., 2003). In contrast to the wide methodological repertoire of available animal stress models, the cytoskeletal effects of different physical and psychological stressors 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. MATERIAL 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. All animal procedures were approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged (approval number: I – 74 – 4/2011.MÁB). 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. Since each stress protocol was done as a separate experiment, each stress model had its own control group. The animals were housed 3 per cage in RS, EFSS and FSS, and 5 per cage in PSS.

The day after the last stress procedure (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 (between 8 a.m. and 1 p.m.) (Pitman et al., 1988).

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 (Tsukada et al., 2003, Robbins and Ness, 2008).

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. The water was so deep that the tails of the swimming or floating animals did not touch the bottom. The water was changed after each animal. Three identical cylinders were used, separated by opaque screens, for simultaneous testing (Porsolt et al., 1978, Takao et al., 1995, Lahmame et al., 1997, Alkadhi et al., 2005).

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 (Gerges et al., 2001, Alkadhi et al., 2005, Aleisa et al., 2006).

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. 0.3 μg of RNase inhibitor 40 U/μl (Fermentas, Glen Burnie, Maryland, USA) was added and the eluted RNA was stored at -80 °C until use.

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. Each reaction tube, with a total volume of 30 μl, contained 2 ng of total RNA in a volume of 15 μl, and 15 μl of transcription mix (3 μl of reverse transcription buffer, 1.2 μl (100 mM) of dNTP mix, 3 μl of random primers, 1.5 μl of Multi Scribe^{TM} reverse transcriptase, 0.75 μl (20 U) of RNase inhibitor and RNA Free Water (Ambion, Austin, TX, USA)). The thermal cycling consisted of three cycles: the first at 25 °C for 10 min, the second at 37 °C for 120 min, and the final one at 85 °C for 5 s. 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. Primer sequences are shown in Table 1.

	forward	reverse
β-ACTIN	CCC GCG GAG TAC AAC CTT CT	CGT CAT CCA TGG CGA AC
COFILIN	GGC GGC TCT GTT CTT CTG T	CTC CAT CAG AGA CAG CCA CA
APP	CCC CAA GAT CCG GTT AAA CT	TAC TTG TCG ACT GCG TCA GG
MAPK-1	CCA AGC TCA ACC GTC TCA TC	GGC TGG TAG GGT AGT TGA TGC
GAPDH	AGA TCC ACA ACG GAT ACA TT	TCC CTC AAG ATT GTC AGC AA

Table 1: List of primers.

30 µl of cDNA solution were diluted with 510 µl of DNase and RNase – free water. qRT – PCR was carried out in a final volume of 20 µl containing 10 µl of SYBR Green MasterMix (Roche, Basel, Switzerland), 0.5 µl of forward primer, 0.5 µl of reverse primer, and 9 µl of template cDNAs. The protocol started with denaturation for 25 s at 95 °C, followed first by annealing for 25 s at 60 °C, and then by extension for 15 s at 72 °C. The relative gene expression was normalized to glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH). The results were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3.5. Western blotting

The brain regions were homogenized in a solution containing 50 mM Tris buffer (pH 7.5), 150 mM NaCl, 0.1% Nonidet – P – 40, 0.1% cholic acid, 2 µg/ml leupeptin, 2 mM PMSF, 1 µg/ml pepstatin and 2 mM EDTA. The homogenates were centrifuged at 10 000 g for 15 min at 4 °C. The supernatants were used for protein assays. Proteins were measured with bicinchoninic acid (Smith et al., 1985).

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 (Santa Cruz, CA, USA, 1:2000), rabbit polyclonal cofilin (D59) antibody (Cell Signaling Technology, MA, USA 1:1000), mouse monoclonal ERK 1/2 antibody (Santa Cruz Biotechnology CA, USA 1:200), mouse monoclonal anti – APP (Millipore, MA, USA 1:33.3) and mouse monoclonal anti – GAPDH (Millipore, MA, USA 1:4000). The next day, after five washes with TBST, horseradish – peroxidase – labelled anti – mouse IgG (Jackson Immunoresearch, West Grove, PA, USA 1:1000), horseradish – peroxidase – labelled anti – rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA 1:1000) secondary antibodies were applied for 1 h. The nitrocellulose membranes were subsequently washed five times with TBST, and then incubated with the Supersignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) 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. 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 order to demonstrate that RS, EFSS, FSS and PSS are representative stressors, the overall BW was measured repeatedly throughout the individual stress experiments. Two – way ANOVA revealed significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (7, 14 and 21 days) in the BW of male rats. There was a significant main effect of stress types and exposure times (Table 2).

	INTERACTION stress type x exposure times	STRESS TYPE	EXPOSURE TIME
Body weights	$F_{(12,6227)}$=9.8, p<0.001	$F_{(3,622)}$=827.86, p<0.001	$F_{(4,622)}$=990.31, p<0.001
Adrenal gland weights	$F_{(9,107)}$=4.055, p<0.001	$F_{(3,107)}$=5.577, p=0.001	$F_{(3,107)}$=4.055, p<0.001
Thymus weights	$F_{(9,105)}$=2.248, p=0.024	$F_{(3,105)}$=7.473, p<0.001	$F_{(3,105)}$=15.372, p<0.001

Table 2.: Two – way ANOVA statistical analysis of the body weights, adrenal gland weights and thymus weights.

In the RS experiment, the relative decrease in BW was significant by day 3 ($p < 0.001$); and the reduction remained significant at all examined time points (on the 7th day group 3: $p = 0.07$; on the 14th day group 4: $p = 0.02$, group 5: $p < 0.001$; on the 21st day group 5: $p = 0.001$) (Fig. 3A). EFSS also caused a significant lack of gain in BW, but only in response to chronic stress (on the 14th day group 5: $p = 0.049$; on the 21st day group 5: $p = 0.019$) (Fig. 3B). However, exposure to FSS and PSS did not provoke any appreciable difference in BW (Fig. 3C,D).

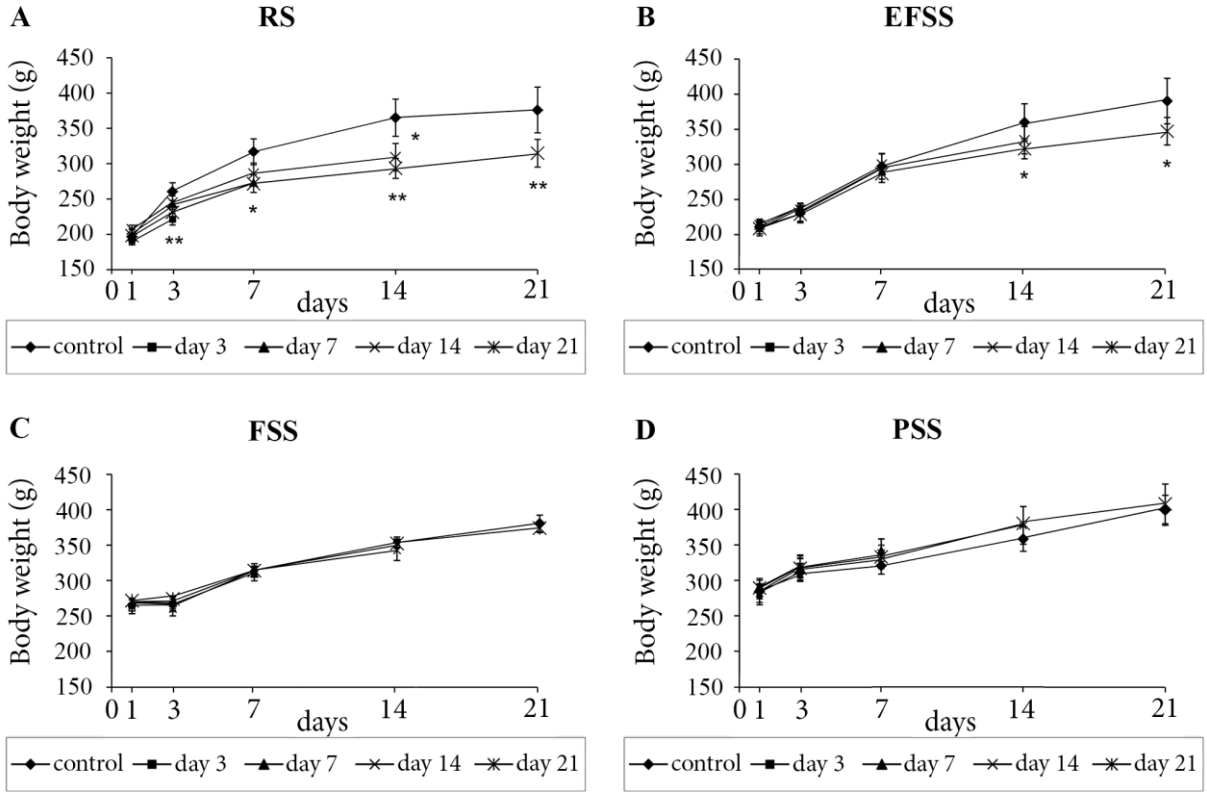

Fig. 3.: Effects of restraint stress (RS) ***(A)****, electric foot shock stress (EFSS)* ***(B)****, forced swimming stress (FSS)* ***(C)*** *and psychosocial stress (PSS)* ***(D)*** *on the overall body weight of rats, measured on days 3, 7, 14 and 21. Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 and **p < 0.001: significant differences as compared to the control.*

The adrenal gland and thymus were weighed at different time points when the animals were sacrificed. Fig. 4A shows effects of RS, EFSS, FSS and PSS on the weights of the adrenal glands of male rats. The adrenal gland weight relative to the BW was significantly elevated by RS and by EFSS (Fig. 4A). RS caused a significant increase after 14 and 21 days (day 14: $p = 0.012$; day 21: $p = 0.002$), EFSS induced a significant increase after 7 and 14 days ($p < 0.001$) (Fig. 4A). In contrast, FSS and PSS did not result in any significant change in the weight of the adrenal glands (Fig. 4A).

Fig. 4B shows effects of RS, EFSS, FSS and PSS on the weights of the thymus of rats. The thymus weight relative to BW was significantly decreased only by day 21 after RS ($p = 0.047$) (Fig. 4B). EFSS and PSS caused a transient increase in the thymus weight, after which a decreasing tendency was detected (Fig. 4B). The two – way ANOVA revealed significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (7, 14

and 21 days) in the weights of adrenal glands, and thymus. There was a significant main effect of stress types and exposure times (Table 2).

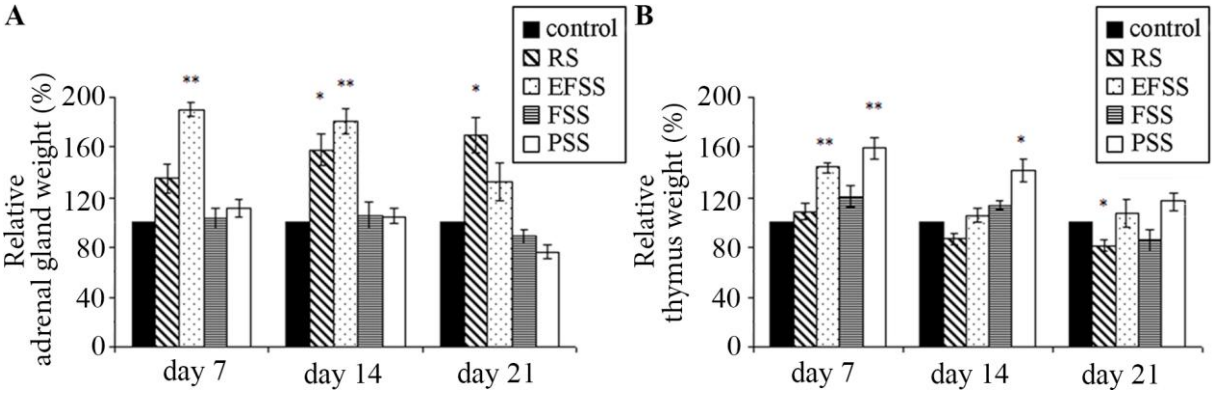

*Fig. 4.: Effects of restraint stress (RS), electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the weights of the adrenal glands **(A)** and the thymus **(B)** of male rats, measured every 7 days. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 and **p < 0.001: significant differences as compared to the control.*

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

Fig. 5 shows the expression of β – actin in the male rat hippocampus (A) and frontal cortex (B). The two – way ANOVA revealed significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the β – actin mRNA expression in the hippocampus. There was a significant main effect of stress types and exposure times (Table 3).

	INTERACTION stress type x exposure times	STRESS TYPE	EXPOSURE TIME
Gene expression in the hippocampus	$F_{(12,121)}$=22.105, p<0.001	$F_{(3,121)}$=22.105, p<0.001	$F_{(4,121)}$=3.584, p<0.001
Gene expression in the frontal cortex	$F_{(12,122)}$=1.273, p=0.243	$F_{(3,122)}$=5.285, p=0.002	$F_{(4,122)}$=1.220, p=0.306

Table 3.: Two – way ANOVA statistical analysis of the gene expressions of β – actin in the rat hippocampus and frontal cortex.

In the hippocampus, RS, EFSS and FSS caused significant increases in β – actin mRNA expression by day 3 (RS p = 0.05, EFSS p = 0.034, FSS p = 0.05) (Fig. 5A). The RS – induced transcription of β – actin mRNA followed a U – shaped time course. Following a 5 – fold elevation in the level of β – actin mRNA on day 3, its expression then decreased somewhat by days 7 and 14, respectively, but again increased significantly by day 21, relative to day 3 (Fig. 5A).

A similar U – shaped time course was detected in the case of EFSS, but with a later appearance. The β – actin mRNA level was found to be elevated on days 3, 7 and 21, respectively, but not on day 14 (Fig. 5A). In comparison with the 10 – fold rise in the expression of β – actin mRNA induced by RS, EFSS caused only a 4 – fold increase on day 21 (Fig. 5A).

In the case of FSS, the time course was not U – shaped: significant elevations were observed on days 3 and 7, but there were no changes at the later time points (Fig. 5A).

In contrast to the physical stressors, PSS did not influence the β – actin mRNA transcription either in the hippocampus nor in the frontal cortex (Figs 5A,B).

The two – way ANOVA did not reveal significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the β – actin mRNA expression in the frontal cortex. There was a significant main effect of stress type, but the two – way ANOVA did not reveal a significant main effect of exposure times (3, 7, 14 and 21 days) in the β – actin mRNA expression in the frontal cortex (Table 3). FSS notably increased the frontal cortical levels of β – actin mRNA expression by day 7 (p = 0.032) (Fig. 5B).

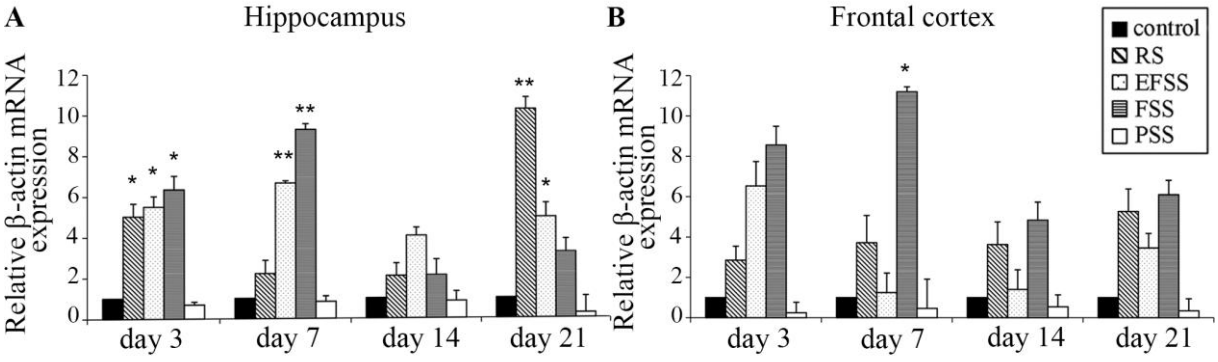

Fig. 5.: Effects of restraint stress (RS), electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the expressions ***(A, B)*** *of β – actin mRNA in the rat hippocampus and frontal cortex. GAPDH was used as reference gene. Values for*

*each group are means ± SEM, n = 6 – 10. *p ≤ 0.05 and **p < 0.001: significant differences as compared with the control.*

Fig. 6A – D show the levels of β – actin protein in the male rat hippocampus and frontal cortex. Fig. 6 A, B depict representative β – actin immunoblots after different types of stress. The β – actin signal of homogenates from the hippocampus and frontal cortex were resolved at approximately 43 kDa.

The two – way ANOVA revealed significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the levels of β – actin in the hippocampus. There was a significant main effect of stress types and exposure times (Table 4).

	INTERACTION stress type x exposure times	STRESS TYPE	EXPOSURE TIME
Protein levels in the hippocampus	$F_{(12,95)}$=3.446, p<0.001	$F_{(3,95)}$ =34.755, p<0.001	$F_{(4,95}$ =13.904, p<0.001
Protein levels in the frontal cortex	$F_{(12,100)}$=1.379, p=0.188	$F_{(3,100)}$=10.708, p<0.001	$F_{(4,100)}$=1.300, p=0.275

Table 4.: Two – way ANOVA statistical analysis of the protein levels of β – actin in the rat hippocampus and frontal cortex.

Western blot experiments revealed statistically significant elevations in the hippocampal β – actin levels by day 3 (p = 0.003) of exposure to RS or EFSS (day 3: p < 0.001). EFSS caused a longer increase in β – actin levels (day 7: p = 0.012); then, following a transient reduction on day 14, the β – actin protein levels were again significantly increased by day 21 of exposure to RS (p = 0.046) or EFSS (p < 0.001) (Fig. 6C). Thus, 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 (Figs 5A, 6C). Neither FSS nor PSS modified the hippocampal β – actin levels significantly (Fig. 6A,C). In the frontal cortex, none of the applied stressors caused any significant changes in the level of β – actin (Fig. 6B,D).

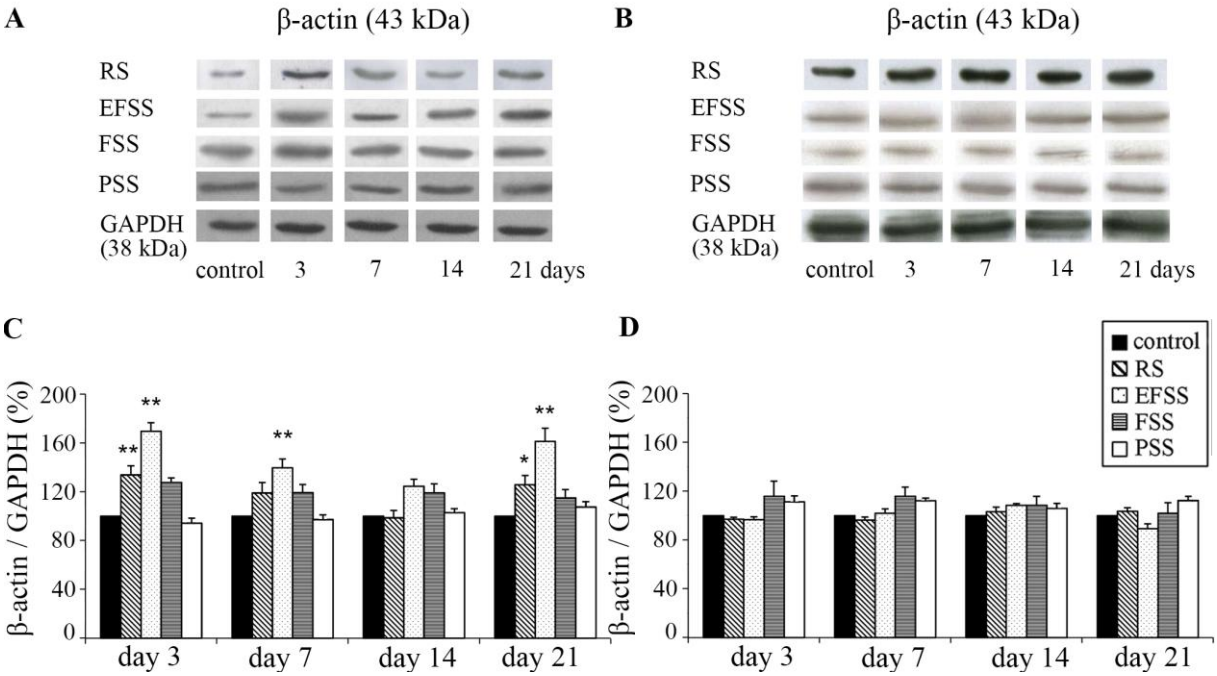

*Fig. 6.: Effects of restraint stress (RS), electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the protein levels of β – actin in the rat hippocampus **(A,C)** and frontal cortex **(B,D)**. GAPDH was used as reference gene. Densitometric analysis of the bands was obtained via Western blotting. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 and **p < 0.001: significant differences as compared with the control.*

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

Fig. 7 shows the expression of cofilin in the male rat hippocampus (A) and frontal cortex (B). The two – way ANOVA revealed significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the cofilin mRNA expression in the hippocampus. There was a significant main effect of stress types and exposure times (Table 5).

	INTERACTION stress type x exposure times	STRESS TYPE	EXPOSURE TIME
Gene expression in the hippocampus	$F_{(12,110)}$=5.838, p<0.001	$F_{(3,110)}$=105.815,p<0.001	$F_{(4,110)}$=5.419, p=0.001
Gene expression in the frontal cortex	$F_{(12,107)}$=4.100, p<0.001	$F_{(3,107)}$=545.267,p<0.001	$F_{(4,107)}$=5.963, p<0.001

Table 5.: Two – way ANOVA statistical analysis of the gene expressions of cofilin in the rat hippocampus and frontal cortex.

Changes in the expression of cofilin mRNA in the hippocampus were observed only in the case of RS, with 5-, 4- and 10 – fold increases on days 3, 7 and 21, respectively (day 3: $p = 0.029$; day 7: $p = 0.033$; day 21: $p < 0.001$). There was no change on day 14, but the cofilin transcription also displayed a U – shaped time course. EFSS, FSS and PSS had no effect on the expression of cofilin mRNA at any tested time point (Fig. 7A).

The two – way ANOVA revealed significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the cofilin mRNA expression in the frontal cortex. There was a significant main effect of stress types and exposure times (Table 5). FSS decreased the cofilin mRNA expression significantly by day 7 ($p = 0.032$) (Fig. 7B).

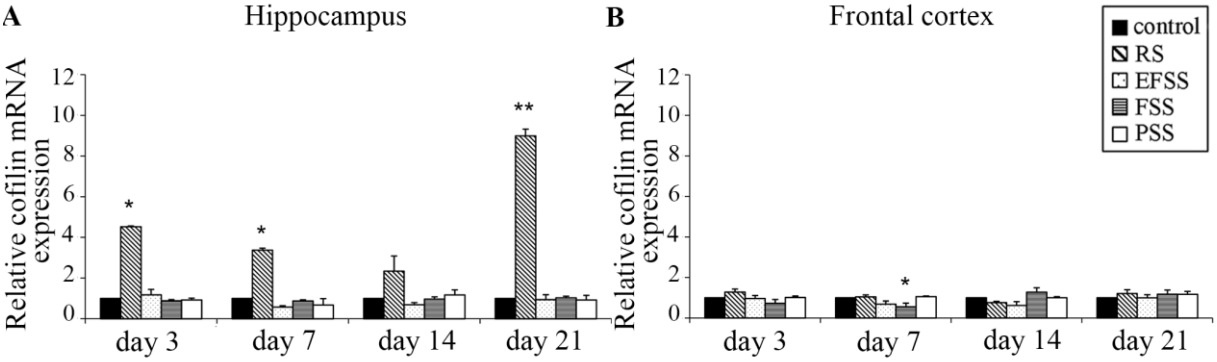

*Fig. 7.: Effects of restraint stress (RS), electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the expression of cofilin in the rat hippocampus **(A)** and frontal cortex (**B**). GAPDH was used as reference gene. Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 and **p < 0.001: significant differences as compared with the control.*

Fig. 8A – D show the levels of cofilin protein in the male rat hippocampus and frontal cortex. Figs 8A, B depict representative cofilin immunoblots after different types of stress. The cofilin signal of homogenates from hippocampus and frontal cortex were resolved at approximately 19 kDa.

The two – way ANOVA revealed significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the levels of cofilin in the hippocampus. There was a significant main effect of stress types and exposure times

(Table 6). The hippocampal cofilin levels increased only in the RS – exposed group, where the 1.5 – fold elevation was significant by day 21 (p = 0.038) (Fig. 8A, C).

	INTERACTION stress type x exposure times	STRESS TYPE	EXPOSURE TIME
Protein levels in the hippocampus	$F_{(12,95)}$=3.581, p<0.001	$F_{(3,95)}$=61.855, p<0.001	$F_{(4,95)}$=6.591, p<0.001
Protein levels in the frontal cortex	$F_{(12,95)}$=3.659, p<0.001	$F_{(3,95)}$=16.245, p<0.001	$F_{(4,95)}$=3.704, p=0.008

Table 6.: Two – way ANOVA statistical analysis of the protein levels of cofilin in the rat hippocampus and frontal cortex.

In the frontal cortex none of the applied stressors caused any significant changes in the levels of cofilin (Fig. 8B, D).

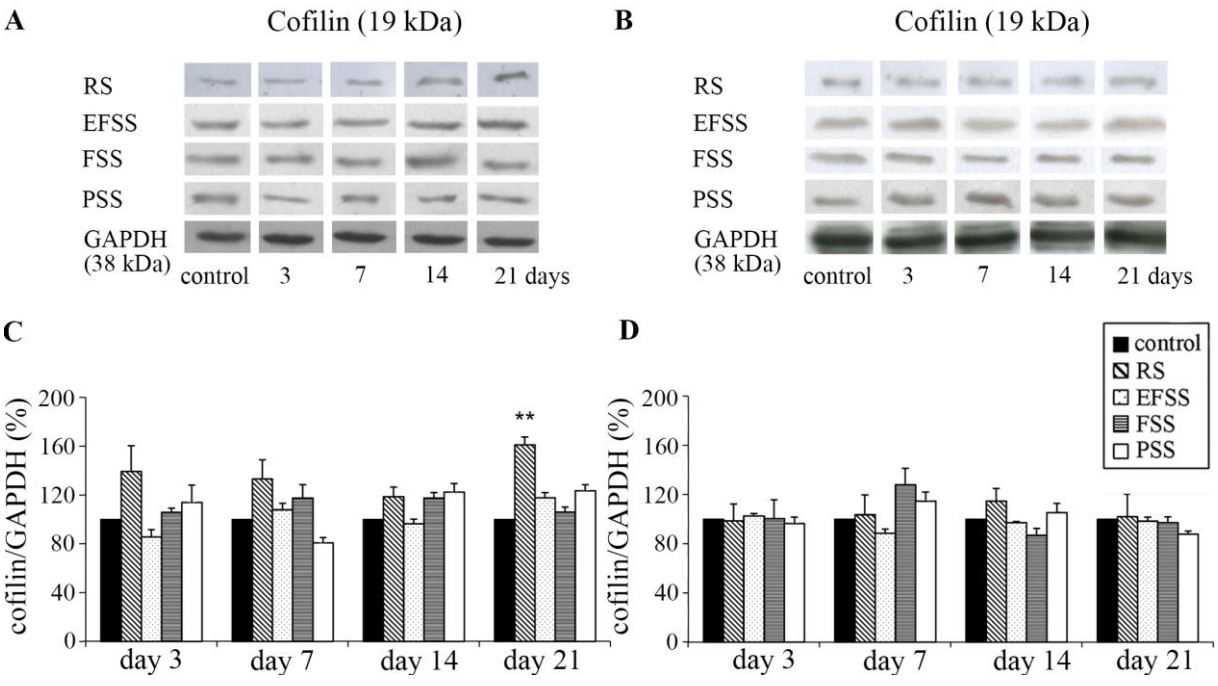

*Fig. 8.: Effects of restraint stress (RS), electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the protein levels of cofilin in the rat hippocampus (**A,C**) and frontal cortex (**B,D**). GAPDH was used as reference gene. Densitometric analysis of the bands was obtained via Western blotting. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 and **p < 0.01: significant differences as compared with the control.*

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

Fig. 9 shows the expression of MAPK – 1 mRNA elevation after different stress types in the male rat hippocampus (A) and frontal cortex (B). There was a significant interaction between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the MAPK – 1 mRNA expression in the hippocampus. The significant main effect of stress type observed, but the two – way ANOVA did not reveal a significant main effect of exposure times (3, 7, 14 and 21 days) in the MAPK – 1 mRNA expression in the hippocampus (Table 7).

	INTERACTION stress type x exposure times	STRESS TYPE	EXPOSURE TIME
Gene expression in the hippocampus	$F_{(12,126)}$=2.762, p=0.002	$F_{(3,126)}$=91.494, p<0.001	$F_{(4,126)}$=2.068, p=0.089
Gene expression in the frontal cortex	$F_{(12,104)}$=0.455, p=0.936	$F_{(3,104)}$=119.253,p<0.001	$F_{(4,104)}$=0.662, p=0.620

Table 7.: Two – way ANOVA statistical analysis of the gene expressions of MAPK – 1 in the rat hippocampus and frontal cortex.

Changes in the expression of MAPK – 1 mRNA in the hippocampus were observed only in the case of RS. Similarly to the β – actin and cofilin mRNA expression, both acute and chronic RS caused significant elevations in MAPK – 1 mRNA expression in the hippocampus: 2 and 2.7 times of the control value on days 3 (p = 0.030) and 21 (p = 0.01), respectively. As in the cases of β – actin and cofilin, the MAPK – 1 data described a U – shaped time course (Fig. 9A). The two – way ANOVA did not reveal significant interactions between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the MAPK – 1 mRNA expression in the frontal cortex (Table 7). RS, EFSS, FSS and PSS had no effect on the expression of MAPK – 1 mRNA at any tested time point (Fig. 9B).

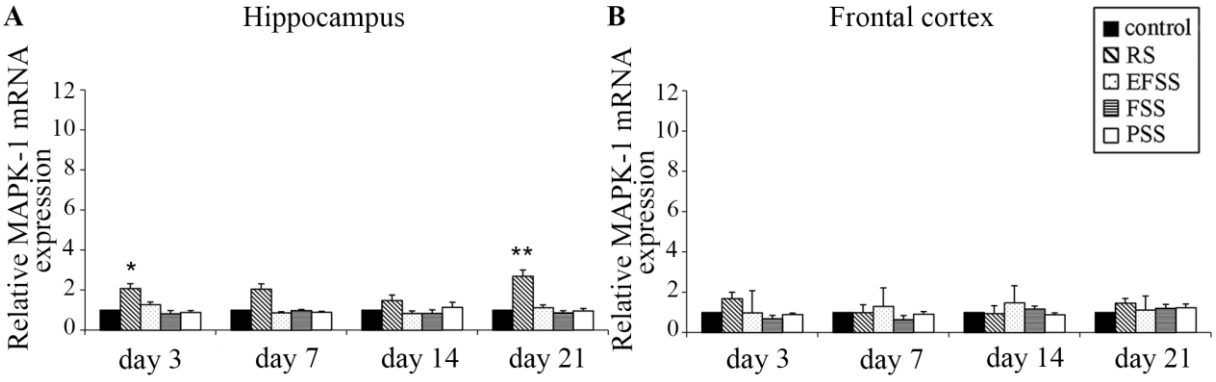

*Fig. 9.: Effects of restraint stress (RS), electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the expression of MAPK – 1 in the rat hippocampus (**A**) and frontal cortex (**B**). GAPDH was used as reference gene. Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 and **p < 0.01: significant differences as compared to the control.*

Western blotting experiments did not reveal significant changes after RS in the amount of MAPK – 1 (ERK 1/2) protein (Fig. 10A – C).

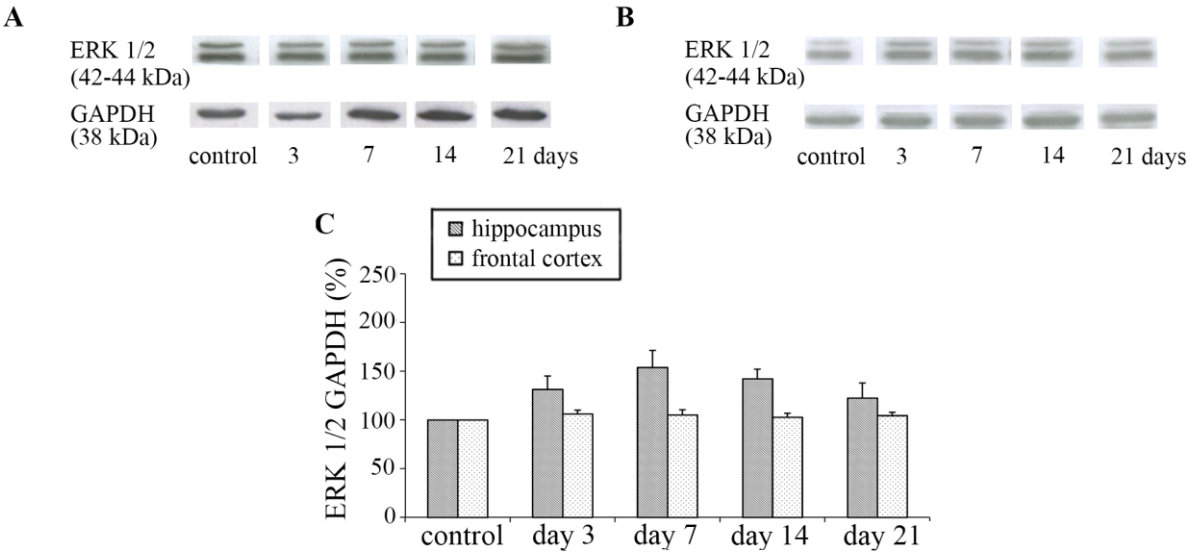

*Fig. 10.: Effects of restraint stress (RS) on the protein level of ERK1/2 (MAPK – 1) in the rat hippocampus (**A,C**) and frontal cortex (**B,C**). GAPDH was used as reference gene. Densitometric analysis of the bands was obtained via Western blotting. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 6 – 10.*

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

Fig. 11 shows the expression of APP mRNA elevation after different stress types in the male rat hippocampus (A) and frontal cortex (B). According to the two – way ANOVA there was a significant interaction between stress types (RS, EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the APP mRNA expression in the hippocampus. There was a significant main effect of stress type, but the two – way ANOVA did not reveal a significant main effect of exposure times (3, 7, 14 and 21 days) in the APP mRNA expression in the hippocampus (Table 8).

	INTERACTION stress type x exposure times	STRESS TYPE	EXPOSURE TIME
Gene expression in the hippocampus	$F_{(12,131)}$=1.974, p=0.031	$F_{(3,131)}$=35.988; p<0.001	$F_{(4,131)}$=2.304; p=0.062
Gene expression in the frontal cortex	$F_{(12,92)}$=0.719, p=0.729	$F_{(3,92)}$=13.762;p<0.001	$F_{(4,92)}$=0.246; p=0.912

Table 8.: Two – way ANOVA statistical analysis of the gene expressions of APP in the rat hippocampus and frontal cortex.

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, when it was 6 times of the control value (p = 0.043) (Fig. 11A).

None of the applied stressors caused any significant changes in the expression of APP mRNA in the frontal cortex (Fig. 11B).

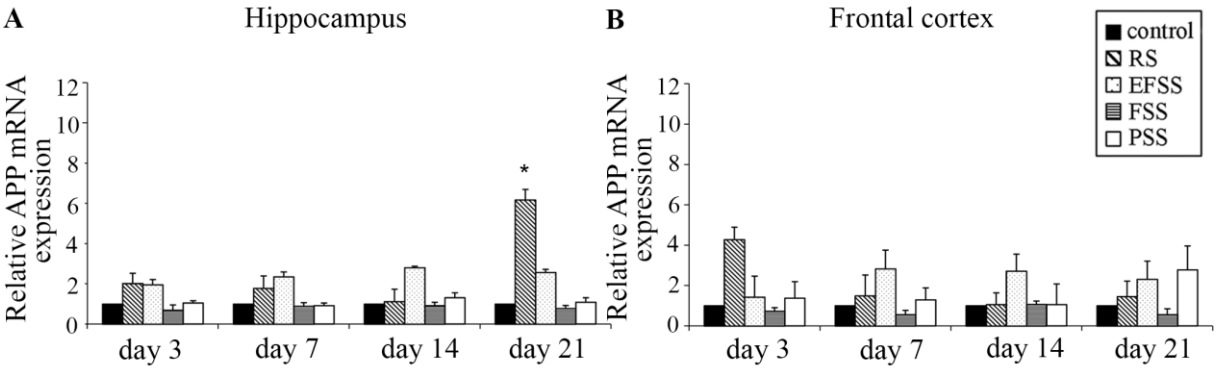

*Fig. 11.: Effects of restraint stress (RS), electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the expression of APP in the rat hippocampus (**A**) and frontal cortex (**B**). GAPDH was used as reference gene. Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 : significant differences as compared to the control.*

Figs 12 A, B depict representative APP immunoblots after RS. The APP signal of homogenates from the hippocampus (A) and frontal cortex (B) were resolved at approximately 110 – 130 kDa. There were significant changes in the hippocampal APP protein levels on days 3, 7 and 21 (day 3: $p = 0.031$; day 7: $p = 0.034$; day 21: $p = 0.001$), respectively. RS did not cause any significant change in the cortical levels of APP (Fig. 12C).

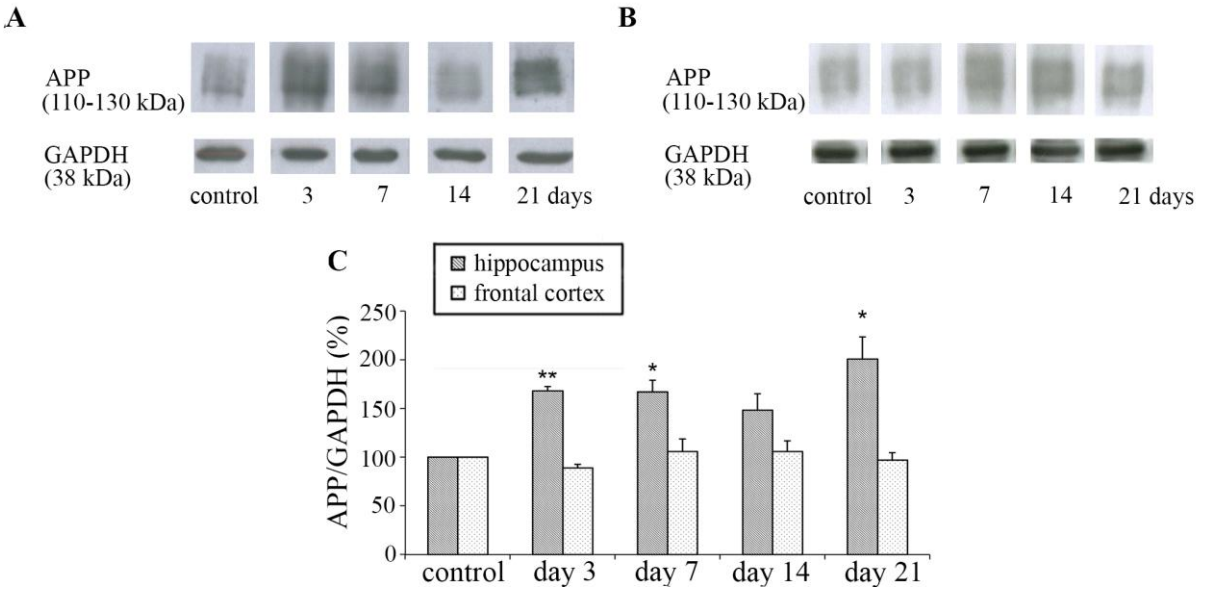

*Fig. 12.: Effects of restraint stress (RS) on protein level of APP in the rat hippocampus (**A,C**) and frontal cortex (**B,C**). GAPDH was used as reference gene. Densitometric analysis of the*

*bands was obtained via Western blotting. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 6 – 10. *p < 0.05 and **p < 0.01: significant differences as compared to the control.*

4.2. Stress – induced alterations in female rats

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

Like in male rats, in order to demonstrate that RS is a representative stressor, BW was measured repeatedly during the course of the experiment in female rats. The stressed animals gained BW at a significantly slower rate than the control group by day 21 ($p = 0.002$) (Fig. 13A). The weight of adrenal glands and the thymus were measured at the different time points when the animals were sacrificed. The adrenal gland weight relative to the BW was not significantly elevated by RS (Fig. 13B). The weight of thymus in the stressed animals was significantly lower on days 14 ($p = 0.003$) and 21 ($p = 0.006$) compared to control (Fig. 13C).

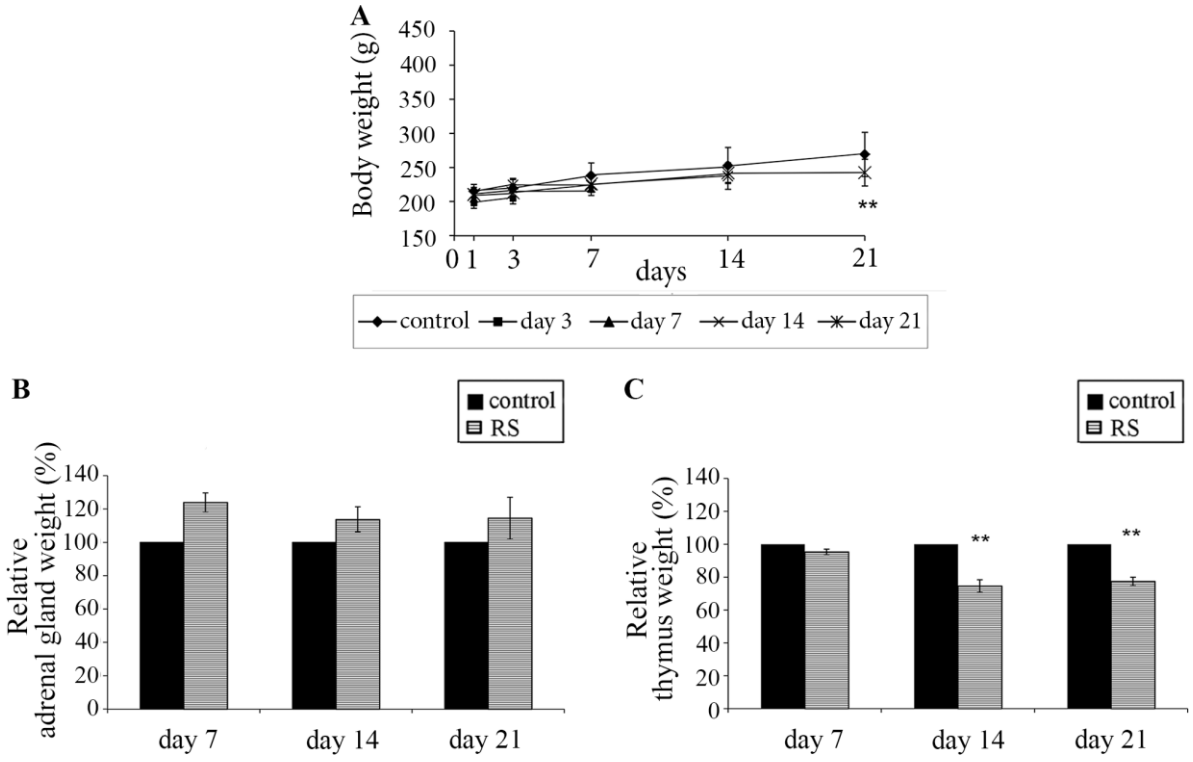

*Fig. 13.: Effects of restraint stress on the overall body weight **(A)** and on the weights of the adrenal glands **(B)** and thymus **(C)** of female rats. Values for each group are means ± SEM, n = 6. **p < 0.01: significant differences as compared to the control.*

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

Fig. 14 shows the expression and the level of β – actin proteins after RS in the female rat hippocampus (A) and frontal cortex (B). 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 ($p = 0.03$) (Fig. 14A). In comparison to the 10 – fold rise induced in the expression of β – actin mRNA by RS in the male hippocampus on day 21 (Fig. 5A), RS caused only a 6 – fold elevation in the female hippocampal region (Fig. 14A). Western blotting experiments revealed that exposure to RS did not elevate the amount of β – actin protein in any of the examined regions (Figs 14B, 15A, B).

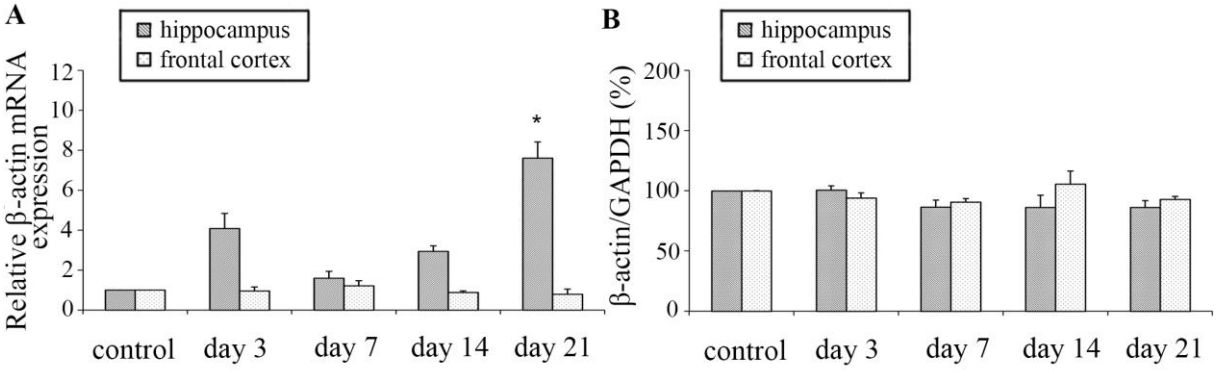

*Fig. 14.: Effects of restraint stress (RS) on the expression **(A)** and the protein levels **(B)** of β – actin in the rat hippocampus and frontal cortex. GAPDH was used as reference gene. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 4 – 6. *p < 0.05: significant differences as compared to the control.*

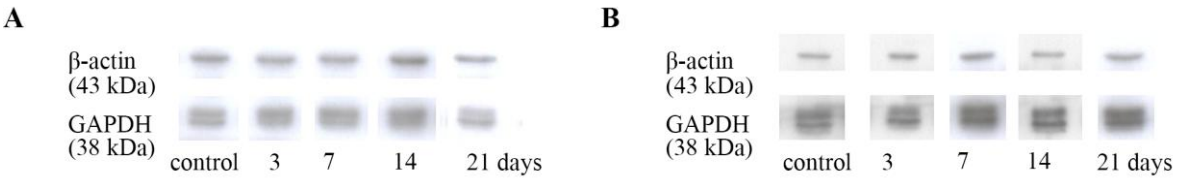

*Fig. 15.: Representative blots of β – actin in the hippocampus **(A)** and frontal cortex **(B)**. Densitometric analysis of the bands was obtained via Western blotting. The β – actin signal of homogenates from hippocampus and frontal cortex were resolved at approximately 43 kDa.*

4.2.3. Effects of RS on cofilin transcription and translation levels

Fig. 16 shows the expression and the level of cofilin protein after RS in the female rat hippocampus (A) and frontal cortex (B). RS caused a significant elevation in the cofilin mRNA expression by day 3 ($p = 0.005$) and 7 ($p = 0.026$) in the frontal cortex only (Fig. 16A).

RS did not cause any significant change in the hippocampal levels of cofilin (Fig. 16B), but there were significant changes in the frontal cortex cofilin protein levels on day 7 ($p = 0.002$) (Fig. 16B, 17B).

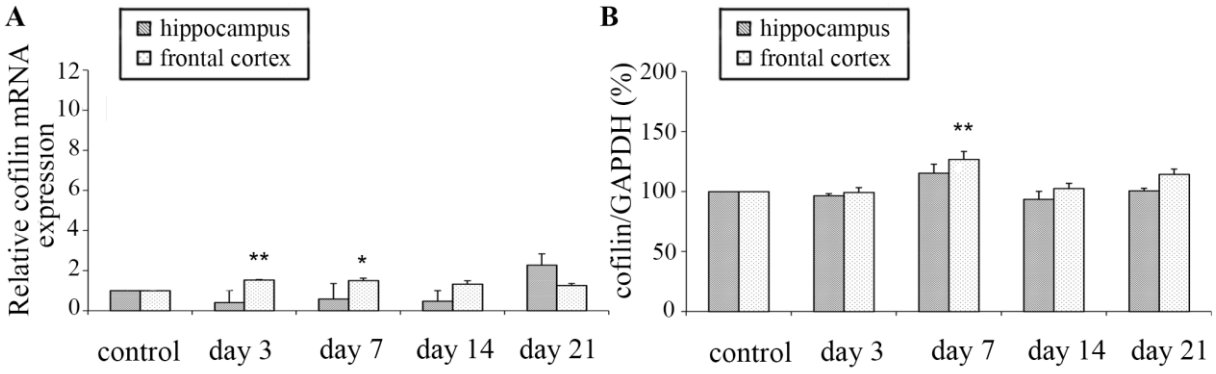

*Fig. 16.: Effects of restraint stress (RS) on the expression **(A)** and the protein levels **(B)** of cofilin in the rat hippocampus and frontal cortex. GAPDH was used as reference gene. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 4 – 6. *p < 0.05 and **p < 0.01: significant differences as compared to the control.*

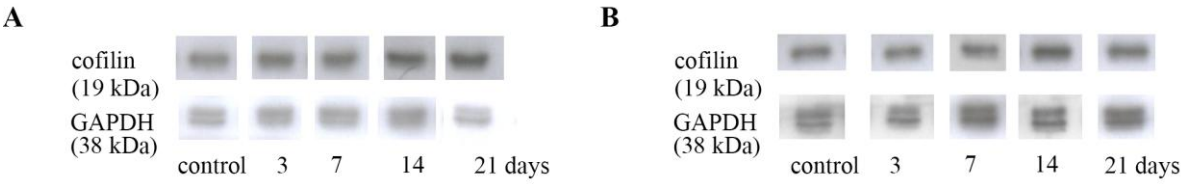

*Fig. 17.: Representative blots of cofilin in the hippocampus **(A)** and frontal cortex **(B)**. Densitometric analysis of the bands was obtained via Western blotting. The cofilin signal of homogenates from the hippocampus and frontal cortex were resolved at approximately 19 kDa.*

5. DISCUSSION

The main goal of our work was to study the biochemical relationships between stress, depression and AD. Therefore, we investigated the expression of those genes and translation of those proteins which are involved in the pathogenesis of AD and are related to neurodegeneration. The focus of our interest was the main cytoskeletal component β – actin and its regulatory protein cofilin. Furthermore we examined other genes, such as MAPK – 1 and APP which play an important role in the development of AD. To evaluate the molecular background of the neuropathological processes characteristic for AD, the effects of four different experimental stressors were compared in two different brain regions.

5.1. Stress – induced cytoskeletal changes

One of interesting findings of our results was that the quantitative pattern of cytoskeletal stress response in the rat brain is unique to the stress model used to trigger it. The various stress models employed in these experiments affected the mRNA expression and protein levels of β – actin differently; of the various physical and psychosocial stressors, RS induced the most pronounced changes in the investigated cytoskeletal markers. Both the transcription and protein translation of the main cytoskeletal component β – actin underwent biphasic dynamic changes in response to RS. Early in the stress period, the level of β – actin mRNA increased; then, following a transient reduction, its expression increased again. This typical stress response could be observed only in the hippocampus implying the sensitivity of this brain region to the stress.

EFSS also caused biphasic β – actin mRNA changes, but there was some difference between the patterns of the stress response after RS and EFSS. EFSS – induced elevation in the β – actin mRNA was longer – lasting than the elevation caused by RS. However, these slower kinetic properties were not reflected on a protein level. A possible explanation for these kinetic characteristics may be that short periods of stress serve an adaptive function, while longer durations may result in more profound changes through the depletion of compensatory mechanisms.

FSS caused only a transient elevation of the β – actin mRNA expression in the initial stages of the experiment in the hippocampus and frontal cortex, which did not induce any

increase in protein levels. The fact that chronic FSS does not exert a substantial impact on the cytoskeletal markers can be explained by the development of resistance to chronic stress exposure. Our results confirm earlier data concerning the adaptation to chronic swimming – induced stress (Armario et al., 1995).

5.2. Stress – induced changes in regulatory proteins of the cytoskeleton

The very important actin – binding protein, cofilin expression or levels in an experimental stress model were not studied before. We found that the cofilin mRNA expression followed a U – shaped curve during the 21 days of RS. A similar pattern in the cofilin mRNA levels and the elevation in cofilin protein levels was not demonstrated after EFSS. Our observation suggests that the changes induced in β – actin transcription and translation by RS and EFSS may be differently regulated. RS may modify the actin dynamics (actin filament assembly/disassembly) and stabilization through regulation of the actin – depolymerizing factor/cofilin family, whereas EFSS is not likely to have the same effect. Further investigations are necessary to clarify the roles of other regulator proteins, such as different kinases or drebrin in stress – induced cytoskeletal changes.

The other regulatory protein of actin filament dynamics is MAPK – 1 (Jafari et al., 2012) which is also responsible for the hyperphosphorylation of the tau protein. Tau protein is a highly – soluble microtubule – associated protein which plays a role in axonal transport. RS significantly altered the mRNA expression of hippocampal MAPK – 1, but this effect was not manifested on a translational level according to our Western blot analysis during the 21 days of RS. In a previous study, the amount of soluble phosphorylated tau in the rat brain was increased at a protein level after 14 – days of RS (Yan et al., 2010). In contrast to RS, the other stress types did not alter either MAPK – 1 mRNA expression or protein levels. These results suggest that RS leads to hyperphosphorylation of the tau protein via an increase in MAPK – 1, whereas EFSS, FSS and PSS do not cause similar alterations in the gene expression. These data suggest that the changes induced in MAPK – 1 transcription by RS, EFSS, FSS and PSS may be differently regulated, but further experiments are necessary to elucidate the precise mechanism of filamentous tau lesion induction by stress.

5.3. Different responses to physical and psychosocial stress

Besides RS and EFSS, FSS and PSS are other commonly used stress types in animal models of depression or work – related stress (Porsolt et al., 1978, Gerges et al., 2001). FSS caused only a transient elevation of the β – actin mRNA expression in the initial stages of the experiment in the hippocampal and the frontal cortex which did not induce any increase in protein levels.

Although PSS has previously been proven to provoke changes in the plasma corticosterone levels and in blood pressure (Gerges et al., 2001, Alberini, 2009), our observations also indicate that neither acute nor chronic PSS caused any significant alterations in the investigated markers. The question arises whether this experimental set – up really did give rise to PSS. Since the body, adrenal gland and thymus weight changes are important indicators of stress, we examined how these parameters varied in response to the different stressors. In fact, the BW gain and the typical stress – related changes of adrenal gland weight or thymus weight did not occur in the case of FSS and PSS. Similar data have been reported earlier (Gerges et al., 2001, Karandrea et al., 2002). For instance, BW gain was generally reduced and the absolute adrenal weight increased following exposure to long – term FSS, but these changes were not significant (Karandrea et al., 2002). Additionally, the BW of psychosocially stressed rats increased in another experiment (Gerges et al., 2001). These earlier reports and our own investigation lead us to conclude that PSS may act as a stressor, but it does not exert strong effects on cytoskeletal markers. Moreover the cytoskeletal changes are less sensitive to FSS or PSS, contrary to the biphasic effects induced by EFSS and RS.

5.4. APP mRNA and protein changes related to AD pathomechanism

Aβ plaque deposition and alterations in APP mRNA expression and metabolism by stress were investigated earlier (Rosa et al., 2005, Catania et al., 2009, Lee et al., 2009, Devi et al., 2010, Ray et al., 2011). Previous studies demonstrate that acute (6 h, single) or chronic (7 or 21 days, repeated) RS significantly increased the APP695 mRNA expression in the rat basolateral amygdaloid nuclei, while no APP isoform changed in the hippocampus after any stress condition (Rosa et al., 2005). In contrast, we observed an increased level of APP mRNA after

chronic RS in the hippocampus. In the cases of EFSS, FSS and PSS we did not see any alteration in APP on a transcriptional level. In agreement with our findings in the case of RS, Catania et al. (2009) demonstrated that chronic, unpredictable (28 days) stress provoked significant increases in the levels of the C99 fragment of APP in the hippocampal and cortical regions. In addition, stress gave rise to the misprocessing of APP, subsequently increasing the levels of C99 and BACE (Catania et al., 2009). Our results confirm these data and the earlier suggestion that stress plays a role in the development of AD via modification of the APP metabolism. These results suggest a stress type – dependent alteration in APP metabolism.

5.5. Stress – induced variabilites in sensitivity of different brain regions

The most studied two brain regions in context with stress and cognition are the frontal cortex and the hippocampus. In addition, these two regions show early and progressive signs of neuropathology (Sotiropoulos et al., 2008). The changes in the examined genes and proteins showed that the effects of acute or chronic RS or EFSS are region – specific. A recent morphological study on cultured hippocampal slices demonstrated that glucocorticoid engaged the cofilin pathway involved in regulating actin polymerization (Jafari et al., 2012). Aside from filamentous actin being the major cytoskeletal component of dendritic spines, the local actin dynamics determine the changes in spine shape, numbers and size (Kojima and Shirao, 2007). Previous reports demonstrated that chronic stress induces dendritic atrophy of the hippocampal pyramidal neurons and reduces the number of hippocampal neurons (Fuchs and Flugge, 1998, Dagyte et al., 2009). Our findings are in agreement with these results and confirm that the hippocampus is one of the most stress – sensitive regions in the brain.

5.6. Sex differences on the stress responses of the main cytoskeletal component, β – actin, and its regulatory protein, cofilin

Stressful life events are one of environmental factors which cause different physiological and behavioral responses to stress. These effects are more highly correlated with symptoms of depression in women than men (Faraday, 2002, Bangasser and Valentino, 2012). In agreement with previous studies, our experiment also proved that stress induces

reduction in BW and thymus weight in male as well as female rats. Surprisingly, the reduction of these parameters were less obvious in female rats.

There was also a gender difference in the stress – induced cytoskeletal responses. In male rats, elevation of both transcription and protein translation of the main cytoskeletal component, β – actin, underwent biphasic dynamic changes following RS. In contrast to this, RS – induced change in β – actin transcription was observed after only 21 days of RS in female rats. However, this alteration was not reflected in the protein levels. In our experiment, in addition to the gender differences in β – actin transcription and protein translation, the cofilin mRNA and protein levels were differently altered in female and male rats. The difference appeared in the time course and the region. In accordance with the results of our *in vivo* study, other papers also proved that in the difference between genders of the post – stress effect, males were more affected than females. Nevertheless, it is still unknown why the presence of depression and AD are higher in women than in men (Faraday, 2002). These finding indicate that it is important to consider the sex of animals when examining and interpreting molecular responses to stress.

6. SUMMARY

Experimental data and clinical studies support that chronic and transient stress types contribute to the development of AD. Many studies describing 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. Our data 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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9. APPENDIX

PUBLICATIONS I.

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ORIGINAL PAPER

Restraint Stress in Rats Alters Gene Transcription and Protein Translation in the Hippocampus

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Abstract Stress is a relatively new and emerging risk factor for Alzheimer's disease (AD). Severe stress can alter brain characteristics such as neuronal plasticity, due to changes in the metabolism of cytoskeletal proteins. In this study, male Wistar rats were exposed to restraint stress (RS) for 5 h daily for different time periods. At the end of the exposure periods, the amounts of β-actin, cofilin, amyloid precursor protein (APP) and mitogen-activated protein kinase 1 (MAPK-1) RNAs and proteins were investigated. The mRNA expressions of β-actin, cofilin and MAPK-1 followed U-shaped time course. Acute (3 days) and chronic (21 days) RS caused a fourfold and tenfold increases, respectively, in hippocampal β-actin mRNA expression. In the case of cofilin mRNA expression, elevations were detected in the hippocampus on days 3, 7 and 21. The APP mRNA level was increased on day 21. On protein level, chronic stress elevated the levels of β-actin, cofilin and APP in the hippocampus. These results suggest that stress causes the induction of some genes and proteins that are also elevated in AD selectively in the hippocampal region of the rat brain.

Keywords Stress · Alzheimer's disease · β-Actin · Cofilin · Amyloid precursor protein · Mitogen-activated protein kinase 1

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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 neuropsychiatric disorders, such as mood and anxiety disorders, schizophrenia and neurodegenerative diseases, e.g. Alzheimer's disease (AD) [1]. The relationships between stress, depression and AD have been examined both under experimental conditions and in epidemiological investigations in humans. In in vitro and in vivo experiments, stress reduced the spine density within hours in vulnerable dendritic domains of the hippocampal pyramidal cells [2]. 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 [3]. An association between stress and the biological markers of AD neuropathogenesis has been demonstrated in amyloid precursor protein (APP) transgenic (Tg2576) mice [4, 5]. Repeated restraints increased amyloid β (Aβ) and plaque formation via the generation of metabolic oxidative stress and matrix metalloproteinase-2 (MMP-2) down-regulation [5]. These results were confirmed by others using another transgenic (5XFAD) mouse model of AD [6]. They also found an acceleration of β-amyloidosis in response to adverse stress, which was accounted for by transcriptional and translational up-regulation of BACE1 [6]. The regulatory role of BACE in stress-induced β-amyloidosis was also proved in an Aβ rat model of AD [7]. It was reported earlier that chronic stress-induced molecular alterations may accelerate the impairment of cognition and synaptic plasticity in this "at-risk" rat model of AD [8].

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 [9]. Moreover, 25–40% of AD patients suffer from

co-morbid depression, possibly making it a significant risk factor for AD [10], while the blood of AD patients displays increased levels of corticosteroids, similar by as in patients suffering from depression [11]. However, certain neuropathological findings concerning the relationship between depression and AD are controversial. Hippocampal atrophy is seen in practically all AD brains, whereas the brains of depressive patients do not exhibit AD neuropathology [12].

The major cytoskeletal component of the dendritic spines is filamentous actin [13]. Its dynamics appears to involve signalling through numerous proteins that control the organization of the actin cytoskeleton [13]. The most important regulators of actin dynamics are members of the actin-depolymerizing factor ADF/cofilin family [14]. Exposure to neurodegenerative stimuli causes hippocampal neurons to reorganize their actin cytoskeleton into rod-like inclusions. These rods can occlude the neurites, by disrupting microtubules, causing neuritic atrophy. They may also participate in amyloid production [15]. The rods have been suggested to be a precursor of protein inclusions, such as Hirano bodies, which are found in the hippocampus of AD brains [16].

One of the central mechanisms hypothesized to contribute to AD is the Aβ cascade. Aβ protein is cleaved from the APP by the secretase proteins [17–19]. More recent studies have demonstrated an alternative representation of the Aβ cascade hypothesis, according to which the fibrillar amyloid is not the only toxic form of Aβ. There are non-fibrillar, soluble Aβ aggregates, which have been called Aβ-derived diffusible ligands (ADDLs), and these aggregates cause neuronal cell death or plaque deposition [20].

Other abnormal accumulations in the brains of AD patients are abundant filamentous tau lesions. In a physiological case, tau protein binds to axonal microtubules and regulates their assembly and transport. In AD, tau is hyperphosphorylated by specific kinases such as mitogen-activated protein kinase 1 (MAPK-1) [21]. Hyperphosphorylated tau is deleterious to neurons, leading to microtubule degeneration and cell death [22].

Since stress may play a key role in the development of AD, we set out to investigate the effects of acute and chronic restraint stress (RS) on the transcription and translation of these genes and proteins in the rat hippocampus and cortex. We additionally examined whether the transcriptional or translational changes induced by stress involve a time-dependent mechanism.

Materials and Methods

Animals

Adult male Wistar rats (n = 44; 200–250 g) were used in the experiments. The animals were housed in a temperature (22 ± 1°C) and humidity (55 ± 5%)-controlled room on a 12-h light–dark cycle (lights on from 08:30 to 20:30) and allowed free access to tap water and rat chow. All experiments on laboratory animals were performed in accordance with the protocol approved by the University Ethics Committee.

Experimental Stressor

We applied RS (an established animal model of stress) by placing the rats into plastic tubes 10 cm in diameter and 25–30 cm long for 5 h daily [23]. The animals were divided into five experimental groups. Group 1 (n = 10) comprised the controls; group 2 (n = 8) underwent RS for 3 days (acute stress); group 3 (n = 8) underwent RS for 7 days; group 4 (n = 8) underwent RS for 14 days; and group 5 (n = 10) underwent RS for 21 days (chronic stress).

Total RNA Isolation and Reverse Transcription

Total cellular RNA was extracted from the frontal cortex and hippocampus by means of the NucleoSpin RNA II Total RNA isolation kit (Macherey–Nagel, Düren, Germany), according to the manufacturer's instructions. 0.3 μg of RNase inhibitor 40 U/μl (Fermentas, Glen Burnie, ML, USA) was added and the eluted RNA was stored at −80°C until use.

Reverse transcription (RT) 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). Two ng of total mRNA was transcribed into cDNA. Each reaction tube contained 2 ng of total RNA in a volume of 15 μl, plus 15 μl of transcription mix [3 μl of reverse transcription buffer, 1.2 μl (100 mM) of dNTP mix, 3 μl of random primers, 1.5 μl of Multi ScribeTM reverse transcriptase, 0.75 μl (20 U) of RNase inhibitor and RNA Free Water (Ambion, Ambion, Austin, TX, USA)]. The total volume was 30 μl. The thermal cycling consisted of three cycles: the first at 25°C for 10 min, the second at 37°C for 120 min, and the final one at 85°C for 5 s. The samples were then cooled down to 4°C, and finally stored at −20°C until Q-PCR.

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. Primer sequences are shown in Table 1.

30 μl of cDNA solution was diluted with 510 μl of DNase and RNase-free water. Q-PCR was carried out with a final volume of 20 μl, containing 10 μl of SYBR Green MasterMix (Roche, Basel, Switzerland), 0.5 μl of forward primer, 0.5 μl of reverse primer, and 9 μl of template cDNAs. The protocol

Table 1 List of primers

	Forward	Reverse
β-Actin	CCC GCG GAG TAC AAC CTT CT	CGT CAT CCA TGG CGA ACT
Cofilin	GGC GGC TCT GTT CTT CTG T	CTC CAT CAG AGA CAG CCA CA
APP	CCC CAA GAT CCG GTT AAA CT	TAC TTG TCG ACT GCG TCA GG
MAPK-1	CCA AGC TCA ACC GTC TCA TC	GGC TGG TAG GGT AGT TGA TG
GAPDH	AGA TCC ACA ACG GAT ACA TT	TCC CTC AAG ATT GTC AGC AA

followed: denaturation (25 s at 95°C), annealing (25 s at 60°C), and extension (15 s at 72°C). The relative gene expression was normalized to glyceraldehyde-3-phosphate-dehydrogenase. The results were analysed by the $2^{-\Delta\Delta CT}$ method [24].

Western Blotting

The brain tissues were homogenized in 50 mM Tris buffer (pH 7.5), 150 mM NaCl, 0.1% Nonidet-P-40, 0.1% cholic acid, 2 μg/ml leupeptin, 2 mM PMSF, 1 μg/ml pepstatin and 2 mM EDTA. The homogenates were centrifuged at 10,000×*g* for 15 min at 4°C. The supernatants were used for protein assays. Proteins were measured with bicinchoninic acid [25].

After denaturation, 20 μg of protein was separated on 12% SDS–polyacrylamide gel and electroblotted onto nitrocellulose membranes. The samples were blocked in 0.1 M Tris-buffer saline containing 0.02% Tween 20 (TBST) supplemented with 5% non-fat milk solution for 1 h. The membranes were then incubated overnight with mouse monoclonal anti-β-actin (Santa Cruz, CA, USA, 1:2,000), rabbit polyclonal cofilin (D59) antibody (Cell Signaling Technology, MA, USA, 1:1,000), mouse monoclonal ERK 1/2 (MK1) antibody (Santa Cruz Biotechnology CA, USA, 1:200), mouse monoclonal anti-GAPDH (Millipore, MA, USA, 1:4,000) and mouse anti-APP (Millipore, MA, USA, 1:33.3). Next day, after five washes with TBST, HRPO-sheep anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA, 1:1,000) and HRPO-goat anti rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA, 1:500) were applied for 1 h. The nitrocellulose membranes were subsequently washed five times with TBST, and then incubated with the Supersignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and exposed to Kodak autography film. The optical densities of the immunoreactive bands were quantified by mean of Scion Image Software. The amounts of examined proteins were calculated by comparison with the optical density of GAPDH.

Statistical Analysis

All data were analysed by one-way ANOVA with SPSS 15.0 Software, followed by the Bonferroni and Dunnett post hoc tests; the level of significance of comparisons was taken as $P < 0.05$. All data are reported as means ± SEM.

Results

In order to demonstrate that RS is a representative stressor, the overall body weight was measured repeatedly during the course of the experiment. The stressed animals gained body weight at a significantly slower rate than that for the control group at all examined time points (Fig. 1a). The weights of the adrenal glands in the stressed animals were higher at all examined time points, but the differences were significant only on days 14 and 21 (Fig. 1b). The thymuses were weighed at different time points when the animals were sacrificed, but a significant decrease in the weight of the thymus in the stressed animals was found only on day 21 (Fig. 1c). The same tendency was reported in previous studies [26, 27].

Our results indicate that the mRNA expression of β-actin followed a U-shaped time course. The acute RS resulted in a significant increase in the hippocampal β-actin mRNA expression by day 3. The β-actin mRNA expression was decreased by days 7 and 14 relative to day 3, and significantly increased by day 21 (Fig. 2a).

The cofilin mRNA expression induced by RS in the hippocampus also followed a U-shaped time course. The elevations were fivefold, fourfold and tenfold by days 3, 7 and 21, respectively (Fig. 3a).

The hippocampal APP mRNA level was increased significantly only on day 21, when it was six times the control value (Fig. 4a).

Similarly to the β-actin and cofilin mRNA expressions, both acute and chronic RS caused significant elevations in MAPK-1 mRNA expression in the hippocampus: 2 and 2.7 times the control value on days 3 and 21, respectively. As in the cases of β-actin and cofilin, the MAPK-1 data described a U-shaped time course (Fig. 5a).

RS did not cause any significant change in the cortical expressions of β-actin, cofilin, APP or MAPK-1 mRNA (Figs. 2a, 3a, 4a, 5a).

Western blotting experiments revealed that exposure to RS significantly elevated the amount of hippocampal β-actin protein by days 3 and 21 (Fig. 2c, d). Similarly to the changes

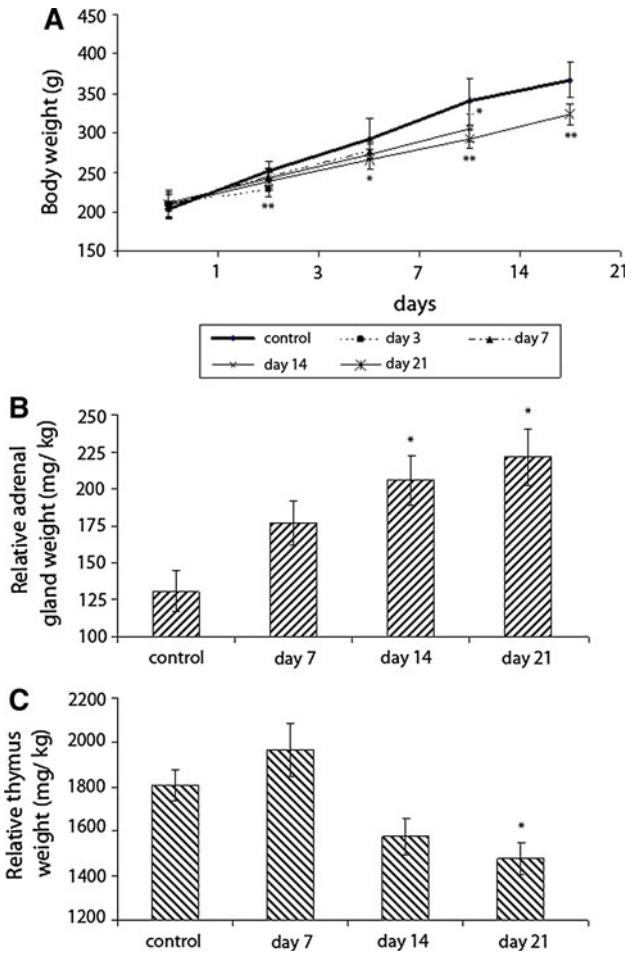

Fig. 1 Effects of restraint stress on the overall body weight (**a**), and on the weights of the adrenal glands (**b**) and the thymus (**c**) of rats. Control (n = 10), day 3 (n = 8), day 7 (n = 8), day 14 (n = 8), day 21 (n = 10). Values are group means ± SEM. $*P < 0.05$ and $**P < 0.01$: significant differences as compared with the control

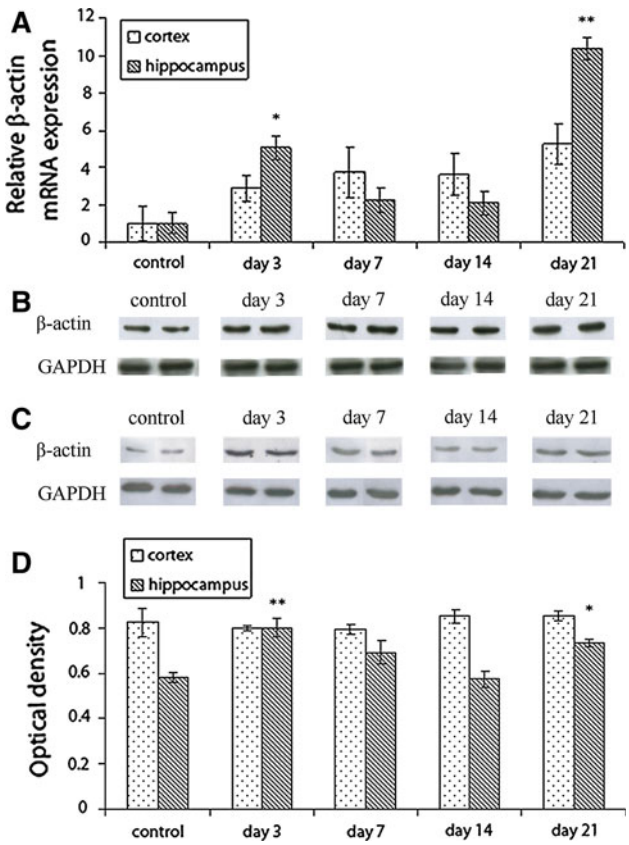

Fig. 2 Effects of restraint stress on the expression of β-actin mRNA in the rat cortex and hippocampus (**a**). Effects of restraint stress on the level of β-actin protein: control (n = 10), day 3 (n = 8), day 7 (n = 8), day 14 (n = 8), day 21 (n = 10). *Representative blots* of the cortical (**b**) and hippocampal (**c**) samples. Semiquantitative evaluation of Western blots (**d**). Values for each individual sample were normalized with the geometric mean of the reference gene GAPDH measurements. Data are group means ± SEM. $*P < 0.05$ and $**P < 0.01$: significant differences as compared with the control

in β-actin mRNA expression, the changes in β-actin protein followed a U-shaped kinetic curve. The elevation in the amount of cofilin protein was significant only on day 21 only (Fig. 3c, d). There were significant changes in the APP protein levels on days 3, 7 and 21 (Fig. 4c, d), but no change in the amount of MAPK-1 protein (Fig. 5c, d).

RS did not cause any significant change in the cortical levels of β-actin, cofilin, APP or MAPK-1 proteins (Figs. 2b, 3b, 4b, 5b, 2d, 3d, 4d, 5d).

Discussion

The main finding of the present study was that RS selectively induced biphasic dynamic changes in β-actin mRNA expression in the rat hippocampus during the examined 3-week period. Early in the stress period, the level of β-actin mRNA increased; then, following a transient reduction, its expression increased again. Another interesting result of this study was that the cofilin mRNA expression followed U-shaped curve during the 21 days of RS. Similar time-dependent change in plasma corticosterone levels has been reported during a 3-week stress period [28]. The Western blotting analysis results suggest that, similarly as for the transcription of the β-actin gene, a biphasic change induced by stress was be detected in the protein level. These results accord nicely with previous morphological findings that chronic stress leading to stress-evoked dendritic spine loss may be a rapid and dynamic process in the hippocampus [2, 29].

There are no studies on the very important actin-binding protein cofilin expressions or levels in an experimental stress model. We found that the level of cofilin was increased only by chronic stress (21 days). Earlier studies demonstrated that the concentration of cofilin relative to actin and actin-binding proteins plays an important role in the function of cofilin in actin assembly or disassembly [30,

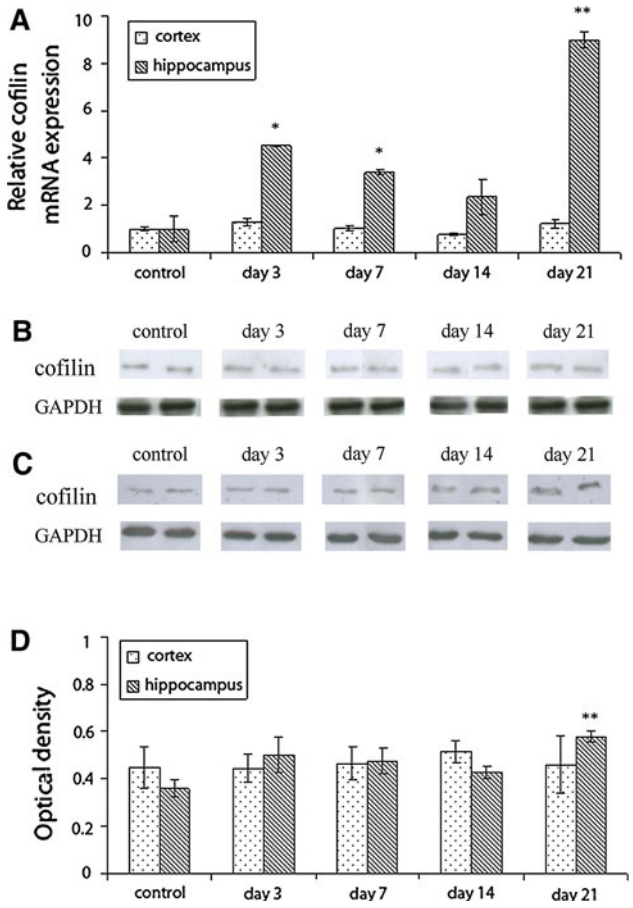

Fig. 3 Effects of restraint stress on the expression of cofilin mRNA in the rat cortex and hippocampus (**a**). Effects of restraint stress on the level of cofilin protein: control (n = 10), day 3 (n = 8), day 7 (n = 8), day 14 (n = 8), day 21 (n = 10). Representative blots of the cortical (**b**) and hippocampal (**c**) samples. Semiquantitative evaluation of Western blots (**d**). Values for each individual sample were normalized with the geometric mean of the reference gene GAPDH measurements. Data are group means ± SEM. $*P < 0.05$ and $**P < 0.01$: significant differences as compared with the control

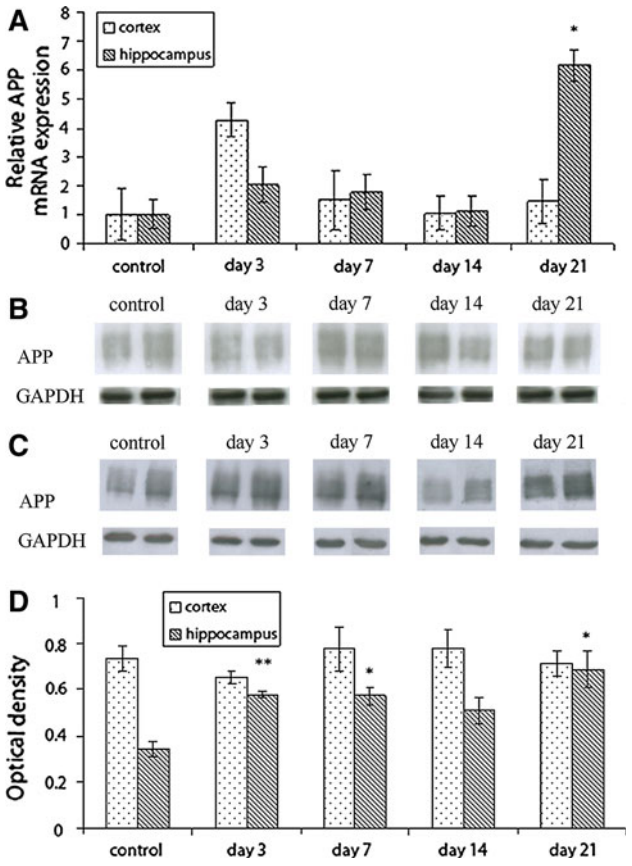

Fig. 4 Effects of restraint stress on the expression of APP mRNA in the rat cortex hippocampus (**a**). Effects of restraint stress on the level of APP protein: control (n = 10), day 3 (n = 8), day 7 (n = 8), day 14 (n = 8), day 21 (n = 10). *Representative blots* of the cortical (**b**) and hippocampal (**c**) samples. Semiquantitative evaluation of Western blots (**d**). Values for each individual sample were normalized with the geometric mean of the reference gene GAPDH measurements. Data are group means ± SEM. $*P < 0.05$ and $**P < 0.01$: significant differences as compared with the control

31]. ADF/cofilin increases the rate of actin dissociation, which is the rate-limiting step of steady-state F-actin treadmilling [13, 32].

If the ADP-actin levels are elevated, the cofilin-saturated F-actin assembles into rod-shaped bundles [33]. The assembly of the cofilin- ADP-actin complex into rods is readily reversible. However, in AD, the rods form aggregates which block transport within the neurites, mainly in the hippocampus and cortex [14]. These earlier reports and our own investigation lead us to conclude that stress-evoked modifications of β-actin and cofilin may play a role in the stress-induced hippocampal cytoskeletal changes.

Stress-provoked Aβ plaque deposition and alterations in APP mRNA expression and metabolism were investigated earlier [5, 6, 34–36]. It was found that the APP695 mRNA expression was significantly increased in the rat basolateral amygdaloid nuclei following acute (6 h, single) or chronic (7 or 21 days, repeated) RS, while no APP isoform was changed in the hippocampus after any stress condition [34]. In contrast, we observed an increased level of APP mRNA after chronic RS in the hippocampus. In agreement with our finding, Catania et al. [35] demonstrated that chronic, unpredictable (28 days) stress gave rise to significant increases in the level of the C99 fragment of APP in the hippocampal and cortical regions. Moreover, stress provoked the misprocessing of APP, subsequently increasing the levels of C99 and BACE [35]. Our results confirm these data and the earlier suggestion that stress plays a role in the development of AD via modification of the APP metabolism.

The tau protein is a highly-soluble microtubule-associated protein, which plays a role in axonal transport. We demonstrated that RS alters the mRNA expression of hippocampal MAPK-1, which is responsible for hyperphosphorylation of

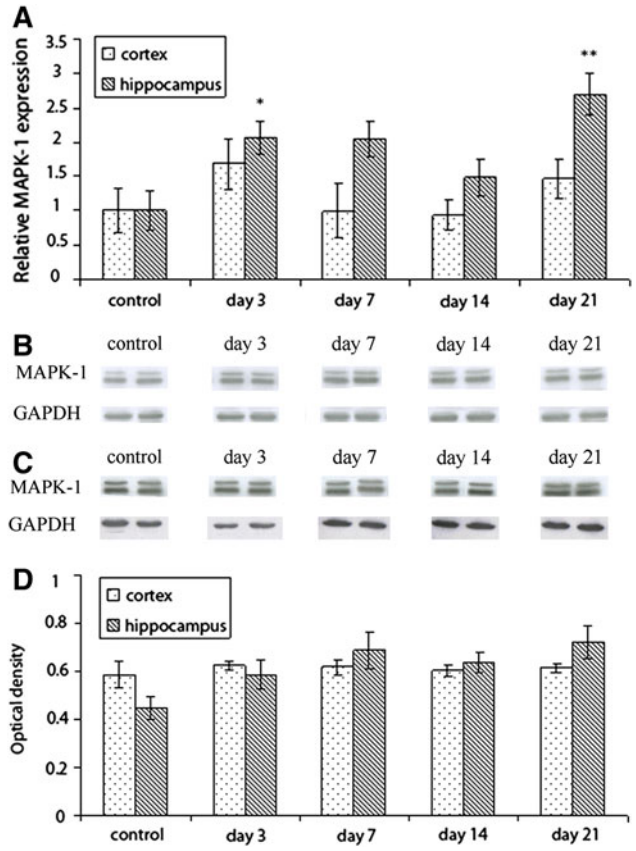

Fig. 5 Effects of restraint stress on the expression of MAPK-1 mRNA in the rat cortex and hippocampus (**a**). Effects of restraint stress on the level of MAPK-1 (ERK 1/2) protein: control (n = 10), day 3 (n = 8), day 7 (n = 8), day 14 (n = 8), day 21 (n = 10). Representative blots of the cortical (**b**) and hippocampal (**c**) samples. Semiquantitative evaluation of Western blots (**d**). Values for each individual sample were normalized with the geometric mean of the reference gene GAPDH measurements. Data are group means ± SEM. $*P < 0.05$ and $**P < 0.01$: significant differences as compared with the control

the tau protein, but this effect was not manifested in translational level according to our Western blot analysis during the 21 days of RS. In a previous study, the amount of soluble phosphorylated tau in the rat brain was increased at a protein level after a 14-day RS [37]. These results suggest that RS leads to hyperphosphorylation of the tau protein via an increase in MAPK-1. Further experiments are necessary to elucidate the precise mechanism of filamentous tau lesion induction by stress.

The changes of the investigated mRNA and protein levels were not parallel in all cases. β-Actin, cofilin and MAPK-1 mRNA expression increase more than they protein levels, APP mRNA expression increased less than its protein levels. This virtual discrepancy can be explained by post-transcriptional and translational regulation factors and processes involving RNA secondary structure, codon bias, ribosome occupancy and translation efficiency or protein half-life [38]. Similar to our findings, chronic RS induced different changes in mRNA and protein levels of vesicle-associated membrane protein, an important contributor of synaptic transmission [39].

Our results indicated that the changes induced in the examined genes and proteins by RS were region-specific. Since there was no significant elevation of the parameters in the rat cortex, the results suggest that the hippocampus may be the most stress-sensitive formation. Our findings are in agreement with earlier data pointing to the selectivity of hippocampal neuronal loss in rats repeatedly injected with glucocorticoids [40, 41].

Our results have methodological consequences. Since the β-actin mRNA and protein levels in the brain change in consequence of RS, we conclude that β-actin in the central nervous system can not be used as an internal standard in QRT-PCR experiments or in Western blotting. The present study confirms previous results and draws attention to need for care in the selection of housekeeping genes and proteins in the neuronal system [42].

In conclusion, we have demonstrated that RS induces biphasic dynamic changes in β-actin, cofilin and MAPK-1 transcription and protein translation selectively in the rat hippocampal region. The effects caused by stress on these genes and proteins may contribute to changes in cell survival, synaptic plasticity, learning and neurogenesis, due to modifications in the cytoskeleton. These alterations may be of relevance as concerns the pathophysiology of stress-related neuropsychiatric conditions such as anxiety and mood disorders, schizophrenia and AD, paving the way for the identification of new biomarkers and novel therapeutic strategies for these devastating disorders.

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Conflict of interest The authors declare that there are no conflicts of interest.

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PUBLICATIONS II.

AZ AKUT ÉS KRÓNIKUS STRESSZ HATÁSA AZ ALZHEIMER-KÓR PATOMECHANIZMUSÁBAN SZEREPET JÁTSZÓ GÉNEK TRANSZKRIPCIÓJÁRA

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ACUTE AND CHRONIC STRESS INDUCED CHANGES IN GENE TRANSCRIPTIONS RELATED TO ALZHEIMER'S DISEASE
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Kapcsolódó cikk online

Az akut és a krónikus stresszhatások jelentős mértékben hozzájárulnak a depresszió és a neurodegeneratív betegségek, például az Alzheimer-kór (AK) kialakulásához. Kutatásunk célja annak vizsgálata volt, hogy a stressz hogyan befolyásolja az AK patomechanizmusában szerepet játszó, illetve a szinaptikus plaszticitásban, a neurodegenerációs folyamatokban részt vevő β-aktin, amyloid prekurzor protein (APP) és a mitogénaktivált proteinkináz-1 enzim (MAPK-1) transzkripcióját patkányok agyában.
Kísérletünk során hím Wistar-patkányokat rövid (három nap) és hosszú (1–3 hét) ideig tartó immobilizációs stressznek (IS) tettünk ki, majd mintát vettünk a hippocampusból és a cortexből, melyekben a β-aktin, APP és MAPK-1 mRNS mennyiségét real-time-PCR technika segítségével határoztuk meg.
Mind a β-aktin, mind az APP mRNS expresszió jellegzetes U alakú időkinetikai görbét mutatott. Az akut, illetve krónikus IS szignifikáns emelkedést eredményezett a β-aktin és a MAPK-1 mRNS expressziójában, valamint 21 nap után az APP mRNS mennyisége is megnőtt.
Eredményeink szerint az IS befolyásolja a cytoskeleton és a szinaptikus plaszticitás normális funkcióinak fenntartásában szerepet játszó β-aktin, APP és MAPK-1 gének transzkripcióját, mely változások hozzájárulhatnak a kognitív funkciók romlásához és az AK kialakulásához.

Kulcsszavak: *Alzheimer-kór, stressz, β-aktin, amyloid prekurzor protein, mitogénaktivált proteinkináz-1*

Preclinical and clinical studies demonstrate that stress may be implicated in the risk of neurodegenerative diseases such as Alzheimer's disease (AD). Our study aimed to investigate the effects of acute and chronic immobilization stress (IS) on the gene transcriptions of β-actin, amyloid precursor protein (APP) and mitogen activated protein kinase-1 (MAPK-1), proteins related to synaptic plasticity and neuronal degeneration.
Male Wistar rats were exposed to IS for five hours daily for 3 days (acute stress) or through 7-14-21 days (chronic stress). At the end of exposure periods, total RNA was purified from the cortex and hippocampus. The amounts of β-actin, APP and MAPK-1 mRNA were determined with real time PCR method.
Our results indicate that the mRNA expression of β-actin and APP followed a U-shaped time-response curve. Both acute and chronic IS caused a significant increase in β-actin and MAPK-1 mRNA expression. Significant APP mRNA elevation was observed only by the 3rd week after RS.
Our findings demonstrate that both acute and chronic IS lead to gene transcriptional changes of β-actin, APP and MAPK-1. These proteins maintain the normal function of the cytoskeleton and the synaptic plasticity. The above changes may lead to cognitive deterioration, and the development of AD.

Keywords: *Alzheimer's disease, stress, β-actin, amyloid precursor protein, mitogen activated protein kinase-1*

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A stressz a mindennapi élet része, életünk természetes velejárója. A *Selye János* által elsőként leírt stresszválasz, az egész szervezetben létrejövő válaszreakció szerteágazó volta miatt részleteiben máig feltérképezetlen. Köztudott, hogy nem minden stresszállapot kóros. A rövid, enyhe, kontrollálható hatások (eustressz) energetizálóak, erőt adnak ahhoz, hogy könnyebben vegyük a kihívásokat. Azonban az erős vagy a hosszú ideig tartó stressz (distressz) pszichiátriai kórállapotokhoz vezethet, melyek közül a leggyakoribb a depresszió. Az utóbbi évek vizsgálatai szerint a stressz nemcsak az affektív zavarok kockázati tényezője, hanem a neurodegeneratív betegségek, köztük az Alzheimer-kór (AK) kialakulásában is szerepet játszik[1]. Epidemiológiai vizsgálatokban bizonyították, hogy a különböző stresszhatások több mint kétszeresére növelik az AK kialakulásának kockázatát[2].

Az AK és a depresszió komorbiditása sem ritka. Az AK-betegek 25–40%-ában figyelhető meg depressziószindróma[3]. Ugyanakkor azt is kimutatták, hogy azok esetében, akik hajlamosak depresszióra, gyakrabban figyelhető meg kognitív hanyatlás és az AK is gyakoribb[2].

A stressz, a depresszió és az AK közötti összefüggést a neuropatológiai vizsgálatok eredményei is alátámasztják. *Post mortem* vizsgálatok szerint a depresszió fokozza az AK-ra jellemző neuropatológiai elváltozások, az amyloidplakkok (AP), a neurofibrillaris kötegek (NFT), a neuronalis degeneráció kialakulását[4]. Állatkísérletekben bizonyították, hogy a tanulási folyamatok romlásának hátterében a stresszhatást követően megjelenő morfológiai változások, mint például a dendrittüskék számának csökkenése, illetve a szinapszisok számának csökkenése áll, az agy hippocampalis és corticalis régióiban[5–7]. A dentrittüskék számának csökkenése az AK korai fázisában is megjelenik, mely szignifikánsan korrelál a mentális funkciók leépülésével[8].

A dendrittüskék fő cytoskeletalis komponense a β-aktin[9], melynek regulációs szerepet tulajdonítanak a szinaptikus plaszticitásban[10]. Neurodegeneratív folyamatok az aktin cytoskeleton átrendeződését eredményezhetik a hippocampusban, kofilin-aktin pálcikák keletkeznek és Hirano-testek (HB) képződnek[11]. Ezek kialakulása hozzájárul az aktinaggregátumok kialakulásához, valamint a neuronok következményes pusztulásához[12].

Az AK-betegek agyában megfigyelhető AP fő komponense a β-amyloid (Aβ) peptid, mely az amyloid prekurzor protein (APP) kóros hasítási terméke. Kóros körülmények között az APP-t a β- és γ-szekretázok hasítják, aminek eredménye a 40–42 aminosavból álló Aβ keletkezése[13, 14]. A keletkező Aβ fémionok (Cu^{2+}, Fe^{2+}, Zn^{2+}) megkötésére képes, és ezt követően β-lemez-konformációt vesz fel, amely aggregációra hajlamosít. Az aggregálódó oligomer depozitumok pedig a szinapszis funkcionális károsodásához és végső soron a sejtek degenerációjához vezetnek[15].

Az AK másik neuropatológiai sajátossága az intracellulárisan kialakuló NFT kialakulásában egy microtubulus-asszociált protein, a τ hiperfoszforilálódása alapvető fontosságú[16]. A τ-nak fiziológiásan szerepe van többek között a neuritnövekedésben és az axonalis transzportban. Kóros esetben ez a fehérje hiperfoszforilálódhat, melynek hatására microtubulusok dezorganizálódnak, a celluláris transzportfolyamatok sérülnek, ami szintén a neuronok pusztulását eredményezheti[17]. A τ foszforilálását többek között a mitogénaktivált proteinkináz-1 (MAPK-1) enzim végzi. A MAPK-1 azokat a funkciós csoportokat foszforilálja, amelyeken létrejön az AK-ra jellemző hiperfoszforiláció-mintázat[18].

A stressz, a depresszió és az AK közötti etiológiai kapcsolat további bizonyítása céljából a neuronalis degenerációval összefüggésbe hozható gének transzkripcióját vizsgáltuk patkányokon, immobilizációs stresszt (IS) követően. Célunk annak vizsgálata volt, hogy az IS miként változtatja meg a β-aktin, APP és MAPK-1 gének transzkripcióját patkányhippocampusban és -cortexben, illetve, hogy a stresszindukált változás mutat-e időfüggést.

Módszerek

VIZSGÁLATI CSOPORTOK

Kísérletünket hím, 180-200 grammos (45–48 napos) Wistar-patkányokon (n=55) végeztük. Az állatokat öt csoportba osztottuk. A kontrollállatok semmilyen kezelésben nem részesültek. A kezelt állatok között elkülönítettünk rövid (háromnapos, akut) és hosszú (egy-, két-, illetve háromhetes, krónikus) ideig stresszelt csoportokat. A kísérlet során standard IS-kezelést alkalmaztunk: napi öt órán át, 25-30 cm hosszú, 10 cm átmérőjű műanyag csövekben tartottuk az állatokat élelem és víz nélkül[19].

A kísérlethez a Szegedi Tudományegyetem Munkahelyi Állatkísérleti Bizottsága adott etikai engedélyt.

MINTAVÉTEL

Az állatokat intraperitonealisan adott 6%-os klorálhidráttal altattuk, majd hideg fiziológiás sóoldattal transcardialisan perfundáltuk. Mindkét hemisphaerium eltávolítása után mintát vettünk a frontális cortexből és a hippocampusból, amelyeket felhasználásig –80 °C-on tároltunk.

TOTÁLIS RNS-IZOLÁLÁS

A frontális cortexből és hippocampusból vett mintákból teljes sejt-RNS-t izoláltunk (Macherey-Nagel; NucleoSpin II protokoll alapján): 30 mg agyszövetet 350 µl RA1 pufferrel homogenizáltunk, majd 3,5 µl merkaptoetanollal lizáltuk a sejteket. A lizátumot centrifugálva szűrtük át az 1. szűrőn (1 min, 11 000 g), majd 350 µl 70%-os etanolt adtunk hozzá és ismét centrifugálva a 2. szűrőn is átszűrtük (30 sec, 11 000 g), ekkor kötöttük meg a szűrőn a nukleinsavakat. A szilikamembránt kisóztuk 350 µl membrán desalting buffer (MDB) segítségével. A kapott oldathoz 10 µl rDNázt, 90 µl rDNáz reakciópuffert és 95 µl rDNáz reakciómixet pipettáztunk, majd szobahőmérsékleten 15 percig inkubáltuk, így a dezoxiribonukleáz enzim a működését segítő puffer és a mix segítségével megemésztette a mintában lévő DNS-t. Ezt követően a membránt centrifugálás közben 200 µl RA2 (30 sec, 11 000 g), majd 600 µl RA3 (30 sec, 11 000 g), végül ismét 250 µl RA3 pufferrel (2 min, 11 000 g) lemostuk és kiszárítottuk. A teljes sejt-RNS-t tartalmazó oldatot 60 µl ribonukleázmentes vízzel oldottuk le a membránról (1 min, 11 000 g). Végül 0,5 µl ribonukleázgátlót adtunk hozzá és az oldatokat felhasználásig –80 °C-on tároltuk.

REAL-TIME POLIMERÁZ LÁNCREAKCIÓ

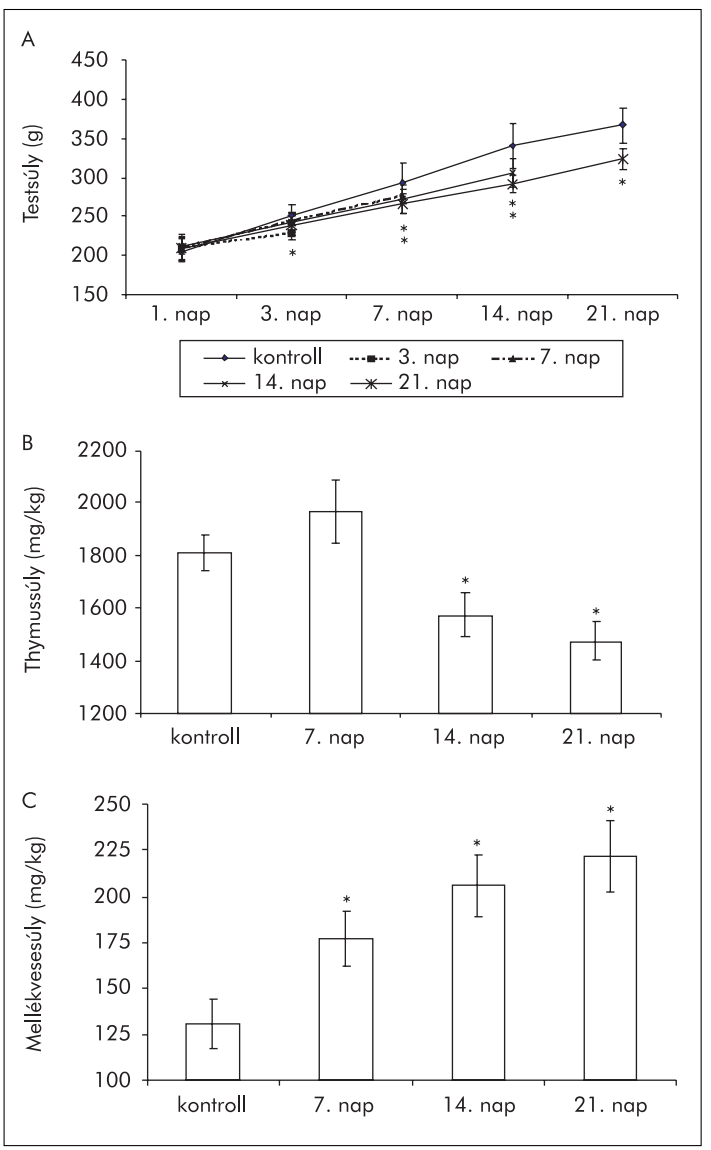
Az oldatok totál mRNS-koncentrációját spektrofotometriás módszerrel Nanodrop készülékkel mértük meg, majd 2 µg/15 µl koncentrációra hígítottuk az oldatunkat. 15 µl reverz transzkripciós reakcióelegyhez [Applied Biosystems: High Capacity cDNS Reverse Transcription Kit: reverz transzkriptáz (1,5 µl), dNTP monomerek (1,2 µl), random primer (3 µl), reverztranszkripciós puffer (3 µl), ribonukleázinhibitor (1,5 µl), ribonukleázmentes víz (4,8 µl)] adtuk a 2 µg mRNS/15 µl-es oldatot. A PCR-gépben a reverztranszkriptáz a totál sejt-mRNS-ről copy DNS-t (cDNS) szintetizált. A következő lépésben 10 µl SYBR Green Mixhez (Roche: Fast Start SYBR Green Master Mix) 1 µl szekvenciaspecifikus primert és 9 µl templát cDNS-oldatot adtunk. A cDNS-ek mennyiségi meghatározása Rotogene® (Corbett) készülékkel történt.

STATISZTIKA

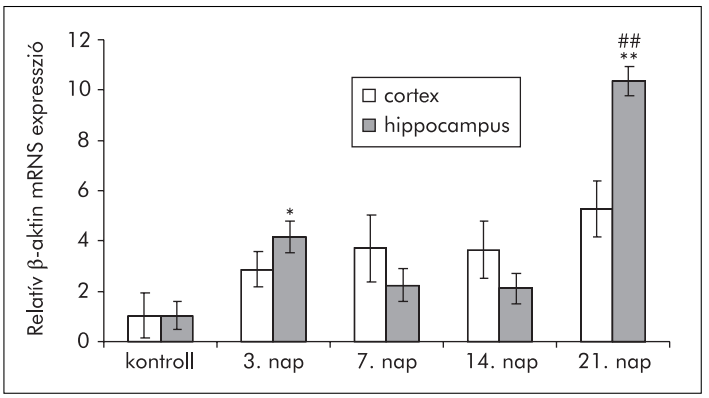
A vizsgált mRNS-expressziós szintek normalizálása glicerinaldehid-3-foszfát-dehidrogenáz (GAPDH) mRNS-meghatározással történt. Az eredmények értékelése során a $2^{-\Delta\Delta CT}$ módszert alkalmaztuk[20]. A statisztikai értékelés során egyszempontos varianciaanalízist, valamint Bonferroni- és Dunnett-féle *post hoc* tesztet végeztünk (SPSS 15.0 program). Az adatokat átlag ± SEM formában jelenítettük meg. A szignifikanciaszint $p<0,05$ volt.

Eredmények

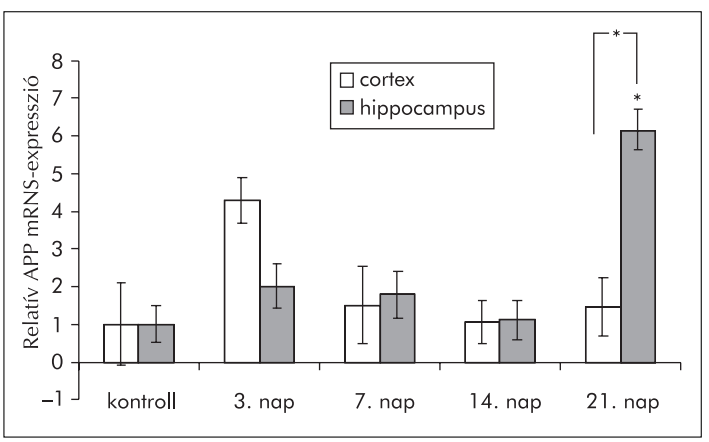
Eredményeink szerint mind az akut, mind a krónikus IS hatására az állatok testsúlya szignifikánsan csökkent **(1A ábra)**. Szignifikáns relatív thymussúly-csökkenést **(1B ábra)**, illetve relatív mellékvesesúly-emelkedést csak krónikus IS esetén tapasztaltunk **(1C ábra)**.



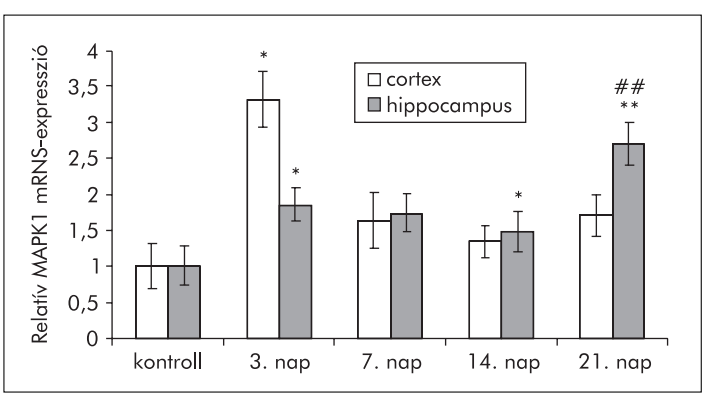
1. ábra. *A testsúly* **(A)**, *a thymus súlya (mg/kg)* **(B)**, *a mellékvese súlya (mg/kg)* **(C)** *változása immobilizációs stressz hatására* *$p<0,05$



2. ábra. *A β-aktin mRNS expressziója immobilizációs stressz hatására. Változás a kontrollcsoporthoz viszonyítva: *p<0,05, **p<0,01. Változás a 14 napos kezelt csoporthoz viszonyítva ##p<0,01*



3. ábra. *Az APP mRNS expressziója immobilizációs stressz hatására. Változás a kontrollcsoporthoz viszonyítva: *p<0,01*



4. ábra. *A MAPK1 mRNS expressziója immobilizációs stressz hatására. Változás a kontrollcsoporthoz viszonyítva: p*<0,05, **p<0,01. Változás a 14 napos kezelt csoporthoz viszonyítva ##p<0,01*

IS hatására a β-aktin és APP mRNS expressziója jellegzetes U alakú görbét mutatott a hippocampalis mintákban. A β-aktin esetében a hippocampalis neuronokban akut stressz (három nap) hatására szignifikáns ($p<0,05$) expressziónövekedés volt megfigyelhető. Krónikus IS hatására, hét, illetve 14 napos kezelést követően, az mRNS-szintek csökkenő tendenciát mutattak a kezdeti, háromnapos emelkedéshez képest, majd a 21. nap után a β-aktin mRNS expressziója ismét megemelkedett és elérte a kontrollérték közel tízszeresét **(2. ábra)**.

Hasonlóan a β-aktinhoz, az egymást követő napokon alkalmazott IS hatására az APP-mRNS-szintek kinetikája is U alakú dózis-idő függést mutatott. A 3. napon a stressz hatására a kontrollérték a kétszeresére emelkedett, a 7. és a 14. napon folyamatos csökkenést tapasztaltunk a kezdeti, emelkedett értékhez képest. A 21 napos stresszkezelés hatására viszont szignifikánsan emelkedett az APP mRNS expressziója (SPSS 15.0 program) **(3. ábra)**.

A hippocampalis MAPK1 mRNS-expresszió szintje mind akut, mind krónikus stressz hatására megemelkedett, s a növekedés a 3., 14., illetve 21. napokon volt szignifikáns **(4. ábra)**.

A corticalis régióban IS hatására nem tapasztaltunk szignifikáns változást a β-aktin és az APP mRNS génexpressziójában sem. A MAPK1 mRNS szintje a háromnapos stresszt követően szignifikáns növekedést mutatott, a későbbi időpontokban viszont nem változott szignifikáns mértékben a kontrollértékekhez képest.

A különböző agyterületek mRNS-expresszióját vizsgálva a cortex és a hippocampus APP-mRNS-expressziós változása szignifikáns, szelektív változást mutat a 21. napon **(3. ábra)**.

Megbeszélés

Kísérletünk fő megfigyelése, hogy az akut és a krónikus IS hatására a β-aktin mRNS expressziója a 3. és a 21. nap után szignifikánsan emelkedett a patkányagy hippocampalis régiójában. Feltételezhető, hogy az aktin expressziós változása hozzájárulhat a szinaptikus plaszticitás zavarához, ami központi folyamat az AK-ban. Az aktin-cytoskeleton átrendeződés szabályozásában az aktinkötő fehérjék kulcsfontosságú szerepet játszanak. Az átrendeződést a kofilin koncentrációja az aktinhoz és más aktinkötő fehérjéhez képest nagyban befolyásolja. A kofilin az ADP-aktinhoz nagyobb hatásfokkal kapcsolódik, míg a koraktin, mely aktinkötő fehérje, főként ATP- és foszforilált ADP-aktin-alegységet köt, és ezáltal versenyzik a kofilinnel az aktinalegységért. Stressz hatására csökken az ATP, mely elősegíti az aktin kapcsolódását kofilinhez és ez által a kofilin-aktin pálcák keletkezését[21]. Ezek a pálcák aggregátumo-

kat képeznek, melyek az axontranszport sérülését idézik elő főként a hippocampusban és a cortexben[22]. Eredményeinkből arra következtethetünk, hogy mind az akut, mind a krónikus IS által indukált β-aktin-mRNS-expresszió-növekedés hozzájárulhat a kofilin-aktin pálcák, illetve a későbbiekben Hirano-testek kialakulásához, a sejtműködés romlásához és ezáltal a szinaptikus plaszticitás csökkenéséhez.

Kísérleteink során az APP expressziója krónikus IS hatására a 21. nap után szignifikánsan emelkedett, amely alapján azt feltételezhetjük, hogy a stressz az APP-expresszió modulálásán keresztül is szerepet játszhat az AK kialakulásában. Számos bizonyíték alátámasztja, hogy az APP kóros amyloidogenhasítása okozati tényező lehet a neurodegeneratív változások kialakulásában, a szinapszisok degenerációjában[15, 23, 24], illetve a long term potentiation csökkenésében[25, 26], melyek jellemző vonásai az AK patomechanizmusának. Az $A\beta_{1-42}$ szerepe összefüggésbe hozható a kofilin-aktin pálcák keletkezésével is. A megnövekedett $A\beta_{1-42}$ serkenti a kofilin-aktin pálcák képződését[22]. A neuronokban megjelenő pálcák ugyanakkor a hiperfoszforilált τ felhalmozására képesek és ezáltal serkentik az NFT-k kialakulását[21].

Kísérleteink során mind a β-aktin, mind az APP mRNS expressziója jellegzetes U alakú kinetikai eloszlást mutat patkányhippocampusban. Akut (három nap) és krónikus (21 nap) IS-hatás többszörösére emelte az említett gének mRNS-expresszióját, míg a 7. és a 14. napon szintjük közel változatlan maradt. Mindezek arra utalnak, hogy az IS-hatás különböző mértékű génexpressziós változást eredményez az idő függvényében, valamint kompenzációs mechanizmus jelenléte feltételezhető, mely a 21. napra kimerül. Ezek az adatok a β-aktin és az APP vonatkozásában egyedülállóak a szakirodalomban. Hasonló kinetikai eloszlást figyeltek meg *Murakami* és munkatársai (2005), akik azt tapasztalták, hogy ismétlődő IS hatására a hippocampus kortikoszteronszint-változása is U alakú görbét mutat[27].

A microtubulus-asszociált protein, a τ fiziológiásan fontos szerepet tölt be neuritnövekedésben és az axonalis transzportban. Kísérleti adataink azt bizonyítják, hogy a τ foszforilálását végző MAPK-1 enzim expressziója akut, illetve krónikus IS hatására szignifikánsan emelkedik. Eredményeink alátámasztják *Yan* és munkatársai adatait, akik az immobilizációs stressz hatását fehérjeszinten vizsgálták. Immuncitokémiai módszerrel bizonyították 14 napos stresszkezelés hatására a τ-foszforilációt patkányhippocampusban és -cortexben[28]. Saját eredményeink és az irodalmi adatok tükrében arra következtethetünk, hogy a stressz valószínűleg a MAPK-1 fokozása révén a τ-fehérje hiperfoszforilálódásához vezet, s az általunk vizsgált molekuláris útvonal is hozzájárul a stressz és az AK patomechanizmusa közötti ok-okozati összefüggéshez. A feltételezés bizonyításához további, fehérjeszintű változások detektálása szükséges.

Az akut és a krónikus IS régióspecifikus géntranszkripciós változást eredményezett patkányhippocampusban. Ebből a szempontból lényeges megjegyezni, hogy ez az agyterület kulcsszerepet játszik egyrészt a stresszválasz szabályozásában[29], másrészt az AK kialakulásában[30].

Eredményeink fontos módszertani vonatkozása, hogy a β-aktin bizonyos kísérleti körülmények között tehát nem tekinthető háztartási génnek. A β-aktint elterjedten használják belső standardnak a real-time PCR-technika során, feltételezve, hogy mint háztartási gén, expressziója a külső behatásoktól nagymértékben független. Ezt az utóbbi időben egyre több adat cáfolja[31, 32], melyeket eredményeink is megerősítenek.

Eredményeinket összefoglalva, az irodalomban elsőként írtuk le, hogy az akut és a krónikus IS hatással van a β-aktin, az APP, valamint a MAPK-1 transzkripciójára, szelektíven a hippocampalis agyi régióban. Az AK patomechanizmusában szerepet játszó mindhárom gén stresszindukált expressziós változásai hozzájárulhatnak a szinaptikus plaszticitásnak az AK-ra jellemző csökkenéséhez, valamint az aktin-cytoskeleton átrendeződés szabályozásához. Másrészt az IS hatás U alakú kinetikai görbét mutat a vizsgált gének mRNS-expressziójában, mely a stresszhatások időfüggésére, illetve krónikus stressz esetén a kompenzációs mechanizmusok kimerülésére, s a mechanizmusok további vizsgálatának szükségességére hívja fel a figyelmet.

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ERRATUM

Az Ideggyógyászati Szemle 2012;65(3–4) számában megjelent „Stroke-prevenció – Lakossági szűrőnap Budapest XII. kerületében" cikk szerzői közül tévesen került feltüntetésre az egyik szerző, Zsiga Katalin munkahelye.

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Cytoskeletal Protein Translation and Expression in the Rat Brain Are Stressor-Dependent and Region-Specific

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Abstract

Stress is an integral component of life that can sometimes cause a critical overload, depending on the qualitative and quantitative natures of the stressors. The involvement of actin, the predominant component of dendritic integrity, is a plausible candidate factor in stress-induced neuronal cytoskeletal changes. The major aim of this study was to compare the effects of three different stress conditions on the transcription and translation of actin-related cytoskeletal genes in the rat brain. Male Wistar rats were exposed to one or other of the frequently used models of physical stress, i.e. electric foot shock stress (EFSS), forced swimming stress (FSS), or psychosocial stress (PSS) for periods of 3, 7, 14, or 21 days. The relative mRNA and protein expressions of β-actin, cofilin and mitogen-activated protein kinase 1 (MAPK-1) were determined by qRT- PCR and western blotting from hippocampus and frontal cortex samples. Stressor-specific alterations in both β-actin and cofilin expression levels were seen after stress. These alterations were most pronounced in response to EFSS, and exhibited a U-shaped time course. FSS led to a significant β-actin mRNA expression elevation in the hippocampus and the frontal cortex after 3 and 7 days, respectively, without any subsequent change. PSS did not cause any change in β-actin or cofilin mRNA or protein expression in the examined brain regions. EFSS, FSS and PSS had no effect on the expression of MAPK-1 mRNA at any tested time point. These findings indicate a very delicate, stress type-dependent regulation of neuronal cytoskeletal components in the rat hippocampus and frontal cortex.

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Introduction

Organisms are often exposed to periods of stress throughout their whole lives; most of these episodes can be controlled and may even be necessary for survival. Stressful stimuli can play a relevant role as environmental factors in psychiatric disorders, such as anxiety, affective disorders and Alzheimer's disease (AD).

Recent papers implicate that stress has profound effects on the reorganization of dendritic spines of the hippocampus and the reduction of synaptic plasticity [1–3]. Dynamic actin cytoskeleton has a unique stress response and it mediates cellular events that underlie changes in synaptic transmission and morphology [4–6]. Proteomic and genomic investigations have demonstrated that cytoskeletal proteins are involved in the neurobiological processes related to stress [7,8]. Filamentous actin (F-actin) is the major cytoskeletal component of the dendritic spines and plays a key role in the morphogenesis, maintenance and plasticity of these spines [6,7,9]. The actin filament dynamics are regulated by several types of proteins [10]. One of the most important is cofilin, which is regulated by the ratio of its concentration to those of actin and other actin-binding proteins [11,12]. Another regulatory factor is the mitogen-activated protein kinase 1 (MAPK-1) which contributes F-actin stabilization and arrangement [13]. MAPK-1 is also responsible for the hyperphosphorylation of tau, leading to microtubule degeneration and cell death in AD [14].

Although β-actin is considered an internal standard gene, recent studies clearly revealed changes in β-actin transcription and translation and imbalanced functioning of the actin-regulator machinery in experimental stress models [8,15]. This cytoskeletal remodeling results in a synaptic dysfunction, which is indicated by different forms of behavioural, cognitive and affective impairments in humans [7]. Further evidence of the involvement of cytoskeletal modification in depressive disorders has emerged from investigations of the response to antidepressant treatment [16–18].

Stressful stimuli lead to a variety of changes in the function, shape and proliferative capacity of brain cells [19,20]. Previous studies have proven that acute and chronic restraint stress (RS) and electric foot shock stress (EFSS) can cause decreases in neurogenesis [21–23], while chronic psychosocial stress (PSS) partially blocks the early long-term potentiation of the CA1 area of the hippocampus [24]. RS can change the status of the microtubular dynamics in the rat hippocampus, causing an involution of structural neuronal plasticity, thereby playing a part in the pathophysiology of stress-related conditions [25]. As we have demonstrated in our recent study, RS induced biphasic dynamic changes in the transcription and protein translation of the

main cytoskeletal component, β-actin, and its regulatory proteins, cofilin and MAPK-1, in an *in vivo* rat model, selectively in the hippocampal region [15].

It has been suggested that the effects of stress are influenced by many factors, including its type or duration, gender, age, individual sensitivity and the brain region [26]. The importance of the various parameters can be established from separate experiments. Relatively few comparative investigations have been made concerning the influence of gender, the duration of stress and the affected brain region [22,23,27–29]. In contrast to the wide methodological repertoire of available animal stress models, the cytoskeletal effects of different physical and psychological stressors have not yet been compared.

As a follow-up study to our previous work on RS, in the present study, we investigated the effects of three widely-used experimental stressors, EFSS, forced swimming stress (FSS) and PSS on mRNA and protein expression of β-actin and cofilin in rat hippocampus and frontal cortex, regions most sensitive to stress-related changes [15,22,27]. Furthermore, the mRNA expression of MAPK-1, a regulator of cytoskeletal components also implicated in stress was examined in these different stress modalities. The acute and chronic effects of these physical and psychological stressors were also compared.

Materials and Methods

Animals

Adult male 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. All animal procedures were approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged (approval number: I-74-4/2011.MÁB). In each of the stress procedures (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 animals were housed 3 per cage in EFSS and FSS, and 5 per cage in PSS. The control animals were left completely undisturbed. Since each stress protocol was done as a separate experiment, each stress model had its own control group.

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.

Stress procedures

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 [30,31].

Forced swimming stress. The FSS protocol described by Porsolt et al. (1978) was used in our experiments. Each rat was placed into 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. The water was so deep that the tails of the swimming or floating animals did not touch the bottom. The water was changed after each animal. Three identical cylinders were used, separated by opaque screens, for simultaneous testing [32–35].

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 [24,35,36].

Total RNA isolation and reverse transcription

Total cellular RNA was extracted from the frontal 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. 0.3 μg of RNase inhibitor 40 U/μl (Fermentas, Glen Burnie, Maryland, USA) was added and the eluted RNA was stored at −80°C until further use.

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. Each reaction tube, with a total volume of 30 μl, contained 2 ng of total RNA in a volume of 15 μl, and 15 μl of transcription mix (3 μl of reverse transcription buffer, 1.2 μl (100 mM) of dNTP mix, 3 μl of random primers, 1.5 μl of Multi Scribe^{TM} reverse transcriptase, 0.75 μl (20 U) of RNase inhibitor and RNA Free Water (Ambion, Austin, TX, USA)). The thermal cycling consisted of three cycles: the first at 25°C for 10 min, the second at 37°C for 120 min, and the final one at 85°C for 5 s. The samples were then cooled down to 4°C, and finally stored at −20°C until qRT-PCR.

Real-time polymerase chain reaction

Reactions were performed with a RotorGene 3000 (Corbett Research, Sydney, Australia). Gene-specific primers designed by using Primer Express software (Applied Biosystems, Foster City, CA, USA) were used. The primer sequences were as follows: β-actin (forward): CCC GCG GAG TAC AAC CTT CT, (reverse): CGT CAT CCA TGG CGA ACT; cofilin (forward): GGC GGC TCT GTT CTT CTG T, (reverse): CTC CAT CAG AGA CAG CCA CA; GAPDH (forward): AGA TCC ACA ACG GAT ACA TT and (reverse): TCC CTC AAG ATT GTC AGC AA; MAPK-1 (forward): CCA AGC TCA ACC GTC TCA TC, (reverse): GGC TGG TAG GGT AGT TGA TG.

30 μl of cDNA solution was diluted with 510 μl of DNase and RNase-free water. Q-PCR was carried out in a final volume of 20 μl containing 10 μl of SYBR Green MasterMix (Roche, Basel, Switzerland), 0.5 μl of forward primer, 0.5 μl of reverse primer, and 9 μl of template cDNAs. The protocol comprised denaturation for 25 s at 95°C, annealing for 25 s at 60°C, and extension for 15 s at 72°C. The relative gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase. The results were analysed by the $2^{-\Delta\Delta CT}$ method [37].

Western blotting

The brain regions were homogenized in a solution containing 50 mM Tris buffer (pH 7.5), 150 mM NaCl, 0.1% Nonidet-P-40, 0.1% cholic acid, 2 μg/ml leupeptin, 2 mM PMSF, 1 μg/ml pepstatin and 2 mM EDTA. The homogenates were centrifuged at 10 000 g for 15 min at 4°C. The supernatants were used for protein assays. Proteins were measured with bicinchoninic acid [38].

After denaturation, 20 μg of protein were separated on 12% 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 (Santa Cruz, CA, USA, 1:2000), rabbit polyclonal cofilin (D59) antibody (Cell Signaling Technology, MA, USA 1:1000) and mouse monoclonal anti-GAPDH (Millipore, MA, USA 1:4000). The next day, after five washes with TBST, horseradish-peroxidase-labelled anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA 1:1000) and horseradish-peroxidase-labelled anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA 1:1000) secondary antibodies were applied for 1 h. The nitrocellulose membranes were subsequently washed five times with TBST, and then incubated with the Supersignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and exposed to Kodak autography film. The optical densities of the immunoreactive bands were quantified by means of Scion Image Software. The amounts of examined proteins were calculated by comparison with the optical density of internal control. For each blot of β-actin and cofilin, the relative protein level was calculated from the ratio of absorbance of β-actin/GAPDH and the ratio of the absorbance of cofilin/GAPDH. This was considered as 100% in the control group and the data of different time points were compared to this ratio.

Statistical analysis

All data are reported as mean ± SEM; they were analyzed by two-way ANOVA with SPSS 15.0 Software: Stress types (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 comparison within the same groups was assessed by Student's t-test and by one-way ANOVA followed by the Bonferroni and Games-Howell *post hoc* tests; the level of significance of comparisons was taken as $p<0.05$.

Results

Body, adrenal gland and thymus weights of the stressed animals

Body weight (BW) was measured repeatedly throughout the individual stress experiments. The two-way ANOVA revealed significant interactions between stress types (EFSS, FSS, PSS) and exposure times (7, 14 and 21 days) [$F_{(8,420)}=14.300$, $p<0.001$] in the BW. There was a significant main effect of stress types [$F_{(2,420)}=1286.413$, $p<0.001$] and exposure times [$F_{(4,420)}=812{,}974$, $p<0.001$]. The rats subjected to EFSS gained significantly less BW than the control animals (Fig. 1A). EFSS caused a significant lack of gain in BW, but only in response to chronic stress [on the 14th day [$F_{(2,17)}=3.026$, $p=0.05$] group 5: $p=0.049$; on the 21st day group 5: [$t_{(10)}=10$, $p=0.019$] (Fig. 1A). However, exposure to FSS and PSS did not provoke any appreciable difference in BW (Fig. 1B,C).

Figure 2A shows effects of EFSS, FSS and PSS on the weights of the adrenal glands of rats. The two-way ANOVA revealed

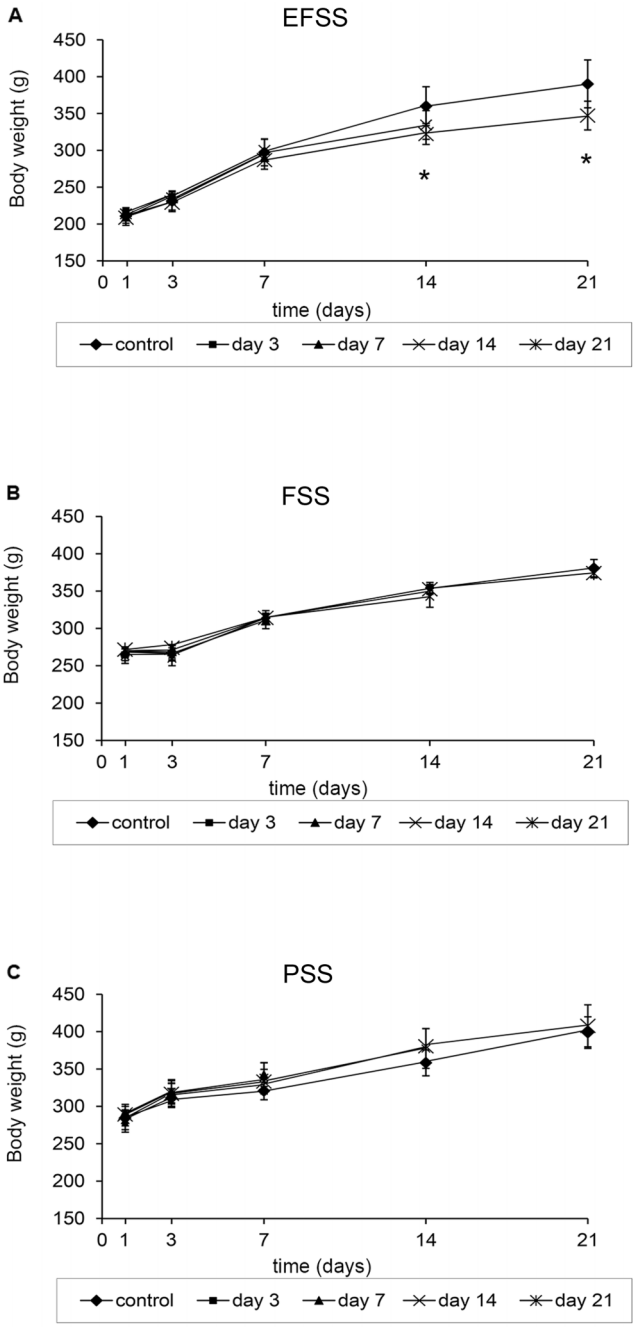

Figure 1. Stress type-dependent body weight alterations. Effects of electric foot shock stress (EFSS) (**A**), forced swimming stress (FSS) (**B**) and psychosocial stress (PSS) (**C**) on the overall body weight of rats, measured on days 3, 7, 14 and 21. Values for each group are means ± SEM, n = 6–10. *$p<0.05$ and **$p<0.01$: significant differences as compared to the control.
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significant interactions between stress types (EFSS, FSS, PSS) and exposure times (7, 14 and 21 days) [$F_{(6,67)}=2.820$, $p=0.017$] in the weights of the adrenal gland. There was a significant main effect of stress types [$F_{(2,67)}=4.039$, $p=0.022$] and exposure times [$F_{(3,67)}=11.037$, $p<0.001$]. The adrenal gland weight relative to the BW was significantly elevated by EFSS (Fig. 2A). EFSS induced a significant increase [$F_{(3,19)}=13.657$, $p<0.001$] within 7 days [$p<0.001$] (Fig. 2A); the adrenal gland weight was also

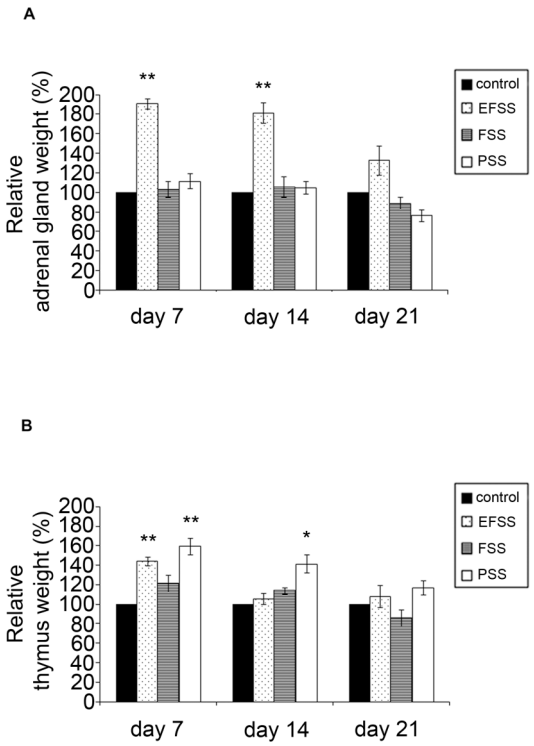

Figure 2. Stress type-dependent alterations of the weights of the adrenal glands and thymus. Effects of electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the weights of the adrenal glands (**A**) and the thymus (**B**) of rats, measured every 7 days. Results are expressed as percentages of the control (unstressed rats). Values for each group are means ± SEM, n = 6–10. *$p<0.05$ and **$p<0.01$: significant differences as compared to the control.
doi:10.1371/journal.pone.0073504.g002

increased on day 14 [$p<0.001$], but there was no significant elevation on day 21 (Fig. 2A). In contrast, FSS and PSS did not result in any significant change in the weight of the adrenal gland (Fig. 2A).

Figure 2B shows effects of EFSS, FSS and PSS on the weights of the thymus of rats. The two-way ANOVA did not reveal significant interactions between stress types (EFSS, FSS, PSS) and exposure times (7, 14 and 21 days) [$F_{(6,67)} = 1.165$, $p = 0.335$] in the weights of the thymus. There was a significant main effect of stress types [$F_{(2,67)} = 10.714$, $p<0.001$] and exposure times [$F_{(3,67)} = 12.230$, $p<0.001$]. EFSS, FSS and PSS caused a transient increase in the thymus weight relative to BW, after which a decreasing tendency was detected (Fig. 2B).

Expressions of β-actin, cofilin and MAPK-1 mRNA in the rat brain after different stressful stimuli

Figures 3A–F show the expression of β-actin (A,B), cofilin (C,D) and MAPK-1 (E,F) mRNA in the rat hippocampus and frontal cortex. The two-way ANOVA revealed significant interactions between stress types (EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the β-actin mRNA expression in the hippocampus [$F_{(8,76)} = 3.64$, $p = 0.01$]. There was a significant main effect of stress types [$F_{(2,76)} = 43.159$, $p<0.001$] and exposure times [$F_{(4,76)} = 5.781$, $p<0.001$]. In the hippocampus, EFSS [$F_{(5,30)} = 4.663$, $p = 0.003$] and FSS [$F_{(4,19)} = 4.510$, $p = 0.01$] caused significant increases in β-actin mRNA expression by day 3 [EFSS $p = 0.034$ and FSS $p = 0.05$] (Fig. 3A). A biphasic U-shaped time course was detected in the case of EFSS. The β-actin mRNA level was found to be elevated on days 3, 7 and 21, respectively, but not on day 14 (Fig. 3A).

In the case of FSS, the time course was not U-shaped: significant elevations were observed on days 3 and 7, but there were no changes at the later time points (Fig. 3A). In contrast to the physical stressors, PSS did not influence the β-actin mRNA transcription in the hippocampus or the frontal cortex (Fig. 3A,B).

The two-way ANOVA revealed significant interactions between stress types (EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the β-actin mRNA expression in the frontal cortex [$F_{(8,82)} = 2.788$, $p = 0.009$]. There was a significant main effect of stress type [$F_{(2,82)} = 13.524$; $p<0.001$], but the two-way ANOVA did not reveal a significant main effect of exposure times (3, 7, 14 and 21 days) in the β-actin mRNA expression in the frontal cortex [$F_{(2,82)} = 1.113$; $p = 0.356$]. FSS [$F_{(4,28)} = 7.266$, $p = 0.001$] caused significant increases in β-actin mRNA expression by day 7 [$p = 0.032$] (Fig. 3B).

The two-way ANOVA did not reveal significant interactions between stress types (EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the cofilin mRNA expression in the hippocampus [$F_{(8,87)} = 1.756$, $p = 0.097$]. EFSS, FSS and PSS had no effect on the expression of cofilin mRNA at any tested time point (Fig. 3C).

The two-way ANOVA revealed significant interactions between stress types (EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the cofilin mRNA expression in the cortex [$F_{(8,85)} = 3.885$, $p = 0.01$]. There was a significant main effect of stress types [$F_{(2,85)} = 712.122$, $p<0.001$] and exposure times [$F_{(4,85)} = 6.461$, $p<0.001$]. FSS decreased the cofilin mRNA expression [$F_{(4,24)} = 7.266$, $p<0.001$] significantly by day 7 [$p = 0.032$] (Fig. 3D).

The two-way ANOVA did not reveal significant interactions between stress types (EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the MAPK-1 mRNA expression in the hippocampus [$F_{(8,84)} = 2.011$, $p = 0.055$] or in the frontal cortex [$F_{(8,84)} = 0.463$, $p = 0.962$] (Fig. 3E,F). EFSS, FSS and PSS had no effect on the expression of MAPK-1 mRNA at any tested time point (Fig. 3E,F).

Levels of β-actin and cofilin protein in the rat brain after different stressful stimuli

Figure 4 depicts representative β-actin or cofilin immunoblots after different types of stress. The β-actin or cofilin signals of the homogenates from the hippocampus were resolved at approximately 43 kDa, and 19 kDa, respectively (Fig. 4).

Figure 4 A–D shows the changes of β-actin and cofilin protein induced by the different stress types in the rat hippocampus. The two-way ANOVA revealed significant interactions between stress types (EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21 days) in the levels of β-actin in the hippocampus [$F_{(8,75)} = 4.196$, $p<0.001$]. There was a significant main effect of stress types [$F_{(2,75)} = 45.983$, $p<0.001$] and exposure times [$F_{(4,75)} = 8.886$, $p<0.001$]. Western blot experiments revealed statistically significant elevations in the hippocampal β-actin levels of exposure to EFSS [[$F_{(4,25)} = 13.288$, $p<0.001$] day 3: $p<0.001$; day 7: $p = 0.012$]; then, following a transient reduction on day 14, the β-actin protein level was again significantly increased by day 21 of exposure to EFSS [$p<0.001$] (Fig. 4C). Thus, similarly to the changes induced by EFSS in the transcription of β-actin mRNA, the protein level changes described a U-shaped time course (Fig. 3A, 4C). Neither FSS nor PSS modified the hippocampal β-actin levels significantly (Fig. 4C).

The two-way ANOVA revealed significant interactions between stress types (EFSS, FSS, PSS) and exposure times (3, 7, 14 and 21

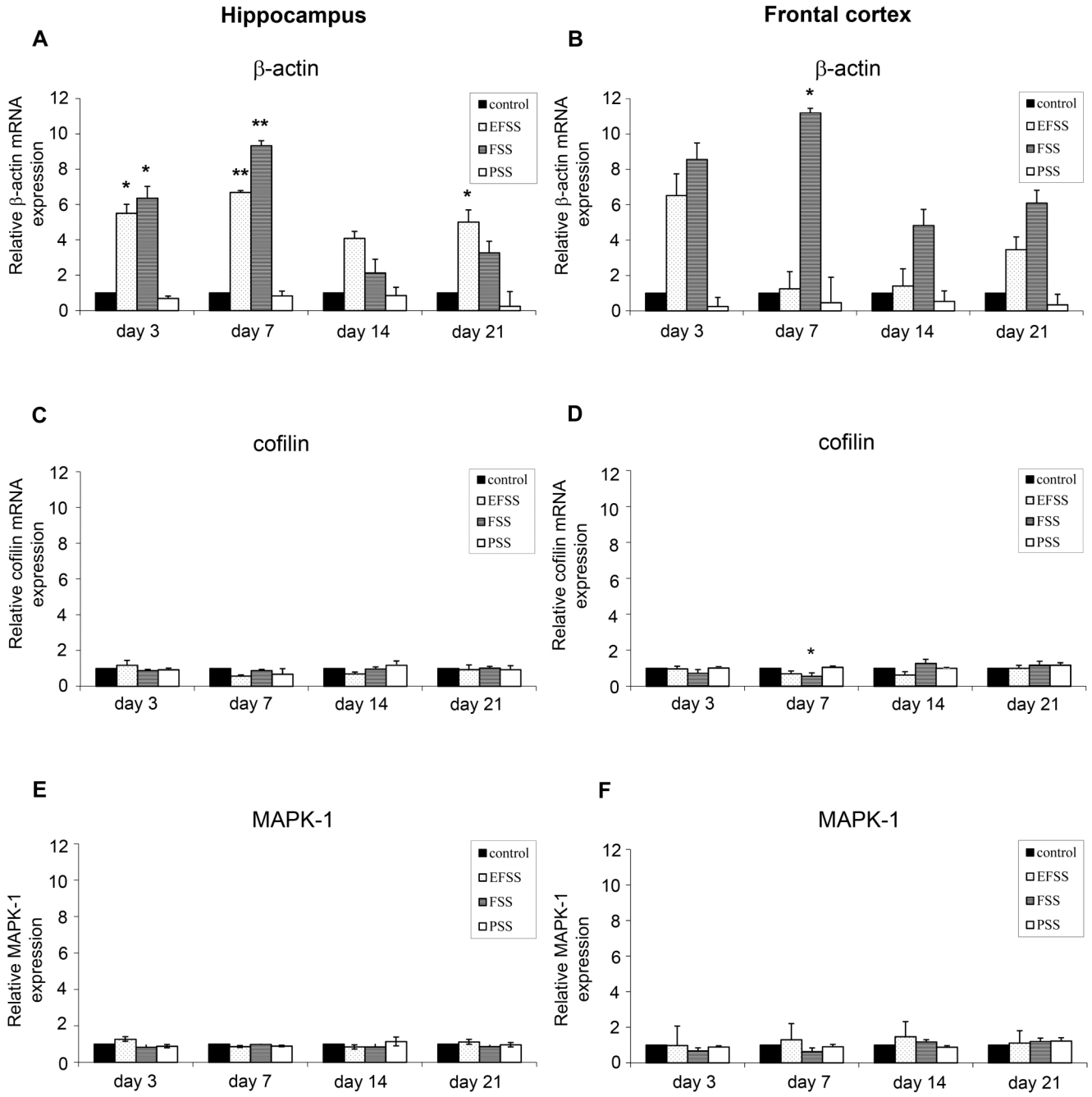

Figure 3. Stress type-dependent transcriptional alterations. Effects of electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the expressions of β-actin (**A, B**), cofilin (**C, D**) and MAPK-1 (**E, F**) mRNA in the rat hippocampus and frontal cortex. GAPDH was used as reference gene. Values for each group are means ± SEM, n = 6–10. *$p<0.05$ and **$p<0.01$: significant differences as compared to the control.

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days) in the levels of cofilin in the hippocampus [$F_{(8,75)} = 4.945$, $p<0.001$]. There was a significant main effect of stress types [$F_{(2,75)} = 99.013$, $p<0.001$] and exposure times [$F_{(4,75)} = 4.665$, $p = 0.02$]. The hippocampal cofilin level increased only in the EFSS-exposed group [$F_{(4,25)} = 8.065$, $p<0.001$], where the elevation was significant by day 21 [$p = 0.026$] (Fig. 4D).

In the frontal cortex, none of the applied stressors caused any significant changes in the level of either β-actin or cofilin (Fig. 4G,H).

Discussion

The main finding of the present study was that the quantitative pattern of cytoskeletal stress response in the rat brain is unique to the stress model used to trigger it. The various stress models employed in this study affected the mRNA expression and protein levels of β-actin and cofilin differently; of the various physical and psychosocial stressors, EFSS induced the most pronounced changes in the investigated cytoskeletal markers. Another impor-

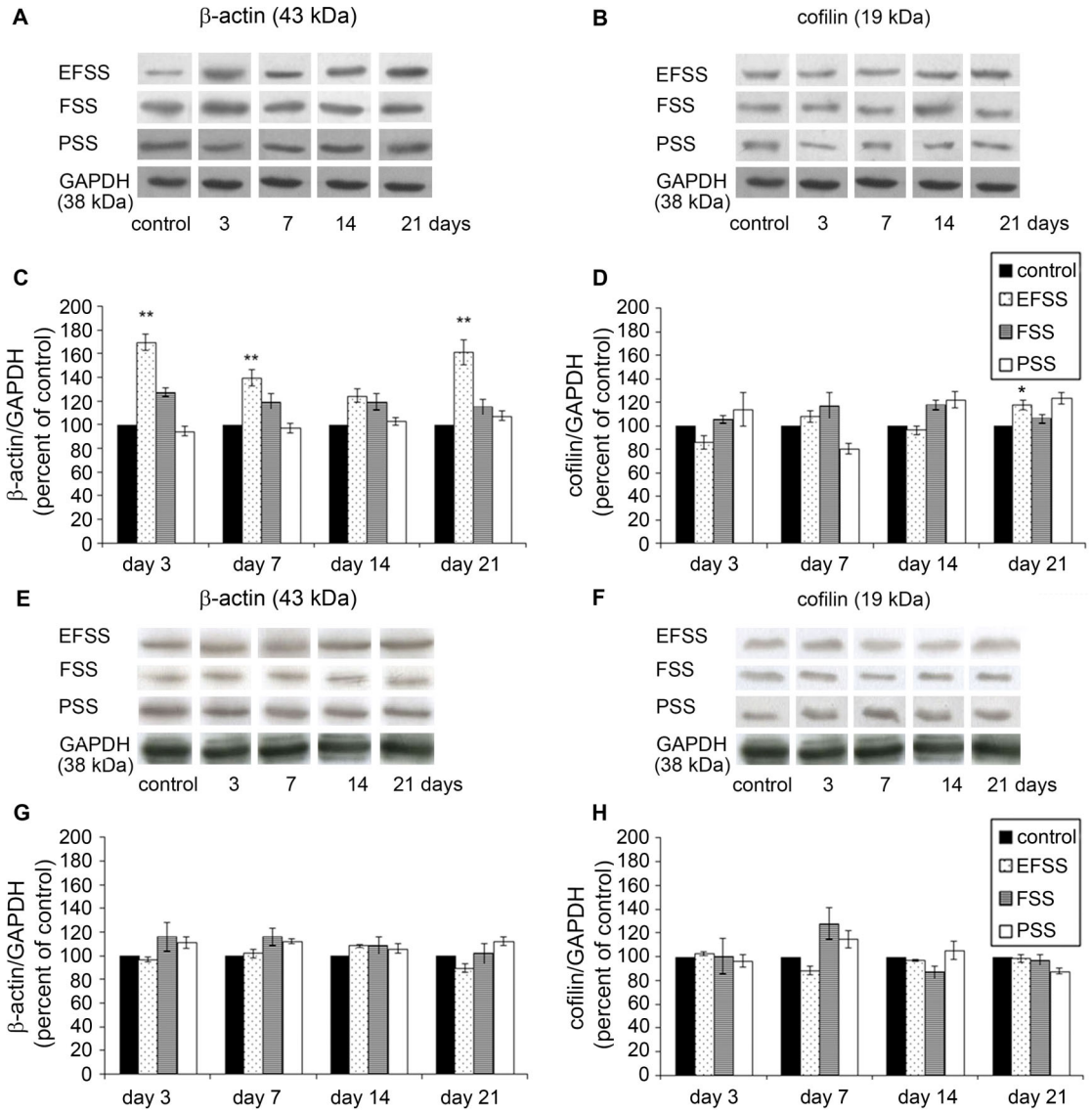

Figure 4. Western blot analysis of β-actin and cofilin after different stressors in hippocampus and frontal cortex. The specific bands for β-actin and cofilin in rat hippocampus (**A, B**) and frontal cortex (**E, F**) appeared at 43 kDa and 19 kDa, respectively. Antibodies used are described in Materials and methods. Semi-quantitative representations of electric foot shock stress (EFSS), forced swimming stress (FSS) and psychosocial stress (PSS) on the levels of β-actin and cofilin protein in the rat hippocampus (**C, D**) and frontal cortex (**G, H**). Results are expressed as percentages of the control (unstressed rats). GAPDH (38 kDa) was used as reference gene. Values for each group are means ± SEM, n = 6–10. *$p<0.05$ and **$p<0.01$: significant differences as compared to the control.
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tant observation was that the effects of this type of stress were region-specific, since changes were detected only in the hippocampus.

Our present results revealed that the levels of both β-actin mRNA and protein underwent biphasic dynamic changes in response to EFSS during the examined 3-week period. Previously, we demonstrated that RS induced biphasic dynamic changes in the transcription and protein translation of the main cytoskeletal component, β-actin, in an *in vivo* rat model, selectively in the hippocampal region [15]. The stress changes in β-actin transcription show a somewhat different pattern after EFSS compared to RS [15]: the initial elevation of the β-actin mRNA level was longer-lasting in the case of EFSS. However, these slower kinetic properties were not reflected in the protein level. In the first week of the stress period, the amount of β-actin in the hippocampus increased, then normalized and subsequently increased again. A possible explanation for these kinetic characteristics may be that short periods of stress serve an adaptive function, while longer durations may result in more profound changes through the depletion of compensatory mechanisms.

Examining the stress induced changes of regulating proteins cofilin and MAPK-1 of the actin filament dynamics, our study interestingly indicated that neither cofilin nor MAPK-1 were altered when the type of stressors, brain regions and time points were compared, in contrast to our previous work [15]. While the β-actin level pattern after EFSS was similar to earlier described stress type-dependent changes [15], the alteration in cofilin and MAPK-1 transcription and translation in response to EFSS differed considerably. The biphasic time course of mRNA levels of regulating factors and the elevation in cofilin protein levels were

not demonstrated after EFSS. Results from a previous study from our group [15] and these newest data suggest that the changes induced in β-actin transcription and translation by RS and EFSS may be differently regulated. RS, but not EFSS may modify the actin dynamics (actin filament assembly/disassembly) and stabilization through regulation of the actin-depolymerizing factor/cofilin family and MAPK-1 [15].

Results from a previous study from our group [15] and these newest data suggest that the changes induced in β-actin transcription and translation by RS and EFSS may be consequences of different regulatory mechanisms. RS may modify the actin dynamics (actin filament assembly/disassembly) and stabilization through regulation of the actin-depolymerizing factor/cofilin family and MAPK-1 [15], whereas EFSS is not likely to have the same effect. Further investigations are necessary to clarify the roles of other regulator proteins, such as different kinases or drebrin in stress-induced cytoskeletal changes.

The elevation in the examined genes and proteins showed that the effects of acute or chronic EFSS are region-specific. The hippocampal cytoskeletal changes have been detected not only in this stress type, but similar changes were observed in a previous study after RS [15]. Recently, a morphological examination performed on cultured rat hippocampal slices demonstrated that glucocorticoid engaged the cofilin signaling pathway involved in regulating actin polymerization [13]. Since the major cytoskeletal component of the dendritic spines is filamentous actin, the local actin dynamics determine the changes in spine shape, numbers and size [7]. Previous reports demonstrated that chronic stress induces dendritic atrophy of the hippocampal pyramidal neurons and reduces the number of hippocampal neurons [23,39]. Our findings are in agreement with these results and confirm that the hippocampus is one of the most stress-sensitive regions in the brain.

Besides EFSS, FSS and PSS are other commonly used animal models of depression or work-related stress [24,32]. In the case of FSS and PSS the BW gain and the typical stress-related changes of adrenal gland weight or thymus weight were not observed. Additionally, FSS caused only a transient elevation of the β-actin mRNA expression only in the initial stages of the experiment in the hippocampal and the frontal cortex which did not induce any increase in protein levels. Our observations also indicate that neither acute nor chronic PSS caused any significant alterations in the investigated markers. These results suggest that the cytoskeletal changes are less sensitive to FSS or PSS, contrary to the biphasic effects induced by EFSS and RS [15].

The master regulator of the actin cytoskeleton expression, including the level of β-actin and cofilin, is a nuclear transcription factor, serum response factor (SRF) [40]. SRF activity is regulated by its co-factors, like myocardin-related transcription factors (MRTFs) [41]. The actin cytoskeleton is both an upstream regulator of MRTFs activity, with monomeric actin directly acting as a signal transducer, and a downstream effector, because of the many cytoskeletal target genes. In a conditional forebrain-specific SRF knockout mouse model shorter neuritic length and alteration of the cytoskeleton dynamics, impairments of growth cones dynamics and downregulation of actin mRNA levels in hippocampal neurons were observed [42], indicating the importance of actin levels in neuronal functions. A recent study identified SRF as a novel upstream mediator of FosB in nucleus accumbens after chronic social defeat stress, and implicated SRF in the development of depressive- and anxiety-like behaviors [43]. Based on these observations we hypothesize that the SRF/MRTFs signaling pathway may be responsible for the stress-induced β-actin changes in the hippocampus seen in our study.

Our experiment has important limitations. First, to confirm the physiological efficacy of the stress procedures, we measured only the body, adrenal gland and thymus weights. Although these stress-markers are often used parameters which represent the impact of stress, adrenocorticotropic hormone and corticosterone levels would be more informative to prove the intensity of the stressors. Second, the use of multiple control groups in each time point was ignored due to ethical reasons. Since the number of animals would have been greatly elevated, we compared the different experimental subgroups to one control group only for each stressor.

In conclusion, our study is the first to demonstrate that the levels of cytoskeleton proteins β-actin and cofilin increased in the hippocampus and frontal cortex of rats in models of electric foot shock and forced swimming stress, but not in psychosocial stress. These results suggest that the different stress models give rise to different quantitative and kinetic changes in the transcription and translation of the main components of cytoskeletal organization. Our results have important implications regarding the need for the careful selection of different stress models and their methodological importance. 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. These changes additionally indicate strong stress-dependent neuronal cytoskeletal regulation in the rat brain, and our results may therefore contribute to the selection of appropriate stress models in connection with certain stress-related human conditions.

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Author Contributions

Conceived and designed the experiments: MP JK ZJ PS EKF. Performed the experiments: PS EKF ÖCF SK JKJr. Analyzed the data: PS MP JK. Contributed reagents/materials/analysis tools: GS MP JK ZJ. Wrote the paper: PS MP JK ZJ.

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