CHRONIC STRESS EFFECTS ON SYNAPTIC VESICLE-ASSOCIATED PROTEINS EXPRESSION: INDICATIONS FOR REGION/GENDER-DEPENDENT REGULATION

Stress-induced neuronal dysfunctions have been associated with altered synaptic protein expression. In this study, chronic stress-induced changes in expression levels of several synaptic vesicle-associated proteins were thus investigated, in the hippocampus and prefrontal cortex of male and female rats, by microarray and RT-PCR analysis. The results illustrate gender- and region-specific regulation of membrane-associated synaptic protein expression in response to stress. Specifically, chronically stressed males revealed increased transcription levels of several synaptic proteins, including synaptophysin, synaptotagmin and synapsin in prefrontocortical and hippocampal areas. Although no changes were observed in stressed females, they consistently expressed lower mRNA levels of these proteins under stress conditions compared to males. These findings support the hypothesis that altered expression of selective synaptic proteins may constitute a potential mechanism underlying stress-induced impairment of neuronal plasticity. Moreover, these data suggest the possibility that differentially affected synaptic protein expression may contribute to gender distinctive mechanisms of dysregulation that characterize certain affective disorders.

tional players. In fact, sole exposure to persistently elevated glucocorticoids is not always enough to induce these effects since chronic stress-induced dendritic remodeling and suppression of hippocampal cell proliferation have also been shown to occur in the absence of prolonged HPA axis hyperactivity 15. During such processes their deleterious effects may thus also depend upon interactions with multiple other mediators of the stress response, including excitatory amino acids such as glutamate.

The “glutamate hypothesis” of neuronal dysfunctions.

Although extracellular glutamate levels are significantly elevated in the hippocampus following stress exposure 16, adrenalectomy can prevent this stress-induced rise 17, thereby suggesting a role for adrenal hormone participation in the modulation of glutamate release 15. The ability of some pharmacotherapeutic agents to interfere with systems as serotonin and glutamate are also in line with this notion and some of the mechanisms governing neuronal dysfunction also share additional common mediators besides glucocorticoids. Excessive glutamate release, for instance, appears to be a fundamental factor underlying inhibition of cell proliferation in the dentate gyrus as well as the remodeling of dendrites 9,18. More importantly, persistently elevated extracellular glutamate levels may also explain some of the neuronal abnormalities observed following prolonged stress exposure such as ERK1/2 hyperphosphorylation in prefrontocortical dendrites and reduced calcineurin immunoreactivity in addition to suppressed hippocampal neurogenesis. Whereas the interactions between HPA axis hyperactivity and alternative theories thus attest the fact that multiple hypotheses act concurrently to induce neuronal defects, glucocorticoids likewise, do not act alone to promote the effects underlying these respective theories but simultaneously with other mediators such as glutamate.

Throughout attempts to identify the intracellular changes that mediate stress-induced neuronal defects, glutamate emerged as a critical player in the modulation of deleterious stress effects on neuronal plasticity. This excitatory amino acid has been suggested to lead to neuronal dysfunctions through excitotoxicity and oxidative stress, both of which are associated with alterations in multiple signaling systems including the MAP kinase cascade and Ca\(^{2+}\)/calmodulin-dependent protein kinase pathway. Excessive extracellular glutamate, for instance, inhibits cystine uptake, a precursor for glutathione synthesis. Cellular glutathione synthesis thus declines and reactive oxygen species (ROS) accumulate, generating a condition of oxidative stress, which in turn activates the MAPK cascade, stimulates ERK1/2 and contributes to cellular dysfunctions as shown in neurons 19. Accordingly, persistent ERK1/2 phosphorylation has been reported in response to increased extracellular glutamate concentrations 20. Furthermore, while prolonged activation following oxidative stress has been associated with neuronal dysfunctions and cell death, inhibition of ERK1/2 phosphorylation by MAPK inhibitors has been shown to block glutamate-mediated neuronal defects 21,22.

Besides prolonged ERK1/2 kinase activity however, a role for abnormal phosphatase activity has also been proposed to mediate neuronal dysfunction, as phosphatase inhibi-
INTRODUCTION

Stress, particularly when prolonged and severe, has been associated with multiple morphological and functional abnormalities in cortical and subcortical structures of both humans and animals. Consistent neuroimaging findings in support of the latter include hippocampal atrophy and reduced prefrontocortical metabolism following chronic stress and stress-related disorders 1-5. Stress-induced structural and functional impairments have in turn been implicated in cognitive deficits and significant behavioral alterations 6-11. Reduced neuronal plasticity however has also been reported in response to stress and is believed to represent a common feature in a wide variety of psychiatric disorders. Since impaired synaptic plasticity may depend upon stress-induced alterations on the molecular level, it is thus notable that investigation of these underlying mechanisms has been only tendentious. Stress, for instance, has been reported to affect the expression of neurotrophic factors as brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) 12,13 and adverse events have also been speculated to impair neuronal plasticity by affecting expression of various synaptic vesicle-associated proteins (SVaPs) 14,15. The latter raises an intriguing possibility that abnormal expression of these proteins may contribute to the molecular basis of stress-induced neuronal plasticity changes as well as behavioral and cognitive alterations associated with prolonged stress exposure. Proper regulation of SVaP expression is in fact essential for efficient modulation of neurotransmitter release while depleted or dysfunctional neurotransmission has been implicated in the occurrence of various affective disorders. Abnormal expression and/or function of SVaP has thus been identified as a possible predisposing factor for the pathophysiology of psychiatric disorders such as schizophrenia and depression 16-18. In this study, we examined chronic footshock-induced alterations in synaptotagmin, synaptophysin, synapsin I and synapsin II expression in the prefrontal cortex and hippocampus, to investigate whether dysregulated transcription of specific synaptic vesicle-associated proteins could account, at least in part, for the often documented neuronal plasticity impairments in response to repeated stress. The experiments were performed in both male and female rats in view of the well-established gender-related prevalence of stress-related psychiatric illnesses, which illustrate that women are more likely to develop depressive or anxiety disorders than men 19,20. Although recent reports have attributed this differential, prevalence of psychopathology to sex-related differences in behavioral response to stress 21, considerably less attention has been given to gender-related differences in the neurobiological mechanisms underlying stress and psychopathology, particularly at a molecular level. The present data illustrate a region- and gender-dependent regulation of SVaP expression in response to repeated stress, which may offer additional insights into the understanding of neurobiological substrates underlying the differential gender-related susceptibility to adverse events and psychopathology.
MATERIAL AND METHODS

Animals

The experiments were performed with male ($n=48$: 225-249 g) and female ($n=48$: 200-224 g) Wistar rats. The animals were individually housed with food and water available ad libitum and maintained on a 12:12 h light/dark cycle at 21°C. All rats were handled daily for 5-8 min to minimize the nonspecific stress response. All handling was performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the guidelines of the Animal Bio-ethics Committee of the University of Groningen (FDC: 2509).

Stress Procedure

The rats were subjected to a daily footshock stress protocol for 3 weeks as previously described. In short, the rats were placed in a rodent test-chamber with a metallic grid floor connected to a shock generator and scrambler. Rats in the stress group ($n=24$; 12 male and 12 female rats) were subjected to a single daily session of varying duration (between 30 and 120 min/day) in the “footshock box” during which five uncontrollable and inescapable footshocks were applied (0.8 mA in intensity and 8 sec in duration). In order to make the procedure as unpredictable as possible, starting time, inter-shock interval, and total time spent in the box were varied on a daily basis. To investigate the cellular and molecular changes induced by sustained footshock stress, non-stressed control rats ($n=24$; 12 male and 12 female rats) followed an identical schedule in a similar setup, but did not receive any footshocks throughout the experiment. On the final (21st) day of the experiment all rats were placed in the footshock box for 15 minutes without receiving any electric shocks. Exposure to the footshock chamber on the final day was essential as it established a link between a harmless stimulus (environment in which footshocks were applied or “footshock box”) and aversive events (“footshocks”). This provided a way to create a stress condition without directly exposing the stressed animals to physical or painful stimuli. The response of control and stress rats to an identical, stimulus could then be investigated by evaluating gene expression changes (microarrays and quantitative RT-PCR). To limit circadian variability in circulating hormone levels, animals were sacrificed on subsequent days within a narrow window of 4 hours.

Physiological parameters

Upon termination, adrenal glands were removed, weighed and recalculated to correct for the body weight of the animals. Blood was also collected upon termination and the serum obtained was stored at -20°C to determine plasma corticosterone levels with HPLC. These parameters served to verify the efficacy of the chronic stress procedure.
Corticosterone assay.

Plasma corticosterone levels were measured by HPLC as previously described \(^2^2,^2^3\). For quantification of corticosterone concentration dexamethasone was used as internal standard. Plasma was extracted with 3ml of diethylether, vortexed for 5 min, and then centrifuged for 5 min at 3000 x g. The extraction procedure was repeated twice. The organic phase was evaporated to dryness in a 50°C waterbath. The detection limit of corticosterone was 10 nM.

Tissue and RNA preparation

Total RNA- and poly(A)+RNA-isolation.

Thirty minutes after the start of the final session, 24 rats were anesthetized with halothane and decapitated. The brains were dissected to separate the prefrontal cortex and the hippocampus, which were subsequently quick-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from these brain areas by using Trizol (Life Technology, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Integrity of total RNA was confirmed on a 2% agarose gel and final concentrations were assessed spectrophotometrically. Poly(A)*RNA was in turn isolated from total RNA using Micro-FastTrack™ 2.0 Kit (Invitrogen) according to the manufacturer’s instructions.

cDNA gene expression arrays

RNA (2-5µg/rat) extracted from the prefrontal cortex (of 4-5 animals per group) was subsequently converted into a \(^3^2\)P-labeled first-strand cDNA and used to hybridize cDNA microarrays (rat atlas cDNA array 1.2; Clontech, Palo Alto, CA, USA). In this microarray, plasmid and bacteriophage DNAs are included as negative controls, along with several housekeeping cDNAs as positive controls. A complete list of genes and controls spotted on the array, as well as array coordinates and GenBank accession numbers, is available at the Clontech web site (http://www.clontech.com). To suppress non-specific background each membrane was prehybridized for 30 min at 68°C in 5ml of hybridization solution (ExpressHyb, Clontech) with continuous agitation. Hybridization was subsequently carried out by the addition of the denatured, labeled cDNA to the prehybridization solution at 68°C for overnight incubation to reach a final probe concentration of 2-5 x 10^6cpm/ml. Membranes were stringently washed with continuous agitation at 68°C in 2 x SSC, 1% SDS (4x30 min) and then in 0.1 SSC, 0.5% SDS (30 min). After a final rinse in 0.1 x SSC (5 min), membranes were mounted on Whatman paper, plastic-wrapped, exposed to x-ray film overnight at -80°C followed by exposure to a phosphoimager screen for 3 days.
PhosphoImaging analysis.

Membranes were scanned using a Molecular Dynamics STORM PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA, USA), and images were analyzed by ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA, USA). According to the Array manufacturer, Clontech, the radioactive cDNA signal is linear for RNAs present at levels of 0.01-3% of the total RNA population. An admonition of quantitative analysis however, is that the accuracy for extremely low abundant genes may not be reliable due to the detection limitation of this technique. Here the expression level for each gene was measured by the phosphoimager in arbitrary signal intensity units and the original raw output was subsequently used to perform the statistical analysis.

Statistical analysis.

A full account of the microarray analysis has been previously described 24. In short, use was made of specific Delphi 5.0 data transformation software and SPSS 11.0. After log transformation, factor analysis was performed on the data points to identify the common component in the (log) data. Subtraction of this common component yields a log ratio value, used for further analysis. After checking for systemic positional effects, a reference set of genes was chosen against which to test hypotheses with regard to the experimental variation. Genes selected for inclusion in the analysis set were those, which displayed the greatest variation compared to other genes; the reference set contained the genes with the least amount of variation between arrays. To verify sensitivity of the results for this selection procedure, the criterion for inclusion of genes in the test set versus the reference set was variation in log expression ratio over a range of standard deviations (>1.5 - >2.5). The common variation or expression of the genes (first principal component) plot against the interarray differences or variation (second principal component) allowed identification of the strongest alterations of individual genes. The outliers, previously published 24 are genes most likely responsible for the observed experimental effects.

Real-time quantitative polymerase chain reaction (qRT-PCR)

First-strand cDNA synthesis.

First-strand cDNA was synthesized from 200 ng poly(A)+RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s instructions. Before first-strand synthesis was performed, contaminating genomic DNA was removed using Deoxyribonuclease I, Amplification Grade (Invitrogen). The first-strand synthesis was primed with oligo(dT), and the RNA template was not removed from the DNA:RNA hybrid.
Primers and PCR Conditions.

All primers were designed with Primer Express® Software v2.0 (Applied Biosystems) using default parameters (sequences are listed in table 1). PCR reactions were performed on an ABI Prism 7900HT Sequence Detection System. Amplification mixtures (25 µL divided over 3 wells, 7 µL per well) for all genes contained 2 ng cDNA from polyA+RNA template, 1 x SYBR Green PCR buffer, 3 mM MgCl2, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP 0.4 mM dUTP, 0.025U/ml Amplitaq Gold, 0.01 U/ml AmpErase UNG and 300 nM of each primer. The cycling conditions comprised of 2 minutes AmpErase UNG activity, 10 minutes Amplitaq Gold activation, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, and a dissociation stage: 15 seconds 95°C, 15 seconds 60°C, 15 seconds 95°C ramp rate 2%. The No Template Control contained all components except the cDNA template.

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Real-time polymerase chain reaction (RT-PCR).

Real time detection was performed using an ABI PRISM 7900HT Sequence Detection System and SYBR Green PCR Core Reagents. Data analysis was performed using software provided with the instrument. The mRNAs of the different genes were relatively quantified across groups with the Comparative CT Method. The CT value, or fractional cycle number at which the defined threshold is crossed above baseline, is predictive of the input amount of target and thus useful in quantification of RNA. The threshold was set...
between 0.4 and 0.6 with a baseline range from 1 to 7. All RNA samples were measured in triplo and the amount of target was normalized to an endogenous reference, the housekeeping gene, RPS29 (ΔCT). An additional reference (β-actin) was included as an extra control.

**Statistical Analysis.**

Data are expressed as means ± standard error (SEM). Statistical significance was determined by performing one-way analysis of variance (ANOVA) and F test of variance on the corresponding parameter measures from experimental and control conditions. To compare values between male and female, and/or control and experimental conditions t tests for equal or unequal variance were performed. P<0.05 was defined as the level of significance between groups. Calculations were made using Jandel SigmaStat statistical software.

**RESULTS**

**Adrenal Weight**

Chronic footshock exposure caused significant adrenal hypertrophy in both male (29%; F=26.41; P<0.0001) and female rats (17%; F=8.32; P=0.014). A gender-related difference in the weight of adrenal glands was also evident as females had heavier glands than males under both basal (107%; F=82.71; P<0.0001) and stress conditions (87%; F=218.14; P<0.0001) (Fig. 1a).

**Plasma Corticosterone**

In line with corresponding adrenal weights, chronic footshock stress resulted in significantly enhanced plasma corticosterone concentrations in both sexes. Males expressed a 45% (F=6.68; P=0.032) increase compared to basal levels and females exhibited a 77% rise (F=12.30; P=0.005). Plasma corticosterone was also higher in females than males and this gender-related difference was found under both control (F=9.40; P=0.012) and stress conditions (F=31.12; P<0.0001) (Fig. 1b).
A significant gender difference appeared with regard to gene expression following repeated stress. Stressed males responded with stronger changes and display an opposite effect compared to stressed females. Whereas females illustrated reduced mRNA transcription (-0.0080 to –0.0252), males demonstrated a strong increase in prefrontocortical gene expression (-0.284 to 0.0594). The stress*sex interaction effect was highly significant (p=0.006) although it explains only a minimal amount of variation (~0.1%).
The expression array data was further analyzed to identify changes in genes that have been reported to modulate synaptic plasticity in the medial prefrontal areas and might be affected by prolonged stress exposure. The genes were grouped according to their biological functions and table 3 highlights a selection of the most stress-responsive genes involved in the synaptic vesicle cycle and related functions (a full list of genes can be found in a previous publication 24). In short, members of various classifications were represented including exocytosis (i.e. synapsin II, SNAP25, syntaxin 2, synaptobrevin 2), targeting (i.e. SNAP25, syntaxin 2), trafficking, intracellular kinase network (i.e. CAMKII, MAPK2, PKC) and cytoskeletal/motility (i.e. synapsin II, GAP43, myelin basic protein), all of which have been reported as synaptic function-related genes.

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* R Squared = .002 (Adjusted R Squared = .002)

Table 2.

Results of the analysis of variance generated by SPSS. Two factors were used for the analysis. Female ("sex1") and male ("sex2") rats were used, in two conditions: non-stress ("stress1") and stress ("stress2"). The effects found indicate that there are significant expression differences between male and female rats in response to stress, with about similar levels of expression in the control conditions and an increase in expression under stress compared to controls, which was stronger for males compared to females.

The expression array data was further analyzed to identify changes in genes that have been reported to modulate synaptic plasticity in the medial prefrontal areas and might be affected by prolonged stress exposure. The genes were grouped according to their biological functions and table 3 highlights a selection of the most stress-responsive genes involved in the synaptic vesicle cycle and related functions (a full list of genes can be found in a previous publication 24). In short, members of various classifications were represented including exocytosis (i.e. synapsin II, SNAP25, syntaxin 2, synaptobrevin 2), targeting (i.e. SNAP25, syntaxin 2), trafficking, intracellular kinase network (i.e. CAMKII, MAPK2, PKC) and cytoskeletal/motility (i.e. synapsin II, GAP43, myelin basic protein), all of which have been reported as synaptic function-related genes.
<table>
<thead>
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<th>TABLE 3.</th>
<th>DIFFERENTIAL GENE EXPRESSION IN PREFRONTAL CORTEX OF MALE AND FEMALE RATS EXPOSED TO CHRONIC STRESS EVALUATED BY CDNA ARRAY</th>
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Synaptic Protein Expression

Significant alterations in the expression of synaptic vesicle-associated proteins were observed following repeated stress. Although males tended to show increased expression, females generally showed tendencies towards unaltered or reduced expression levels. The specific changes appear region-specific however and vary considerably between genders.

Prefrontal Cortex

Synaptotagmin mRNA levels. Prefrontal synaptotagmin mRNA levels showed gender-distinctive stress effects as males illustrated significantly increased expression (p<0.021; F=8.198) and females a trend towards decreased expression (p<0.088; F=3.579). This opposite stress response between genders reached significance when males and females were compared under stress conditions (p<0.002; F=19.612) (fig. 2).

Synaptophysin mRNA levels. Following chronic stress, males also indicated a strong trend towards increased synaptophysin mRNA levels (p<0.053; F=4.955). Although females did not illustrate a significant stress effect, their trend towards reduced expression reached significance when compared to stressed male levels (p<0.006; F=11.932) (fig. 2).

Synapsin I mRNA levels. Although a gender effect was observed under stress conditions (p<0.005; F=12.811), males and females illustrated only a trend towards increased (p<0.079; F=3.917) and decreased (p<0.189; F=1.99) synapsin I expression respectively (fig. 2).

Synapsin II mRNA levels. In contrast to the other synaptic proteins, synapsin II expression revealed neither a stress effect nor a gender effect in the prefrontal cortex (fig. 2).

Figure 2.
Stress effects on synaptic vesicle-associated protein expression in the prefrontal cortex. Chronic stress increased synaptotagmin and synaptophysin mRNA in the prefrontal cortex of males whereas it did not significantly affect SvaP expression in females. Stressed females illustrated significantly less synaptotagmin, synaptophysin, and synapsin I mRNA levels compared to stressed males however. mRNA levels on the y axis are depicted by the inverse fractional cycle number at which the defined threshold is passed (1/ΔCT). Values are shown as mean ± SEM. * represents a significant difference between control and stress conditions (p<0.05); # represents a significant difference between male and female rats (p<0.05).
Hippocampus

**Synaptotagmin mRNA levels.** Following stress hippocampal synaptotagmin mRNA levels were increased in males (p<0.035; F=5.968) and unaltered in females such that a gender difference was only observed under stress conditions (p<0.016; F=8.503) (fig. 3).

**Synaptophysin mRNA levels.** Like synaptotagmin, synaptophysin mRNA levels were also increased in males (p<0.041; F=5.456) and unaffected in females following stress. Similarly, a gender effect was evident under stress conditions with females expressing lower levels than males (p<0.019; F=7.784) (fig. 3).

**Synapsin I mRNA levels.** Following stress, hippocampal synapsin I expression was elevated in males (p<0.014; F=8.750) as well as females although the latter did not reach significant levels (p<0.059; F=4.536) (fig. 3).

**Synapsin II mRNA levels.** In contrast to the other synaptic proteins, synapsin II expression following stress was unaltered in males as well as females. A gender effect was evident however with females expressing lower levels than males under both control (p<0.037; F=5.834) and stress conditions (p<0.004; F=14.370) (fig. 3).

![Figure 3.](image)

Figure 3.

Stress effects on synaptic vesicle associated proteins expression in the hippocampus. Chronic stress increased synaptotagmin, synaptophysin, and synapsin I mRNA in the hippocampus of males whereas it did not significantly affect SvaP expression in females. Stressed females illustrated significantly less synaptotagmin, synaptophysin, and synapsin II mRNA levels compared to stressed males however. Values are shown as mean ± SEM. * represents a significant difference between control and stress conditions (p<0.05); # represents a significant difference between male and female rats (p<0.05).
DISCUSSION

An important mechanism by which nerve cells modulate the amount of neurotransmitter release in response to discrete stimuli is through controlled coordination of the relative number of vesicles in the releasable and reserve pool 15. Action potential arrival at a synapse triggers the opening of voltage-gated Ca2+ channels, transient calcium influx into the presynaptic terminal, repolarization of the invading action potential, synaptic vesicle fusion with the presynaptic plasma membrane, exocytosis and finally neurotransmitter release 15. Stress has been reported to disturb this delicate equilibrium, affecting neurotransmitter release and causing neuronal defects 18. These observations have led to the hypothesis that stress-induced disruption of neurotransmitter release at the synaptic terminals, possibly mediated by attendant calcium overload, may play a key role in the development of neuronal impairments. In the present investigation, microarray analysis revealed the ability of chronic stress to influence transcriptional activity in a gender-related manner. Specifically, male rats illustrated a generally increased level of mRNA transcription following stress, whereas females revealed relatively decreased gene expression. This gender difference in the ability of chronic stress to affect transcriptional activity, especially in male rats, is in line with previous data illustrating sex-related dimorphism in patterns of protein expression (calcineurin), phosphorylation (phospho-CREB) and possibly neuronal activity (c-fos) in response to chronic stress (Kuipers et al., unpublished observations) 24. In support of recent studies that document atrophy of prefrontocortical dendrites in response to chronic stress 26, our findings also substantiate abnormal gene expression of prefrontocortical MAPK1/2-CREB signaling cascade members in chronically stressed rats (Table 3) 22,23. Given the pivotal role of this cascade in neuronal plasticity, the expression arrays were further analyzed to identify changes in genes that have been reported to modulate synaptic plasticity in the medial prefrontal areas and might be affected by prolonged stress exposure. Interestingly, “stress-responsive genes” underly this marked sex-dependent influence of stress on gene expression codify for selective proteins that play a central role in the regulation of specific synaptic functions including synaptophysin, synaptotagmin and synapsin I. After microarray identification of these genes, RT-PCR was applied to further investigate their specific transcriptional changes and confirm these potential targets of stress. The data suggest that stress represents a major factor in the regulation of the expression of these specific genes. More importantly, their abnormal regulation could account, at least in part, for reduced neuronal plasticity and neuronal dysfunctions observed in response to repeated stress. Synaptophysin and synaptotagmin are major integral proteins of synaptic vesicles and are essential for vesicle-cytosolic membrane fusion and neurotransmitter release 18,27-29. Similarly, synapsins belong to a family of highly conserved phosphoproteins that are specifically associated with the cytosolic surface of the synaptic vesicle membrane. By means of changes in their phosphorylation state, they control the fraction of synaptic vesicle available for release, thereby regulating synaptic vesicle life cycle 30 and the efficiency of neurotransmitter release 31. Abnormal expression of these
three intrinsic synaptic proteins is consistent with the idea that stress-induced neuronal impairments might be associated with abnormal neurotransmitter release. This is supported by the fact that several stress-related psychiatric illnesses have been associated with altered synaptic vesicular docking protein levels specifically in the hippocampal and prefrontal regions 16-18.

Given the importance of a well-regulated synaptic vesicle life cycle for efficient neurotransmitter release, stress-induced alterations in the expression of such proteins in critical brain regions thus support their potential involvement in stress-related psychopathology. Dysfunctions in prefrontocortical and hippocampal areas have been reported in response to repeated stress 23,26,32,33 and have also been associated with cognitive and emotional deficits observed in stress-related disorders such as depression 19 or schizophrenia 34. Some of the most consistent neuroimaging findings in support of this hypothesis include decreased hippocampal volume 35,36 and reduced prefrontocortical metabolism 2,37. Studies aiming to identify the molecular and cellular correlates underlying stress-induced pathology have identified both a reduction in the number of cells as well as atrophy of apical dendrites in these regions 9,26,38. Although such studies have provided suggestions for various accountable mechanisms, definite consensus on this matter is still lacking. In view of stress-mediated effects on synaptic integrity, the nature of these presynaptic components and their potential role in neurotransmitter release, an interesting possibility hence holds that stress-induced disruption of synaptic protein expression and functions may represent a critical contributing factor in the occurrence of cortical-limbic dysfunctions. Interestingly and in support of the latter, microarray analysis also revealed stress-responsivity of closely related genes such as various kinases implicated in the modulation of cytoskeleton integrity and synaptic traffic (CAMK-II), as well as various cytoskeletal proteins essential for the structural synapse integrity (GAP-43) and regulation of the synaptic vesicle cycle and exocytosis (members of SNARE complex) (table 3). Calcium/calmodulin-dependent protein kinase II (CAMKII), suggested as a potential calcium sensor, modulates the phosphorylation of synaptic proteins 15,39-41 and serves as an essential regulator of the stress response in the brain. Synaptic proteins such as GAP-43 and the SNARE complex are crucial for proper synaptic functioning as they interact with microtubules and neurofilaments, which define shape and are important for the guidance of organelles toward synaptic terminals where they accumulate 29,42. Since synaptic proteins define the basic machinery for bridging membranes, mediate the fusion between synaptic vesicle and cell membrane and, ultimately, regulate neurotransmitter release, dysregulation in their expression might account for some of the deleterious consequences of stress on the brain, including disturbed neuronal plasticity 43.

Neuronal abnormalities could thus be attributable to a stress-induced decrease in the expression of specific synaptic elements as this may impair synaptic integrity, cause abnormal neurotransmission and compromise neuronal plasticity. Considering the general increase in SVaPs expression observed in males (and unchanged levels in females) however, it is intriguing to speculate that these changes could reflect a compensatory
increase in synaptic protein transcription to recompense reduced SVaP levels lowered by the persistent exposure to adverse conditions. In schizophrenia for instance, reduced presynaptic proteins such as SNAP25, synaptophysin, and synapsins have been reported in several brain regions including the prefrontal cortex and hippocampus.\textsuperscript{16,44-47} Moreover, preclinical reports have also revealed reduced synaptophysin mRNA levels in response to stress.\textsuperscript{18} Abnormal synapsin, synaptophysin and synaptotagmin expression observed in this study may support the deleterious influence of stress on expression of these synaptic proteins. Notably, this effect was more evident in male rats, as illustrated by their increased synaptic protein expression while females demonstrated an overall lack of change. If reduced synaptic protein expression in such disorders reflects decreased synaptic integrity, it thus raises an interesting possibility that the increased expression seen in males and not females might represent gender-divergent forms of adaptation and/or sensitivity to stress. If we assume that the latter reflects gender-differential sensitivity to stress, then discrepant gene expression of synaptic proteins might represent an interesting underlying mechanism. It must also be noted however that clarity regarding the nature of these protein changes remains unclear. Despite preclinical and post-mortem studies documenting decreased SVaP expression in response to stress and psychopathology, reports of increased expression have also been made. A post-mortem study, for instance, has illustrated increased levels of SNAP25 in the hippocampus of depressed subjects, whereas preclinical investigations have shown increased synaptotagmin expression following stress.\textsuperscript{18} The present data thus illustrate the complex role of stress in regulating the transcription of these proteins. One could assume that changes in the level of expression of specific genes codifying for selective SVaPs might reflect potentially impaired neurotransmission efficacy and disrupted neuronal plasticity. Alternatively however, if increased transcription of synaptophysin, synaptotagmin and synapsin mRNA levels seen here in males reflects a compensatory adaptation to correct for decreased protein levels, then this response may also represent an important aspect of an efficacious adaptation to counter stress-induced plasticity impairments. Given the discrepancy observed in gene expression analysis in which stressed males respond with increased transcriptional activity relative to stressed females, these gender-related activity changes may also account for the increased synapsin I but not synapsin II expression observed in stressed males. High levels of synapsin II relative to synapsin I expression has been associated with excitatory synapses, while relatively high level of synapsin I expression is thought to be associated with inhibitory synapses.\textsuperscript{41,48,49} This would suggest that defects in turnover rate of synaptic vesicles at inhibitory synapses but not at excitatory synapses can produce disintegration of certain neural circuits culminating in presumable removal of inhibitory transmission as has been suggested in mutant mice lacking synapsin I.\textsuperscript{50} Considering the overall increased transcriptional activity observed in males, their enhanced synapsin I expression might thus represent a compensatory inhibitory response, to possibly limit excessive stress-induced excitation.

Together these data provide evidence for distinct gender-dependent synaptic regulatory responses to stress. More specifically these findings demonstrate that synaptic vesicle pro-
teins are stress-responsive genes that are regulated in a sex- and region-dependent manner. If negatively influenced, disturbed regulation and altered expression of these or other synaptic vesicle-associated proteins might be related to some of the physiological and pathophysiological effects of stress in the prefrontal cortex and hippocampus. In turn, stress-induced impairment of synaptic protein regulation could represent one of the molecular mechanisms underlying disorders such as depression and schizophrenia, psychiatric conditions characterized by abnormal neuronal plasticity. Although many questions remain, the investigation of putative regulators of synaptic formation in parallel with broad coverage analysis of implicated gene expression provides a useful approach to further investigate impaired synaptic functions following chronic stress. Clearly there is much to be learned about gender-related mechanisms underlying the regulation of neuronal plasticity under prolonged stressful conditions and it remains an open question whether synaptic proteins are mechanistically related to differential neurotransmission in males and females. This work provides strong indications in support of gender-distinctive synaptic protein expression upon stress and future studies will no doubt continue to elucidate the role of these synaptic protein interactions following sustained stress.

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