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Chronic corticosterone exposure alters postsynaptic protein levels of PSD-95, NR1, and synaptopodin in the mouse brain

Julia W. Cohen¹, Natalia Louneva¹, Li-Ying Han¹, Georgia Hodes¹, Robert S. Wilson², David A. Bennett², Irwin Lucki¹, and Steven E. Arnold^{1,*}

¹Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania, Philadelphia, Pennsylvania 19104

²Rush Alzheimer's Disease Center, Rush University Medical Center, Chicago, Illinois 60612

Abstract

Animal models provide compelling evidence that chronic stress is associated with biochemical and morphological changes in the brain, many of which are mediated by corticosterone, a principal glucocorticoid synthesized in the rodent adrenal cortex and secreted in response to stress. To better characterize the effects of chronic corticosterone at the synaptic and sub-synaptic level, we implanted 3-month-old male C57B/6 mice with 2 × 5 mg corticosterone pellets (CORT group, n=14), 21 day release formulation (20 mg/kg/day dose) or placebo pellets (Placebo group, n=14), 21 day release formulation. After 20 days, brains were removed. One hemisphere was frozen for biochemical analysis by synaptosomal fractionation with Western blotting, and the other hemisphere was fixed for immunohistochemistry. Localization and expression levels for PSD-95, NR1, and synaptopodin proteins were assessed. Biochemical analysis revealed lower protein levels of PSD-95 (32% decrease, $p<0.001$), NR1 (47%, $p=0.01$), and synaptopodin (65%, $p<0.001$) in the postsynaptic density subsynaptic fraction of the CORT group. Optical densitometry in immunohistochemically labeled sections also found lower levels of PSD-95 in synaptic fields of the dentate gyrus (PSD-95, 33% decrease, $p<0.001$; NR1, 31%, $p<0.001$; synaptopodin, 40%, $p<0.001$) and the CA3 stratum lucidum (36%, $p<0.001$, 40%, $p<0.001$, and 35%, $p<0.001$) of the CORT group. While mechanistic relationships for these changes are not yet known, we speculate that synaptopodin, which is involved in regulation of spine calcium kinetics and post-translational modification and transport of locally synthesized proteins, may play an important role in the changes of PSD-95 and NR1 protein levels and other synaptic alterations.

Keywords

stress; glucocorticoid; hippocampus; synaptosome; dendritic spine; postsynaptic density

INTRODUCTION

Corticosterone is the major glucocorticoid synthesized in the rodent adrenal cortex and secreted in response to stress. It has been established that glucocorticoid receptors are present in the hippocampal formation at relatively high concentrations. (Han et al., 2005; Herman et al., 1989; Reul and de Kloet, 1985; Sapolsky et al., 1983; Van Eekelen et al., 1988) Because of this and because of the important role corticosterone plays in memory and complex behaviors, the hippocampus has been a principal brain area to examine the impact

*Correspondence to: Professor of Psychiatry and Neurology 2205 Translational Research Laboratories 125 South 31st Street Philadelphia, PA 19104 USA Tel. 215-349-8225 Fax 215-349-8540 steven.arnold@uphs.upenn.edu.

of corticosteroids and the physiological response to acute and chronic stress in the brain. There have been a wide array of studies describing the behavioral, physiological, and molecular consequences of glucocorticoid action over the lifespan in this brain area. (McEwen, 2007) There are significant similarities between the effects of glucocorticoid exposure on the hippocampus and hippocampal features associated with various neuropsychiatric conditions. (Bridges et al., 2008; Sapolsky, 2001; Shin et al., 2004)

Various stress paradigms have been employed to characterize myriad effects of stress responses on the structure and function of the brain. In select subfields of the hippocampus and other brain regions, chronic stress leads to retraction of apical dendrites, loss of spines, and abnormal density and distribution of synaptic vesicles in axon terminals. The mechanisms by which these changes occur are not well understood, although roles for glucocorticoids, BDNF, NMDA and serotonin have been described. (McEwen, 1999; McEwen, 2007; McEwen and Milner, 2007; Sapolsky et al., 2000)

Among the effects of chronic corticosterone exposure are CA3 dendritic retraction, (Magarinos et al., 1999; Sousa et al., 2000; Woolley et al., 1990) reduction in hippocampal neuropil volume, alterations in LTP/LTD, and on a behavioral level, impairment of hippocampal-dependent memory tasks. (Arbel et al., 1994; Bardgett et al., 1996; Bardgett et al., 1994; Bodnoff et al., 1995; Coburn-Litvak et al., 2003; Dachir et al., 1993; Endo et al., 1996; McLay et al., 1998) We undertook this study to advance a biochemical understanding of the synaptic effects of corticosterone exposure and its impact on important components of dendrites, spines, as well as neurotransmission.

MATERIALS AND METHODS

These experiments used adult male C57BL6 mice (24-32 gm; Jackson Laboratory, Bar Harbor, ME) aged 3 months, were allowed one week to habituate in an environment controlled for temperature, lighting, and food and water administration. Animals were housed four per cage.

Animals were anesthetized using isoflurane and implanted subcutaneously either with two 5 mg corticosterone (CORT group, n=14) or two placebo pellets (Placebo group, n=14), 21 day release formulation (Innovative Research of America, Sarasota, FL), in accordance with the manufacturer's instructions. Accordingly, each corticosterone-implanted animal received a dose of 20mg/kg/day.

After 20 days, the animals were euthanized, their brains removed and bisected, with one hemisphere stored at -80°C , and the other fixed in 4% paraformaldehyde. All procedures were conducted according to IACUC-approved protocols.

Synaptosomes were prepared using a one-step synaptosome preparation based on the method of Phillips and colleagues, (Phillips et al., 2001) as described previously. (Louneva et al., 2008) In brief, synaptosomes were isolated using a sucrose gradient, in which the synaptosomal fraction forms a band at the 1.25/1.0 mol/L sucrose interface. This fraction was collected and stored at -80°C . Half of this material was used for Western blotting and another half for further fractionation.

Synaptosomes were further fractionated to separate pre- and postsynaptic elements according to previously described methods. (Louneva et al., 2008) Briefly, synaptosomes were solubilized in 20 mmol/L Tris-HCl, pH 6.0, 1% Triton X-100, 0.1 mmol/L CaCl_2 , incubated on ice for 30 minutes, and centrifuged at $40,000 \times g$, yielding the supernatant as the synaptic vesicle fraction (SV). The pellet contains the pre- and postsynaptic membranes, and this material was solubilized in 20 mmol/L Tris-HCl, pH 8.0, 1% Triton X-100, 0.1

mmol/L CaCl_2 , incubated on ice for 30 minutes, and centrifuged at $40,000 \times g$ resulting in the supernatant containing the presynaptic fraction (PrS) and the pellet containing the post synaptic density fraction (PSD). Ultimately, all subsynaptic fractions (synaptic vesicle, presynaptic, and PSD fractions) were dissolved in 5% SDS. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL).

For Western blot analysis of synaptosomes, one ml of synaptosome material was washed twice in 0.1 mmol/L CaCl_2 and twenty μg of each protein sample in Laemmli loading buffer were separated on 12% Tris-glycine gels (Novex Invitrogen, Carlsbad, CA). All 28 samples were run at one time on two gels with seven placebo and seven treated mice brains on one gel and the same design on the other gel. Fresh Western blots were run for PSD-95, NR1 and synaptopodin.

The gels were transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% milk and incubated with primary antibody overnight at 4 °C. In order to be in linear range primary antibodies were used at the following dilutions in 3% milk in Tris-buffered saline with 0.1% Tween-20: PSD-95, 1:10,000 (MAB1598; Chemicon/Millipore, Billerica, MA); NR1, 1:500 (sc-9058; Santa Cruz Biotechnology, Santa Cruz, CA), synaptopodin, 1:1,500 (S9567; Sigma, St. Louis, MO) and synaptophysin, 1:20,000 (MSB5258; Chemicon, Millipore, Billerica, MA). After primary antibody incubation, membranes were incubated with a horseradish peroxidase-coupled secondary antibody (HRP-conjugated mouse or rabbit IgG, NA931 or NA934 [Amersham, Piscataway, NJ]) for one hour at room temperature and processed with the ECL or ECL Plus chemiluminescence system (Amersham, Piscataway, NJ). All membranes were stripped and re-probed with β -actin, 1:4,000 (A3853; Sigma, St. Louis, MO) for normalization purposes. Band densities were quantified by densitometric analysis with the GS-800 calibrated densitometer and Quantity One 1-D analysis software (Bio-Rad, Hercules, CA). All data is presented in relative units (ratio protein of interest to β -actin). The same Western blot analysis was used for measurement of the 28 PSD fraction samples.

Fixed, paraffin-embedded tissues were cut in the coronal plane at 6 μm on a rotary microtome that had been tested to assure invariant section thickness and then mounted on APES-coated slides. De-waxed sections were immersed in 5% hydrogen peroxide dissolved in absolute methanol for 30 minutes to quench endogenous peroxidase activity. For antigen retrieval, the sections were boiled in 1 mmol/L ethylenediaminetetraacetic acid in 0.1 mol/L Tris buffer, pH 8.0, for 10 minutes. After cooling for 20 minutes and rinsing in water, followed by two changes of Tris-Triton (0.01% Triton X-100 in 0.1 mol/L Tris-HCl buffer, pH 7.6), sections were blocked for 45 minutes in 2% horse serum dissolved in Tris-Triton and incubated in the primary antibody, PSD-95, 1:200 (MAB1598; Chemicon/Millipore, Billerica, MA), NR1, 1:500 (sc-9058; Santa Cruz Biotechnology, Santa Cruz, CA), and synaptopodin, (1:1,000-(S9567; Sigma, St. Louis, MO); 1:4,000[163 002; Synaptic Systems, Goettingen, Germany]) overnight at 4 °C. After Tris-Triton rinses, sections were incubated in a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for an hour at room temperature. Sections were then treated for another hour at room temperature with an avidin-biotin-peroxidase complex made from a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and developed for 10 minutes in a solution containing 0.05% diaminobenzidine (Biogenex, San Ramon, CA) and 0.03% hydrogen peroxidase in Tris-Triton, supplemented with 0.25% $\text{NiSO}_4 \cdot \text{H}_2\text{O}$ to amplify the immunohistochemical signal. After clearing in xylenes, all tissue sections were coverslipped under Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

Immunolabeled slides were first qualitatively examined, and then immunoreactivity was semiquantitatively measured in the molecular layer of the dentate gyrus and the stratum

lucidum of the CA3 region of the hippocampal formation by net optical density (OD), defined as the OD of the region of interest minus the OD of the background (obtained from blood vessel walls in the regions of interest). OD analyses were performed on high resolution, gray-scale photomontages acquired on a Leica DMRBE microscope equipped with a motorized microscope stage using Image-Pro Plus software, version 6.2 (Media Cybernetics, Silver Spring, MD). OD comparisons were performed on sections photographed at the same, verified light intensity. The operator was blind to any identifying information throughout data accrual and analysis.

Statistical significance of differences between diagnostic groups was assessed with two-tailed Student's *t*-test, using JMP 7.0.2 software (SAS, Cary, NC). Statistical significance was defined as $P < 0.05$.

RESULTS

Biochemical Localization of PSD-95, NR1, and Synaptopodin in Postsynaptic Density Subsynaptic Fraction

Synaptosomal extracts were fractionated into subsynaptic compartments, and Western blotting was used to validate the efficiency of the methods (Figure 1). (Louneva et al., 2008; Phillips et al., 2001) The proteins used as markers for the different subsynaptic fractions were synaptophysin for synaptic vesicles (SV), and NR1 and PSD-95 for postsynaptic density (PSD). As expected, the synaptic vesicle fractions showed a positive signal only for synaptophysin, while PSD-95, NR1 were observed only in PSD. Synaptopodin was present in the synaptosomal extracts for all samples and exclusively concentrated in the PSD subsynaptic fractions similarly to PSD-95 and NR1.

The proteins PSD-95, NR1, and Synaptopodin were measured in synaptosomes and in PSD fractions. In the synaptosomal fractions, all three postsynaptic proteins were present at significantly lower levels (PSD-95: 23% decrease, $p=0.03$; NR1: 42%, $p=0.004$; synaptopodin: 72%, $p=0.01$), and this decrease was similarly demonstrated in the postsynaptic density subsynaptic fraction protein levels (PSD-95: 32% decrease, $p<0.001$; NR1: 47%, $p=0.01$; synaptopodin: 65%, $p=0.001$) (Figure 2, A-F).

Immunohistochemical Expression of PSD-95, NR1 and Synaptopodin in Hippocampal Formation

The cellular, laminar, and neuropil expression of PSD-95, NR1, and synaptopodin were examined in the hippocampal formation of control and CORT animals with immunohistochemistry (Figures 3-5). For all three postsynaptic proteins, immunolabeled puncta, suggestive of spines or postsynaptic densities, were distributed in neuropil of synaptic terminal fields of gray matter. Subtle sublaminal gradations of immunolabeled puncta density were evident in the dentate gyrus and CA3, the principal hippocampal formation subfields of interest. Puncta densities were greater in the inner molecular layer of the dentate gyrus compared to the outer molecular layer. In CA3, stratum radiatum and stratum moleculare exhibited more intense labeling relative to stratum lucidum. Overall, labeling intensity was visually greater in control than CORT animals in almost all instances.

Optical densitometry in immunohistochemically labeled sections verified significantly lower levels of PSD-95 in the synaptic fields of the dentate gyrus molecular layer (PSD-95, 33% decrease, $p<0.001$; NR1, 31%, $p<0.001$; synaptopodin, 40%, $p<0.001$) and the CA3 stratum lucidum (36%, $p<0.001$, 40%, $p<0.001$, and 35%, $p<0.001$) of the CORT group (Figure 6, A-C).

DISCUSSION

This study examined the effects of chronic corticosterone exposure on a panel of postsynaptic proteins concentrated in dendritic spines. Specifically, the postsynaptic proteins PSD-95, NR1, and synaptopodin were selected due to their essential roles in the structure and function of the spine apparatus.(Alvarez et al., 2007; Bourne and Harris, 2008; Deller et al., 2007; Ehrlich et al., 2007; Jedlicka et al., 2008; Kremerskothen et al., 2005; Sala et al., 2008) Both Western blotting and immunohistochemical experiments demonstrated significantly lower protein levels of PSD-95, NR1, and synaptopodin in the brains of mice with chronic corticosterone exposure. It was found by Western blotting experiments that in synaptosomes, and specifically within the postsynaptic density synaptic fraction, that there were significantly lower levels of all three proteins. By immunohistochemistry, protein expression levels in the synaptic fields of the dentate gyrus molecular layer and CA3 stratum lucidum were assessed and found to be significantly lower in the hippocampus of CORT animals.

Previous studies have characterized many regional, cellular, and subcellular structural changes in the brain in response to various stress paradigms, including chronic glucocorticoid exposure. These include atrophy of the hippocampus and other brain regions, reductions in neuron size, and reversible atrophy of dendrites and spines in some, but not all regions .(Isgor et al., 2004; Magarinos et al., 1998; McEwen, 1999; McEwen, 2007) However, the biochemical changes resulting from elevated glucocorticoid levels in the brain have been much less well characterized.

To understand the potential roles of corticosterone-induced changes in protein levels of PSD-95, NR1, and synaptopodin, it is worthwhile to consider the relationships among these proteins in mechanisms of synaptic plasticity as they relate to physiological stress responses. Neuronal NMDARs stably co-localize with PSD-95, an essential scaffolding protein of the post-synaptic density, at excitatory synapses,(Lin et al., 2006) concentrated at the tips of dendritic spine heads.(Jedlicka et al., 2008) The use of RNA interference (RNAi) to knock down NMDAR results in increased dendritic spine motility and eventual spine elimination.(Alvarez et al., 2007) In cultured hippocampal CA1 neurons, it has been found that chronic restraint stress induces loss of dendritic spines and NMDA receptor subunits.(Pawlak et al., 2005) In addition, many types of stress, both acute and chronic, suppress neurogenesis and/or cell survival in the dentate gyrus, and this suppression is partially mediated by NMDA receptors.(Cameron et al., 1998; Gould et al., 1997; Nacher and McEwen, 2006)

Our interest in synaptopodin was prompted by our recent observation that synaptopodin-immunoreactive spine density is decreased in postmortem hippocampus from humans who have experienced greater psychological distress.(Soetanto et al., 2010) Synaptopodin is an actin-binding protein that is associated with the spine apparatus of dendritic spines and also plays a role in synaptic plasticity.(Deller et al., 2000; Jedlicka et al., 2008; Mundel et al., 1997; Okubo-Suzuki et al., 2008; Vlachos et al., 2009) The dendritic spine apparatus has been implicated in local calcium trafficking,(Fifkova et al., 1983; Korkotian and Segal, 1998; Sharp et al., 1993) and spine calcium is essential to induction of synaptic plasticity.(Bliss and Collingridge, 1993) Following the induction of long-term potentiation *in vivo* of dentate granule cells of the hippocampus, there is increased synaptopodin expression, which is NMDA receptor-dependent.(Yamazaki et al., 2001)

Alpha-actinin-2, included in the spectrin/dystrophin family of actin-binding proteins, has been identified as a postsynaptic density protein colocalizing in dendritic spines with NMDA receptors and PSD-95.(Wyszynski et al., 1997) Furthermore, synaptopodin has been found to regulate the actin-bundling activity of alpha-actinin.(Asanuma et al., 2005) It has

been hypothesized that the spine apparatus modulates the delivery of AMPAR and NMDAR glutamate receptors to synapses, considering that AMPARs and NMDARs have been localized to dendritic spines and that synaptopodin can bind to synaptic NMDARs via alpha-actinin (Jedlicka et al., 2008). In such a way, synaptopodin could be a significant determinant of dendritic receptor trafficking and/or synthesis and modification and induction of LTP. While mechanistic relationships for these changes are not yet known, we speculate that synaptopodin, which is involved in regulation of spine calcium kinetics and post-translational modification and transport of locally synthesized proteins, may play an important role in the changes of PSD-95 and NR1 protein levels and other synaptic alterations that are induced by corticosterone and other chronic stress manipulations.

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Figure 1.

Western blotting demonstrates synaptic fractionation efficiency in whole mouse brain. Lanes were loaded with equal protein amounts from whole synaptosome preparation of three C57BL/6 mice (1-3) (S), postsynaptic density (PSD), presynaptic (PrS), and synaptic vesicle (SV) fractions. Blots were immunolabeled with antibodies to specific, compartmentalized synaptic protein constituents, including NR1 and PSD-95 (postsynaptic density), and synaptophysin (synaptic vesicles). Synaptopodin is here shown to be compartmentalized in the PSD fraction of synaptosomes and is a component of the postsynaptic density.

Figure 2.

Densitometric analysis (mean + SD bars) of PSD-95, NR1, and synaptopodin protein levels in synaptosomes (A-C) and PSD subsynaptic fractions (D-F) in Placebo and CORT groups. (G) Representative immunoblots of PSD subsynaptic fractions in Placebo and CORT groups.

Figure 3.

Immunohistochemical expression of PSD-95. **A** and **B**: Hippocampal formation at 4X magnification of a control C57B/6 mouse (**A**) and CORT mouse (**B**). Shown for both **A** and **B** are insets of CA3 stratum lucidum (····) (**C** and **E**) and dentate gyrus (—) (**D** and **F**) at high magnification (100X). The boxed areas shown on **A** and **B** designate the approximate area shown at high magnification in **C-F**.

Figure 4.

Immunohistochemical expression of NR1. **A** and **B**: Hippocampal formation at 4X magnification of a control C57B/6 mouse (**A**) and CORT mouse (**B**). Shown for both **A** and **B** are insets of CA3 stratum lucidum (···) (**C** and **E**) and dentate gyrus (—) (**D** and **F**) at high magnification (100X). The boxed areas shown on **A** and **B** designate the approximate area shown at high magnification in **C-F**.

Figure 5.

Immunohistochemical expression of synaptopodin. **A** and **B**: Hippocampal formation at 4X magnification of a control C57B/6 mouse (**A**) and CORT mouse (**B**). Shown for both **A** and **B** are insets of CA3 stratum lucidum (····) (**C** and **E**) and dentate gyrus (—) (**D** and **F**) at high magnification (100X). The boxed areas shown on **A** and **B** designate the approximate area shown at high magnification in **C-F**.

Figure 6. Densitometric analysis (mean + SD bars) of PSD-95 (A), NR1 (B), and synaptopodin (C) protein levels in dentate gyrus molecular layer (DGml) and CA3 stratum lucidum (sl) in Placebo and CORT groups. There are significant between group differences for all three proteins in DGml and CA3 sl.