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EFFECTS OF RESTRAINT STRESS ON THE EXPRESSION OF PROTEINS INVOLVED IN SYNAPTIC VESICLE EXOCYTOSIS IN THE HIPPOCAMPUS

Y. GAO,[a1] Y. B. BEZCHLIBNYK,[a,c] X. SUN,[a] J.-F. WANG,[a,b] B. S. McEWEN[d] AND L. T. YOUNG[a,b]*

[a]*The Vivian Rakoff Mood Disorders Laboratory, Centre for Addiction and Mental Health, 250 College Street, Toronto, ON, Canada M5T 1R8*

[b]*Department of Psychiatry, University of Toronto, Toronto, ON, Canada*

[c]*Department of Psychiatry and Behavioural Neuroscience, McMaster University, Hamilton, ON, Canada*

[d]*The Harold and Margaret Milliken Hatch Laboratory of Neuroendocrinology, The Rockefeller University, New York, NY, USA*

Abstract—Chronic restraint stress has been associated with induction of morphological changes in the hippocampus. Postsynaptically, these changes include decreased length and branching of apical dendrites from CA3 pyramidal neurons, while presynaptically, depletion and clustering of synaptic vesicles have been observed. However, the molecular correlates of these changes remain poorly defined; while some studies have identified changes in the levels of some presynaptic proteins, none have assessed the coordinate expression of components of the membrane fusion complex, including synaptobrevin, syntaxin, and synaptosomal-associated protein 25 kDa, and their major regulatory molecules synaptotagmin, synaptophysin, and synapsin. Therefore, we undertook to assess the immunoreactivity of these proteins in hippocampal slices obtained from rats subjected to either acute (one 6 h session) or chronic (21 days at 6 h per day) of restraint stress. Specifically, we observed a significant increase in synaptobrevin immunoreactivity in the inner molecular layer of the dentate gyrus (54.2%; $P=0.005$), the stratum radiatum in the CA1 subfield (55.5%; $P=0.007$), and a region including the stratum lucidum and the proximal portion of the stratum radiatum in the CA3 subfield (52.7%; $P=0.002$); we also observed a trend toward increased synaptophysin levels in the stratum lucidum/radiatum of the CA3 subfield (8.0%; $P=0.051$) following chronic, but not acute, restraint stress. In that synaptobrevin has been associated with replenishment of the "readily-releasable" pool of synaptic vesicles and the efficiency of neurotransmitter release, the present results suggest that stress-induced changes in synaptobrevin may at least in part underlie the previously observed changes in synaptic and neuronal morphology.

Key words: synaptobrevin, synaptophysin, syntaxin, SNAP-25, synaptotagmin, synapsin.

The hippocampus is a mediotemporal structure that plays a critical role in spatial learning and memory. It is characterized by a relative abundance of glucocorticoid (GC) receptors (McEwen et al., 1968; Eichenbaum, 1997; De Kloet et al., 1998; McEwen, 1999), and functions in negative feedback regulation of the stress response through inhibition of the hypothalamic–pituitary–adrenal axis (Jacobson and Sapolsky, 1991; McEwen, 1999). As such, the hippocampus is especially vulnerable to prolonged changes in the levels of GCs, for while basal levels are necessary for cellular sufficiency, chronically elevated levels lead to structural and functional alterations of cells, including long-term potentiation and depression, synaptic reorganization, synaptogenesis, neurite outgrowth or retraction, and neurogenesis (Jacobson and Sapolsky, 1991; McEwen and Sapolsky, 1995; McEwen, 1999). Indeed, it has been suggested that long-term disruption of the hippocampus by stress may impinge on its ability to regulate the stress-response, thus perpetuating the deleterious effects of GCs on hippocampal neuroanatomy (McEwen and Chattarji, 2004).

The chronic restraint stress (CRS) paradigm is an especially robust and well-characterized model for studying the effects of stress-induced morphological changes in the hippocampus. Subjecting rats to physical restraint daily for a period of 3–4 weeks leads to significant, but reversible, atrophy and reduced branching of apical dendrites on pyramidal neurons in the CA3 region (Watanabe et al., 1992c; Magarinos and McEwen, 1995; Conrad et al., 1999), and decreased neurogenesis of granule cells in the dentate gyrus (DG) (Pham et al., 2003). These changes can be blocked by GC inhibitors and *N*-methyl-D-aspartate (NMDA) receptor agonists, underscoring the roles of GCs and excitatory amino acid (EAA) neurotransmission in these phenomena (Magarinos and McEwen, 1995).

The retraction of dendritic arbors strongly suggests associated functional changes at the synaptic level. Indeed, a number of studies have described changes in synaptic plasticity, including a suppression of long-term potentiation in DG granule cells and CA3 pyramidal neu-

[1] Present address: Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China.

*Correspondence to: L. Trevor Young, Centre for Addiction and Mental Health, Room 814, 250 College Street, Toronto, ON, Canada M5T 1R8. Tel: +1-416-979-4749; fax: +1-416-260-4189.
E-mail address: trevor_young@camh.net (L. T. Young).

Abbreviations: ANOVA, analysis of variance; CA1or, CA1 stratum oriens; CA1ra, CA1 stratum radiatum; CA3lu/ra, CA3 stratum lucidum/radiatum; CA3or, CA3 stratum oriens; CA3ra, CA3 stratum radiatum; CORT, corticosterone; CRS, chronic restraint stress; DG, dentate gyrus; DGhi, hilus of the dentate gyrus; DGin, inner molecular layer of the dentate gyrus; EAA, excitatory amino acid; GC, glucocorticoid; NMDA, *N*-methyl-D-aspartate; OD, optical density; PBS, phosphate-buffered saline; ROI, region of interest; SNAP-25, synaptosomal-associated protein 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor.

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rons (Pavlides et al., 1993, 2002), and altered number and density of dendritic spines (Sunanda et al., 1995; Sandi et al., 2003; Stewart et al., 2005), subsequent to CRS. Moreover, Magarinos et al. (1997) observed a striking reorganization of synaptic vesicles within mossy fiber terminals, including an apparent depletion of these vesicles, and increased packing density of clear synaptic vesicles about the active zone within these terminals. Synaptic vesicles are the intracellular organelles responsible for neurotransmitter release, a process that involves a number of specialized proteins involved in targeting vesicles to the active zone, mediating vesicle fusion with the presynaptic membrane, and various regulatory proteins that act to control these events. As such, the above studies strongly suggest corresponding changes in the molecular mechanisms of intercellular signaling.

With the aim of identifying the molecular correlates of previously observed ultrastructural, functional, and synaptic changes subsequent to restraint stress, we undertook to evaluate the immunohistochemical expression of critical components of synaptic vesicle exocytosis. Synapsin is believed to anchor synaptic vesicles to the cytoskeletal framework of the presynaptic terminal, thus enabling vesicles to cluster near release sites in the reserve pool (Valtorta et al., 1992; Greengard et al., 1993). Syntaxin and synaptosomal-associated protein 25 kDa (SNAP-25) are integral membrane proteins localized to the presynaptic membrane. Together with synaptobrevin/vesicle-associated membrane protein, an integral membrane protein localized to synaptic vesicles (Sollner et al., 1993; Rothman, 1994), these proteins constitute the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Although formation of the fusion complex is sufficient to allow for a certain level of constitutive membrane fusion events, regulated release occurs when calcium concentrations in the cell rise during an action potential. Synaptotagmin, another vesicle-associated integral membrane protein, detects elevations in calcium concentration that provide the final force necessary to overcome the energy barrier of lipid rearrangement during the fusion process. Finally, synaptophysin is a synaptic vesicle-associated integral membrane protein that regulates the assembly of the SNARE complex by binding to synaptobrevin, thereby preventing its binding to syntaxin and SNAP-25. This is thought to preclude synaptobrevin from entering non-functional SNARE complexes with itself or other SNARE proteins localized to the same membrane (Hinz et al., 2001). Moreover, it has been widely used as a general marker protein of pre-synaptic nerve endings in previous studies, some of which have observed changes in its expression subsequent to various stressors or chronic administration of corticosterone (CORT) (Thome et al., 2001; Xu et al., 2004; Grillo et al., 2005). Herein, we analyzed the coordinate expression of these proteins in the hippocampus of rats subjected to either acute or chronic (21 days) of restraint stress.

EXPERIMENTAL PROCEDURES

Subjects

Male Sprague–Dawley rats (Charles River Canada, St. Constant, QC, Canada) weighing 275–300 g were housed individually in standard-sized rectangular polypropylene cages contained within a micro-isolator unit. The unit was located in a colony room maintained at a constant temperature of 22±1 °C, with a standard 12-h light/dark cycle (lights on 07:00 h, lights off 19:00 h) with food and water freely available. All rats were allowed to acclimatize to the laboratory facility for one week, during which time they were handled three to five times to allow habituation to the experimenter. All manipulations were carried out during the light period of the light/dark cycle. All experiments conformed to the guidelines of the Centre for Addiction and Mental Health Animal Care Committee and the Canadian Council on Animal Care. All efforts were made to minimize stress to animals and the number of animals used.

Restraint stress

Restraint stress was conducted using wire-mesh restrainers secured at either end with bulldog clips as previously described (Watanabe et al., 1992c), in the home cage within the micro-isolator unit. These restrainers can be adjusted to conform to the rat's body size throughout the experiment, while permitting only minimal movement. Rats were randomly assigned to one of four groups: 1) CRS (n=10), 2) acute restraint stress (n=10), or non-stressed rats matched to animals in the 3) chronic (n=8) or 4) acute stress groups (n=8). In the CRS experiments, rats were restrained for 6 h/day for a period of 21 days. During this period, rats in the non-stressed groups were handled once daily. In the acute restraint stress experiments, rats were restrained once for 6 h. Eight additional non-stressed rats were used as controls, and were removed from their cages and handled simultaneously to the application of acute stress. Food and water intake was monitored and all rats were weighed daily.

Eighteen hours after the final (or single in the acute experiments) restraint, all animals were anesthetized with sodium pentobarbital (Somnotol; 75 mg/kg i.p.) and perfused transcardially with 100 mM phosphate-buffered saline (PBS; pH 7.5) at room temperature, followed by 4% paraformaldehyde in PBS. The brains were removed from the skull, postfixed overnight in the same fixative at 4 °C, and transferred into 30% sucrose until the brains sank to the bottom of the container. Subsequently, brains were transferred into cold 2-methylbutane (Sigma, St. Louis, MO, USA) and stored at −80 °C. Sections were obtained from the dissected brains using a cryostat maintained at −20 °C. Brains were cut into coronal slices 20 μm thick, and the slices placed in cryoprotectant (50 mM PBS, 25% ethylene glycol, 25% glycerol). Subsequently, sections were mounted on superfrost/plus slides (Fisher Scientific, Ottawa, ON, Canada) such that each slide contained sections from both control and stress groups, in order to ensure equivalent staining. The slides were then air-dried for 1 h at 42 °C, washed with PBS three times, and immediately used in the immunohistochemistry experiments as described below; shrinkage due to fixation was evaluated at approximately 30% for each section.

Immunohistochemistry

Sections were washed three times in PBS for 5 min, exposed for 2 min to boiling 0.01 M citric acid in a microwave to retrieve antigens, and incubated for 10 min in 0.1% H_2O_2/methanol to block endogenous peroxidase activity. Next, the sections were pre-incubated for 1 h with 2.5% normal horse serum at room temperature. Subsequently, serial sections were incubated overnight at 4 °C with the appropriate primary antibody (all obtained from Chemicon International, Inc., Temecula, CA, USA), diluted in