Neuroscience 236 (2013) 47–54

THE DIFFERENTIAL EFFECTS OF ACUTE VS. CHRONIC STRESS AND THEIR COMBINATION ON HIPPOCAMPAL PARVALBUMIN AND INDUCIBLE HEAT SHOCK PROTEIN 70 EXPRESSION

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Abstract—The hippocampus plays a central role in stress-related mood disorders. The effects of acute vs. chronic stress on the integrity of hippocampal circuitry in influencing the vulnerability to, or resiliency against, neuronal injury are poorly understood. Here we investigated whether acute vs. chronic psychosocial isolation stress or a combination of the two (chronic stress followed by acute stress) influences the expression of the interneuronal marker parvalbumin (PV) and the chaperone-inducible heat shock protein 70 (Hsp70i) in different subregions of the hippocampus. Low levels of the Ca^{2+}-binding protein (PV) may increase the vulnerability to neuronal injury, and Hsp70i represents an indicator of intense excitation-induced neuronal stress. Adult male Wistar rats were exposed to 2 h of immobilization (IM) or cold (4 °C) (acute stressors), 21 d of social isolation (chronic stress), or a combination of both acute and chronic stress. Both chronic isolation and the combined stressors strongly decreased the PV-immunoreactive cells in the CA1, CA3 and dentate gyrus (DG) region of the hippocampus, while acute stress did not affect PV expression. The combination of acute and chronic stress induced a dramatic increase in Hsp70i expression in the DG, but Hsp70i expression was unaffected in acute and chronic stress alone. We also monitored serum corticosterone (CORT) levels as a neuroendocrine marker of the stress response. Acute stress increased CORT levels, while chronic isolation stress compromised hypothalamic–pituitary–adrenocortical (HPA) axis activity such that the normal stress response was impaired following subsequent acute stress. These results indicate that in contrast to acute stress, chronic isolation compromises the HPA axis and generates a considerable reduction in PV expression, representing a decrease in the calcium-buffering capacity and a putatively higher vulnerability of specific hippocampal interneurons to excitotoxic injury. The induction of Hsp70i expression in response to acute and chronic isolation reveals that neurons in the DG are particularly vulnerable to an acute stressor following a chronic perturbation of HPA activity. **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: chronic social stress, vulnerability, neuroprotection, parvalbumin, Hsp70i, dentate gyrus.

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Abbreviations: ANOVA, analysis of variance; C, cold stress; CORT, corticosterone; DG, dentate gyrus; GC, Glucocorticoid; HPA, hypothalamic–pituitary–adrenocortical; Hsp70i, inducible heat shock protein 70; IM, immobilization; IS, chronic isolation; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PV, parvalbumin.

INTRODUCTION

Stressful life events are a major predisposing risk factor for the development of depression (McEwen, 2005). Patients suffering from depression often display profound neuroendocrine alterations, such as hypo- or hypercortisolism, resulting from dysregulation of the hypothalamic–pituitary–adrenocortical (HPA) axis (de Kloet et al. 1998; Holsboer and Ising, 2010). Glucocorticoids (GCs) are the effectors of the HPA axis and mediate the response of an organism to stress. The effects of GCs following acute stress can be classified as adaptive and protective (Munck et al., 1984; McEwen, 2000a,b; Sapolsky et al., 2000), while chronic stress, especially chronic psychological stress, is primarily maladaptive. Numerous studies have demonstrated that stress may affect the hippocampal GABAergic system (Bowers et al., 1998; Orchinik et al., 2001). For example, both preclinical and clinical studies suggest that an abnormal GABAergic system is part of the pathophysiology of depressive disorders (Sanacora et al., 1999; Krystal et al., 2002; Brambilla et al., 2003; Gronli et al., 2007). In animal studies, changes in the amino acid-based neurotransmitter systems in the learned helplessness model of depression have shown that an increased ratio of glutamate/GABA in the hippocampus may lead to defective neuroprotection against excitotoxicity and thus contribute to the disorder (Sartorius et al., 2007).

Parvalbumin (PV)-containing cells represent a subpopulation of GABAergic interneurons that have been demonstrated to provide the most powerful inhibitory perisomatic input to principal cells (Freund, 2003). In the dentate gyrus (DG) and CA3 region of the hippocampus, PV-containing interneurons receive most of their excitatory input from granule cells (Seress et al., 2001). Thus, PV-positive interneurons are subjected to the deleterious effects of excessive excitatory amino acid release from mossy fiber terminals during stress exposure (Magarinos et al., 1997). Many studies have

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http://dx.doi.org/10.1016/j.neuroscience.2013.01.033

shown stress-induced effects on the morphology and number of principal neurons of the hippocampus (Sapolsky et al., 1985; Uno et al., 1989), but few data are available regarding alterations in interneurons (Czeh et al., 2005).

In response to various stress conditions, cells synthesize a group of highly-conserved proteins called heat shock proteins (Hsps) that are classified into families based on their molecular weight (Lindquist and Craig, 1998). Hsp70 is involved in cellular repair and other protective mechanisms (Georgopoulos and Welch, 1993; Morimoto et al., 1994; Lindquist and Craig, 1998). Inducible Hsp70 (Hsp70i) is an inducible form of Hsp70 triggered by physiological, pathological and environmental stressors (Kiang and Tsokos, 1998). Interestingly, Hsp70i induction protects neurons from apoptosis (Kelly et al., 2002; Arieli et al., 2003; Belay and Brown, 2003), as overexpression of Hsp70 attenuates caspase-dependent and caspase-independent pathways and inhibits neuronal apoptosis (Sabirzhanov et al., 2012). Elevated expression of the Hsp70i protein also occurs in specific brain regions after the administration of *N*-methyl-D-aspartate (NMDA) receptor antagonists. In these areas, including the retrosplenial cortex, the increased expression of Hsp70i has been demonstrated to correlate with its reversible neurotoxic effect, thus Hsp70i has been referred to as an immunohistochemical "marker of neuronal injury" (Sharp et al., 1992; Inta et al., 2012). Nevertheless, Hsp70i expression has clear neuroprotective effects under conditions of intense stress.

The aim of the present study was to investigate whether acute vs. chronic stress, or a combination of the two, influences the expression of PV and Hsp70i, markers associated with the vulnerability to injury in the hippocampal formation. The 21 d of chronic psychosocial isolation was used as a model of potential maladaptive stress, while the combination of stressors was used to examine whether chronic stress resulted in irreversible alterations in the stress response.

EXPERIMENTAL PROCEDURES

Animal treatments

Adult male Wistar rats (2–3 months old, body weight 330–400 g) were housed in groups of four per cage in a temperature-controlled environment (21–23 °C) on a 12-h/12-h light/dark cycle (lights on between 07:00 h and 19:00 h), with food (commercial rat pellets) and water available *ad libitum*. All procedures complied with the European Communities Council Directive (86/609/EEC) and were approved by the Ethics Committee for the Use of Laboratory Animals according to the guidelines of the EU-registered Serbian Laboratory Animal Science Association (SLASA). Animals were randomly divided into four groups. Group I comprised of the unstressed animals (control group, $n = 6$). Group II was exposed to one of two acute stressors, either 2 h of immobilization (IM) or cold (C, 4 °C) stress ($n = 6$ per stressor). Group III was exposed to chronic isolation via individual housing for 21 d (IS, $n = 6$–7). In this condition, animals had relatively normal auditory and olfactory experiences but no visual or tactile exposure to other animals. Group IV was exposed to chronic IS followed by a single 2-h exposure to acute stress, either IM or C (4 °C), representing the combined stressors (IS + IM, IS + C, $n = 6$, respectively). Experiments with acute stressors were performed between 8:00 and 10:00, in order to minimize possible hormonal interference by circadian rhythms. Rats were exposed to IM by introducing them in a prone position with all four limbs fixed to a board with adhesive tape. The head was fixed with a metal loop over the neck area to limit head movement (Kvetnansky and Mikulaj, 1970). Animals exposed to C stress were initially kept at an ambient temperature (20 ± 2 °C) and then carefully transferred into a cold room at 4 °C. Following the stress procedure, stressed animals and controls were deeply anesthetized with ketamine/xylazine 100/20 mg/kg (i.p.), transcardially perfused with 0.9% physiological saline followed by 4% paraformaldehyde (pH 7.4), and then sacrificed by guillotine decapitation (Harvard Apparatus, South Natick, MA, USA). The brains were removed and post-fixed overnight in the same solution at 4 °C.

Serum corticosterone assay

Trunk blood was collected and the serum obtained by centrifugation at 1500*g* for 10 min at 4 °C, was kept at −70 °C until assay. The blood for the controls and all experimental groups was collected within 3 min of touching the cage. The OCTEIA corticosterone enzyme-linked immunosorbent assay (ELISA) kit (REF AC-14F1; Immunodiagnostics Systems-IDS, UK IDS) was used for measuring serum corticosterone (CORT) levels (ng/ml) in all experimental groups. All samples were analyzed in a single assay to avoid inter-assay variation. The variation between duplicates of samples was less than 7%. The lower detection limit for CORT in this assay system was 25 ng/ml.

Immunohistochemistry

Immunohistochemical stainings were carried out to reveal PV- and Hsp70i-positive cells. Brains were cut into coronal sections of 40-μm thickness on a vibratome (VT 100 S; Leica, Bensheim, Germany), and the sections were processed using immunofluorescence. After three washes in phosphate-buffered saline (PBS), pH 7.4, the sections were incubated for 1 h at room temperature in a blocking solution containing 3% normal donkey serum and 0.3% Triton X-100 in PBS. The slices were then incubated with primary antibody prepared in blocking solution (anti-PV, polyclonal rabbit, SWANT, 1:1000) overnight at 4 °C, followed by incubation with Alexa-Fluor 555-conjugated anti-rabbit secondary antibodies (Molecular Probes, Invitrogen, Eugene, OR) for 2 hr at room temperature. After three washes in PBS, pH 7.4, sections were mounted on gelatin-coated glass slides and coverslipped with fluorescent mounting medium (Dako). For Hsp70i immunohistochemistry, slices were incubated with primary antibody prepared in blocking solution (anti-Hsp70i, monoclonal mouse; Enzo Life Sciences, 1:1000) overnight, followed by incubation with a secondary antibody (biotinylated IgG, Jackson ImmunoResearch) for 2 h at room temperature and then with the standard avidin–biotinylated peroxidase complex (ABC kit, Vector Laboratories) for 1 h at room temperature. Peroxidase activity was revealed by 0.02% diaminobenzidine with 0.01% H_2O_2 and enhanced with 0.04% nickel ammonium sulfate. Finally, the sections were placed on gelatinized glass slides and coverslipped with Eukit.

Statistical evaluation

CORT levels and the number of PV- and Hsp70i-expressing cells were analyzed using a two-way analysis of variance (ANOVA) [the factors were acute (levels: none, IM and C) or chronic (levels: no stress and isolation (IS) stress]. Duncan's *post hoc* test was used to evaluate differences between groups.

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