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EXPRESSION OF FIBROBLAST GROWTH FACTOR-2 AND BRAIN-DERIVED NEUROTROPHIC FACTOR MRNA IN THE MEDIAL PREFRONTAL CORTEX AND HIPPOCAMPUS AFTER UNCONTROLLABLE OR CONTROLLABLE STRESS

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Abstract—Neurotrophic factors, including basic fibroblast growth factor (FGF-2) and brain-derived neurotrophic factor (BDNF) are known to be affected by exposure to stressful experiences. Here, we examine the effects of behaviorally controllable (escapable tailshock, ES) or uncontrollable (inescapable tailshock, IS) stress on the expression of FGF-2 and BDNF mRNA in subregions of the medial prefrontal cortex (mPFC) and the hippocampal formation (HF) of male Sprague–Dawley rats. ES rats were placed in Plexiglas boxes equipped with a free spinning wheel and IS rats were placed in identical boxes with the wheels fixed. ES and IS rats were yoked such that they received the same tailshocks, but the ES rat could terminate each shock for both rats. No stress controls (NS) remained in their home cages. Rats were killed 0, 2, 24, or 72 h after termination of the stress session. *In situ* hybridization was performed to measure FGF-2 and BDNF mRNA in the mPFC and HF. In the mPFC, ES produced a significant increase in FGF-2 mRNA expression at 0 and 2 h post-stress. In the HF, ES produced a greater increase in FGF-2 mRNA expression than IS and NS only in CA2. ES also produced an increase in BDNF mRNA expression in the anterior cingulate at 0 h post-stress. No effects of stressor controllability on BDNF were observed in the HF, although both ES and IS decreased BDNF mRNA in the DG. FGF-2 in the mPFC may be involved in emotional regulation ("coping") during stressful experiences. **© 2006 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: stress, learned helplessness, prefrontal cortex, hippocampus, growth factors, in situ hybridization.

A perceived lack of control over adverse experiences has been proposed to be a predisposing factor in the development of stress-related disorders such as post-traumatic stress disorder (PTSD) (Livanou et al., 2002; Van Der Kolk, 2006) and depression (Wardle et al., 2004). Work with animal models has revealed that behavioral control over a stressor can confer resilience to many of the negative effects of stressor exposure (Maier and Seligman, 1976). The influence of stressor controllability has been studied in animals by comparing the effects of escapable shocks (ES) with exactly identical yoked inescapable shocks (IS) (Maier, 1984). IS produces a constellation of behavioral changes that do not follow ES, a phenomenon called learned helplessness (Maier and Seligman, 1976). Electric shocks are used as the stressor because it is difficult to manipulate the behavioral control that an organism has over other stressors in a manner such that the animals with and without control are exposed to physically identical events. Inhibition of the medial prefrontal cortex (mPFC) during exposure to ES or IS completely blocks the protective effects of control over the stressor on subsequent behaviors that are known to be differentially affected by IS and ES (Amat et al., 2005), suggesting that the mPFC mediates the protective effects of control. The hippocampal formation (HF) is also differentially affected by IS, compared with ES. We have observed that IS (but not ES) reduces neurogenesis in the HF, and that ES (but not IS) increases protein levels of the neurotrophic factor basic fibroblast growth factor (FGF-2) in the HF (Bland et al., 2006). Moreover, uncontrollable swim stress reduces LTP in the CA1 region of the HF to a greater extent than does controllable swim stress (Kavushansky et al., 2006).

Neurotrophic factors, including both FGF-2 and brain-derived neurotrophic factor (BDNF), have been implicated in stress-related mood disorders such as depression (Duman and Monteggia, 2006; Riva et al., 2005), and FGF-2 mRNA is decreased in the HF of patients with major depressive disorder (MDD) (Gaughran et al., 2006). FGF-2 (Molteni et al., 2001a) and BDNF (Smith et al., 1995) are known to be sensitive to acute stressor exposure, in particular in the hippocampus. For example, FGF-2 mRNA levels increase in the HF, but not the PFC, in response to acute restraint stress (Molteni et al., 2001a). Hippocampal BDNF has been proposed to be involved in mediating the effects of IS and learned helplessness (Shirayama et al., 2002; Vollmayr et al., 2001).

Less is known about how stress influences neurotrophic factor expression in the mPFC, or how neurotropic factors in the mPFC might be influenced by the controllability of the stressor. We (Bland et al., 2005) have previ-

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Abbreviations: AC, anterior cingulate; BDNF, brain-derived neurotrophic factor; DG, dentate gyrus; ES, escapable shock; FGF, fibroblast growth factor; HF, hippocampal formation; IL, infralimbic; IS, inescapable shock; mPFC, medial prefrontal cortex; NS, no stress; PL, prelimbic; SSC, saline sodium citrate.

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ously observed that in male (but not female) rats BDNF mRNA expression is increased in the mPFC after IS. BDNF mRNA expression is also known to increase in the mPFC in response to restraint (Molteni et al., 2001b) and immobilization stress (Lee et al., 2006), but acute restraint stress does not alter FGF-2 mRNA levels in the PFC (Fumagalli et al, 2005). However, the role of FGF-2 in the PFC is of particular clinical interest because patients with major depressive disorder exhibit decreased FGF-2 mRNA in both the dorsolateral PFC and the anterior cingulate (AC), and this decrease is attenuated by antidepressant treatment (Evans et al., 2004). The mPFC is known to mediate the impact of stressor controllability on both brain and behavior (Amat et al., 2005), yet no studies to date have examined the impact of stressor controllability on the expression of neurotrophins in the mPFC.

To investigate the effects on controllable and uncontrollable stressors on expression of FGF-2 and BDNF mRNA in subdivisions of the HF and mPFC, we exposed rats to yoked IS or ES or to no-stress control treatment (NS). Rats were killed 0, 2, 24, or 72 h after the end of the stressor session. Using *in situ* hybridization we examined BDNF and FGF-2 mRNA expression in the AC, prelimbic (PL), and infralimbic (IL) regions of the mPFC and in the CA1, CA2, and dentate gyrus (DG) subfields of the HF. Despite the interest in BDNF and FGF-2, there are no prior studies that have directly compared the effects of physically equated controllable and uncontrollable stressors on these mRNAs.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were 75 male Sprague–Dawley rats (Harlan Laboratories, Madison WI, USA) weighing 275–325 g. and housed two per cage on a 12-h light/dark cycle (on at 07:00 and off at 19:00 h). Experiments were conducted between 08:00 and 12:00 h. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder; approvals can be provided upon request. Procedures were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). All efforts were taken to minimize the number of animals used and to minimize suffering.

Wheel-turn escape/yoked IS procedure

Each rat was placed in a Plexiglas box (14×11×17 cm) with a wheel mounted in the front and a Plexiglas rod extending from the back. The rat's tails were taped to the Plexiglas rod and affixed with copper electrodes. Rats received shocks in yoked pairs (ES and IS). The treatment consisted of 80 trials with an average inter-trial interval of 60 s. Shocks began simultaneously for both rats in a pair and terminated for both whenever the ES rat met a response criterion. Initially the shock was terminated by a one-quarter turn of the wheel. The response requirement was increased by one-quarter turn when each of three consecutive trials was completed in less than 5 s. Subsequent latencies under 5 s increased the requirement by 50% up to a maximum of four full turns. If the requirement was not reached in less than 30 s, the shock was terminated and the requirement reduced to a single one-quarter turn. This procedure was used to insure that the ES animals learned an operant response. Any rat (along with its yoked partner) that did not learn the response within 10 trials was eliminated from the experiment. Shock intensity was 1.0 mA for the first 27 trials, 1.3 mA for the second 27 trials, and 1.6 mA for the last 26 trials. We have previously used this procedure (Amat et al., 2005; Bland et al., 2006) to insure that ES rats do not habituate to the shocks and so continue to turn the wheel. Using this procedure, no rats failed to adequately learn the escape response. Non-shocked control (NS) rats remained undisturbed in the colony.

Tissue collection

Rats were killed 0, 2, 24, or 72 h following the stress session, or at equivalent times for the NS controls. Rats in the 2, 24, and 72 h groups were returned to their home cages before kill. All animals were assigned to groups as cage-mate pairs such that, upon removal from or return to the home cage, no rat was left isolated. Rats were killed by rapid decapitation. Trunk blood was collected in heparinized tubes and placed in wet ice. Brains were immediately removed, rapidly frozen in dry ice chilled isopentane, and placed in a −80 °C freezer for later analysis. Brain sections (10 μm) were taken using a −20 °C cryostat and thaw mounted on poly-L-lysine-coated slides. Brain sections were returned to the −80 °C freezer after thaw-mounting.

In situ hybridization

Sections were fixed in a buffered 4% paraformaldehyde solution for 1 h at room temperature. Slides were washed in 2× saline sodium citrate (SSC) and acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min to minimize nonspecific hybridization by reducing positive charge on tissue and poly-lysine coated slides. Slides were then washed again in distilled water, dehydrated in a series of graded ethyl alcohol concentrations, and air dried. ^{35}S-UTP/^{35}S-CTP-labeled cRNA probes were generated for FGF-2 or BDNF mRNA from cDNA subclones in transcription vectors using standard *in vitro* transcription methodology. FGF-2 and BDNF cDNA was kindly provided by Dr. Huda Akil (University of Michigan School of Medicine, Ann Arbor, MI, USA) and Dr. James Herman (University of Cincinnati Medical Center, Cincinnati, OH, USA), respectively.

To generate ^{35}S-labeled complementary RNA to FGF-2 OR BDNF mRNA, 1 μg of linearized plasmid DNA; 1× T3 transcription buffer (Promega Corp., Madison, WI, USA); 125 μC of ^{35}S-UTP (Amersham/GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA); 4 μl of H_2O; 12.5 mM dithiothreitol (DTT); 150 μM GTP, CTP, and ATP (Promega Corp.); 20 U of RNase inhibitor (Promega Corp.); and 6 U T3 polymerase (Promega Corp.) in a total volume of 25 μl were incubated for ~2 h at 37 °C. To isolate the complete complementary RNA from single nucleotides, a Sephadex G50-50 column was used. The ^{35}S-labeled probe was diluted in hybridization buffer to yield an approximate concentration of 1×10^6 cpm/65 μl. The hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 2× SSC, 50 mM sodium phosphate buffer (pH=7.4), 1× Denhardt's solution, and 0.1 mg/ml yeast tRNA. The radiolabeled probe/hybridization mixture (65 μl) was applied to each slide, and sections were coverslipped. Slides were placed in covered plastic boxes lined with filter paper moistened with 50% formamide/50% H_2O and incubated for 12–16 h at 55 °C. Coverslips were floated off in 2× SSC, and slides were rinsed three times in 2× SSC. Slides were incubated in RNase A (200 μg/ml) for 60 min at 37 °C, followed by successive washes in 2×, 1×, 0.5×, and 0.1× SSC for 2–3 min each, with an additional incubation in 0.1× SSC for 60 min at 70 °C. Slides were rinsed in distilled H_2O, dehydrated in alcohols, and exposed to Biomax MR X-ray film (Kodak, Rochester, NY, USA) for approximately 8 days.

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