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DISTRIBUTION OF FOS-IMMUNOREACTIVE CELLS IN RAT FOREBRAIN AND MIDBRAIN FOLLOWING SOCIAL DEFEAT STRESS AND DIAZEPAM TREATMENT

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Abstract—The anxiolytic diazepam selectively inhibits psychological stress-induced autonomic and behavioral responses without causing noticeable suppression of other central performances. This pharmacological property of diazepam led us to the idea that neurons that exhibit diazepam-sensitive, psychological stress-induced activation are potentially those recruited for stress responses. To obtain neuroanatomical clues for the central stress circuitries, we examined the effects of diazepam on psychological stress-induced neuronal activation in broad brain regions. Rats were exposed to a social defeat stress, which caused an abrupt increase in body temperature by up to 2 °C. Pretreatment with diazepam (4 mg/kg, i.p.) attenuated the stress-induced hyperthermia, confirming an inhibitory physiological effect of diazepam on the autonomic stress response. Subsequently, the distribution of cells expressing Fos, a marker of neuronal activation, was examined in 113 forebrain and midbrain regions of these rats after the stress exposure and diazepam treatment. The stress following vehicle treatment markedly increased Fos-immunoreactive (IR) cells in most regions of the cerebral cortex, limbic system, thalamus, hypothalamus and midbrain, which included parts of the autonomic, neuroendocrine, emotional and arousal systems. The diazepam treatment significantly reduced the stress-induced Fos expression in many brain regions including the prefrontal, sensory and motor cortices, septum, medial amygdaloid nucleus, medial and lateral preoptic areas, parvicellular paraventricular hypothalamic nucleus, dorsomedial hypothalamus, perifornical nucleus, tuberomammillary nucleus, association, midline and intralaminar thalami, and median and dorsal raphe nuclei. In contrast, diazepam increased Fos-IR cells in the central amygdaloid nucleus, medial habenular nucleus, ventromedial hypothalamic nucleus and magnocellular lateral hypothalamus. These results provide important information for elucidating the neural circuitries that mediate the autonomic and behavioral responses to psychosocial stressors.

Key words: emotion, mapping, psychological stress-induced hyperthermia, psychogenic fever, psychosocial stress, stress circuit.

INTRODUCTION

Psychological stress induces a variety of autonomic, neuroendocrine and behavioral responses. The development of stress responses involves central neural processes to perceive and integrate stress signals as well as to output autonomic and somatic (behavioral) motor commands to cope with stressors. However, the brain circuitry mechanism underlying stress responses is poorly understood. With the recent introduction of optogenetics, it is now possible to manipulate activities of specific populations of neurons *in vivo*, which is a powerful approach for probing the central neural circuitries recruited for stress responses (Sparta et al., 2013). However, a prerequisite to those optogenetic strategies is to know if the activity of the neurons of interest is indeed affected by stress signals under physiological conditions. A mapping of stress-activated neurons in the brain would provide candidate brain regions and neuronal populations that can be analyzed in such physiological studies to determine their functions in the central stress mechanism.

Social defeat stress has been widely used as a rodent stress model that is caused by social interaction and as such may be closer to stress in human society than more artificial stressors, such as needle injection, restraint or placement into a new cage (Vinkers et al., 2009), although it is not entirely analogous to heterogeneous interpersonal conflicts in human social conditions (Huhman, 2006). This stress model has become more widely used to determine the pattern of stress-induced neuronal activation in the brain (Kollack-Walker and

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Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; BAT, brown adipose tissue; BNST, bed nucleus of the stria terminalis; IR, immunoreactive; LPS, lipopolysaccharide; PG, prostaglandin; PSH, psychological stress-induced hyperthermia; T_c, body core temperature.

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Newman, 1995; Matsuda et al., 1996; Kollack-Walker et al., 1997; Martinez et al., 1998; Chung et al., 1999; Miczek et al., 1999). However, the results from these studies are inconsistent, probably due to non-psychogenic effects caused by the variations in the procedure, conditions and species (reviewed by Martinez et al., 2002).

Among responses induced by acute psychological stress, an increase in body temperature, known as psychological stress-induced hyperthermia (PSH), is a basic autonomic stress response observed in many mammalian species (Oka et al., 2001). Although most of such acute stress responses are beneficial in stress coping, intense and long-lasting stressors often cause stress disorders and mental illness, such as psychogenic fever and depression (de Kloet et al., 2005; Oka and Oka, 2012). For the treatment of stress-related symptoms, benzodiazepine anxiolytics, represented by diazepam, have been clinically used (Ashton, 1994). We have shown that systemic administration of diazepam in rats reduces hyperthermia induced by social defeat stress (Lkhagvasuren et al., 2011). Diazepam may exert such clinical and experimental effects through its nonselective action facilitating GABAergic inhibition of neurons that occurs ubiquitously in broad brain areas. However, clinical dosage of diazepam highly selectively alleviates psychogenic symptoms without causing noticeable suppression of other central performances (e.g., sensory and motor systems). This pharmacological property of diazepam indicates that this drug selectively influences the neuronal populations that function in the central stress mechanism. Therefore, neurons that exhibit diazepam-sensitive, stress-induced activation in the brain are potentially involved in the central stress mechanism, and the distribution of such neurons would be important information for understanding not only the central stress circuitries for the development of PSH, but also those for many other autonomic, behavioral and neuroendocrine stress responses.

In the present study, we sought to identify the patterns of neuronal activation in rat forebrain and midbrain following social defeat stress and/or diazepam administration using immunohistochemistry for Fos, a marker of neuronal activation (Sagar et al., 1988). The stress-relief effect of diazepam in the animals used for this Fos study was physiologically confirmed by monitoring stress-induced increases in their body core temperature (T_c) following diazepam administration. Then, we performed detailed quantification of Fos expression in 113 forebrain and midbrain regions and classified them into four types based on the patterns of Fos expression after the stress/diazepam treatments: (1) stress increased Fos expression, which was decreased by diazepam; (2) stress increased Fos expression, which was unaffected by diazepam; (3) stress increased Fos expression, which was further increased by diazepam; and (4) stress did not increase Fos expression.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats weighing 190–290 g and male Long-Evans rats weighing 400–550 g (SLC, Kurume, Japan) were used as intruders and residents, respectively. Wistar rats were individually caged and Long-Evans rats were pair-caged with age-matched females. Both strains were housed in separate rooms air-conditioned at 24 ± 2 °C with a standard 12-h light–dark cycle (lights on 7:00–19:00 h) and allowed *ad libitum* access to food and water.

All procedures conformed to the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and to those of the NIH in the USA regarding the care and use of animals for experimental procedures, and were approved by the Ethics Committees of Kyushu University (A22-165-0) and by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyo11095).

Surgery and body temperature monitoring

We measured T_c of Wistar rats using a telemetry system (Data Sciences International, St Paul, MN, USA). A battery-operated telemetric transmitter (TA10TA-F40) was implanted into the peritoneal cavity of each rat via a midline incision under anesthesia with a mixture of medetomidine (0.15 mg/kg), midazolam (2 mg/kg) and butorphanol 2.5 mg/kg (0.1 ml/10 g weight, i.p.). After closure of the cavity with suture, the animals were housed individually for 1 week to recover from the surgery under regular health checks. T_c signals were received by an antenna below the rat cage and relayed to a signal processor (Dataquest A.R.T. System, Data Sciences International) connected to a server computer. At least 1 day before the experiment, the telemetric transmitters were activated using a magnet to start recording T_c every 5 min. Only rats that showed stable diurnal changes in T_c were used for the following experiments.

Drug injection and social defeat stress

On the experimental day, the Wistar rats received an i.p. injection of diazepam (4 mg/kg, 0.4–0.6 ml; Wako, Osaka, Japan) or its vehicle. Diazepam was dissolved in physiological saline with 40 mM hydrochloric acid (Shannon and Herling, 1983). In our pilot experiments, we determined the minimum effective dose of diazepam based on its inhibitory effect on social defeat stress-induced increase in T_c and found that 4 mg/kg consistently gave a significant inhibitory effect on the PSH without causing noticeable muscle relaxation or hypothermia. To minimize stress from the injection procedure, the solution was quickly injected into the abdominal cavity through the lower abdominal skin, which was exposed to the experimenter by gently bending the lower back backward with the base of the tail lifted up. This procedure was performed within the home cages of the Wistar rats, and during the procedure, their forelegs were touching the floor with little movement of the head.

Sixty minutes after the injection, the Wistar rats were exposed to social defeat stress (Stress group) or left undisturbed in their home cages (Control group). The social defeat stress procedure followed our established method (Lkhagvasuren et al., 2011), which was modified