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PROLONGED BEHAVIORAL STRESS ENHANCES SYNAPTIC CONNECTIVITY IN THE BASOLATERAL AMYGDALA

A. VYAS,[1] S. JADHAV[2] AND S. CHATTARJI*

National Centre for Biological Sciences, GKVK Campus, Bangalore 560065, India

Abstract—Recently identified cellular and molecular correlates of stress-induced plasticity suggest a putative link between neuronal remodeling in the amygdala and the development of anxiety-like behavior. Rodent models of immobilization stress, applied for 10 consecutive days, have been reported to enhance anxiety, and also cause dendritic elongation and spine formation in the basolateral amygdala (BLA). Paradoxically, longer exposure to stress, which is also anxiogenic, fails to affect key molecular markers of neuronal remodeling in the BLA. This has raised the possibility of homeostatic mechanisms being triggered by more prolonged stress that could potentially dampen the morphological effects of stress in the BLA. Therefore, we examined the cellular and behavioral impact of increasing the duration of stress in rats. We find that prolonged immobilization stress (PIS), spanning 21 days, caused significant enhancement in dendritic arborization of spiny BLA neurons. Spine density was also enhanced along these elongated dendrites in response to PIS. Finally, this striking increase in synaptic connectivity was accompanied by enhanced anxiety-like behavior in the elevated plus-maze. Thus, we did not detect any obvious morphological correlate of adaptive changes within the BLA that may have been activated by prolonged and repeated application of the same stressor for 21 days. These findings add to accumulating evidence that structural encoding of aversive experiences, through enhanced availability of postsynaptic dendritic surface and synaptic inputs on principal neurons of the BLA, may contribute to the affective symptoms of stress disorders.

Key words: anxiety, basolateral amygdala, dendritic remodeling, spinogenesis, stress disorders, synaptic plasticity.

The amygdala, in addition to playing a pivotal role in processing emotional information, is an important component of the neural circuitry mediating stress responses (LeDoux, 1994; Herman and Cullinan, 1997). Accumulating evidence also suggests that, when exposed to stress, the amygdala itself undergoes plastic changes, the effects of which are evident at multiple levels of neural organization. At the behavioral level, chronic stress facilitates fear and anxiety (Conrad et al., 1999; Vyas and Chattarji, 2004). At the level of individual pyramidal neurons, chronic stress elicits dendritic remodeling in the basolateral amygdala (BLA) (Vyas et al., 2002). Further, recent reports have identified key molecular markers, which are activated by stress and have also been implicated in neuronal remodeling (Pawlak et al., 2003; Cordero et al., 2005). Taken together, these findings have contributed to an emerging framework for exploring the cellular and molecular correlates of stress-induced changes that may link structural plasticity in the amygdala with the affective symptoms of chronic anxiety disorders.

These studies in the amygdala also highlight several key properties of structural plasticity elicited by stress. First, stress-induced dendritic remodeling in the BLA differs from its hippocampal counterpart in two important ways: the actual direction of dendritic remodeling and its reversibility (McEwen, 1999; Vyas et al., 2002, 2004). With respect to the direction of dendritic remodeling, it has previously been reported that unlike stress-induced hippocampal atrophy, dendrites of BLA pyramidal cells grow bigger in response to chronic stress (Vyas et al., 2002). Further, stress-induced hippocampal atrophy is reversible, while amygdalar hypertrophy persists for a number of weeks after termination of the stressor (Vyas et al., 2004). Second, although repeated restraint stress has been used in earlier studies on the hippocampus (McEwen, 1999), there is growing appreciation of the fact that repeated application of the same stressor can lead to habituation in the stress response (Melia et al., 1994). This raises the possibility that although 10 days of chronic immobilization stress is adequate for triggering enduring amygdalar dendritic remodeling, repeated presentation of the same stressor for a longer duration may elicit adaptive changes that dampen the initial effects of stress on dendritic morphology. Third, it is also possible that such adaptive plasticity, if it exists in the BLA, may not necessarily be manifested as modulation in dendritic morphology alone. A recent report has shown that variations in stress duration can elicit spine plasticity independent of dendritic remodeling in BLA (Mitra et al., 2005). This dissociation between stress-induced modulation in spines and dendrites, in turn, raises yet another possibility wherein homeostatic mechanisms triggered by prolonged stress could be mediated by a decrease in the number of spines, thereby regulating the overall synaptic connectivity in the BLA. Finally, the importance of challenging the amygdala with longer periods of

[1] Present address: Department of Biological Sciences, 428 Gilbert Hall, Stanford University, Stanford, CA 94305-5020, USA.
[2] Present address: Computational Neurobiology Program, 9500 Gilman Drive, University of California–San Diego, La Jolla, CA 92093, USA.
AV and SJ contributed equally to this work.
*Corresponding author. Tel: +91-80-23636421; fax: +91-80-23636662.
E-mail address: shona@ncbs.res.in (S. Chattarji).
Abbreviations: BLA, basolateral amygdala; CeA, central amygdala; MeA, medial amygdala; NMDA, *N*-methyl-D-aspartate; PIS, prolonged immobilization stress; PSA-NCAM, polysialylated neural cell adhesion molecule; tPA, tissue-plasminogen activator.

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stress is also highlighted by the finding that in the BLA, 21-days of prolonged stress fails to cause any change in the polysialylated neural cell adhesion molecule (PSA-NCAM), a key mediator of structural plasticity (Nacher et al., 2002; Cordero et al., 2005). This has led to speculation that prolonged stress may cause BLA hypertrophy that is eventually followed by a retraction process, akin to the biphasic modulation in PSA-NCAM observed in the dentate gyrus as a function of stress duration. Therefore, in the present study we examined if administration of more prolonged immobilization stress (PIS) dampens its effects on remodeling of BLA principal neurons.

EXPERIMENTAL PROCEDURES

Experimental animals

Male Wistar rats were used for PIS. At the termination of the experiments, animals were between 2 and 2.5 months of age. All animals (National Centre for Biological Sciences, Bangalore, India) were housed in groups of three with *ad libitum* access to food and water. Control animals, which were littermates of the stress-treated animals, were housed in separate cages. Animals were maintained in a temperature-controlled room, with a 12-h light/dark cycle (lights on at 7:00 A.M.). All procedures related to animal maintenance and experimentation were approved by the Institutional Animal Ethics Committee. All experiments conformed to U.S. National Institutes of Health (NIH) and India Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSEA) guidelines on the ethical use of animals. Experiments were designed with the aim of minimizing the number of animals used and their suffering. Separate sets of animals were employed for behavioral testing in elevated plus-maze and for morphological analysis.

Experimental treatment groups

Rats, randomly assigned to experimental groups, were subjected to PIS for 21 consecutive days. PIS consisted of complete immobilization (2 h/day, 10 A.M. to noon) in rodent immobilization bags without access to either food or water. Restraint stress paradigms of similar number of days are known to cause dendritic atrophy in CA3 hippocampal neurons, in addition to causing abnormalities in synaptic transmission and dysregulation of hypothalamic-pituitary-adrenal axis (Magarinos and McEwen, 1995; McEwen et al., 1995; McEwen and Sapolsky, 1995; McEwen and Magarinos, 1997; McEwen, 1999). Control animals were housed in the same room together with stressed animals and were not subjected to any type of stress.

Elevated plus-maze

The elevated plus-maze, consisting of two opposite open arms (60×15 cm) and two enclosed arms (60×15 cm, surrounded by a 15-cm-high opaque wall), was elevated 75 cm from the ground. The animals were tested on the maze 24 h after the termination of the stress paradigm. Individual trials lasted for 5 min each and were videotaped for subsequent off-line analysis. At the beginning of each trial, animals were placed at the center of the maze, facing an enclosed arm. All trials were conducted between 10:00 A.M. and 2:00 P.M., and the maze was cleaned with 5% ethanol solution (v/v) after each trial. Number of entries in open and enclosed arms was quantified from videotapes. For each individual animal, open-arm exploration was computed in terms of percentage open-arm time (time-spent in open arms relative to total duration of trial) and percentage open-arm entries (entries in open arms relative to entries in open and enclosed arms). Open-arm exploration data for both control and PIS groups were then normalized to mean of control group.

Tissue preparation

Both groups of rats were killed under deep anesthesia 24 h after termination of stress. The brain was removed quickly, and blocks of tissue containing the amygdala were dissected and processed for rapid Golgi staining technique as described earlier (Vyas et al., 2002). Coronal sections (120 μm thick) were prepared as described earlier (Vyas et al., 2002). Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed.

Analysis of dendritic arborization

To be selected for analysis, Golgi-impregnated neurons had to satisfy the following criteria: (1) presence of untruncated dendrites, (2) consistent and dark impregnation along the entire extent of all dendrites, and (3) relative isolation from neighboring impregnated neurons to avoid interfering with analysis. Both spiny pyramidal-like and stellate neurons from the BLA were selected for analysis on the basis of morphological criteria described in the literature (McDonald, 1982; Vyas et al., 2002) As described earlier (Vyas et al., 2002), our analysis of BLA neurons was restricted to those located between bregma −2.0 mm and −3.2 mm. Three-dimensional reconstruction of dendrites of the selected neurons was accomplished using motorized microscope stage and dendritic length and number of branch points were computed, using the NeuroLucida software (MicroBrightfield, Colchester, VT, USA).

Analysis of dendritic spine density

A subset of the neurons, whose dendritic trees were reconstructed for the purpose of quantifying dendritic arborization, was selected randomly for measuring spine density. For the purpose of this study, dendrites directly originating from cell soma were classified as primary dendrites, and those originating from primary dendrites were classified as secondary dendrites. Dendritic spines were counted manually at a total magnification of 1000×. A dendritic segment was picked that extended at least 80 μm or more from the origin of the branch, and possessed consistent and dark impregnation along the entire extent of the dendrite. All protrusions, irrespective of their morphological characteristics, were counted as spines if they were in direct continuity with the dendritic shaft. Starting from the origin of the branch, and continuing away from the cell soma, we counted the number of spines for a total of 80 μm. One 80-μm segment of primary dendrite and one 80-μm segment of secondary dendrite from each BLA neuron were analyzed for spine density. A total of 36 neurons from six control animals and 42 neurons from seven stressed animals were subjected to spine density analysis.

Statistical analysis

Statistical significances were calculated using Student's *t*-test. Values are reported as mean±S.E.M. Statistical analysis was carried out on individual neurons (i.e. n=number of neurons) and measures were averaged across all neurons within a particular experimental group. In cases where multiple *t*-tests were used to compare morphological parameters, Bonferroni correction was used to control for spurious type I errors.

RESULTS

Previously we demonstrated that chronic immobilization stress (2 h/day for 10 days) leads to a significant increase in dendritic arborization of principal neurons of the BLA,