PROLONGED BEHAVIORAL STRESS ENHANCES SYNAPTIC CONNECTIVITY IN THE BASOLATERAL AMYGDALA

A. VYAS,1 S. JADHAV2 AND S. CHATTARJI*
National Centre for Biological Sciences, GKVVK 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. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: anxiety, basolateral amygdala, dendritic remodeling, spino genesis, 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; Vy as 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...
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 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,
manifested as enhanced dendritic length and number of branch points (Vyas et al., 2002). Therefore, we first examined if extending this stress paradigm, by more than doubling its duration in the form of PIS (2 h/day for 21 days), in any way influences its efficacy in causing dendritic remodeling in the BLA.

**Effects of prolonged stress on dendritic remodeling of spiny BLA neurons**

Morphometric analysis of BLA spiny pyramidal neurons revealed that the total dendritic length of BLA spiny neurons from PIS animals was significantly greater than their control counterparts (control: 1001±48 μm, n=38 neurons from six animals; PIS: 1450±43 μm, n=42 neurons from seven animals; P<0.0001; Fig. 1A; 45% increase relative to control). A more detailed segmental analysis of dendritic arborization of spiny BLA neurons, in incremental steps of 20 μm from the soma, indicates that a statistically significant increase in mean dendritic length extended across a fairly wide range of distances, with the most striking differences elicited between 60 and 200 μm from the soma (Fig. 1B). Moreover, the PIS-induced dendritic hypertrophy was evident throughout a wide range of total dendritic length values for all the BLA neurons analyzed (Fig. 1C). This is borne out as a clear rightward shift in the cumulative frequency plot for the entire database of neurons from the PIS group compared with controls. PIS also induced a statistically significant increase in the total number of branch points (Fig. 1D), resulting in an overall rightward shift in the cumulative frequency distribution (Fig. 1E). The magnitude of this effect, however, was smaller compared with that observed for dendritic length (total number of branch points, control: 12.8±0.6; PIS: 16.6±0.6; P<0.0001; Fig. 1D; 30% increase relative to control). Further, the increase in number of branch points was not as extensive and was restricted between 60 and 100 μm from soma (Fig. 1F). Camera lucida tracings of representative Golgi-impregnated spiny BLA neurons, from control as well as PIS animals, are illustrated in Fig. 1G. Also, the relative location of these sample tracings (Fig. 1G) with respect to the entire database of BLA neurons analyzed from the two groups (control and PIS) is indicated with arrows in Fig. 1C and 1E. Thus, these results indicate that, even with a more prolonged exposure to immobilization stress, BLA neurons continue to exhibit significantly greater dendritic arborization relative to unstressed controls. This is in contrast to earlier reports showing that after 21 days of repeated stress, CA3 pyramidal neurons in the hippocampus undergo dendritic retraction (Magarinos and McEwen, 1995; McEwen, 1999).

**Effects of prolonged stress on dendritic spine density in BLA neurons**

Thus, increasing the duration of the stressor in itself does not appear to trigger adaptive mechanisms capable of attenuating the effects of stress on dendritic morphology. However, this does not rule out more subtle homeostatic changes taking place at the level of dendritic spines. In other words, after exposure to PIS, do the elongated dendrites receive a greater or fewer numbers of synaptic inputs? Previous morphological studies in the hippocampus point to one possible scenario. It has been reported that dendritic atrophy in hippocampal CA3 pyramidal neurons, caused by repeated restrained stress, is accompanied by a numerical increase in spines (Sunanda et al., 1995). This is indicative of an adaptive mechanism that may compensate for the loss of dendritic area for synaptic inputs to terminate. If similar adaptive plasticity mechanisms are activated in the BLA by prolonged application of stress, then one would predict a decrease in the number of spines along the elongated dendrites. Hence, we quantified spine-density in BLA neurons in control and PIS animals (Fig. 2).

Some, but not all, cells had up to fourth-order branches. However, all the selected neurons had at least primary and secondary dendrites. Therefore, to avoid any bias caused by this variability in branch orders across neurons, we restricted our spine density analysis to primary and secondary dendrites irrespective of the presence/absence of higher order branches. Strikingly, PIS caused a significant increase in the density of spines along both primary and secondary dendrites of spiny BLA neurons. PIS caused increase in the spine-density along the primary branch by 31% (total number of spines on 80 μm dendritic segment, control: 42±1.6, n=36 neurons from six animals; PIS: 55±0.9, n=42 neurons from seven animals; P<0.0001; Fig. 2A), compared with control. The effects of PIS on spine density along the secondary branch were equally pronounced (total number of spines on 80 μm dendritic segment, control: 54±1.2; PIS: 71±0.5; P<0.0001; Fig. 2A; 31% increase relative to control). This increase in spine density is depicted in photomicrographs of representative segments of primary dendritic branches of BLA pyramidal neurons (Fig. 2B). Thus, contrary to a scenario that would be consistent with a homeostatic regulation of synaptic connectivity in the BLA following prolonged exposure to stress, dendritic hypertrophy is accompanied by an equally robust increase in spine-density.

**Effects of prolonged stress on anxiety-like behavior**

The data presented thus far indicate that extending the length of the stress protocol does not lead to any obvious cellular manifestation of homeostatic regulation in dendritic arborization and spine-density. On the contrary, the cumulative effect of longer dendrites and greater number of spines per unit length of dendrite would cause a significant increase in the physical substrate for synaptic connectivity on principal neurons of the BLA. To examine the behavioral consequence of such widespread increase in synaptic connectivity in the BLA, we measured anxiety-like behavior of control and PIS-treated animals on the elevated plus-maze. PIS caused significant (P<0.01) reduction in open-arm exploration relative to control animals (Fig. 3). This was manifested as a 24% decrease in the percentage open arm entries and a 43% decrease in the percentage time spent in open arms (Fig. 3 left and middle). For example, during a 5 min session on the plus-maze, the number of entries into the open arms made by control animals was 54% of the total number of entries made in the
Fig. 1. PIS induces growth in dendritic arborization of spiny BLA neurons. (A) Mean (±S.E.M.) values for total dendritic length of BLA spiny neurons from PIS and control groups. *** P < 0.0001, compared with control, Student’s t-test; control, n = 38 neurons; PIS, n = 42 neurons. (B) Segmental analysis of total dendritic length (mean ±S.E.M.) for each successive 20 µm segment as a function of the radial distance of that segment from the soma. *** P < 0.001, compared with control, Student’s t-test. (C) Cumulative frequency plots for all BLA neurons illustrating the rightward shift (i.e. increase) across the database of total dendritic lengths for PIS neurons relative to control neurons. The 50% mark (dashed line) for the total n represents the median values, which are marked with vertical lines (control, black; PIS, gray). Values of dendritic length marked on the plots by arrows (black, control; gray, PIS) and arrowheads (black, control; gray, PIS) correspond to the two pairs of sample BLA neurons depicted in Fig. 2G. (D) Mean (±S.E.M.) values for total number of branch points of BLA spiny neurons from PIS and control groups. (E) Cumulative frequency plots for all BLA neurons illustrating the rightward shift (i.e. increase) across the database of total number of branch points for PIS neurons relative to control neurons. (F) Segmental analysis of total number of branch points (mean ±S.E.M.) for each successive 20 µm segment as a function of the radial distance of that segment from the soma. ** P < 0.05, *** P < 0.001. (G) Representative drawings of BLA neurons from control and PIS groups. For the purpose of illustration, tracings have been collapsed in two-dimensions from original 3-D reconstructions. Scale bar = 50 µm.
open and enclosed arms together. This percentage of entries in the open arms came down to 41% following PIS (Fig. 3, $P<0.01$). The reduction in open arm exploration was even more pronounced in terms of the mean time spent in the open arms (control: 99±7 s, $n=32$; PIS: 56±12 s, $n=12$ animals; $P<0.01$). Finally, the reduction in open-arm exploration observed in PIS animals was not due to a net decrease in locomotor activity in the maze, as measured by the number of entries into the enclosed arms (Fig. 3 right). The mean number of entries into the enclosed arms made by control animals (4.4±0.3) was not significantly different from PIS (4.8±0.7, $P>0.6$). Thus, consistent with its facilitating effects at the cellular level, PIS also caused a significant potentiation in anxiety-like behavior.

**DISCUSSION**

The primary goal of this study was to explore the implications of recent evidence indicating that stress-induced structural plasticity in amygdalar neurons may provide a cellular substrate for enhanced anxiety (Conrad et al., 1999; Vyas et al., 2002, 2003; Pawlak et al., 2003). These recent studies point to several features of stress-induced neuronal remodeling in the amygdala, which are distinct from those observed earlier in the hippocampus (McEwen, 1999). In particular, some of the temporal characteristics of amygdalar structural plasticity elicited by various stressors are quite striking. At one end of the temporal spectrum, a single 2 h episode of acute stress has been reported to trigger delayed and localized formation of spines 10 days later, without any effect on dendritic morphology (Mitra et al., 2005). At the other end, when the 2-h stressor is repeated for 10 consecutive days, it causes more extensive spine formation alongside dendritic elongation. Further, stress-induced BLA dendritic hypertrophy persists for up to 21 days after the end of the 10-day stress protocol (Vyas et al., 2004), which is in contrast to hippocampal CA3 dendritic atrophy that is reversible within 7–10 days after the end of a 21-day restraint stress (Conrad et al., 1999). Despite these findings on stress-induced structural plasticity in the BLA, rodent models of more prolonged...
stress (e.g. for 21 days), used extensively in earlier studies on the hippocampus (McEwen, 1999), have no significant effect in the BLA on key molecular mediators of neuronal remodeling, such as PSA-NCAM (Cordero et al., 2005). Taken together these observations have raised the possibility that more prolonged exposure to the same immobilization stress that elicits neuronal remodeling after shorter periods of application, may trigger homeostatic mechanisms leading to biphasic modulation of such structural plasticity. To explore this possibility in greater detail, we measured the cellular and behavioral effects of increasing the duration of a previously used model of immobilization stress (2 h/day) from 10 days to 21 days. Since the 10-day paradigm is known to elicit a significant increase in both dendritic arbors and spine density in BLA (Vyas et al., 2002; Mitra et al., 2005), we investigated two specific scenarios by which extending the duration of this stress protocol could lead to homeostatic mechanisms. First, prolonged stress could induce biphasic modulation directly at the level of dendritic morphology, i.e. dendritic growth could be attenuated or even reversed at the end of PIS. We found no evidence for such regulation since PIS caused a significant increase in both dendritic length (45%) and the number of branch points (30%) of BLA principal neurons, at levels that are greater than what has earlier been reported using a 10-day protocol (Vyas et al., 2002). Second, we also explored the possibility that the effect of dendritic growth is counterbalanced by a numerical reduction in spines. Contrary to this scenario, PIS also induced a significant increase in BLA spine-density. Thus, we detected no obvious morphological sign of homeostatic regulation that attenuates the impact of PIS on dendritic remodeling and spinogenesis. Hence, these findings on enhanced dendritic arborization, combined with greater density of spines along these elongated arbors, argue for a possible increase in synaptic efficacy that could lead to an overall increase in functional output from the amygdala following prolonged stress. Consistent with this idea, we find that PIS also facilitates anxiety-like behavior in the plus-maze, a finding that is in agreement with earlier studies showing a potentiating effect of 21-days of repeated restraint stress on anxiety and fear-conditioning in rats (Conrad et al., 1999).

What may be the molecular underpinnings of prolonged stress-induced structural plasticity that are manifested as a significant remodeling of the dendritic architecture and synaptic composition in the BLA? Accumulating evidence is providing valuable insight into possible mechanisms. For example, N-methyl-D-aspartate (NMDA) receptor signaling in the BLA, which mediates long-term synaptic potentiation (LTP), dendritic development and activity-dependent spinogenesis, has also been reported to play a role in fear memory formation and anxiety (Adamec et al., 1999; Cline, 2001; Bauer et al., 2002; Goosens and Maren, 2004; Rainnie et al., 2004). Prior exposure to stress has been shown to facilitate various forms of classical fear conditioning and anxiety-like behavior in rats (Shors et al., 1992; Conrad et al., 1999). Importantly, local infusion of NMDA receptor antagonists into the BLA prevents these facilitatory effects of stress (Shors and Mathew, 1998). Thus, stress-induced spine plasticity could also be mediated by NMDA receptor-dependent mechanisms.

More recent reports are also bringing into focus challenging issues by going beyond the traditional focus on the BLA to investigate the molecular correlates of stress-induced neuronal remodeling in other amygdalar nuclei. For instance, tissue-plasminogen activator (tPA), a plasticity-related serine protease, has been implicated in stress-induced neuronal remodeling in the central (CeA) and medial amygdala (MeA), as well as the development of anxiety-like behavior (Pawlak et al., 2003). The particularly striking result from this study is that, tPA immunoreactivity and its upregulation by stress, are confined to the MeA and CeA and almost completely absent in the BLA (Pawlak et al., 2003). Interestingly, restraint stress also increased the expression of GAP-43, a presynaptic protein that has served as a key marker of axonal plasticity (Benowitz and Routtenberg, 1997). These data suggest that stress could also cause remodeling of axonal inputs impinging on CeA and MeA neurons. Since these nuclei receive inputs from the BLA, it will be interesting to examine if axons originating from BLA projection neurons also undergo structural changes after exposure to prolonged stress. The importance of examining the potential downstream effects of stress-induced plasticity in the BLA, especially in projections from the BLA to non-cortical nuclei of the amygdala, is also highlighted by a recent study reporting significant reduction in PSA-NCAM immunoreactivity in the CeA and MeA after 21-days of restraint stress (Cordero et al., 2005). This observation, taken together with data on a predominantly postsynaptic localization of PSA-NCAM in these nuclei (Nacher et al., 2002), raises the intriguing possibility that stress-induced strengthening of the synaptic wiring in the BLA, as described here, could in turn cause its target nuclei such as the MeA or CeA to reduce its available postsynaptic substrate, lower PSA-NCAM levels being a molecular indicator of such synaptic plasticity. Moreover, these findings also suggest that prolonged stress could induce forms of structural plasticity in the MeA and CeA that are distinct from those exhibited by the BLA, the precise nature of which will require further investigation. Therefore, it will be particularly interesting to examine if enhancement in BLA synaptic connectivity caused by prolonged stress can have a cascading effect on structural remodeling, and its underlying molecular mechanisms, in the output nuclei of the amygdala that are not only further removed from the input interface of the amygdala, but are closer to brain areas that exert a profound influence on the stress response.

Acknowledgments—This work was supported by funds from the Wellcome Trust and NCBS.

REFERENCES

Adamec RE, Burton P, Shallow T, Budgell J (1999) NMDA receptors mediate lasting increases in anxiety-like behavior produced by the


(Accepted 1 August 2006)
(Available online 8 September 2006)