Neuroscience 144 (2007) 8–16

STRESS-INDUCED SPINE LOSS IN THE MEDIAL AMYGDALA IS MEDIATED BY TISSUE-PLASMINOGEN ACTIVATOR

S. BENNUR,[a] B. S. SHANKARANARAYANA RAO,[b] R. PAWLAK,[c] S. STRICKLAND,[d] B. S. McEWEN[e] AND S. CHATTARJI[a]*

[a]*National Centre for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bangalore 560065, India*

[b]*Department of Neurophysiology, National Institute of Mental Health and Neuro Sciences, Bangalore 560029, India*

[c]*Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN, UK*

[d]*Laboratory of Neurobiology and Genetics, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA*

[e]*Laboratory of Neuroendocrinology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA*

Abstract—The amygdala, which exerts a regulatory influence on the stress response, is itself affected by stress. It has been reported that the serine protease tissue-plasminogen activator (tPA), a key mediator of spine plasticity, is required for stress-induced facilitation of anxiety-like behavior. Importantly, tPA is also involved in stress-induced activation of molecular signals that have the potential to contribute to neuronal remodeling in the medial amygdala (MeA). However, little is known about the precise nature of, and specific role played by tPA in, stress-induced structural plasticity in the MeA. Hence, we compared the impact of chronic restraint stress on spine density of medium spiny stellate neurons in MeA in wild-type mice with mice in which the tPA gene is disrupted ($tPA^{-/-}$). In wild-type mice, chronic stress caused significant reduction in MeA spine density, which was in contrast to enhanced spine density in the neighboring basolateral amygdala (BLA). Strikingly, $tPA^{-/-}$ mice exhibited significant attenuation of stress-induced spine retraction in the MeA, but BLA spinogenesis was not affected. Therefore, tPA-dependence of stress-induced modulation in spine density was restricted to the MeA. Further, MeA neurons in $tPA^{-/-}$ mice, even when challenged with repeated stress, were able to maintain levels of spine density that were comparable to that of wild-type mice without stress. Our findings provide novel evidence for a permissive role for tPA in amygdalar spine plasticity elicited by behavioral stress.

Key words: anxiety, chronic stress, structural plasticity, basolateral amygdala, extracellular matrix, neuronal remodeling.

*Corresponding author. Tel: +91-80-23636421; fax: +91-80-23636662.
E-mail address: shona@ncbs.res.in (S. Chattarji).

Abbreviations: ANOVA, analysis of variance; BLA, basolateral amygdala; CRF, corticotropin-releasing factor; ERK, extracellular signal-related kinase 1/2; GAP, growth-associated protein 43; HPA axis, hypothalamic–pituitary–adrenal axis; MD, monocular deprivation; MeA, medial amygdala; PSA-NCAM, polysialylated-neural cell adhesion molecule; tPA, tissue-plasminogen activator; $tPA^{+/+}$, wild-type C57/BL/6 mice; $tPA^{-/-}$, tPA knockout mice; WT, wild-type.

The amygdala, a brain area involved in the formation of emotional memories, plays a pivotal role in the neural circuitry of stress (LeDoux, 2000). In addition to its regulatory influence on the stress response, the amygdala itself is affected by stress (Herman et al., 1996; Vyas et al., 2002; Pawlak et al., 2003; Mitra et al., 2005). The impact of stress on amygdalar function has been studied at multiple levels of neural organization. At the behavioral level, animal models of stress potentiate fear and anxiety (Conrad et al., 1999; Vyas and Chattarji, 2004; Cordero et al., 2003). At the cellular level, in addition to causing persistent dendritic growth, chronic stress triggers formation of dendritic spines (Mitra et al., 2005). At the molecular level, a recent study has identified a key role for the serine protease tissue-plasminogen activator (tPA) in the amygdala in the development of anxiety-like behavior after stress (Pawlak et al., 2003). This study showed that tPA, in addition to being upregulated by stress, activates molecular signaling mechanisms that could mediate structural aspects of neuronal remodeling, such as extracellular signal-regulated kinase 1/2 (ERK1/2) and growth-associated protein 43 (GAP-43). Importantly, mice with a genetic disruption of tPA did not exhibit an increase in anxiety, or activation of these molecular markers of neuronal remodeling, after exposure to chronic restraint stress (Pawlak et al., 2003).

These findings on tPA, in addition to providing a framework that integrates molecular and behavioral correlates of stress-induced amygdalar plasticity, also raise several important issues. First, recent observations point to a key role for tPA in experience-dependent plasticity, especially spine dynamics, in the visual cortex (Berardi et al., 2004). In the amygdala, however, it is not known whether tPA-dependent neuronal remodeling caused by stress is manifested as spine plasticity. Second, the role of tPA in influencing spines is particularly relevant in light of studies showing that chronic stress leads to spine formation in the basolateral amygdala (BLA) (Mitra et al., 2005). Further, stress-induced BLA spinogenesis is accompanied by enhanced anxiety-like behavior, which is also a consequence of stress-induced tPA activation in the amygdala. Paradoxically, tPA immunoreactivity, and its modulation by stress, are confined to the medial amygdala (MeA) and almost completely absent in the BLA (Pawlak et al., 2003). Third, the MeA, a major efferent nucleus of the amygdala that projects to various forebrain regions involved in regulating the hypothalamic–pituitary–adrenal axis (HPA axis) (Feldman et al., 1994; Canteras et al., 1995; Dayas et al., 1999; Ma and Morilak, 2005), also exhibits Fos expression in response to a variety of stressors (Arnold et al., 1992;

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2006.08.075

Pezzone et al., 1992; Cullinan et al., 1995; Campeau and Watson, 1997; Chen and Strickland, 1997; Bhatnagar and Dallman, 1998; Li and Sawchenko, 1998; Ma and Morilak, 2004). Thus, these results also highlight the importance of looking beyond the BLA, which has been the primary focus of earlier reports on amygdalar plasticity. Taken together these findings raise the possibility that stress leads to the development of enhanced anxiety by leaving its mark in the MeA through structural modifications in synaptic connectivity. Therefore, the present study tests the hypothesis that in the MeA, but not the BLA, stress triggers spine plasticity that depends on tPA. To this end we examined the precise nature of chronic stress-induced morphological plasticity in the MeA, and how such plasticity is affected by a disruption of the tPA gene in mice.

EXPERIMENTAL PROCEDURES

Experimental animals

Three-month old wild-type (WT) C57/BL/6 ($tPA^{+/+}$) and tPA knockout mice ($tPA^{-/-}$) (Pawlak et al., 2003) were back-crossed to C57/BL/6 for nine generations. The mice were housed in cages of four to five animals each. All the mice in a given cage were subjected to the same treatment (i.e. Stress or no stress). We did not use one subject from each litter for each experimental condition, but $tPA^{-/-}$ mice have been backcrossed with C57/BL/6 for nine generations therefore they are highly isogenic. The restraint began at 11:00 a.m. and all experiments were performed during the light phase of the circadian cycle. All mice were in the same room, but unstressed mice were in a different soundproof hood than stressed mice. Control animals (N=3–5 per genotype) were left undisturbed, and stressed animals (N=4 per genotype) were subjected to daily 6-h restraint stress for 3 weeks in a separate room. The mice were placed in their home cages in wire mesh restrainers secured at the head and tail ends with clips. The experiments were carried out in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Animals (NIH publication No. 80-23, revised 1996), and the Rockefeller University ethics committee approved the experimental protocols. All efforts were made to minimize both the suffering and the number of animals used.

Golgi staining

After completion of experiments, animals were anesthetized using 2.5% avertin (0.02 mg/kg body weight) and then killed. The brain was removed quickly, and blocks of tissue containing the amygdala were dissected and processed for the Golgi-Cox technique at room temperature (Ramon-Moliner, 1970). The blocks of tissue remained in Golgi-Cox fixative for a period of 8–10 weeks (Ramon-Moliner, 1970), after which 120 μm thick sections were obtained from the fixed tissue using a rotary microtome (Jung RM 2055; Leica, Rueil-Malmaison, France). Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene and coverslipped. Slides were coded prior to quantitative analysis. The experimenter was blind to the code, which was broken only after the morphological analysis was completed.

Analysis of dendritic spine density

Our analysis of MeA neurons was restricted to those located between Bregma: −2.3 mm and −3.2 mm. To be selected for morphological analysis, Golgi-impregnated neurons had to satisfy the following criteria that have been applied in similar morphometric analyses in earlier reports in the literature (Watanabe et al., 1992; Conrad et al., 1999; Vyas et al., 2002; Mitra et al., 2005): (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 (e.g. avoid extensive dendritic overlap). Using these criteria in the MeA, we analyzed medium spiny stellate neurons because in earlier anatomical studies (reviewed in McDonald, 1992) this class of neurons was the most predominant cell type in the MeA. Further, these MeA neurons analyzed were selected randomly without any bias and the first six neurons in a given brain that met the above selection criteria were used. In agreement with earlier reports, we also observed a small proportion of MeA neurons with thick dendrites that exhibit a very dense covering of spines (Millhouse and DeOlmos, 1983; McDonald, 1992). These were excluded from our analysis. For every MeA neuron selected, spine density analysis was carried out for only one dendrite. To this end, we selected any one of the main dendritic shafts, originating from the soma, with the following features: (i) extending for a length of at least 100 μm or more from the soma; (ii) possessed consistent and dark impregnation along the entire extent of the dendrite. In the BLA, spiny pyramidal-like neurons were selected for analysis on the basis of morphological criteria outlined above and previously published reports (McDonald, 1992; Vyas et al., 2002; Mitra et al., 2005). Our analysis of BLA neurons was restricted to those located between bregma −2.0 mm and −3.2 mm.

By using the NeuroLucida image analysis system (MicroBrightField, Williston, VT, USA) attached to an Olympus BX61 microscope (100×, 1.3 N.A., Olympus BX61; Olympus, Shinjuku-Ku, Tokyo, Japan), all protrusions, irrespective of their morphological characteristics, were counted as spines if they were in direct continuity with the dendritic shaft (Fig. 1A and 1E, inset; Fig. 1C). For MeA neurons, spines were counted on dendrites directly originating from cell soma (Fig. 1A) while for BLA neurons we always selected the first branch that emerged from the main apical shaft, i.e. primary apical dendrite (Fig. 1E). Starting from the origin of the branch, and continuing away from the cell soma, we counted the number of spines in successive steps of 10 μm each, for a total of 10 steps in MeA neurons (i.e. extending a total length of 100 μm) and five steps in BLA neurons (for a total of 50 μm). The values for number of spines from each 10-μm segment, at a given distance from the origin of the branch, were then averaged across all neurons in a particular experimental group.

Statistical analysis

Values are reported as mean±S.E.M. In all cases, "n" refers to the number of neurons used for morphometry, and "N" refers to the number of mice used. Effects of chronic restraint stress and genetic deletion of tPA on segmental spine density were analyzed using the Student's t-test. Effects of stress and genetic deletion of tPA on total spine density were analyzed by one-way analysis of variance (ANOVA) with the number of neurons as the "n." Significant effects were further analyzed by Tukey-HSD post hoc test. Effects of stress and tPA disruption, and their interaction, on total spine density were also analyzed using a two-way ANOVA with stress and tPA disruption as between-subject factors and the number of neurons as the "n." Similarly, the effect of stress and tPA disruption on segmental spine density, were also analyzed using a ANOVA with stress, tPA disruption and "dendritic location" as between-subject factors and the number of neuronal segments as the "n."

RESULTS

As a first step toward examining the role of tPA in stress-induced neuronal remodeling, we searched for a morphological metric of the effects of repeated stress in MeA. To this end we focused our attention on dendritic spines since

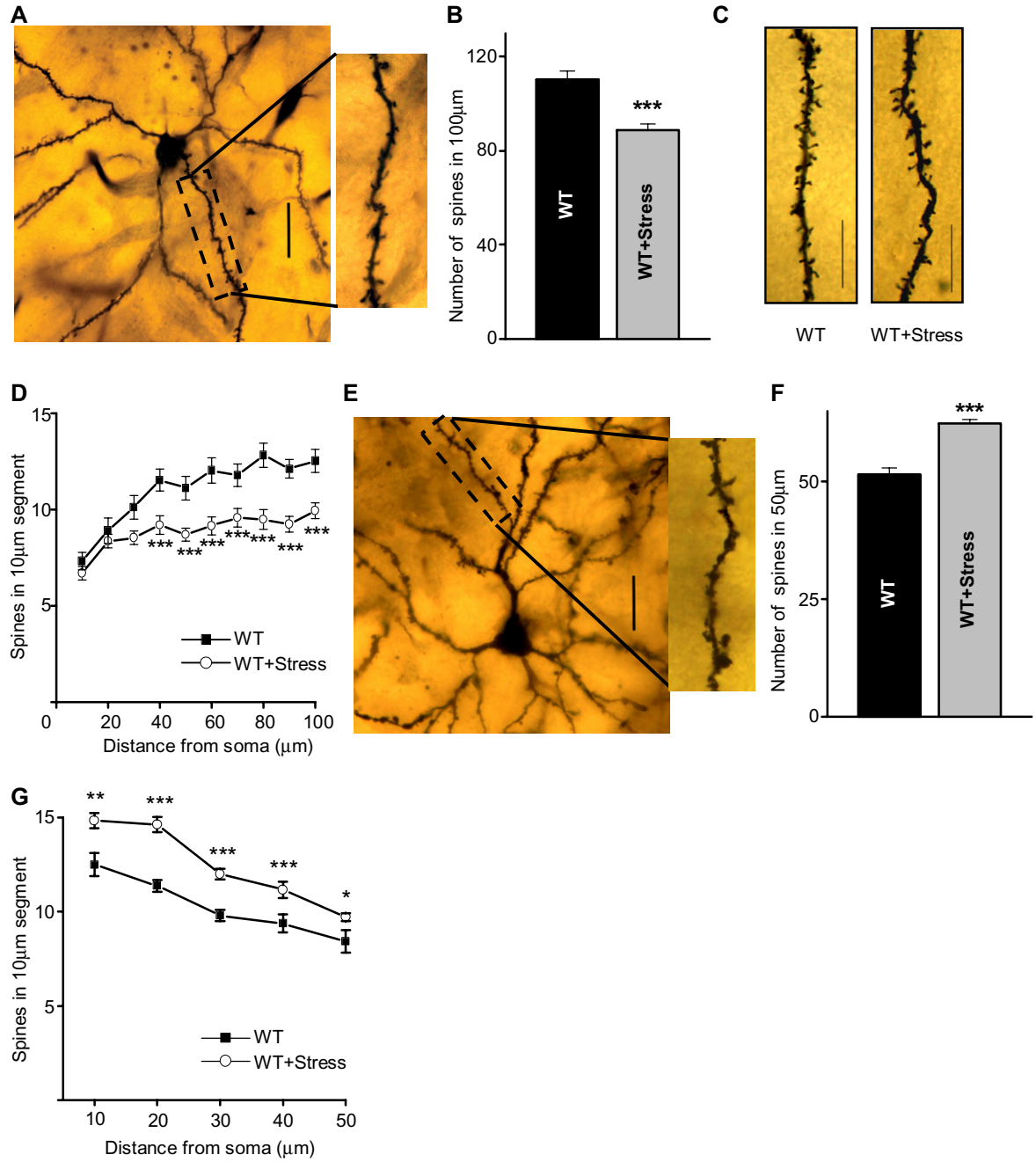

Fig. 1. Spine density is reduced in the MeA, but enhanced in the BLA, in WT mice exposed to chronic restraint stress (Stress). (A) Low-power photomicrograph of a Golgi stain-impregnated medium spiny neuron in the MeA. (Scale bar=10 μm.) (*Inset*) High-power image of spines on a dendritic segment from the same neuron. (B) Mean (±S.E.M.) values for spine-density (calculated as average number of spines per 100 μm) of primary branches of spiny MeA neurons from WT and WT+Stress groups, demonstrating decrease in the number of spines. *** $P<0.001$, compared with WT, Tukey-HSD; WT, $n=24$ neurons; WT+Stress, $n=24$ neurons. (C) Photomicrographs of representative segments of primary branches from unstressed (WT, *left*) and stressed (WT+Stress, *right*) mice. (Scale bar=10 μm.) (D) Segmental analysis of the mean (±S.E.M.) number of spines in each successive 10-μm segment along dendritic branches of WT (*filled square*) and WT+Stress (*open circle*) neurons in MeA as a function of the distance of that segment from the origin of the branch. *** $P<0.005$, compared with WT, Tukey-HSD. (E) Low-power photomicrograph of a Golgi stain-impregnated pyramidal-like spiny neuron in the BLA (scale bar=10 μm). (*Inset*) High-power image of spines on a dendritic segment from the same neuron. (F) Mean (±S.E.M.) values for spine-density (calculated as average number of spines per 50 μm) of primary branches of spiny BLA neurons from WT and WT+Stress groups, demonstrating increase in the number of spines. *** $P<0.005$, compared with WT, Student's *t*-test; WT, $n=24$ neurons; WT+Stress, $n=24$ neurons. (G) Segmental analysis of the mean (±S.E.M.) number of spines in each successive 10-μm segment along dendritic branches of WT (*filled square*) and WT+Stress (*open circle*) neurons in BLA as a function of the distance of that segment from the origin of the branch. * $P<0.05$; ** $P<0.01$; *** $P<0.005$, compared with WT, Student's *t*-test.

tPA has previously been shown to play a role in experience-dependent spine plasticity in other brain areas (Berardi et al., 2004).

Chronic restraint stress causes a decrease in MeA spine density in WT mice

Chronic restraint stress caused a significant reduction in the mean number of spines on a 100-μm segment of primary dendrite in medium-spiny stellate neurons (Fig. 1A) of the MeA in WT mice (F=23.971, df=3, P<0.001). These MeA neurons from stress-treated WT animals exhibited a 20% decrease in spine density ("WT+Stress," Fig. 1B and C; number of spines per 100 μm: 88.5±2.7, n=24 neurons, N=4 animals) compared with unstressed controls (WT, Fig. 1B and C; number of spines per 100 μm: 110.4±3.6, n=24 neurons, N=3 animals). These data on stress-induced decrease in spine density in MeA were obtained from a more detailed segmental analysis wherein the number of spines was counted in 10 consecutive steps of 10 μm each, starting from the soma and radiating outward along the dendrite. This segmental analysis shows that spine density exhibited a clear variation along the proximal–distal axis of the dendrite, as revealed by a factorial ANOVA (F=15.842, df=9, P<0.001; Fig. 1D). Moreover, spine density along the dendrite was significantly decreased by stress in a location dependent manner as indicated by a significant interaction between "stress" and "dendritic location" factors (F=2.040; df=9, P<0.04; Fig. 1D). Furthermore, post hoc Tukey analyses indicated, after an initial segment of 20 μm, a clear difference in spine density across the length of the primary dendrite in stressed WT neurons (Fig. 1D).

Although this spine loss in MeA is in striking contrast to chronic stress-induced increase in BLA spine-density reported earlier (Mitra et al., 2005), the previous finding on BLA spinogenesis was obtained in rats using a chronic immobilization stress protocol spanning 10 days (2 h/day). Hence, to rule out potential effects of differences in species or stress paradigms, we also examined the effects of our 21-day chronic restraint stress protocol on BLA spine-density in WT mice. In agreement with the earlier report using rats, we observed a significant increase in spine density (+21.1%, P<0.005, Student's t-test) along primary dendrites of stressed BLA spiny pyramidal neurons ("WT+Stress," Fig. 1F; number of spines per 50 μm: 62.3±0.8, n=24 neurons, N=4 animals) compared with their unstressed counterparts (WT, Fig. 1F; number of spines per 50 μm: 51.4±1.4, n=24 neurons, N=4 animals). A more detailed segmental analysis also shows a significant increase in the numbers of BLA spines across 10-μm segments along the primary dendrite, as revealed by factorial ANOVA (F=41.029, df=4, P<0.001; Fig. 1G). However, in contrast to the MeA, there was no significant interaction between "stress" and "dendritic location" factors in the BLA (F=1.472, df=4, P=0.211).

Therefore, we conclude that in the MeA, chronic stress-induced neuronal remodeling is manifested as a reduction in spine density in medium spiny stellate neurons, an effect which is in stark contrast to stress-induced spinogenesis in the BLA.

MeA spine density in tPA$^{-/-}$ mice

In our effort to investigate a potential role for tPA in chronic stress-induced reduction in MeA spine density, we next focused on mice in which the tPA gene has been disrupted (tPA$^{-/-}$ mice). In an earlier study, stress-induced upregulation of molecular mediators of neuronal remodeling in the MeA was prevented in the tPA$^{-/-}$ mice. Hence, we measured basal levels of spine-density in medium spiny stellate neurons of the MeA in tPA$^{-/-}$ mice (Fig. 2). We observed a small (+9%, P<0.03) increase in MeA spine density in the tPA$^{-/-}$ mice ("tPA$^{-/-}$," Fig. 2A; number of spines per 100 μm: 121.1±2.9, n=30 neurons, N=5 animals) relative to WT mice (Fig. 2A; number of spines per 100 μm: 110.4±3.6, n=24 neurons, N=3 animals). Further, segmental analysis along the dendrite indicates that

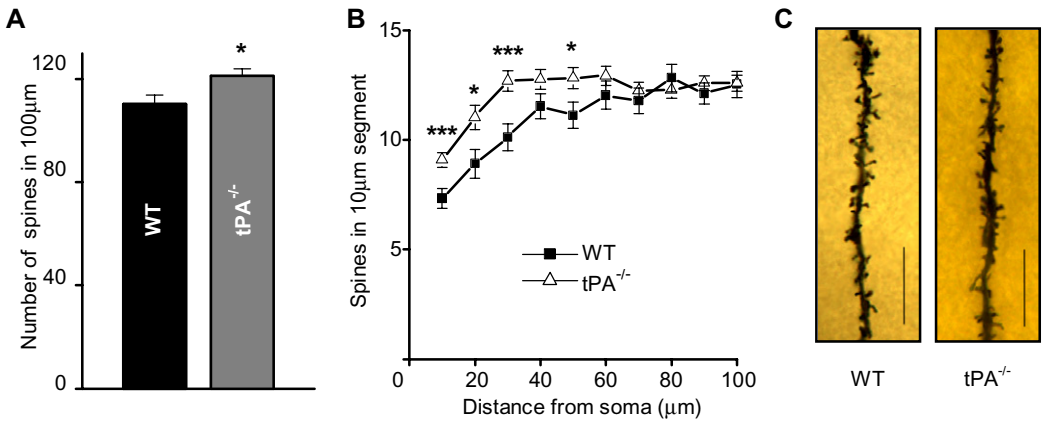

Fig. 2. Disruption of the tPA gene has no significant effect on MeA spine density. (A) Mean (±S.E.M.) values for spine-density (calculated as average number of spines per 100 μm) of primary branches of spiny MeA neurons from WT and tPA$^{-/-}$ mice, demonstrating a significant difference in the number of spines. * P<0.05, compared with WT, Tukey-HSD; WT, n=24 neurons; tPA$^{-/-}$, n=30 neurons. (B) Segmental analysis of the mean (±S.E.M.) number of spines in each successive 10-μm segment along dendritic branches of WT (*filled square*) and tPA$^{-/-}$ (*open triangle*) neurons in MeA as a function of the distance of that segment from the origin of the branch. * P<0.05, *** P<0.005, compared with WT, Tukey-HSD. (C) Photomicrographs of representative segments of primary branches from WT (*left*) and tPA$^{-/-}$ (*right*) mice. (Scale bar=10 μm.)

genetic background reduced spine density in a location dependent manner as indicated by a significant interaction between "genotype" and "dendritic location" ($F=2.228$; $df=9$; $P<0.019$; Fig. 2B). However, unlike the effects of stress on WT neurons, post hoc Tukey analyses reveal that barring a few 10-μm segments proximal to the soma, genetic deletion of tPA did not elicit a consistent pattern of robust increase in spine density across the length of dendrites in the mutant mice (Fig. 2B).

Chronic stress-induced spine loss in the MeA is attenuated in tPA$^{-/-}$ mice

The results described thus far provide a morphometric framework for examining a role for tPA in mediating stress-induced neuronal remodeling, i.e. spine loss, in the MeA. Hence, the impact of chronic restraint stress on tPA$^{-/-}$ mice was compared next with that of WT mice (Fig. 3). An overall decrease was observed ($P<0.027$) in spine density in stressed tPA$^{-/-}$ mice (number of spines per 100 μm: 110.2±1.6, $n=24$ neurons, $N=4$ animals; Fig. 3A) relative to their unstressed counterparts (number of spines per 100 μm: 121.1±2.9, $n=30$ neurons, $N=5$ animals). However, the percentage of loss of spines in stress-treated tPA$^{-/-}$ mice is significantly smaller than that observed in stress-treated WT mice ($P<0.001$, Student's t-test). This attenuation in spine loss is evident from a direct comparison of the percentage decrease in spine-density (normalized to their respective unstressed counterparts) observed in tPA$^{-/-}$ mice (−9%, $P<0.027$, Fig. 3A) versus WT mice (−20%, $P<0.001$, Fig. 3C). Importantly, in striking contrast to the effects of stress on MeA neurons in WT mice, spine density along the dendrite was not significantly reduced in a location dependent manner as indicated by the lack of a significant interaction between "stress" and "dendritic location" in the tPA$^{-/-}$ mice ($F=0.821$, $df=9$, $P>0.5$; Fig. 3B). Thus, the absence of the tPA gene appears to dampen the

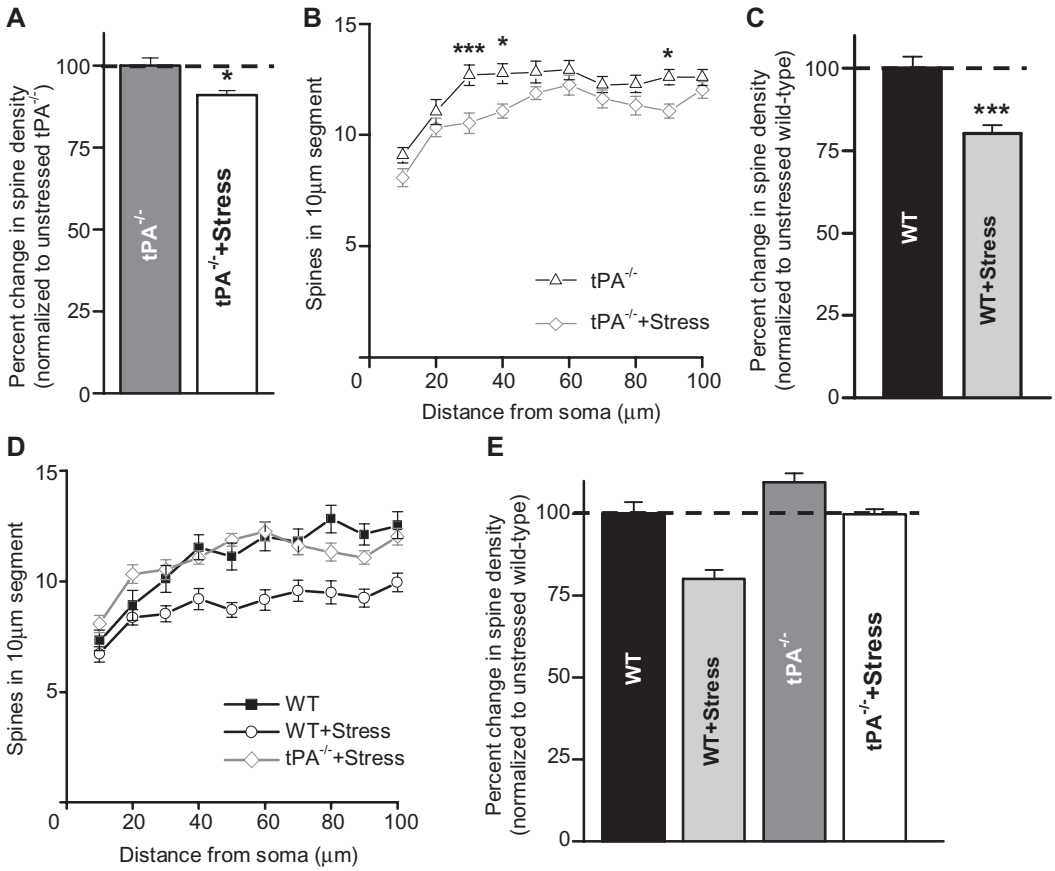

Fig. 3. Stress-induced spine loss in MeA is attenuated in tPA$^{-/-}$ mice. (A) Percent change in spine density elicited by stress in tPA$^{-/-}$ mice. * $P<0.05$, one-way ANOVA followed by Tukey-HSD post hoc test. (B) Segmental analysis of the mean (±S.E.M.) number of spines in each successive 10-μm segment along dendritic branches of tPA$^{-/-}$ (*open triangle*) and tPA$^{-/-}$+Stress (*open diamond*) neurons in MeA as a function of the distance of that segment from the origin of the branch. * $P<0.05$, *** $P<0.005$, compared with tPA$^{-/-}$, Tukey-HSD; tPA$^{-/-}$, $n=30$ neurons; tPA$^{-/-}$+Stress, $n=24$ neurons. (C) Percent change in spine density elicited by stress in WT mice (normalized to unstressed WT mice) is greater compared that seen in tPA$^{-/-}$ mice. *** $P<0.001$, one-way ANOVA followed by Tukey-HSD post hoc test. (D) Overlay of segmental plots (depicted earlier in Figs. 1D and 3A of the mean (±S.E.M.) number of spines in each successive 10-μm segment along dendritic branches from MeA neurons in WT (*closed square*), WT+Stress (*open circle*), and tPA$^{-/-}$+Stress (*open diamond*) mice as a function of the distance of that segment from the origin of the branch, demonstrating considerable overlap between WT and tPA$^{-/-}$+Stress neurons. (E) Comparison of percent changes in MeA spine density in all four groups (WT, WT+Stress, tPA$^{-/-}$ and tPA$^{-/-}$+Stress) normalized to unstressed WT, demonstrating comparable levels of spine density in WT and tPA$^{-/-}$+Stress neurons.

impact of chronic stress in terms of spine loss in the MeA. Furthermore, it was previously reported that stress-induced modulation of tPA signaling was localized to the MeA, and the BLA was not affected. In agreement with this finding, there was no difference in the percentage increase in BLA spine-density in $tPA^{-/-}$ mice (+20.8%) and WT mice (+21.2%, Fig. 1E). Therefore, the tPA-dependence of stress-induced modulation in spine-density was restricted to the MeA.

The attenuation of stress-induced spine loss in the $tPA^{-/-}$ mice is also evident in our detailed segmental analysis of spine-density. As depicted earlier in Fig. 1D, stress elicits significant spine loss across a majority of dendritic segments in WT mice, a pattern that is in contrast to what we observed in $tPA^{-/-}$ mice (Fig. 3B). Strikingly, a superposition of these segmental data (Fig. 3D) demonstrates that chronic stress, unlike in WT neurons ("WT+Stress," Fig. 3D), leads to a pattern of spine density in $tPA^{-/-}$ neurons ("$tPA^{-/-}$+Stress," Fig. 3D) that exhibits a remarkable degree of overlap with unstressed WT neurons (Fig. 3D). This result is also borne out by the fact that the average spine density in stressed $tPA^{-/-}$ mice (number of spines per 100 μm: 110.2±1.6, n=24 neurons, N=4 animals) is nearly the same as that measured in unstressed WT mice (number of spines per 100 μm: 110.4±3.6, n=24 neurons, N=3 animals).

In an attempt to obtain a comprehensive overview of the relative impact of chronic stress and the genetic deletion of tPA on MeA spine-density, Fig. 3E presents the average spine density in all four experimental groups, by normalizing the respective mean values relative to that of WT unstressed controls. This analysis shows that chronic stress causes an average decrease of ~20% in spine density in WT mice (WT versus WT+Stress, Fig. 3E). Disruption of the tPA gene leads to a small increase (~9%) in spine-density in the unstressed mutant mice ($tPA^{-/-}$), which is reversed by stress to a level that is virtually identical to WT spine-density (dotted line, Fig. 3E). Therefore, we conclude that in the absence of the tPA gene, even when challenged with repeated stress, MeA neurons are able to maintain levels of spine-density that are comparable to those seen in WT mice without stress.

DISCUSSION

The primary objective of this study was to test the hypothesis that the serine protease tPA mediates a specific form of stress-induced neuronal remodeling in the MeA, where it has previously been shown to play a role in triggering anxiety-like behavior and activating molecular markers of structural plasticity (Pawlak et al., 2003). To this end, evidence in support of three specific facets of this hypothesis is presented here. First, morphological data are presented for chronic stress-induced structural plasticity being manifested as a reduction in spine density in medium spiny stellate neurons of the MeA. Further, stress-induced spine loss in MeA is in stark contrast to stress-induced spine formation in the adjacent BLA. Second, absence of the tPA gene dampens the impact of chronic stress in terms of spine loss in the MeA. This attenuation in stress-induced spine loss, conferred by genetic deletion of tPA, is evident in MeA neurons from the stress-treated $tPA^{-/-}$ mice, which are able to maintain levels of spine-density comparable to those seen in WT neurons without stress. Third, the tPA-dependence of stress-induced modulation in spine density was restricted to the MeA because BLA spinogenesis was not affected in $tPA^{-/-}$ mice. This is in agreement with earlier observations that predicted stress-induced modulation of tPA to be specific to MeA, but not the BLA (Pawlak et al., 2003), and that morphological plasticity caused by chronic stress in the MeA may exhibit characteristics that are different from those observed in the BLA.

tPA and experience-dependent plasticity of spines

Results presented here suggest that in the absence of tPA, chronic stress fails to cause the significant spine loss that is observed in WT mice. Interestingly, while the absence of tPA has a small effect on basal levels of MeA spine density, it has a more pronounced impact on stress-induced loss of MeA spines. Earlier studies reported comparable levels of molecular markers of post- and pre-synaptic plasticity, such as ERK1/2 and GAP-43, in the WT and $tPA^{-/-}$ mice in the unstressed condition (Pawlak et al., 2003). Thus, future studies will be required to further examine the possibility that in the adult amygdala, tPA may be more important for mediating the stress-induced retraction of MeA spines, and less for maintaining the integrity of basal synaptic connectivity under normal unstressed conditions.

The sign or direction of this tPA-dependent change in MeA spine density is also relevant in light of recent data showing that tPA plays a pivotal role in controlling experience-dependent plasticity, especially spine dynamics, in the developing visual cortex (Mataga et al., 2004; Oray et al., 2004). In particular, our findings on stress-induced spine retraction are reminiscent of a recent report showing that tPA is required for the decrease in spine density in layer III pyramidal neurons caused by monocular deprivation (MD) (Mataga et al., 2004). Importantly, this MD-induced spine retraction was not present in tPA knockout mice. These data suggest that tPA-dependent plasticity does not necessarily lead to an increase in the number of spines, thereby shedding new light on earlier work demonstrating that tPA is important for activity-dependent growth of synapses and perforated spines (Neuhoff et al., 1999), and enhanced long-term potentiation (Huang et al., 1996; Baranes et al., 1998; Zhuo et al., 2000). In fact, recent data from cortical studies suggest a role for tPA in experience-induced structural remodeling that, rather than specifying the sign of spine changes, creates an environment permissive for such plasticity (Muller and Griesinger, 1998; Mataga et al., 2002). These findings suggest a scenario where the overall balance of input activity on to a neuron, along with the local molecular environment, determines whether experience-dependent activation of tPA will culminate in the loss or gain of dendritic spines (Berardi et al., 2004). Interestingly, our segmental analysis suggests that stress-induced variations in spine density of MeA neurons depend on dendritic location and this modulation is

also dependent on tPA. Although the precise dendritic distribution of synaptic inputs and receptors on MeA neurons is unknown, such tPA-dependent modulations in spine density that vary with dendritic location may reflect a finer regulation of the balance of input activity on these neurons. It is also important to note that, while the data contributing to this view on tPA-dependent plasticity have been gathered primarily from the hippocampus and cortex, our results provide the first evidence for a permissive role for tPA in spine retraction in a non-cortical nucleus of the amygdala, a structure with a non-laminar cytoarchitecture. Moreover, this cortex-based model has been proposed to describe the regulation of structural plasticity during the critical period of development. In the adult visual cortex, however, the extracellular matrix becomes strongly inhibitory for structural plasticity and tPA is no longer activated by MD (Mataga et al., 2004). In contrast, our observations point to a role for tPA in amygdalar spine plasticity in the adult brain. Thus, future studies will be required to investigate the precise nature of the role played by tPA in experience-induced plasticity in the amygdala.

What could be the mechanism by which tPA promotes stress-induced spine retraction in the MeA? It is known that tPA can modulate neuronal activity either in a proteolytic or non-proteolytic manner. The latter has been attributed to its interaction with either the NR2B subunit of *N*-methyl-D-aspartate receptor (Pawlak et al., 2005), or with the low-density lipoprotein receptor-related protein (LRP) receptor (Zhuo et al., 2000). These interactions facilitate various forms of neuronal activity, including seizures (Tsirka et al., 1995; Pawlak and Strickland, 2002; Yepes et al., 2002; Pawlak et al., 2005) and long-term potentiation (Huang et al., 1996; Baranes et al., 1998; Zhuo et al., 2000) and therefore could affect dendritic spine plasticity. As a protease, tPA has narrow substrate specificity, but its effects can be amplified by activation of a zymogen plasminogen to a broad-spectrum protease plasmin. Plasmin-mediated proteolysis can render the environment more permissive for structural plasticity, by acting upon a wide variety of substrates, including extracellular matrix proteins (Chen and Strickland, 1997), growth factors and neurotrophins (Mars et al., 1993; Pang et al., 2004), membrane receptors (Matys and Strickland, 2003; Pawlak et al., 2005), and cell adhesion molecules (Endo et al., 1998). Recent studies suggest that extracellular matrix degradation by plasmin is critical for dendritic spine plasticity in other brain regions (Mataga et al., 2004; Oray et al., 2004). Further studies are needed to clarify if similar mechanisms operate in the amygdala to facilitate stress-induced remodeling, fear and anxiety.

Stress-induced neuronal remodeling in the MeA: functional implications

In our study chronic stress led to a retraction in spines in MeA, in contrast to an increase observed in the BLA (Mitra et al., 2005). What could be the mechanism of such dissociation? First, we have examined stellate medium spiny neurons in MeA, which according to earlier anatomical studies (reviewed in McDonald, 1992) are the predominant cell type in the MeA. Interestingly, earlier Golgi studies (Millhouse and DeOlmos, 1983; McDonald, 1992) observed these cells to be very similar to the principal cell type found in the adjacent central nucleus, the other amygdalar nucleus where stress enhances tPA expression (Pawlak et al., 2003). However, in contrast to the moderately spiny MeA neurons analyzed in our study, a small proportion of MeA neurons are reported to have thick dendrites that exhibit a very dense covering of spines (Millhouse and DeOlmos, 1983; McDonald, 1992). Hence, we cannot rule out the possibility that chronic stress elicits different patterns of spine plasticity in this other cell type. Second, stress-induced spine retraction in the MeA could be a homeostatic response to enhanced synaptic connectivity in the BLA, from which it receives direct inputs (McDonald, 1992). This contrast between stress-induced plasticity in the MeA and the BLA is also highlighted by a recent study examining expression patterns in the amygdala of polysialylated-neural cell adhesion molecule (PSA-NCAM), which can be a substrate for tPA-mediated proteolysis (Endo et al., 1998) and also plays an important role in morphological plasticity and synaptic reorganization. This study demonstrates that PSA-NCAM immunoreactivity within the amygdala, present in somata and neuronal processes, has a regional gradient with the central and medial amygdaloid nuclei showing the highest levels of expression (Cordero et al., 2005). Further, chronic restraint stress caused a significant down-regulation in these nuclei, but not the BLA. In addition to chronic stress, contextual fear conditioning and corticosterone treatment modulate PSA-NCAM expression in the dentate gyrus (Sandi et al., 2001, 2003; Pham et al., 2003), as well as the frontal and piriform cortex (Sandi and Loscertales, 1999; Nacher et al., 2002). Studies in the amygdala also suggest a predominantly postsynaptic localization of PSA-NCAM in the MeA (Nacher et al., 2002; Cordero et al., 2005), which is consistent with a postsynaptic effect of repeated stress in the MeA reported here. More detailed anatomical data, on how specific classes of neurons in the BLA and MeA are connected, will be necessary to investigate post- and pre-synaptic aspects of this plasticity.

tPA mediated stress-induced spine changes in the MeA, but not in the BLA, which is consistent with its expression pattern in these structures. How does this reconcile with earlier reports on the effect of tPA on stress-induced plasticity and anxiety-like behavior (Pawlak et al., 2003; Matys et al., 2004)? We have previously shown that tPA is liberated during stress into the extracellular space in the MeA, as a result of corticotropin-releasing factor (CRF) receptor stimulation by CRF (Matys et al., 2004). This extracellular tPA facilitates neuronal activity in MeA, and mediates stress-induced anxiety (Pawlak et al., 2003; Matys et al., 2004). This is not surprising, since MeA is a major efferent nucleus of the amygdala, and there is anatomical evidence for a substantial projection from the MeA to the paraventricular nucleus, the apex of the HPA axis (Silverman et al., 1981; Tribollet and Dreifuss, 1981; Sawchenko and Swanson, 1983). Stimulation of MeA triggers activation of HPA axis and could thereby contribute to

behavioral effects of stress (Dunn and Whitener, 1986). Therefore, the effect of tPA on spine plasticity in MeA is consistent with the role of this protease in behavioral response to stress. Further studies are needed to gather more detailed data on the microcircuitry and molecular composition of the specific cell types and projections exhibiting structural plasticity in response to stress of different duration and intensity. Finally, it will be important to understand which of these multiple cellular events are causally related to stress-induced anxiety.

Acknowledgments—S.C. is supported by the Wellcome Trust. Research in R.P. laboratory is supported by Medical Research Council and by Alcoholic Beverage Medical Research Foundation. The authors thank Dr. Arvind Govindarajan for helpful discussions and help in statistical analysis of the data.

REFERENCES

Arnold FJ, De Lucas Bueno M, Shiers H, Hancock DC, Evan GI, Herbert J (1992) Expression of c-fos in regions of the basal limbic forebrain following intracerebroventricular corticotropin-releasing factor in unstressed or stressed male rats. Neuroscience 51:377–390.

Baranes D, Lederfein D, Huang YY, Chen M, Bailey CH, Kandel ER (1998) Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. Neuron 21:813–825.

Berardi N, Pizzorusso T, Maffei L (2004) Extracellular matrix and visual cortical plasticity: freeing the synapse. Neuron 44:905–908.

Bhatnagar S, Dallman M (1998) Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. Neuroscience 84:1025–1039.

Campeau S, Watson SJ (1997) Neuroendocrine and behavioral responses and brain pattern of c-fos induction associated with audiogenic stress. J Neuroendocrinol 9:577–588.

Canteras NS, Simerly RB, Swanson LW (1995) Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. J Comp Neurol 360:213–245.

Chen ZL, Strickland S (1997) Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. Cell 91: 917–925.

Conrad CD, LeDoux JE, Magarinos AM, McEwen BS (1999) Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. Behav Neurosci 113: 902–913.

Cordero MI, Venero C, Kruyt ND, Sandi C (2003) Prior exposure to a single stress session facilitates subsequent contextual fear conditioning in rats. Evidence for a role of corticosterone. Horm Behav 44:338–345.

Cordero MI, Rodriguez JJ, Davies HA, Peddie CJ, Sandi C, Stewart MG (2005) Chronic restraint stress down-regulates amygdaloid expression of polysialylated neural cell adhesion molecule. Neuroscience 133:903–910.

Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ (1995) Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64:477–505.

Dayas CV, Buller KM, Day TA (1999) Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. Eur J Neurosci 11:2312–2322.

Dunn JD, Whitener J (1986) Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: cytoarchitectural specificity. Neuroendocrinology 42:211–217.

Endo A, Nagai N, Urano T, Ihara H, Takada Y, Hashimoto K, Takada A (1998) Proteolysis of highly polysialylated NCAM by the tissue plasminogen activator-plasmin system in rats. Neurosci Lett 246:37–40.

Feldman S, Conforti N, Itzik A, Weidenfeld J (1994) Differential effect of amygdaloid lesions on CRF-41, ACTH and corticosterone responses following neural stimuli. Brain Res 658:21–26.

Herman JP, Prewitt CM, Cullinan WE (1996) Neuronal circuit regulation of the hypothalamo-pituitary-adrenocortical stress axis. Crit Rev Neurobiol 10:371–394.

Huang YY, Bach ME, Lipp HP, Zhuo M, Wolfer DP, Hawkins RD, Schoonjans L, Kandel ER, Godfraind JM, Mulligan R, Collen D, Carmeliet P (1996) Mice lacking the gene encoding tissue-type plasminogen activator show a selective interference with late-phase long-term potentiation in both Schaffer collateral and mossy fiber pathways. Proc Natl Acad Sci U S A 93:8699–8704.

LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23:155–184.

Li HY, Sawchenko PE (1998) Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: a comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels. J Comp Neurol 393:244–266.

Ma S, Morilak DA (2004) Induction of FOS expression by acute immobilization stress is reduced in locus coeruleus and medial amygdala of Wistar-Kyoto rats compared to Sprague-Dawley rats. Neuroscience 124:963–972.

Ma S, Morilak DA (2005) Norepinephrine release in medial amygdala facilitates activation of the hypothalamic-pituitary-adrenal axis in response to acute immobilisation stress. J Neuroendocrinol 17: 22–28.

Mars WM, Zarnegar R, Michalopoulos GK (1993) Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. Am J Pathol 143:949–958.

Mataga N, Mizuguchi Y, Hensch TK (2004) Experience-dependent pruning of dendritic spines in visual cortex by tissue plasminogen activator. Neuron 44:1031–1041.

Mataga N, Nagai N, Hensch TK (2002) Permissive proteolytic activity for visual cortical plasticity. Proc Natl Acad Sci U S A 99: 7717–7721.

Matys T, Pawlak R, Matys E, Pavlides C, McEwen BS, Strickland S (2004) Tissue plasminogen activator promotes the effects of corticotropin-releasing factor on the amygdala and anxiety-like behavior. Proc Natl Acad Sci U S A 101:16345–16350.

Matys T, Strickland S (2003) Tissue plasminogen activator and NMDA receptor cleavage. Nat Med 9:371–372; author reply 372–373.

McDonald AJ (1992) Cell types and intrinsic connections of the amygdala. In: The amygdala: Neurobiological aspects of emotion, memory, and mental dysfunction (Aggleton JP, ed), pp 67–96. New York: Wiley-Liss, Inc.

Millhouse OE, DeOlmos J (1983) Neuronal configurations in lateral and basolateral amygdala. Neuroscience 10:1269–1300.

Mitra R, Jadhav S, McEwen BS, Vyas A, Chattarji S (2005) Stress duration modulates the spatio-temporal patterns of spine formation in the basolateral amygdala. Proc Natl Acad Sci U S A 102:9371–9376.

Muller CM, Griesinger CB (1998) Tissue plasminogen activator mediates reverse occlusion plasticity in visual cortex. Nat Neurosci 1:47–53.

Nacher J, Lanuza E, McEwen BS (2002) Distribution of PSA-NCAM expression in the amygdala of the adult rat. Neuroscience 113:479–484.

Neuhoff H, Roeper J, Schweizer M (1999) Activity-dependent formation of perforated synapses in cultured hippocampal neurons. Eur J Neurosci 11:4241–4250.

Oray S, Majewska A, Sur M (2004) Dendritic spine dynamics are regulated by monocular deprivation and extracellular matrix degradation. Neuron 44:1021–1030.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306:487–491.

Pawlak R, Magarinos AM, Melchor J, McEwen B, Strickland S (2003) Tissue plasminogen activator in the amygdala is critical for stress-induced anxiety-like behavior. Nat Neurosci 6:168–174.

Pawlak R, Melchor JP, Matys T, Skrzypiec AE, Strickland S (2005) Ethanol-withdrawal seizures are controlled by tissue plasminogen activator via modulation of NR2B-containing NMDA receptors. Proc Natl Acad Sci U S A 102:443–448.

Pawlak R, Strickland S (2002) Tissue plasminogen activator and seizures: a clot-buster's secret life. J Clin Invest 109:1529–1531.

Pezzone MA, Lee WS, Hoffman GE, Rabin BS (1992) Induction of c-Fos immunoreactivity in the rat forebrain by conditioned and unconditioned aversive stimuli. Brain Res 597:41–50.

Pham K, Nacher J, Hof PR, McEwen BS (2003) Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. Eur J Neurosci 17:879–886.

Ramon-Moliner E (1970) The Golgi-Cox technique. In: Contemporary research methods in neuroanatomy (Nauta WJH, Egbesson SOE, eds), p 32. New York: Springer-Verlag.

Sandi C, Loscertales M (1999) Opposite effects on NCAM expression in the rat frontal cortex induced by acute vs. chronic corticosterone treatments. Brain Res 828:127–134.

Sandi C, Merino JJ, Cordero MI, Kruyt ND, Murphy KJ, Regan CM (2003) Modulation of hippocampal NCAM polysialylation and spatial memory consolidation by fear conditioning. Biol Psychiatry 54:599–607.

Sandi C, Merino JJ, Cordero MI, Touyarot K, Venero C (2001) Effects of chronic stress on contextual fear conditioning and the hippocampal expression of the neural cell adhesion molecule, its polysialylation, and L1. Neuroscience 102:329–339.

Sawchenko PE, Swanson LW (1983) The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. J Comp Neurol 218:121–144.

Silverman AJ, Hoffman DL, Zimmerman EA (1981) The descending afferent connections of the paraventricular nucleus of the hypothalamus (PVN). Brain Res Bull 6:47–61.

Tribollet E, Dreifuss JJ (1981) Localization of neurones projecting to the hypothalamic paraventricular nucleus area of the rat: a horseradish peroxidase study. Neuroscience 6:1315–1328.

Tsirka SE, Gualandris A, Amaral DG, Strickland S (1995) Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. Nature 377:340–344.

Vyas A, Chattarji S (2004) Modulation of different states of anxiety-like behavior by chronic stress. Behav Neurosci 118:1450–1454.

Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci 22:6810–6818.

Watanabe Y, Gould E, McEwen BS (1992) Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. Brain Res 588:341–345.

Yepes M, Sandkvist M, Coleman TA, Moore E, Wu JY, Mitola D, Bugge TH, Lawrence DA (2002) Regulation of seizure spreading by neuroserpin and tissue-type plasminogen activator is plasminogen-independent. J Clin Invest 109:1571–1578.

Zhuo M, Holtzman DM, Li Y, Osaka H, DeMaro J, Jacquin M, Bu G (2000) Role of tissue plasminogen activator receptor LRP in hippocampal long-term potentiation. J Neurosci 20:542–549.

(Accepted 29 August 2006)
(Available online 16 October 2006)