Tissue plasminogen activator in the amygdala is critical for stress-induced anxiety-like behavior

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Although neuronal stress circuits have been identified, little is known about the mechanisms that underlie the stress-induced neuronal plasticity leading to fear and anxiety. Here we found that the serine protease tissue-plasminogen activator (tPA) was upregulated in the central and medial amygdala by acute restraint stress, where it promoted stress-related neuronal remodeling and was subsequently inhibited by plasminogen activator inhibitor-1 (PAI-1). These events preceded stress-induced increases in anxiety-like behavior of mice. Mice in which the tPA gene has been disrupted did not show anxiety after up to three weeks of daily restraint and showed attenuated neuronal remodeling as well as a maladaptive hormonal response. These studies support the idea that tPA is critical for the development of anxiety-like behavior after stress.

The adaptive response to stress includes behavioral and physiological processes directed toward maintaining homeostasis[1]. Severe or sustained stress can compromise this response and lead to the development of behavioral disorders associated with cognitive impairments, depression, fear and anxiety[2]. The amygdala and the hippocampus are two critical components of the neuroanatomical stress circuit. The amygdala facilitates the stress response[3] and mediates aggression, fear and anxiety[4]. The hippocampus, on the other hand, attenuates stress responses by shutting off the hypothalamic-pituitary-adrenal (HPA) axis[5] and is involved in forming episodic, spatial and contextual memories[6]. Both the amygdala and hippocampus show dendritic remodeling in response to repeated stress[7,8], but the mechanisms underlying this neuronal plasticity are poorly understood.

Extracellular proteolysis provides an attractive mechanism by which the axons could remodel their synaptic connections. Precisely orchestrated exocytosis of a protease could facilitate axonal plasticity by degrading extracellular matrix proteins[9,10], interacting with membrane receptors[11] or activating latent growth factors[12]. One likely candidate for such a molecule is tissue plasminogen activator (tPA), a plasticity-related serine protease highly expressed in the hippocampus and amygdala[13,14]. Although the role of tPA in the hippocampus has been studied previously[9,14–18], its role in the amygdala has not been addressed. Here we show that tPA promotes stress-induced neuronal remodeling in this region and that tPA is critical for the development of anxiety-like behavior after stress.

Results

To investigate whether tPA has a role in the amygdala, we examined its expression pattern within this structure. tPA immunoreactivity was confined to the central and medial amygdala and was almost completely absent in the basolateral amygdala (**Fig. 1**). The central and medial amygdala have extensive connections to the paraventricular nucleus of the hypothalamus[19], which implicate these structures in the response of animals to stress[20]. Therefore, to determine if tPA participates in the stress response, we subjected mice to restraint stress and monitored changes in either extracellular or total tPA activity in the amygdala and hippocampus by *in situ* or SDS-PAGE zymography[13], respectively. Acute stress affected extracellular tPA activity in the medial and central amygdala ($F_{2,12} = 96.52$; $P < 0.0001$), which was elevated fourfold 30 minutes after the beginning of the restraint (**Fig. 2a–c** and **g**; $P < 0.001$). This extracellular increase was accompanied by a two-fold elevation of total tPA activity (**Fig. 3a** and **b**; $P < 0.05$). tPA activity returned to normal 18 hours after the restraint was completed. This induction of tPA activity was spatially specific, as there was no increase in tPA activity in the hippocampus (**Figs. 2a–c** and **6a**). In addition, the other form of plasminogen activator, urokinase-type plasminogen activator (uPA), was unaffected in the amygdala or hippocampus by stress (**Figs. 3a** and **b** and **6a**).

After the increase in the extracellular and total tPA activity, extracellular tPA activity in the medial amygdala was markedly attenuated 6 hours after the beginning of restraint (**Fig. 2c** and **g**), which could reflect its inhibition or clearance. Neuroserpin is a specific inhibitor of tPA found in the brain[21], and we therefore examined the regulation of its expression by stress using immunoblotting. This protein was not upregulated by restraint stress in the amygdala (data not shown); therefore it could not be responsible for inhibiting tPA activity. Another possible inhibitor was plasminogen activator inhibitor-1 (PAI-1), a major protein upregulated after restraint stress in most tissues[22]. PAI-1 protein was upregulated in the areas showing reduced tPA

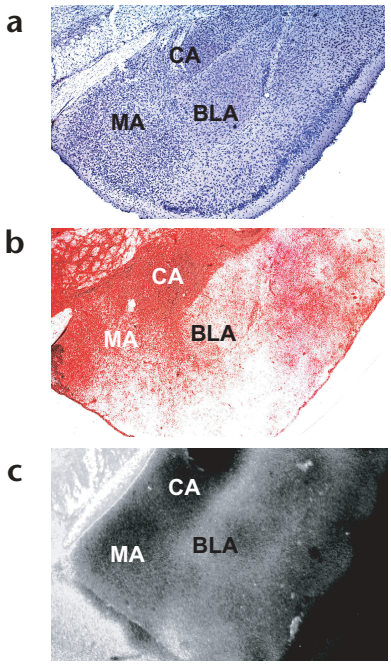

Fig. 1. The expression of tissue plasminogen activator (tPA) in the amygdala. (**a**) The anatomical organization of the amygdala visible on cresyl violet–stained section helps identify tPA immunoreactivity (red in **b**) and activity (dark lytic zones on *in situ* zymography in **c**) in central (CA) and medial (MA), but not in basolateral (BLA) amygdala.

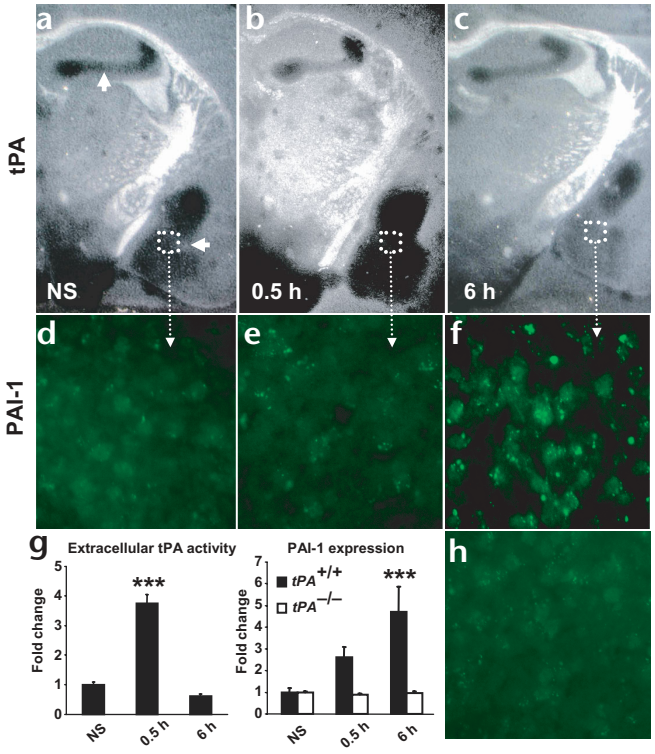

Fig. 2. The regulation of extracellular tissue plasminogen activator (tPA) activity by restraint stress in the hippocampus and amygdala. (**a**–**c**) *In situ* zymography of extracellular tPA activity (dark lytic zones) shows that in basal conditions (**a**) tPA is active in the hippocampal mossy fiber pathway (upper arrowhead in **a**) and in the central and medial amygdala (lower arrowhead in **a**). Extracellular tPA activity increased after 30 min of restraint stress (**b**) and was subsequently inhibited 6 h later (**c**) (quantification in **g**). This inhibitory factor was identified as plasminogen activator inhibitor-1 (PAI-1), as shown by immunohistochemistry performed on adjacent brain sections (green in **d**–**f** and quantification in **g**). The increase in PAI-1 was absent in $tPA^{-/-}$ mice after 6 h of stress (**h**, quantified in **g**). NS, no stress; 0.5 and 6 h indicate the duration of the restraint. ***$P < 0.001$ versus NS.

activity (**Fig. 2c**, **f** and **g**; $P < 0.001$), suggesting that PAI-1 was responsible for this decrease. The upregulation of PAI-1 was absent in $tPA^{-/-}$ mice (**Fig. 2g** and **h**), showing that tPA directly, or via its effect on other molecules, induces PAI-1 expression.

Given the role of tPA in mediating neuronal plasticity and the stress-induced increase in tPA activity in the medial and central amygdala, it was possible that tPA participated in stress-induced neuronal remodeling in these regions. To study this possibility, we examined phosphorylation of extracellular signal–regulated kinase 1/2 (ERK1/2), a key event linking synaptic activity with postsynaptic plasticity and learning[23,24]. In $tPA^{+/+}$ mice, ERK1/2 phosphorylation in the amygdala was evident after 5 minutes of stress ($P < 0.01$) and returned to normal by 15 minutes (**Fig. 4a** and **b**). The latter suggests rapid activation of a phosphatase, serving as a negative feedback loop that controls dephosphorylation of ERK1/2 (ref. 25). Phosphorylation of ERK1/2 was not observed in $tPA^{-/-}$ mice at any time point (5, 15 min, **Fig. 4a** and **b**; 30 min, 2 h and 6 h, data not shown). This result shows an essential role of tPA in triggering mechanisms crucial for postsynaptic plasticity.

To study if axonal remodeling was similarly affected by stress, we examined the expression of GAP-43, a presynaptic protein used as a marker of axonal plasticity[26]. In wild-type mice, the expression of GAP-43 in the amygdala increased considerably after six hours of restraint stress (**Fig. 4c**, **d**, **f** and **h**), overlapping spatially with changes in tPA activity. In contrast, there was no increase in GAP-43 in the hippocampus (**Fig. 6b**). To further confirm the role of tPA in stress-induced plasticity in the amygdala, we checked if stress similarly increases the expression of GAP-43 in $tPA^{-/-}$mice. Depending on the method of detection (western blotting or immunohistochemistry), the stress-induced increase in GAP-43 was either abolished (**Fig. 4c** and **d**) or attenuated in the medial amygdala of $tPA^{-/-}$ compared with $tPA^{+/+}$ animals (**Fig. 4f–i**). Taken together, these results suggest that tPA promotes stress-induced neuronal plasticity in this region.

As neuronal plasticity may underlie the behavioral changes observed after stress, we explored whether tPA affects stress-induced behavior modifications. To this end, we subjected $tPA^{+/+}$ and $tPA^{-/-}$ mice before and after stress to the elevated-plus maze, a test which requires an intact amygdala[27] and measures the level of fear and anxiety. This maze takes advantage of the natural tendency of rodents to prefer closed spaces to open spaces, which is exacerbated when their anxiety level is high. This model is known to depend on the central amygdala where tPA is expressed, but is less dependent on the basolateral amygdala[27], where we did not observed any tPA protein. ANOVA revealed a strong effect of the genotype on the number of entries to open arms of the elevated-plus maze after stress ($F_{1,45} = 11.44$; $P = 0.001$). Acute stress resulted in a decrease in open arm entries in $tPA^{+/+}$ mice (Tukey *post-hoc* comparison, $P = 0.037$; **Fig. 5a**), whereas 21 days of daily restraint led to some habituation. The number of entries to the closed arms as well as head dips to the open arms were

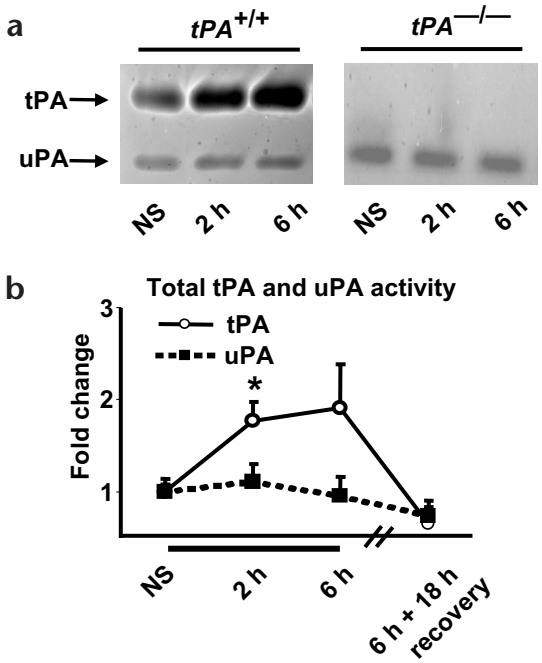

Fig. 3. Stress increases total tPA activity in the amygdala. (**a**, **b**) SDS-PAGE zymography shows that tPA activity (extracellular plus intracellular) increased in amygdala homogenates 2 h after the beginning of stress and returned to normal levels 18 h after the stress was completed. (**b**) No corresponding changes in urokinase-type plasminogen activator (uPA) were observed. NS, no stress; solid line under *x*-axis indicates the duration of the restraint. *$P < 0.05$ versus NS.

similarly altered by stress in these animals (**Fig. 5b** and **c**). In $tPA^{-/-}$ mice, however, none of these parameters was altered by stress (**Fig. 5a–c**). This finding shows that tPA participates in the increase in anxiety after stress, probably through facilitating neuronal plasticity in the medial and central amygdala, the importance of which for various kinds of the anxiety-like behavior is well-documented[27,28]. Although the elevated-plus maze might also require the hippocampus, it seems unlikely that it affected our study, as acute stress did not have any effect on either tPA activity (**Figs. 2a–c** and **6a**) or neuronal plasticity (**Fig. 6b**) within this structure.

A primary substrate of tPA is plasminogen: tPA converts plasminogen to the broad-spectrum protease plasmin. To investigate if the effect of tPA is plasminogen-dependent, we subjected *plasminogen*$^{-/-}$ mice to restraint stress and monitored their level of anxiety in the elevated-plus maze. *Plasminogen*$^{-/-}$ mice reacted to stress similarly to wild-type animals (significant effect of stress on the open arm entries, $F_{2,41} = 7.29$; $P < 0.01$; lack of effect of the genotype; **Fig. 5d**), indicating that the observed effect of tPA did not depend on plasminogen.

One possible explanation for the lack of anxiety in $tPA^{-/-}$ mice after restraint is that this procedure is not stressful to them for some reason. A useful indicator of stress level is the activation of the HPA axis, which can be estimated by the plasma concentration of corticosterone. We therefore measured this hormone in $tPA^{+/+}$ and $tPA^{-/-}$ mice before, immediately after, and 90 minutes after a 30-minute restraint period. Pre-stress corticosterone plasma levels did not differ between the two genotypes. Both $tPA^{+/+}$ and $tPA^{-/-}$ mice responded to a 30-minute restraint stress with a significant increase in corticosterone secretion (Tukey *post-hoc* comparisons; $P < 0.001$ for both $tPA^{+/+}$ and $tPA^{-/-}$, compared with the respective pre-stress levels), but the corticosterone levels of $tPA^{-/-}$ mice were 30% higher than those of $tPA^{+/+}$ animals ($P < 0.005$). In addition, and unlike $tPA^{+/+}$ mice, corticosterone levels in $tPA^{-/-}$ mice did not return to pre-stress levels after a recovery period ($P < 0.001$ and $P < 0.005$ compared with pre-stress levels in $tPA^{-/-}$ mice and levels in $tPA^{+/+}$ at the same time point, respectively; **Fig. 7**).

Adaptation to chronic restraint stress was investigated by measuring plasma corticosterone levels in mice immediately before their 21st day of restraint stress. At that point, corticosterone levels in $tPA^{+/+}$ mice had returned to pre-stressed, basal levels. In contrast, $tPA^{-/-}$ mice tended to show elevated corticosterone levels before their final restraint stress session, compared with naive $tPA^{+/+}$ and $tPA^{-/-}$ mice, but no statistical significance was reached (**Fig. 7**). These results show that tPA is not necessary for the stress-induced activation of the HPA axis, but does participate in the extent and duration of the hormonal response to stress.

Discussion

Prolonged, intense stress can cause functional and morphological changes in the brain[29,30] and can trigger pathological anxiety-like behavior, such as that observed in posttraumatic stress syndrome[31]. The amygdala is critical for the processing of various kinds of emotions including fear and anxiety[4,32]. Both neuronal plasticity[4] and long-term potentiation (LTP)-like events[33] have been observed in the amygdala of animals undergoing fear conditioning, suggesting that the development of these emotions involves mechanisms similar to learning. Moreover, stress causes neuronal remodeling within the amygdala[8], which could either be adaptive and aimed to attenuate the traumatic impact of stress on the brain or could reflect the disruption of putative protective mechanisms. The mechanisms underlying this stress-induced neuronal plasticity and anxiety have not yet been identified.

Activity-evoked extracellular proteolysis is an attractive mechanism that could translate electrophysiological events into more permanent structural remodeling of the synaptic connections. The serine protease tPA, which is released from neurons upon excitation[10,34,35], can facilitate learning[17] by mediating axonal plasticity[10] and formation of new synapses[10,36] in the hippocampus. The presence of tPA in the medial and central amygdala, regions showing strong connections with the paraventricular hypothalamus and hypothalamic-pituitary-adrenal (HPA) axis[19,20], suggests that it could be important for appropriate processing of stress-related information.

Our work identifies tPA in the amygdala as a key player in a sequence of events which may link experience-dependent plasticity with the development of anxiety. First, tPA is released from neurons into the extracellular space in a spatially restricted manner. This release is followed by the elevation of intracellular tPA, most likely reflecting an increase in its transcription or translation. The liberated tPA provides a signal to the postsynaptic machinery to phosphorylate ERK1/2, a trigger for plasticity-like events[23,24]. Respective axonal counterparts are also structurally modified at later time points. Finally, tPA forms a complex with its inhibitor PAI-1 upon completion of neuronal remodeling. These events precede the development of anxiety after stress. Our findings are consistent with previous reports showing the release of tPA into the extracellular space upon neuronal depolarization[10,34,35], subsequent upregulation of tPA mRNA following various forms of neuronal activity[14] and upregulation of PAI-1 after restraint stress[22].

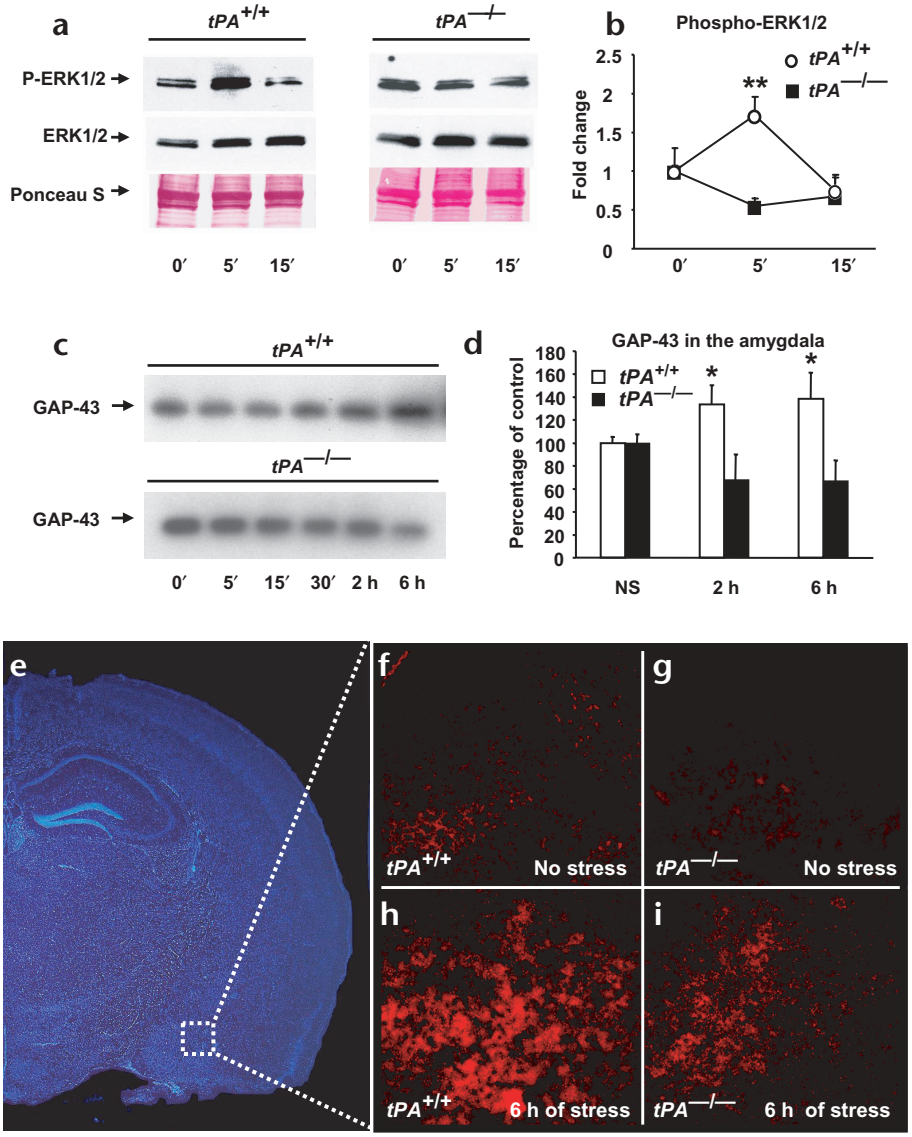

Fig. 4. tPA mediates synaptic plasticity in the amygdala. (**a** and quantification in **b**) Stress resulted in phosphorylation of postsynaptic extracellular signal-regulated kinase (P-ERK1/2) at 5 min (5′; $^{**}P < 0.01$ versus baseline), which was not observed in $tPA^{-/-}$ mice. ERK1/2 phosphorylation returned to normal at 15 min (15′) after stress. (**c**–**i**) The expression of GAP-43, a marker of axonal plasticity, was analyzed in the whole amygdala by western blotting (**c**, **d**) and in the medial amygdala (indicated by the box in **e**, a representative, DAPI-stained section) by immunohistochemistry (red in **f**–**i**). GAP-43 was upregulated by stress in $tPA^{+/+}$ mice (**c**, **d**, **f**, **h**), and this upregulation was either absent (**c**, **d**) or attenuated (**g**, **i**) in $tPA^{-/-}$ mice. ANOVA revealed a major effect of the genotype ($F_{1,17} = 14.81$, $P < 0.01$) and significant genotype × time interaction ($F_{2,17} = 3.8$; $^{*}P < 0.05$ versus $tPA^{-/-}$) in (**d**). NS, no stress; 5′, 15′, 30′, 2 h and 6 h indicate the duration of the restraint.

These results raise the question of the identity of a substrate/receptor that tPA acts upon to promote neuronal remodeling. A primary substrate of tPA is plasminogen, which it converts to the broad-spectrum protease plasmin. As plasmin can modulate neuronal activity[37] and promote neuronal death[9], we investigated whether the effect of tPA in the amygdala depends on plasminogen activation. The action of tPA on neuronal plasticity did not require plasminogen, similar to facilitation of electrical activity by tPA within the limbic circuit[38].

If plasminogen is not involved, what is the mechanism by which tPA facilitates neuronal plasticity and promotes anxiety? It is possible that tPA could facilitate LTP in the medial amygdala, as it does in the hippocampus[39,40]. The mechanism of LTP in the medial amygdala, where tPA is expressed, seems to differ from LTP in the lateral nuclei[41], where tPA is not present. For example, LTP in the medial amygdala is more dependent on the activation of NMDA receptors than that in the lateral amygdala[41]. Thus, the role of tPA could involve the modification of the NR1 subunit[11], leading to potentiation of NMDA receptor signaling. Alternatively, the effect of tPA could be non-proteolytic[42] and mediated by the low-density lipoprotein receptor–related protein (LRP)[43]. LRP is abundantly expressed in the brain and is a major clearance receptor for both tPA and tPA/PAI-1 complexes. The interaction between tPA and LRP is crucial for the main-

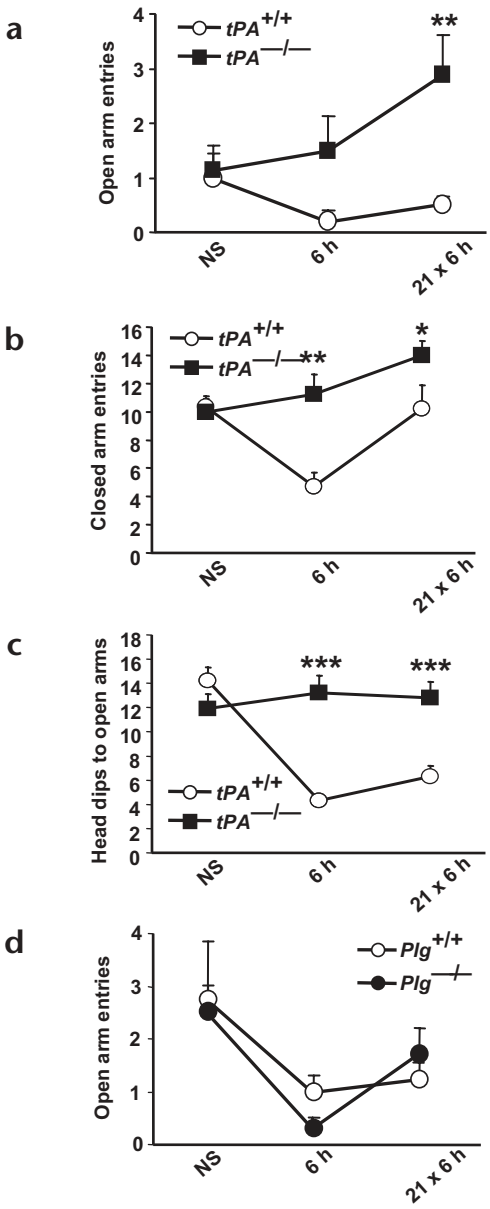

Fig. 5. Lack of stress-induced anxiety in the absence of tPA but not in the absence of plasminogen. The day after indicated periods of stress, the mice were subjected to the elevated-plus maze, which measures anxiety level. Stress elevated the level of anxiety, as judged by the decrease in the number of entries to the open arms in wild-type mice (*tPA*$^{+/+}$ and *plasminogen*$^{+/+}$; open circles in **a** and **d**). (**b**, **c**) Other behavioral measures were also altered by stress. These responses were not observed in *tPA*$^{-/-}$ mice (black squares in **a**–**c**). Unlike *tPA*$^{-/-}$ mice, *plasminogen*$^{-/-}$ mice showed the usual response to stress (black circles in **d**), showing that tPA must use a substrate different from plasminogen to promote stress-induced anxiety (see statistical analysis in the text). NS, no stress; 21 × 6 h indicates 6 hours of daily restraint for 21 consecutive days. *$P < 0.05$, **$P < 0.01$,***$P < 0.001$ versus *tPA*$^{+/+}$.

tenance of LTP in the hippocampus[43]. It is possible that LRP could serve a similar physiological role in the amygdala, promoting LTP and neuronal remodeling, as well as the development of region-specific behaviors such as contextual learning in the hippocampus and anxiety in the amygdala.

The conclusion that tPA acts in the amygdala, and not in the hippocampus, to facilitate stress-induced anxiety-like behavior is based on the fact that acute stress upregulates tPA, PAI-1 and GAP-43 specifically in the amygdala. Although these proteins are also present in the hippocampus, none of them was modulated by stress. These findings strongly suggest that after acute restraint-stress, an increase in anxiety is mediated primarily by tPA in the amygdala.

Our results show that tPA is not necessary for activation of the HPA axis, and therefore functions at some other point to modulate stress. There are presumably many factors that converge to generate stress-induced plasticity, and tPA is one critical, but not the only, element.

It is well documented that the basolateral amygdala is involved in classical fear conditioning and serves as a putative site of emotional learning[4,32]. Consistent with the fact that tPA is not expressed in the basolateral amygdala, *tPA*$^{-/-}$ mice do not have deficits in fear conditioning[39]. It is possible that tPA gene disruption could affect the ability of stress to modulate fear conditioning through its influence on the central amygdala, in which tPA is expressed. Other serine proteases such as neuropsin, which is expressed in the basolateral amygdala[44], could directly facilitate neuronal plasticity related to conditioned fear, serving a function similar to that of tPA in other amygdala regions. Therefore, synaptic mechanisms activated in response to conditioned and unconditioned stimuli may differ according to the region involved (basolateral versus medial amygdala), the molecular machinery used (tPA versus other proteases) and/or the time of its activation. In the present study, for example, phosphorylation of ERK1/2 occurred more rapidly than typically observed after fear conditioning and was more transient in nature[45].

In summary, our study shows that tPA is an important mediator of neuronal remodeling in the medial amygdala and is essential for the development of stress-induced anxiety, relevant to that observed in posttraumatic stress syndrome. tPA and/or its molecular collaborators could thus be new pharmacological targets for the development of drugs for anxiety disorders.

Methods

Restraint stress. Three-month old wild-type C57/BL/6 (*tPA*$^{+/+}$, *plasminogen*$^{+/+}$) and tPA or plasminogen knockout mice (*tPA*$^{-/-}$ and *plasminogen*$^{-/-}$; generous gift of P. Carmeliet and D. Collen) were back-crossed to C57/BL/6 for nine generations. Experiments were performed during the light period of the circadian cycle. Control animals (n = 4–10 per genotype at each time point) were left undisturbed, and stressed animals (n = 4–10) were subjected to a single 30-min, 2-h or 6-h restraint or 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. All the results are expressed as mean ± s.e.m. The experiments were approved by the Rockefeller University Laboratory Animal User's Committee.

***In situ* and SDS-PAGE zymography.** *In situ* and SDS-PAGE zymography was performed as previously described[13]. For SDS-PAGE zymography, the hippocampus was dissected as previously described[46]. The amygdala (–0.5 to –2.5 mm from bregma) was dissected using coronal mouse brain template (ASI Instruments, Warren, Michigan), homogenized in 0.1 M Tris 0.1% Triton X-100 (pH 7.2), and the protein concentration was adjusted to 2 mg/ml with the same buffer. The samples (15 µl) were electrophoresed on 7.5–10% SDS-polyacrylamide gels without reduction. After soaking in 2.5% Triton X-100, the gels were incubated overnight on casein-agar indicator films containing 10 mM Tris, 10 mg/ml of agarose, 2% skim milk and 4 µg/ml of human Glu-plasminogen, in a humid chamber at 37 °C and photographed.

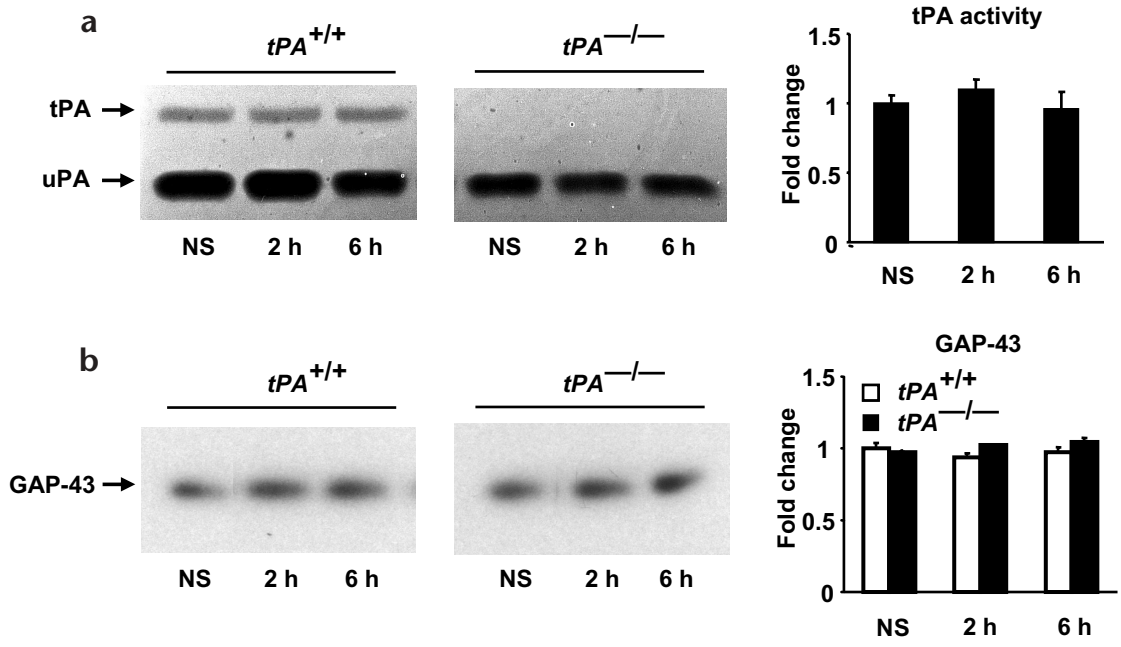

Fig. 6. Acute stress does not affect tPA, uPA or GAP-43 levels in the hippocampus. (**a**) SDS-PAGE zymography of hippocampal homogenates shows similar total tPA and uPA activity before and after stress. Extracellular tPA activity was also unaffected (quantification in **a**; see **Fig. 2a–c** for examples). (**b**) Western blot analysis showed that stress did not affect GAP-43 in the hippocampus of $tPA^{+/+}$ and $tPA^{-/-}$ mice. NS, no stress; 2 h and 6 h indicate the duration of the restraint.

For *in situ* zymography, mice were anesthetized and transcardially perfused with ice-cold isotonic saline, their brains were removed, frozen and cut at 15 μm-thick sections. The overlay mixture (identical as for SDS-PAGE zymography) was prepared at 42 °C and 300 μl were applied to the prewarmed brain sections mounted on glass slides and spread evenly under glass coverslips. The slides were incubated at 37 °C in humid chambers and the developed zymograms were examined under dark-field illumination.

The optical density of the bands (SDS-PAGE zymography or western blotting) or area of lysis (*in situ* zymography) were measured using NIH Image.

Immunohistochemistry and western blotting. Immediately after the restraint session, mice were anesthetized with 2.5% avertin. The animals were perfused transcardially with PBS (with 10 mM NaF and 10 mM β-glycerophosphate for the phosphorylation study), their brains removed and frozen. Coronal brain sections (15 μm) were cut, collected on silane-coated slides and stored in –80 °C until analyzed. The sections were then fixed with 4% paraformaldehyde in PBS for 25 min at 4 °C, rinsed, blocked with 1% BSA and 1% goat serum and incubated with primary antibodies: rabbit anti-tPA (1:1,500, Molecular Innovations, Southfield, Michigan), rabbit anti-GAP-43 (1:5,000, Chemicon, Temecula, California), rabbit anti-PAI-1 (1:1,000, American Diagnostica, Greenwich, Connecticut) and rabbit anti-PAI-1 (1:1,000, gift from D. Loskutoff) for 2 h at room temperature. For tPA and GAP-43, the sections were then rinsed and incubated with biotinylated goat anti-rabbit secondary antibody (1:1,000, Vector Laboratories, Burlingame, California) followed by either streptavidin-HRP (1:500, Vector) and NovaRed (Vector) or ExtrAvidin-Cy3 conjugate (1:500, Sigma, St. Louis, Missouri). For PAI-1, FITC-conjugated anti-rabbit secondary antibody was used (Vector, 1:500). The images were obtained using a Zeiss Axioscope 2 equipped with appropriate fluorescent filters and connected to a digital camera. For quantification, the fluorescent pictures were converted to grayscale with Adobe Photoshop and the signal intensity was measured using NIH Image. Western blotting (the samples homogenized in 0.1 M Tris, 10 mM NaF and 10 mM β-glycerophosphate, 0.1% Triton-X, pH 7.2 and the protein concentration adjusted to 2 mg/ml, 5 and 25 μg of total protein per lane, respectively) was performed using rabbit anti-GAP-43 (Chemicon, 1:2,500) or rabbit anti-phospho-ERK1/2 (1:1,000, Cell Signaling, Beverly, Massachusetts) and, after stripping, re-blotted with rabbit anti-ERK1/2 (1:1,000) followed by HRP-conjugated goat anti-rabbit IgG (1:1,000, Vector). For quantification, the level of phosphorylated form of ERK1/2 was normalized to total ERK1/2.

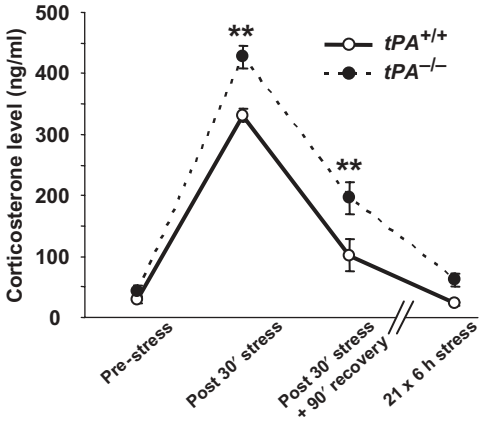

Fig. 7. The effect of acute and chronic stress on plasma corticosterone (CORT) levels (ng/ml) in $tPA^{+/+}$ and $tPA^{-/-}$ mice. Two-way ANOVA revealed a significant genotype × time interaction ($F_{2,24} = 3.80$, $P = 0.037$). Circulating CORT levels were higher in $tPA^{-/-}$ mice immediately after 30-min acute restraint stress, compared with those detected in $tPA^{+/+}$ animals. After 90-min recovery, hormonal levels of $tPA^{+/+}$ mice did not differ from pre-stress levels, whereas $tPA^{-/-}$ mice showed an impaired shutoff of the hormonal stress response. See Results for detailed statistical analysis. $^{**}P < 0.005$ versus $tPA^{+/+}$.

Elevated-plus maze. Stressed animals were tested the following morning, and subjected to the maze only once. The apparatus was made of four wooden arms (two enclosed arms, $67 \times 7 \times 17$ cm, that formed a cross shape with the two open arms 67×7 cm). The maze was 55 cm above the floor and dimly illuminated. The mice were placed on the central platform facing an open arm and allowed to explore the apparatus for 5 min. The sessions were videotaped for subsequent analysis. The numbers of entries of the animal from the central platform to closed or open arms was counted.

Corticosterone measurement. Blood was obtained by tail clipping in less than 30 s after removing the mice from their home cages. Heparinized tubes were centrifuged and the plasma separated and analyzed for corticosterone levels using I^{125} RIA-based kit (ICN Diagnostics, Orangeburg, New York) according to manufacturer instructions.

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Competing interests statement

The authors declare that they have no competing financial interests.

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