

ARTICLE



PKC δ -positive GABAergic neurons in the central amygdala exhibit tissue-type plasminogen activator: role in the control of anxiety

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Tissue plasminogen activator (tPA) is a serine protease expressed in several brain regions and reported to be involved in the control of emotional and cognitive functions. Nevertheless, little is known about the structure-function relationships of these tPA-dependent behaviors. Here, by using a new model of constitutive tPA-deficient mice (tPA^{null}), we first show that tPA controls locomotor activity, spatial cognition and anxiety. To investigate the brain structures involved in these tPA-dependent behavioral phenotypes, we next generated tPA^{flox} mice allowing conditional tPA deletion (cKO) following stereotaxic injections of adeno-associated virus driving Cre-recombinase expression (AAV-Cre-GFP). We demonstrate that tPA removal in the dentate gyrus of the hippocampus induces hyperactivity and partial spatial memory deficits. Moreover, the deletion of tPA in the central nucleus of the amygdala, but not in the basolateral nucleus, induces hyperactivity and reduced anxiety-like level. Importantly, we prove that these behaviors depend on the tPA present in the adult brain and not on neurodevelopmental disorders. Also, interestingly, our data show that tPA from Protein kinase-C delta-positive (PKC δ) GABAergic interneurons of the lateral/ capsular part of adult mouse central amygdala controls emotional functions through neuronal activation of the medial central amygdala. Together, our study brings new data about the critical central role of tPA in behavioral modulations in adult mice.

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INTRODUCTION

In humans, emotional and cognitive impairments are symptoms of several psychiatric diseases, exacerbated by concomitant events such as stress, aging and/or chronic diseases, leading to a dramatic deterioration in the quality of life [1]. The precise molecular and cellular mechanisms that underlie these abnormal behaviors are still not well understood, and deserve further works aimed at developing accurate targeted therapies.

The tissue-type plasminogen activator (tPA) is a pleiotropic serine protease initially characterized for its ability to activate plasminogen into plasmin and thus to promote vascular and tissue fibrinolysis [2, 3]. In the central nervous system, tPA is expressed in many brain areas including the hippocampus, the entorhinal cortex and the amygdala [4–8] and reported to influence physiological mechanisms such as synaptic plasticity [9–12]. Thus, tPA contributes, either directly or indirectly, to the modulation of some learning and memory processes [8, 10, 13–17] as well as emotional responses [4, 18–21], and locomotor activity [22, 23]. tPA-dependent effects on synaptic plasticity can be attributed to the modulation of *N*-Methyl D-Aspartate receptor, the type I Low Density Lipoprotein Related Receptor (LRP-1) signaling or to the plasmin-dependent conversion of the pro-

Brain Derived Neurotrophic Factor (pro-BDNF) into its mature form [8–10, 12, 15, 24, 25]. Up to now, all the studies aiming at investigating the role of tPA in behaviors were performed from constitutive tPA-deficient mice, without information about whether these impairments could be linked to developmental alterations or not. Similarly, structure-function respective relationships in these tPA-dependent functions were indirectly determined in the majority of cases. In addition, all the data today available were obtained from C57BL/6J tPA-deficient mice harboring the 129-derived chromosomal segment that co-segregates with the targeted tPA (Plat) allele, leading to a differential expression of a set of genes which could interfere with tPA's functions [26].

In the present study, we have first characterized the behavioral phenotype of a novel tPA-deficient mouse strain (named tPA^{null} mice) generated by a unique deletion of the exon-3 of the Plat gene, to avoid possible off-target effects. Then, the region-specific tPA's deletion was induced through stereotaxic delivery of AAV-Cre-GFP either in the dentate gyrus of the hippocampus (DG), the amygdala (basolateral amygdala; BLA and central amygdala; CeA) or the prefrontal cortex (PFC) of adult tPA^{flox} mice (flanking exon-3 of the Plat gene), also newly generated by our group. Using these new

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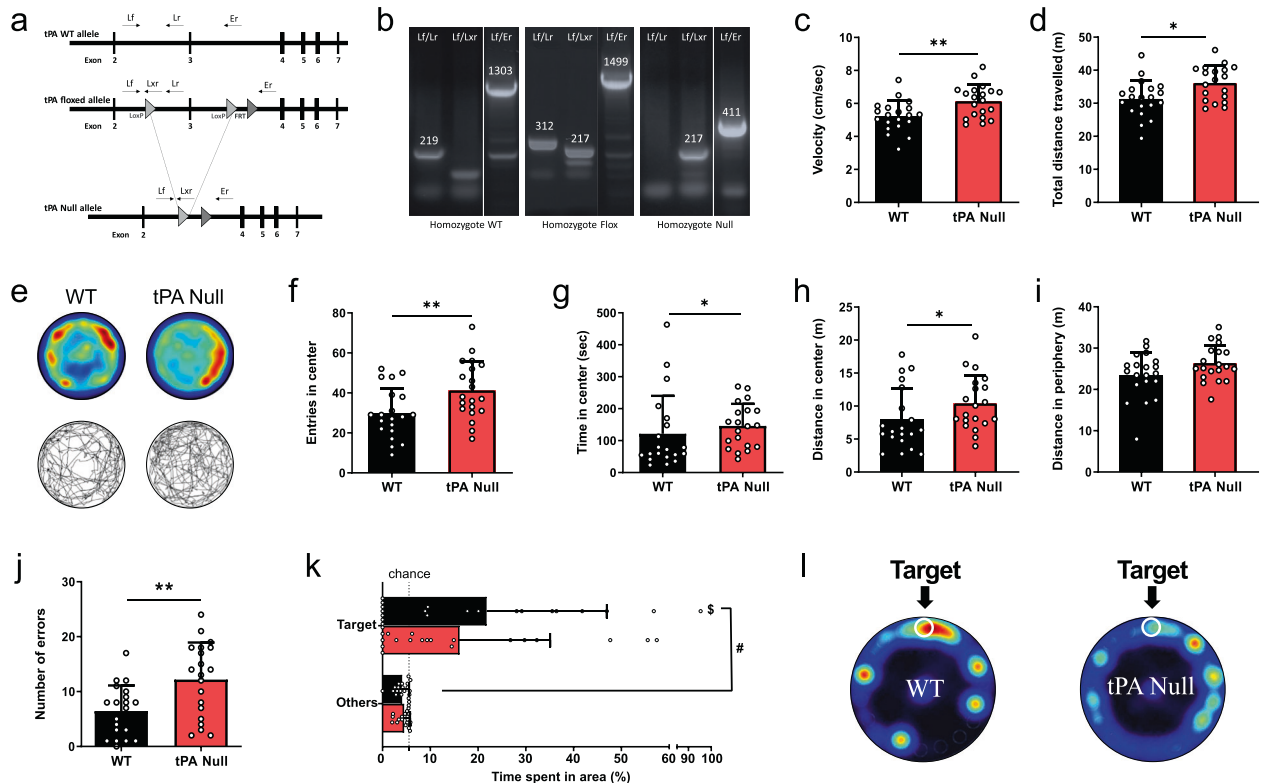


Fig. 1 Constitutive tPA-deficient mice (tPA^{null}) display locomotor, emotional and cognitive deficits. **a, b** Generation of tPA^{null} mice and the corresponding tPA^{lox} mice. **c–e** tPA^{null} mice display locomotor hyperactivity in the open-field test. Intergroup comparisons of the velocity (**c**) and distance (**d**); Mann–Whitney *U* tests, **p* < 0.05, ***p* < 0.01. Averaged heat maps and representative trajectories of tPA^{null} and WT mice (**e**). **f–i** tPA^{null} mice show decreased anxious responses in the open-field test. Intergroup comparisons of entries (**f**), time (**g**) and distance in the center (**h**), or peripheral (**i**), area; Mann–Whitney *U* tests, **p* < 0.05, ***p* < 0.01. **j–l** tPA^{null} mice display impaired cognition in the Barnes maze test. Intergroup comparisons of the number of errors (**j**); Student's *t* test, ***p* < 0.01. Intragroup comparisons of the time spent in areas (**k**); Wilcoxon test, #*p* < 0.05. Comparison with chance level (5.5 %), Wilcoxon signed-rank test, §*p* < 0.05. Averaged heat maps of tPA^{null} (*n* = 10) and WT mice (*n* = 10) from the same training group (**l**). *n* = 20 per genotype. Data are presented as mean ± SD.

experimental models, we first found that the conditional tPA deletion in the dentate gyrus of adult mice leads to hyperactivity and mild cognitive deficits. Then, we revealed that deletion of the tPA contained in PKCδ-positive GABAergic neurons of the adult CeA promotes hyperactivity as well as reduced anxiety-like behavior. Finally, we demonstrate that tPA expressed by PKCδ interneurons of the lateral/ capsular part (CeL/C) of the CeA modulates neuronal activity within the central medial amygdala (CeM).

MATERIALS AND METHODS

Animals

Experiments were performed on 8 weeks old males tPA^{null} mice, tPA^{lox} mice and their wild-type (WT) littermates (20–25 g; Animal care facilities: Centre Universitaire de Ressources Biologiques, Normandy University, Caen, France; Approval #A14118015). Animals were housed with a 12-h light/12-h dark cycle in standard polypropylene cages (22 × 37 × 19 cm, Charles River, L'Arbresle, France; 5 mice per cage). All experiments were conducted in accordance with the French ethical law (Decree 2013-118; Approval #10959) and the European Community Council guidelines (2010/63/EU).

Generation of transgenic mice

tPA^{lox} mice (C57BL/6NTac background) in which exon-3 of Plat gene is flanked by loxP sites was generated as previously described (see [27]). tPA^{null} mice in which exon-3 of Plat gene is constitutively excised through the Cre-lox system were generated by the Mouse Clinical Institute (ICS, Illkirch, France) by crossing tPA^{lox} mice with the ubiquitous Cre-expressing mouse line, Rosa26Cre [28].

Mice were genotyped by PCR analysis and southern blots, using tail genomic DNA samples, for the presence of loxP sequences and the presence of the third exon.

Behavioral assessment

Behavioral tests were performed 3 weeks after the AAV injections in the 11–13 week old mice.

Open-field test. The open-field test (OF) was used to assess general locomotor activity and anxiety-like behavior as previously described [29, 30].

Barnes maze test. The Barnes maze test (BM) was used to assess long-term reference memory [31, 32].

c-Fos analysis

For c-Fos quantification, the number of c-Fos positive cells was counted manually in five independent animals for which the AAV injections were validated by the GFP expression in the CeA (Supplementary Fig. 1) using ImageJ software. Three to four slices per mouse, spanning the CeL/C, the CeM and the bed nucleus of the stria terminalis (BNST) in each of the two hemispheres were used for quantifications.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0). Analysis of the datasets via parametric approaches turned out to be inappropriate in some cases due to violation of normality (Shapiro–Wilk tests). Therefore, parametric or non-parametric approaches were used whenever appropriated.

RESULTS

Constitutive tPA^{null} mice display hyperactivity, reduced anxiety, and impaired spatial memory

To avoid possible off-target effects of tPA knockout strategies [26], we used here a new strain of tPA-deficient mouse

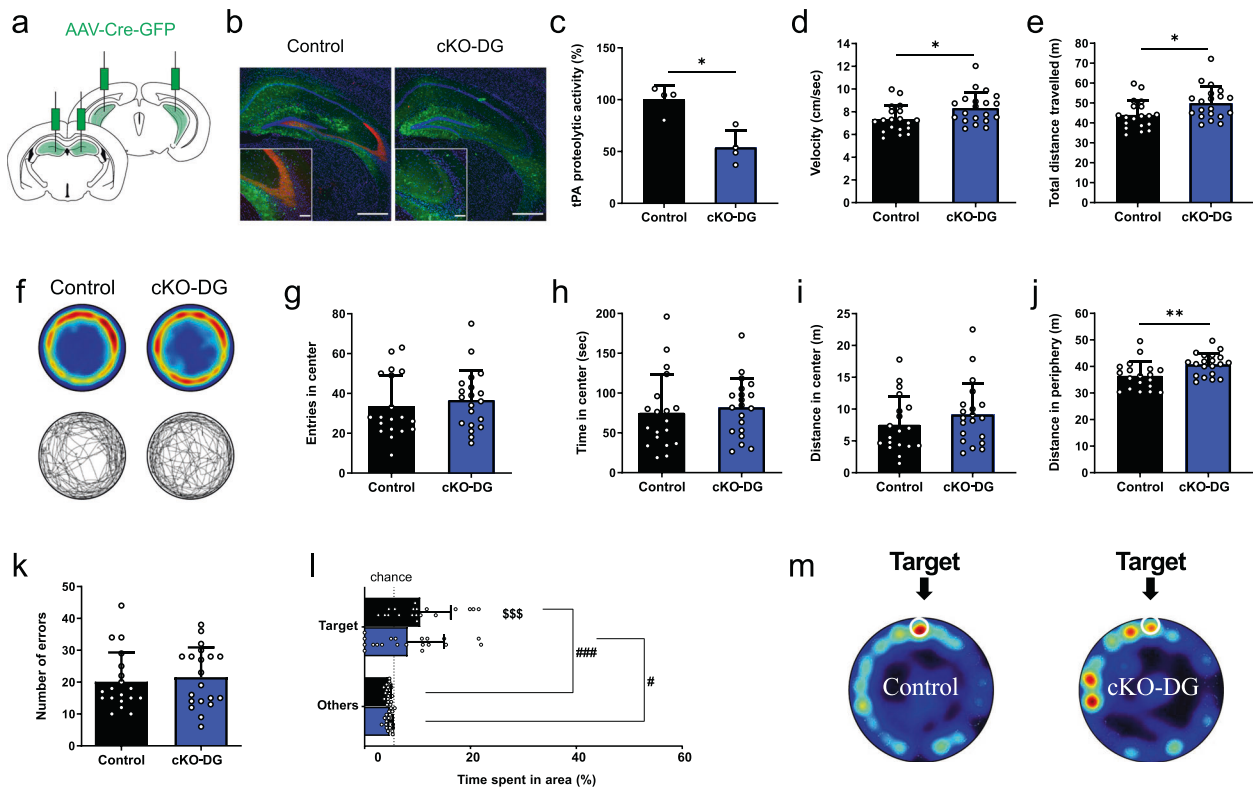


Fig. 2 Conditional deletion of tPA in the hippocampus (cKO-DG) leads to locomotor hyperactivity and moderate spatial cognitive deficits. **a** Bilateral stereotaxic injections of AAV-Cre-GFP in the dentate gyrus of WT and tPA^{fllox} mice. **b** Immunostaining for GFP (green), tPA (red) and DAPI (blue); Coronal sections; White scale bar: 500 μm (High magnification scale bar: 100 μm). **c** Fibrin-agar zymography showing tPA activity in the hippocampus of Control and cKO-DG mice; Mann–Whitney U test, $*p < 0.05$, $n = 4$ per group. **d–f** cKO-DG mice show locomotor hyperactivity in the open-field test. Intergroup comparisons of the velocity (**d**) and distance (**e**); Mann–Whitney U tests, $*p < 0.05$. Averaged heat maps and representative trajectories of Control and cKO-DG mice (**f**). **g–j** tPA deletion in the hippocampus does not affect anxious responses in the open-field test. Intergroup comparisons of entries (**g**), time (**h**) and distance in the center (**i**), or peripheral (**j**), area; Mann–Whitney U test, $**p < 0.01$. **k–m** cKO-DG mice display moderate spatial deficits in the Barnes maze test. Intergroup comparisons of the number of errors (**k**); Student's t test, $p > 0.05$. Intragroup comparisons of the time spent in areas (**l**); Wilcoxon signed-rank test, $^{55}p < 0.001$. Comparison with chance level (5.5 %), Wilcoxon signed-rank test, $^{55}p < 0.001$. Averaged heat maps of Control ($n = 6$) and cKO-DG ($n = 6$) mice from the same training group (**m**). Control mice: $n = 19$, cKO-DG mice, $n = 20$. Data are presented as mean \pm SD.

(tPA^{null} ; Fig. 1a, b) subjected to a battery of behavioral tests ($n = 20$ per group).

The OF was first used to measure general locomotion (Fig. 1c–e) and anxiety-related behavior (Fig. 1f–i). Mutant mice displayed significant higher velocity (Fig. 1c, +17.5%, $p < 0.01$) and distance traveled (Fig. 1d, +17.5%, $p < 0.05$) than WT animals. Moreover, mutant mice showed an increase in number of entries (Fig. 1f, +31.65%, $p < 0.01$), time spent (Fig. 1g, +20%, $p < 0.05$) and distance traveled (Fig. 1h, +12.8%, $p < 0.05$) in the aversive center area of the apparatus compared to controls. Note that the locomotor behavior cannot explain the observed results as the total distance was similar for both groups in periphery (Fig. 1i, $p > 0.05$). Thus, data from OF indicate hyperactivity and reduced anxiety in tPA^{null} mice. We then used the BM to study possible spatial cognition deficits due to the lack of tPA. During the training session, both groups showed good acquisition performance in spite of the greater traveled distance exhibited by mutant mice (Supplementary Fig. 2a, $p < 0.001$). Interestingly, tPA^{null} mice did not succeed in the probe test as they made more errors to find the target area than control mice (Fig. 1j, +89%; $p < 0.01$). Furthermore, they spent similar time in the target area compared to others (Fig. 1k, l, $p > 0.05$; Supplementary Fig. 3a', $p > 0.05$) unlike control mice that explored more time the target than any other areas (Fig. 1k, l, $p < 0.05$; Supplementary Fig. 3a, $p < 0.001$). Consistent with these results, the time spent in the target area was significantly higher than chance level in WT mice (Fig. 1k, l; $p < 0.05$) contrary to tPA^{null} mice (Fig. 1k, l; $p > 0.05$). These data

show that the tPA deficiency leads to an impairment of long-term spatial memory.

Altogether, we reveal here that tPA is involved in a range of behavioral functions, including locomotor activity, anxiety and spatial memory.

Region-specific deletion of tPA reproduces a subset of phenotypes observed in tPA^{null} mice

To characterize the respective structure-function relationships in the tPA-dependent behaviors characterized above, we induced the deletion of the target protein in several brain areas (DG, BLA, CeA, PFC; Supplementary Fig. 4) of adult mice.

First, AAV-Cre-GFP was bilaterally injected in the dentate gyrus of newly generated tPA^{fllox} mice (Fig. 2a) for a conditional deletion of tPA in the hippocampal mossy fibers (cKO-DG, $n = 19$). The same AAV construct was injected in WT littermate mice (Control, $n = 20$). The efficacy of the recombination was confirmed by immunohistochemistry for GFP and tPA to unmask the specific depletion of endogenous tPA in the hippocampal mossy fibers (Fig. 2b). These data were confirmed by fibrin-agar zymography showing reduced levels of active tPA in hippocampal homogenates of the injected tPA^{fllox} mice compared to the injected tPA^{WT} animals (Fig. 2c, $n = 4$ per group, $p < 0.05$). As performed for the tPA^{null} mice (Fig. 1), the same set of behavioral tests was conducted with the cKO-DG and control mice three weeks post-AAV-Cre-GFP injections. In the OF, both the velocity and the distance traveled in the arena were significantly increased in

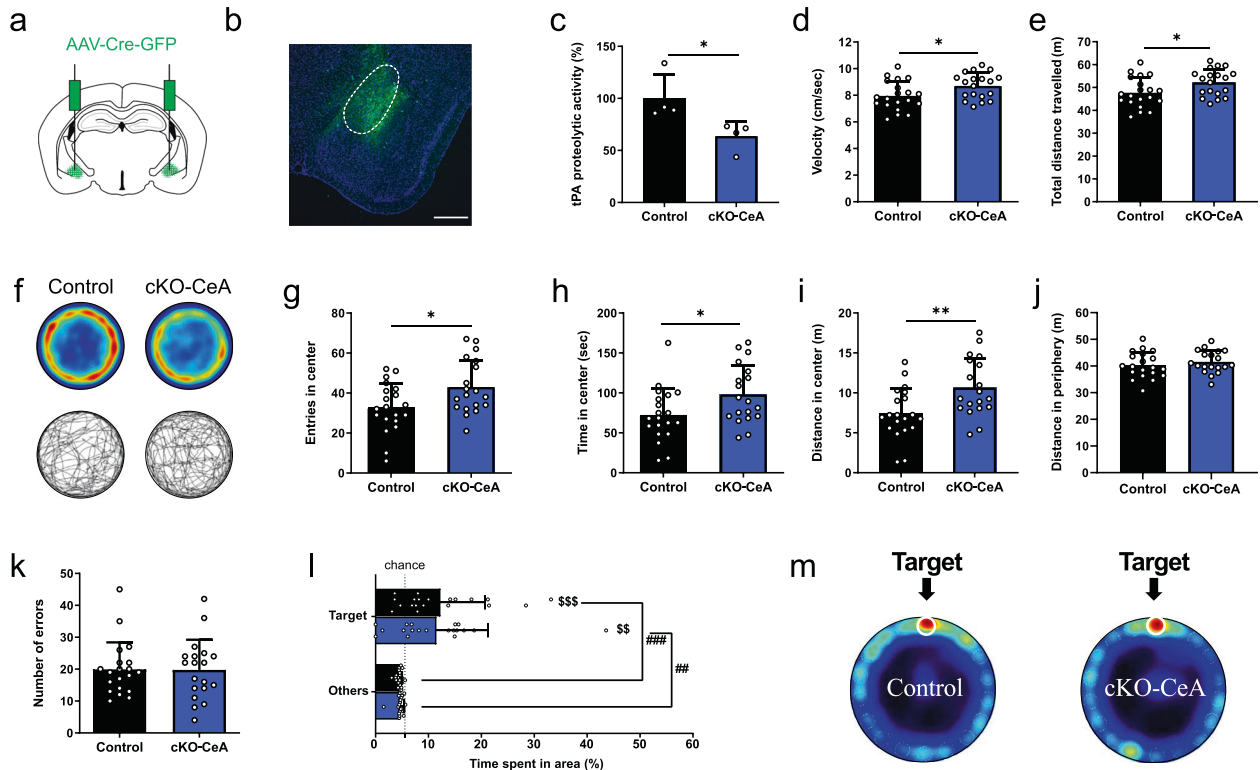


Fig. 3 Conditional deletion of tPA in the central amygdala (cKO-CeA) leads to locomotor hyperactivity and reduced anxiety. **a** Bilateral stereotaxic injections of AAV-Cre-GFP in the central amygdala of WT and tPA^{fllox} mice. **b** Immunostaining for GFP (green) and DAPI (blue); Coronal sections; White scale bar: 500 μ m. **c** Fibrin-agar zymography showing tPA activity in the central amygdala of Control and cKO-CeA mice; Mann-Whitney *U* test, * $p < 0.05$, $n = 4$ per group. **d-f** cKO-CeA mice show locomotor hyperactivity in the open-field test. Intergroup comparisons of the velocity (**d**) and distance (**e**); Student's *t* tests, * $p < 0.05$. Averaged heat maps and representative trajectories of Control and cKO-CeA mice (**f**). **g-j** tPA deletion in the central amygdala induces decreased anxious responses in the open-field test. Intergroup comparisons of entries (**g**), time (**h**) and distance in the center (**i**), or peripheral (**j**), area; Student's *t* tests, * $p < 0.05$, ** $p < 0.01$. **k-m** cKO-CeA mice display similar cognition performance to controls in the Barnes maze test. Intergroup comparisons of the number of errors (**k**); Mann-Whitney *U* test, $p > 0.05$. Intragroup comparisons of the time spent in areas (**l**); Wilcoxon test, ## $p < 0.01$, ### $p < 0.001$. Comparison with chance level (5.5%), Wilcoxon signed-rank test, ^{ss} $p < 0.01$, ^{sss} $p < 0.001$. Averaged heat maps of Control ($n = 8$) and cKO-CeA ($n = 8$) mice from the same training group (**m**). Control mice: $n = 20$, cKO-CeA mice, $n = 19$. Data are presented as mean \pm SD.

cKO-DG mice compared to controls (Fig. 2d-f; velocity: +12.7%, $p < 0.05$; distance traveled: +17%, $p < 0.05$). This locomotor hyperactivity of conditional mice was particularly seen in the periphery (Fig. 2j, $p < 0.01$). By contrast, the anxiety-like behavior was similar in the two groups with the same number of entries, time spent and distance traveled in the center area (Fig. 2g-i, $p > 0.05$). In the cognitive task, both groups displayed an equal number of errors to discriminate the target box (Fig. 2k, $p > 0.05$; Supplementary Fig. 3). However, cKO-DG mice performance was equivalent to random exploration (Fig. 2l, $p > 0.05$; compared with chance level) unlike that of the control mice which was significantly greater than chance level (Fig. 2l, \$\$\$ $p < 0.001$, compared with chance level). Altogether, these data demonstrate that tPA present in the hippocampus of adult mice is involved in the modulation of locomotor activity and partly in spatial memory.

The targeted suppression of tPA in the PFC, which is known to modulate decision making and anxiety responses [33–36], did not affect locomotor activity, anxiety-like behaviors or spatial cognition (Supplementary Fig. 5).

Then, AAV-Cre-GFP was bilaterally injected in the CeA of the tPA^{fllox} mice for a conditional deletion of tPA (Fig. 3a; cKO-CeA, $n = 19$). The same AAV construct was injected in the WT littermate mice (Control, $n = 20$). The efficacy of the recombination was confirmed as above (Fig. 3b, c, $n = 4$ per group, $p < 0.05$). In the OF, cKO-CeA mice displayed a hyperactive phenotype (Fig. 3d-f; velocity: +9.4%, $p < 0.05$; distance traveled: +9.4%, $p < 0.05$).

A reduced anxiety-like behavior was also observed in conditional mutant mice compared to controls (Fig. 3g-j; entries to center area: +31.5%, $p < 0.05$; time in the center area: +35.5%, $p < 0.05$; distance traveled in the center area: +43.2%, $p < 0.01$; distance traveled in periphery $p > 0.05$). In the BM, the conditional knockout mice succeeded in the task since spatial performance was similar in both groups (Fig. 3k-m).

In a second time, parallel bilateral injections of AAV-Cre-GFP were performed in the basolateral part of the amygdala (BLA) of tPA^{fllox} mice and their WT littermates (Fig. 4a; cKO-BLA: $n = 18$; Control: $n = 18$). The site of injection and efficacy of the recombination was confirmed as above (Fig. 4b, c, $n = 4$ per group, $p < 0.05$). In the OF, the locomotor activities were equal in cKO-BLA and control mice (Fig. 4d-f; $p > 0.05$). Likewise, the anxious responses were similar for the two groups (Fig. 4g-j; $p > 0.05$). The BM test revealed that both groups displayed analogous performance (Fig. 4k-m). Altogether, these data reveal that the tPA present in the CeA of adult mice, but not in the BLA, is involved in the control of anxiety-like behavior, with tPA promoting anxiety. Furthermore, we demonstrate that central amygdalar tPA modulates locomotor activity.

In order to determine which cell types of the CeA are involved in the functions of tPA described above, immunohistochemistry was performed from coronal brain sections of WT mice using a set of neuronal markers (protein kinase-C delta: PKC δ ; somatostatin: SOM; vesicular GABA transporter: VGAT; vesicular glutamate transporter 2: VGLUT2) combined with tPA immunostaining. Our data revealed positive neuronal fibers for tPA colocalized with

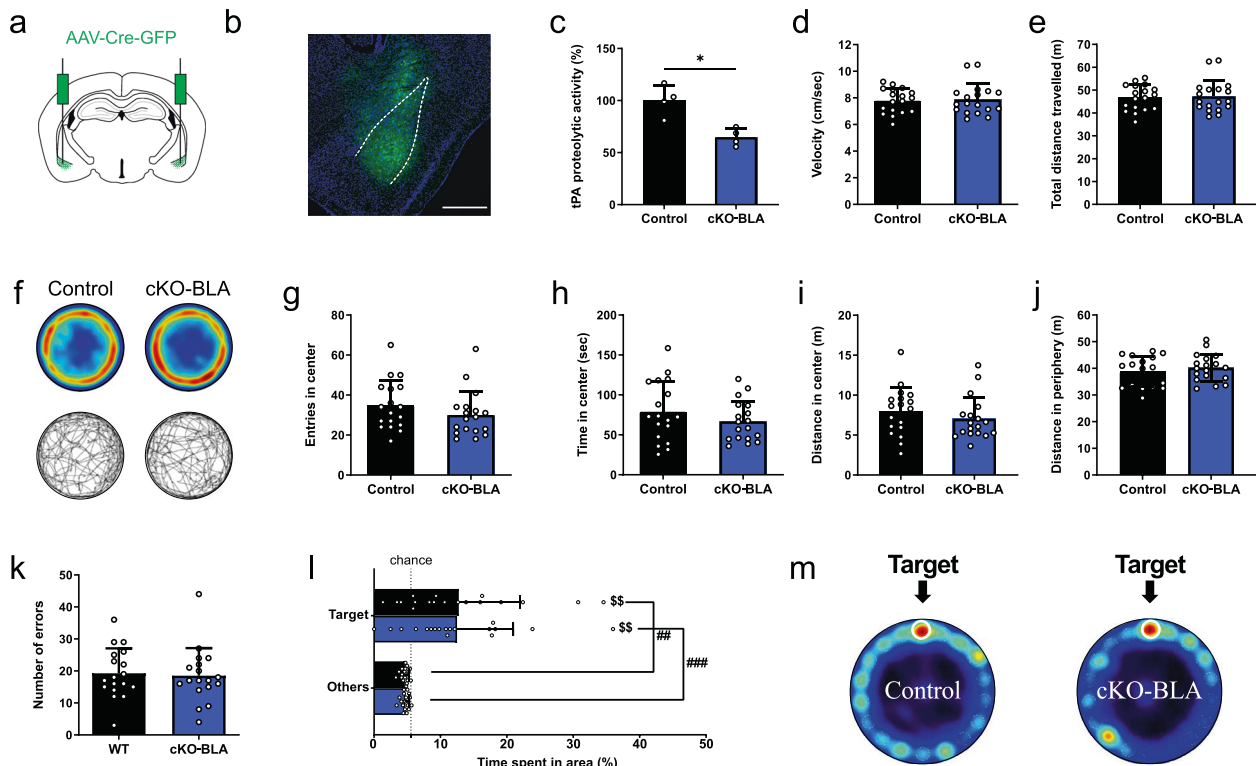


Fig. 4 Conditional deletion of tPA in the basolateral amygdala (cKO-BLA) does not affect locomotor, emotional or cognitive performance. **a** Bilateral stereotaxic injections of AAV-Cre-GFP in the basolateral amygdala of WT and tPA^{fl^{ox}} mice. **b** Immunostaining for GFP (green) and DAPI (blue); Coronal sections; White scale bar: 500 μ m. **c** Fibrin-agar zymography showing tPA activity in the basolateral amygdala of Control and cKO-BLA mice; Mann-Whitney *U* test, * $p < 0.05$, $n = 4$ per group. **d–f** Deletion of tPA in the basolateral amygdala does not affect locomotor activity in the open-field test. Intergroup comparisons of the velocity (**d**) and distance (**e**); Mann-Whitney *U* tests, $p > 0.05$. Averaged heat maps and representative trajectories of Control and cKO-BLA mice (**f**). **g–j** tPA deletion in basolateral amygdala does not affect anxious phenotype in the open-field test. Intergroup comparisons of entries (**g**), time (**h**) and distance in the center (**i**), or peripheral (**j**), area; Mann-Whitney *U* tests, $p > 0.05$. **k–m** cKO-BLA mice display similar cognition performance to controls in the Barnes maze test. Intergroup comparisons of the number of errors (**k**); Mann-Whitney *U* test, $p > 0.05$. Intragroup comparisons of the time spent in areas (**l**); Wilcoxon test, ## $p < 0.01$, ### $p < 0.001$. Comparison with chance level (5.5%), Wilcoxon signed-rank test, \$\$ $p < 0.01$. Averaged heat maps of Control ($n = 10$) and cKO-BLA ($n = 10$) mice from the same training group (**m**). $n = 18$ for both genotypes. Data are presented as mean \pm SD.

VGAT but not with VGLUT2 (Fig. 5a). Co-immunostaining of these tPA-positive GABAergic interneurons revealed that they were also positive for the PKC δ in the central lateral/capsular part (CeL/C), but not for SOM (Fig. 5b, c; $p = 0.05$). The expression of tPA mRNA (Plat) was also confirmed by fluorescent in situ hybridization which shows that Plat mRNA are abundantly present in PKC δ neurons and in a lesser extent in SOM neurons (Fig. 5d, e; $p < 0.001$). In order to assess the effect of tPA expressed by CeA's PKC δ interneurons on neuronal activation, we then investigated c-Fos expression in cKO-CeA and control mice. Quantifications were performed in the CeL/C (where PKC δ interneurons are found). Therefore c-Fos measurements were also performed in the central medial part (CeM) of CeA, and in the BNST which were previously described as output of CeA PKC δ neurons [37, 38]. We found a selective decrease of c-Fos-positive cells in the CeM of cKO-CeA mice compared to controls (Fig. 5f; $p < 0.05$). In contrast, tPA deletion in the CeA had no significant effect in the CeL/C nor in the BNST (Fig. 5f; $p > 0.05$). Altogether, these data show that the tPA expressed in CeL/C PKC δ neurons influences neuronal activation of the CeM.

DISCUSSION

In the present study, we reveal that tPA of PKC δ -positive GABAergic interneurons of the CeL/C in adult mouse is involved

in the control of emotional functions through neuronal activation of CeM.

The current literature investigating roles of tPA, including its brain functions, were obtained using C57BL/6J tPA-deficient mice harboring the 129-derived chromosomal segment that co-segregates with the targeted Plat allele [26]. In this model, some others genes than tPA are altered (*Arhgef18*, *Mcf2l* and *Mcp1*) which can lead to confusion in the data interpretation [39, 40]. The tPA^{null} mouse strain generated by our group and used in the present study is the consequence of a specific deletion of the exon-3 of the *Plat* gene, with no possibly of off-target effects. Our behavioral experiments confirm most of the previous reports about the functional roles of tPA such as its involvement in spatial memory processes [8, 10, 15, 41] or locomotor activity [23]. Importantly, our study is the first to show that tPA deficiency also leads to decreased anxiety-like responses under basal conditions. Nevertheless, as the phenotypes found in tPA^{null} mice could be linked to neurodevelopmental disorders [27, 42, 43], we then completed our work by generating a novel model allowing conditional deletion of tPA at adult stages.

Using this new molecular tool, the tPA^{fl^{ox}} mice targeting the exon-3 of the *Plat* gene, we demonstrate tPA-dependent structure-function respective relationships. We thus evidence that tPA of the dentate gyrus, in adult mice, is involved in the regulation of locomotor activity and partly in spatial cognition.

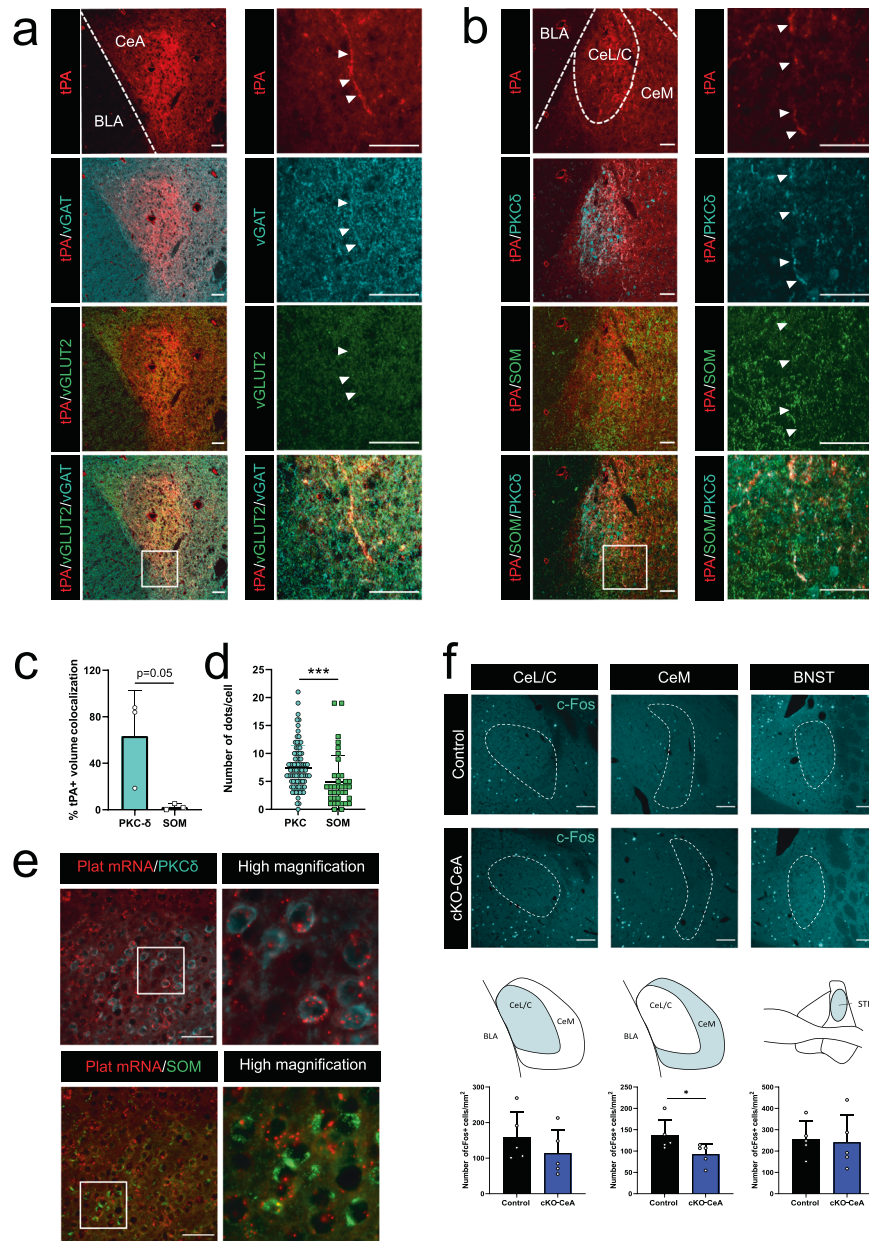


Fig. 5 tPA expressed in CeL/C PKC δ interneurons influences neuronal activation of the CeM. **a** Immunostaining for tPA (red), vGAT (cyan) and vGLUT2 (green) in the CeA of WT mice reveal the colocalization of tPA and vGAT interneurons. Arrows indicates the colocalization of tPA and vGAT along a tPA-positive neurite. White scale bar: 50 μ m. **b** Immunostaining for tPA (red), PKC δ (cyan) and somatostatin (SOM; green) interneurons in the central amygdala lateral/capsular part (CeL/C) of WT mice reveal the colocalization of tPA and PKC δ interneurons. Arrows indicates the colocalization tPA and PKC δ staining along the along a tPA-positive neurite. White scale bar: 50 μ m. **c** Quantification of the percentage of tPA colocalized volume over PKC δ or SOM immunostainings in the CeA ($n = 3$ coronal slices from 3 WT mice; unpaired t -test; $p = 0.05$). **d** Quantification of the number of tPA mRNA (Plat) dots per cell showing that Plat mRNA are abundantly present in PKC δ neurons and in a lesser extent in SOM neurons in the CeA ($n = 85$ PKC δ -positive neurons; $n = 37$ SOM-positive neurons; Mann–Whitney U tests, $p < 0.001$; data from two coronal slices). **e** Representative images of Plat mRNA (red dots) colocalization with PKC δ positive neurons (cyan) and SOM (green) positive neurons in the CeA. **f** Quantification of c-Fos-positive cells in the lateral/capsular (CeL/C) and the medial (CeM) parts of the CeA, and in the dorsal part of the bed nucleus of the stria terminalis (BNST) of Control and cKO-CeA mice; Mann–Whitney U tests, $*p < 0.05$, $n = 5$ for both genotypes. Data are presented as mean \pm SD.

Furthermore, we show that tPA expressed in CeA of adult mice controls both locomotor activity and anxiety-like behaviors.

It was previously reported that tPA displays functions in learning and memory processes [8, 10, 13–17, 41]. For example, over-expression of tPA in the central nervous system has been associated with improved spatial performance when using both Morris Water Maze (MWM) and Homing hole board tests [13]. Accordingly, tPA deficiency has been shown to induce spatial deficits in both MWM

and T-maze [8, 41], as well as alterations in responsiveness to a spatial change during an object placement test [10]. However, other studies using tPA-deficient mice did not report a particular phenotype when using MWM or BM [9, 44]. Regarding tPA and locomotor activity, data in literature are again contradictory. Indeed, Pothakos and collaborators reported a locomotor hyperactivity in tPA-deficient mice while Calabresi and collaborators did not [10, 23]. One notes that these studies were led on constitutive

tPA-deficient mice, with distinct behavioral experimental conditions. In this study, we highlight for the first time that the tPA in the adult dentate gyrus plays a role in the modulation of locomotor activity. Since CA3 neurons are the major input of dentate gyrus granular cells, immunohistochemical analysis should be performed in a future study in order to assess whether tPA released from mossy fibers may modulate the activation of CA3 neurons and downstream locomotor effects. We also reveal moderate cognitive deficits in the BM following tPA removal in the dentate gyrus, suggesting that tPA synthesized in other brain areas could influence spatial processing. Indeed, tPA expression was also reported in CA1 and CA3 regions of the hippocampus and in parahippocampal regions such as the entorhinal cortex which are also involved spatial processing [7, 8, 45, 46]. In a previous work, we have showed that tPA plays a key role in entorhinal cortex-dependent processing of spatial information in a gender specific manner [8]. Our present study was only conducted in males to avoid heterogeneity related to the estrous cycle. Thus, we cannot exclude that the behavioral responses observed here could be differentially affected in tPA^{null} female mice.

tPA activity in the amygdala is critical for both stress-induced synaptic plasticity and anxiety-like behavior [4, 18, 47]. Accordingly, tPA-deficient mice exhibit reduced anxiety-like behavior under stress conditions [4, 18]. Our present data thus provide new insights about tPA and anxiety, demonstrating that conditional deletion of tPA in the adult amygdala central nucleus leads to reduced anxiety-related behavior, regardless of any stressor. It is important to note that CeA plays a key role in the integration of emotional sensory information coming from the BLA, and can modulate emotional responses through intrinsic circuit within the CeA and projections to different brain areas such as the BNST [48–52]. The CeA consists of CeL, CeC and CeM. One of the peculiarities of CeL and CeM neurons is that they synthesize GABA. Moreover, PKC δ -positive interneurons is one of the major neuronal subtype found in the CeL/C with SOM-positive interneurons [51, 53]. These two populations, largely non-overlapping, display distinct firing patterns and connectivity [38, 51, 54]. Indeed, PKC δ and SOM interneurons can form a local and reciprocal inhibitory circuit within the CeL while PKC δ neurons can also inhibit CeM neurons and send long range projections to the BNST [37, 38, 51, 54, 55]. Optogenetic approaches already reported that CeL PKC δ neurons modulate anxiety-like behaviors, with their activation that lead to differential responses depending on the experimental design [37, 56]. In addition, a recent study demonstrates that CeL PKC δ neuronal activity is necessary to induce the anxiolytic effects of benzodiazepines [57]. Here, we evidence that tPA is expressed by PKC δ -positive GABAergic interneurons in the CeA. Our data also reveal that tPA depletion in the CeA induces a decrease of c-Fos positive cells in the CeM demonstrating that tPA expressed by PKC δ interneurons in the CeL could influence CeM activation. In neurons, tPA is transported along dendrites and axons and can be released at the synapse in a constitutive or activity-dependent manner [58, 59]. tPA activity is increased in limbic regions such as the CeA, the medial amygdala (MeA) and the BNST under stress conditions [4, 18, 60]. Based on our present results, we can hypothesize that the tPA released from CeL PKC δ neurons influences anxiety responses with projections to the CeM. This result suggests that tPA expressed by PKC δ interneurons projecting on CeM neurons may induce a process of disinhibition by positively-modulating neurons of the CeM. However, we did not identify the exact molecular mechanism by which tPA can influence CeM neurons activation. In the brain, tPA is susceptible to have neuromodulatory effects through multiple pathways, including modulations of NMDA receptors or LRP receptors signaling [9, 25, 61], control of synaptic plasticity and behavioral responses through a plasmin-dependent conversion of pro-BDNF into its mature form [17, 24]. Interestingly, calcium imaging recently revealed that the anxiolytic effect of benzodiazepine was linked to a decreased activity of CeM+ neurons without effect on PKC δ + neurons [57]. Since tPA can modulate

NMDA-dependent calcium influx [12, 61], we can speculate that tPA deletion in PKC δ + could reduce post-synaptic activation of CeM by decreasing NMDA receptor signaling. Further investigations will be necessary to assess whether tPA-dependent modulation of such pathways could affect central amygdala activation and emotional responses. Our study also shows that tPA deficiency in the CeA increases locomotor activity. Accordingly, a previous study revealed that CeL PKC δ neuronal activity can modulate locomotor activity [56]. In addition, targeting monosynaptic connections between the CeA and the mesencephalic locomotor region was reported to regulate spontaneous locomotion [62]. Further investigations will be needed to describe the projections of tPA-expressing PKC δ interneurons.

In our study we cannot exclude that other subpopulations of interneurons, such as those expressing neuropeptide markers such as neuropeptide Y, corticotropin-releasing factor (CRF) or neurotensin found in the CeA, could synthesize tPA [53].

Indeed, in the amygdala, tPA was shown to promote the effect of CRF of neuronal activation and anxiety-related responses [18]. Given that reciprocal inhibition may occur between interneurons of the CeA, we can hypothesize that tPA may differentially influence neuronal activity in the CeA depending on its expression by CRF-positive interneurons or other interneuron subpopulations. Similarly, tPA detection in the CeA could also originate from projecting axons coming from other brain areas such as the BLA or the BNST, two brain areas which are well-known inputs of the CeA [38, 49, 50] and reported to express tPA [7, 60].

In conclusion, we demonstrate that tPA deficiency (tPA^{null} mice) leads to hyperactivity, spatial memory deficits and reduced anxious responses. Using a corresponding conditional knockout model allowing structural brain-specific deletion of tPA in the adult mice, we reveal that 1/ selective loss of tPA in the hippocampus leads to hyperactivity and moderate spatial memory deficits, 2/ tPA deficiency in the CeA induces both hyperactivity and anxiolytic-like phenotype. In addition, we unveil that the expression of tPA in PKC δ -positive GABAergic interneurons of the CeA is associated to tPA-dependent emotional behaviors involving the activation of neurons within the CeM.

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AUTHOR CONTRIBUTIONS

DV and VA designed and supervised the study. SD, EL, and DV performed the data analysis. SD, EL, YH, LL, and EM performed the experiments. AB and CJ designed and produced AAV constructs. SD, VA, and DV wrote the manuscript. All authors have read and have approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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