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Urokinase receptor and tissue plasminogen activator as immediate early genes in pentylenetetrazole-induced seizures in the mouse brain

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Abstract

Epileptogenesis progressively leads to the rearrangement of normal neuronal networks into more excitable ones and can be viewed as a form of neuroplasticity the molecular mechanisms of which still remain obscure. Here we studied pentylenetetrazole seizure-induced regulation of genes for plasminogen activator system in the mouse brain. We found that tissue plasminogen activator (tPA) and urokinase receptor (uPAR) mRNA expression was strongly increased in the mouse cerebral cortex, hippocampus, striatum and amygdala as early as three hours after pentylenetetrazole seizures. Such early activity-induced expression of uPAR in the central nervous system has not been demonstrated before. uPAR mRNA accumulation was followed by elevation of uPAR protein, indicating a complete transcription-translation process. Both tPA and uPAR gene induction was independent of the protein synthesis, suggesting that they are regulated by neural activity as immediate early genes. In contrast to tPA and uPAR genes, the expression of which returned to the basal level 6 hours following seizures, urokinase and plasminogen activator inhibitor-1 gene expression showed a delayed activation only at three days after seizures. In conclusion, our results suggest an important sensitivity of the brain plasminogen activator system to seizure activity which raises the question of its role in activity-dependent neural tissue remodeling in pathological and normal conditions.

1. Introduction

Epilepsy comprises the second most common neurological pathology after stroke (MacDonald *et al.*, 2000) and many cases of it are acquired, i.e. develop gradually after another neurological disease or acute brain injury. According to International League Against Epilepsy structural, genetic, infectious, metabolic, and immune disturbances can cause epilepsy (Fisher *et al.*, 2017; Scheffer *et al.*, 2017). Though the etiology of acquired epilepsy is often known, the exact molecular mechanisms underlying its progression are still largely unclear (Pitkänen *et al.*, 2015). One of hypotheses is that this process exploits activity-dependent long-term neural plasticity (Scharfman, 2002). The prime genes for such plasticity are immediate early genes (IEGs) and late response genes that are induced in episodes of synchronous neural activity and mediate subsequent reorganization of cellular networks that can decrease the threshold of next seizure generation (Qureshi & Mehler, 2010).

Since GABAergic system is heavily involved in epileptogenesis(Ben-Ari, 2006), an important role in these remodeling processes can belong to genes of plasminogen activator (PA) system. The PA system comprises urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), which are serine proteases that activate plasmin; uPA receptor (uPAR), which is glycosylphosphatidylinositol-anchored (GPI-anchored) and lacks transmembrane or cytosolic domains, and plasminogen activator inhibitors (PAI-1 and PAI-2)(Iyer *et al.*, 2010; Liu *et al.*, 2010). Though initially studied in angiogenesis and vascular remodeling, the PA system is now being increasingly implicated in brain development and regulation of neural functions (Archinti *et al.*, 2011; Bruneau & Szepetowski, 2011). The study in uPAR knockout mice revealed that among the plethora of its functions is the regulation of migration and differentiation of interneurons in the developing brain. It was shown that uPAR knockout results in the loss of up to 50% of GABAergic interneurons in the neocortex in mice(Powell *et al.*, 2001, 2003; Eagleson *et al.*, 2005). uPAR-deficient mice exhibit a greater susceptibility to pentylenetetrazole-induced seizures(Powell *et al.*, 2001, 2003). Seizures in uPAR-knockout mice proceed longer and more severe than in wild-type mice, cause greater neurodegeneration and a delayed and increased inflammatory response (Ndode-Ekane & Pitkänen, 2013). Noteworthy, uPA-deficient mice have the same seizure severity as wild-type ones (Lahtinen *et al.*, 2010; Rantala *et al.*, 2015), while tPA-/- mice are less susceptible to seizures (Tsirka *et al.*, 1995; Yepes *et al.*, 2002). At present, no specific phenotype, which occurs due to uPAR mutation or deficiency,

has been described in humans. However, several polymorphisms of the uPAR gene associated with CNS diseases have been identified: a migraine without aura (Zandifar *et al.*, 2014), autism spectrum disorders (Campbell *et al.*, 2008). Mutation of one of the possible uPAR ligands – SRPX2, results in rolandic epilepsy and speech-language dyspraxia (Roll *et al.*, 2006; Royer-Zemmour *et al.*, 2008).

Two studies have shown that the brain samples from patients with focal epilepsies of different etiologies exhibit elevated expression of plasmin activator system components (uPA, uPAR, tPA, PAI-1) in neurons, glial cells, and blood vessels compared to control (Iyer *et al.*, 2010; Liu *et al.*, 2010). Serum uPAR level was also elevated in patients with focal epilepsy and it was consistently normalized after epileptogenic brain region was surgically removed (Quirico-Santos *et al.*, 2013). Whether this increase in expression might be due to seizures themselves or requires the chronic state can be found out via experimental studies. Overall, the experimental studies of epileptic lesions in rodents are relevant to human pathology, inspiring research with the use of experimental animal models. Additionally, chemically and electrically induced seizures result in significant increase in expression of uPA(Lukasiuk *et al.*, 2003; Gorter *et al.*, 2006, 2007; Lahtinen *et al.*, 2006), uPAR (Lahtinen *et al.*, 2009), tPA(Gorter *et al.*, 2006, 2007). A dramatic increase in uPAR expression has been noticed particularly in interneurons, including previously mentioned parvalbumin-expressing GABA interneurons (Lahtinen *et al.*, 2009).

The above-mentioned data suggest that PAs may critically influence neuronal activity, inflammatory response, as well as the reorganization of neuronal tissue following seizures and during the epileptic process itself. However, the patterns of their temporal and spatial induction of in the brain after seizures is characterized only partially. In order to study this progression in more details we analyzed the mRNA expression of PA system components (uPAR, uPA, PAI-1, tPA) at different times after pentylenetetrazole-induced acute seizures that are known to induce immediate early and late effector genes involved in neuronal plasticity. Our initial hypothesis was that the genes of PA system will behave as late response genes consistent with their role in cell and tissue remodeling. Unexpectedly we found that not only the tPA gene, previously shown to be an immediate early gene, but also the uPAR gene was strongly induced in the first hours after seizures. Furthermore, pentylenetetrazole-induced mRNA expression of both genes was not suppressed by protein synthesis inhibitor suggesting that they behave as classical immediate early genes. This finding raises new questions not only on the role of PA system in seizure-induced

pathology, but also about possible function of uPAR in normal activity-induced neuronal plasticity.

2. Materials and methods

2.1 Animals

Adult male C57BL/6J wild-type mice (RRID:IMSR_JAX:000664), 12–14 week old, 28,2± 3,7 g (Pushchino, Russia) were enrolled into the study. The mice were housed in individual cages and maintained under a standard 12-h light cycle, with constant temperature (22±1 °C) and humidity (50–60%).Water and food were available *ad libitum*. Mice were handled on daily basis before the onset of experiments. The ARRIVE guidelines were followed (Kilkenny *et al.*, 2012).

A careful consideration was given to the number of animals: a cohort of 144 animals was used, the minimum number required to obtain valid results.Due to the exploratory nature of this study we couldn't know the expected variance, thus a sample size calculation wasn't performed. Particular effort was made to minimize the animals' pain and distress(Weary *et al.*, 2006). For the following series of experiments, mice were randomly assigned to experimental and control groups by a web-basedresearchrandomizer(https://www.randomizer.org/).

The study was completed in accordance with the Council Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. All manipulations with animals were carried out in accordance with the requirements of Order No. 267 of the Ministry of Health of the Russian Federation "On Approval of Laboratory Practice Rules" (June 19, 2003 No. 4809) and were approved by the local ethical committee in accordance with internal requirements of the Commission on Bioethics of the Faculty of Medicine ofLomonosov Moscow State University.

2.2 Materials

Seizures were induced with pentylenetetrazole (PTZ) (Sigma-Aldrich, cat. # P6500). Protein synthesis was inhibited with cycloheximide (CHX) (Sigma-Aldrich, cat. # C7698). Both drugs were dissolved in 0.9% NaCl solution.Trizol Reagent for RNA extraction was purchased in Invitrogen, USA (cat. # 15596026). MMLV RT kit (cat. # SK021) for reverse transcription and qPCRmix-HS SYBR (cat. # PK147L) for qPCR were purchased in Evrogen, Russia. The following antibodies were used: mouse anti-uPAR (Sigma-Aldrich, cat. #SAB4200412), rabbit anti-tPA (Cloud-Clone Corp., USA,cat. #PAA525Mu01), mouse anti-vinculin (Sigma-Aldrich,

cat. # V9131), goat anti-rabbit (Imtek, Russia, cat. #P-GAR Iss), goat anti-mouse (Imtek, Russia, cat. #P-GAM Iss).

2.3 Drug injections, brain dissection and blood collection

Three series of experiments were performed (Fig 1). First, to analyze the seizure-produced changes in gene expression in mouse brain,acute seizures were induced by injecting PTZ (a single dose of 75 mg/kg body weight, intraperitoneally) as described previously(Zhu *et al.*, 2007). Seizures were visually inspected, all mice had pronounced behavioral symptoms of seizures. Time points for the analysis of gene expression were: 30', 1h, 3h, 6h, 24h and 72h after PTZ treatment. Saline-injected mice were used as a control (later referred as saline group). Each time point group comprised4-6 mice.

To answer the question ifthe early gene induction after seizures depends on the protein synthesis, mice were injected intraperitoneally with PTZ (75 mg/kg body weight) along with CHX 90 mg/kg body weight, a dose sufficient for brain protein synthesis inhibition in mice (Gold & Wrenn, 2012). CHX was chosen as a protein synthesis inhibitor that is traditionally used to test the compliance of gene expression response to criterion of immediate early gene, i.e. independence of gene transcription on de novo protein synthesis (Worley *et al.*, 1990; Maciejak *et al.*, 2010). CHX is used in these tests either prior or along with the examined simulation (Schreiber *et al.*, 1993; Feldman *et al.*, 1998). CHX effect was investigated only for the genes that had an early and potent induction of expression after seizures in the first series of experiments. Time points for gene expression analysis in mouse brain were: 15', 30', 1h and 3h after PTZ treatment. Mice treated only with PTZ for the same period of time were used for relative comparison. Saline-injected mice were used as a control (saline group). For each time point, 4-6 mice were included in a group (biological replicates).The sample size variations were due to mice death prior to time points evaluated andRNA degradation, evaluated as described further.

In the third experiment we collected brain tissue samples 3h and 6h after PTZ injection to analyze uPAR and tPA protein expression in mouse brain by western blot; to exclude the impact of mRNA from blood cells in brain samples we collected mice whole blood 3h, 6h and 72hafter PTZ injection. For each time point, 4-7 mice were included in a group.

In each case, mice were treated between 10:00 and 12:00 in the laboratory; mice were treated and analyzed in the same order: first, experimental group, then control group. At times required,mice weresacrificed by cervical dislocation and decapitated, thebrains were removed.

Anterior cortex, posterior cortex, right and left hippocampus and striatum were dissectedas described in the previously published protocol (Spijker, 2011). Right and left amygdalae were dissected from the bregma -1 to -2.75 brain sections (Franklin & Paxinos, 1997). Samples were quickly frozen in liquid nitrogen using 2 ml-microcentrifuge tubes and stored at -80°C prior to total RNA or protein extraction. In the third experiment prior to decapitation a cardiac puncture was performed to collect approximately 500 µl of mouse blood as measured by 1 ml insulin syringe. The blood was immediately placed in 15 ml tubes, containing 5µl of 0.5 M EDTA, and thoroughly mixed with 2 ml of Trizol Reagent (Invitrogen, USA).

Control and experimental groups were blinded for further RNA or protein extraction, reverse transcription and qPCR analysis by assigning codes for each group, such that the experimenter who performed the analysis did not know what treatment the animals received.

2.4 RNA isolation from mouse brain and blood, reverse transcription and qPCR

Total RNA was isolated from the frozen brain tissue and from blood by Trizol Reagent following manufacturer'sinstruction. The quantity and quality of total RNA were measured using NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, USA); 1% agarose gel with ethidium bromide was used to assess RNA integrity. 1,5 µg of total RNA was reverse-transcribed using oligo(dT) and random $(dN)_{10}$ primers with MMLV RT kit (Evrogen, Russia). PCR was carried out using qPCRmix-HS SYBR (Evrogen, Russia) on a DT-96 real-time PCR device (DNA-technology, Russia). The murine cDNA primers (Supplementary table 1) were obtained from Evrogen (Russia). The thermal cycling program was as follows: a 5-minute denaturing step at 95 °C followed by 40 amplification cycles consisting of 15 seconds denaturing at 95 °C, 15 seconds of annealing at 62 °C and 20 seconds of extension at 72 °C. qPCR reactions for each sample were performed in duplicates (technical replicates). A relative transcript level was calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin as a reference gene;normalization was done assuming as 1 the mean level of each transcript in saline group (control). The outliers would be excluded by robust regression and outlier removal (the ROUT) method.

In order to confirm that the changes in gene expression that we observe in brain samples aren't caused by the presence of blood and correspond to brain tissue, we analyzed the level of expression of the same genes in the blood at relevant time points. uPAR, tPA, uPA and PAI-1 expression in blood was not affected after PTZ-induced seizures (Supplementary Fig 1).

2.5 Protein isolation from mouse brain, electrophoresis and western blot

Brain tissue was lysed in anice-cold RIPA lysis buffer (150 мM NaCl, 25 mM Tris, 0.5 % sodium deoxycholate, 1% Nonidet P-40, 0,1% SDS, pH 7.4) containing protease inhibitor cocktail (Thermo Fisher Scientific, USA)diluted 1:100 (100 µl of RIPA lysis buffer per 10 mg of brain tissue). Brain tissue was further homogenized by hand grinding with a cold pestle and passing 10 times through 27G needle of 1ml insulin syringe. The lysates were vortexed 3 times during 20-minute incubationon ice and centrifuged at +4°C for 20 minutes at 16000 g. The supernatant was transferred into a new pre-cooled microcentrifuge tube and cell pellet was discarded. 5 µl of supernatant diluted 1:1000 was used for quantification of protein concentration via Bradford assay. Finally, after measuring the concentration, the lysates were dissolved in the equal volume of 2X Laemmli buffer containing 10% β-mercaptoethanol and heated at 95°C for 10 min.

Proteins (45 µg) were resolved in 10% SDS-PAGE gels and transferred to PVDF membrane (GEHealthcare, USA) in transfer buffer (1.92 M Tris/glycine buffer, 10% SDS and 20% methanol). Nonspecific bindingwas blocked in 5% non-fat dried milk in phosphate-buffered saline (PBS, Sigma-Aldrich), containing 0.1%Tween-20 at +4°C overnight. Proteins were probed with the following primary antibodies in 1:1000 dilution: mouse anti-uPAR, rabbit anti-tPA, mouse anti-vinculin (control of protein load) for 2 h at room temperature. Membranes were washed with PBS, containing 0.1% Tween-20, and incubated with appropriate peroxidase-conjugated secondary antibodies in 1:10000 dilution at room temperature for 1.5 h, followed by washing in PBS, containing 0.1% Tween-20. Proteins were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, USA) and ChemiDoc™ XRS+System (BioRad) for western blotting imaging and analysis.Densitometric analysis of blots at non-saturating exposureswas performed using ImageJ.Values of uPAR and tPA proteins were normalized to vinculin.

2.6Data and statistical analysis

The study wasn't pre-registered. All data were assessed for normal distribution using the D'Agostino-Pearson normality test. Data are presented as a mean ± standard error of the mean (SEM). Data were analyzed using GraphPad Prism 7 software (GraphPad Software Inc., USA).The unit of analysis is a single animal. No outliers were detected by performing robust

regression the ROUT method. Differences in gene expression were determined within one brain region between different time points using one-way analysis of variance (ANOVA), with the null hypothesis of equal means between the groups. The test follows the Fisher-Snedecor distribution (F-distribution) and when the F value was significant, interactions of each experimental group with control were further analyzed using Dunnett post hoc test to take into account the problem of multiple-to-one comparisons. Student's unpaired t-tests were used to compare data when there were only two groups.To test for linear relationships between gene hyperexpressionof two genes in each RNA sample from a separate brain region we used the Pearson's correlation coefficient.The level of significance was set at $P< 0.05$.

3. Results

3.1 uPAR and tPA are immediate early genes in acute seizures: their induction occurs in the first hours after seizures and is not dependent on protein synthesis

The results of uPAR expression analysis at different time points after seizure induction with PTZ in the anterior cortex, posterior cortex, hippocampus, amygdala, striatum are shown in Fig2 ($F_{6, 27}$ = 14.306, P<0.0001; $F_{6, 28}$ = 34.89, P<0.0001; $F_{6, 28}$ = 10.5, P< 0.0001; $F_{6, 25}$ = 13.79, P<0.0001; $F_{6, 27}$ = 33.92, P<0.0001, respectively). We observed an increase in uPAR expression as early as 1h after seizure induction in the anterior and posterior cortex (Post hoc Dunnett testsaline group vs. 1h q = 3.113, P = 0.0203 and$q$ =4.768, P = 0.0003, respectively) and a pronounced increase in uPAR expression in all examined regions 3h post-seizure (Post hoc Dunnett test saline group vs. 3h in anterior cortex q = 6.939, posterior cortex q = 11.390, hippocampus$q$ = 6.409, amygdala q = 6.865 and striatum q = 11.390,P< 0.0001 for all groups).6 hours after induction of seizures, the expression level of uPAR was normalized to control values and did not show any significant elevation at 24 and 72 hours. The increase in uPAR protein expression was also observed 3h and 6h afterseizures in different brain regions (Fig 3).

CHX administration had no effect on uPAR expression. 3h after the treatment uPAR expression was still significantly increased in both groups: PTZ ($F_{4, 20}$ = 18.2, P< 0.0001, Post hoc Dunnett test saline group vs. 3h q = 6.293, P< 0.0001) and PTZ + CHX ($F_{4, 20}$ = 13.32, P< 0.0001, Post hoc Dunnett test saline group vs. 3h q = 5.307, P = 0.0001), suggesting that the observed upregulated uPAR expression was independent of *de novo* protein synthesis (Fig 4).

Expression of tPA genewas also activated early after seizures induction (Fig5) ($F_{6, 24}$ = 34.6, P< 0.0001 for anterior cortex; $F_{6, 24}$ = 16.12, P < 0.0001 for hippocampus). As in the case of uPAR, we observeda dramatic increase in tPAgene expression in all analyzed brainregions at 3h after PTZ-induced seizures: in anterior cortex (Post hoc Dunnett test saline group vs. 3h q = 11.63, P< 0.0001), in posterior cortex(Unpaired t-test saline group vs 3h t_7 = 9.854, P< 0.0001), in hippocampus (Post hoc Dunnett test saline group vs. 3h q = 7.4, P< 0.0001), in striatum (Unpaired t-test saline group vs 3h t_8 = 3.172, P = 0.0132), and in amygdala (Unpaired t-test saline group vs 3h t_7 = 5.585, P = 0.0008). Moreover, there was even more rapid tPAmRNA increase in the hippocampus –mRNA level was elevated there as early as 30 minutes after PTZ treatment (Post hoc Dunnett test saline group vs. 30' q = 3.743, P= 0.0051). 6 hours after induction of seizures,the level of tPAexpression was normalized to control values without any further increase. We didn't observe any significant increase in tPA protein expression 3h and 6h after seizures in different brain regions (Fig 6). tPA protein expression in amygdala was lower the limit of detection.

As with uPAR, the protein synthesis inhibition with CHX had no effect on tPA expression at 3h (Fig 7) – tPA was still significantly increased 3h after treatment in both groups: PTZ ($F_{4, 13}$ = 11.37, P = 0.0003, Post hoc Dunnett test saline group vs. 3h q = 3.882, P = 0.0066) and PTZ + CHX ($F_{4, 19}$ = 2.931, P = 0.0481, Post hoc Dunnett test saline group vs. 3h q = 2.692, P = 0.0475). Thus, in our experiments uPAR and tPA demonstrated the same early activation pattern upon seizure induction and exhibited immediate early gene (IEGs) behavior, as their mRNA expression was not dependent upon *de novo* protein synthesis.

The correlation analysis of uPAR and tPAmRNA expression 3 hours after the induction of seizures showed a negative correlation (Pearson correlation coefficient r = -0.4793, r^2 = 0.2297, P = 0.0114).uPAR and tPAexpressionhad a specific spatial distribution: while in anterior and posterior cortex tPA expression was the highest among the evaluated regions, uPAR expression was the lowest; the opposite pattern was found in hippocampus and striatum, where uPAR expression substantially predominated over tPA expression (Fig6, see also Supplementary table 2).

3.2 uPA and PAI-1 are late response genes, which are induced several days after seizures

uPA expression analysis revealed its delayed activation 72h post-seizures in anterior cortex($F_{5, 22}$ = 8.433, P = 0.0001, Post hoc Dunnett test saline group vs. 72h q = 5.447, P< 0.0001) and hippocampus($F_{5, 22}$ = 12.08, P< 0.0001, Post hoc Dunnett test saline group vs. 72h q = 5.287,

$P = 0.0001$) (Fig9 a, b). Expression of PAI-1 was increased following PTZ-induced seizures in the anterior cortex($F_{4, 17} = 4.054$, $P = 0.0173$, Post hoc Dunnett test saline group vs. 72h $q = 2.952$, $P = 0.0304$),but not in the hippocampus (Fig9 c, d).

4. Discussion

Neuronal activity-regulated gene transcription is generally classified into two stages: a rapid expression of IEGs, which is protein synthesis independent, followed by the expression of late response genes that depend on the synthesis of transcription factors encoded by the IEGs (Tullai *et al.*, 2007; Pérez-Cadahía *et al.*, 2011). In the present study the obtained results on the early uPAR gene expression in the mouse brain following PTZ seizure induction were quite unexpected. Based on the preceding findings we predicted that uPAR expression will follow the pattern of lately regulated genes. The previous study on seizure-induced uPAR expression in rats examined such late time points:one, two, four, or 14 days after status epilepticus (SE) produced by electrical stimulation of amygdala (Lahtinen *et al.*, 2009).It was found that uPAR mRNA wasincreased 25-fold at 24 hours and 14-fold at 48 hours after SE.Immunohistochemistry revealed that uPAR protein expression peaked at one and fourdays after SE and was localized to parvalbumin, somatostatin and neuropeptide Y positive interneurons of the hippocampus. In addition, uPAR immunoreactivity was observed in blood vessel endothelium throughout the hippocampus (Lahtinen *et al.*, 2009). However, it should be noted that this study did not address the question of uPAR mRNA or protein expression at earlier times. Accumulation of uPAR protein in microglial cells in the mouse brain was also detected threedaysafter kainic acid-induced SE (Cunningham *et al.*, 2009).In humans, uPAR protein expression was elevated in the brain tissue of chronic patients with focal intractable frontal lobe epilepsy (Liu *et al.*, 2010). uPAR increase was detected in the soma of NeuN-positive neurons, and to a lower degree in microglial cells and astrocytes. The elevated levels of uPAR mRNA and uPAR protein were alsoverifiedby qPCR and Western blot analysis within epileptogenic brain lesions of patients with focal epilepsy caused by several forms of chronic epileptogenic pathologies (Iyer *et al.*, 2010). This late-onset and long-term increase in uPAR expression suggests a possible involvement of uPAR in neural tissue remodeling at the post-injury phase in epilepsy. The accumulating evidence indicate the involvement of proteolytic enzymes in long-term alterations of neuronal circuitry during neurodegeneration, gliosis, angiogenesis and rearrangement of the extracellular matrix and are also consistent with the role of uPAR in the conversion of pro-uPA to active uPA(Lukasiuk *et al.*, 2011; Pitkänen *et al.*, 2014).

Our results unexpectedly demonstrated an early but not late expression of uPAR mRNA following acute PTZ seizures. There was aneight-nine-fold uPAR mRNA increase in the hippocampus, amygdala, striatum and cerebral cortex already three hours after the onset of acute seizures(Fig2). The elevation of uPAR expression in cerebral cortex occurred even earlier, one hour after the onset of seizures (Figs2a, 2b). This early uPAR mRNA accumulation was followed by elevation of uPAR protein, indicating a full transcription-translation process (Fig 3). Furthermore, this early increase of uPAR transcription was not prevented by administration of protein synthesis inhibitor CHX (Fig4). These data indicate that expression of the uPAR gene can be regulated by neural activity more rapidly than it was generally considered before and in the manner typical to the IEGs.

The products of neuronal activity-regulated IEGs can be classified into different types including transcription factors (c-Fos), synaptic proteins (Arc), signaling molecules (Cox-2), secretory factors (BDNF or tPA). Importantly, many of them are involved in regulation of long-term plasticity changes triggered by neuronal activity (Minatohara *et al.*, 2015; Kim *et al.*, 2018). For example, tPA, a well-known neuronal IEG(Qian *et al.*, 1993), was demonstrated to be induced by various forms of seizures. The six-fold increase in tPA mRNA expression throughout the brain was reported in the convulsive seizure model at one-four hours after seizures and it was independent of*de novo*protein synthesis(Qian *et al.*, 1993). In rats, RNA gel blot and *in situ* hybridization analysis showed a three-four-fold elevated levels of tPA mRNA in the hippocampus one hour after the onset of PTZ seizures(Popa-Wagner *et al.*, 2000; Schmoll *et al.*, 2001). Expression of tPA mRNA was also strongly increased by single and repeated electroconvulsive seizures in the rat hippocampus at one and two hours but not at eight hours after seizure induction (Segawa *et al.*, 2013). Our results on the elevation of tPA mRNA in the hippocampus 30 minutes after PTZ-induced seizures and in the hippocampus, cerebral cortex and striatum at three hours are in accordance with the above data and confirm the independence of tPA gene induction on *de novo* protein synthesis(Figs 5, 7). However, the increase in tPA mRNA wasn't followed by an elevation in protein expression at three or six hours after seizures (Fig 6). The differential transcription and translation of IEGs might be specific to certain brain zones and requires further investigation (Kiessling *et al.*, 1993).

Taken together, our data suggest that both,tPA and uPAR genes are rapidly activated and can behave as IEGs in response to seizures. However, the activity-induced expression of these two

genes of the PA-interactome system may not be tightly co-regulated, since a correlation analysis revealed a negative correlation between tPA and uPAR mRNA levels in the brain samples from individual mouse subjected to PTZ seizures (Fig 8).

The role of thisneuronal activity-dependent upregulation of tPA and uPAR in neuronal plasticity and epileptogenic pathology is not yet clear.tPA-mediated extracellular proteolysis is has been shown to have an important role in long-term neuronal plasticity, since the overexpression of tPA enhances hippocampal long-term potentiation and learning ability, while tPA knockout in mice results in impaired neural plasticity and long-term memory(Huang *et al.*, 1996; Madani *et al.*, 1999; Nakagami *et al.*, 2000; Samson & Medcalf, 2006). tPA knockout mice also demonstrate a higher threshold for seizure onset(Tsirka *et al.*, 1995), suggesting the involvement of tPA in this process, possibly via direct cleavage of the NR1 subunit and/or non-proteolytic interaction with the NR2 subunit of the NMDA receptor (Nicole *et al.*, 2001; Fernández-Monreal *et al.*, 2004; Pawlak *et al.*, 2005). uPAR knockout mice exhibita more severe epilepsy phenotype in kainic acid epileptogenesis model (Ndode-Ekane & Pitkänen, 2013). UsinguPAR knockout mice it has been shown that uPAR deficiency results in comorbidity-modifying effects following traumatic brain injury, particularlyin motor recovery and in contextual fear conditioning (Bolkvadze *et al.*, 2016). These studies indirectly point to a possible involvement of uPAR in the central nervous system plasticity. However, the specific role of early activity-induced uPAR gene expressionin epileptogenic pathology and experience-dependent neuronal plasticity has yet to be investigated.uPAR itself and uPAR-mediated downstream effector proteins involved in intracellular signal transmission(Irigoyen *et al.*, 1999) can be a promising target for such a study.

We also observed a four- to five-fold increase in the expression of uPA mRNA in the mouse hippocampus and anterior cerebral cortex three days after PTZ seizures. Our data are in accordance with the previously published results that demonstrated the increased uPA gene expression in the hippocampus and temporal lobe in the rat brain on the first, fourth and 14th day after electrically triggered epileptogenesis and on the 14th day after the onset of SE (Lukasiuk *et al.*, 2003). SE in rats also has been found to produce an elevation of uPA protein in hippocampus on the firstand on the fourth day after its onset (Lahtinen *et al.*, 2006). Increased uPA was observed in astrocytes, neurons and neuropil, as well as in blood vessels(Lahtinen *et al.*, 2006). In mice kainic acid seizures led to accumulation of uPA mRNA in the hippocampus as early as two-four hours after the treatment; theelevated expression persisted for at least three days as has been

shown by *in situ* hybridization (Masos & Miskin, 1997). In the present study no early (three hours) seizure-induced expression of uPA gene could be detected (Fig9 a, b). These data discrepancy can be due to the difference in the mechanisms of regulation of gene expression in PTZ and kainate seizure models (Rubio *et al.*, 2010; Lévesque *et al.*, 2016; Becker, 2018).

Functionally, post-seizure uPA expression is supposed to be neuroprotective (Lahtinen *et al.*, 2010; Cho *et al.*, 2012; Gur-Wahnon *et al.*, 2013). The pivotal role of uPA system in brain recovery was also shown for ischemic injury(Diaz *et al.*, 2017, 2018; Merino *et al.*, 2018). Hypoxic injury resulted in prominentuPA release from neurons as well as uPAR exposureon the astrocytic plasma membranes(Diaz *et al.*, 2017). This uPA/uPAR coupling is assumed to be crucial in astrocytic activation after ischemia and the subsequent crosstalk between glial cells and injured neurons that promotes recovery of normal synapses in injured brain(Diaz *et al.*, 2017). Altogether, the delayed neuroprotective effects of uPA interactome are important for the synaptic recovery and can be considered as a long-term mechanism of brain restoration after traumatic events, including epileptic states.

We observed a two-fold increase in PAI-1 expression in the anterior cortex, but not in the hippocampus three days after PTZ seizures (Fig9 c, d). PA inhibitors were shown to be actively induced together with PAs after SE(Gorter *et al.*, 2007). PAI-1 mRNA expression after seizure induction by kainic acid in mice was recorded mostly in large brain blood vessels and in the hypothalamus at two-six hours post-seizures. It was also observed in cortex, hippocampus, and amygdala with the temporal pattern similar to that of uPA(Masos & Miskin, 1997). These similarities suggest a synchronous function of urokinase and its inhibitor in balancing the proteolytic cascades.

To summarize, in the present study we demonstrate the acute seizure-induced upregulation of severalPA-interactomegenesin the central nervous system. Particularly we identified a much earlier increase in uPAR mRNA and protein expression in the animal brain after seizures than reported before. This finding calls attention to a possible role of PA-interactome in pathological activity-dependent neural plasticity and raises the question about the functionof such early transcriptional uPAR responsein the normal neuronal functions, particularly learning and memory consolidation.

Declarations

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Conflict of interest

The authors declare no conflict of interests regarding the publication of this paper.

Author contributions

Semina E. and Anokhin K. conceived and designed the experiments. Shmakova A., Rysenkova K., Ivashkina O. and Gruzdeva A. performed the experiments. Shmakova A. analyzed the data and performed the statistical analyses. Shmakova A., Semina E., Rubina K. and Anokhin K. contributed to the writing of the manuscript. Tkachuk V. and Semina E. made a substantial contribution to the conception of the manuscript.

Data accessibility statement

All gene expression files, western blot full images, western blot densitometry data are available from the Open Science Framework database (DOI 10.17605/OSF.IO/H2ZYT, accession URL https://osf.io/h2zyt/?view_only=dd1fe69b78b34508be7abcac345949e6).

Abbreviations

': minute; ANOVA: analysis of variance; BDNF: brain-derived neurotrophic factor; bp: base pairs; cDNA: complementary deoxyribonucleic acid; CHX: cycloheximide; CNS: central nervous system; GABA: γ-aminobutyric acid; GPI: glycosylphosphatidylinositol; kg: kilogram; h: hour; HGF: hepatocyte growth factor; IEG: immediate early gene; LDL: low-density lipoprotein; μg: microgram; mg: milligram; ml: milliliter; mRNA: messenger ribonucleic acid; NMDA-receptor: N-methyl-D-aspartate receptor;PA: plasminogen activator; PAI: plasminogen activator inhibitor; PTZ: pentylenetetrazole; qPCR: quantitative polymerase chain reaction; RNA: ribonucleic acid; tPA: tissue-type plasminogen activator; SE: status epilepticus; SEM: standard error of the mean;

SRPX2: sushi-repeated protein X-linked 2; uPA: urokinase-type plasminogen activator, urokinase; uPAR: urokinase receptor.

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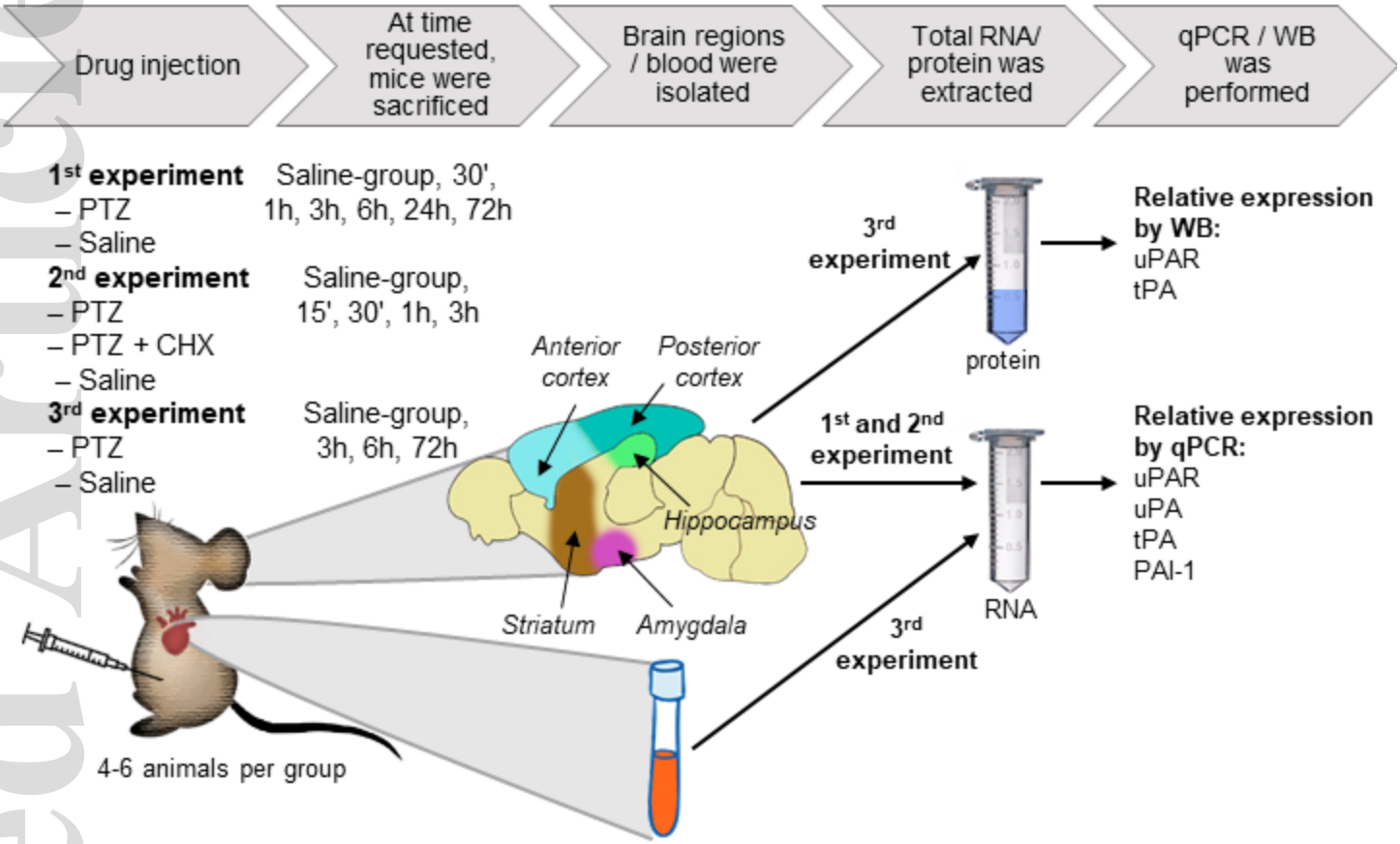

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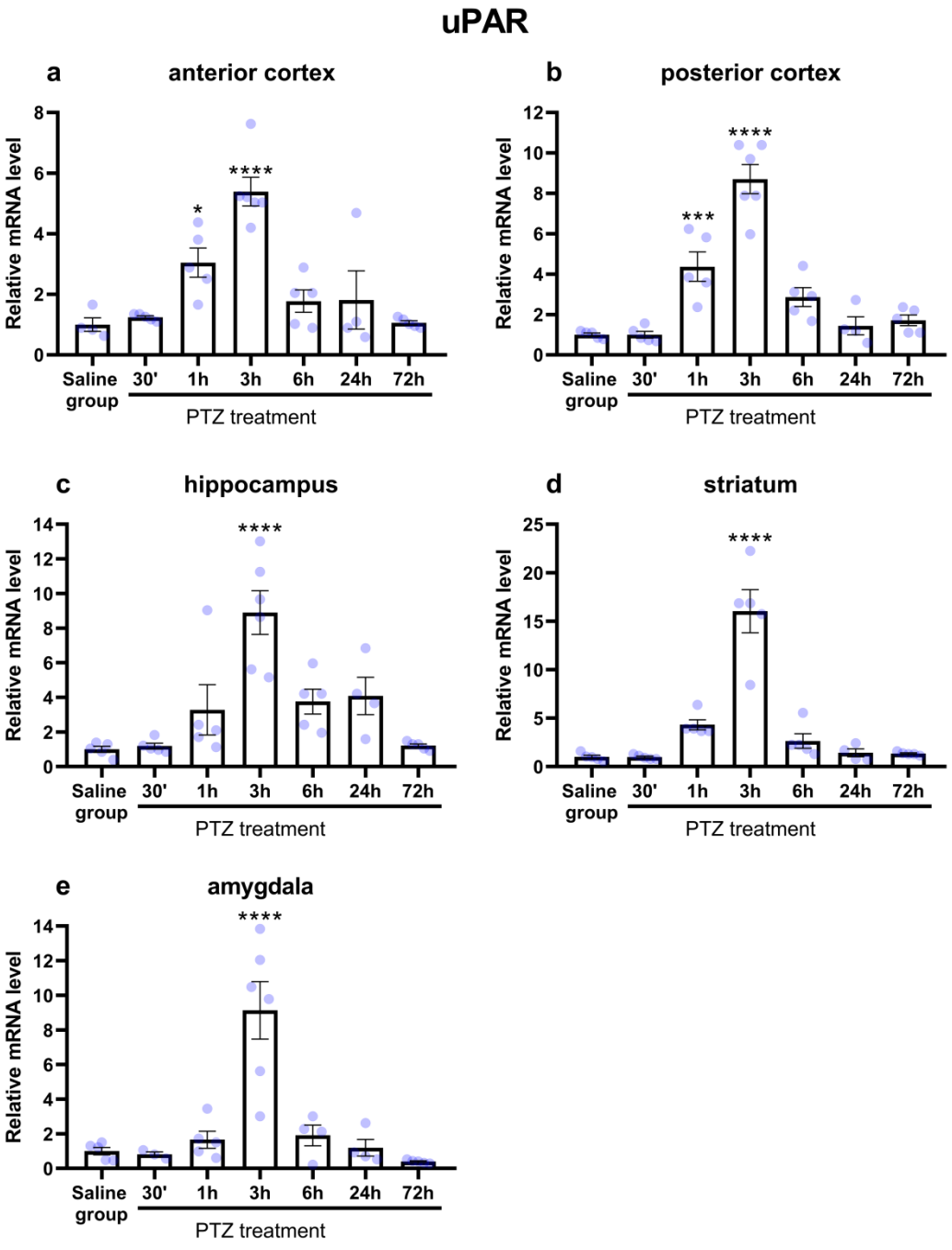

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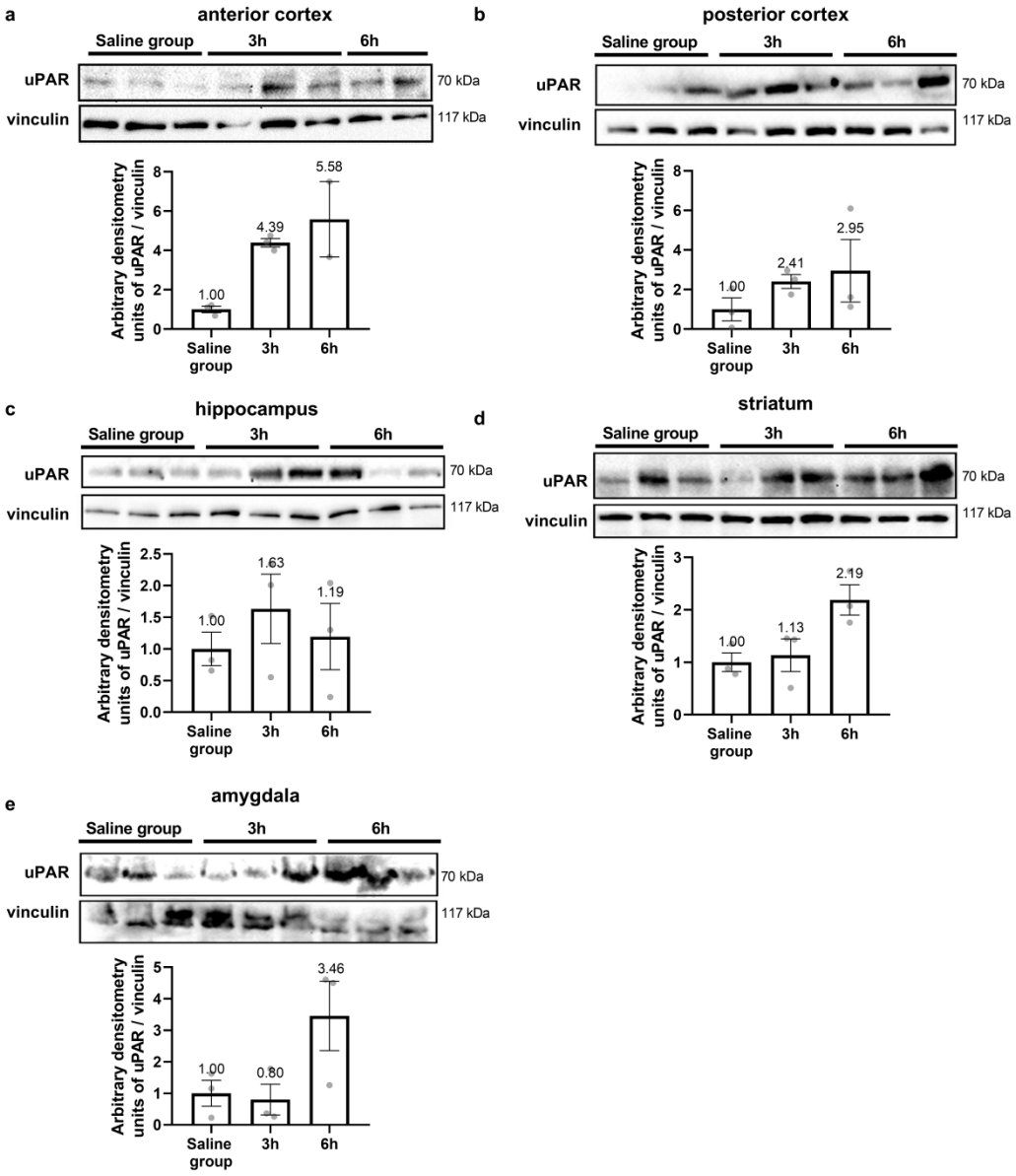

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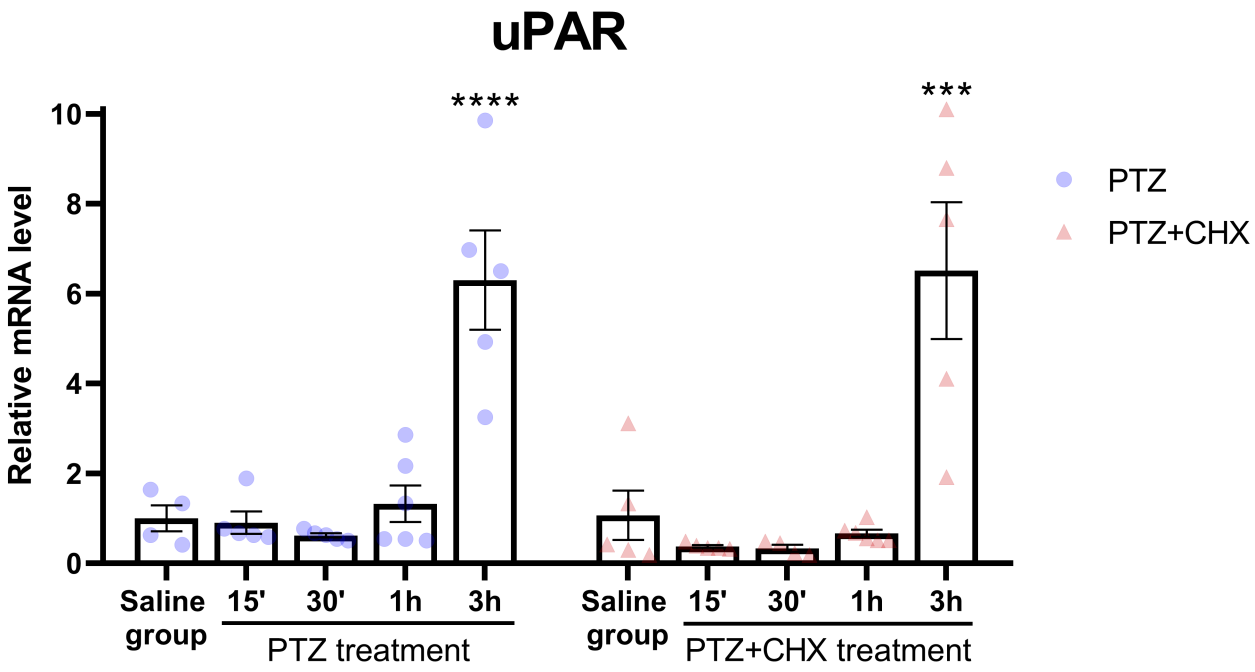

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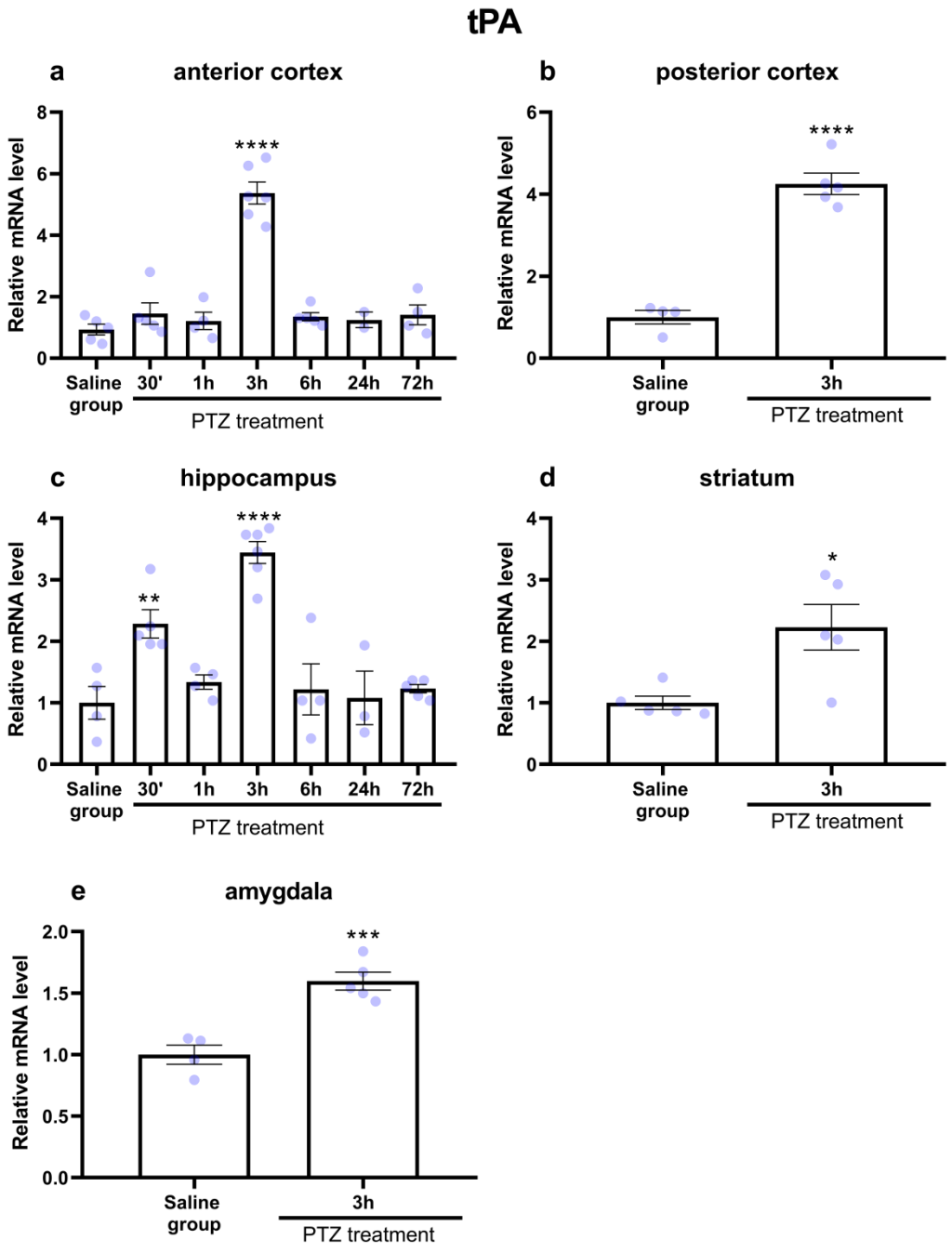

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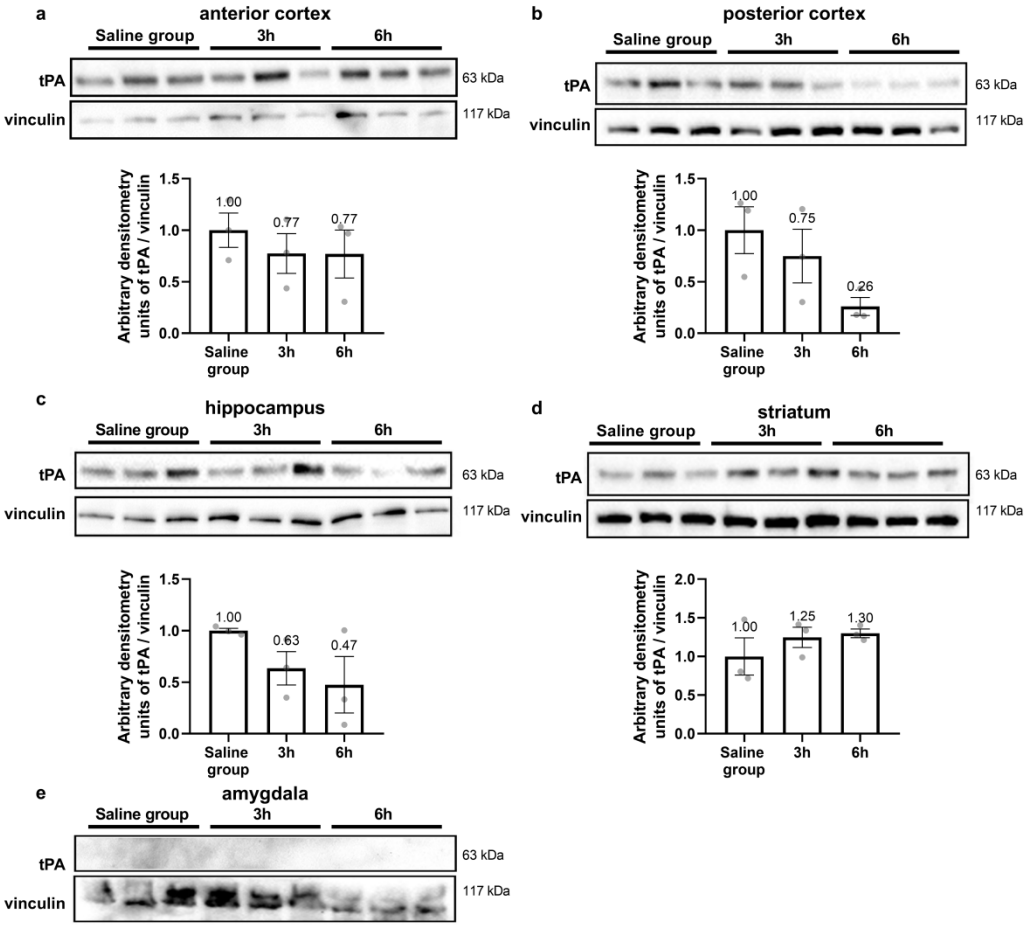

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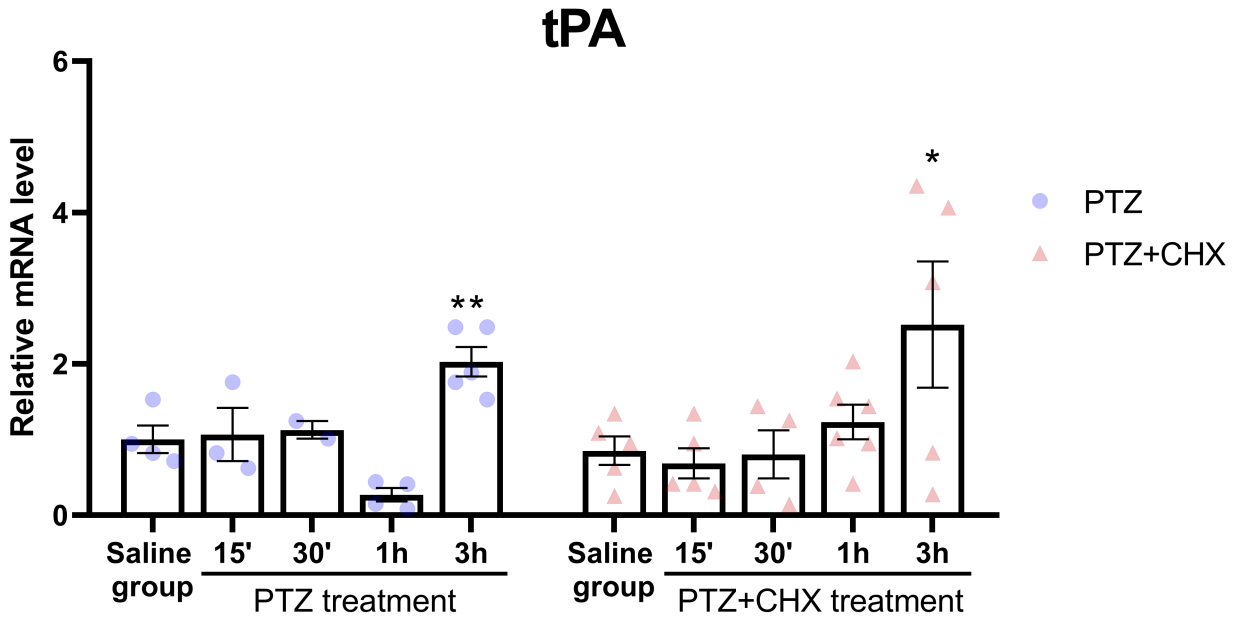

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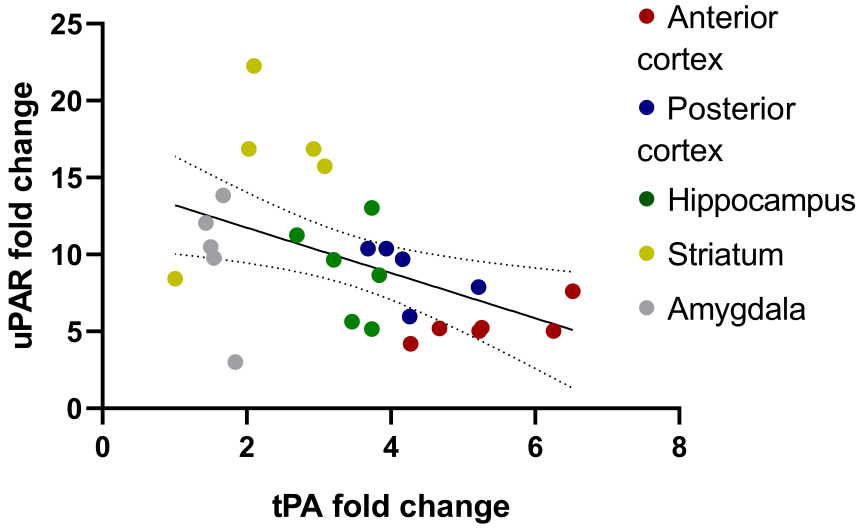

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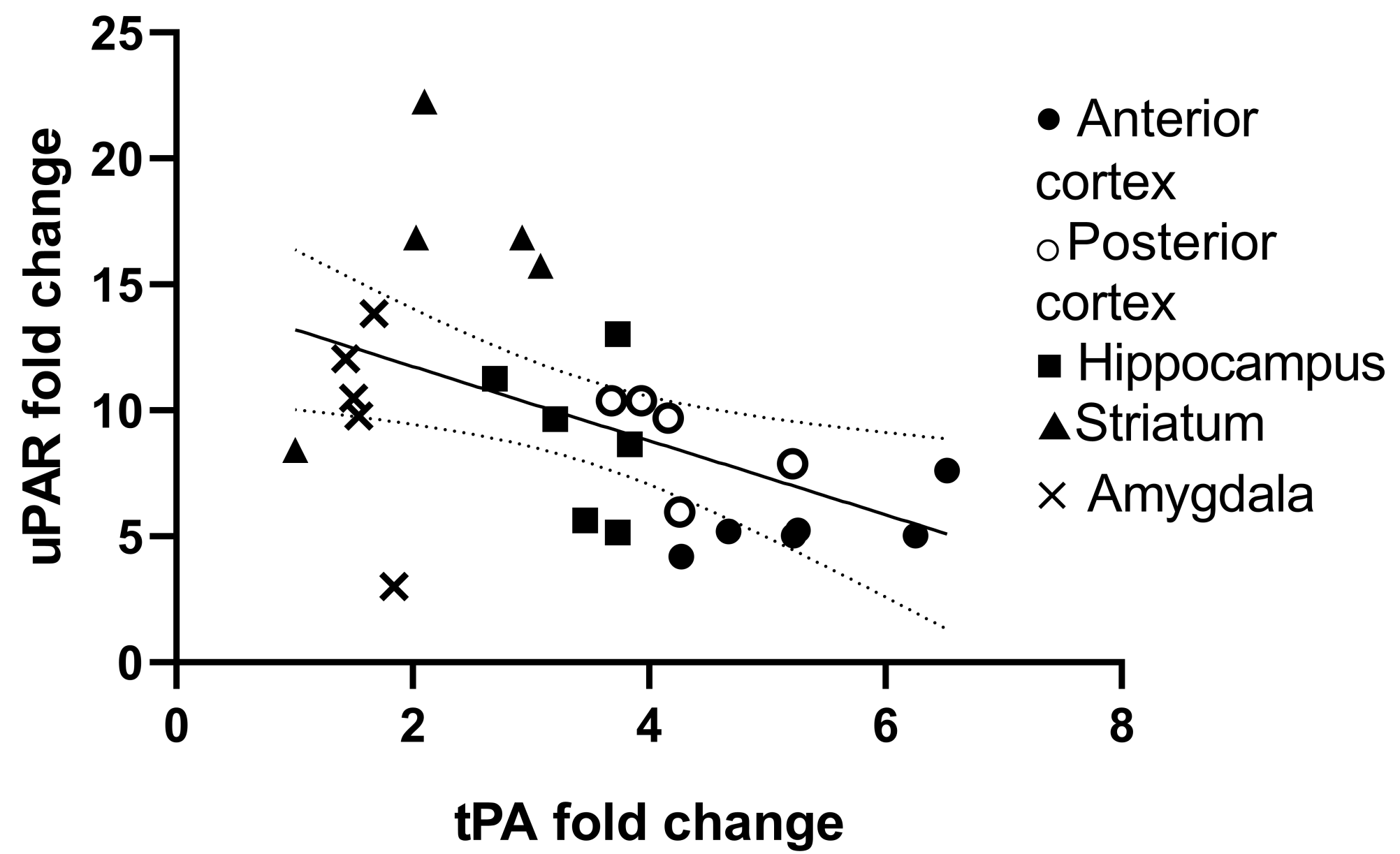
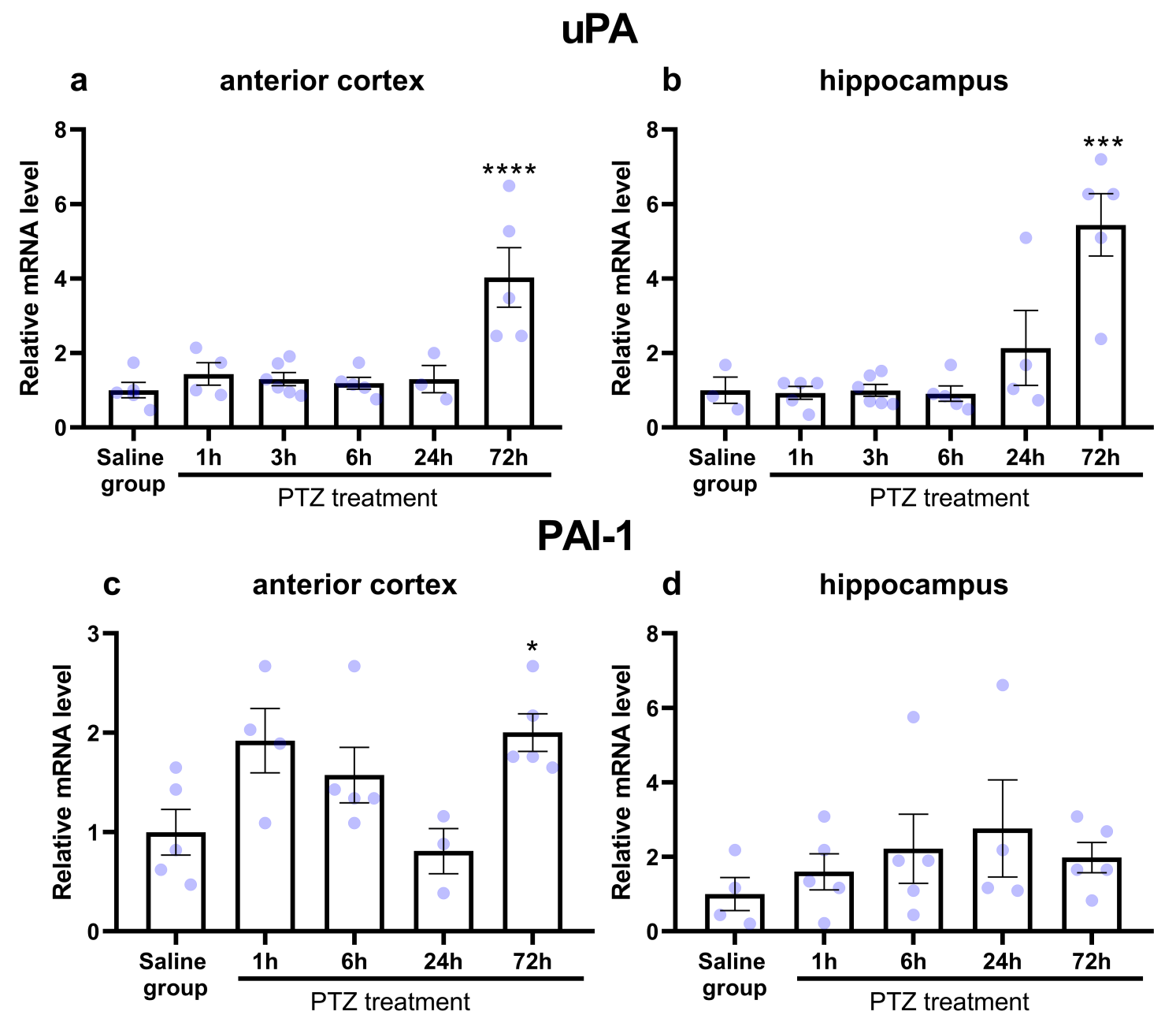

25
20
15
10
5
0
uPAR fold change
0
2
4
6
8
tPA fold change
Anterior cortex
Posterior cortex
Hippocampus
Striatum
Amygdala


uPA
a
anterior cortex
Relative mRNA level
8
6
4
2
0
****
Saline group
1h
3h
6h
24h
72h
PTZ treatment
b
hippocampus
Relative mRNA level
8
6
4
2
0
***
Saline group
1h
3h
6h
24h
72h
PTZ treatment
PAI-1
c
anterior cortex
Relative mRNA level
3
2
1
0
*
Saline group
1h
6h
24h
72h
PTZ treatment
d
hippocampus
Relative mRNA level
8
6
4
2
0
Saline group
1h
6h
24h
72h
PTZ treatment