GluN2D Subunit-Containing NMDA Receptors Control Tissue Plasminogen Activator-Mediated Spatial Memory

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Tissue plasminogen activator (tPA) is a serine protease with pleiotropic actions in the CNS, such as synaptic plasticity and neuronal death. Some effects of tPA require its interaction with the GluN1 subunit of the NMDA receptor (NMDAR), leading to a potentiation of NMDAR signaling. We have reported previously that the pro-neurotoxic effect of tPA is mediated through GluN2D subunit-containing NMDARs. Thus, the aim of the present study was to determine whether GluN2D subunit-containing NMDARs drive tPA-mediated cognitive functions. To address this issue, a strategy of immunization designed to prevent the *in vivo* interaction of tPA with NMDARs and GluN2D-deficient mice were used in a set of behavioral tasks. Altogether, our data provide the first evidence that tPA influences spatial memory through its preferential interaction with GluN2D subunit-containing NMDARs.

Introduction

Tissue plasminogen activator (tPA) is a serine protease that promotes thrombolysis in the vascular compartment by converting the zymogen plasminogen into active plasmin (Collen and Lijnen, 1991). In the brain parenchyma, tPA is involved in physiological processes, including synaptic plasticity (Huang et al., 1996; Calabresi et al., 2000; Zhuo et al., 2000; Mataga et al., 2002; Pang et al., 2004) and behaviors (Seeds et al., 1995, 2003; Madani et al., 1999; Pawlak et al., 2002, 2003; Matys et al., 2004; Yamada et al., 2005). tPA also displays important roles in models of acute and chronic brain disorders, such as ischemic brain injury, seizure, and multiple sclerosis (Tsirka et al., 1995; Chen and Strickland 1997; Wang et al., 1998; Wu et al., 2000; Gveric et al., 2001; Lu et al., 2002; Liot et al., 2006).

Several plasminogen-dependent- and -independent mechanisms have been described to explain these multifaceted roles of tPA. For instance, tPA influences some brain functions and dysfunctions by activating plasminogen into plasmin and subsequent degradation of the extracellular matrix (Plow et al., 1995; Chen and Strickland 1997; Wu et al., 2000; Hu et al., 2006) or conversion of the precursor form of brain-derived neurotrophic factor (BDNF) to its mature form (Pang et al., 2004). tPA was also reported to mediate some of its effects through a direct interac-

tion with the NMDA receptor (NMDAR) (Nicole et al., 2001; Benchenane et al., 2007).

Seven NMDAR subunits have been characterized in the CNS of mammals: the ubiquitously expressed GluN1 subunit, four GluN2 subunits (GluN2A–GluN2D), and two GluN3 subunits (GluN3A and GluN3B) (Cull-Candy et al., 2001; Kew and Kemp, 2005). More often, NMDARs are hetero-tetrameric assemblies composed of two GluN1 subunits and at least one GluN2 subunit. The GluN3 subunit can coassemble with GluN1/GluN2 complexes. The functional properties of NMDAR channels are in part determined by their subunit composition, in particular, the GluN2 type subunits (Cull-Candy et al., 2001).

We have demonstrated previously that tPA interacts with the GluN1 subunit of NMDARs, a necessary step to enhance NMDAR signaling and subsequent neurotoxicity (Nicole et al., 2001; Fernández-Monreal et al., 2004). Interestingly, a strategy preventing *in vivo* this tPA/GluN1 interaction in mice [active immunization against the amino-terminal domain (ATD) of the NMDAR GluN1 subunit] led to a reduced sensitivity to ischemic and excitotoxic neuronal death and impaired cognitive functions, such as spatial memory deficits (Benchenane et al., 2007; Macrez et al., 2010). More recently, we have evidenced by pharmacological and molecular approaches that tPA selectively promotes NMDAR signaling and subsequent neurotoxicity through GluN2D subunit-containing NMDARs (Baron et al., 2010; Jullienne et al., 2011). Thus, to mediate its deleterious functions, tPA interacts with the GluN1 subunit of the NMDAR, a phenomenon occurring preferentially in GluN2D subunit-containing NMDARs.

The aim of the present study was to determine whether GluN2D subunit-containing NMDARs drive tPA-mediated cognitive functions. To address this question, we performed a set of behavioral tasks in wild-type (WT) and GluN2D-deficient mice actively immunized or not to prevent the interaction of tPA with NMDARs.

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Materials and Methods

Animal experiments

Experiments were performed in accordance with the French (Decree 87/848) and the European Communities Council (Directive 86/609) guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and the number of animals used.

Subjects

Homozygous male mutant mice lacking the GluN2D subunit of NMDAR were generated by Prof. Mishina (University of Tokyo, Tokyo, Japan) (Ikeda et al., 1995) and provided by the RIKEN BioResource Center. These homozygous GluN2D mutant mice came from the GluN2D knock-out (KO) strain (RBRC01840), which has a 99.99% pure C57BL/6 genetic background. The genotype of mice was controlled by PCR using specific primers. Primers 1 and 2 recognize WT allele, and primers 2 and 3 recognize the mutant allele (1, CTTTCAGGGATCTGC-CACAAC; 2, CAGACAGTGCCGCGAGTCG; 3, TGATATTGCTGAA-GAGCTTGG). All WT mice (C57BL/6) and GluN2D-deficient mice (GluN2D KO) used for the behavioral studies were 3-month-old males (20–25 g). Molecular analyses were also performed on 3-month-old male mice weighing 25–30 g (Swiss mice; Janvier). All mice were housed in standard polypropylene cages (37 × 23.5 × 18 cm; Charles River) with access water and food *ad libitum* (SDS Dietex).

Behavioral tests

Previous studies have shown that tPA does not influence locomotor activity (Pawlak et al., 2002) but is implicated in emotional and spatial memories (Calabresi et al., 2000; Benchenane et al., 2007; Obiang et al., 2011). Accordingly, we used these behavioral tasks to determine whether the effect of tPA could be mediated by GluN2D subunit-containing NMDARs. All the behavioral tests were performed by an experimenter blinded to the treatment and genotype of the mice.

Locomotor activity. Spontaneous locomotor activity was quantified by using activity cages equipped with horizontal infrared beams located across the long axis of the cage (IMETRONIC). Mice were placed in individual acrylic chambers (30 × 20 × 20 cm) for 60 min. The number of horizontal movements was determined by breaks in movement-sensitive photobeams that were then converted into locomotor activity counts.

Contextual fear conditioning. Contextual emotional memory was tested in a conditioning chamber (67 × 53 × 55 cm; Bioseb) constructed from black methacrylate walls and a Plexiglas front door. Floor of the chamber consisted of 22 stainless steel bars (3 mm in diameter, spaced 11 mm apart, center-to-center) wired to a shock generator with scrambler for the delivery of footshock unconditioned stimulus. Signal generated by the mice movement was recorded and analyzed through a high-sensitivity weight transducer system. The analogical signal was transmitted to the Freezing software module through the load cell unit for recording purposes and posterior analysis in terms of activity/immobility. An additional interface associated with corresponding hardware allowed controlling the intensity of the shock from the Freezing software. On the training day, the mice were placed into the conditioning chamber, and they received two shocks in the feet at 2 min intervals after a 2 min acclimatizing period. Each shock was 0.4 mA and 2 s duration. Thirty seconds after the final shock, the mice were returned in their home cages. Forty-eight hours or 1 month after the conditioning session, contextual fear memory was assessed by returning the mice to the conditioning chamber and measuring freezing behavior during a 4 min retention test (Obiang et al., 2011). The measurement of fear was performed by considering the freezing time, defined as immobility (i.e., the absence of all movements with the exception of those related to respiration) for a period of at least 500 ms. Contextual fear memory was assessed through the comparison of the percentage of freezing during the retention test normalized to the percentage of freezing during the acclimatizing period of the training session.

Place recognition test. Spatial memory was tested in a gray plastic Y-maze with three identical arms (34 × 8 × 15 cm). Mice were tested after a two-session procedure with a 2h30 intersession interval. The length of the rest interval was chosen according to previous studies that

have shown that retention of the recognition place task does not last longer than a few hours in mice (Dellu et al., 2000; Obiang et al., 2011). During the acquisition session, one arm was randomly closed with a guillotine door. The position of the closed arm was chosen randomly among the three arms. Each mouse was placed in one of the two other arms (arms 1 and 2, with its head facing away from the center of the maze) and allowed to visit the two accessible arms for 5 min. Mice were then returned to their home cage for 2h30, before being subjected to the retention test, in which they had free access to all three arms for 5 min. The number of visits to each arm (considered only when the mouse passed two-thirds of the arm) was recorded for each session. Spatial memory was assessed through the comparison of the percentage of visits in each arm for the 5 min of the retention test.

Active immunization to prevent the interaction of tPA with the NMDAR GluN1 subunit

As previously described (Fernández-Monreal et al., 2004; Benchenane et al., 2007), the region of the ATD of the GluN1–1a subunit (amino acids 19–371), corresponding to the domain of interaction with tPA (designed rATD GluN1), was produced from the full-length rat GluN1–1a. rATD was purified from inclusion bodies of isopropyl 1-thio- β -galactopyranoside-induced bacterial cultures (*Escherichia coli*, M15 strain) on a nickel affinity matrix (Qiagen). When GluN2D-deficient and WT mice were 2 months old, immunization began by intraperitoneal injection of immunogenic mixtures: complete Freund's adjuvant (first injection) and incomplete Freund's adjuvant (once a week during 3 weeks) containing crude lysate of bacterial cultures transformed with a vector containing rATD GluN1 (crude ATD groups) or not (crude control groups). Behavioral tests were performed 2 weeks after the last injection.

Transcardiac perfusion

Animals were deeply anesthetized with 5% isoflurane and, thereafter, maintained with 2.5% isoflurane in a 70%/30% mixture of NO₂/O₂. A transcardiac perfusion was performed with ice-cold 0.9% NaCl with 3% heparin. Then, cortices and hippocampi were carefully harvested for protein and mRNA analyses.

Immunoblotting

Tissues were dissociated in ice-cold TNT buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% Triton X-100) at 1 mg/ml. Debris was removed by centrifugation (13,000 × g at 4°C, 15 min). Protein quantification was performed according to the BCA method (Pierce). Proteins (20 μ g) were resolved on 15% polyacrylamide gel under denaturing conditions and transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with Tris-buffered saline (10 mM Tris and 200 mM NaCl, pH 7.4) containing 0.05% Tween 20 and 5% nonfat dry milk. Blots were incubated overnight with primary antibodies: our mouse α ATD–GluN1 (Macrez et al., 2011) or control IgGs (1:2000), a goat anti-C-terminal domain (CTD)–GluN1 (α CTD–GluN1, 1:200; Santa Cruz Biotechnology), and a goat anti-CTD–GluN2D (α CTD–GluN2D, 1:200; Santa Cruz Biotechnology). After incubation with the appropriate peroxidase-conjugated secondary antibodies, proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (PerkinElmer Life and Analytical Sciences).

Primary cultures of cortical neurons

Cultures were prepared from E15–E16 mouse embryos (Swiss mice; Janvier) as described previously (Baron et al., 2010). Microdissection of cortices was followed by a dissociation of the tissue in a 37°C DMEM (Sigma-Aldrich). Cells grew on plates coated with poly-D-lysine (0.1 mg/ml) and laminin (0.02 mg/ml) in DMEM supplemented with 5% horse serum, 5% fetal bovine serum, and 2 mM glutamine (Invitrogen). Cells were maintained in a humidified 5% CO₂ atmosphere at 37°C. To inhibit glial proliferation, cytosine β -D-arabinoside (10 μ M) was added after 3 d *in vitro*. Neurons were used after 12 d *in vitro*.

Calcium video microscopy

Experiments were performed at room temperature on the stage of a Nikon Eclipse inverted microscope equipped with a 75 W xenon lamp

and a Nikon 40×, 1.3 numerical aperture epifluorescence oil-immersion objective. Cell cultures were transferred into a serum-free medium (HBSS) and loaded with 10 μ M fura-2 AM (Invitrogen) for 45 min at 37°C. Neurons were washed, and NMDA treatment (25 μ M for 30 s) was applied using a peristaltic pump. Neurons were then treated for 45 min with the GluN2D antagonist (2R*, 3S*)-1-(9-bromophenanthrene-3-carbonyl) piperazine-2, 3-dicarboxylic acid (UBP145; 0.2 μ M) (Costa et al., 2009), α ATD–GluN1 (0.01 mg/ml), and tPA (20 μ g/ml) either alone or in combination (directly applied in the bathing medium), and again, neurons were exposed to NMDA (25 μ M for 30 s). Fura-2 (excitation, 340 and 380 nm; emission, 510 nm) ratio images were acquired with a CCD camera (Princeton Instruments) and digitized (256 × 512 pixels) using Metafluor 4.11 software (Universal Imaging Corporation).

Quantitative real-time PCR

Total RNAs were extracted using Nucleospin RNA II columns (Macherey-Nagel). Then, RNAs (1 μ g) were reverse-transcribed using the iScript Select cDNA synthesis kit (reverse transcription: 42°C for 1.5 h; Bio-Rad). Primers were designed for each gene using the Beacon Designer software (Bio-Rad). Sequence alignments were performed with the BLAST database to ensure the specificity of primers. The following sequences were used: GluN1 forward primer (F), 5'-CTCTAGCCAGGTCTACGCTATCC-3'; GluN1 reverse primer (R), 5'-GACGGGGATTCTGTAGAAGCCA-3'; GluN2A (F), 5'-ACATCCACGTTCTTCCAGTTGG-3'; GluN2A (R), 5'-GACATGCCAGTCATAGTCCTGC-3'; GluN2B (F), 5'-CCAGAGTGAGAGATGGGATTGC-3'; GluN2B (R), 5'-TGGGCTCAGGGATGAACTGT-3'; GluN2D (F), 5'-CTGTGTGGGTGATGATGTTCTGT-3'; and GluN2D (R), 5'-GTGAAGGTAGAGCCTCCGGG-3'.

PCR solutions were prepared with RNase-free water containing primers and IQ SYBR Green Supermix (Bio-Rad). For PCR amplification, 20 μ l of mix were added to 5 μ l of reverse transcription reaction previously diluted (1:20). Two negative controls were performed during each experiment: reactions with no added reverse transcriptase to confirm absence of genomic DNA contamination, and samples with no added cDNA template to confirm absence of primer dimers. Assays were run in triplicate on the iCycler iQ real-time PCR detection system (Bio-Rad). The amplification conditions were as follows: Hot Goldstar enzyme activation, 95°C for 3 min; 40 cycles of PCR (denaturation, 95°C, 15 s; hybridization/extension, 60°C, 1 min). Relative mRNA transcription was expressed in $2^{-(Ct \text{ gene of interest})}$, in which Ct is the threshold cycle value.

Data analyses

Data analyses were conducted with the Systat software package (version 5.02). An α level of $p < 0.05$ was used for determination of significance in all statistical tests. All p values are two tailed. For behavioral and molecular analyses, Friedman's tests were used for intragroup multiple comparisons. In significant cases, Wilcoxon's signed-rank tests for matched samples were performed as *post hoc* tests (Siegel and Castellan 1988). Kruskal–Wallis tests were used for intergroup multiple comparisons. When significant, Mann–Whitney U tests were performed as *post hoc* tests (Siegel and Castellan 1988). For calcium video microscopy, statistical analyses were performed using paired Student's t test.

Results

Behavioral phenotype of GluN2D-deficient mice

Deficiency in GluN2D subunit reduces spontaneous locomotor activity

GluN2D-deficient mice showed significantly less horizontal movements than their WT littermates (Fig. 1; number of movements, 614 ± 212 for WT mice and 466 ± 105 for GluN2D KO mice; Mann–Whitney U test, $U = 109.500$, $p < 0.05$). These results show a reduced spontaneous locomotor activity in GluN2D-deficient mice.

Deficiency in GluN2D subunit alters emotional memory

The percentage of freezing in both WT and GluN2D-deficient mice was similar during the acclimatizing period of the training session (Fig. 2A; Mann–Whitney U test, $p > 0.05$). This means that the emotional state in the two groups was homogeneous.

Figure 1. Deficiency in GluN2D subunit reduces spontaneous locomotor activity. Spontaneous locomotor activity was assessed, during 60 min, as the total number of horizontal movements determined by breaks in movement-sensitive photobeams converted into locomotor counts. GluN2D KO mice, $n = 9$; WT mice (WT), $n = 10$. Mann–Whitney U test, $\#p < 0.05$. Vertical bars indicate SD.

Figure 2. Deficiency in GluN2D subunit alters emotional memory. **A**, Percentage of freezing during the acclimatizing period of the training session of the fear conditioning test. **B**, Retention tests conducted 48 h and 1 month after conditioning. Contextual emotional memory was assessed as the percentage of freezing during the retention test normalized to the percentage of freezing during the acclimatizing period of the training session. GluN2D KO mice, $n = 9$; WT mice (WT), $n = 10$. Mann–Whitney U tests, $\#p < 0.05$. Vertical bars indicate SD.

Forty-eight hours or 1 month after the training session, contextual emotional memory was assessed by returning the mice in the conditioning chamber and by measuring the freezing behavior during the 4 min of the retention test. GluN2D-deficient mice showed less fear because they took significantly less time to freeze than the WT mice (Fig. 2B; Mann–Whitney U tests: 48 h after training, $U = 69$, $p = 0.05$; 1 month after training, $U = 71$, $p < 0.05$).

Figure 3. Deficiency in GluN2D subunit alters spatial memory. Total number of visits (**A**) and percentage of visits (**B**) in arms 1 and 2 during the acquisition session of the place recognition task. **C**, Retention test was conducted after a 2h30 rest interval. Spatial memory was assessed through the comparison of the percentage of visits in each arm for 5 min of the retention test. GluN2D KO mice, $n = 9$; WT mice (WT), $n = 10$. Wilcoxon's signed-rank tests, $*p < 0.05$, $**p < 0.01$. Vertical bars indicate SD.

0.05). These results indicate that deficiency in GluN2D subunit impairs contextual fear memory.

Deficiency in GluN2D subunit alters spatial memory

During the acquisition session, the total number of visits in the two free access arms of the Y-maze was similar between GluN2D-deficient mice and their WT littermates (Fig. 3A; Mann–Whitney U test, $p > 0.05$). Moreover, both groups visited arm 1 as often as arm 2 (Fig. 3B; Wilcoxon's signed-rank tests: WT mice, arm 1 vs arm 2, $p > 0.05$; GluN2D KO mice, arm 1 vs arm 2, $p > 0.05$). However, during the retention session, although WT mice correctly discriminated the newly open arm and the two familiar arms (Fig. 3C; Wilcoxon's signed-rank tests: arm 1 vs new arm, $Z = 2.677$, $p < 0.01$; arm 2 vs new arm, $Z = 2.539$, $p < 0.05$), GluN2D-deficient mice failed to discriminate arm 2 and the newly open arm (Fig. 3C; Wilcoxon's signed-rank tests: arm 1 vs new arm, $Z = 2.399$, $p < 0.05$; arm 2 vs new arm, $p > 0.05$). These results show a deficit of spatial memory in GluN2D-deficient mice.

Active immunization selectively prevents tPA/GluN1 interaction and tPA-induced enhancement of NMDAR signaling without altering the basal NMDAR signaling

Here, we used a strategy of active immunization raised against the recombinant form of the ATD of the NMDAR GluN1 subunit (ATD–GluN1; Fig. 4A), reported previously to prevent the interaction of tPA with GluN1 subunit, and subsequent action of tPA on NMDAR signaling. IgGs purified from plasma harvested from both control and ATD–GluN1 immunized mice (control IgGs and α ATD–GluN1, respectively) were used to reveal immunob-

Figure 4. Active immunization selectively prevents tPA/GluN1 interaction and tPA-induced enhancement of NMDAR signaling without altering the basal Ca^{2+} conductivity. **A**, Schematic representation of NMDAR composed of GluN1/GluN2D subunits, including binding sites of α ATD–GluN1, α CTD–GluN1, and α CTD–GluN2D antibodies, UB145, and tPA. **B**, ATD–GluN1 immunized mice display antibodies specifically targeting the GluN1 subunit of NMDAR. Proteins extracts from naive mouse brain ($n = 3$) were subjected to immunoblots revealed with IgGs purified from either control mice (control IgGs) or ATD–GluN1 (120 kDa) immunized mice (α ATD–GluN1). Parallel immunoblottings were performed and revealed with antibodies raised against either CTD–GluN1 (named α CTD–GluN1), known to reveal a band at ~ 120 kDa, or CTD–GluN2D (named α CTD–GluN2D), known to reveal a band at ~ 165 kDa. **C**, After immunization, mice display circulating antibodies against GluN1, capable of preventing the potentiating effect of tPA on GluN1/GluN2D subunit-containing NMDARs. NMDA induces Ca^{2+} influx in cortical neurons as measured by fura-2 video microscopy ($N = 3$, $n = 150$ cells). Coapplication of tPA (20 $\mu\text{g}/\text{ml}$; 45 min) potentiates the NMDA-evoked Ca^{2+} influx by 47% ($N = 3$, $n = 108$ cells). Neither UB145 alone (0.2 μM ; $N = 3$, $n = 150$ cells) nor α ATD–GluN1 antibodies alone (0.01 mg/ml; $N = 3$, $n = 108$ cells) alter NMDA-induced Ca^{2+} influx. Both UB145 (0.2 μM) and α ATD–GluN1 (0.01 mg/ml) are capable of blocking this potentiating effect of tPA ($N = 3$, $n = 150$ cells and $N = 3$, $n = 108$ cells, respectively). Ctrl, Control; HBSS, serum-free medium. Paired Student's t test (before vs after treatment), $*p < 0.001$. Vertical bars indicate SD.

lotings using naive mouse brain extracts as sample. Figure 4B shows that ATD–GluN1 antibodies (α ATD–GluN1) reveal full-length GluN1 (and not GluN2D). Control immunoblottings revealed with commercially available antibodies for GluN1 (α CTD–GluN1) and GluN2D (α CTD–GluN2D) were performed in parallel as controls (Fig. 4B).

We then determined whether these ATD–GluN1 antibodies were capable to block the interaction and the potentiating effect of tPA on

Figure 5. Immunization targeting the tPA/GluN1 interaction does not affect spontaneous locomotor activity in WT (**A**) and GluN2D-deficient (**B**) mice. Spontaneous locomotor activity was assessed, during 60 min, as the total number of horizontal movements determined by breaks in movement-sensitive photobeams converted into locomotor counts. WT Crude ATD mice, $n = 11$; Crude Control mice, $n = 10$; GluN2D KO Crude ATD mice, $n = 13$; GluN2D KO Crude Control mice, $n = 12$. Vertical bars indicate SD.

GluN1/GluN2D subunit-containing NMDARs. Thus, NMDA-induced calcium influx was recorded on primary cultures of cortical neurons (Fig. 4C). Although tPA promotes NMDA-induced calcium influx (+47%; Fig. 4C; paired Student's t test, $p < 0.001$), coincubation of tPA with ATD–GluN1 antibodies completely prevented the potentiating effect of tPA (Fig. 4C; paired Student's t test, $p > 0.05$), as observed with a GluN2D receptor antagonist (UBP145; Fig. 4C; paired Student's t test, $p > 0.05$). Importantly, the ATD–GluN1 antibodies, when applied alone, did not influence basal NMDAR activity (Fig. 4C; paired Student's t test, $p > 0.05$).

Immunization targeting the tPA/GluN1 interaction affects neither spontaneous locomotor activity nor emotional memory in WT and GluN2D-deficient mice

Spontaneous locomotor activity was similar between control (WT crude control) and immunized WT (WT crude ATD; Fig. 5A; number of movements, 848 ± 154 for WT crude control and 750 ± 211 for WT crude ATD; Mann–Whitney U test, $p > 0.05$) mice. These results indicate that the tPA/GluN1 interaction is not involved in spontaneous locomotor activity. Similar results were obtained for GluN2D-deficient mice (Fig. 5B; number of movements, 644 ± 197 for GluN2D KO crude control and 736 ± 204 for GluN2D KO crude ATD; Mann–Whitney U test, $p > 0.05$).

WT and GluN2D-deficient mice, immunized or not, showed similar percentages of freezing during the acclimatizing period of the training session (Fig. 6A; Mann–Whitney U test, $p > 0.05$) showing that the emotional state in the control and immunized groups was homogeneous. Interestingly, 48 h or 1 month after training, immunized WT mice (WT crude ATD) submitted to the retention tests

Figure 6. Immunization targeting the tPA/GluN1 interaction does not affect emotional memory in WT and GluN2D-deficient mice. **A**, Percentage of freezing during the acclimatizing period of the training session of the fear conditioning test. **B**, **C**, Retention tests conducted 48 h and 1 month after conditioning. Contextual emotional memory was assessed as the percentage of freezing during the retention test normalized to the percentage of freezing during the exploration of the training session. WT Crude ATD mice, $n = 11$; WT Crude Control mice, $n = 10$; GluN2D KO Crude ATD mice, $n = 13$; GluN2D KO Crude Control mice, $n = 12$. Vertical bars indicate SD.

showed a time of freezing similar to their control counterparts (WT crude control; Fig. 6B; Mann–Whitney U tests: 48 h after training, $p > 0.05$; 1 month after training, $p > 0.05$). These results indicate that the tPA/GluN1 interaction is not involved in contextual fear memory. Similar results were obtained for GluN2D-deficient mice (Fig. 6C; Mann–Whitney U tests: 48 h after training, $p > 0.05$; 1 month after training, $p > 0.05$).

Altogether, these results show that locomotor activity and contextual fear memory, reported previously to be mediated by GluN2D subunit-containing NMDARs (Figs. 1, 2), are not impaired as a consequence of the immunization protocol targeting GluN1 subunit.

Although immunization targeting tPA/GluN1 interaction specifically alters spatial memory in WT mice, it fails to affect spatial memory in GluN2D-deficient mice

During the acquisition session, the total number of visits in the two free access arms of the Y-maze was similar between WT control (WT crude control) and immunized WT (WT crude ATD; Fig. 7A; Mann–Whitney U test, $p > 0.05$) mice. In addition, both groups visited the two arms equally (Fig. 7B; Wilcoxon's signed rank tests: WT crude control, arm 1 vs arm 2, $p > 0.05$; WT

Figure 7. Immunization targeting the tPA/GluN1 interaction does not affect acquisition performance of WT and GluN2D-deficient mice in the place recognition task. **A**, Total number of visits in arms 1 and 2. **B**, Percentage of visits in arms 1 and 2. WT Crude ATD mice, $n = 11$; WT Crude Control mice, $n = 10$; GluN2D KO Crude ATD mice, $n = 13$; GluN2D KO Crude Control mice, $n = 12$. Vertical bars indicate SD.

4crude ATD, arm 1 vs arm 2, $p > 0.05$). Similar results were obtained in control (GluN2D KO crude control) and immunized (GluN2D KO crude ATD) GluN2D-deficient mice (Fig. 7A; Mann–Whitney U test, $p > 0.05$; Fig. 7B; Wilcoxon’s signed-rank tests: GluN2D KO crude control, arm 1 vs arm 2, $p > 0.05$; GluN2D crude ATD, arm 1 vs arm 2, $p > 0.05$).

Interestingly, spatial memory of WT mice was totally inhibited after immunization, preventing the interaction of tPA with NMDAR. Indeed, immunized WT mice (WT crude ATD) did not discriminate between the three arms, whereas control animals (WT crude control) were able to recognize the familiar arms from the newly open arm (Fig. 8A; Wilcoxon’s signed-rank tests: WT crude control, arm 1 vs new arm, $Z = 2.812$, $p < 0.01$; arm 2 vs new arm, $Z = 2.325$, $p < 0.05$; WT crude ATD, arm 1 vs new arm, $p > 0.05$; arm 2 vs new arm, $p > 0.05$). These results suggest that the tPA/GluN1 interaction is required for the achievement of the spatial memory. In contrast, the blockage of the tPA/GluN1 interaction in GluN2D-deficient mice (GluN2D KO crude ATD) did not aggravate the deficit of spatial memory observed previously in the non-immunized GluN2D-deficient mice (GluN2D KO crude control; Fig. 8B; Wilcoxon’s signed-rank tests: GluN2D KO crude control, arm 1 vs new arm, $Z = 2.666$, $p <$

Figure 8. Although immunization targeting tPA/GluN1 interaction alters spatial memory in WT mice (**A**), it fails to affect spatial memory in GluN2D-deficient mice (**B**). Retention test was conducted after a 2h30 rest interval. Spatial memory was assessed through the comparison of the percentage of visits in each arm for the 5 min of the retention test. Immunized WT mice (WT Crude ATD), $n = 11$; control WT mice (WT Crude Control), $n = 10$; GluN2D KO Crude ATD mice, $n = 13$; GluN2D KO Crude Control, $n = 12$. Wilcoxon’s signed-rank tests, $*p < 0.05$; $**p < 0.01$. Vertical bars indicate SD.

Figure 9. Regulation of NMDAR subunits (GluN1, GluN2A, GluN2B, GluN2D) in hippocampus after active immunization against the ATD of the NMDAR GluN1 subunit. Relative mRNA quantity, estimated by RT-qPCR, was expressed in 2^{-Ct} (Ct gene of interest), in which Ct is the threshold cycle value. **A**, GluN1 subunit mRNA expression. **B**, GluN2A subunit mRNA expression. **C**, GluN2B subunit mRNA expression. **D**, GluN2D subunit mRNA expression. WT Crude ATD mice, $n = 5$; WT Crude Control mice, $n = 5$. Mann–Whitney U test, $*p < 0.05$. Vertical bars indicate SD.

0.05; arm 2 vs new arm, $p > 0.05$; GluN2D KO crude ATD, arm 1 vs new arm, $Z = 2.198$, $p < 0.05$; arm 2 vs new arm, $p > 0.05$). These data reveal that mice lacking GluN2D subunit are resistant to inhibition of tPA/GluN1 interaction-induced spatial

memory impairment, suggesting that tPA-dependent impairment of spatial memory involves a GluN2D subunit-dependent mechanism.

These results cannot be explained by nonspecific effects of the immunization strategy because the expression of NMDAR was not changed in the hippocampus, a cerebral structure involved in spatial memory. Indeed, the *in vivo* distribution of the different NMDA subunits was determined by quantitative PCR in hippocampi of control and ATD–GluN1 immunized mice (Fig. 9). The data show that our strategy of immunization against the NMDAR GluN1 subunit failed to influence the endogenous expression of GluN1, GluN2A, and GluN2B (Fig. 9; Mann–Whitney *U* test, $p > 0.05$). As a feedback response to the blockage of the interaction of tPA with GluN1/GluN2D subunit-containing NMDAR, a weak increase in the expression of GluN2D was observed (Fig. 9; Mann–Whitney *U* test, $U = 2$, $p < 0.05$). This increased expression of GluN2D could be a tentative mechanism of compensation. However, it does not modify the previously reported GluN2D-dependent behaviors in immunized WT mice when compared with their non-immunized littermates (i.e., horizontal locomotor activity, Figs. 1, 5A; contextual fear conditioning, Figs. 2, 6A,B).

Figure 10 provides a summary of the behavioral data obtained. A–C depict, respectively, the behaviors influenced by tPA, those affected by the blockage of the interaction of tPA with the GluN1/GluN2D subunit-containing NMDAR in WT mice, and those affected by the blockage of the interaction of tPA with NMDAR in the GluN2D-deficient mice. Together, our data show for the first time that the GluN1/GluN2D subunit-containing NMDAR drives the effects of tPA on spatial memory.

Discussion

In the brain parenchyma, tPA has been identified as a key player in numerous physiological and pathological processes. For example, tPA is involved in synaptic plasticity processes (Mataga et al., 2002), such as long-term potentiation (Huang et al., 1996; Pang et al., 2004) and long-term depression (Calabresi et al., 2000). Accordingly, tPA has been implicated in numerous behaviors, including various forms of learning and emotional behaviors (Seeds et al., 1995; Madani et al., 1999; Calabresi et al., 2000; Pawlak et al., 2002, 2003; Seeds et al., 2003; Benchenane et al., 2007). Apart from these physiological functions, tPA was reported to influence neuronal, oligodendrocytic, and endothelial death/survival after excitotoxic, apoptotic, and/or inflammatory challenges (Chen and Strickland 1997; Wang et al., 1998; Nicole et al., 2001; Liu et al., 2004a; Liot et al., 2006; Correa et al., 2011).

Among the mechanisms advanced to explain these actions of tPA, its interaction with the GluN1 subunit of the NMDAR, leading to a potentiation of NMDA-mediated calcium influx, has been evidenced by several groups (Nicole et al., 2001; Fernández-Monreal et al., 2004; Kvajo et al., 2004; Samson et al., 2008; Nassar et al., 2010). Alternatively, it has been proposed that tPA could modulate NMDAR-mediated signaling through the

	A	B		C	
	tPA-influenced Behavior	WT		GluN2D KO	
		tPA/NMDAR interaction		tPA/NMDAR interaction	
		NORMAL	INHIBITION	NORMAL	INHIBITION
Locomotor activity (Pawlak et al., 2002)	NO	✓	✓	↓	↓
Emotional Memory (Calabresi et al., 2000)	YES	✓	✓	↓	↓
Spatial Memory (Benchenane et al., 2007)	YES	✓	⇓	↓	↓

✓ = no deficit ↓ = mild deficit ⇓ = severe deficit

Figure 10. GluN1/GluN2D subunit-containing NMDARs drive tPA-influenced spatial memory. **A**, Previous studies have evidenced that tPA was not involved in locomotor activity (Pawlak et al., 2002). However, tPA is known to influence both emotional (Calabresi et al., 2000) and spatial memories (Benchenane et al., 2007). **B**, Our present experiments reveal that inhibition of the tPA/NMDAR interaction prevents neither locomotor activity nor emotional memory in mice. In addition, our results show that the tPA/NMDAR interaction is a critical mechanism underlying tPA-influenced spatial memory. **C**, In agreement with Ikeda et al. (1995), we observe a decrease in spontaneous locomotor activity in GluN2D-deficient mice. Furthermore, our present study reveals impairments of both emotional and spatial memories in this strain. In addition, we also show that the inhibition of tPA/NMDAR interaction does not impair the spatial memory in GluN2D KO mice. Together, these results demonstrate that tPA influences spatial memory through an increased affinity for NMDAR when associated with GluN2D subunit. Arrows point to the behavioral deficit.

GluN2B subunit (Pawlak et al., 2005) or lipoprotein receptor-related protein (Samson et al., 2008).

Because we have identified the exact location of the interaction of tPA within GluN1 in cultured cortical neurons (Fernández-Monreal et al., 2004), we performed active immunization leading to the production of antibodies recognizing the ATD of GluN1 (Benchenane et al., 2007; Macrez et al., 2010). Importantly, these antibodies do not alter NMDA-induced basal neurotransmission (toxicity, calcium influx), but they specifically block the potentiating effect of tPA on these receptors. Accordingly, we have shown that this strategy of blockage of the tPA/GluN1 interaction leads to behavioral deficits in Swiss mice and also prevents the pro-neurotoxicity of tPA both *in vitro* and *in vivo* (Benchenane et al., 2007; Macrez et al., 2010). In addition, in recent studies, we evidenced both *in vitro* and *in vivo* that the pro-neurotoxic effect of tPA was mediated by GluN2D subunit-containing NMDAR (Baron et al., 2010; Jullienne et al., 2011), suggesting that, although tPA interacts with the GluN1 subunit, this mechanism occurs preferentially in GluN2D subunit-containing NMDAR. Obviously, this interesting new question will need to be clarified in future studies.

Thus, the first step of our present study was to confirm (Ikeda et al., 1995; Miyamoto et al., 2002; Hagino et al., 2010) and extend the behavioral phenotype of GluN2D-deficient mice by the use of various tests, such as actimetry, contextual fear conditioning, and place recognition task. Our results show a decrease in spontaneous locomotor activity and in emotional memory in GluN2D-deficient mice as described previously by other authors (Ikeda et al., 1995; Miyamoto et al., 2002; Hagino et al., 2010). In addition, we evidenced that GluN2D-deficient mice exhibited low performances in the place recognition task because they do not discriminate the newly open arm and one familiar arm. We thus provide

here the first evidence linking the GluN2D subunit of the NMDAR to the regulation of spatial memory.

We then tested the effects of immunization preventing *in vivo* the tPA/GluN1 interaction (Benchenane et al., 2007) in WT and GluN2D-deficient mice in the same behavioral tasks. Our data demonstrate that the blockage of the interaction of tPA with NMDAR influences neither locomotor behavior nor emotional memory, suggesting that tPA/GluN1 interaction is not involved in such behaviors in mice. In contrast, in the Y-maze task, immunized WT mice (WT crude ATD) do not discriminate between the newly open arm and the two familiar arms, showing a high alteration of spatial memory. These results demonstrate that the tPA/GluN1 interaction mediates the place recognition task used in this study. These data obtained in C57BL/6 mice are in agreement with our previous work performed on Swiss mice (Benchenane et al., 2007), showing then the phenotypic consistency of the mechanism across these mouse strains. In addition, our present data reveal that, although NMDARs are involved in emotional memory (Levenson et al., 2002; Bardgett et al., 2003; Gao et al., 2010), the influence of tPA on this process cannot be explained by its ability to promote NMDAR-dependent signaling. It is well admitted that the establishment of emotional memory also involves BDNF (Liu et al., 2004b). Interestingly, the maturation of BDNF critically depends on tPA activity (Pang et al., 2004; Obiang et al., 2011). Thus, it is possible that the influence of tPA on emotional memory is based only on its implication in the maturation of BDNF.

Finally, based on our proposed mechanism of action in which tPA mediates some of its effects through GluN2D subunit-containing NMDARs (Baron et al., 2010; Jullienne et al., 2011), we used the same strategy of immunization in GluN2D-deficient mice and studied the functional consequences in the place recognition task. Interestingly, blockage of the interaction of tPA with NMDAR does not affect further the spatial memory deficits of the GluN2D-deficient mice (GluN2D KO crude ATD) compared with non-immunized GluN2D-deficient mice (GluN2D KO crude control). Moreover, the memory performances of immunized GluN2D-deficient mice are better than those of immunized WT mice, which are not able to discriminate the three arms. Thus, these results provide *in vivo* evidences that the impairment found in WT mice in the Y-maze behavioral task is a consequence of the lack of tPA interaction with GluN1/GluN2D subunit-containing NMDARs.

Altogether, we provide here the first evidence that binding of tPA to GluN2D subunit-containing NMDARs mediates some tPA-influenced behaviors. The relevance of the link between tPA and GluN2D might require additional evidences, such as further studies using specific GluN2D antagonists, or genetic means, but none of those strategies are available yet. Future studies should contribute to the characterization of neuronal networks underlying the functional consequences of the interaction of tPA with the GluN1/GluN2D subunit-containing NMDARs.

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