Anti-NR1 N-terminal-domain vaccination unmasks the crucial action of tPA on NMDA-receptor-mediated toxicity and spatial memory

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Accepted 21 November 2006
Journal of Cell Science 120, 578-585 Published by The Company of Biologists 2007
doi:10.1242/jcs.03354

Summary

Fine-tuning of NMDA glutamatergic receptor signalling strategically controls crucial brain functions. This process depends on several ligands and modulators, one of which unexpectedly includes the serine protease tissue-type plasminogen activator (tPA). In vitro, tPA increases NMDA-receptor-mediated calcium influx by interacting with, and then cleaving, the NR1 subunit within its N-terminal domain. Owing to lack of in vivo evidence of the relevance and contribution of this mechanism in physiological and pathological brain processes, active immunisation was developed here in mice, to allow transient and specific prevention of the interaction of tPA with the NR1 subunit. Immunisation significantly reduced the severity of ischemic and excitotoxic insults in the mouse brain. Cognitive function was altered in some, but not all behavioural tasks affected in tPA-deficient mice. Our data demonstrate that in vivo, tPA controls neurotoxicity and the encoding of novel spatial experiences by binding to and cleaving the NMDA receptor NR1 subunit. Interesting therapeutic possibilities for several brain pathologies that involve excitotoxicity may now be envisaged.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/4/578/DC1

Key words: Excitotoxicity, Stroke, Immunisation, NMDA receptor, Spatial memory, Tissue-type plasminogen activator

Introduction

Tissue-type plasminogen activator (tPA) is one of the two mammalian serine proteases that activates plasminogen into plasmin, the primary plasmatic function being fibrinolysis. Moreover, a growing body of data suggests that parenchymal tPA plays a crucial role in brain function (Benchenane et al., 2004). For instance, tPA is a major immediate-early gene activated by neuronal firing during cortico-striatal long-term depression (LTD) and hippocampal long-term potentiation (LTP). Accordingly, deletion of the tPA gene in mice results in selective interference of the hippocampal late-phase LTP (L-LTP) and in both cortico-striatal LTP and LTD (Huang et al., 1996; Calabresi et al., 2000). Whereas exogenous tPA enhances L-LTP, tPA inhibitors reduce this effect (Baranes et al., 1998). Furthermore, mice overexpressing tPA display increased and prolonged L-LTP (Madani et al., 1999). Along with these electrophysiological data, tPA is involved in anxiety-related behaviour, spatial memory tasks and the induction of cerebellar motor learning (Huang et al., 1996; Calabresi et al., 2000; Pawlak et al., 2003; Seeds et al., 2003).

Apart from these physiological functions, tPA controls neuronal death. For example, tPA-deficient mice are resistant to excitotoxin-induced degeneration of hippocampal neurons (Tsirka et al., 1995). In some stroke models, an aggravating role of tPA was reported (Benchenane et al., 2004): tPA-deficient mice display smaller ischemic brain lesions than their normal counterparts, although their infarct volumes can be restored to control levels by a systemic administration of tPA (Wang et al., 1998). Thus, although tPA-induced thrombolysis remains the only efficient clinical treatment for stroke, results from animal models suggest potential side effects of tPA in this condition.

The first proposed mechanism of action of tPA, relies on its ability to degrade extracellular matrix components, through plasmin activation (Benchenane et al., 2004). It was also proposed that the effect of tPA on L-LTP is initiated by the binding to its cell surface receptor LRP (low density lipoprotein receptor-related protein), which, in turn, activates PKA (Zhuo et al., 2000). Recently, tPA has been shown to modulate LTP through the plasmin-dependent activation of pro-BDNF (Pang et al., 2004). We have demonstrated that tPA binds to, and then cleaves, the N-terminal domain (NTD) of

the NMDA receptor NR1 subunit at Arg260: a necessary step to enhance NMDA receptor signalling into neurons (Nicole et al., 2001; Fernandez-Monreal et al., 2004). Thus, tPA acts as a positive neuromodulator of NMDA-receptor-dependent glutamatergic transmission. Nevertheless, demonstration and relevance of this in brain functions and dysfunction in vivo has not yet been established.

To address this issue, we developed a strategy to selectively prevent the tPA-NR1 subunit interaction. We immunised mice against the NTD of NR1. We hypothesised that the antibodies generated would physically prevent the interaction with, and cleavage of, NR1 by tPA (Fig. 1A). This should reveal the potential involvement of a tPA-NR1 interaction on neuronal death or survival and in behavioural tasks.

Results

Immunisation prevents both the interaction with and cleavage of NR1 by tPA

To characterise our model, sera were harvested and tested by immunoblotting for their ability to reveal increasing concentrations of recombinant NTD-NR1. Sera from immunised mice recognised recombinant NTD-NR1, whereas that taken from control mice (CFA only) did not (Fig. 1B). tPA zymography assays revealed that the immunisation protocol did not affect tPA activity in the cortex or in the striatum (Fig. 1C). In agreement with previous work (Hazama et al., 2005), IgG immunoreactivity was evident in the parenchyma, supporting the hypothesis of immune responses in the brain (Fig. 1D).

We then tested whether the antibodies produced in the immune response could physically prevent tPA from interacting with and then cleaving the NMDA receptor NR1 subunit in vivo. Immunoprecipitations with antibodies directed against either tPA or the C-terminus of the NR1 subunit, followed by immunoblotting analysis with the NR1 antibody were thus performed from cortical extracts harvested from both groups (Fig. 1E, n=3). In support of our hypothesis, immunisation led to reduced amounts of tPA bound to NR1 (lanes 2 and 3), without significant alteration in the total amount of NR1 (lanes 4 and 5). Interestingly, we could not only detect the cleaved form of NR1 in vivo (lanes 2 and 4), but also evidence that immunisation reduces the amount of the cleaved form of NR1 (lanes 3 and 5). Altogether, these data demonstrate that endogenously produced antibodies raised against the NTD of NR1 block both tPA interaction with and subsequent cleavage of NR1, two processes responsible for the ability of tPA to promote NMDA receptor signalling.

Immunisation prevents tPA-induced exacerbation of excitotoxic neuronal death

We previously reported that (1) tPA within the cerebral parenchyma, exacerbates excitotoxic neuronal death (Nicole et al., 2001), an effect demonstrated in vitro to result from a direct interaction with the NR1 subunit of NMDA receptors (Nicole et al., 2001; Fernandez-Monreal et al., 2004) and that (2) intravenously injected tPA (as performed in stroke patients) can penetrate the brain (Benchenane et al., 2005a; Benchenane et al., 2005b). Thus, we postulated that antibodies preventing the tPA-NR1 interaction might improve the current acute management of stroke.

Excitotoxic lesions were induced by injecting NMDA into the striatum. Thirty minutes later, tPA (1 mg/kg) or the vehicle alone was injected intravenously over 15 minutes and the subsequent brain lesion measured 24 hours later. The dose of tPA was chosen based on its relevance to the clinical recommendations from the NINDS for stroke patients and on our previous studies (Benchenane et al., 2005a). In CFA-

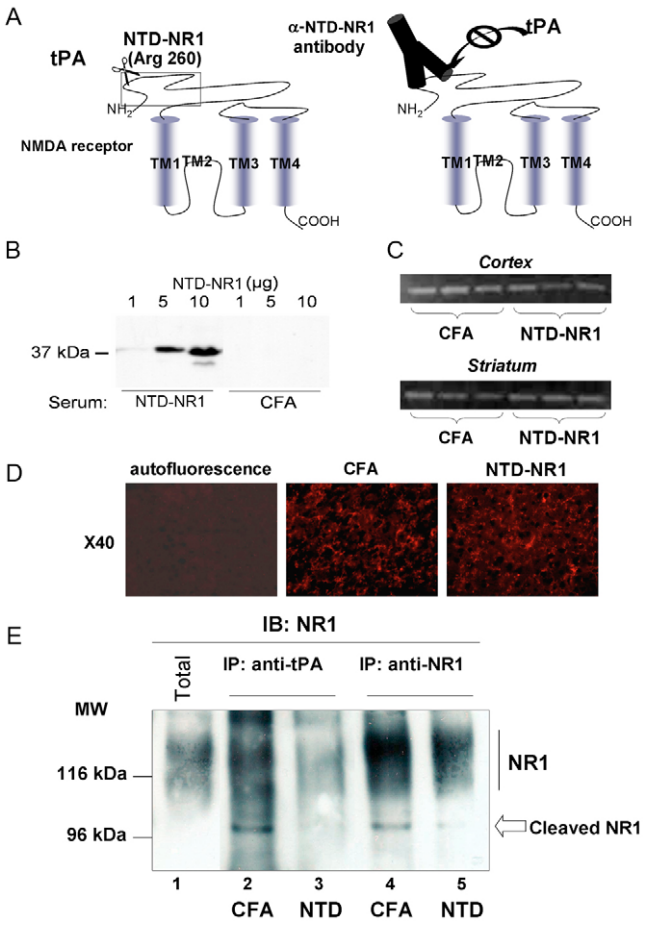

Fig. 1. Immunisation against the NTD of the NMDA receptor NR1 subunit prevents both the interaction of tPA with NR1 and subsequent cleavage of NR1 in vivo. (A) Schematic representation of the hypothesis: antibodies raised against the NTD-NR1 should interact with endogenous NR1 and thus prevent the potentiating effect of tPA on NMDA receptor signalling. (B) 1, 5 or 10 μg of recombinant N-terminal domain of NR1 (NTD-NR1, amino acids 19-371) was separated by SDS-PAGE prior to detection with sera collected from NTD-NR1- or CFA-immunised mice. Immunoblot is representative of three independent experiments performed with sera from three different animals for each group. (C) tPA activity in cortical and striatal extracts measured by zymography assay in CFA-treated mice and vaccinated mice (n=3). (D) Evidence of IgG immunoreactivities in the cerebral parenchyma of NTD-NR1- or CFA-immunised mice (representative photomicrographs taken from the same anatomical level of the cortex) (E) Protein extracts from cerebral cortices of control and immunised mice were subjected to immunoprecipitation against tPA or NR1 before immunoblotting against NR1 as described in the Materials and Methods section. Immunoblot is representative of three independent experiments. IB, immunoblot; IP, immunoprecipitation; CFA, CFA-injected; NTD, NTD-NR1-injected.

injected mice, NMDA alone produced an excitotoxic lesion of 12.9±2.7 mm^3, NMDA plus the administration of tPA, when injected intravenously, doubled the sise of the lesion (29.5±3.1 mm^3; P=0.008). By contrast, intravenous tPA was unable to potentiate NMDA-induced excitotoxic injury in NTD-NR1-vaccinated mice (Fig. 2A; P=0.372). No difference was observed between basal NMDA-induced lesions in CFA- and NTD-NR1-vaccinated mice (Fig. 2A; P=0.819), an indication that the generated antibodies do not affect basal NMDA-mediated neurotransmission.

Immunisation protects against permanent focal cerebral ischemia

The therapeutic value of vaccination was investigated in a model of permanent focal cerebral ischemia induced by MCA occlusion, known to involve a deleterious component driven by endogenous tPA (Nagai et al., 1999). There was no statistical difference between CFA- or NTD-NR1-vaccinated mice, regarding body weight (mean ± s.d.; 35.33±1.97 g vs 33.88±2.80 g, respectively) or rectal temperature (mean ± s.d.; 36.3±0.3°C vs 36.3±0.4°C, respectively). At 24 hours post MCA occlusion, the area of infarcted tissue was smaller in immunised mice (n=8) compared with CFA-injected mice (n=7) (Fig. 2B). There was a 32% protection of the brain against MCA occlusion in NTD-NR1-vaccinated mice (Fig. 2C; infarct volumes, mean ± s.e.m., were 51.53±7.0 mm^3 vs 34.84±2.7 mm^3 in CFA- and NTD-NR1-treated mice, respectively; Student's t-test, P=0.036). Interestingly, the beneficial effect of immunisation was also observed in male mice (see supplementary material Fig. S1).

Immunisation has no effect on tPA-independent behaviour tasks

We used behavioural tasks in which tPA-deficient mice are known to perform normally (Pawlak et al., 2002). As expected, in the activity cage, no difference in locomotor activity was observed between tPA-deficient and WT mice or between NTD-NR1- and CFA-vaccinated mice (Fig. 3A, P=0.67, Fig. 3B, P=0.13), nor in the number of entries into the arms of the plus maze (Fig. 3A, P=0.30, Fig. 3B, P=0.80). Anxiety-related behaviour in the plus maze was also similar in each group, as quantified by the number of entries into the open arms (Fig. 3A, P=0.34, Fig. 3B, P=0.65). These data suggest that immunisation has no effect on tPA-independent processes.

Similarly to tPA deficiency, NTD-NR1 immunisation impairs spatial memory

The total number of entries into the arms of the Y maze was similar between the groups tested (naive, CFA-injected, NTD-NR1-immunised, tPA-deficient mice and corresponding littermates; data not shown). However, spatial memory (as assessed by the time spent in the new arm compared with the two others) was clearly impaired in tPA-deficient female mice when compared with WT littermates (Fig. 4A). The same deficit was observed in NTD-NR1-immunised female mice when compared with control or CFA-injected female mice (Fig. 4B). Moreover, plotting all the parameters used to quantify performances in this task (time spent and number of entries in each arm), reveals that all the parameters affected by tPA deletion are also affected by vaccination against the NTD of NR1 (Fig. 4C; R^2=0.67, P<0.001). These results show that

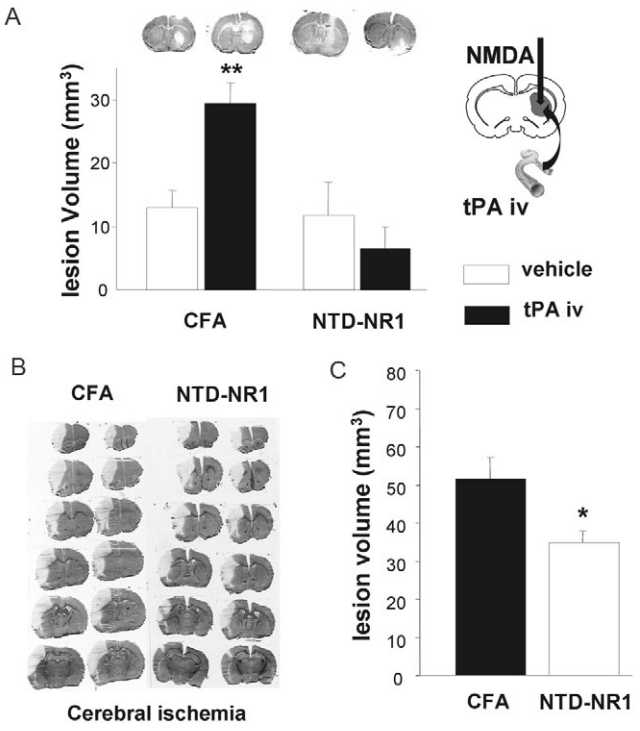

Fig. 2. Immunisation against the NTD of the NMDA receptor NR1 subunit prevents the neurotoxic effect of endogenous and exogenous tPA. (A) Excitotoxic lesions were performed, 4-5 days after the last inoculation, by injecting NMDA (10 nmol) into the striatum. After 30 minutes, tPA (1 mg/kg) or vehicle was injected over 15 minutes. Brains were harvested 24 hours later, and cerebral lesions were measured by analysing thionine-stained cryostat sections (see representative section for each group). Lesion volume was analysed by a two-way ANOVA and Bonferroni/Dunn (n=9-11 mice per group, mean ± s.e.m.; **P<0.01). (B,C) Focal permanent ischemia was induced by MCA occlusion and cerebral lesions were measured after 24 hours of injury. (B) Representative staining and (C) lesion volumes analysed by Student's t-test (n=7-8 mice per group, mean ± s.e.m.; *P<0.05).

the impairment of spatial memory observed in tPA-deficient female mice depends on the ability of tPA to promote NMDA receptor signalling. Interestingly, the deficit observed in the NTD-immunised mice was transient, because it disappeared 5 weeks after the last injection (supplementary material Fig. S2), suggesting that this impairment was not due to irreversible deficits such as neuronal loss. Also, there was no influence of gender on performances in the test in the Swiss (supplementary material Fig. S3) and tPA-deficient (supplementary material Fig. S4) mouse strains.

The impairment of performance on object recognition observed in tPA-deficient mice is not mediated by the tPA-NR1 interaction

No difference in total exploration was found between tPA-deficient mice and WT littermates (3.5±2.3 vs 4.6±3.6; P=0.26) in the object recognition test. However, although WT mice significantly explored the new object more than the familiar one (Fig. 5A; 64±22% vs 36±23%, P<0.05), tPA-deficient mice did not (Fig. 5A; 49±25% vs 51±26%, P=0.9).

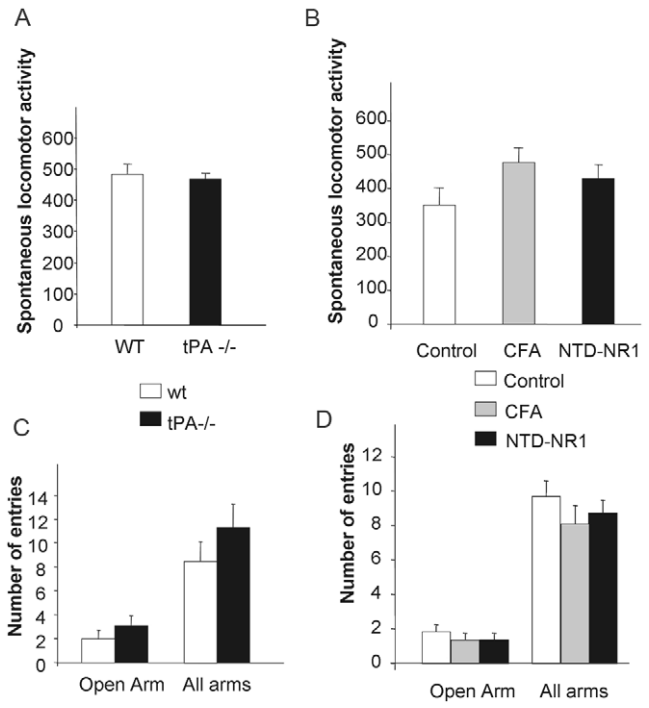

Fig. 3. Immunisation has no effect on tPA-independent behaviour tasks. (A,B) Spontaneous locomotor activity in an activity cage. No difference was seen in either group. (C,D) Mice were subjected to an elevated plus-maze, which allows the measurement of the anxiety level. (C) The activity of tPA-deficient mice in the closed or open arms of the maze did not differ from that of WT mice. (D) Accordingly no behaviour difference was seen in the plus-maze between control, CFA-treated and NTD-NR1-vaccinated mice.

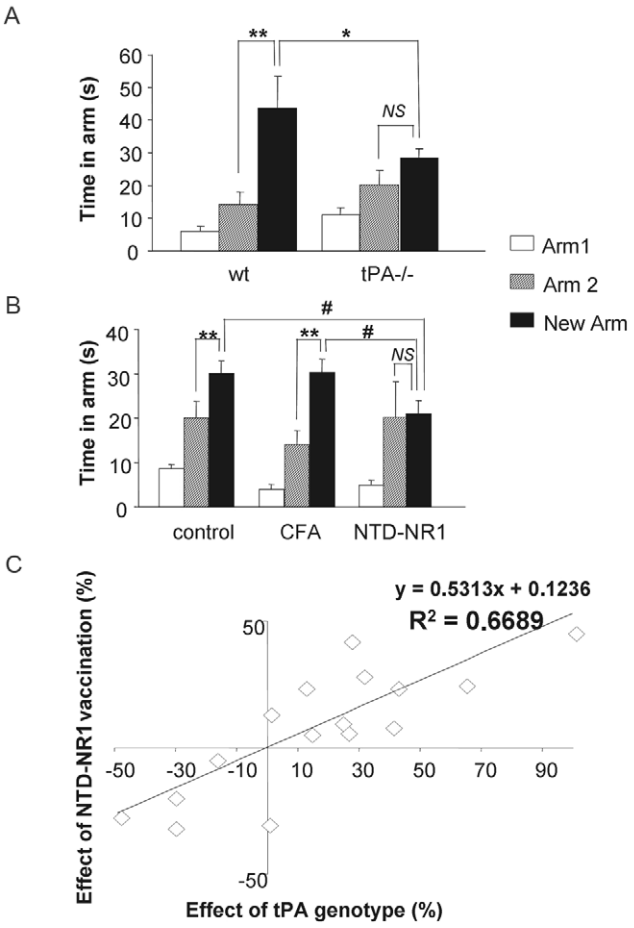

Fig. 4. Immunisation leads to an impaired spatial memory comparable to that observed in tPA-deficient mice. (A) In the Y-maze task, spatial memory was clearly impaired in tPA-deficient mice when compared with WT littermates as assessed by the time spent in the new arm compared with the two others. (B) A similar deficit was observed in NTD-NR1-immunised mice when compared with control or CFA-injected mice. (C) Plot of all the parameters used to quantify the performance in this task (time spent and number of entries in each arm). There is a positive correlation between the effect of tPA deletion and that of vaccination (R^2=0.67, P<0.001).

No differences in the total explorations or recognition of the new object were found in control, CFA-treated mice or vaccinated mice (Fig. 5B; total exploration: 9.6±7.38, 7.75±5.97 and 7.18±5.33, for control, CFA-treated mice and NTD-NR1 vaccinated mice, respectively; P=0.40; new object recognition: controls 61±11% vs 39±11%, P<0.0001; CFA-treated mice 60±17% vs 40±17%, P<0.001; NTD-NR1 vaccinated mice 72±18% vs 28±18%, P<0.0001). This finding suggests that the defect in object recognition observed in tPA-deficient mice involves a tPA-dependent effect that is not related to its ability to interact with the NR1 subunit of NMDA receptors.

Discussion

We have previously shown in vitro that tPA exacerbates NMDA receptor-mediated signalling, by interacting with and then cleaving the NTD of NR1 (Nicole et al., 2001; Fernandez-Monreal et al., 2004). Nevertheless, the relevance of this remains a matter of debate (Matys and Strickland, 2003), although a recent report suggests that tPA interacts with NR1 in mice to participate in learning processes (Kvajo et al., 2004). As we have identified the exact location of the interaction of tPA at Arg260 within the NTD of NR1 (Fernandez-Monreal et al., 2004), we have immunised mice against the NTD of NR1, postulating that the generated antibodies could act as competitive inhibitors of the binding to NR1.

Though the brain has generally been considered to be an immunoprivileged organ, increasing evidence supports the possibility of immune responses to brain markers. Several observations, including ours, demonstrate the presence of immunoglobulins in the cerebral parenchyma, although their origin remains unclear. Indeed, several hypotheses can be proposed: leakage through a compromised blood-brain barrier (BBB) (for instance because of CFA or pathological inflammatory conditions), local synthesis by infiltrated B cells or uptake of serum IgG through Fcγ receptors (Bard et al., 2000; Wilcock et al., 2004; Hazama et al., 2005). In addition, immunisation protocols have been successfully used before. For instance, oral vaccination against the entire NMDA receptor NR1 subunit protects neurons against experimental stroke and epilepsy (During et al., 2000). Moreover, immunisation with a GluR3B peptide in mice (with no experimental injury) leads to a humoral response associated

with multiple brain pathologies and behavioural abnormalities that partially resemble those of Rasmussen's encephalitis (Levite and Hermelin, 1999). In addition, passive immunisation against Aβ reduces plaque burden in a mouse model of Alzheimer's disease, suggesting that antibodies can cross the BBB (Bard et al., 2000). Here, we show that vaccination with a recombinant form of the NTD of NR1 leads to the appearance in the serum of antibodies that recognise recombinant NTD-NR1. Histological analysis showed no macroscopic abnormality in the brain of vaccinated mice (data not shown). Moreover, no alteration of motility was detected in immunised mice, as assessed by spontaneous locomotor activity, total number of entries in the three arms of a Y maze (followed up to 5 weeks) or performances in a rotarod test and in a linear swimming task, excluding severe syndromes (data not shown).

Binding of tPA to NR1 mediates some CA3-dependent behavioural tasks

Calabresi and collaborators found that tPA-deficient mice do not react to spatial changes and have no habituation of object exploration when compared with wild-type littermates (Calabresi et al., 2000). Nevertheless, results obtained in tPA-knockout mice are controversial. In the Morris water maze, tPA-deficient mice exhibit significantly longer swim paths during both acquisition learning and reversal learning (Huang et al., 1996). However, the authors suggest that these effects could be due to an increased thigmotaxis and not impaired spatial memory. This suggestion is based on the fact that tPA-deficient mice cross the old platform location as frequently as the wild-type mice during the reversal learning session (Huang et al., 1996).

We used the Y-maze task and a two-trial memory task that does not require previous learning. tPA-knockout mice do not discriminate between the newly open arm and the other one. Interestingly, the same impairment was observed in the NTD-immunised mice compared to both control and CFA-treated mice. Moreover, the parameters used to assess the performance in the Y maze, which were affected by the tPA gene deletion, were also those affected by immunisation against NTD-NR1. This observation implies that the impairment found in tPA-deficient mice in the Y-maze behavioural task is a consequence of the lack of tPA interaction with NR1. The performance on this behavioural task relies on the involvement of the hippocampus, especially the CA3 region (Stupien et al., 2003; Florian and Roullet, 2004). Moreover, a role for NMDA receptors in the effect of tPA in the Y maze is consistent with the fact that mice knocked out for NR1 in the CA3 region (NR1-CA3$^{-/-}$) display deficits in spatial tasks that require a rapid acquisition of novel information (Nakazawa et al., 2003) as shown in the two-trial Y-maze memory task. These NR1-CA3$^{-/-}$ transgenic mice acquire and retrieve spatial reference memory in the classical Morris water maze but are impaired in retrieving this memory when presented with a fraction of the original clues (Nakazawa et al., 2002). Interestingly, tPA is strongly detected in the CA3 region of the hippocampus (Salles and Strickland, 2002). Thus, the selective action of tPA on NR1 in the CA3 region could explain the lack of impairment of tPA-deficient mice in the classical Morris water maze (Huang et al., 1996).

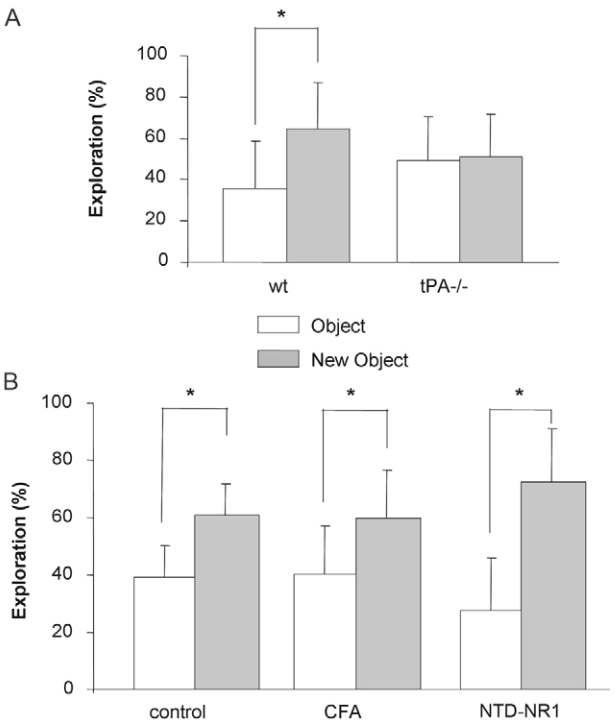

Fig. 5. Impairment of performance on object recognition in tPA-deficient mice is not mediated by the tPA/NR1 interaction. (A) In the novel-object-recognition test, WT mice exhibit good recognition performances as indicated by a strong preference towards the novel objects. By contrast, recognition memory is impaired in tPA-deficient mice, as indicated by the absence of preference towards the novel object. (B) In the same test, vaccination does not affect recognition memory. No differences were found among control, CFA-treated and NTD-NR1-vaccinated mice. *P<0.05.

The tPA/NR1 interaction is not involved in all tPA-driven functions

tPA-knockout mice exhibit a radically different behaviour in an object recognition task when compared with NTD-NR1-immunised mice; however, this observation should be discussed along with other studies that show that this specific task can be independent of NMDA receptor signalling. Indeed, the performance of animals in the object recognition task is unaffected by the reversible inhibition of CA3 neurons induced by a permeable calcium chelator (Stupien et al., 2003). Inducible and reversible knockout mice for NR1 also fail to show alterations in object recognition tasks (Cui et al., 2004). Object recognition was shown to depend on both the globus pallidus (Ennaceur, 1998) and a LTD in the perirhinal cortex by a mechanism that involved the cholinergic system (Warburton et al., 2003). Interestingly, tPA alters both cortico-striatal LTP and LTD and striatal-related behavioural tasks by a dopamine-dependent (D1 receptor) and NMDA-independent activation of cholinergic-releasing striatal interneurons (Calabresi et al., 2000; Centonze et al., 2002). In addition, tPA was also shown to promote the release of dopamine in the nucleus accumbens (Nagai et al., 2004) and to induce the release of acetylcholine in the striatum (Nagai et al., 2005). However, the involvement of other mechanisms cannot be excluded [e.g. the plasmin-dependent activation of pro-BDNF

in BDNF (Pang et al., 2004) that is involved in LTP in the perirhinal cortex and in different behavioural processes].

Vaccination to prevent tPA-mediated toxicity: proof of a concept

tPA is the only approved current emergency treatment for stroke patients. However, despite an overall neurological benefit from its thrombolytic activity, there is evidence to suggest that this therapeutic approach has its limitations (Benchenane et al., 2004). First, only a sub-population of patients is eligible for thrombolysis, because recommendations state that tPA has to be administered within three hours and solely for cases of ischemic stroke. Nonetheless, considerable efforts have been made to increase the awareness of the general population of the emergency status of brain attacks and the development of stroke units has offered a clear advance in terms of patient management. Second, a more serious side effect of tPA-induced thrombolysis is related to the promotion of brain haemorrhage. Finally, because tPA has direct toxic effects within the cerebral parenchyma, thrombolysis could result in an additional neuronal death. Although controversial, this latter hypothesis has received considerable attention since the pioneering work of Wang and colleagues, who showed that the intravenous injection of tPA can exacerbate focal cerebral ischemic lesions in mice (Wang et al., 1998). In support of the foregoing study, we demonstrated that during experimental stroke, vascular tPA can access the brain by two routes: an LRP-dependent transcytotic mechanism in the acute stage of stroke (when the BBB remains intact) and at a later stage, an exacerbated LRP-independent passage (during BBB disruption) (Benchenane et al., 2005a; Benchenane et al., 2005b). Equally important is the fact that the passage of vascular tPA increases NMDA-induced striatal neuronal loss. This finding fully supports the concept that intravenous tPA potentiates neuronal death (in concurrence with endogenous tPA) once it reaches the brain parenchyma (Benchenane et al., 2004; Benchenane et al., 2005a; Benchenane et al., 2005b) and this is independent of its described vasoactive effects, as discussed earlier (Lopez-Atalaya et al., 2007).

The absence of effect of immunisation on the basal NMDA-induced striatal lesion fully supports our previous observation that PAI-1, a natural inhibitor of tPA activity, has no effect on NMDA-induced striatal lesion per se, but prevents the potentiating effect of an intravenous injection of exogenous tPA in the same paradigm (Benchenane et al., 2005a). This suggests that in this model, there is no endogenous tPA-dependent component and that the generated antibodies do not affect NMDA receptor basal activity. By contrast, the model of ischemia used in this study, which affects mostly cortical areas, is known to involve endogenous tPA (Nagai et al., 1999). Accordingly, through these two models, we have been able to determine the efficiency of immunisation for both exogenous (NMDA striatal injection coupled to intravenous administration of tPA) and endogenous (model of cerebral ischemia) tPA. We demonstrated here that the potentiating effect of tPA on NMDA-induced excitotoxic lesions was abolished in NR1-NTD-immunised mice. Thus tPA can exacerbate excitotoxic lesions by cleaving the NMDA receptor in vivo.

Moreover, in an ischemic stroke model mostly affecting the cortex, in which endogenous tPA is shown to be deleterious (Nagai et al., 1999), specific immunisation against the NTD of NR1 decreased the ischemic lesion size by 32%. Vaccination using the whole NR1 protein was shown to protect against stroke (During et al., 2000), but this might alter too many regulatory steps of NMDA receptor activity. This group emphasised the difficulty in choosing the right immunogenic motif, because analyses showed that immunisation with different parts of NR1 can be either efficient, ineffective or indeed, deleterious (Symes et al., 2002). In our hands, immunisation does not seem to affect NMDA receptor function directly as it has no effect on the lesion induced by NMDA alone. All the effects reported in this study thus reflect the effect of tPA on NMDA receptors and not unspecific alterations of NMDA receptors per se.

Absence of gender specificity in the effects of immunisation

A last noteworthy point is the fact that females were used here in most of the experiments, on the rationale that a well-established sexual dimorphism exits within the immune system. Females have higher levels of immunoglobulins, higher incidence of autoimmune diseases and most importantly, a greater antibody response to antigens, than males (Olsen and Kovacs, 1996; Grossman, 1984). Accordingly, one could wonder whether our findings can apply to males. In the context of stroke, a controversial debate is currently emerging regarding the efficiency of thrombolysis with tPA (Savitz et al., 2005; Kent et al., 2005). However, the potential sex-specific effect of tPA in terms of neurotoxic processes has never been studied. Here, we thus show that the deleterious effects of endogenous and exogenous tPA during excitotoxic/ischemic injuries occur in females, as they do in males, as previously reported (Benchenane et al., 2004) and as confirmed here (supplementary material Fig. S1). Regarding physiological processes, some but not all studies have suggested subtle gender differences in some behavioural tasks, including spatial memory in the Y-maze task (Conrad et al., 2003; Conrad et al., 2004). In our hands, the involvement of the tPA-NR1 interaction in spatial memory was not influenced by sexual dimorphism (Fig. 4 and supplementary material Figs S3 and S4).

In conclusion, we provide proof for the concept that antibodies to prevent tPA-NR1 interaction might be valuable as a potent auxiliary therapy for stroke patients. Indeed, in addition to the prevention of NMDA receptor cleavage and the consequent potentiation of excitotoxic necrosis (induced by both endogenous and blood-derived tPA in the cerebral parenchyma), this strategy would not modify the beneficial thrombolytic activity of this serine protease. Accordingly, investigating the effect of such antibodies in a thromboembolic stroke model (closer mimicking the clinical situation) should help validating this hypothesis.

We showed that binding of tPA to the NMDA receptor NR1 subunit and the subsequent increase in NMDA receptor signalling are crucial events in both physiological and pathological CNS processes. These molecular mechanisms should be considered as original targets to design new therapeutics for brain disorders including stroke. This study represents a solid basis for the development of passive immunisation (i.e. co-administration of purified antibodies concomitant with thrombolysis) to comply with the emergency nature of the clinical management of stroke patients.

Materials and Methods

Animal experiments

Studies were carried out according to the Quality Reference system of INRA

(www.international.inra.fr/content/download/947/11111/file/requirements.pdf), in accordance with the framework of the European Directives and the French Law on Animal Experimentation and were approved by the Region Aquitaine Poitou-Charente ethical committee. Every effort was made to minimise suffering and the number of animals used.

Behavioural analyses

Sexually naive female mice were studied (n=11-20 per group) with an identical sequence (over 11 days) for each group (i.e. elevated plus-maze test, spontaneous locomotor activity measurement, place recognition test and object recognition test). The mice were housed in groups of 5-10 and first acclimated to the animal facilities for a minimum of 2 weeks. They had free access to water and food (SDS Dietex, St-Gratien, France). Studies were conducted during the animal's light phase (12 hours light/dark cycle; lights on between 8:00 and 20:00). For each test, the experimenter and observer were blinded to the genotype or immunisation procedures.

Elevated plus-maze test

Anxiety behaviour was assessed with a plus-maze elevated 50 cm above the floor, composed of a white central area (8×8 cm) connecting four perpendicular arms (22×8 cm; two white-open and two black-closed arms). The apparatus was illuminated by a single 40 W bulb placed 70 cm above its centre. Thirty minutes after habituation to the test room, each mouse was placed in the central square area facing an open arm. The number of entries into each arm (when all four paws entered into the arms) and time spent in each arm were recorded for 5 minutes (Pellow et al., 1985; Lister, 1987). Between each test, the apparatus was cleaned with 70% ethanol to avoid instinctive odorant clues.

Spontaneous locomotor activity

Spontaneous locomotor activity was recorded for 10 minutes through an activity cage (LETICA LE 886) with automatic counting of movements across the bars on the cage floor.

Place recognition test

Spatial recognition memory was tested in a grey plastic Y-maze with three identical arms (34×8×13 cm). The maze was illuminated by a single 100 W bulb placed 100 cm above its centre and its floor was covered with wood shavings, which were redistributed after each trial in order to minimise the influence of olfactory clues. After 30 minutes of habituation to the test room, mice were tested following a two-trial procedure with a 1 hour inter-trial interval (Dellu et al., 2000). During the acquisition session, one arm was randomly closed. Each mouse was placed in one of the two other arms (with its head facing away from the centre of the maze) and allowed to visit the two accessible arms for 5 min. Mice were then returned to their home cage for 60 minutes, before being subjected to the retention test, in which they had free access to all three arms for 2 minutes. The number of visits (considered only when the mouse passed two-thirds of the arm) and the time spent in each arm were recorded for each trial. Effects of genotype or immunisation procedures on spatial recognition memory were assessed through the comparison of the time spent in each arm and the use of a spatial recognition index (delta response to the novel arm) based on the absolute function of the average time spent in arms 1 and 2 minus the time spent in the novel arm (Dellu et al., 2000).

Object recognition test

Before the actual test, each mouse was handled regularly and habituated to a black open-field box (30×30×30 cm) for 3 days (two daily sessions of 5 minutes separated by 1 hour) under a low illumination (one 40 W bulb placed 70 cm above the centre). Training and retention phases (with a 1 hour inter-session interval) were assessed on the fourth day (Ennaceur, 1998). In the training session, two novel identical objects (various objects differing in their shape and colour but similar in global size) were placed symmetrically in the box and each animal was allowed to explore freely for 3 minutes. After this, mice were returned immediately to their home cage and the open-field box and objects were cleaned with 70% ethanol to avoid instinctive odorant clues. In the retention session, mice were allowed to explore the open-field for 3 minutes in the presence of two objects: one familiar object and a novel object. All sessions were recorded using an overhead video camera. The time spent exploring each object was recorded separately and expressed as a percentage of the overall time of object exploration. The animals were considered to be exploring when their head was facing the object within 1 cm of the object or any rostral part of the body was touching the object. The effects of genotype or immunisation procedures on recognition memory were assessed through the comparison of exploratory preferences for the familiar, or novel, object at the retention session. Throughout the experiments, the sets of familiar/new objects and the right/left position of the new object were exchanged randomly within each group to avoid object- and side-preferences.

Production of recombinant NTD

The region of the NR1-1a subunit encoding amino acids 19-371 corresponding to the NTD was amplified from the full-length rat NR1-1a cDNA, as described previously (Fernandez-Monreal et al., 2004). Recombinant proteins were purified from inclusion bodies of isopropyl 1-thio®-D-galactopyranoside-induced bacterial cultures (*Escherichia coli*, M15 strain) on a nickel affinity matrix as described by the manufacturer (Qiagen, Courtaboeuf, France).

Active immunisation

Active immunisation was performed either on Swiss mice (CERJ, Le Genest Saint Isle, France) or in tPA-deficient mice and their corresponding WT littermates (C57Black6) weighing 25-30 g. Complete Freund's Adjuvant (CFA) or 30 μg NTD-NR1 in CFA, were intraperitoneally injected once a week for four weeks. Weight and temperature were controlled each week the day after injection.

SDS-PAGE, immunoprecipitation and immunoblotting

Increasing amounts of NTD-NR1 were subjected to electrophoresis in a 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane. Membranes were blocked with 5% dried milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 and incubated with sera from immunised mice (1:500) in the same buffer. After incubation with a biotinylated antibody (anti-mouse IgG, Vector Laboratories, UK) and horseradish-peroxidase-conjugated streptavidin reagent (ExtrAvidin®, Sigma), proteins were visualised with an enhanced chemiluminescence ECL Plus detection system (Perkin Elmer-NEN, Paris, France). The same protocol was performed on immunoprecipitated samples, except that SDS-PAGE was performed with a 6% SDS-polyacrylamide gel. Protein levels were evaluated before immunoprecipitation and the same amount of total protein was then used for each sample. The primary antibody was directed against NR1 (1:200, NMDAξ1; Santa Cruz, Heidelberg, Germany) and the secondary antibody was a horseradish-peroxidase-conjugated anti-goat IgG (1:10,000, Vector Laboratories, UK).

SDS-PAGE zymography assay

Each extract (cortex or striatum; 2.5 μg) was subjected to electrophoresis in a 15% SDS-polyacrylamide gel containing 1 mg/ml casein and 4.5 μg/ml plasminogen. After removal of SDS by washing with buffer containing 2.5% Triton X-100, the gels were incubated in a Glycine-EDTA buffer (pH 8.3) at 37°C for 1.5 hours. Caseinolytic bands indicating proteolytic activities were visualised as clear bands after Coomassie Blue staining.

IgG immunostaining

Chloral hydrate anesthetised mice were perfused transcardially with 0.9% sodium chloride (30 ml) followed by 100 ml of fresh 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were dissected, post-fixed in the same fixative for 24 hours and cryoprotected in 0.1 M veronal buffer, pH 7.4, containing 20% sucrose. Brains were then embedded in Tissue-Tek (Miles, Elkart, IN), frozen and stored at –80°C until used. 12-μm-thick coronal sections were collected on gelatin-coated slides and stored at –80°C. The detection of mouse IgGs was carried out using F(ab′)2 fragments of donkey anti-mouse IgG linked to tetramethyl rhodamine isothiocyanate (TRITC) (Jackson ImmunoResearch, West Grove, PA). Sections were incubated overnight with one of these affinity-purified antibodies diluted 1:400 in Verona1 buffer containing 0.25% Triton X-100. Images were digitally captured using a camera (Photometrics, coolSnap ES) mounted on a DM 6000 (Leica) microscope and Metavue™ software.

Excitotoxic lesions

Excitotoxic lesions were performed under sevoflurane-induced anesthesia in Swiss females (30-35 g; CERJ, Le Genest Saint Isle, France). Four to five days after the last inoculation, 0.3 μl of NMDA (total of 10 nmol) was injected into the right striatum (coordinates: 0.0 mm posterior, 2.0 mm lateral, 4.0 mm ventral to the bregma). 5 minutes after the needle insertion, the solution was injected through the use of a Hamilton syringe pump (0.5 μl/minute). The needle was removed 5 minutes later. After 30 minutes, tPA (1 mg/kg; Boehringer Ingelheim, Paris, France) or the vehicle (containing arginine and Tween 80 as found in Actilyse) was injected intravenously over 15 minutes.

Focal cerebral ischemia

Focal cerebral ischemia was induced 4-5 days after the last inoculation, in female Swiss mice (30-35 g; CERJ, Le Genest Saint Isle, France), by permanent occlusion of the left middle cerebral artery (MCA) under sevoflurane anesthesia. Briefly, a skin incision was made between the orbit and ear and the temporal muscle incised. The left lateral aspect of the skull was exposed by reflecting the temporal muscle and surrounding soft tissue. The distal course of the MCA was then visible and a small craniotomy was performed with a cooled dental drill. The MCA was then coagulated by bipolar diathermy, the muscle and soft tissue were replaced and the incision was sutured. Temperature was monitored and controlled over the whole surgical procedure.

Histological analysis of infarct volume

After 24 hours, mice were killed by an anaesthetic overdose and the brains were removed and frozen in isopentane. Coronal brain sections (20 μm) were stained with thionine and analysed with an image analyser (BIOCOM RAG 200, Paris, France). For volume analysis, one section in ten was taken (covering the whole lesion). Regions of interest were delineated with reference to a stereotaxic atlas for the mouse and correspond to the non-stained areas. Results are expressed as mean ± s.e.m. Statistical tests were, for excitotoxicity experiments, two-way ANOVA with Bonferroni

correction (n=5-7 in each group, $^{**}P<0.01$) and Student's t-test for ischemia experiments (n=7-10 animals per group, $^{*}P<0.05$).

We thank D. Divoux for his technical contribution and A. R. Young for helpful comments. This work was supported by the INSERM, the CNRS, the University of Caen, the European Council, FP6-projectDiMI-LSHB-CT-2005-512146, the Fondation Paul Hamel, the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche, the Regional Council of Lower Normandy and the Fondation pour la Recherche Médicale.

References

Baranes, D., Lederfein, D., Huang, Y. Y., Chen, M., Bailey, C. H. and Kandel, E. R. (1998). Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. *Neuron* **21**, 813-825.

Bard, F., Cannon, C., Barbour, R., Burke, R. L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K. et al. (2000). Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.* **6**, 916-919.

Benchenane, K., Lopez-Atalaya, J. P., Fernandez-Monreal, M., Touzani, O. and Vivien, D. (2004). Equivocal roles of tissue-type plasminogen activator in stroke-induced injury. *Trends Neurosci.* **27**, 155-160.

Benchenane, K., Berezowski, V., Ali, C., Fernandez-Monreal, M., Lopez-Atalaya, J. P., Brillault, J., Chuquet, J., Nouvelot, A., MacKenzie, E. T., Bu, G. et al. (2005a). Tissue-type plasminogen activator crosses the intact blood-brain barrier by low-density lipoprotein receptor-related protein-mediated transcytosis. *Circulation* **111**, 2241-2249.

Benchenane, K., Berezowski, V., Fernandez-Monreal, M., Brillault, J., Valable, S., Dehouck, M. P., Cecchelli, R., Vivien, D., Touzani, O. and Ali, C. (2005b). Oxygen glucose deprivation switches the transport of tPA across the blood-brain barrier from an LRP-dependent to an increased LRP-independent process. *Stroke* **36**, 1065-1070.

Calabresi, P., Napolitano, M., Centonze, D., Marfia, G. A., Gubellini, P., Teule, M. A., Berretta, N., Bernardi, G., Frati, L., Tolu, M. et al. (2000). Tissue plasminogen activator controls multiple forms of synaptic plasticity and memory. *Eur. J. Neurosci*. **12**, 1002-1012.

Centonze, D., Napolitano, M., Saulle, E., Gubellini, P., Picconi, B., Martorana, A., Pisan, A., Gulino, A., Bernardi, G. and Calabresi, P. (2002). Tissue plasminogen activator is required for corticostriatal long-term potentiation. *Eur. J. Neurosci*. **16**, 713-721.

Conrad, C. D., Grote, K. A., Hobbs, R. J. and Ferayorni, A. (2003). Sex differences in spatial and non-spatial Y-maze performance after chronic stress. *Neurobiol. Learn. Mem*. **79**, 32-40.

Conrad, C. D., Jackson, J. L., Wieczorek, L., Baran, S. E., Harman, J. S., Wright, R. L. and Korol, D. L. (2004). Acute stress impairs spatial memory in male but not female rats: influence of estrous cycle. *Pharmacol. Biochem. Behav*. **78**, 569-579.

Cui, Z., Wang, H., Tan, Y., Zaia, K. A., Zhang, S. and Tsien, J. Z. (2004). Inducible and reversible NR1 knockout reveals crucial role of the NMDA receptor in preserving remote memories in the brain. *Neuron* **41**, 781-793.

Dellu, F., Contarino, A., Simon, H., Koob, G. F. and Gold, L. H. (2000). Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice. *Neurobiol. Learn. Mem*. **73**, 31-48.

During, M. J., Symes, C. W., Lawlor, P. A., Lin, J., Dunning, J., Fitzsimons, H. L., Poulsen, D., Leone, P., Xu, R., Dicker, B. L. et al. (2000). An oral vaccine against NMDAR1 with efficacy in experimental stroke and epilepsy. *Science* **287**, 1453-1460.

Ennaceur, A. (1998). Effects of lesions of the Substantia Innominata/Ventral Pallidum, globus pallidus and medial septum on rat's performance in object-recognition and radial-maze tasks: physostigmine and amphetamine treatments. *Pharmacol. Res*. **38**, 251-263.

Fernandez-Monreal, M., Lopez-Atalaya, J. P., Benchenane, K., Cacquevel, M., Dulin, F., Le Caer, J. P., Rossier, J., Jarrige, A. C., Mackenzie, E. T., Colloc'h, N. et al. (2004). Arginine 260 of the amino-terminal domain of NR1 subunit is critical for tissue-type plasminogen activator-mediated enhancement of NMDA receptor signalling. *J. Biol. Chem*. **279**, 50850-50856.

Florian, C. and Roullet, P. (2004). Hippocampal CA3-region is crucial for acquisition and memory consolidation in Morris water maze task in mice. *Behav. Brain Res*. **154**, 365-374.

Grossman, C. J. (1984). Regulation of the immune system by sex steroids. *Endocr. Rev*. **5**, 435-455.

Hazama, G. I., Yasuhara, O., Morita, H., Aimi, Y., Tooyama, I. and Kimura, H. (2005). Mouse brain IgG-like immunoreactivity: strain-specific occurrence in microglia and biochemical identification of IgG. *J. Comp. Neurol*. **492**, 234-249.

Huang, Y. Y., Bach, M. E., Lipp, H. P., Zhuo, M., Wolfer, D. P., Hawkins, R. D., Schoonjans, L., Kandel, E. R., Godfraind, J. M., Mulligan, R. et al. (1996). Mice lacking the gene encoding tissue-type plasminogen activator show a selective interference with late-phase long-term potentiation in both Schaffer collateral and mossy fiber pathways. *Proc. Natl. Acad. Sci. USA* **93**, 8699-8704.

Kent, D. M., Price, L. L., Ringleb, P., Hill, M. D. and Selker, H. P. (2005). Sex-based differences in response to recombinant tissue plasminogen activator in acute ischemic stroke: a pooled analysis of randomized clinical trials. *Stroke* **36**, 62-65.

Kvajo, M., Albrecht, H., Meins, M., Hengst, U., Troncoso, E., Lefort, S., Kiss, J. Z., Petersen, C. C. and Monard, D. (2004). Regulation of brain proteolytic activity is necessary for the in vivo function of NMDA receptors. *J. Neurosci*. **24**, 9734-9743.

Levite, M. and Hermelin, A. (1999). Autoimmunity to the glutamate receptor in mice–a model for Rasmussen's encephalitis? *J. Autoimmun*. **13**, 73-82.

Lister, R. G. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology Berl.* **92**, 180-185.

Lopez-Atalaya, J. P., Roussel, B. D., Ali, C., Maubert, E., Petersen, K.-U., Berezowski, V., Cecchelli, R., Orset, C. and Vivien, D. (2007). Recombinant *Desmodus rotundus* salivary plasminogen activator (desmoteplase) crosses the blood-brain barrier through a LDL receptor-related protein (LRP)-dependent mechanism without exerting neurotoxic effects. *Stroke* In Press.

Madani, R., Hulo, S., Toni, N., Madani, H., Steimer, T., Muller, D. and Vassalli, J. D. (1999). Enhanced hippocampal long-term potentiation and learning by increased neuronal expression of tissue-type plasminogen activator in transgenic mice. *EMBO J*. **18**, 3007-3012.

Matys, T. and Strickland, S. (2003). Tissue plasminogen activator and NMDA receptor cleavage. *Nat. Med*. **9**, 371-372.

Nagai, N., De Mol, M., Lijnen, H. R., Carmeliet, P. and Collen, D. (1999). Role of plasminogen system components in focal cerebral ischemic infarction: a gene targeting and gene transfer study in mice. *Circulation* **99**, 2440-2444.

Nagai, T., Yamada, K., Yoshimura, M., Ishikawa, K., Miyamoto, Y., Hashimoto, K., Noda, Y., Nitta, A. and Nabeshima, T. (2004). The tissue plasminogen activator-plasmin system participates in the rewarding effect of morphine by regulating dopamine release. *Proc. Natl. Acad. Sci. USA* **101**, 3650-3655.

Nagai, T., Kamei, H., Ito, M., Hashimoto, K., Takuma, K., Nabeshima, T. and Yamada, K. (2005). Modification by the tissue plasminogen activator-plasmin system of morphine-induced dopamine release and hyperlocomotion, but not anti-nociceptive effect in mice. *J. Neurochem*. **93**, 1272-1279.

Nakazawa, K., Quirk, M. C., Chitwood, R. A., Watanabe, M., Yeckel, M. F., Sun, L. D., Kato, A., Carr, C. A., Johnston, D., Wilson, M. A. et al. (2002). Requirement for hippocampal CA3 NMDA receptors in associative memory recall. *Science* **297**, 211-218.

Nakazawa, K., Sun, L. D., Quirk, M. C., Rondi-Reig, L., Wilson, M. A. and Tonegawa, S. (2003). Hippocampal CA3 NMDA receptors are crucial for memory acquisition of one-time experience. *Neuron* **38**, 305-315.

Nicole, O., Docagne, F., Ali, C., Margaill, I., Carmeliet, P., MacKenzie, E. T., Vivien, D. and Buisson, A. (2001). The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. *Nat. Med.* **7**, 59-64.

Olsen, N. J. and Kovacs, W. J. (1996). Gonadal steroids and immunity. *Endocr. Rev*. **17**, 369-384.

Pang, P. T., Teng, H. K., Zaitsev, E., Woo, N. T., Sakata, K., Zhen, S., Teng, K. K., Yung, W. H., Hempstead, B. L. and Lu, B. (2004). Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* **306**, 487-491.

Pawlak, R., Nagai, N., Urano, T., Napiorkowska-Pawlak, D., Ihara, H., Takada, Y., Collen, D. and Takada, A. (2002). Rapid, specific and active site-catalyzed effect of tissue-plasminogen activator on hippocampus-dependent learning in mice. *Neuroscience* **113**, 995-1001.

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

Pellow, S., Chopin, P., File, S. E. and Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* **14**, 149-167.

Salles, F. J. and Strickland, S. (2002). Localization and regulation of the tissue plasminogen activator-plasmin system in the hippocampus. *J. Neurosci*. **22**, 2125-2134.

Savitz, S. I., Schlaug, G., Caplan, L. and Selim, M. (2005). Arterial occlusive lesions recanalize more frequently in women than in men after intravenous tissue plasminogen activator administration for acute stroke. *Stroke* **36**, 1447-1451.

Seeds, N. W., Basham, M. E. and Ferguson, J. E. (2003). Absence of tissue plasminogen activator gene or activity impairs mouse cerebellar motor learning. *J. Neurosci*. **23**, 7368-7375.

Stupien, G., Florian, C. and Roullet, P. (2003). Involvement of the hippocampal CA3-region in acquisition and in memory consolidation of spatial but not in object information in mice. *Neurobiol. Learn. Mem*. **80**, 32-41.

Symes, C. W., Jiao, X. Y., Young, D., Klugmann, M., Oshinsky, M. L. and During, M. J. (2002). Critical epitopes of a NR1 vaccine for stroke and epilepsy. Washington, DC: Society for Neuroscience Abstracts, Program No: 600.4.

Tsirka, S. E., Gualandris, A., Amaral, D. G. and Strickland, S. (1995). Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. *Nature* **377**, 340-344.

Wang, Y. F., Tsirka, S. E., Strickland, S., Stieg, P. E., Soriano, S. G. and Lipton, S. A. (1998). Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. *Nat. Med.* **4**, 228-231.

Warburton, E. C., Koder, T., Cho, K., Massey, P. V., Duguid, G., Barker, G. R., Aggleton, J. P., Bashir, Z. I. and Brown, M. W. (2003). Cholinergic neurotransmission is essential for perirhinal cortical plasticity and recognition memory. *Neuron* **38**, 987-996.

Wilcock, D. M., Rojiani, A., Rosenthal, A., Levkowitz, G., Subbarao, S., Alamed, J., Wilson, D., Wilson, N., Freeman, M. J., Gordon, M. N. et al. (2004). Passive amyloid immunotherapy clears amyloid and transiently activates microglia in a transgenic mouse model of amyloid deposition. *J. Neurosci*. **24**, 6144-6151.

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