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The human immunodeficiency virus-1 protein Tat upregulates NMDA receptor function by acting at mGluR1 receptors coexisting on human and rat brain noradrenergic neurones

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Running title: Tat mediates mGluR1-NMDA receptor interactions

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ABBREVIATIONS: ACh, acetylcholine; AIDS, acquired immunodeficiency syndrome; Ant-AIP-II, Autocamtide-2 related Inhibitory peptide II; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; 7-Cl-KYNA, 7-Chloro-4-hydroxyquinoline-2-carboxylic acid; CNS, central nervous system; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; GBR12909, 1-(2-[bis(4-fluorophenyl)-[methoxy]ethyl)-4-(3-phenylpropyl) piperazine; GF109203X, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide; gp120, glycoprotein 120; H89, N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride; HAD, human immunodeficiency virus-1 associated dementia; HIV-1, human immunodeficiency virus-1; LY 367385, (S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid; mGluR1, metabotropic glutamate receptor 1; MK-801, dizocilpine; MPEP, 2-Methyl-6-(phenylethynyl)pyridine hydrochloride; NE, norepinephrine; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptors; PKA, protein kinase A; PKC, protein kinase C; PLC, phospolipase C; PP2, 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; PTx, Pertussis toxin; Src, cytosolic tyrosine kinase; Tat, transactivator of transcription; U 73122, 1-(6-[([17β]-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione. Recommended section: Neuropharmacology

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ABSTRACT

We investigated the effects of the human immunodeficiency virus-1 Tat on the release of norepinephrine (NE) from human and rat brain synaptosomes. Tat could not evoke directly release of [^{3}H]norepinephrine ([^{3}H]NE). In presence of Tat (1 nM), NMDA concentrations unable to release (human synaptosomes) or slightly releasing (rat synaptosomes) [^{3}H]NE became very effective. The NMDA/Tat-evoked release depends on NMDA receptors (NMDARs), since it was abolished by MK-801. Tat binding at NMDARs was excluded. The NMDA-induced release of [^{3}H]NE in presence of glycine was further potentiated by Tat. The release evoked by NMDA/glycine/Tat depends on metabotropic glutamate receptor 1 (mGluR1) activation, since it was halved by mGluR1 antagonists. Tat seems to act at the glutamate recognition site of mGluR1. Recently, Tat was shown to release [^{3}H]acetylcholine from human cholinergic terminals: here we demonstrate that also this effect is mediated by presynaptic mGluR1. The peptide sequence Tat_{41-60}, but not Tat_{61-80}, mimicked Tat. Phospolipase C, protein kinase C and Src are involved in the NMDA/glycine/Tat-evoked [^{3}H]NE release. To conclude: Tat can represent a potent pathological agonist at mGlu1 receptors able to i) release acetylcholine from human cholinergic terminals; ii) upregulate NMDARs mediating NE release from human and rat noradrenergic terminals.

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Introduction

Acquired immunodeficiency syndrome (AIDS) is often accompanied by neuropsychiatric symptoms known as the human immunodeficiency virus-1 (HIV-1)-associated dementia (HAD; Navia et al. 1986; Diesing et al. 2002; Lawrence and Major 2002) the origins of which are poorly understood. Neuroinflammation and neuronal death (collectively termed HIV-1 encephalopathy) are common in AIDS patients and have been implicated in the pathogenesis of HAD (Masliah et al. 1992; Everall et al. 1999). Reducing the viral load by antiretroviral therapy does not impede HAD development and neuronal damage (Lawrence and Major 2002). Since HIV-1 encephalopathy is a gradual process, dramatic neurodegenerative events may not be necessary to explain neuropsychiatric deficits which, in some patients, represent early manifestations of AIDS (Lawrence and Major 2002). In fact, subtle changes in neurotransmission induced by viral components, including proteins gp120 and Tat, could underlie various functional impairments.

Evidence has been provided that HIV-1 proteins can affect neuronal functions through glutamate receptor activation (Savio and Levi 1993; Pittaluga and Raiteri 1994; Magnuson et al. 1995; Pittaluga et al. 1996, 2001; Kaul and Lipton 1999; Feligioni et al. 2003; Self et al. 2003; Behnisch et al. 2004; Gelman et al. 2004), although the exact targets for gp120 and Tat remain undetermined.

If subtle changes in neurotransmission, independent of or preceding neurotoxicity, underlie the development of HAD, it is worthwhile to investigate if and how HIV-1 proteins alter neurotransmitter systems potentially involved in functions that can be impaired during HIV-1 infection. We already found that gp120 acts as a potent glycine-site agonist at glutamate NMDA receptors (NMDARs) mediating norepinephrine (NE) release from human and rat central nervous system (CNS) nerve endings (Pittaluga and Raiteri 1994; Pittaluga et al. 1996, 2001; Pattarini et al. 1998). More recently, we reported that Tat releases acetylcholine (ACh) from human neocortical

cholinergic terminals by targeting group I metabotropic glutamate receptors (mGluRs; Feligioni et al. 2003).

Tat has been reported to positively modulate NMDARs in neuronal and organotypic slices (Haughey et al. 2001; Prendergast et al. 2002; Self et al. 2003; Song et al. 2003), but the site of action, whether on NMDARs or on receptive sites that can cross-talk with NMDARs, or on both, is not well understood. To shed light on this question, we here investigated if and how Tat can affect the function of NMDARs known to exist on noradrenergic terminals of human and rat brain.

Our results show that Tat is a potent agonist at mGluR1 localized on human and rat noradrenergic terminals. Tat is ineffective on its own, but acts in cooperation with coexisting NMDARs. The mGluR1-NMDAR interaction, triggered by concomitant exposure to Tat and NMDA, leads to enhancement of NE release. In addition, we report that the release-enhancing group I mGluRs, previously found to exist on human neocortical cholinergic terminals and to be targeted by Tat (Feligioni et al. 2003), also belong to the mGluR1 subtype.

Materials and methods

Human brain tissue samples. Samples of human cerebral cortex were obtained from informed and consenting HIV-1 negative patients undergoing neurosurgery, each on a different day, to reach deeply seated tumours. The samples represented parts of frontal (n = 20) and temporal (n = 24) lobes obtained from 16 women and 28 men (age 30-70 years). Immediately after removal, the tissue was placed in a physiological salt solution at 2-4°C and crude synaptosomes were prepared within 90 min. The experimental procedures were approved by the Ethical Committee of the University of Genoa.

Rat brain tissue samples. Adult male rats (Sprague Dawley; 200-250 g) were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light/dark schedule (light, 7.00 A.M. to 7.00 P.M.). Food and water were freely available. The animals were killed by decapitation and hippocampi or cortices were rapidly dissected at 0-4°C. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC).

Preparation of synaptosomes. Crude synaptosomes were prepared according to Raiteri and Raiteri (2000). Briefly, the tissue was homogenized in 40 volumes of 0.32 M sucrose, buffered at pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was first centrifuged at 1000 x g for 5 min, to remove nuclei and cellular debris, and synaptosomes were then isolated by centrifugation at 13,000 x g for 20 min. The synaptosomal pellet was resuspended in a physiological medium having the following composition (mM): NaCl, 125; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 22; glucose, 10 (aeration with 95% O_2 and 5% CO_2); pH 7.2-7.4. When indicated, brain tissue was homogenized in buffered sucrose containing 5 nM Pertussis Toxin

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or 20 µg of antibody anti-phosphotyrosine in order to entrap these agents into subsequently isolated synaptosomes (Raiteri et al. 2000).

Release experiments. Human or rat synaptosomes were labelled with [^{3}H]norepinephrine ([^{3}H]NE; final concentration: 30-50 nM) in the presence of the transporter blockers 6-nitroquipazine (0.1 µM) and GBR 12909 (0.1 µM) to avoid false labelling with [^{3}H]NE of serotonergic and dopaminergic nerve terminals, respectively. In a set of experiments, human cortical synaptosomes were preloaded with [^{3}H]choline (final concentration 60 nM). Incubation was performed at 37°C, for 15 min, in a rotary water bath and in an atmosphere of 95% O_2 and 5% CO_2. After the labelling period, identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel superfusion chambers thermostated at 37°C (Raiteri and Raiteri 2000). Synaptosomes were superfused at 0.5 ml/min with standard physiological solution aerated with 95% O_2 and 5% CO_2, at 37°C. When indicated, the medium was replaced, at t = 20 min, with a medium from which Mg^{2+} ions were omitted, to permit NMDAR activation. The system was first equilibrated during 38 min of superfusion; subsequently, eight consecutive 1-min fractions were collected. Synaptosomes were exposed to agonists at the end of the first fraction collected (t = 39 min) till the end of the superfusion, while antagonists were added eight min before agonists. Fractions collected and superfused synaptosomes were counted for radioactivity.

Calculations and statistics. The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). Drug effects were expressed as percent increase over basal release and were evaluated as the ratio between the percentage of tritium released into the fraction where the maximal releasing effect was observed and that in the first fraction collected; this ratio was compared with the corresponding ratio obtained under control conditions (no drug added). Analysis of variance was performed by ANOVA followed by Dunnett's test multiple-comparison test or Student's *t*-test as appropriate. Data were considered significant for $p < 0.05$ at least. Appropriate controls with antagonists and inhibitors were always run in parallel.

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Materials. 1-[7, 8-^{3}H]NE (specific activity 39 Ci/mmol) and [methyl-^{3}H]Choline (specific activity, 83 Ci/mmol) were from Amersham Radiochemical Centre (Buckinghamshire, UK). N-methyl-D-aspartate (NMDA), 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2), 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), (S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid (LY 367385), 7-Chloro-4-hydroxyquinoline-2-carboxylic acid (7-Cl-KYNA), 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide (GF 109203X) were obtained from Tocris Cookson (Bristol, UK). Glycine, arcaine, 1-(6-[([17β]-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione (U 73122), N-(2-[p-Bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H89) were purchased from Sigma Chemical Co. (St. Louis, MO). Ac-RQIKIWFQNRRMKWKKKKKLRRQEAFDAL-OH (Ant-AIP-II) and Pertussis Toxin (PTx) were from Calbiochem (La Jolla, CA). Mouse anti-phosphotyrosine (clone 4G10) was from Upstate Biotechnology (Lake Placid, NY). The recombinant HIV-1 protein gp120 (strain$_{IIIB}$) from AMS Raggio Italgene (Milan, Italy) The following compounds were gifts: 6-nitroquipazine maleate (Duphar, Amsterdam, The Netherlands), GBR 12909 (Gist-Brocades, Delft, The Netherlands), MK801 (Merck-Sharp and Dohme, Whitehouse Station, NJ). Recombinant Tat (HIV-1 Clade B HAN2), Tat peptide 41-60 (HIV-1 strain LAI) and Tat peptide 61-80 (HIV-1 strain LAI) were kindly donated by European program EVA (779.5-779.1-7) (NIBSC, Hertfordshire, UK). The amount of contaminant glycine or glutamate in the protein solution (at the final concentration of 1nM) amounted to 65 ± 7.8 glycine and 53 ± 4.3 nM for glutamate.

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Results

Tat upregulates presynaptic NMDARs mediating NE release without binding at the NMDAR complex. Human cortical noradrenergic nerve endings are endowed with release-enhancing NMDARs (Fink et al. 1992; Pittaluga et al. 1996). In the absence of extracellular Mg^{2+} and of exogenously added glycine, neither NMDA (1 mM) nor Tat (1 nM) elicited significant release of [^{3}H]NE from superfused human neocortical synaptosomes prelabelled with the [^{3}H]catecholamine (Fig. 1**A**). Concomitant addition of glycine (3 or 10 μM) permitted NMDA to evoke significant release of [^{3}H]NE [Fig 1A, $F(7, 30) = 22.1$; $p < 0.05$ and $p < 0.001$ respectively]. On the contrary, Tat remained ineffective in the presence of 10 μM glycine (Fig. 1**A**).

Release-enhancing NMDARs also exist on rat hippocampal noradrenergic nerve endings (Fink et al. 1990; Pittaluga and Raiteri 1990). Since NMDARs mediating NE release in the hippocampus has been the object of a number of our investigations (Pittaluga and Raiteri 1990, 1992, 1994; Pittaluga et al. 2001, 2005), the present work was carried out with rat hippocampal synaptosomes with some confirmatory experiments performed with rat Hippocampal preparations (see below). In absence of extracellular Mg^{2+} and of exogenously added glycine, NMDA (100 μM), but not Tat (1 nM), elicited small but significant [^{3}H]NE release (~25% over basal release) from rat hippocampal synaptosomes (Fig. 1**B**). It had been reported that this effect of NMDA alone is due to unavoidable small glycine contamination (40 ± 10 nM; see also Kew et al. 1998) in the solutions and could only be observed in experiments with rodent synaptosomes (cf. Pittaluga and Raiteri 1990 and Pittaluga et al. 1996), probably due to their better viability. Fig. 1**B** also reports that Tat (1 nM) was ineffective when added together with 1 μM glycine.

When human synaptosomes were exposed to NMDA plus Tat (1 nM), without adding glycine, a dramatic release of [^{3}H]NE was observed [Fig. 1**A**; $F(7, 30) = 22.1$, $p < 0.001$]. This effect was entirely dependent on NMDAR activation since it was almost eliminated by the NMDA receptor antagonist MK-801 [Fig 1A; $F(7, 30) = 22.1$, $p < 0.001$].

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In rat synaptosomes, Tat increased in a concentration-dependent manner the release of tritium caused by 100 μM NMDA in absence of exogenously added glycine; the maximal effect was reached when Tat was present at 1nM [Fig. 1**C**; $F(4, 24) = 14.75$, $p < 0.01$]. As in human synaptosomes, the effect of NMDA/Tat depended on NMDAR activation since it was abrogated by MK-801 [Fig. 1**B**; $F(6, 36) = 23.78$, $p < 0.01$].

Based on these results, the effects of Tat would appear compatible with a potent action at the glycine site of the NMDARs. It should be reminded that gp120 was previously found to behave as an extremely potent glycine-like agonist, capable (similarly to glycine) to competitively reverse and surmount the antagonism by 7-chloro kynurenic acid (7-Cl-KYNA, a selective blocker of the glycine site) of the NMDA-evoked [^{3}H]NE release (Pittaluga and Raiteri 1994; Pattarini et al. 1998). In contrast, table 1 shows that Tat failed to revert and surmount the 7-Cl-KYNA antagonism of the NMDA/Tat effects both in human [$F(5, 22) = 15.76$, $p < 0.001$] and rat [$F(5, 23) = 79.93$, $p < 0.001$] synaptosomes, a result inconsistent with an action of Tat at the NMDAR glycine site.

Importantly, the potentiation by 3 μM (human) or by 1μM (rat) glycine (Fig. 1**A** and **B)** as well as by gp120 (Fig. 2**A** and **B**) of the NMDA-evoked [^{3}H]NE (see Pittaluga and Raiteri 1994; Pittaluga et al. 1996) could be further increased by 1 nM Tat [human synaptosomes, $F(2, 12) = 70.15$, $p < 0.01$; rat synaptosomes, $F(2, 10) = 52.02$, $p < 0.05$] results consistent with Tat and glycine (or gp120) acting at different sites on noradrenergic terminals.

Studies with neuronal and organotypic slice cultures of rat hippocampus, aimed at understanding the mechanisms of NMDAR upregulation by Tat, reported that the neurotoxicity of the protein may require action at an NMDAR polyamine-sensitive site (Prendergast et al. 2002; Self et al. 2003). These authors found that both the neurotoxicity produced by Tat in the CA1 and CA3 pyramidal cell layers (Prendergast et al. 2003) and the elevations in intracellular Ca^{2+} caused by the protein in the same regions (Self et al. 2003) could be significantly attenuated by the NMDAR polyamine-site antagonist arcaine. As shown in table 2, the potentiation by Tat of the NMDA/glycine-evoked release of [^{3}H]NE in human and rat synaptosomes was insensitive to 30 μM

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arcaine, suggesting that the Tat effects on [^{3}H]NE release do not involve the polyamine sites of the NMDARs located on noradrenergic axon terminals.

The effects of Tat on NMDAR functions are mediated by activation of a Pertussis toxin-sensitive mechanism. Since Tat failed to mimic NMDA, glycine or polyamines, the possibility existed that it could bind to a "receptor" coexisting with NMDARs on noradrenergic terminals. Receptor-receptor interactions are diffuse mechanisms generally occurring postsynaptically (see, for review, Agnati et al. 2003). However, interactions between presynaptic receptors, both ionotropic and metabotropic, have also been described (Desce et al. 1992; Raiteri et al. 1992; Diaz-Hernandez et al. 2004; Risso et al. 2004; Pittaluga et al. 2005). One interesting aspect of receptor-receptor interactions is that receptors sometimes unable to mediate responses when exposed to selective agonists become functional when other receptors coexisting on the same membrane are activated.

We first investigated if Tat activates G protein-coupled receptors and started by verifying the possible involvement of Pertussis toxin (PTx)-sensitive G proteins in the control by Tat of NMDAR function. To avoid excessively long incubations with PTx, we prepared synaptosomes by homogenizing brain tissues in presence of PTx, a technique previously shown to successfully entrap the toxin into subsequently isolated synaptosomes (Raiteri et al. 2000). Figure 3**B** shows that entrapped PTx did not modify the release of [^{3}H]NE elicited from rat synaptosomes either by 100 μM NMDA alone or in presence of 1 μM glycine. On the contrary, PTx totally prevented the Tat-mediated component of the NMDA-evoked release either in absence or in presence of glycine [$F(7, 28) = 12.11$, $p < 0.05$ and $p < 0.001$, respectively].

Similar results were obtained in experiments with human synaptosomes. As shown in Fig. 3**A**, entrapped PTx prevented the Tat-mediated component of the release elicited by NMDA/glycine/Tat [$F(2, 17) = 18.3$, $p < 0.01$]. The inhibition produced by PTx was higher in human than in rat synaptosomes, in line with the larger contribution by Tat observed in human neocortex. Entrapping PTx failed to modify the basal release of tritium (not shown).

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The Tat-mediated effects involve the mGlu1 receptor subtype. In a recent work (Feligioni et al. 2003), Tat was shown to directly activate release-enhancing group I mGluRs presynaptically located on human neocortex cholinergic nerve endings. This finding prompted us to verify whether the indirect Tat-mediated effect on the function of NMDARs sited on noradrenergic terminals also could involve mGluRs.

Human synaptosomes were exposed to NMDA/glycine/Tat in the presence of CPCCOEt or MPEP, two non-competitive antagonists at mGluR1 or mGluR5, respectively. As shown in Fig. 4**A**, CPCCOEt (5 μM) totally prevented the Tat-mediated component of the NMDA/glycine/Tat-evoked release [$F(4, 23) = 10.39$, $p < 0.001$], whereas MPEP (1 μM) only exerted a slight non-significant inhibition. To confirm the involvement of a mGlu1 receptor subtype, we tested LY367385, a selective competitive mGluR1 antagonist. As reported in Fig. 4**A**, LY367385 (1 μM) abrogated the Tat-mediated component of the NMDA/glycine/Tat-evoked [^{3}H]NE release from human nerve terminals [$F(4, 23) = 10.39$, $p < 0.001$].

When rat synaptosomes were exposed to NMDA/glycine/Tat in the presence of mGluR antagonists, CPCCOEt (5 μM) totally prevented the Tat-mediated component of the evoked release [Fig. 4**B**, $F(6, 38) = 13.9$, $p < 0.001$]. Moreover, LY367385 concentration-dependently (10 nM-1 μM) inhibited the Tat-mediated release causing complete inhibition when added at 0.1 μM, [$F(6, 38) = 13.9$, $p < 0.001$].

At the maximal concentration applied, CPCCOEt, MPEP and LY367385 failed to affect the basal release of tritium or that evoked by NMDA/glycine from human and rat synaptosomes (not shown).

Since LY367385 is a competitive antagonist of mGluR1, we investigated if Tat could indeed compete with the drug. As shown in table 3, 0.1 μM LY367385 inhibited the Tat-mediated component of the NMDA/glycine/Tat-evoked [^{3}H]NE release [$F(5, 14) = 31{,}21$, $p < 0.001$]; increasing Tat concentration up to 10 nM did not further potentiate the NMDA/glycine-evoked release, but it reversed completely the antagonism by LY367385 [$F(5, 14) = 31{,}21$, $p < 0.001$].

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Tat activates mGlu1 receptors also in human cortical cholinergic nerve endings. As mentioned above, group I mGluRs were found to exist on human (but not rat) neocortex cholinergic axon terminals whose activation by Tat (in absence of NMDA) brings about ACh release (Feligioni et al. 2003). It was therefore important to better characterize the pharmacology of these presynaptic mGluRs. Synaptosomes from human neocortex were labelled with [^{3}H]choline and exposed during superfusion (with Mg^{2+}-containing medium) to Tat (1 nM), in absence or in the presence of the selective mGlu1 receptor antagonist LY367385 (df = 5, t = 5.99, $p < 0.05$). Figure 5 shows that the Tat-induced release of [^{3}H]acetylcholine ([^{3}H]ACh) was completely abolished by 1 μM LY367385.

The Tat peptide fragment 41-60 mimics the intact protein. In our previous work (Feligioni et al. 2003), the aminoacid sequence 41-60 of Tat could mimic the intact protein at the mGluRs mediating ACh release from human cortical nerve endings. Differently, the effect of Tat on ACh release from rat cortical synaptosomes (mediated by an unidentified "receptor") was reproduced by the sequence 61-80, indicating species-specificity.

Experiments were performed to test the effects of Tat_{41-60} and Tat_{61-80} on the NMDA/glycine-evoked release of [^{3}H]NE from human and rat noradrenergic axon terminals. As reported in table 4, Tat_{41-60} potentiated the evoked release from both human [$F(2, 9) = 8.77$, $p < 0.01$] and rat [$F(2, 9) = 21.83$, $p < 0.001$] synaptosomes, whereas the sequence 61-80 was ineffective.

Tat upregulation of NMDARs occurs through a phospholipase C/protein kinase C/Src pathway. In rat hippocampal noradrenergic terminals, the [^{3}H]NE release evoked by NMDA/glycine/Tat was significantly diminished by U73122, a selective phospholipase C (PLC) inhibitor, by GF109203X, a selective protein kinase C (PKC) blocker and by PP2, the selective inhibitor of cytosolic tyrosine kinases (Src) [$F(7, 23) = 5.05$, $p < 0.05$]. On the contrary, H89, a protein kinase A (PKA) inhibitor and Autocamtide-2 related Inhibitory peptide II (Ant-AIP-II), a selective inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), left unaffected the evoked release (Fig. 6**B**). The effective compounds appear to block the Tat-mediated component of

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the NMDA/glycine/Tat-evoked release since U73122 or PP2 did not inhibit significantly the release evoked by NMDA plus glycine (not shown).

The involvement of Src implies phosphorylation of tyrosine residues. Accordingly, entrapping of antibodies against phosphotyrosines into rat synaptosomes inhibited the release of [^{3}H]NE elicited by NMDA/glycine/Tat to the same extent as PP2 [Fig. 6**B**; $F(7, 23) = 5.05$, $p < 0.05$].

Finally, U73122 and PP2 produced significant inhibition of the [^{3}H]NE release provoked by NMDA/glycine/Tat in human synaptosomes [$F(3, 12) = 23.61$, $p < 0.001$], suggesting participation of a PLC/Src pathway also in human neocortex noradrenergic nerve endings (Fig. 6**A**). At the concentrations applied, antagonists or enzyme inhibitors did not affect, on their own, the release of tritium from human and rat synaptosomes (not shown).

Tat activates presynaptic mGlu1 receptors co-localized with NMDA receptors on rat cortical cholinergic nerve endings. In rat central nervous system, cortical noradrenergic nerve endings are endowed with NMDA receptors, whose activation elicits the release of preloaded [^{3}H]NE: previous results suggested that NMDARs located on cortical axon terminals display a pharmacological profile similar to that of NMDARs sited on hippocampal nerve endings (Pittaluga and Raiteri, 1994). However, these similarities do not allow to assume that the Tat-NMDAR interaction characterized in the hippocampus also occurs in the neocortex. A set of experiments was therefore was therefore carried out with rat cortical synaptosomes. Table 5 shows that enhancement of [^{3}H]NE release provoked by 100 μM NMDA + 1 μM glycine in rat cortical synaptosomes is significantly increased by 1 nM Tat [$F(3, 8) = 10.31$; $p < 0.01$, data from three experiments run in triplicate]. Moreover, LY367385 (1 μM) but not MPEP(1 μM) significantly prevented the Tat-mediated potentiation of the NMDA/glycine-induced [^{3}H]NE release.

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Discussion

The main result of this work is that the HIV-1 protein Tat can potentiate NMDAR-mediated responses, also in conditions of glycine deficiency, by acting at mGlu1 receptors coexisting with NMDARs on the same neuronal membrane.

The technique used is particularly suitable to identify receptor coexistence and to study receptor-receptor interactions. Mono-layers of synaptosomes, plated on microporous filters in parallel chambers, are up-down superfused and transmitters released are measured in the superfusate fractions collected. In these conditions, the compounds released are immediately removed by the superfusing solution before they can activate targets on the releasing nerve endings or on adjacent particles, so that indirect effects are prevented (see Raiteri and Raiteri 2000). In the present work, synaptosomes are selectively prelabelled with [^{3}H]NE which therefore can only be released from noradrenergic terminals; moreover, release modifications by added agents reflect direct action on noradrenergic terminals. If NMDA or Tat alone is unable to elicit NE release, whereas NMDA and Tat added together provoke release sensitive to different receptor antagonists, it can be concluded that the two corresponding receptors coexist and cross-talk on the same noradrenergic terminal.

It is well known that activation of NMDARs requires either glutamate (or NMDA) and glycine/D-serine. Concomitant addition of NMDA (without glycine) and Tat, inactive when added alone, produced dramatic release of NE from human noradrenergic terminals, suggesting that Tat could mimic glycine, being however 3 to 4 orders of magnitude more potent than the natural cotransmitter of glutamate at NMDARs.

If Tat mimics glycine, it would behave like gp120, previously found to be a potent agonist at the NMDAR glycine site on human and rat noradrenergic terminals (Pittaluga and Raiteri 1994; Pittaluga et al. 1996; Pattarini et al. 1998). However, gp120 was able to reverse and surmount the antagonism of the NMDA-evoked NE release provoked by 7-Cl-KYNA (Pittaluga et al. 1996;

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Pattarini et al. 1998), whereas Tat failed to compete with 7-Cl-KYNA for the glycine site, indicating that in human and rat brain, gp120/glycine and Tat act at different sites on noradrenergic nerve terminals, a view strengthened by the ability of Tat to further enhance the maximal effects of gp120 (see Pittaluga and Raiteri 1994; Pittaluga et al. 1996; Fig. 2).

The release evoked by NMDA/glycine/Tat was PTx sensitive, whereas that of NMDA/glycine was insensitive to the toxin, suggesting that Tat acts through G protein-coupled receptors. We previously found that Tat could directly activate group I mGluRs located on human cortical cholinergic nerve terminals to elicit ACh release (Feligioni et al. 2003). Since group I mGluRs have been reported to up-regulate NMDAR currents (Nicoletti at al. 1996; Skeberdis et al. 2001), it was of interest to investigate if Tat could activate mGluRs and interact with NMDARs possibly coexisting on noradrenergic terminals. This seems to be the case, as shown in Fig. 4; more precisely, our pharmacological characterization with selective group I mGluR antagonists suggests that Tat activates mGluRs of subtype 1. Interestingly, we here find (Fig. 5) that the mGluRs present on human cholinergic terminals also belong to the mGluR1 subtype. Moreover, results with the competitive mGluR1 antagonist LY367385 are consistent with the idea that Tat is a mGluR1 agonist able to bind the glutamate recognition site on the outer side of mGluR1.

The effect of Tat as a mGluR1 agonist on both human and rat noradrenergic terminals is retained by the peptide fragment Tat_{41-60}, but not by Tat_{61-80}. Of note, Tat_{41-60} contains the arginine-rich basic region (Tat_{49-57}) that is required to induce depolarization and internal Ca^{2+} mobilization in human fetal neurones (Nath et al. 1996). Accordingly, Tat_{41-60} had been found to imitate the intact protein and to directly release ACh from human (but not rat) neocortex cholinergic terminals (Feligioni et al. 2003). Altogether the results with mGluR antagonists and with Tat peptide fragments lead to the following conclusions: a) Tat is an extremely potent mGluR1 agonist; b) mGlu1 receptors exist on human cholinergic terminals and on human and rat noradrenergic terminals; c) mGluR1 on human cholinergic terminals directly mediates ACh release through internal Ca^{2+} mobilization from IP_3-sensitive stores (Feligioni et al. 2003), whereas mGluR1 on

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noradrenergic terminals indirectly upregulates NMDAR-evoked NE release ; d) the rodent model of NMDAR-mediated NE release appears appropriate to study effects of Tat and fragments on glutamate receptors; e) Tat_{41-60}, or shorter fragments, might be of help in drug design projects aimed at developing selective potent mGluR1 ligands.

The Tat effect on NE release thus requires a mGluR1-NMDAR cross-talk through intra-terminal pathways. The release of NE elicited by NMDA/glycine/Tat was significantly reduced when PLC, PKC or Src activity was inhibited by selective blockers. The release also was reduced in synaptosomes entrapped with antiphosphotyrosine antibodies. All these agents inhibited to a similar extent the NE release elicited by NMDA/glycine/Tat, the sensitive portion of the release probably representing the Tat-mediated component of the evoked NE efflux. Thus PLC activation by Tat acting at mGluR1 linked to PTx-sensitive G proteins plays an important role in the mGluR1-NMDAR interaction. The downstream signalling pathway includes PKC- and Src-mediated tyrosine phosphorylation.

The involvement of Src is supported by the inhibition of the evoked NE release observed in the presence of the Src blocker PP2, as well as by the finding that anti-phosphotyrosine antibodies prevented the effect of NMDA/glycine/Tat. Src is associated with NMDARs and phosphorylation by Src was reported to upregulate NMDAR currents (Yu et al. 1997) Notably, activation of Src family kinase can be triggered by PKC (Salter and Kalia 2004). Thus, the PLC/PKC/Src pathway seems to couple mGluR1 activation and NMDAR function in brain noradrenergic terminals. This cascade has been proposed to mediate upregulation of NMDA currents by various G protein-coupled receptors, including group I mGluRs (Salter and Kalia 2004).

Experiments concerning NMDAR function are usually performed with Mg^{2+}-free solutions. However, NMDA was reported to elicit NE release in presence of physiological Mg^{2+} concentrations during concomitant activation of AMPA receptors which was found to coexist with NMDARs on noradrenergic terminals (Raiteri et al. 1992). The following scenario could be envisaged: glutamate reaching noradrenergic terminals activates depolarizing AMPA receptors

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which in turn permit Mg^{2+} removal and NMDAR activation; if Tat mimics glutamate and binds at mGluR1, the NMDAR-mediated response could be strongly enhanced. In addition Tat might also contribute by releasing ACh (Feligioni et al. 2003) onto α7 nicotinic receptors located on glutamatergic terminals and which mediate glutamate release (Risso et al. 2004).

The lack of releasing effect of Tat added alone suggests that the mere activation of mGluR1 stimulates the above enzymes insufficiently to trigger NE release. Nonetheless, mGluR1 activation can upregulate the functions of NMDARs, an event particularly impressive when these receptors, almost silent in human terminals when glycine is not added, become fully responsive in presence of 1 nM Tat (see Fig. 1**A**) as in human terminals, is almost silent in absence of added glycine (Fig. 1**A**). If glycine is an obligatory coagonist, a reasonable explanation for the Tat effect could be that the protein causes, through mGluR1 activation and the above phosphorylative pathway, an increase in the affinity of the glycine site for its natural ligand. Assuming a glycine contamination in the solutions of ~50 nM and considering the results with human neocortex (Fig. 1**A**) showing quantitatively comparable effects of NMDA/glycine(3 μM)/Tat(1 nM) and NMDA/Tat(1 nM), it could be concluded that activation of mGluR1 by Tat leads to ~100-fold increase in the affinity of the glycine site for glycine. Unfortunately this hypothesis can not be verified by glycine binding experiments or by morphological techniques, due to the very low density of noradrenergic terminals in the CNS.

The concentrations of Tat here used are lower than those causing overt neurotoxicity. It is therefore possible that mGluR1 represents a major target through which Tat produces early impairments in neurotransmission independent of cell death. Circulating levels of Tat have been measured to be ~2.5 nM in AIDS patients (Westendorp et al. 1995) but, according to Nath and Geiger (1998), concentrations are likely to be higher in microenvironments around brain cells. Since Tat or its fragments cannot be removed as easily as glutamate in the CNS, mGluR1 could be ”pathologically” activated. The persistent effects of Tat may provoke release of ACh and NE outside of the physiological range and with abnormal kinetic characteristics, potentially contributing

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to the cognitive impairments occurring in HAD. The situation could become particularly serious if gp120 is also present, considering that this HIV-1 coat protein is an agonist at the glycine site of NMDARs and that the effects of Tat and gp120 can be additive (Fig. 2**A**; see also Nath et al. 2000). Assuming that Tat and its fragments are 'pathological' mGluR1 agonists, HAD symptoms might be controlled, in part, by mGluR1 antagonists.

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References

Agnati LF, Ferre S, Lluis C, Franco R and Fuxe K (2003) Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA Neurons. *Pharmacol Rev* **55**:509-550.

Behnisch T, Francesconi W and Sanna PP (2004) HIV secreted protein Tat prevents long-term potentiation in the hippocampal CA1 region. *Brain Res* **1012**:187-189.

Desce JM, Godeheu G, Galli T, Artaud F, Chèramy A and Glowinski J (1992) L-Glutamate-evoked release of dopamine from synaptosomes of the rat striatum: involvement of AMPA and NMDA receptors. *Neuroscience* **47**:335-339.

Diaz-Hernandez M, Sanchez-Nogueiro J, Pintor J and Miras-Portugal MT (2004) Interaction between dinucleotide and nicotinic receptors in individual cholinergic terminals. *J Pharmacol Exp Ther* **311**:954-967

Diesing TS, Swindells S, Gelbard H and Gendelman HE (2002) HIV-1-associated dementia: a basic science and clinical perspective. *AIDS Read* **12**:358-368.

Everall IP, Heaton RK, Marcotte TD, Ellis RJ, McCutchan JA, Atkinson JH, Grant I, Mallory M and Masliah E (1999) Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. *Brain Pathol* **9:**209-217.

Feligioni M, Raiteri L, Pattarini R, Grilli M, Bruzzone S, Cavazzani P, Raiteri M and Pittaluga A (2003) The human immunodeficiency virus-1 protein Tat and its discrete fragments evoke selective release of acetylcholine from human and rat cerebrocortical terminals through species-specific mechanisms. *J Neurosci* **23**:6810-6818.

Fink K, Bönisch H and Göthert M (1990) Presynaptic NMDA receptors stimulate noradrenaline release in the cerebral cortex. *Eur J Pharmacol* **185**:115-117.

Fink K, Schultheiss R and Göthert M (1992) Stimulation of noradrenaline release in human cerebral cortex mediated by N-methyl-D-aspartate (NMDA) and non-NMDA receptors. *Br J Pharmacol* **106**:67-72.

Gelman BB, Soukup VM, Schuenke KW, Keherly MJ, Holzer C 3rd, Richey FJ and Lahart CJ (2004) Acquired neuronal channelopathies in HIV-associated dementia. *J Neuroimmunol* **157**:111-119.

Haughey NJ, Nath A, Mattson MP, Slevin JT and Geiger JD (2001) HIV-1 Tat through phosphorylation of NMDA receptors potentiates glutamate excitotoxicity. *J Neurochem* **78**:457-467.

Kaul M and Lipton SA (1999) Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. *Proc Natl Acad Sci USA* **96**:8212-8216.

Kew JNC, Richards JG, Mutel V and Kemp JA (1998) Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct population of NMDA receptors in individual rat cortical neurons. *J Neurosci* **18**:1935-1943.

Lawrence DM and Major EO (2002) HIV-1 and the brain: connections between HIV-1-associated dementia, neuropathology and neuroimmunology. *Microbes Infect* **4**:301-308.

Magnuson DSK, Knudsen BE, Geiger JD, Brownstone RM and Nath A (1995) Human immunodeficiency virus type 1 Tat activates non-N-methyl-D-aspartate excitatory amino acid receptors and causes neurotoxicity. *Ann Neurol* **37**:373-380.

Masliah E, Achim CL, Ge N, DeTeresa R, Terry RD and Wiley CA (1992) Spectrum of human immunodeficiency virus-associated neocortical damage. *Ann Neurol* **32**:321-329.

Nath A and Geiger JD (1998) Neurobiological aspects of HIV infections: neurotoxic mechanisms. *Prog Neurobiol* **54**:19-33.

Nath A, Psooy K, Martin C, Knudsen B, Magnuson DSK, Haughey N and Geiger JD (1996) Identification of a human immunodeficiency virus type 1 Tat epitope that is neuroexcitatory and neurotoxic. *J Virol* **70**:1475-1480.

Nath A, Haughey NJ, Jones M, Anderson C, Bell JE and Geiger JD (2000) Synergistic neurotoxicity by human immunodeficiency virus proteins Tat and gp120: protection by memantine. *Ann Neurol* **47**:186-194.

Navia BA, Jordan BD and Price RW (1986) The AIDS dementia complex: I. Clinical features. *Ann Neurol* **19**:517-524.

Nicoletti F, Bruno V, Copani A, Casabona G and Knöpfel T (1996) Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? *Trends Neurosci* **19**:267-271.

Pattarini R, Pittaluga A and Raiteri M (1998) The human immunodeficiency virus-1 envelope protein gp120 binds through its V3 sequence to the glycine site of N-methyl-D-aspartate receptors mediating noradrenaline release in the hippocampus. *Neuroscience* **87**:147-157.

Pittaluga A and Raiteri M (1990) Release-enhancing glycine-dependent presynaptic NMDA receptors exist on noradrenergic terminals of hippocampus. *Eur J Pharmacol* **191**:231-234.

Pittaluga A and Raiteri M (1992) N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. I. Location on axon terminals and pharmacological characterization. *J Pharmacol Exp Ther* **260**:232-237.

Pittaluga A and Raiteri M (1994) HIV-1 envelope protein gp120 potentiates NMDA-evoked noradrenaline release by a direct action at rat hippocampal and cortical noradrenergic nerve endings. *Eur J Neurosci* **6**:1743-1749.

Pittaluga A, Pattarini R, Severi P and Raiteri M (1996) Human brain N-methyl-D-aspartate receptors regulating noradrenaline release are positively modulated by HIV-1 coat protein gp120. *AIDS* **10**:463-468.

Pittaluga A, Pattarini R, Feligioni M and Raiteri M (2001) N-methyl-D-aspartate receptors mediating hippocampal noradrenaline and striatal dopamine release display differential sensitivity to quinolinic acid, the HIV-1 envelope protein gp120, external pH and protein kinase C inhibition. *J Neurochem* **76**:139-148

Pittaluga A, Feligioni M, Longordo F, Arvigo M and Raiteri M (2005) Somatostatin-induced activation and upregulation of N-methyl-D-aspartate receptor function: mediation through

calmodulin-dependent protein kinase II, phospholipase C, protein kinase C and tyrosine kinase in hippocampal noradrenergic nerve endings. *J Pharmacol Exp Ther* **313**:242-249.

Prendergast MA, Trent Rogers D, Mulholland PJ, Littleton JM, Wilkins LH Jr, Self RL and Nath A (2002) Neurotoxic effects of the human immunodeficiency virus type-1 transcription factor Tat require function of a polyamine sensitive-site on the N-methyl-D-aspartate receptor. *Brain Res* **954**:300-307.

Raiteri L and Raiteri M (2000) Synaptosomes still viable after 25 years of superfusion. *Neurochem Res* **25**:1265-1274.

Raiteri M, Garrone B and Pittaluga A (1992) N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. II. Evidence for functional cooperation and for coexistence on the same axon terminal. *J Pharmacol Exp Ther* **260**:238-242.

Raiteri M, Sala R, Fassio A, Rossetto O and Bonanno G (2000) Entrapping of impermeant probes of different size into nonpermeabilized synaptosomes as a method to study presynaptic mechanisms. *J Neurochem* **74**:423-431.

Risso F, Grilli M, Parodi M, Bado M, Raiteri M and Marchi M (2004) Nicotine exerts a permissive role on NMDA receptor function in hippocampal noradrenergic terminals. *Neuropharmacology* **47**:65-71.

Salter MW and Kalia LV (2004) Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci* **5**:317-328.

Savio T and Levi G (1993) Neurotoxicity of HIV coat protein gp120, NMDA receptors, and protein kinase C: a study with rat cerebellar granule cell cultures. *J Neurosci Res* **34**:265-272.

Self RL, Mulholland PJ, Nath A, Harris BR and Prendergast MA (2003) The human immunodeficiency virus type-1 transcription factor Tat produces elevation in intracellular Ca^{2+} that require function of an N-methyl-D-aspartate receptor polyamine-sensitive site. *Brain Res* **995**:39-45.

Skeberdis VA, Lan JY, Opitz T, Zheng X, Bennett MVL and Zukin RS (2001) mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. *Neuropharmacology* **40**:856-865.

Song L, Nath A, Geiger JD, Moore A and Hochman S (2003) Human immunodeficiency virus type 1 Tat protein directly activates neuronal N-methyl-D-aspartate receptors at an allosteric zinc-sensitive site. *J Neurovirol* **9**:399-403.

Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM and Krammer PH (1995) Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* **375**:497-500.

Yu XM, Askalan R, Keil GJ 2nd and Salter MW (1997) NMDA channel regulation by channel-associated protein tyrosine kinase SRc. *Science* **275**:674-678.

Footnote

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Legends for Figures

Fig. 1 Effects of Tat, NMDA or glycine, alone or in combination, on the release of [^{3}H]NE from human and rat nerve endings: antagonism by MK-801. Superfusion was carried out in absence of Mg^{2+} ions. Synaptosomes were exposed to Tat, glycine and NMDA at the end of the first fraction collected. MK-801 was present from eight minutes before agonists. Panel **A**: release of [^{3}H]NE from human synaptosomes. Panel **B**: release of [^{3}H]NE from rat synaptosomes. Panel **C**: effects of different concentrations of Tat on the NMDA (100 μM)-evoked [^{3}H]NE release from rat synaptosomes. Results are expressed as percent increase over basal release; data are means ± S.E.M. of three to eight (panel **A**) and three to eight (panel **B**) experiments run in triplicate (three superfusion chambers for each experimental condition). * $p < 0.05$ versus respective control; ** $p < 0.01$ versus respective control; *** $p < 0.001$ versus respective control.

Fig. 2 Effects of Tat on the release of [^{3}H]NE evoked by NMDA/gp120 from human and rat nerve terminals. Proteins were applied concomitantly with NMDA. The gp120 concentrations are those previously fond to be maximally effective on the same system (Pittaluga and Raiteri 1994, Pittaluga et al. 1996). Panel **A**: effects of NMDA/gp120 or NMDA/gp120/Tat on the release of [^{3}H]NE from human synaptosomes. Panel **B**: effects of the NMDA/gp120 or NMDA/gp120/Tat on the release of [^{3}H]NE from rat synaptosomes. Results are expressed as percent increase over basal release; data are means ± S.E.M. of four to six (panel **A**) or three to six (panel **B**) experiments run in triplicate. * $p < 0.001$ versus NMDA; # $p < 0.05$ versus NMDA + gp120; ## $p < 0.01$ versus NMDA + gp120.

Fig. 3 Effects of Pertussis toxin on the release of [^{3}H]NE evoked by NMDA/glycine/Tat from human and rat nerve terminals. Pertussis toxin was entrapped during tissue homogenization. Glycine and Tat were applied concomitantly with NMDA. Panel **A**: release of [^{3}H]NE from human synaptosomes. Panel **B**: release of [^{3}H]NE from rat synaptosomes White filled bars: control

synaptosomes; grey filled bars: PTx-containing synaptosomes. Results are expressed as percent increase over basal release; data are means ± S.E.M. of four to eight (panel **A**) and three to eight (panel **B**) experiments run in triplicate. * $p < 0.05$ versus respective control;** $p < 0.01$ respective control *** $p < 0.001$ respective control.

Fig. 4 Effects of I group mGluR antagonists on the [^{3}H]NE release evoked by NMDA/glycine/Tat from human and rat nerve terminals. NMDA, glycine and Tat were added together to the superfusion medium. Antagonists were present from eight minutes before agonists. Panel **A**: effects of the antagonists on the (1 mM NMDA + 3 μM glycine + 1 nM Tat)-evoked release of [^{3}H]NE from human synaptosomes. Panel **B**: effects of the antagonists on the (100 μM + 1 μM glycine + 1 mM Tat)-evoked release of [^{3}H]NE from rat synaptosomes. Results are expressed as percent increase over basal release; data are means ± S.E.M. of four to seven (panel **A**) or five to eight (panel **B**) experiments run in triplicate. * $p < 0.05$ versus NMDA/glycine; ** $p < 0.01$ versus NMDA/glycine; *** $p < 0.001$ versus NMDA/glycine; # $p < 0.05$ at least versus NMDA/glycine/Tat; ## $p < 0.001$ at least versus NMDA/glycine/Tat.

Fig. 5 The mGluR1 antagonist LY367385 prevents the Tat-evoked release of [^{3}H]ACh from human neocortex cholinergic nerve terminals. Human cortical synaptosomes were prelabelled with [^{3}H]choline and then superfused with a medium containing physiological amount (1.2 mM) of Mg^{2+} ions. Synaptosomes were exposed to Tat at the end of the first fraction collected; LY367385 was added eight minutes before Tat. Results are expressed as percent increase over basal release; data are means ± S.E.M. of six experiments run in triplicate. * $p < 0.05$ versus Tat.

Fig. 6 Effects of enzyme inhibitors on the release of [^{3}H]NE evoked by NMDA/glycine/Tat from human and rat nerve terminals. Enzyme inhibitors were present from eight minutes before agonists and maintained till the end of the superfusion. Panel **A**: effect of the enzyme inhibitors on the

(1 mM NMDA + 3 μM glycine + 1 nM Tat)-evoked release of [^{3}H]NE from human cortical synaptosomes. Panel **B**: effect of the enzyme inhibitors on the (100 μM + 1 μM glycine + 1 mM Tat)-evoked release of [^{3}H]NE from rat hippocampal synaptosomes. Results are expressed as percent increase over basal release; data are means ± S.E.M. of four (panel **A**) and four to five (panel **B**) experiments run in triplicate. *p < 0.05 versus NMDA+ glycine; **p < 0.01 versus NMDA+ glycine; ***p < 0.001 versus NMDA + glycine; # p < 0.05 versus NMDA + glycine + Tat; ## p < 0.001 versus NMDA + glycine + Tat.

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Table 1. Tat does not revert the antagonism by 7-Cl-KYNA of the NMDA-evoked [^{3}H]NE release from human and rat nerve endings.

	Human		Rat	
	NMDA	NMDA+ 7-Cl-KYNA	NMDA	NMDA+ 7-Cl-KYNA
Control	8.99 ± 3.03	11.69 ± 2.76	32.30 ± 3.05	11.21 ± 2.30[a]
1nM Tat	91.46 ± 13.18[a]	18.26 ± 15.87[b]	79.32 ± 6.05[a]	18.44 ± 6.02[b]
10nM Tat	86.32 ± 8.93[a]	15.54 ± 12.78[b]	74.34 ± 5.99[a]	19.31 ± 7.55[b]

Human and rat synaptosomes were superfused with Mg^{2+}-free medium and exposed respectively to 1 mM NMDA (human synaptosomes) or to 100 μM NMDA (rat synaptosomes). When indicated, Tat was added concomitantly with NMDA, at the end of the first fraction collected. 7-Cl-KYNA (1 μM) was added eight minutes before agonists and maintained till the end of superfusion. Results are expressed as percent of increase over basal release; data are means ± S.E.M. of three to six (human) and four to six (rat) experiments run in triplicate (three superfusion chambers for each experimental condition). [a] $p < 0.001$ versus NMDA; [b] $p < 0.001$ versus NMDA + Tat .

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Table 2. Arcaine does not affect the release of [^{3}H]NE evoked by NMDA/glycine in absence or in presence of Tat from human and rat nerve endings.

	Human		Rat	
	NMDA + glycine	NMDA + glycine + Tat	NMDA + glycine	NMDA + glycine + Tat
Control	21.22 ± 3.05	72.02 ± 11.78[a]	56.09 ± 1.31	89.74 ± 8.50[a]
+ arcaine (30μM)	18.46 ± 3.46	75.83 ± 12.41[a]	55.28 ± 3.52	97.45 ± 11.20[a]

Human and rat synaptosomes were superfused with Mg^{2+} free medium and exposed respectively to 1 mM NMDA + 3 μM glycine (human synaptosomes) or to 100 μM NMDA + 1 μM glycine (rat synaptosomes). When indicated, 1 nM Tat was added together with agonists while arcaine was present from eight minutes before agonists. Results are expressed as percent increase over basal release; data are means ± S.E.M. of five (human) and three to six (rat) experiments run in triplicate. Arcaine did not affect on its own the spontaneous release of tritium (not shown). [a] $p < 0.05$ versus respective control [human: $F(3, 16) = 8.29$; rat: $F(3, 23) = 9.71$].

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Table 3. Tat reverses the antagonism by LY367385 of the [^{3}H]NE release evoked by NMDA/ glycine/Tat from rat noradrenergic nerve endings

	NMDA + glycine	NMDA + glycine + LY 367385
Control	64.06±0.41	72.03±3.06
Tat (1nM)	127.02±6.59[a]	79.79±6.36[a]
Tat (10nM)	126.91±5.32[a]	129.83±7.45[a]

Rat synaptosomes were superfused with Mg^{2+} free medium and exposed to 100 µM NMDA + 1 µM glycine in absence or in presence of Tat (concentration as indicated). Agonists were added at the end of the first fraction collected, while LY367385 (0.1 µM) was present from eight minutes before agonists and maintained till the end of superfusion. Results are expressed as percent increase over basal release; data are means ± S.E.M. of at least three experiments run in triplicate. [a] $p < 0.001$ versus respective control.

Table 4. Effects of Tat fragments 41-60 and 61-80 on the [^{3}H]NE release evoked by NMDA/glycine from human and rat noradrenergic nerve endings

	Human	Rat
	NMDA + glycine	NMDA + glycine
Control	38.37±10.86	63.49±10.36
Fragment 41-60	73.10±8.15[a]	111.47±7.40[b]
Fragment 61-80	33.34±1.82	69.53±1.66

Synaptosomes were superfused with Mg^{2+} free medium; human synaptosomes were exposed to 1 mM NMDA+3 μM glycine, while rat synaptosomes were exposed to 100 μM NMDA + 1 μM glycine. Tat fragments (final concentration 1 nM) were added concomitantly with NMDA/glycine. Results are expressed as percent increase over basal release; data are means ± S.E.M. of four (human) and four (rat) experiments run in triplicate. [a] $p < 0.01$ versus respective control, [b] $p < 0.001$ versus respective control.

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Table 5. Effects of Tat on the [^{3}H]NE release evoked by NMDA/glycine from rat cortical noradrenergic nerve endings: effects of I group mGluR antagonists

	NMDA + glycine- induced [^{3}H]NE release % of increase
Control	33.50±3.20
+Tat	55.00±3.24[a]
+Tat + LY367385	28.86±3.22[a]
+Tat + MPEP	47.83±2.33

Synaptosomes were superfused with Mg^{2+} free medium; rat synaptosomes were exposed to NMDA (100 μM) + glycine (1 μM). Tat (final concentration 1 nM) was added concomitantly with NMDA/glycine. I group mGluR antagonists MPEP (1 μM) and LY367385 (1 μM) were added eight minutes before agonists till the end of the superfusion period. Results are expressed as percent increase over basal release; data are means ± S.E.M. of three experiments run in triplicate. [a] $p <$ 0.001 versus respective control.

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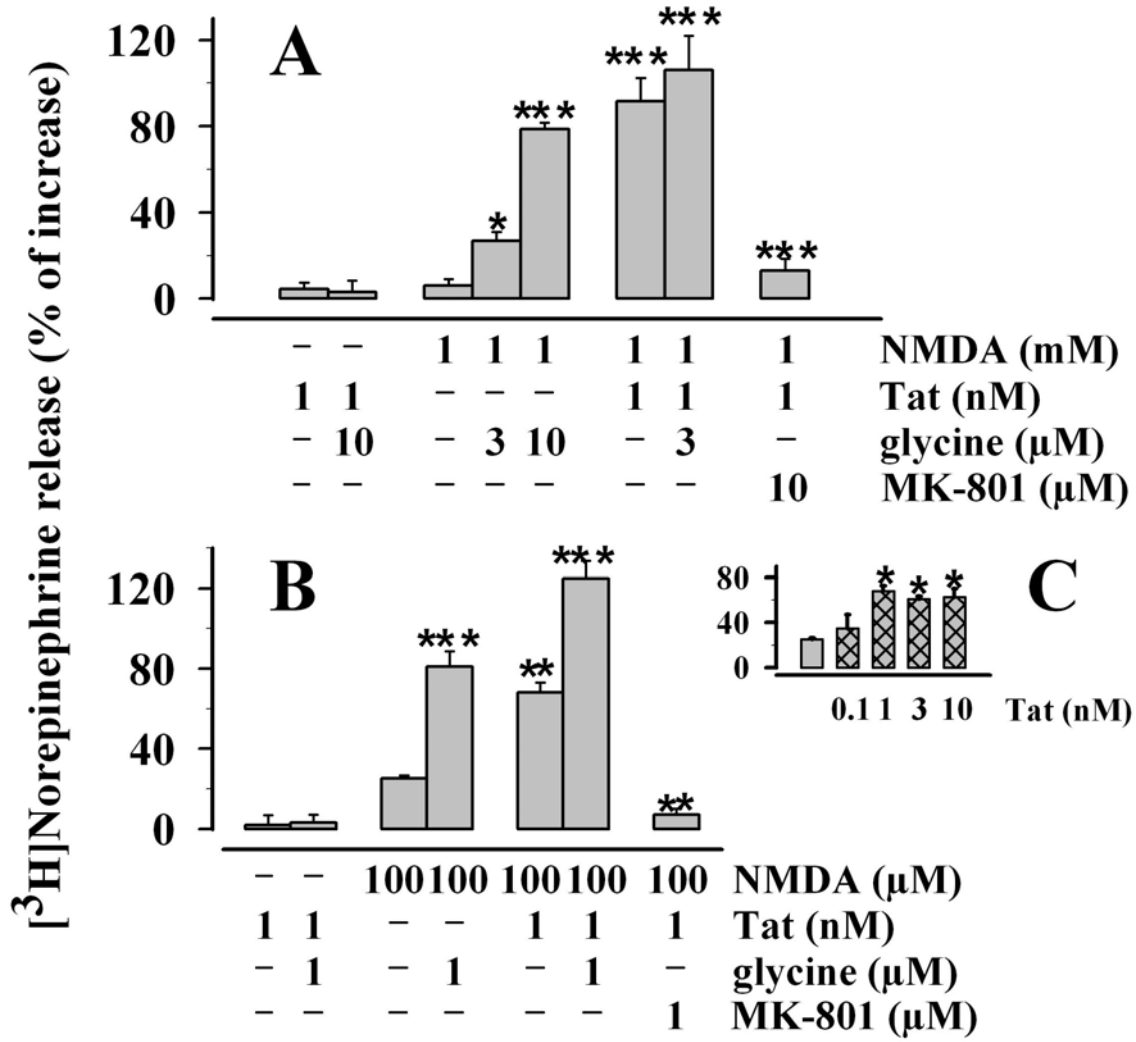

Figure 1

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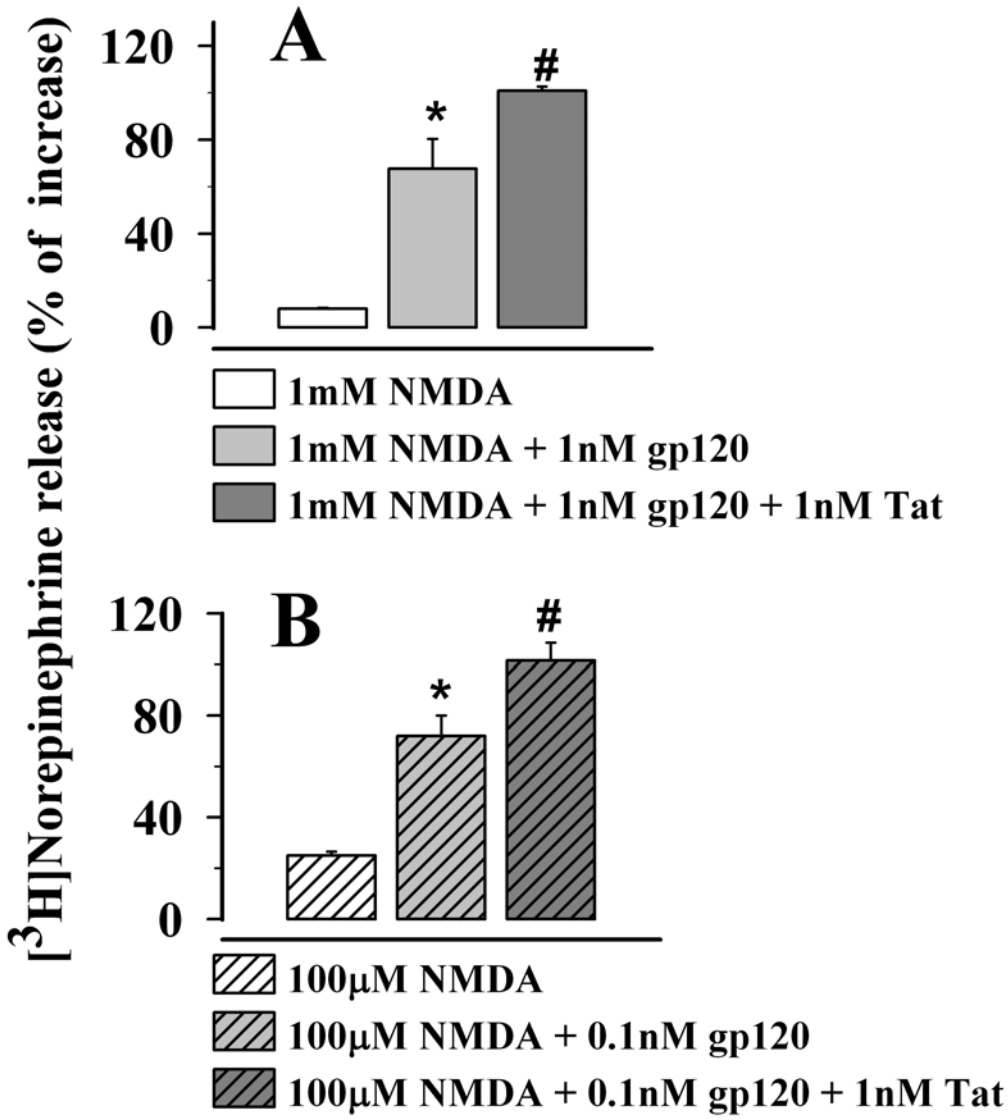

Figure 2

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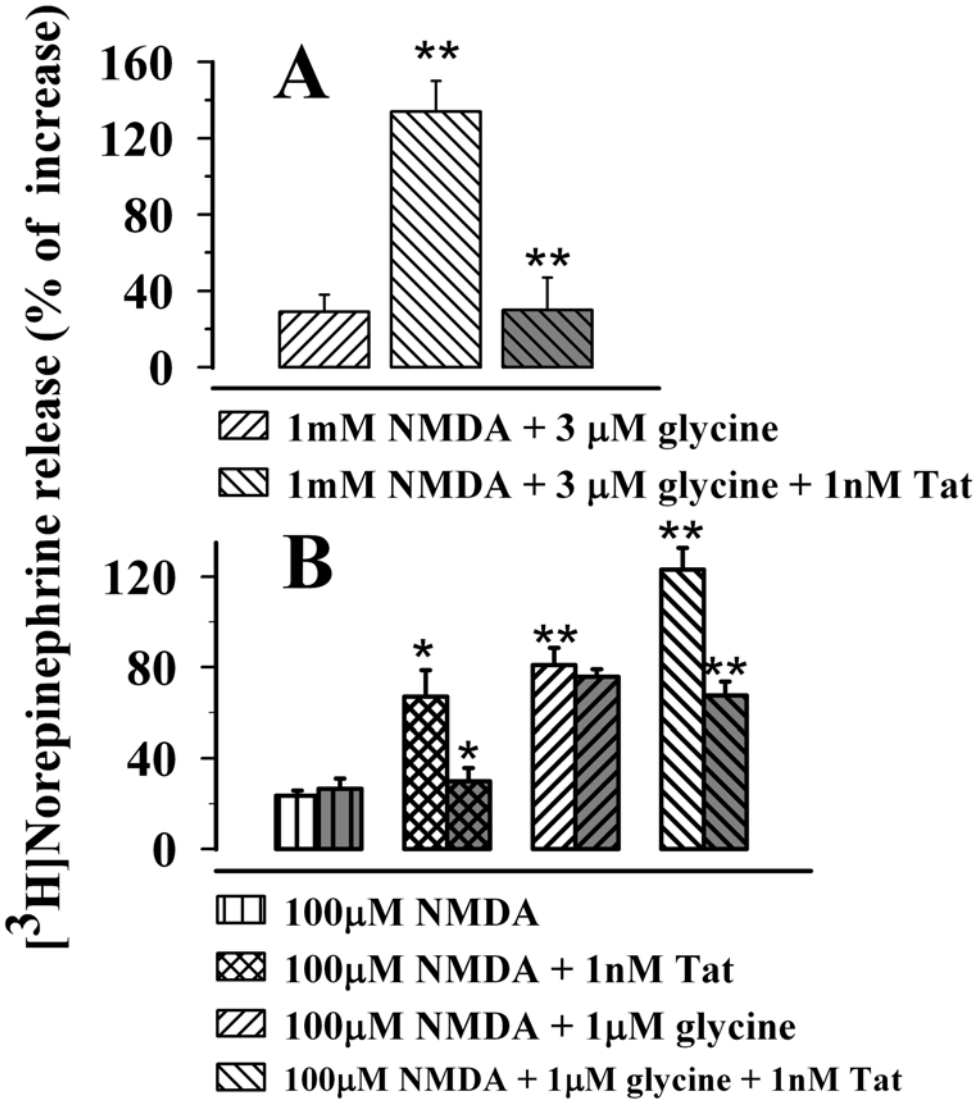

Figure 3

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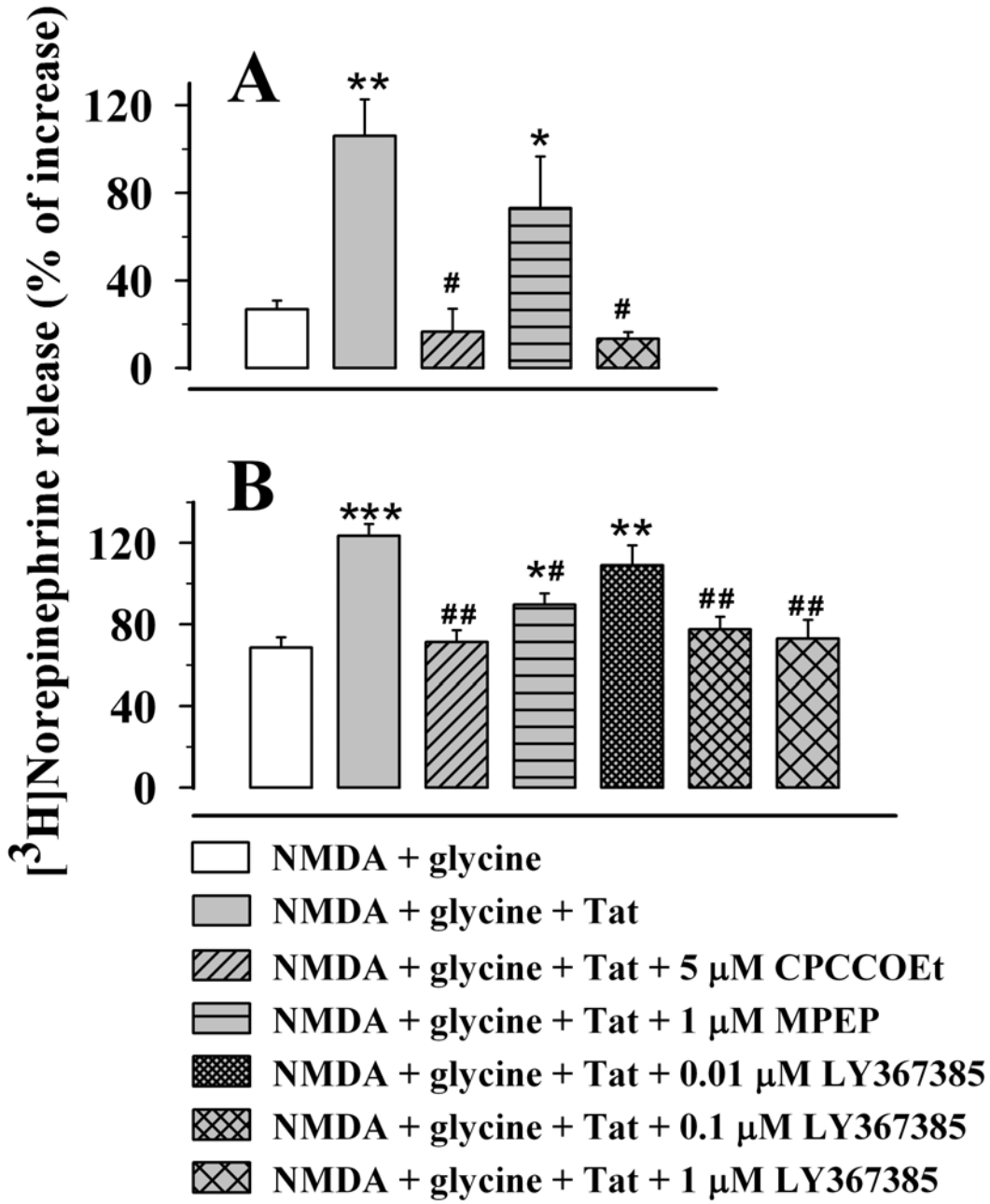

Figure 4

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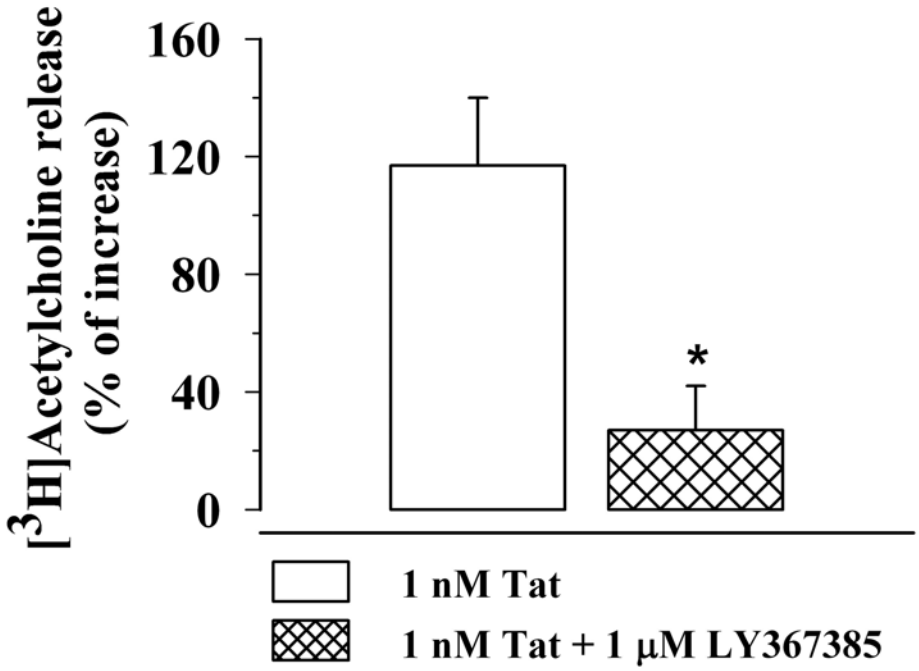

Figure 5

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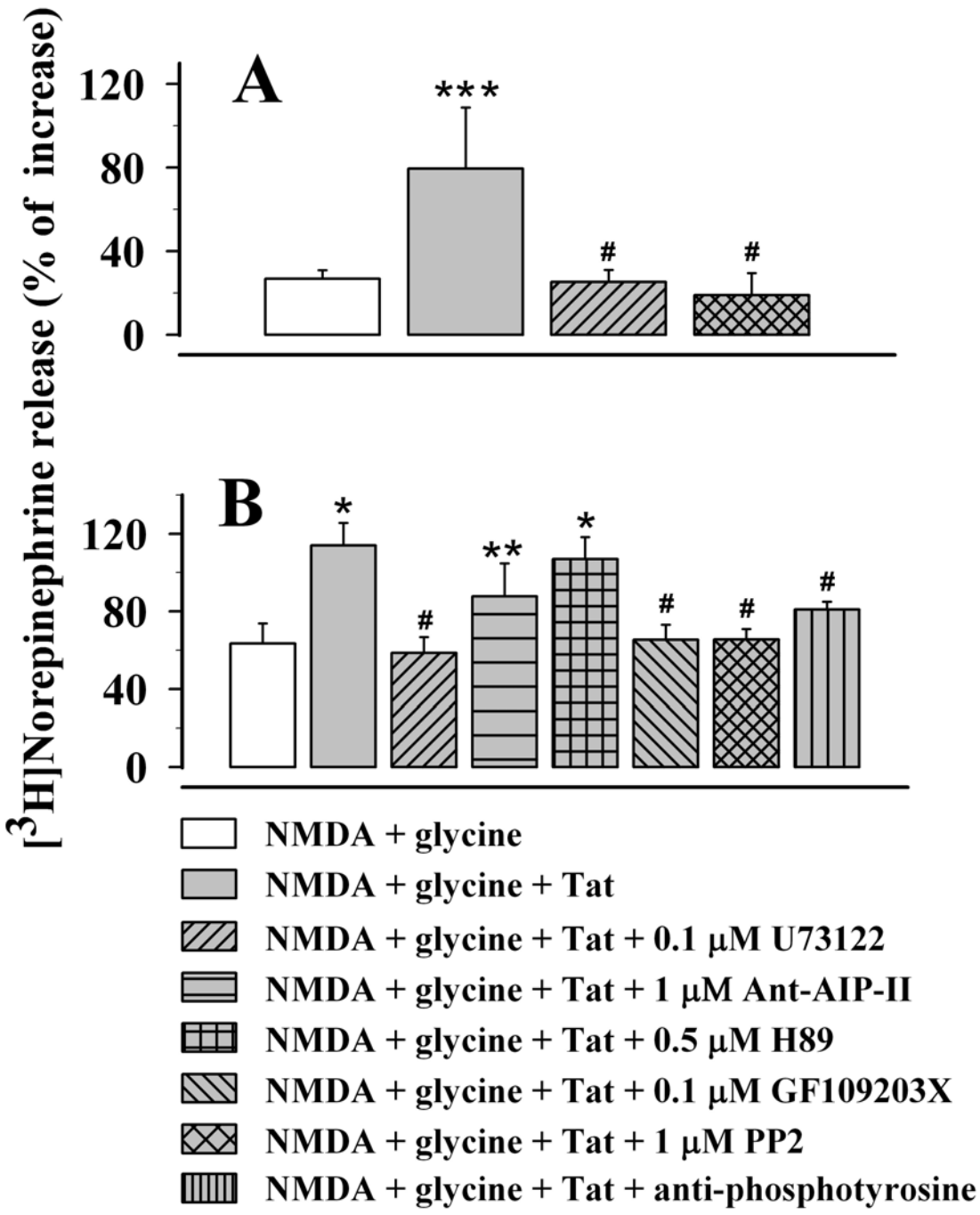

Figure 6