HIV-1 Tat protein inhibits neurosecretion by binding to phosphatidylinositol 4,5-bisphosphate

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Summary

HIV-1 transcriptional activator (Tat) enables viral transcription and is also actively released by infected cells. Extracellular Tat can enter uninfected cells and affect some cellular functions. Here, we examine the effects of Tat protein on the secretory activity of neuroendocrine cells. When added to the culture medium of chromaffin and PC12 cells, Tat was actively internalized and strongly impaired exocytosis as measured by carbon fiber amperometry and growth hormone release assay. Expression of Tat mutants that do not bind to phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] did not affect secretion, and overexpression of phosphatidylinositol 4-phosphate 5-kinase (PIP5K), the major PtdIns(4,5)P_2 synthesizing enzyme, significantly rescued the Tat-induced inhibition of neurosecretion. This suggests that the inhibition of exocytosis may be the consequence of PtdIns(4,5)P_2 sequestration. Accordingly, expression of Tat in PC12 cells interfered with the secretagogue-dependent recruitment of annexin A2 to the plasma membrane, a PtdIns(4,5)P_2-binding protein that promotes the formation of lipid microdomains that are required for exocytosis. In addition Tat significantly prevented the reorganization of the actin cytoskeleton necessary for the movement of secretory vesicles towards plasma membrane fusion sites. Thus, the capacity of extracellular Tat to enter neuroendocrine cells and sequester plasma membrane PtdIns(4,5)P_2 perturbs several PtdIns(4,5)P_2-dependent players of the exocytotic machinery, thereby affecting neurosecretion. We propose that Tat-induced inhibition of exocytosis is involved in the neuronal disorders associated with HIV-1 infection.

Key words: HIV-1 Tat, Neurosecretion, PtdIns(4,5)P_2

Introduction

The human immunodeficiency virus type I (HIV-1) transcriptional activator (Tat) is essential for viral gene expression and replication (Berkhout and Jeang, 1989; Gaynor, 1995). Tat is an ~11 kDa basic protein that is actively released by infected T-cells into the extracellular medium (Ensoli et al., 1990; Rayne et al., 2010a). Tat does not have a signal sequence and is unconventionally secreted (Rayne et al., 2010b). Circulating Tat in the serum of HIV patients can reach nanomolar concentrations (Westendorp et al., 1995; Xiao et al., 2000). Tat is also present within the central nervous system (CNS) since it can cross the blood brain barrier and be produced *in situ* by HIV-1-infected cells such as macrophages and microglia. Thus, Tat may be involved in HIV-1 neuropathogenesis (Banks et al., 2005). HIV-1-associated neuronal symptoms include motor or cognitive dysfunctions and behavioral changes affecting nearly 30% of patients undergoing intensive chemotherapy. Moreover, 10% of patients develop HIV-1-associated dementia at the late stage of AIDS (Ghafouri et al., 2006). The molecular details underlying these CNS perturbations during HIV-1 infection are scarce.

It is generally believed that extracellular Tat operates as a viral toxin (Huigen et al., 2004; Rubartelli et al., 1998) that can enter several types of uninfected cells and affect their physiology (Li et al., 2009). Tat internalization seems to involve different pathways depending on cell type. Indeed, Tat can enter HeLa cells using caveolar endocytosis (Tyagi et al., 2001), and T-cells using clathrin/AP-2-dependent uptake (Vendeville et al., 2004). In neurons, extracellular Tat interacts with the low-density lipoprotein receptor-related protein (LRP) and is then internalized into endosomal structures (Liu et al., 2000), where Tat concentration can reach 2 pg/ml (Deshmane et al., 2011). Upon delivery to endosomes, the amino acid tryptophan 11 initiates Tat insertion into the late endosome membrane (Yezid et al., 2009). Tat transmembrane transport is then catalyzed by the cytosolic chaperone Hsp90 that helps Tat to refold on the *trans* side of the endosomal membrane (Vendeville et al., 2004).

Phosphoinositides control the activity of many membrane protein complexes implicated in a variety of signaling and trafficking pathways including neurosecretion (Balla and Varnái, 2009). PtdIns(4,5)P_2 is a key phospholipid concentrated on the cytoplasmic leaflet of the plasma membrane where it functions as a recruiting platform for proteins that are essential components of the exocytotic or endocytotic machineries (Di Paolo and De Camilli, 2006; van den Bout and Divecha, 2009). The $PH_{PLC\delta}$–GFP fusion protein has been widely used to visualize PtdIns(4,5)P_2 at exocytotic sites in neuroendocrine cells and $PH_{PLC\delta}$–GFP overexpression inhibits exocytosis in chromaffin cells (Holz et al., 2000). Since Tat was recently shown to bind to PtdIns(4,5)P_2 with a high affinity (Rayne et al., 2010b), we examined whether Tat might affect neurosecretion by altering PtdIns(4,5)P_2-dependent processes. We show here that extracellular Tat is able to enter chromaffin and PC12 cells.

Once internalized, Tat strongly inhibits hormone secretion. We observed that Tat modifies the localization of annexin A2, a major organizer of the exocytotic platform in neuroendocrine cells (Umbrecht-Jenck et al., 2010). Tat also affects the reorganization of the cortical actin cytoskeleton required for exocytosis in neuroendocrine cells. Thus, through its capacity to bind PtdIns(4,5)P_2, Tat profoundly disorganizes the exocytotic machinery leading to the inhibition of neurosecretion.

Results

Exogenous Tat is internalized by endocytosis in neurosecretory cells

We first examined whether extracellular Tat could enter neuroendocrine cells. After a 30 min incubation on ice with recombinant Tat, PC12 cells were chased at 37℃ for different periods of time. At time zero, Tat was mostly observed at the cell periphery, showing continuous staining along the cell membrane (Fig. 1A). This Tat labeling at the cell periphery was also detected in the absence of cell permeabilization (data not shown), suggesting that at this early time point, Tat is mostly associated with the extracellular face of the plasma membrane. After a 20 min chase at 37℃, Tat was not detected anymore at the cell periphery but on early endosomal structures labeled with EEA1 (Fig. 1A). Quantification revealed colocalization of Tat with EEA1, being maximal (50±1.8%) after a 20 min chase and thereafter decreasing (Fig. 1B). At later chase times, Tat was observed in vesicles labeled with the late endo-lysosomal marker Lamp-1: Tat/Lamp-1 colocalization peaked at 2 hours and then decreased to be barely significant after 4–6 hours (supplementary material Fig. S1). Interestingly, after 4–6 hours of chase Tat could be detected again at the plasma membrane (Fig. 1A, arrowheads).

To confirm that Tat transits to the plasma membrane, we performed double staining experiments with syntaxin 1A, a specific plasma membrane marker in neurosecretory cells (Fig. 1C,D). At 20 min, there was almost no colocalization between Tat and syntaxin 1A (9±1.4%). However, after 4 hours of chase, the presence of Tat at the plasma membrane was revealed by a significant increase in Tat/syntaxin colocalization (45±2%; Fig. 1C,D). Additional experiments were designed to analyze the inner face of PC12 native plasma membrane sheets. Tat clusters were detected on these membrane lawns only when cells were incubated with Tat and chased for 4 hours (Fig. 1E),

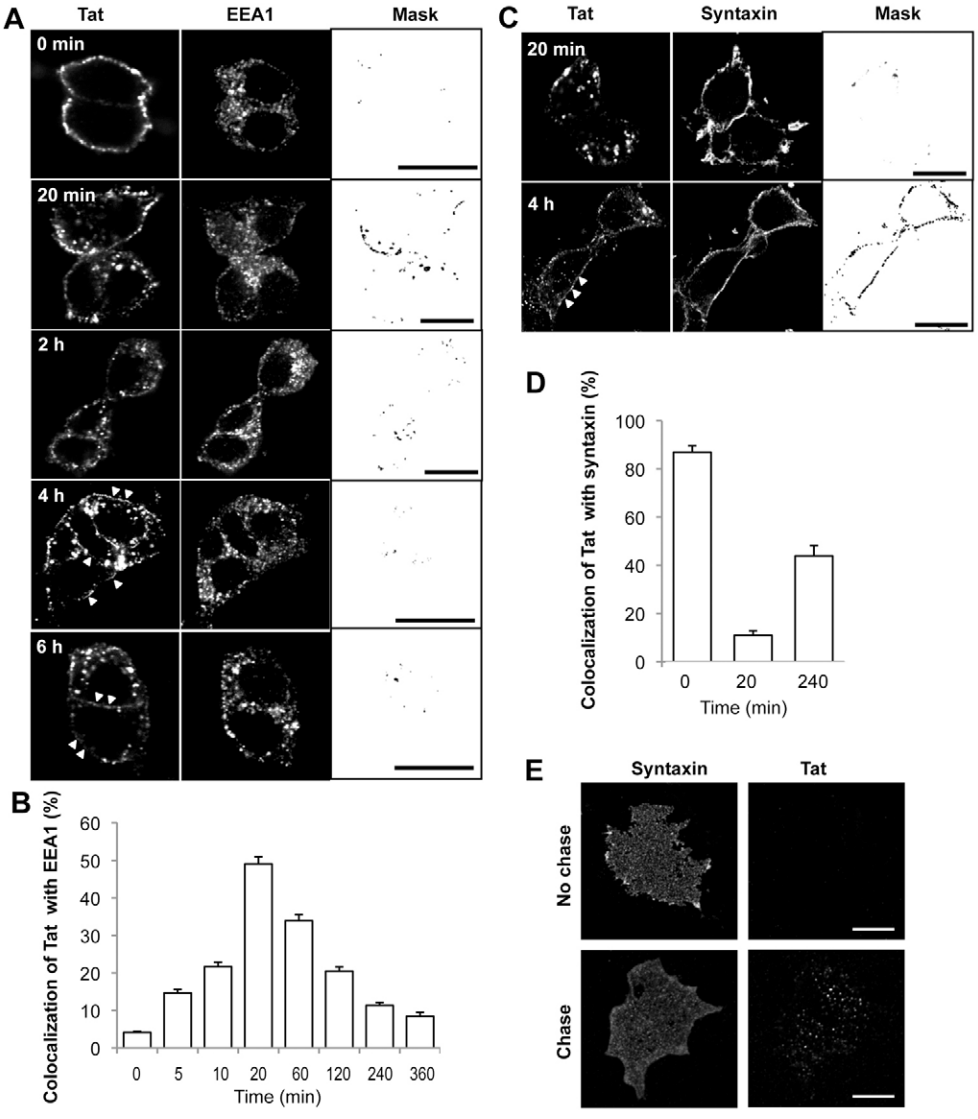

Fig. 1. Extracellular Tat is internalized by endocytosis in neurosecretory cells. PC12 cells were treated with exogenous recombinant Tat protein (50 nM) for 30 min on ice. Cells were washed and either immediately fixed (0 min) or chased at 37℃ for the indicated times and then fixed. Cells were then permeabilized with saponin and processed for Tat and EEA1 (**A**) or syntaxin 1A (**C**) immunolabeling. Representative median confocal sections of at least 20 cells per condition are shown. Mask images highlight the colocalization staining of Tat and syntaxin 1A or EEA1 at different time points. Arrowheads indicate thin plasma membrane Tat staining. Scale bars: 10 µm. Quantification of the colocalization level between Tat and EEA1 (**B**) or syntaxin 1A (**D**) was measured at different time points of chase ($11 \leq n \leq 20$ cells for each time point). Quantification revealed maximal colocalization of Tat with EEA1 after a 20 min chase [B; Pearson's correlation coefficient (PCF): 0.62±0.01], and of Tat with syntaxin after 4 hours of chase (D; PCF: 0.57±0.02). (**E**) Plasma membrane sheets prepared from PC12 cells incubated for 30 min with 50 nM Tat on ice (no chase) or from cells incubated with Tat and subsequently chased for 4 hours at 37℃ (chase). Membrane sheets were then processed for Tat and syntaxin 1A immunolabeling. Scale bars: 10 µm.

suggesting that Tat associates with the inner leaflet of the plasma membrane. In agreement, permeabilization with saponin was required to detect Tat staining on intact fixed cells after 20 min or longer chase periods. Together, these data suggest that in neuroendocrine cells (similar observations were obtained with bovine chromaffin cells; data not shown), exogenous Tat can be internalized by endocytosis into early endosomal structures, reach late endosomes and subsequently escape to bind to the plasma membrane inner leaflet, with kinetics that are similar to those described in neurons (Liu et al., 2000).

Tat inhibits chromaffin and PC12 cell exocytosis

Because Tat binds with high affinity to PtdIns(4,5)P_2 (Rayne et al., 2010b), a key lipid for exocytosis, we examined the effect of Tat on chromaffin cell catecholamine secretion using carbon-fiber amperometry. Bovine chromaffin cells in primary culture were incubated in the presence of Tat for 4 hours and then stimulated by a local application of nicotine. We found that the number of amperometric spikes reflecting exocytotic fusion events was strongly decreased in Tat-exposed cells compared to control cells (Fig. 2A). Tat decreased by more than 50% the number of spikes per cell evoked by stimulation with either nicotine or a depolarizing concentration of K^+ (Fig. 2B). The kinetic parameters (rising and decay time, half-width) of the remaining spikes were, however, unaffected (Fig. 2C), indicating that Tat inhibited catecholamine secretion by decreasing the frequency of fusion events. We verified using TRM labeling, that Tat did not trigger apoptosis under these experimental conditions (data not shown).

The effect of Tat on PC12 cell exocytosis was assessed in cells expressing human growth hormone (GH) (Vitale et al., 2001). Because GH is stored in secretory granules and released by exocytosis, it can be used as a secretory activity reporter. Extracellular Tat inhibited ATP-evoked GH release from PC12 cells in a time- and dose-dependent manner (Fig. 2D,E). At 10 nM, Tat produced a significant inhibitory effect on GH secretion whereas the boiled Tat protein was without effect (Fig. 2E). Similar observations were obtained with PC12 cells stimulated with a depolarizing K^+ concentration (data not shown). Note that the GH expression level (~400 pg/10^5 cells) was not affected by the Tat concentrations used in these studies (supplementary material Fig. S2).

We then examined the effect of transient Tat expression on ATP-evoked GH release from PC12 cells. Transfections enabled us to use various Tat mutants that were unable to penetrate neurosecretory cells. When transiently expressed, Tat inhibited ATP-evoked GH release from PC12 cells (see Fig. 5), suggesting that expressed Tat reproduces the effect of exogenous Tat incubation on regulated exocytosis. Transfected Tat also strongly inhibited GH release triggered by 20 μM free calcium in PC12 cells permeabilized with digitonin to bypass the secretagogue-evoked

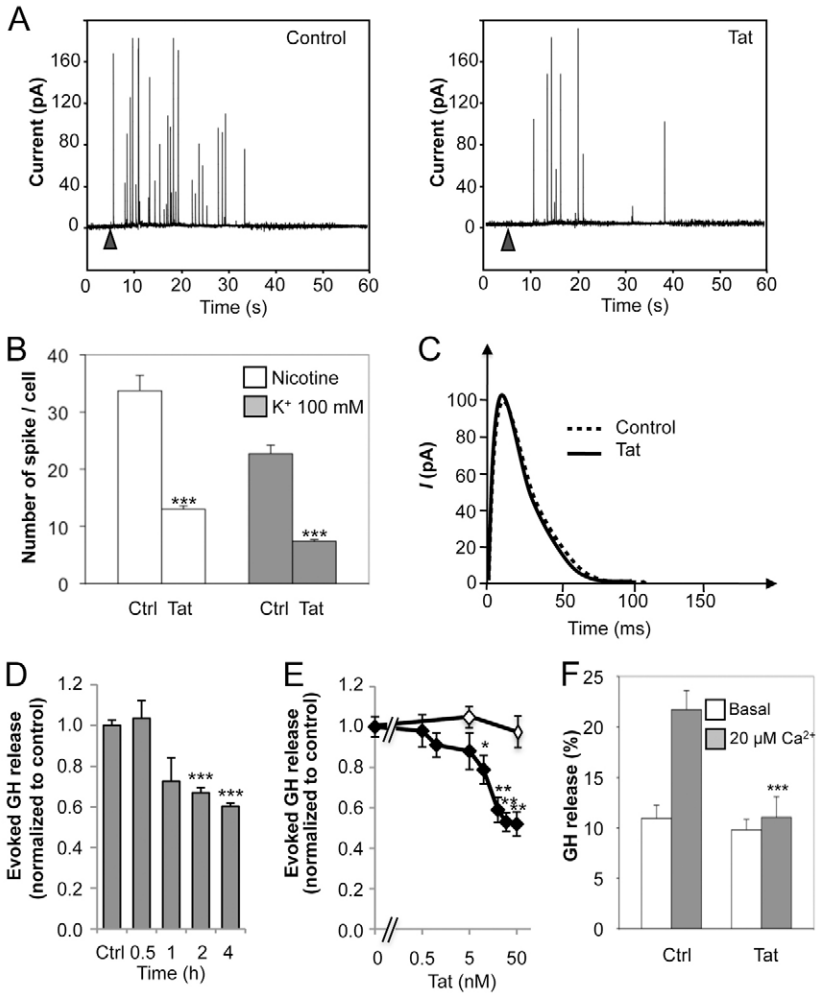

Fig. 2. Tat inhibits neurosecretion from bovine primary chromaffin and PC12 cells. (**A**) Amperometric recordings of catecholamine release in response to a local application of nicotine in control or Tat-treated (50 nM for 4 hours) chromaffin cells. A typical amperometric profile is shown for each condition. Control cells received vehicle only (citrate buffer). (**B**) Quantification of the number of fusion events/cell in response to 100 μM nicotine or 100 mM K^+ stimulation. The number of spikes was quantified for 60 sec after stimulation in $14 \leq n \leq 25$ cells from three different cell cultures. Values are means ± s.e.m., ***$P<0.001$ compared to the corresponding control condition. (**C**) Average individual spike from control (dotted trace) and Tat-treated cells (black trace). (**D**) PC12 cells expressing GH were incubated in the presence of 50 nM Tat protein (30 min on ice then chased for 0.5 to 4 hours). GH secretion was then triggered by 300 μM ATP and released hormone was quantified after 5 min of stimulation. Control cells received vehicle only. Data are means ± s.e.m. from four experiments performed in quadruplicate on three independent cell cultures (***$P<0.001$ compared to the control condition). (**E**) Dose-dependent effect of exogenously supplied Tat (filled diamonds) or boiled Tat (open diamonds) on ATP-evoked GH secretion from PC12 cells. Data are means ± s.e.m. of two experiments performed in triplicate obtained from two different cell cultures (*$P<0.05$, **$P<0.01$ compared to the control condition). (**F**) PC12 cells were co-transfected with a plasmid expressing GH and a bicistronic vector expressing EGFP (ctrl) or Tat and EGFP (Tat). 24 hours after co-transfection, cells were permeabilized with digitonin and GH release estimated in calcium-free medium for resting condition (basal, open bars) or in response to 20 μM free calcium for stimulated condition (20 μM Ca^{2+}, grey bars). Data are means ± s.e.m. of four experiments performed in triplicate, obtained from three different cell cultures (***$P<0.001$ compared to the stimulated control condition).

calcium rise (Fig. 2F). Thus, Tat inhibits secretion at a step located downstream of the rise in cytosolic calcium, probably through a direct perturbation of the exocytotic machinery. Accordingly, using Fluo-4 dye to measure cytosolic calcium levels, we found that the transient calcium rise in response to cell stimulation was similar in control and Tat-expressing PC12 cells (supplementary material Fig. S3).

Tat requires binding to PtdIns(4,5)P_2 to inhibit secretion

When expressed in PC12 cells using a bicistronic plasmid that encodes both EGFP and Tat, Tat was found to colocalize with the plasma membrane marker SNAP-25 (Fig. 3A) and with PtdIns(4,5)P_2 at the cell periphery (Fig. 3B). A similar localization was observed in chromaffin cells (supplementary material Fig. S4). Interestingly, Tat was efficiently displaced from the plasma membrane to the cytosol by neomycin, a PtdIns(4,5)P_2 masking drug (Fig. 3C). A semi-quantitative analysis revealed that neomycin reduced by nearly 50% the peripheral Tat (Fig. 3C), indicating that the association of Tat with the plasma membrane depends on PtdIns(4,5)P_2 binding.

To further support the notion that Tat localization at the cell periphery is dependent on PtdIns(4,5)P_2 level, we co-expressed in PC12 cells Tat and a drug-inducible type IV 5-phosphatase (Béglé et al., 2009) or the PtdIns(4,5)P_2 5-phosphatase synaptojanin to reduce PtdIns(4,5)P_2 levels. In both type of experiments, reduction of PtdIns(4,5)P_2 levels strongly affected the peripheral distribution of Tat, which became largely cytosolic (Fig. 4). Altogether, these observations indicate that, in neuroendocrine cells, Tat either expressed or applied exogenously and internalized by endocytosis, tends to associate in a PtdIns(4,5)P_2-dependent manner to the plasma membrane.

Next, we investigated whether the inhibitory effect of Tat on neurosecretion might be linked to its binding to PtdIns(4,5)P_2. To this end, PC12 cells were transfected with plasmids encoding different Tat mutants and probed for GH secretion. The expression levels of Tat mutants were similar to that of the wild-type Tat protein (supplementary material Fig. S5). As illustrated in Fig. 5, expression of Tat(49–51)A that does not bind to PtdIns(4,5)P_2 (Rayne et al., 2010b) did not affect secretagogue-evoked GH secretion, whereas expression of Tat(55–57)A, also mutated in the basic region but still able to bind to PtdIns(4,5)P_2, inhibited GH release like wild-type Tat (Fig. 5A). Moreover, secretory activity was unaffected by the expression of the TatW11Y mutant that preserves the native structure and transactivation activity of Tat (Yezid et al., 2009), but does not bind PtdIns(4,5)P_2 (Rayne et al., 2010b) (Fig. 5A). Note that Tat(49–51)A accumulated mostly in the cytoplasm, whereas Tat(55–57)A was found at the cell periphery like the wild-type Tat protein (supplementary material Fig. S4). Thus, the interaction of Tat with PtdIns(4,5)P_2 at the plasma membrane seems to be necessary for Tat to block exocytosis.

To confirm these results, we examined whether increasing the intracellular PtdIns(4,5)P_2 concentration by overexpressing the phosphatidylinositol 4 phosphate 5-kinase Iγ (PIP5KIγ) isoform could counteract the inhibitory effect of Tat on secretion. PIP5KIγ is the major PtdIns(4,5)P_2 synthesizing enzyme in neurosecreting cells, and its overexpression has previously been shown to increase the amount of PtdIns(4,5)P_2 at the plasma membrane by ~5-fold (Béglé et al., 2009; van den Bout and Divecha, 2009). As illustrated in Fig. 5B, overexpression of PIP5KIγ did not significantly modify GH release from PC12 cells, indicating that PtdIns(4,5)P_2 concentration is not a limiting factor for secretion. However, overexpression of PIP5KIγ did significantly reduce the inhibitory effect of Tat on exocytosis (Fig. 5B). Note that this rescuing effect on Tat-mediated inhibition of secretion was not due to a downregulation of Tat expression (Fig. 5C,D). Altogether, these data strongly suggest that impairment of secretion by Tat is linked to its binding capacity to PtdIns(4,5)P_2 at the plasma membrane.

Tat interferes with PtdIns(4,5)P_2-dependent components of the exocytotic machinery

Neuronal exocytosis requires the presence of PtdIns(4,5)P_2 at the plasma membrane (Aoyagi et al., 2005; Milosevic et al., 2005;

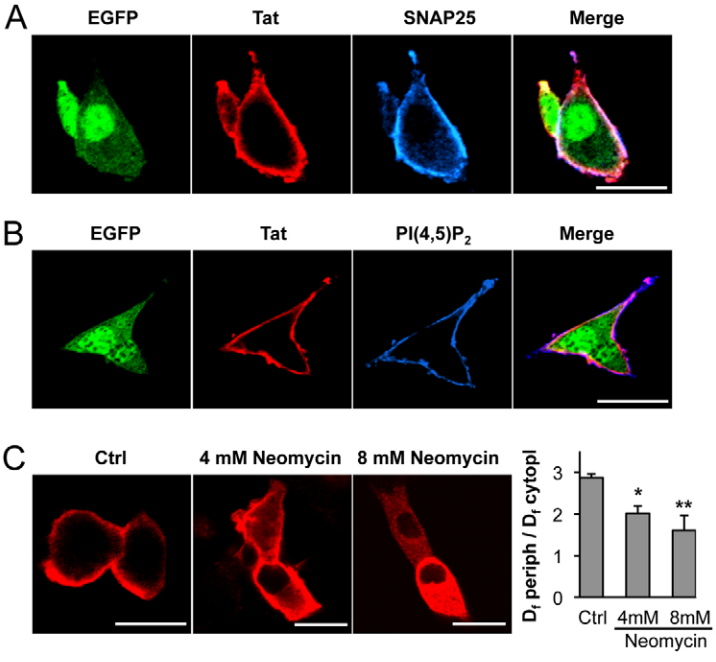

Fig. 3. Intracellular localization of transfected Tat in PC12 cells. Cells were transfected with a bicistronic plasmid expressing Tat and EGFP proteins separately. (**A**) Distribution of Tat and the plasma membrane marker SNAP-25 immunostaining. (**B**) Distribution of Tat and PtdIns(4,5)P_2 [PI(4,5)P_2] immunolabeling. Representative, median confocal sections are shown. (**C**) Localization of Tat in PC12 cells after neomycin treatment. Cells were transfected as above and 24 hours after transfection, cells were treated with 4 or 8 mM neomycin for 1 hour. The subcellular localization of Tat was assessed by immunofluorescence. Representative confocal micrographs are shown. Scale bars: 10 µm. The graph shows the quantification of Tat fluorescence at cell periphery in confocal sections, expressed as a ratio of fluorescence densities (D_f) at cell periphery compared to cytoplasm. Mean values ± s.e.m. are shown (n>9 cells in each condition; *P<0.05; **P<0.01 compared to the control condition).

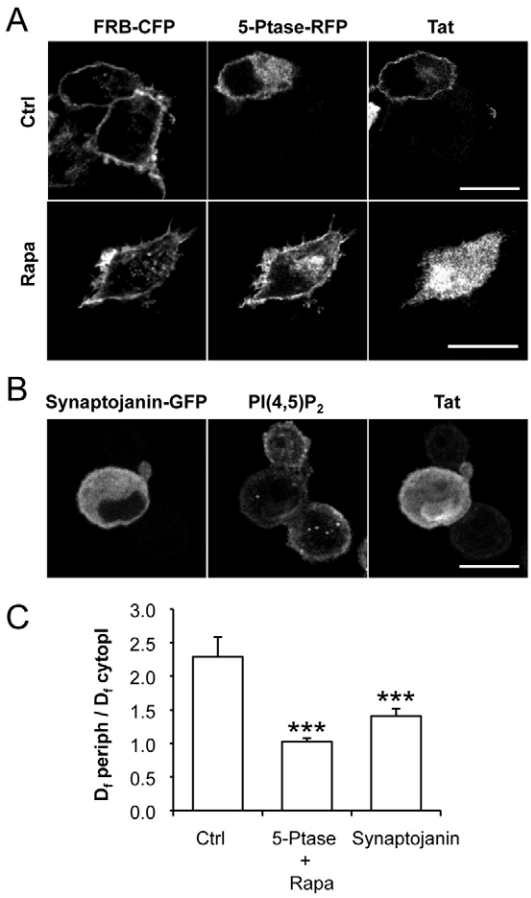

Fig. 4. Tat is partially redistributed to the cytoplasm upon reduction of the PtdIns(4,5)P_2 level. (**A**) PC12 cells were transfected with type IV 5-phosphatase fused to FKBP-12–mRFP (5-Ptase-RFP) and FRB–CFP. 24 hours after transfection, cells were incubated for 6 minutes with control buffer (Ctrl) or 100 nM rapamycin (Rapa) to induce 5-phosphatase translocation to the plasma membrane. After rapamycin treatment, Tat displayed a cytoplasmic distribution. Scale bar: 10 μm. (**B**) PC12 cells co-expressing synaptojanin-GFP and Tat were stained for Tat and PtdIns(4,5)P_2. Expression of synaptojanin reduced PtdIns(4,5)P_2 signal and partially relocated Tat to the cytoplasm. Scale bar: 10 μm. (**C**) Quantification of Tat fluorescent signal at cell periphery in confocal sections expressed as ratio of fluorescence densities (D_f) at cell periphery compared to cytoplasm. Mean values ± s.e.m. are shown (n>21 cells in each condition; ***P<0.001 compared to the control condition).

James et al., 2008; Wen et al., 2011). The amount of plasma membrane located PtdIns(4,5)P_2 determines various parameters of exocytosis, most likely through the interaction with proteins involved in docking, priming and/or fusion (Umbrecht-Jenck et al., 2010; Di Paolo and De Camilli, 2006; James et al., 2008; Rescher et al., 2004; van den Bout and Divecha, 2009). Thus, one hypothesis might be that Tat interferes with the PtdIns(4,5)P_2-mediated recruitment of constituent(s) of the exocytotic machinery to the plasma membrane. Annexin A2 is a well known PtdIns(4,5)P_2-binding protein recruited to the plasma membrane at early stages of exocytosis in chromaffin and PC12 cells (Umbrecht-Jenck et al., 2010). Annexin A2 is cytosolic in resting cells. Secretagogue-evoked stimulation triggers its rapid translocation to the plasma membrane (Fig. 6A), where it stabilizes lipid micro-domains required for exocytosis (Chasserot-Golaz et al., 2005). In Tat-expressing PC12 cells, neither ATP (Fig. 6A) nor Ba^{2+} or high K^+ (data not shown) were able to induce the translocation of annexin A2 to the cell periphery. Semi-quantitative analysis (Fig. 6C) confirmed that annexin A2 recruitment to the plasma membrane was severely impaired in cells expressing Tat whereas expression of the Tat(49–51)A mutant which is unable to bind PtdIns(4,5)P_2 had no effect (Fig. 6B,C). These experiments suggest that, through its ability to bind PtdIns(4,5)P_2, Tat may also affect the lipid organizing activity of annexin A2 required for exocytosis.

Because PtdIns(4,5)P_2 is also able to bind and modulate several actin regulatory proteins known to mediate the actin redistribution required for exocytosis in neuroendocrine cells (Trifaró et al., 2008), we monitored actin depolymerization at the cell periphery using fluorescent phalloidin. As illustrated in Fig. 7, PC12 cell stimulation reduced peripheral phalloidin staining in line with the transient actin reorganization occurring in cells undergoing exocytosis. Expression of Tat impaired peripheral actin depolymerization (Fig. 7A,C), whereas Tat(49–51)A had no effect (Fig. 7B,C). In other words, Tat through its binding to PtdIns(4,5)P_2 interfered with the subplasmalemmal actin reorganization in stimulated cells, an effect that may also be involved in its capacity to impair the exocytotic response.

Discussion

Tat protein plays a pivotal role in the control of transcription and replication of HIV-1 (Coiras et al., 2010). Although Tat function has been thought for a long time to be restricted to the control of viral RNA production (Chiu et al., 2002), recent findings indicated that Tat binds to PtdIns(4,5)P_2 with a very high affinity at the plasma membrane. This binding is required for Tat release to extracellular space (Rayne et al., 2010b). Strikingly, the capacity of Tat to be endocytosed and reach the cytosol in many cell types (Debaisieux et al., 2012) suggests that circulating Tat might interfere with key cellular processes relying on PtdIns(4,5)P_2 such as endocytosis, phagocytosis, exocytosis, cell migration and division (Di Paolo and De Camilli, 2006).

We describe here that Tat taken up by an endocytotic pathway or expressed by transient transfection inhibits secretion from neuroendocrine chromaffin and PC12 cells, probably through its binding to PtdIns(4,5)P_2 at the plasma membrane. We found that Tat affected the PtdIns(4,5)P_2-mediated recruitment of annexin A2, a key component of the exocytotic machinery. Tat also interferes with the peripheral actin cytoskeleton reorganization upon neuroexocytosis, thereby compromising the secretory response at a step following the rise in cytosolic calcium.

Tat uptake by neurosecretory cells

HIV-1-infected cells massively export Tat (Ensoli et al., 1990; Rayne et al., 2010a; Rayne et al., 2010b) and extracellular Tat can enter into various cell types via multiple endocytic routes (Liu et al., 2000; Tyagi et al., 2001). We report here that like in neurons (Liu et al., 2000), Tat is efficiently internalized in neurosecretory chromaffin and PC12 cells, making these two models suitable to study the effect of Tat on neurosecretion. Consistent with the Tat uptake pathway observed in other cell types (Liu et al., 2000; Vendeville et al., 2004), our results show that internalized Tat is first found in early endosomal structures as revealed by colocalization with EEA1, then in Lamp-1-positive structures. After 4 hours, Tat-containing vesicular

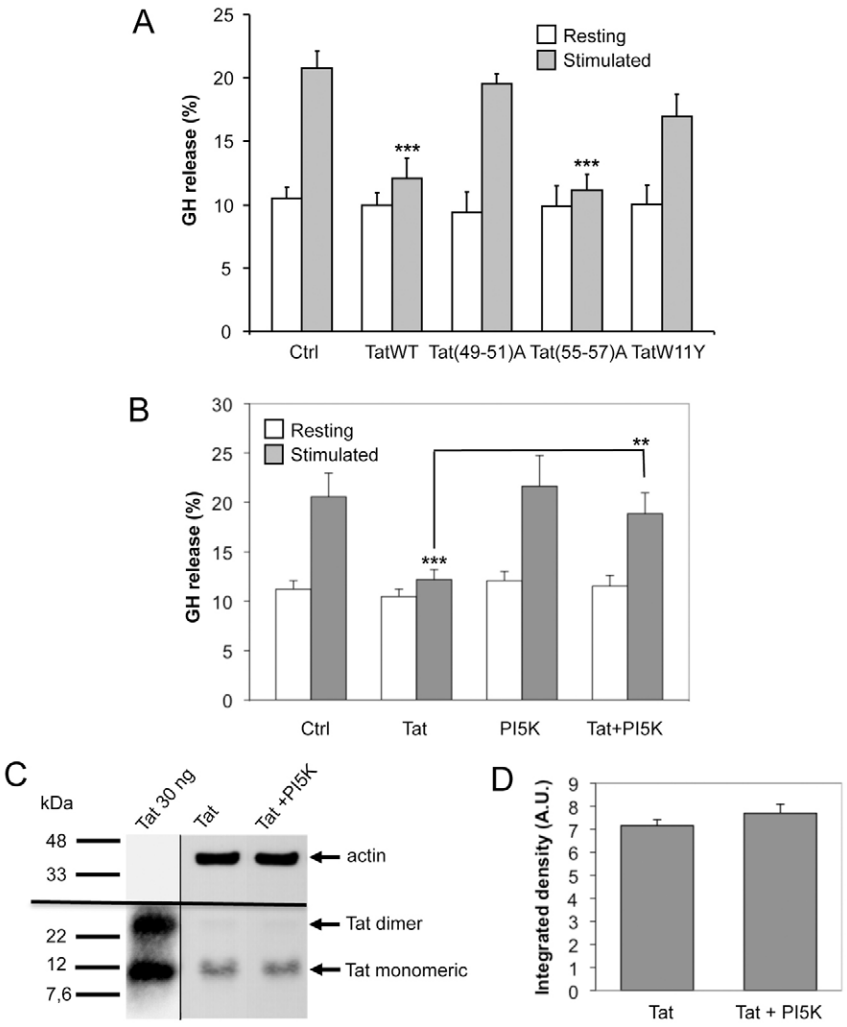

Fig. 5. Tat inhibitory effect on neurosecretion relies on PtdIns(4,5)P_2 **interaction.** (**A**) PC12 cells were transfected with plasmids encoding GH and Tat or the indicated Tat mutants. 24 hours after transfection, GH secretion was evoked with ATP and quantified. Results are means ± s.e.m. of three experiments performed in triplicate on cells derived from three different cell cultures (***$P<0.001$ compared to the stimulated control condition). (**B**) Rescue of Tat inhibitory effect on GH release by overexpression of PIP5Kγ. Cells were transfected as indicated using an empty vector (ctrl), a Tat or a PIP5Kγ-expressing plasmid. The total amount of DNA was kept constant using the empty vector. GH release is represented as the percentage of total GH expressed by the cells. Data are means ± s.e.m. of three to five experiments performed in triplicate on independent cell cultures (***$P<0.001$ compared to the stimulated control condition; **$P<0.005$ when compared to the stimulated control condition in cells coexpressing PIP5Kγ). (**C**) Western blot analysis showing Tat expression level in control and PIP5Kγ-expressing PC12 cells. PC12 cells were transfected with Tat or PIP5Kγ-expressing vectors as indicated. 40 μg of total cellular protein extracts were loaded on gels and membranes were processed for anti-actin and anti-Tat immunolabeling. As positive control for Tat detection, 30 ng of recombinant Tat were loaded. (**D**) Quantification of western blots shown in C, expressed as integrated density per surface unit in arbitrary units (A.U.) and normalized to actin levels. Results are means ± s.e.m. of three experiments performed in duplicate on cells derived from three different cell cultures.

structures were barely detectable suggesting that endocytosed Tat progressively dissipates into the cytosol, due to translocation across the endosome membrane (Vendeville et al., 2004; Yezid et al., 2009). Concomitantly, Tat signal became evident on the inner leaflet of the plasma membrane as revealed on plasma membrane sheets prepared from Tat-exposed cells. When transfected and expressed in PC12 cells, Tat localized essentially at the plasma membrane. The binding of Tat to PtdIns(4,5)P_2 is most likely responsible for this recruitment of Tat to the plasma membrane because both neomycin, a PtdIns(4,5)P_2 masking drug, and PtdIns(4,5)P_2 5-phosphatases, which reduce cellular PtdIns(4,5)P_2 levels when overexpressed, efficiently displaced Tat from the plasma membrane to the cytosol.

Molecular basis underlying the inhibitory effect of Tat on neurosecretion

We investigated the effect of Tat on neurosecretion using chromaffin cells to monitor exocytosis by amperometry (Bader et al., 2002; Bader and Vitale, 2009). In this cell type, Tat strongly inhibited the number of exocytotic events induced by two different secretagogues (nicotine, high K^+), as revealed by a reduction in amperometric spike frequency. Tat also inhibited hormone release from intact PC12 cells stimulated by various secretagogues and from permeabilized PC12 cells in which the intracellular calcium level was buffered at 20 μM. These data indicate that the impairment of secretion was not the consequence of an altered calcium signal, but most likely resulted from a direct effect of Tat on the exocytotic machinery. Accordingly, using a fluorescent calcium dye, we found that Tat did not modify the secretagogue-evoked rise in intracellular calcium.

Using Tat mutants unable to bind PtdIns(4,5)P_2, we demonstrated that inhibition of secretion was directly linked to the interaction of Tat with PtdIns(4,5)P_2 at the plasma membrane. TatW11Y was especially informative since this mutant shows native transcriptional activity (Yezid et al., 2009), but no significant binding to PtdIns(4,5)P_2 (Rayne et al., 2010b). The fact that it did not modify secretion indicated that Tat transcriptional activity was not involved in the inhibition of secretory activity. Additionally, PIP5K was able to rescue secretion from cells exposed to Tat, confirming the idea that Tat blocks secretion through its capacity to bind to the plasma membrane-associated PtdIns(4,5)P_2 with high affinity.

We attempted to identify the PtdIns(4,5)P_2-binding components of the exocytotic machinery that might be displaced by Tat when present at the plasma membrane. Translocation of the PtdIns(4,5)P_2-binding protein annexin A2 to the plasma membrane following cell stimulation is a hallmark

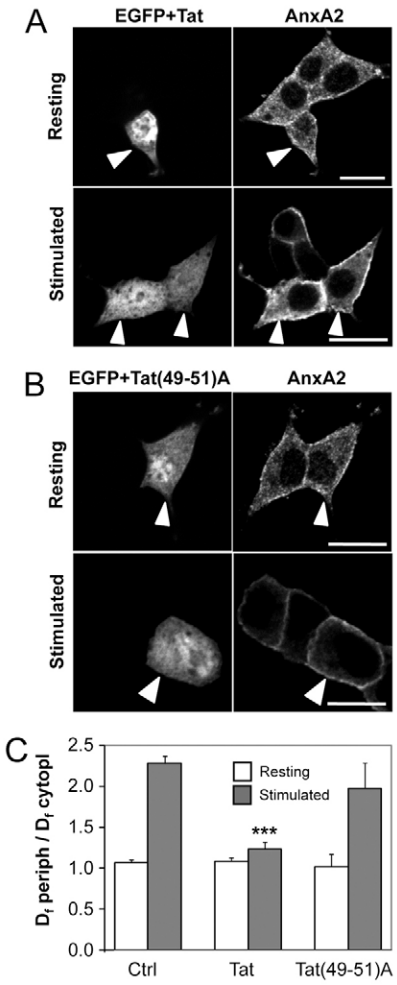

Fig. 6. Tat inhibits annexin A2 recruitment to the plasma membrane in stimulated PC12 cells. PC12 cells were transfected with a vector encoding Tat and EGFP (**A**) or Tat(49–51)A and EGFP (**B**). After 24 hours, cells were stimulated with 300 μM ATP for 3 min before fixation and immunostaining for endogenous annexin A2. Representative confocal micrographs are shown. Scale bars: 10 μm. (**C**) Quantification of annexin A2 fluorescence at the cell periphery compared to the cytoplasm. Results are expressed as the ratio of fluorescence densities (D_f) at the cell periphery versus the cytoplasm. Mean values ± s.e.m. are shown [$n=27$ for Ctrl and Tat, $n=14$ for Tat(49–51)A; ***$P<0.001$ compared to the control stimulated condition].

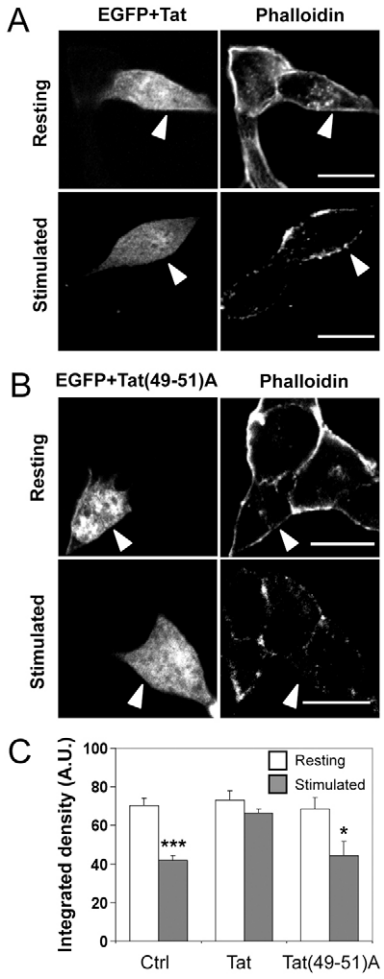

Fig. 7. Tat inhibits cortical actin depolymerization in stimulated PC12 cells. PC12 cells were transfected with a vector encoding Tat and EGFP (**A**) or Tat(49–51)A and EGFP (**B**). After 24 hours, cells were stimulated with 300 μM ATP for 3 min before fixation and phalloidin staining. Scale bars: 10 μm. (**C**) Quantification of fluorescent phalloidin signal intensities at the cell periphery. Results are expressed as integrated density per surface unit in arbitrary units (A.U.). Mean values ± s.e.m. are plotted [$n=22$ for Ctrl and Tat, $n=12$ for Tat(49–51)A]. *$P<0.05$; ***$P<0.001$ when compared to the respective resting condition.

of chromaffin cell exocytosis. Annexin A2 plays an essential role in calcium-regulated exocytosis by promoting ganglioside GM1/cholesterol-containing lipid regions in the plasma membrane (Chasserot-Golaz et al., 2005; Chasserot-Golaz et al., 2010; Umbrecht-Jenck et al., 2010). We recently described that, at the plasma membrane, annexin A2 is found in the vicinity of docked secretory granules, colocalizing with syntaxin-1A in PtdIns(4,5)P_2-enriched domains (Umbrecht-Jenck et al., 2010), and proposed that annexin A2 is a promoter or stabilizer of genuine exocytotic platforms in neuroendocrine cells. As shown here, Tat is able to displace annexin A2 from the cell periphery in stimulated cells, suggesting that the high affinity binding of Tat to PtdIns(4,5)P_2 interferes with the recruitment of annexin A2. Annexin A2 could in principle bind to other acidic lipids present at the inner surface of the plasma membrane such as phosphatidylserine (PS). However, compared to PtdIns(4,5)P_2, Tat does not significantly interact with PS (Rayne et al., 2010b). The fact that annexin A2 recruitment is severely impaired in Tat-expressing cells is also an indication that binding to PtdIns(4,5)P_2 is most likely the trigger for the recruitment of annexin A2 to the plasma membrane in cells stimulated for exocytosis. Impairment of annexin A2 translocation in cells exposed to Tat might led to a reduction in the number of functional exocytotic sites and, as observed here, to a concomitant decrease in the number of exocytotic events.

The cortical F-actin network constitutes a negative clamp on the movement of secretory vesicles to release sites, and it must be locally disassembled and reorganized to allow translocation of secretory vesicles in preparation for exocytosis (Vitale et al., 1995). During stimulation, the entry of Ca^{2+} activates scinderin and gelsolin, which in turn provoke the disassembly of the cortical F-actin network. The activity and the binding to actin of

these two F-actin severing proteins that are constituents of the exocytotic machinery have been shown to be regulated by PtdIns(4,5)P_2 (Trifaró et al., 2008). Thus the observation that Tat hampers the actin cytoskeleton reorganization during neurosecretion may also be a direct consequence of Tat binding to PtdIns(4,5)P_2.

Altogether, our findings suggest that the inhibitory effect of Tat on neurosecretion is linked to its high affinity binding to PtdIns(4,5)P_2, which interferes with important components of the exocytotic machinery relying on PtdIns(4,5)P_2. Inhibition of secretion was observed at nM concentrations of Tat, comparable with the concentrations reported for circulating Tat (Westendorp et al., 1995; Xiao et al., 2000), thereby arguing for the physiological significance of our findings. Yet, the ability of Tat to inhibit the exocytotic machinery, once present at the inner face of the plasma membrane, remains an intriguing observation. Intracellular PtdIns(4,5)P_2 concentrations have been estimated in the micromolar range (McLaughlin et al., 2002). It is therefore difficult to postulate that Tat may titrate the entire pool of cellular PtdIns(4,5)P_2 and impact its availability, especially when exogenously supplied. One hypothesis is that Tat efficiently binds and concentrates at specific location of the plasma membrane, like the exocytotic sites characterized by a specific protein and lipid composition. This would confer a preferential titration of the PtdIns(4,5)P_2 at these sites and directly impair the exocytotic machinery. Alternatively, Tat may efficiently activate PtdIns(4,5)P_2-catalyzing pathways or inhibit the synthesis of PtdIns(4,5)P_2. Either way, these combined effects at exocytotic sites during stimulation may efficiently starve the exocytotic machinery from the required PtdIns(4,5)P_2.

Pathological relevance

HIV-1 infection frequently leads to neurological disorders such as depression, motor dysfunction, and cognitive problems (Li et al., 2009). Because HIV-1 does not infect neurons, indirect mechanisms are probably responsible for neurological problems (King et al., 2006) and likely involve viral proteins like Tat (Ghafouri et al., 2006). Indeed, Tat can be toxic to neurons via glial cell activation (Ghafouri et al., 2006; King et al., 2006). Tat can also induce reversible synapse loss in primary neuron cultures (Kim et al., 2008) and completely capsize neuron/glia ratio in the hippocampus (Fitting et al., 2010). In addition, it is becoming increasingly apparent that Tat can directly alter neuronal functions (Neri et al., 2007). For instance, Tat is able to activate NMDA receptors by receptor phosphorylation (King et al., 2010; Li et al., 2008; Neri et al., 2007). Tat also appears to have an impact on dopamine (Ferris et al., 2009), norepinephrine (Longordo et al., 2006), glutamate and GABA release (Musante et al., 2010). Moreover, it was recently shown that Tat application increased dorsal root ganglion neuron excitability (Chi et al., 2011). Although these findings suggest that Tat is able to affect neurotransmission, direct evidence of a Tat effect on the molecular process of exocytosis has been missing.

We demonstrate here for the first time that Tat directly affects exocytosis of large dense core secretory granules in neuroendocrine cells as early as a few hours after application. Using the classical synaptic vesicle (SV) dye FM1-43, we observed that Tat prevented vesicle loading in primary neurons (supplementary material Fig. S6). Since SV loading requires efficient exocytosis and subsequent endocytosis, these findings suggest that Tat also affects the exo-endocytosis cycle in neurons. Therefore, it is likely that Tat perturbs SV trafficking and thus influences neurotransmitter release, as suggested earlier (Neri et al., 2007).

HIV-1-infected patients not only develop AIDS, but also several other serious related pathologies. These include lypohypertrophy due to decrease in growth hormone secretion (Leung and Glesby, 2011), pubertal delay caused by the dysregulation of hypothalamic-pituitary axis (Majaliwa et al., 2009) and impaired thyroid functions (Hoffmann and Brown, 2007). All of these aberrant functions are linked to impaired hormone secretion, in line with the results presented here.

In conclusion altered neurotransmission and hormone secretion may contribute to the development of neurological dysfunctions in HIV-infected patients. Tat-mediated inhibition of neurosecretion is likely involved in the development of HIV-1-associated disorders that arise even in patients treated by highly active antiretroviral therapy (Ghafouri et al., 2006).

Materials and Methods

Reagents, expression vectors and antibodies

Fluo-4 direct calcium assay kit and FM1-43FX styryl dye were purchased from Molecular Probes. hGH ELISA kits were from Roche Applied Science, Lipofectamine 2000 from Invitrogen, and basic Nucleofector Kit from Lonza/Amaxa Biosystem. Recombinant Tat (BH10 isolate, 86 amino acids) was produced and purified from *E. coli* as described (Vendeville et al., 2004). Expression vectors coding for HIV-1 Tat or Tat mutants (BH10 isolate, 86 residues) were as described (Rayne et al., 2010a).

Mouse monoclonal anti-HIV-1 Tat antibody (sc-65912) and rabbit polyclonal anti-syntaxin 1A were from Santa Cruz Biotechnology. Mouse monoclonal and rabbit polyclonal anti-SNAP-25, and rabbit polyclonal anti-human GH were obtained as indicated (Béglé et al., 2009). Monoclonal anti-PtdIns(4,5)P_2 antibody (IgM) was purchased from Abcam (2C11). Rabbit polyclonal antibody against early endosomal antigen (EEA1) was from Abcam. Alexa-Fluor-488, -555 or -647-labeled secondary anti-mouse and anti-rabbit antibodies were obtained from Molecular Probes. The GFP-synaptojanin and inducible 5-phosphatase plasmids were kindly provided by Drs P. De Camilli (Yale School of Medicine) and T. Balla (NICHD, NIH), respectively.

Cell cultures

PC12 cells were cultured as described previously (Zeniou-Meyer et al., 2007). Freshly dissected primary bovine chromaffin cells were cultured in DMEM in the presence of 10% foetal calf serum, 10 µM cytosine arabinoside, 10 µM fluorodeoxyuridine and antibiotics as described previously (Vitale et al., 1993).

Cell incubation with recombinant Tat

Treatment of PC12 or chromaffin cells with exogenous recombinant HIV-1 Tat protein or citrate buffer (vehicle, 50 mM sodium citrate, 100 mM NaCl, pH 7) was performed the day after cell seeding, or 72 hours after plating, respectively. Cells were incubated with 50 nM Tat for 30 min on ice to allow Tat binding to cells. Cells were then washed three times with Locke's buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 15 mM HEPES and 11 mM glucose, pH 7.4) and either immediately fixed (time 0 min or no chase) or put into the incubator at 37°C (chase) for various periods of time before fixation or cell stimulation. Using the *in situ* cell death detection kit, TMR red (Roche), we found that Tat treatment and overexpression did not induce apoptosis in PC12 cells.

Transfection

pBi-Tat, phGH, and pPIP5KIγ expression vectors (0.8 to 1 µg total DNA, 15×10^4 cells, 24-well plate) were introduced into PC12 cells using Lipofectamine 2000. Chromaffin cells were transfected by Amaxa Nucleofector (Lonza/Amaxa Biosystem) according to the manufacturer's protocol (3 µg DNA, 5×10^6 cells, 24-well plate). Under these conditions, transfection efficiency ranged from 60 to 75% for PC12 cells and from 20 to 40% for chromaffin cells. Co-transfection efficacy was systematically verified by immunostaining and was between 80 and 90%. Experiments were performed 24 hours after transfection.

Plasma membrane sheet preparation

Plasma membrane sheets were prepared according to the following protocol. After incubation with exogenous recombinant Tat for 30 min on ice, cells on coverslips were washed four times with Locke's solution and, when indicated, incubated for 4 hours at 37°C to allow Tat internalization. Cells were then submitted to hypotonic shock with water to burst cells following the lysis-squirting procedure

described previously (Ziegler et al., 1998). Plasma membrane sheets attached on the coverslip were fixed with 2% paraformaldehyde at 4℃ during 2 hours and labeled with anti-Tat or anti-syntaxin 1A antibodies.

Immunofluorescence and confocal microscopy

PC12 or chromaffin cells were washed twice with warm Locke's solution and stimulated 5 min with Locke's solution containing a depolarizing concentration of potassium (59 mM) or 300 μM ATP before fixation by 4% paraformaldehyde in 120 mM phosphate buffer and permeabilization with 0.025% saponin in phosphate-buffered saline containing 0.1% BSA at room temperature. Cells were further processed for anti-Tat (1:200), anti-EEA1 (1:500), anti-GH (1:200), anti-SNAP-25 (1:200), anti-syntaxin 1A (1:150) or anti-annexin A2 (1:200) immunofluorescence as described previously (Béglé et al., 2009). Fixation and anti-PtdIns(4,5)P_2 (1:100) staining were performed on ice as described (Hammond et al., 2006; Hammond et al., 2009). Stained cells were visualized using a SP5II Leica or LSM 510 Zeiss confocal microscope equipped with a 63× objective. Median confocal sections were recorded and quantified as indicated below. Images of membrane sheets were obtained using SP5II Leica with 63× objective (NA1.4) and pinhole adjustment of 0.68 Airy units.

Amperometry

Electrochemical measurements of catecholamine secretion were performed 4–6 hours after treatment with recombinant Tat protein or vehicle (citrate buffer) as described previously (Chasserot-Golaz et al., 2005). Catecholamine secretion was evoked by applying K^+ (100 mM) or nicotine (100 μM) in Locke's solution without ascorbic acid for 5 seconds to single cells by mean of a glass micropipette positioned at a distance of 40–50 μm from the cell.

Growth hormone release assay

PC12 cells (15×10^4 cells, 24-well plates, 80% confluence) were transfected with pBi-Tat (0.6 μg/well) and a plasmid encoding GH (0.3 μg/well) using Lipofectamine 2000. 24 hours after transfection, cells were washed four times for 5 min with Locke's solution and then incubated for 5 min in Locke's solution (basal release) or stimulated for 5 min with a depolarizing concentration of K^+ or with 300 μM ATP. Supernatants were collected and cells were harvested by scrapping in 10 mM phosphate-buffered saline and lyzed by three freeze and thaw cycles. The amounts of GH secreted into the medium or retained within the cells were measured using an ELISA assay (Roche Applied Science). GH secretion is expressed as a percentage of total GH present in the cells before stimulation (Lam et al., 2008). GH release from permeabilized cells was processed as described previously (de Barry et al., 2006).

Western blotting

Confluent PC12 cells grown in 3.5 cm diameter dishes were transfected with pBi-Tat and either an empty vector or a PIP5K-expressing plasmid (4 μg total DNA). The day after transfection, cells were harvested on ice by scraping in lysis buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate and protease inhibitors). Total cellular protein (30–40 μg) extracts were analyzed on 4–20% polyacrylamide–SDS gels. Immunoblots were then revealed with anti-actin and anti-Tat antibodies. Blots were processed using the Super Signal detection system (Pierce) as described previously (Corrotte et al., 2006). Immunoreactive bands were quantified using ImageJ. Tat band density was normalized to that of actin from the same lane and expressed as means ± s.e.m. of three experiments that were performed in duplicates using three different cell cultures.

Data and image analysis

Mask images were created with the LasAF Software 2.3.5 (Leica) using the colocalization application provided. After background threshold determination to eliminate low intensity pixels, colocalized pixels were visualized on black and white overlay images (Masks). Immunofluorescence images showing Tat (Figs 3, 4), annexin A2 (Fig. 6) or phalloidin (Fig. 7) staining were quantified using ImageJ. The amount of Tat or annexin A2 was measured as the average fluorescence intensity normalized to the corresponding surface area (D_f) at the cell periphery (plasma membrane level) and in the cytoplasm. Results are expressed as ratios of D_f (cell periphery)/D_f (cytoplasm). The amount of phalloidin staining was quantified at the level of cell periphery and expressed as fluorescence intensities normalized to surface units. This allows a quantitative cell-to-cell comparison of the fluorescent signal. Colocalization analyses were performed with LasAF Software 2.3.5 (Leica) using the colocalization application provided. Precisely, the threshold of each channel was set individually and independently from the other channel. Cut-off settings of the background fluorescence were generally around 20%, except for the EEA1 antibody, where they were around 30%. The overlay image and the scatter plot is automatically generated by the software algorithm resulting in a table that included colocalization percentages and Pearson's correlation coefficient (PCF) values.

Statistical significance was estimated using Student's *t*-test and data were considered significantly different when $P<0.05$. Gaussian distribution of the data was verified.

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Supplementary Fig. 1. Extracellular Tat progressively colocalizes with LAMP1. A. PC12 cells were treated with exogenous recombinant Tat protein (50 nM) for 30 min on ice. Cells were washed and either immediately fixed (0 min) or chased at 37 °C for the indicated times then fixed. They were then processed for Tat and LAMP1 immunolabeling. Representative median confocal sections of at least 25 cells per condition are shown. Mask images highlight the co-localization staining of Tat and LAMP1. Bar: 10 µm. **B.** Quantification of the colocalization level between Tat and LAMP1 was measured at different time points of chase (n > 15 cells for each time point).

Supplementary Fig. 2. Effect of Tat on GH expression level. Cells were co-transfected with a plasmid expressing GH and a bicistronic plasmid expressing EGFP (Ctrl) or Tat and EGFP (Tat). The day after transfection cells were incubated for 5 min in Locke's solution (resting, open bars) or stimulated for 5 min with Locke's solution containing 300 µM ATP (stimulated, grey bars). The amounts of GH secreted into the medium or retained within cells were measured using an ELISA assay. Total GH of each well was calculated as the sum of secreted and retained GH. Values are means ± S.E.M obtained from 6 independent experiments performed in triplicates.

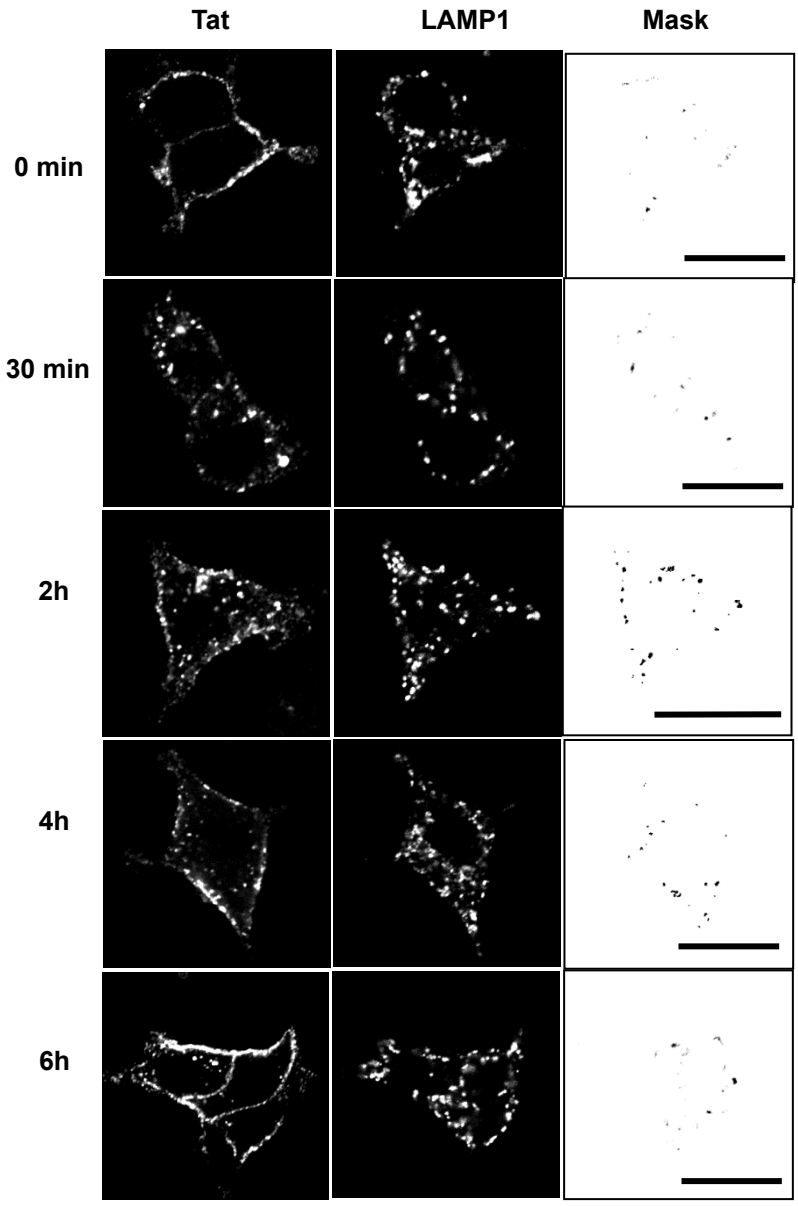
Supplementary Fig. 3. Effect of Tat on calcium level in PC12 cells. Cells were transfected with a bicistronic vector expressing only EGFP (ctrl) or Tat and EGFP (Tat). After 24 h cells were incubated with Fluo-4 dye, stimulated with depolarizing concentration of potassium and intracellular calcium level was quantified as described in materials and methods. Measurement was repeated every 30 sec over several min. Values are means ± S.E.M. obtained from 3 independent experiments performed in duplicate. Fluorescence values were normalized to non-stimulated cells at time zero.

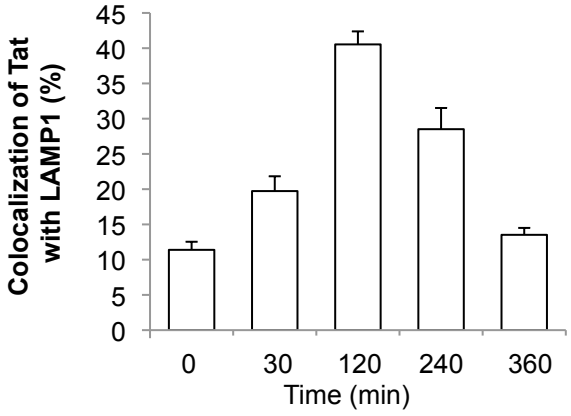
Supplementary Fig. 4. Intracellular localization of expressed Tat, Tat(49-51)A, and Tat(55-57) in chromaffin cells. Cells were transfected with a bicistronic plasmid encoding EGFP and either TatWT, Tat(49-51)A, or Tat(55-57)A separately. **A.** Tat was localized by immunostaining 24 hours post-transfection together with $PI(4,5)P_2$. Representative, median confocal sections are shown. Bars, 10 µm. **B.** Quantification of the colocalization level between TatWT or the indicated Tat mutant and PIP2 was measured. Data are shown as mean ± S.E.M. of n > 15 cells for each experimental condition and from 2 independent cell cultures (***$P < 0.001$).

Supplementary Fig. 5. Expression level of Tat and Tat mutants in PC12 cells. A. Western blot analysis showing Tat and mutants expression levels. PC12 cells were transfected with TatWT or Tat mutant encoding vectors as indicated. 30 µg of total cellular protein extracts were loaded on gels and membranes were processed for anti-actin and anti-Tat immunoblot. As positive control for Tat detection, 30 ng of recombinant Tat were loaded. **B.** Quantification of Western blots are expressed as integrated density per surface unit in arbitrary units (A.U.) and normalized to actin levels. Results are means ± S.E.M. of 2 experiments performed in duplicates derived from 2 different cell cultures.

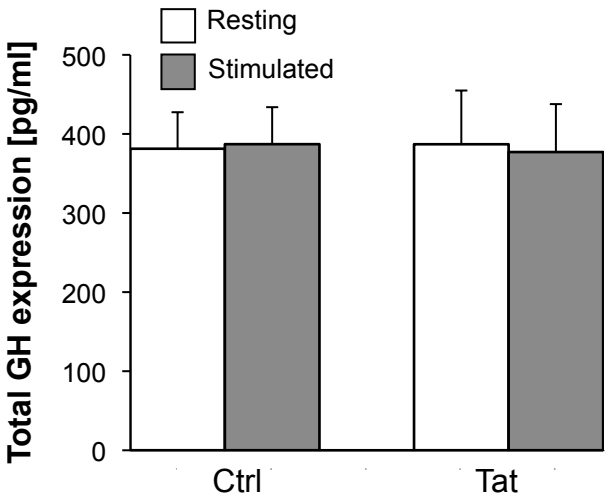
Supplementary Fig. 6. Extracellular HIV-1 Tat affects FM1-43FX dye loading in presynaptic vesicles in primary neuron cultures. A. FM1-43FX dye and synaptotagmin staining in control and Tat treated (50 nM Tat for 30 min at 37°C and then chased for 4 h at 37°C) mouse primary cortical neurons in resting or stimulated conditions are shown. **B.** Quantification of colocalization between FM1-43FX dye and synaptotagmin staining is shown as mean ± S.E.M. of 15-27 cells for each experimental condition and from 3 independent cell cultures (***$P < 0.001$). In control cells, FM1-43FX dye unloads due to efficient exocytosis leading to much lower colocalisation rate (~ 20%) between Tat and synaptotagmin, while Tat treated neurons exhibit FM1-43FX dye loading deficiency demonstrating that synaptic vesicle exo-endocytosis cycle is affected by Tat.

Tryoen-Tóth et al. Supplementary Fig. 1





Tryoen-Tóth et al. Supplementary Fig. 2



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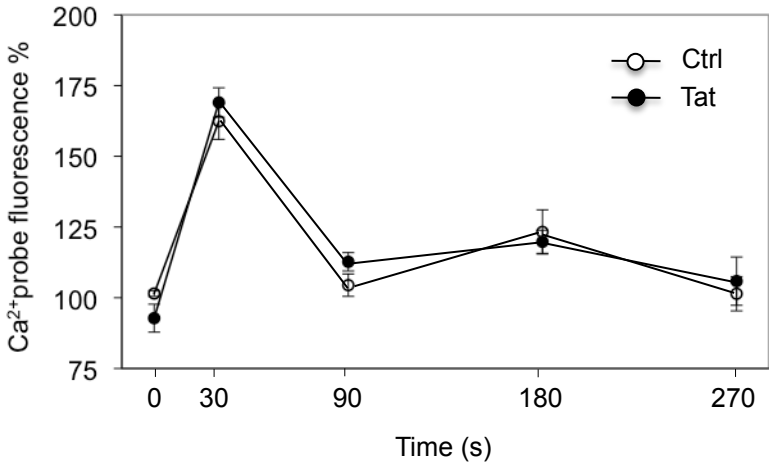

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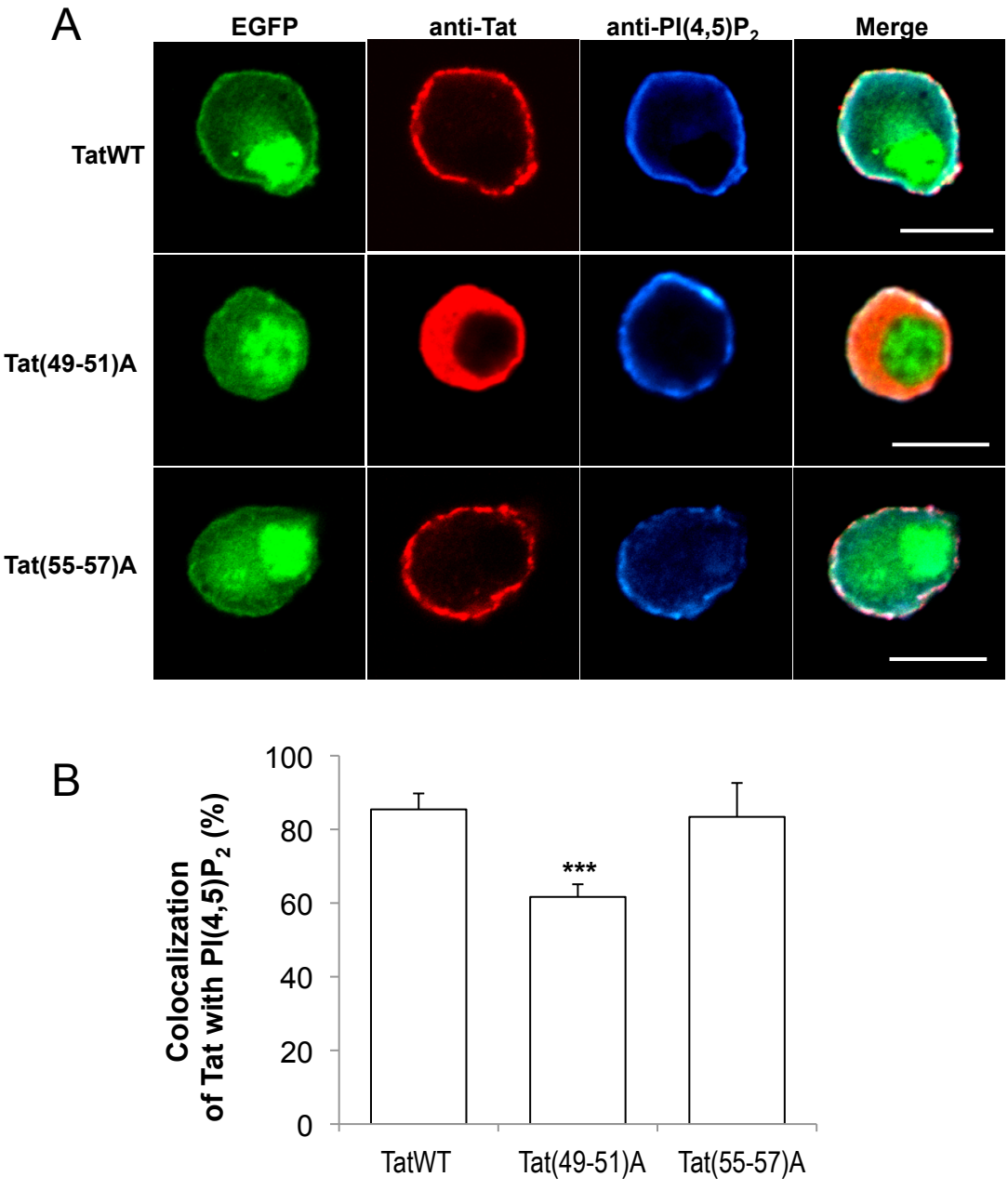
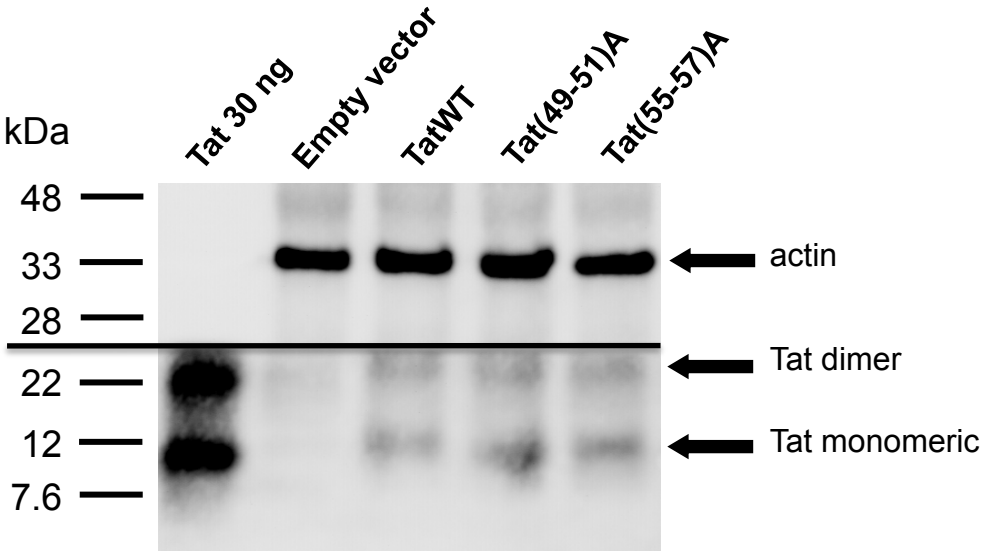


Tryoen-Tóth et al. Supplementary Fig. 5

A



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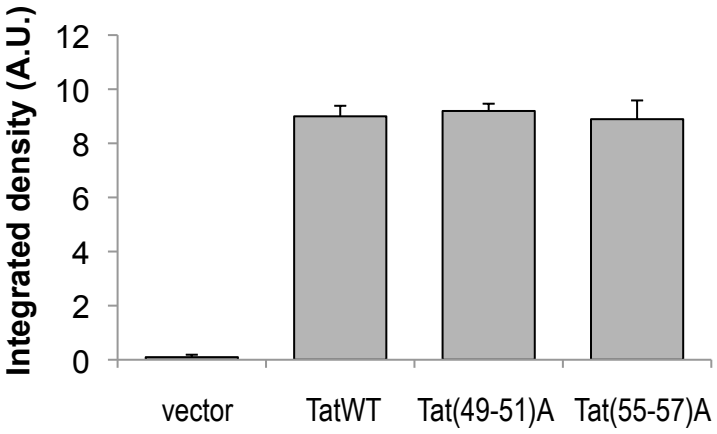

Tryoen-Tóth et al. Supplementary Fig. 6.