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HIV Tat excites D1 receptor-like expressing neurons from rat nucleus accumbens

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Abstract

BACKGROUND—HIV-1 infection and drug abuse are frequently co-morbid and their association greatly increases the severity of HIV-1-induced neuropathology. While nucleus accumbens (NAcc) function is severely perturbed by drugs of abuse, little is known about how HIV-1 infection affects NAcc.

METHODS—We used calcium and voltage imaging to investigate the effect of HIV-1 trans-activator of transcription (Tat) on rat NAcc. Based on previous neuronal studies, we hypothesized that Tat modulates intracellular Ca^{2+} homeostasis of NAcc neurons.

RESULTS—We provide evidence that Tat triggers a Ca^{2+} signaling cascade in NAcc medium spiny neurons (MSN) expressing D1-like dopamine receptors leading to neuronal depolarization. Firstly, Tat induced inositol 1,4,5-trisphosphate (IP_3) receptor-mediated Ca^{2+} release from endoplasmic reticulum, followed by Ca^{2+} and Na^+ influx via transient receptor potential canonical channels. The influx of cations depolarizes the membrane promoting additional Ca^{2+} entry through voltage-gated P/Q-type Ca^{2+} channels and opening of tetrodotoxin-sensitive Na^+ channels. By activating this mechanism, Tat elicits a feed-forward depolarization increasing the excitability of D1-phosphatidylinositol-linked NAcc MSN. We previously found that cocaine targets NAcc neurons directly (independent of the inhibition of dopamine transporter) only when IP_3 -generating mechanisms are concomitantly initiated. When tested here, cocaine produced a dose-dependent potentiation of the effect of Tat on cytosolic Ca^{2+} .

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CONCLUSION—We describe for the first time a HIV-1 Tat-triggered Ca^{2+} signaling in MSN of NAcc involving TRPC and depolarization and a potentiation of the effect of Tat by cocaine, which may be relevant for the reward axis in cocaine-abusing HIV-1-positive patients.

Keywords

calcium imaging; intracellular calcium mobilization; calcium influx; depolarization; TRPC; medium spiny neurons

1. INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) infection and drug abuse are interlinked epidemics (WorldHealthOrganization, 2009) and the poor neurological outcome of this association has been previously documented (Hauser et al., 2007; McCabe et al., 2014; Nath, 2010; Purohit et al., 2013; Purohit et al., 2011).

HIV-1 invades the brain soon after systemic infection, targeting microglia, perivascular macrophages and astrocytes (Gonzalez-Scarano and Martin-Garcia, 2005; Mocchetti et al., 2012). Neurons are resistant to HIV-1 infection, but subject to the neurotoxic action of viral proteins and proinflammatory molecules released by HIV-1-infected glial cells (Gonzalez-Scarano and Martin-Garcia, 2005; Hauser et al., 2007). Although efficient in reducing the viral load, antiretroviral therapy cannot prevent the production of viral proteins (Rumbaugh et al., 2008). HIV-1 proteins are critical neuropathological factors in HIV-1-infected individuals (Nath, 2002) and their neurotoxicity is enhanced in the presence of drugs of abuse, including cocaine (Aksenov et al., 2006; Meyer et al., 2013; Wayman et al., 2015a; Wayman et al., 2015b).

The trans-activator of transcription (Tat) protein, an important contributor to HIV-1-associated neurological impairment (Romani et al., 2010), is expressed at high levels in the brains of HIV-1 infected individuals (Li et al., 2009) and remains present in the cerebrospinal fluid of persons with suppressed viremia due to efficient antiretroviral treatment (Johnson et al., 2013). HIV-1 Tat regulatory protein is essential for viral transcription and replication; it is encoded by two exons, with several well-conserved protein domains found on the first exon: the acidic domain (1–21), the cysteine rich domain (22–37), the core/hydrophobic region (38–48), and the basic domain (49–72) (Jeang et al., 1999; Kuppuswamy et al., 1989; Ruben et al., 1989). Full length Tat protein has 101 amino acids, with residues 1–72 encoded by the first exon and residues 73–101 encoded by a second exon (Jeang et al., 1999). The first 56 residues of Tat are well-conserved, indicating important functional roles (Debaisieux et al., 2012; Romani et al., 2010). The cysteine rich region and the basic domain play major roles in Tat-induced neurotoxic and excitatory effects (Li et al., 2009).

Previous studies have investigated functional interactions between Tat and drugs of abuse (particularly cocaine) in an attempt to identify potential mechanisms of the accelerated and exacerbated cognitive dysfunction seen in drug-abusing HIV-1-infected individuals who engage in substance abuse as compared with those who do not (Aksenov et al., 2006; Ferris et al., 2008; Meyer et al., 2013; Wayman et al., 2016; Wayman et al., 2015a; Wayman et al.,

2015b). Prior studies have focused investigations on the hippocampus and the frontal cortex due to their established roles in memory and cognition. Interestingly, the nucleus accumbens (NAcc), a key node controlling reward-directed behavior including substance abuse, has received less attention in this regard despite the fact that HIV-1 aggregates in the caudate nucleus and NAcc, with the highest concentration occurring in the latter (Wiley et al., 1998).

We have previously identified excitatory effects of Tat on cortical and nucleus ambiguus neurons and demonstrated that several neuropathological consequences of HIV-1 infection are mediated by Tat interference with Ca^{2+} signaling mechanisms (Brailoiu et al., 2006; Brailoiu et al., 2014; Brailoiu et al., 2008). In the present study, we used a similar approach to examine whether and how Tat may alter neuronal function in the rat NAcc. A second aim of the study was to determine if there is a significant interaction between the effects of Tat and cocaine on NAcc medium spiny neurons.

2. MATERIALS AND METHODS

2.1. Ethical approval

Animal protocols were approved by the Institutional Animal Care and Use Committees from Temple University and Thomas Jefferson University. Seventy-two neonatal (1–2 days old) Sprague Dawley rats (Ace Animal Inc., Boyertown, PA) of both sexes were used in these studies. The present study followed the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines and the National Institutes of Health guide for the care and use of Laboratory animals (Institute for Laboratory Animal Research, 2011).

2.2. Chemicals

All chemicals were from Sigma Aldrich (St. Louis, MO), unless otherwise mentioned. HIV-1 Tat clade-B recombinant (1–86) was from ProspeC (East Brunswick, NJ). In control experiments, HIV-1 Tat was heat-inactivated by repeated (10 times) heating (75°C for 30 s) and cooling (4°C for 1 min), as previously reported (Brailoiu et al., 2014).

2.3. Neuronal cell culture

NAcc neurons were dissociated from neonatal (1–2 day old) Sprague Dawley rats (Ace Animal Inc., Boyertown, PA) of both sexes as previously described (Barr et al., 2015). Newborn rats were decapitated and the brains quickly removed and immersed in ice-cold Hanks balanced salt solution (HBSS) (Mediatech, Herndon, VA). The nAcc was identified, removed, minced and subjected to enzymatic digestion (papain, 37°C), followed by mechanical trituration in presence of total medium – Neurobasal A (Invitrogen, Carlsbad, CA) containing 1% GlutaMax (Invitrogen), 2% penicillin-streptomycin-amphotericin B solution (Mediatech) and 10% fetal bovine serum. Cells were cultured on round 25 mm glass coverslips coated with poly-L-lysine (Sigma-Aldrich) in six-well plates. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 . The mitotic inhibitor cytosine β -arabinofuranoside (1 μM) (Sigma-Aldrich) was added to the culture on the third day to inhibit glial cell proliferation. Cells were used after 5 days in culture.

2.4. Calcium imaging

The intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, was measured as previously described (Barr et al., 2015). Cells were incubated with 5 μM Fura-2 AM (Invitrogen, Carlsbad, CA) in HBSS at room temperature for 45 min, in the dark, washed three times with dye-free HBSS, and then incubated for another 45 min to allow for complete de-esterification of the dye. Coverslips (25 mm diameter) were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon Inc., Melville, NY). The microscope is equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ). During the experiments, the Perfect Focus System was activated. Fura-2 AM fluorescence (emission = 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using NIS-Elements AR 3.1 software (Nikon Inc.). After appropriate calibration with ionomycin and CaCl_2 , and Ca^{2+} free and EGTA, respectively, the ratio of the fluorescence signals (340/380 nm) was converted to Ca^{2+} concentrations. In Ca^{2+} -free experiments, CaCl_2 was omitted.

2.5. Measurement of membrane potential

The relative changes in membrane potential of single neurons were evaluated using bis-(1,3-dibutylbarbituric acid) trimethine oxonol, DiBAC₄(3), a slow response voltage-sensitive dye, as previously described (Barr et al., 2015). Upon membrane hyperpolarization, the dye concentrates in the cell membrane, leading to a decrease in fluorescence intensity, while depolarization induces the sequestration of the dye into the cytosol, resulting in an increase of the fluorescence intensity. Cultured accumbens neurons were incubated for 30 min in HBSS containing 0.5 μM DiBAC₄(3) and the fluorescence monitored at 0.17 Hz, excitation/emission: 480 nm/540 nm. Calibration of DiBAC₄(3) fluorescence following background subtraction was performed using the Na^+ - K^+ ionophore gramicidin in Na^+ -free physiological solution and various concentrations of K^+ (to alter membrane potential) and N-methylglucamine (to maintain osmolarity). Under these conditions, the membrane potential was approximately equal to the K^+ equilibrium potential determined by the Nernst equation. The intracellular K^+ and Na^+ concentrations were assumed to be 130 mM and 10 mM, respectively.

2.6. Measurement of intracellular Na^+ levels

Cells grown on 25 mm diameter coverslips were incubated with 10 μM sodium-binding benzofuran isophthalate-AM (SBFI-AM), (Invitrogen, Carlsbad, CA) in HBSS at room temperature for 30 min in the dark, washed three times with dye-free HBSS, and then incubated for another 30 min to allow for complete de-esterification of the dye. SBFI fluorescence (emission = 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using NIS-Elements AR 3.1 software (Nikon Inc.). $[\text{Na}^+]_i$ was calculated according to the equation previously reported: $[\text{Na}^+]_i = K_d (R - R_{\min}) / (R_{\max} - R)$ (Harootunian et al., 1989). The parameters $R_{\min}$ and $R_{\max}$ were obtained from the values of the ratio of 0 Na^+ and 140 mM Na^+ , respectively.

2.7. Data analysis

Data are expressed as mean and standard error of mean. One-way ANOVA, followed by post-hoc Bonferroni and Tukey tests (Origin 7, OriginLab Corporation, Northampton, MA), were used to assess significant differences between groups; $P < 0.05$ was considered statistically significant. N's indicate the number of cells tested in each treatment group.

3. RESULTS

3.1. Selection of neurons of interest

Cultured NAcc medium spiny neurons (MSN) were selected for study based on their responsiveness to D1-like dopamine receptor agonist SKF83959 (10 μ M). Only the neurons that responded to application of SKF83959 were considered D1-like receptor positive MSN, signaling via Gq-coupled pathways (Chun et al., 2013; Jin et al., 2003) and used in further experiments. Depending on the experimental approach, responses to SKF83959 consisted of either an increase in cytosolic Ca^{2+} levels (Fig. 1A), a depolarization (Fig. 1B) or an increase in intracellular Na^+ (Fig. 1C). A recent study used *in vivo* Ca^{2+} imaging to demonstrate that it is the D1-like receptor positive NAcc MSN that encode context-reward associations, are dysregulated by prior cocaine exposure, and are important in relapse to drug use (Calipari et al., 2016). Thus, we assessed the effects of Tat on this particular neuronal type in the NAcc.

3.2. HIV-1 Tat elevates cytosolic Ca^{2+} concentration in D1-like receptor-positive NAcc MSN

In D1-like receptor-expressing NAcc MSN, Tat (500 nM) induced a fast increase in intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, followed by a relatively slow decrease to baseline, while heat-inactivated Tat (500 nM) was ineffective (Fig. 2A, B). The amplitudes of the Ca^{2+} elevations produced by increasing concentrations of Tat (5, 50, 500 and 5000 nM) were 38 ± 2.8 nM, 276 ± 3.4 nM, 619 ± 5.4 nM and 723 ± 7.9 nM, respectively (Fig. 2C; $n = 8$ for each treatment group). Characteristic examples of changes in 340 nm/380 nm Fura-2 fluorescence ratio of cultured NAcc MSN in response to Tat (500 nM) and heat-inactivated Tat (500 nM) are shown in Fig. 2A. Previous *in vitro* studies evaluated the effects of Tat on a variety of endpoints with effective concentrations found between 100 and 500 nM (Badou et al., 2000; Brailoiu et al., 2014; Eletto et al., 2008; Pocernich et al., 2005; Sui et al., 2006). Since robust responses were elicited with 500 nM Tat, similar to the above-cited reports, we used this concentration in our following experiments.

3.3. Tat mobilizes Ca^{2+} from endoplasmic reticulum Ca^{2+} stores

In absence of extracellular Ca^{2+} , Tat (500 nM) triggered a fast and transient $[\text{Ca}^{2+}]_i$ elevation (Fig. 3A) by 264 ± 4.2 nM ($n = 6$) at the peak of the response, and an area under curve (A.U.C.) of 177 ± 2.9 nM $\times$ min (Fig. 3B). This response was significantly reduced as compared to the effect of Tat in Ca^{2+} -containing saline (with 619 ± 5.4 nM amplitude and 2443 ± 46 nM $\times$ min A.U.C., Fig. 2 and Fig. 4A, B). Blocking inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3 Rs) with xestospongin C (XeC, 10 μ M, 15 min) and 2-aminoethoxydiphenyl borate (2-APB, 100 μ M, 15 min), but not inhibition of ryanodine receptors with ryanodine (Ry, 10 μ M, 1h) or of lysosomal NAADP-sensitive two pore

channels with Ned-19 (5 μ M, 15 min) (Naylor et al., 2009), abolished the Ca^{2+} response of NAcc MSN to Tat, indicating that the response was IP_3R -mediated (Fig. 3A,B). In the presence of these blockers, the effect of Tat on $[\text{Ca}^{2+}]_i$ measured 16 ± 2.3 nM (XeC + 2-APB; A.U.C. of 15 ± 1.6 nM $\times$ min, $n = 6$ cells), 251 ± 3.9 nM (Ry; A.U.C. of 161 ± 3.4 nM $\times$ min, $n = 6$) and 257 ± 4.3 nM (Ned-19; A.U.C. of 168 ± 2.7 nM $\times$ min, $n = 6$), respectively (Fig. 3B).

3.4. Tat promotes Ca^{2+} influx in NAcc MSN

Because we observed that mobilization of intracellular Ca^{2+} stores only partially accounted for the Tat-induced Ca^{2+} increase in D1-like receptor-expressing NAcc MSN, we further tested the involvement of a Ca^{2+} entry mechanism. Inhibition of N-type of voltage-activated Ca^{2+} channels with ω -conotoxin GVIA (100 nM, 20 min) did not significantly modify the kinetics of the Ca^{2+} signal produced by Tat (500 nM) (Fig. 4A). The amplitude and the A.U.C. were similarly unaffected: the amplitude of the Tat-induced response was 619 ± 4.8 nM ($n = 6$) in the absence versus 608 ± 5.6 nM ($n = 6$) in the presence of N-type Ca^{2+} channel blocker (Fig. 4B); the A.U.C. were 2443 ± 46 nM $\times$ min and 2398 ± 51 nM $\times$ min, respectively (Fig. 4B). Pretreatment of neurons with ω -conotoxin MVIIC (100 nM, 20 min), a blocker of P/Q-type of Ca^{2+} channels, slightly reduced the amplitude of the Tat-dependent Ca^{2+} elevation ($[\text{Ca}^{2+}]_i$ was 453 ± 3.7 nM, $n = 6$), but had a more profound effect on the A.U.C, which was $\sim 50\%$ reduced (1216 ± 38 nM $\times$ min, Fig. 4B), indicating a reduction in the decay time.

Since we found Tat to trigger IP_3 -mediated signaling and since this is known to induce opening of transient receptor potential canonical nonselective cation channels, TRPC, (Clapham, 2003; Deliu et al., 2014), we further tested the effect of TRPC inhibition on Tat-mediated increase in $[\text{Ca}^{2+}]_i$ in D1-like receptor-expressing NAcc MSN. Upon treatment of neurons with SKF96365 (2 μ M, 20 min), a TRPC blocker (Clapham et al., 2005; Zhu et al., 1998), Tat elicited a significantly blunted Ca^{2+} response, with a $\sim 50\%$ reduced peak and rather fast return to baseline (Fig. 4A); the amplitude measured 271 ± 3.9 nM ($n = 6$) and the A.U.C. was 203 ± 17 nM $\times$ min (Fig. 4B). In presence of BAPTA-AM (200 μ M, 30 min), Tat no longer elicited a significant increase in $[\text{Ca}^{2+}]_i$ (amplitude of 49 ± 1.9 nM, A.U.C. of 55 ± 18 nM $\times$ min, $n = 6$, Fig. 4A, B).

These results suggest that Tat induces Ca^{2+} entry via plasmalemmal P/Q-type of voltage-activated Ca^{2+} channels and TRPC, which is contingent on previous $[\text{Ca}^{2+}]_i$ release from endoplasmic reticulum.

3.5. Cocaine potentiates the effects of Tat on $[\text{Ca}^{2+}]_i$

Since we have previously found that cocaine directly enhances the effect of IP_3 -generating molecules in D1-like receptor expressing NAcc neurons (Barr et al., 2015), we next tested whether this was also true in the case of Tat. Similar to our previous study, we found that cocaine by itself did not alter the $[\text{Ca}^{2+}]_i$ of cultured NAcc MSN when used at concentrations of up to 100 μ M (Barr et al., 2015). Fig 5A illustrates the lack of effect on $[\text{Ca}^{2+}]_i$ of cocaine (10 μ M), the fast increase in $[\text{Ca}^{2+}]_i$ produced by Tat (500 nM) and the potentiation of the effect of Tat by co-administration of increasing concentrations of cocaine

(1 μ M, 5 μ M, 10 μ M and 15 μ M). The comparison of the amplitude and A.U.C. of the neuronal responses induced in each condition are illustrated in Fig. 5B. In D1-like receptor expressing NAcc MSN, cocaine (10 μ M) alone failed to promote a Ca^{2+} response ($[\text{Ca}^{2+}]_i$ of 7 ± 2.27 nM; A.U.C. of 23 ± 2.4 nM $\times$ min, $n = 6$ cells), while Tat (500 nM) increased $[\text{Ca}^{2+}]_i$ by 619 ± 5.4 nM and A.U.C. by 2443 ± 46 nM $\times$ min ($n = 6$). The amplitudes of the Ca^{2+} elevations produced by Tat (500 nM) in the presence of increasing concentrations of cocaine (1 μ M, 5 μ M, 10 μ M, and 15 μ M) were 634 ± 7.8 nM, 706 ± 11 nM, 829 ± 8.5 nM, and 903 ± 14.9 nM, respectively (Fig. 5B; $n = 8$ for each treatment group) demonstrating a dose-dependent effect of cocaine in the presence of Tat. Increases in A.U.C. produced by Tat (500 nM) and increasing concentrations of cocaine (1 μ M, 5 μ M, 10 μ M and 15 μ M) were 2557 ± 56 , 2849 ± 65 , 3938 ± 83 , and 4206 ± 82 nM $\times$ min, $n = 8$ cells tested (Fig. 5B). For example, co-administration of cocaine (10 μ M) and Tat (500 nM) resulted in a potentiation of the effect of Tat, which was increased in amplitude and A.U.C. by ~25% (the amplitude increased from 619 ± 5.4 nM in absence to 829 ± 8.5 nM in presence of cocaine, and the A.U.C. from 2443 ± 46 nM min for Tat alone and 3938 ± 83 nM min for Tat and cocaine, Fig. 5A,B).

The concentrations of cocaine tested here are well in the range of the levels reached by cocaine in the brain after systemic administration. For example, 5 min after 10 mg/kg cocaine is given to mice systemically, a peak value for cocaine of 2.6 μ g/g (~7.6–8.5 μ M) is seen in the brain, whereas after a 25 mg/kg the peak value is 6.7 μ g/g (~19.7–22.1 μ M) (Benuck et al., 1987); moreover, cocaine concentrations in the brain are always higher than those in the plasma, with an average brain/plasma ratio of 7 (Benuck et al., 1987).

3.6. Tat elicits concentration-dependent depolarization of D1-like receptor-expressing NAcc MSN

Tat (500 nM) produced a robust depolarization of D1-like receptor positive NAcc MSN, followed by a plateau phase and a slow return to baseline levels (Fig. 6A). Conversely, heat-inactivated Tat (500 nM) produced negligible effects on membrane potential (voltage was 0.57 ± 0.33 mV, Fig. 6A, B). When testing several concentrations of Tat (5, 50, 500 and 5000 nM) we observed a concentration-dependent increase in neuronal depolarization, with amplitudes of 0.46 ± 0.28 mV, 3.71 ± 0.65 mV, 8.24 ± 0.75 mV and 10.38 ± 0.82 mV, respectively (Fig. 6B, $n = 6$ neurons per each condition).

3.7. Tat-induced depolarization involves TRPC opening and is Ca^{2+} -contingent

Blocking voltage-gated Na^+ channels (VGNaC) with tetrodotoxin (TTX, 0.5 μ M, 20 min) produced a marked reduction of the amplitude and duration of Tat-induced depolarization (Fig. 7A). Treatment of the MSN with BAPTA-AM (200 μ M, 20 min), a fast Ca^{2+} chelator, largely abolished Tat-induced neuronal depolarization (Fig. 6A). Likewise, in the presence of TRPC inhibitor SKF96365 (2 μ M, 20 min), Tat produced a negligible response (Fig. 7A). The amplitude of the depolarization elicited by Tat (500 nM) was reduced from 8.24 ± 0.75 mV to 3.48 ± 0.4 mV in presence of TTX, to 0.43 ± 0.29 with BAPTA and to 0.93 ± 0.37 mV upon TRPC blockade, while A.U.C. decreased from 53.47 ± 0.37 mV $\times$ min to 5.11 ± 0.08 mV $\times$ min (TTX), to 0.87 ± 0.01 mV $\times$ min (BAPTA) and to 0.93 ± 0.08 mV $\times$ min (SKF96365), respectively (Fig. 7B).

3.8. Tat-triggered depolarization is reflected by Na⁺ currents in D1-like receptor-positive NAcc MSN

In Na⁺-imaging experiments, MSN responsive to the D1-like receptor agonist SKF83959, were further subjected to Tat (500 nM) application in absence and presence of pretreatments described in the section above. Using a slow-response dye, we detected an increase in intracellular Na⁺ concentration in response to Tat, measuring 8.94 ± 0.7 mM in amplitude and with an A.U.C. of 47.19 ± 0.37 mM $\times$ min (Fig. 8A, B). This experiment largely followed the design of the voltage-imaging experiment and allowed the detection of a significant decrease in Tat-induced Na⁺ entry after VGNaC blockade with TTX (amplitude 2.52 ± 0.43 mM; A.U.C. 3.24 ± 0.06 mM $\times$ min), a near complete abolishment with BAPTA (amplitude 0.26 ± 0.265 mM; A.U.C. 0.34 ± 0.08 mM $\times$ min) and a reduction to insignificant levels after TRPC inhibition with SKF96365 (0.64 ± 0.24 mM; A.U.C. 0.69 ± 0.06 mM $\times$ min, Fig 8A, B).

The use of slow-response voltage (and sodium) imaging dye(s) likely prevented us from recording fast changes in membrane potential, neuronal firing and associated large Na⁺ currents in response to Tat, which may have been evident with the use of electrophysiological techniques. Accordingly, we found TTX to significantly diminish the neuronal response to Tat both in voltage and sodium-imaging experiments. The remaining response in the presence of TTX might be attributed to sodium entry via TRP channels, whose signals parallel that of membrane depolarization; therefore, our results indicate that sodium entry via TRP channels plays an essential role in the effect of Tat in NAcc MSN.

4. DISCUSSION

Tat is a major neurotoxic protein produced by HIV-1 that promotes oxidative stress, Ca²⁺ responses and depolarization of neurons, not otherwise infected by the virus itself (Aksenov et al., 2006; Brailoiu et al., 2006; Brailoiu et al., 2014; Brailoiu et al., 2008; Li et al., 2009; Wayman et al., 2016). Deleterious effects of Tat are potentiated by cocaine in hippocampal (Aksenov et al., 2006) and medial prefrontal cortex pyramidal neurons (Napier et al., 2014; Wayman et al., 2016) and by ethanol in cortical neurons (Brailoiu et al., 2006), observations that correlate well with the exacerbated neurocognitive impairment in HIV-1 patients that use psychostimulants (Bauer, 2013; Meyer et al., 2013; Nath et al., 2001).

In the current study, we found that Tat promoted a concentration-dependent increase in intracellular Ca²⁺ levels in NAcc MSN expressing phosphatidylinositol-linked D1-like dopamine receptors, a robust effect with rapid onset and relatively slow return to baseline. Using pharmacological blockers, we further dissected the Ca²⁺ pathway activated by Tat, identifying an IP₃R-mediated component mobilizing Ca²⁺ from endoplasmic reticulum stores, as well as a Ca²⁺ influx mechanism occurring via TRPC nonselective cation channels and P/Q-type voltage-gated Ca²⁺ channels. In voltage and Na⁺-imaging experiments, we demonstrate a depolarizing effect of Tat with kinetics similar to that of the Ca²⁺ response and further reveal the contribution of the Ca²⁺ pathway to neuronal excitation: Tat induces an initial increase in intracellular Ca²⁺ by releasing IP₃-sensitive pools that further allows activation of TRPC (Clapham, 2003; Deliu et al., 2014). TRPC opening promotes Ca²⁺ and Na⁺ entry, thus depolarizing the neuron sufficient for TTX-sensitive Na⁺ channel opening

and P/Q activation (proposed mechanism illustrated in Fig. 9). This pathway promotes a feed-forward depolarization increasing the excitability of NAcc MSN expressing D1-like dopamine receptors.

We found it very striking that this mechanism has a large degree of overlap with a recently identified cocaine activated pathway in the same population of NAcc MSN, an effect of cocaine that is independent of the inhibition of dopamine transporter (Barr et al., 2015). In our previous study, the association of cocaine and an IP₃-generating factor induced TRPC opening and further depolarized NAcc MSN, thus increasing their excitability. The effect of cocaine on TRPC opening could not be promoted by cocaine alone (i.e. in the absence of IP₃) and played an important functional role *in vivo*, in cocaine-induced hyperlocomotion and sensitization (Barr et al., 2015). As shown by our data presented herein, cocaine similarly potentiated the effect of Tat on NAcc MSN.

Both HIV-1 Tat (Ferris et al., 2009; Zhu et al., 2009) and cocaine (Medvedev et al., 2013) are known to inhibit the function of the dopamine transporter. However, combined Tat and cocaine administration promotes a hyperdopaminergic tone in the NAcc that is not evident following cocaine or Tat alone (Ferris et al., 2010). Our present results may provide a possible explanation for the previously reported neurochemical changes in NAcc produced by Tat and cocaine (Ferris et al., 2010). Our findings reveal that Tat triggers the IP₃-mediated pathway previously demonstrated to be critical for the direct enhancement of NAcc neuronal activity produced by cocaine (Barr et al., 2015), serving further in the elucidation of the mechanisms underlying the neuropathological complications of HIV-1 infection in drug abusers.

Two additional implications may be inferred from our present study. One is that cocaine dependence may be more difficult to treat and relapse to drug use more prominent in HIV-1-positive patients as compared with non-infected cocaine users. Another implication is that NAcc function may be impaired in HIV-1 patients and that drug use may enhance this deleterious effect. Supporting this idea, Booze and colleagues demonstrated synaptodendritic alterations in NAcc MSN of HIV-1 transgenic female rats as a consequence of chronic, low-level exposure to HIV-1-associated proteins (Roscoe et al., 2014).

A dysfunction at the level of the NAcc can be manifested as depression (Nestler and Carlezon, 2006) and apathy (Levy and Dubois, 2006). Interestingly, the clinical picture of HIV-1-associated neurocognitive disorders includes mood disturbances, disorientation and psychomotor slowing, which are symptoms reminiscent of apathy (Alfahad and Nath, 2013). Noteworthy, apathy is significantly associated with high HIV-1 plasma levels and poor neurocognitive performance in HIV-1-infected individuals (Shapiro et al., 2013). Moreover, depression and apathy are hallmarks of HIV-1 patients with a history of cocaine or alcohol abuse (Bryant et al., 2015).

Collectively, our present findings provide the first evidence that NAcc neuronal function is perturbed by Tat via IP₃R- and TRPC-mediated signaling, a pathway that is enhanced by cocaine (Barr et al., 2015). Our results provide support for a potential mechanism underlying

modulation of the effect of Tat by cocaine in NAcc neurons, with possible implications for drug abuse in HIV-1-infected patients.

ABBREVIATIONS

2-APB	2-aminoethoxydiphenyl borate
A.U.C	area under curve
[Ca²⁺]_i	intracellular Ca ²⁺ concentration
DiBAC₄(3)	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
HBSS	Hank's balanced salt solution
HIV-1	human immunodeficiency virus 1
IP₃	inositol 1,4,5-trisphosphate
IP₃R	inositol 1,4,5-trisphosphate receptors
MSN	medium spiny neurons
NAcc	nucleus accumbens
Ry	rynaodine
SBFI-AM	sodium-binding benzofuran isophthalate-AM
TRPC	transient receptor potential canonical channels
VGNaC	voltage-gated Na ⁺ channels
TTX	tetrodotoxin
XeC	xestospongine C

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Figure 1. Responses of cultured NAcc MSN to D1-like dopamine receptor agonist SKF83959
 Averaged tracings illustrating SKF83959-induced Ca^{2+} elevation (A), membrane depolarization (B), and cytosolic Na^+ increase (C), in NAcc MSN. Neurons in which SKF83959 was effective were considered D1-like receptor-expressing and further used in the characterization of Tat-induced signaling pathways.

Figure 2. HIV-1 Tat increases $[Ca^{2+}]_i$ of phosphatidylinositol-linked D1-like receptor expressing receptor NAcc MSN in a concentration-dependent manner

A, Changes in Fura-2 fluorescence ratio (340 nm/380 nm) of SKF83959-responsive neurons upon administration of 500 nM Tat (top) or 500 nM boiled Tat (bottom). **B,** Averaged Ca^{2+} responses upon administration of Tat and boiled Tat. **C,** Comparison of average maximal amplitudes of Ca^{2+} elevations promoted by increasing Tat concentrations (5–5000 nM) or by heat-inactivated Tat (500 nM) in cultured NAcc neurons; * $P < 0.05$ compared to basal $[Ca^{2+}]_i$ and among indicated treatment groups; ** $P < 0.05$, 500 nM Tat versus 500 nM heat-inactivated Tat.

Figure 3. Tat releases Ca^{2+} from IP_3 -sensitive intracellular stores

A, Averaged traces depicting increases in $[Ca^{2+}]_i$ produced by Tat (500 nM) in Ca^{2+} -free saline-incubated NAcc MSN, in the absence and presence of IP_3 R inhibitors xestospongine C (XeC) and 2-APB, ryanodine receptor blocker ryanodine (Ry), and of lysosomal two pore channel inhibitor Ned-19. **B**, Comparison of mean amplitudes (top) and mean areas under curves (bottom) of $[Ca^{2+}]_i$ changes induced by indicated treatments; * $P < 0.05$ as compared with Tat alone.

Figure 4. Tat promotes Ca^{2+} entry via TRPC and P/Q-type of voltage-activated Ca^{2+} channels
A, Averaged Ca^{2+} responses triggered by Tat in the absence and presence of blockers of voltage-activated Ca^{2+} channels (N-type, ω -conotoxin GVIA; P/Q-type, ω -conotoxin MVIIC), of the nonselective cation channel TRPC (SKF96365, SKF), or of the rapid Ca^{2+} chelator BAPTA. **B**, Comparison of the changes in amplitude (top) and in area under curve (bottom) of the Ca^{2+} effects produced by Tat in the conditions mentioned in A (*, **, *** P < 0.05; means comparison among indicated treatment groups).

Figure 5. Potentiation of the effect of Tat on $[Ca^{2+}]_i$ by cocaine in NAcc MSN expressing phosphatidylinositol-linked D1 receptors

A, Averaged Ca^{2+} responses elicited by cocaine alone (10 μ M, black), Tat alone (500 nM, blue) and by combined administration of cocaine (1 μ M, 5 μ M, 10 μ M and 15 μ M) and 500 nM Tat. **B**, Comparison of the changes in amplitude (top) and in area under curve (bottom) of the Ca^{2+} effects produced by the treatments mentioned in A (* $P > 0.05$; ** $P < 0.05$; comparisons among indicated groups).

Figure 6. Tat depolarizes cultured NAcc MSN

A, Time-course of averaged changes in membrane potential elicited by Tat (500 nM) or heat-inactivated Tat (500 nM) in phosphatidylinositol-linked D1-like dopamine receptor expressing NAcc MSN. **B**, Comparison of the mean amplitude of the depolarizing effects promoted by increasing Tat concentrations (5–5000 nM) and ineffectiveness of heat-inactivated Tat (500 nM) on NAcc neurons; $P < 0.05$ compared with resting membrane potential and among indicated Tat concentrations (*) or when comparing the mean effect of 500 nM Tat with that of 500 nM boiled Tat (**).

Figure 7. Tat-induced depolarization is mediated by Na^+ and Ca^{2+} currents and is contingent on intracellular $[\text{Ca}^{2+}]_i$ release

A, Averaged traces illustrating Tat (500 nM)-induced depolarizations when applied alone or in the presence of voltage-gated Na^+ channel blocker tetrodotoxin (TTX), of Ca^{2+} chelator BAPTA or of TRPC blocker SKF96365 (SKF). **B**, Comparison of the amplitudes (top) and areas under curve (bottom) of Tat-triggered depolarizations in the conditions mentioned in **A**; *,** $P < 0.05$, comparisons among indicated groups.

Figure 8. Tat promotes Na^+ entry via TRPC and tetrodotoxin-sensitive voltage-gated Na^+ channels

A, Averaged traces characterizing Tat (500 nM)-triggered depolarizations when applied alone or in the presence of voltage-gated Na^+ channel blocker tetrodotoxin (TTX), of Ca^{2+} chelator BAPTA or of TRPC blocker SKF96365 (SKF). **B**, Comparison of the amplitudes (top) and areas under curve (bottom) of depolarizations elicited in the conditions mentioned in A; *,** $P < 0.05$, comparisons among indicated groups.

Figure 9. Proposed mechanism of Tat-induced effects on NAcc MSN expressing phosphatidylinositol-linked D1-like dopamine receptors

Tat promotes activation of inositol 1,4,5-trisphosphate receptor (IP₃R) followed by Ca²⁺ release from the endoplasmic reticulum (ER). Consequent opening of TRPC at the plasma membrane induces entry of Ca²⁺ and Na⁺, thus depolarizing the neuron sufficiently to trigger Ca²⁺ influx via P/Q-type of voltage-activated Ca²⁺ channels and Na⁺ influx via voltage-gated Na⁺ channels (VGNaC). The cumulative effect is an augmented depolarization.