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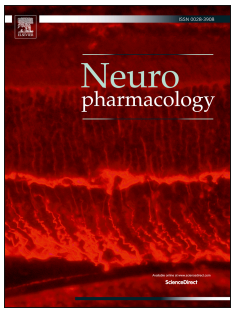

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PII: S0028-3908(17)30472-0

DOI: 10.1016/j.neuropharm.2017.10.005

Reference: NP 6890

To appear in: *Neuropharmacology*

Received Date: 17 July 2017

Revised Date: 15 September 2017

Accepted Date: 3 October 2017

Please cite this article as: Mannangatti, P., Ramamoorthy, S., Jayanthi, L.D., Interference of norepinephrine transporter trafficking motif attenuates amphetamine-induced locomotor hyperactivity and conditioned place preference, *Neuropharmacology* (2017), doi: 10.1016/j.neuropharm.2017.10.005.

Interference of Norepinephrine Transporter Trafficking Motif Attenuates Amphetamine-induced Locomotor Hyperactivity and Conditioned Place Preference*

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Running title: Catecholamine transport regulation in amphetamine behavior

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Abstract:
Amphetamine (AMPH)-mediated norepinephrine transporter (NET) downregulation requires NET-T258/S259 trafficking motif. The present study utilizes cell permeable NET-T258/S259 motif interfering peptide, which blocks AMPH-induced NET downregulation, to explore the role of this form of NET regulation in AMPH-mediated behaviors. In rats receiving intra-accumbal microinjections of TAT-conjugated peptides encompassing NET-T258/S259 motif, acute systemic AMPH failed to inhibit NE transport in the TAT-NET-T258/S259 wild-type (WT) peptide injected hemisphere but not in the vehicle or scrambled peptide injected hemisphere. Acute AMPH-induced hyperactivity was significantly reduced in rats receiving intra-accumbal TAT-NET-T258/S259 WT peptide compared to those receiving intra-accumbal vehicle or TAT-NET-T258A/S259A mutant peptide or corresponding TAT-conjugated scrambled peptide. Basal locomotor activity was not altered by peptide infusions alone. Similarly AMPH-induced locomotor sensitization was significantly reduced in rats receiving intra-accumbal TAT-NET-T258/S259 WT peptide prior to AMPH challenge and not in rats receiving the mutant or scrambled peptide. In conditioned place preference (CPP) paradigm, a single bilateral intra-accumbal microinjection of TAT-NET-T258/S259 WT peptide prior to CPP testing significantly reduced AMPH-induced CPP expression. Likewise, a single bilateral intra-accumbal microinjection of TAT-NET-T258/S259 WT peptide prior to drug-challenge significantly attenuated AMPH-primed CPP reinstatement. On the other hand, bilateral intra-accumbal microinjection of scrambled peptide did not affect AMPH-induced CPP expression or reinstatement. These data demonstrate a role for T258/S259-dependent NET regulation in AMPH-induced hyperactivity and sensitization as well as AMPH-induced CPP expression and reinstatement.

Key words: norepinephrine; catecholamines; transporters; amphetamine; psychostimulants; behavior.

Highlights:

- Amphetamine targets norepinephrine transporter contributing to drug-abuse potential.
- NET-T258/S259 trafficking motif is required for AMPH mediated NET downregulation.
- Intra-accumbal TAT-NET-T258/S259 peptide infusion blocks NET inhibition by AMPH.
- In-vivo interference of NET-T258/S259 motif attenuates AMPH-induced behaviors.

Abbreviations: NET, Norepinephrine transporter; AMPH, Amphetamine; NAc, Nucleus accumbens

1. Introduction

The psychostimulant mechanism of action is either blockade or reversal of monoamine transporters. The psychostimulant, amphetamine (AMPH) acts as a substrate for the monoamine transporters, the norepinephrine transporter (NET), the dopamine transporter (DAT) and the serotonin transporter (SERT) and facilitates transport reversal resulting in the efflux of the amines (1-3). The AMPH-induced DA efflux and resulting spike in extracellular DA is believed to mediate psychostimulation (3). Interestingly, DAT-KO mice exhibit indifference to stimulants and intact cocaine CPP (4,5), where as NET-KO mice exhibit super-sensitivity to psychostimulants and enhanced cocaine CPP (6). Other studies have shown blunted cocaine self-administration by DAT-KO mice (7). In the prefrontal cortex (PFC), NE controls accumbal DA release, which is critical for AMPH reward (8), and NET in the PFC is a major determinant of DA clearance (9). In the nucleus accumbens (NAc), NET plays a crucial role in striatal DA clearance in the absence of DA innervation (10,11). In the hyppocampus where very little DAT is expressed, DA clearance is mediated by the NET (12). These studies underscore the importance of NET in regulating DA-dependent psychostimulant effects. Studies by us and other investigators in the field have shown that NE clearance is a highly orchestrated process involving regulation of NET function by second messenger-linked signaling pathways mediated by receptors (13-16) and psychostimulants (17-21). NET contains multiple consensus sites for several kinases, and our laboratory has demonstrated phosphorylation dependent regulation of NET by PKC and p38 MAPK (16,21,22). We have also shown that activation of NK1R with Substance-P (SP) similarly downregulates NET via PKC phosphorylation of T258/S259 motif and raft-specific protein-protein interactions (16,23). Importantly, we demonstrated that the AMPH-mediated downregulation of NET requires the same PKC-directed T258/S259 trafficking motif (20). In this report, we investigate a potential role for AMPH regulation of NET via this T258/S259 trafficking motif in manifesting behavioral consequences of AMPH administration.

The goal of the present study was to test the hypothesis that the T258/S259-dependent regulation of NET contributes to AMPH-induced behaviors. We demonstrate that acute AMPH administration downregulates NET function in the rat accumbens and stimulates locomotor activity. We show that infusion of TAT-NET-T258/S259 WT peptide, but not the scrambled peptide in the NAc blocks this acute AMPH-mediated NET downregulation as well as locomotor stimulation. Following repeated AMPH administration and drug-withdrawal, AMPH challenge at a lower dose elicits locomotor sensitization that is blocked by TAT-NET-T258/S259 WT peptide infusion. Importantly, neither TAT-NET-T258A/S259A mutant peptide nor the scrambled peptide affected AMPH-induced hyperactivity or sensitization. In addition, AMPH-induced CPP expression and reinstatement are attenuated by intra-accumbal microinjection of TAT-NET-T258/S259 WT peptide but not the scrambled peptide. Thus, T258/S259-dependent NET regulation having control over AMPH-mediated behavior suggests that this regulation may have implications in the neuronal adaptations underlying AMPH-abuse.

2. Materials and Methods

2.1. Materials.

D-Amphetamine hemisulfate (AMPH) was purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]NE was from PerkinElmer (Waltham, MA). All other chemicals were from Sigma Chemical

Co (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise indicated. The sequence LYVSLWKGVKTSGKVVWITATL (amino acids 248-269) is selected encompassing T258/S259 motif (underlined) of rat NET. The WT, mutant, and scrambled peptides were conjugated with the cell membrane transduction domain of the HIV-1 Tat protein. The peptides TAT-NET-T258/S259 WT: YGRKKRRQRRRLYVSLWKGVKTSGKVVWITATL, TAT-NET-T258A/S259A mutant: YGRKKRRQRRRLYVSLWKGVKAAGKVVWITATL and the corresponding TAT-scrambled peptide: YGRKKRRQRRRGKWTAVSWGLITVTLFLKVSKV are synthesized by Thermo Scientific/Pierce Biotechnology, Inc (Rockford, IL) and are of more than 95% purity.

2. 2. Animal surgeries and drug administrations

2.2.1. Guide cannulae implantation. Male Sprague Dawley rats (200-250 gms) were used for the experiments. Rats were maintained on a 12 h light/12 h dark cycle at an ambient temperature of 22°C and 42% humidity. All animal procedures were in accordance with the National Institutes of Health guide (NIH Publication No. 8023, revised 1978) for the Care and Use of Laboratory animals. The protocols of this study were approved by Virginia Commonwealth University Institutional Animal Care and Use Committee. Aseptic rat surgeries were performed under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia. Stainless steel cannulae (23 gauge) lowered 1 mm above the region of interest (Nucleus Accumbens or NAc) using the coordinates +1.6 mm AP, ±1 mm ML and -6.6 mm DV from Bregma with a level skull. Rats are allowed 5-7 day recovery period prior to experimentation.

2.2.2 Microinjections and drug administrations. Membrane permeable TAT-NET peptides (1 µg) or the vehicle (aCSF) were infused via preimplanted cannula in a volume of 1 µl/hemisphere using micro-infusion pump (Harvard Apparatus). Amphetamine was dissolved in injectable grade isotonic saline solution (0.9% NaCl). Saline or AMPH (0.5 or 1 mg/kg) was administered i.p. in a volume of 10 µl/g body weight.

2.3. NE Transport measurements

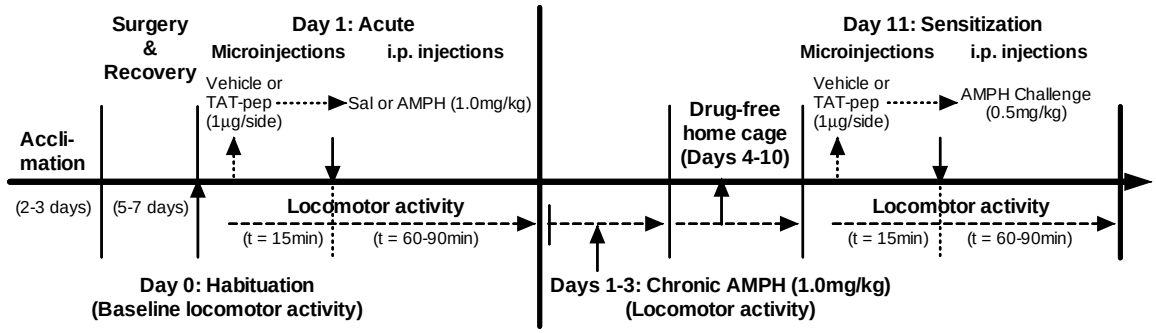
2.3.1. Synaptosome preparations. To examine the effect of TAT-NET peptides, rats received intra-accumbal microinjections of the vehicle in one hemisphere and either the WT or mutant peptide in the other hemisphere 15 min prior to saline or AMPH administration. Following 60 min of drug-administrations, rats were decapitated, and the brains were collected in ice-cooled dishes. Brain tissues from NAc were dissected and collected in 10 volumes (wt/vol) of cold 0.32 M sucrose. The tissue was immediately homogenized using a Teflon-glass homogenizer and centrifuged at 1000 x g for 10 min at 4˚C. The resulting supernatant was centrifuged at 12,000 x g for 20 min and the pellet was washed by resuspending in 0.32 M sucrose (24). Protein concentration was determined by DC protein assay (BioRad) using bovine serum albumin as standard.

2.3.2. NE transport assay. NE uptake assay was performed as described previously (21,23) using 40 nM [^{3}H]NE (35.0 Ci/mmol L-[7,8-^{3}H] norepinephrine for 5 min. Synaptosomes from NAc were preincubated with the NET inhibitor desipramine (DMI) (100 µM) at 37˚C for 5 min followed by the addition of [^{3}H]NE to determine the nonspecific NE uptake. Nonspecific uptake was defined as uptake in the presence of 100 µM DMI and subtracted from total accumulation to yield specific NET mediated NE uptake. Uptake was terminated by addition of 1 ml ice-cold

KRH buffer followed by rapid filtration over 0.3% polyethylenimine coated GF-B filters on a Brandel Cell Harvester (Gaithersburg, MD). Filters were washed rapidly with 15 ml cold PBS and radioactivity bound to filters was quantified by liquid scintillation counting using MicroBeta2 LumiJet (PerkinElmer Health Sciences Inc., Shelton, CT). Mean values of specific uptake ± SEM of at least three separate experiments were determined.

2.4. Behavioral testing

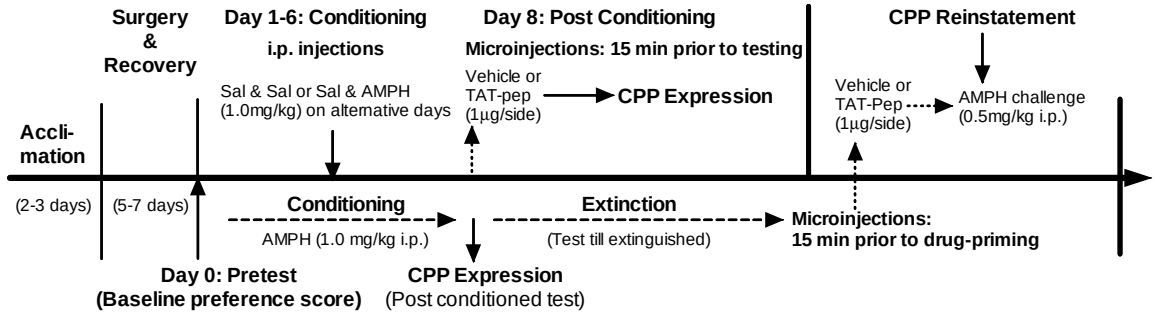
2.4.1. Locomotor or Open-Field Activity monitoring following acute AMPH. The locomotor activity of rats is monitored in open-field activity chambers (MED Associates, St. Albans, VT) (Model ENV-515, 17.0" L x 17.0" W x 12" H). The movements of the animals are tracked using 16 evenly spaced infra-red (I/R) sources and sensors juxtaposed around the periphery of the four sides of the chamber. Each chamber is enclosed in a sound-attenuating shell with artificial ventilation. Detailed time-line of experimental protocol for measuring the locomotor activity and sensitization is shown in Scheme 1. Briefly, on day 0, rats were exposed to locomotor boxes for 60 min period of habituation/acclimation and baseline activity was recorded. On day 1, rats received intra-accumbal microinjection of either vehicle or TAT-NET-T258/S259 peptide 15 min prior to saline or AMPH (1 mg/kg, acute treatment). Thus, we had 4 groups of rats: vehicle/saline; vehicle/AMPH; TAT-NET-T258/S259/saline; TAT-NET-T258/S259/AMPH. Horizontal activity, vertical activity and stereotypy of each rat were recorded for 90 min starting immediately from the time of microinjections in a computer with DIG-729USB software provided by the manufacturer. Data are plotted as total distance traveled (horizontal activity) or vertical counts or stereotypy against time. As controls, rats receiving TAT-NET-T258A/S259A mutant or the scrambled peptide prior to AMPH were tested in a similar manner.



Scheme 1: Time-line of experimental protocol involving surgeries, microinjections and drug administrations for locomotor activity and sensitization.

2.4.2. AMPH-induced locomotor sensitization. On the day of habituation (day 0), rat locomotor activity was recorded and taken as baseline activity. On day one, rats were given AMPH (1.0 mg/kg i.p.) and locomotor activity was recorded for 90 min. Amphetamine administration was repeated for another two days (total three days) and locomotor activity was recorded everyday for 90 min. Rats were then kept in home cages for one week with out any further drug injections. On day 11, rats were given intra-accumbal microinjections of either vehicle or TAT-NET-T258/S259 WT peptide 15 min prior to AMPH (0.5 mg/kg i.p.) challenge and the locomotor activity was recorded for a total of 90 min. Two separate groups of rats received TAT-NET-T258A/S259A mutant or the scrambled peptide prior to AMPH challenge to serve as controls.

2.4.2. AMPH-induced CPP expression. Conditioned place preference was tested using CPP boxes (MED Associates, St. Albans, VT) (Model ENV-517, 17.0" L x 17.0" W x 12" H) with inserts containing two equal sized compartments (black with vertical rod flooring and white with grid flooring) separated by a wall with a guillotine door to open or close the access between the two chambers. Detailed time-line of experimental protocol for measuring CPP expression and reinstatement is shown in Scheme 2. We followed biased CPP protocol where rats were conditioned with AMPH in less preferred chambers and with saline in more preferred chambers. This type of biased CPP has been used in studies including those measuring psychostimulant-induced CPP (25-31). Rats were conditioned for eight days alternating between AMPH and saline. Animals were conditioned for 30 min with AMPH (1 mg/kg i.p.) on days 1, 3, 5 and 7, and with saline on days 2, 4, 6 and 8. On day 9, rats were tested for preference by placing them in saline conditioned chamber with guillotine door completely opened for the rat to explore both chambers for 15 min.



Scheme 2: Time-line of experimental protocol involving surgeries, microinjections and drug administrations for CPP expression and reinstatement.

2.4.3. AMPH-induced CPP reinstatement. Rats were first tested for CPP as described above following conditioning with AMPH. Following the test, rats were extinguished by repeatedly exposing to the drug-paired chambers for 3 weeks without any further injections (twice per week) and tested for CPP at the end of every week until CPP was completely extinguished. Although several methods have been used to extinguish CPP (39, 40), in our experiments the above-described method was more effective in extinguishing AMPH-induced CPP than repeated exposure to the drug-paired chambers following saline injections. Rats exhibiting CPP scores close to their baseline score or significantly below the initial AMPH-induced CPP score are considered extinguished. Reinstatement was carried out by a priming injection of AMPH (0.5 mg/kg i.p.) and testing for place preference by measuring the CPP score. One group of the animals from the extinguished group received vehicle and the other received the WT or the scrambled peptide microinjections into prior to AMPH challenge. Thus, we had three groups to compare: vehicle, TAT-NET-T258/S259 WT or the scrambled peptide

2.5. Histology

Nissl staining to view cannula placements. Following behavioral tests, rats were rapidly decapitated and the brains were quickly removed and flash frozen in isopentane at -25˚C to -30˚C by keeping under dry ice. Frozen frozen brains were stored at -80˚C. Coronal sections (50

micron thickness) of the frozen brains were cut using a freezing microtome and stained with cresyl violet and viewed under microscope for the verification of cannula placements.

2.6. Statistical analysis

Statistical analyses of the data were performed using GraphPad Prism software (La Jolla, CA). A total of 113 rats were used. 12 for NE uptake, 26 for acute AMPH locomotor activity, 38 for locomotor sensitization (actual number is 29 since 9 are from the veh/AMPH group continued following acute locomotor activity measurement), 30 for CPP expression and 24 for CPP reinstatement (actual number is 16 since 8 are from the veh/AMPH group continued following CPP measurement). If cannulas placements were found outside the target brain region or cannulas are lost during an experiment, then the data from those rats were not included. A total of 5 rats were excluded from this study and not included in the total number given above.

3. Results

3.1. TAT-NET-T258/S259 WT but not the scrambled peptide microinjection blocks inhibition of NET by acute AMPH – In order to test our hypothesis that AMPH-mediated T258/S259-dependent NET regulation plays a role in eliciting AMPH psychostimulation, it is important to demonstrate that in-vivo interference of T258/S259 motif indeed prevents AMPH-from inhibiting NET function. One group of rats received intra-accumbal microinjections of TAT-NET-T258/S259 WT peptide (1 µg in 1 µl) into one hemisphere and vehicle into the other hemisphere. In another group, rats received intra-accumbal microinjections of TAT-NET-T258/S259 scrambled peptide (1 µg in 1 µl) into one hemisphere and vehicle into the other hemisphere. Fifteen min later, half of the rats from each group received saline (1ml/kg, i.p.) and the other half received AMPH (1 mg/kg, i.p.). This way we had controls within and across the subjects. One hour later, synaptosomes were prepared from the NAc tissue and NE uptake was measured. In saline administered rats, NE uptake was similar in the vehicle infused hemisphere and in the TAT-NET-T258/S259 WT peptide infused hemisphere (Fig. 1A: panel 1, n=3). Compared to saline administered rats, we found significantly reduced NE uptake in the vehicle infused hemisphere, but not in the TAT-NET-T258/S259 WT peptide infused hemisphere of AMPH administered rats (Fig. 1A: panel 1, n=3). The results show that the AMPH-mediated NET inhibition is effectively blocked by the TAT-NET-T258/S259 WT peptide microinjection. In a different group of saline administered rats, NE uptake was similar in the vehicle infused hemisphere as well as in the scrambled peptide infused hemisphere (Fig. 1A: panel 2, n=3). Compared to saline group, we found significantly reduced NE uptake in both vehicle and scrambled peptide infused hemispheres of the AMPH administered rats (Fig. 1A: panel 2, n=3) indicating that the scrambled peptide is not effective in preventing AMPH-mediated NET inhibition. Collectively, the results presented in figure 1A demonstrate that while TAT-NET-T258/S259 WT or the scrambled peptide does not affect basal NE uptake, TAT-NET-T258/S259 WT peptide specifically blocks AMPH-mediated NET inhibition. In order to ensure that TAT-NET-T258/S259 peptide is stable following microinjections, brains were collected by sacrificing the animals at different time periods following TAT-NET-T258/S259 peptide microinjections and accumbal extracts containing equal proteins were examined for the presence of peptide at ~ 4 kDa using gel electrophoresis followed by Coomassie Brilliant Blue staining. The results show that the peptide was stable over a period of 1 to 6 hrs and slowly degraded afterwards (Fig. 1B). To ensure that microinjections are given at the NAc, histological microscopic examinations were

carried out on the brain sections. A representative brain section stained with Nissl staining shows microinjection sites (locations of the microinjector tips) in the accumbens (Fig. 1C). Coronal section (Paxinos & Watson Rat Brain Atlas, 5th Edition 2005) depicting bilateral microinjection sites as indicated by closed circles is shown in figure 1D.

3.2. Intra-accumbal TAT-NET-T258/S259-WT peptide microinjection attenuates hyperactivity induced by acute AMPH – Since AMPH-mediated NET regulation requires T258/S259 motif, first we asked whether AMPH-induced hyperactivity is affected by interference of T258/S259 motif using TAT-NET-T258/S259 peptide microinjections into the NAc. Figure 2 shows the locomotor activity of rats measured over a 90 min time period including the locomotor activity measured for 15 min immediately following microinjection of vehicle or TAT-NET-T258/S259 WT peptide, but prior to i.p. saline or AMPH administration. Horizontal locomotion measured in 1 min bins over a 90 min time period is given as the distance traveled against time in figure 2A. Rats receiving AMPH exhibited hyperlocomotion when compared to those receiving saline. However, rats microinjected with TAT-NET-T258/S259 WT peptide prior to AMPH administration exhibited reduced hyperlocomotion compared to those microinjected with vehicle prior to AMPH (Fig. 2A). There was no significant change in the locomotor activity following saline injections both in vehicle or TAT-NET-T258/S259 WT peptide microinjected rats (Fig. 2A). Total activity measured for 90 min is shown in the bar graphs (Fig. 2B). The total distance traveled by the rats in vehicle/AMPH group or TAT-WT/AMPH group was significantly higher compared to vehicle/saline group or TAT-WT/saline group (Fig. 2B). However, the total distance traveled by the rats in TAT-WT/AMPH group was significantly less compared to that of vehicle/AMPH group (Fig. 2B). Moreover the total distance traveled by the rats in TAT-WT/saline group did not differ significantly from that of vehicle/saline group suggesting that TAT-NET-T258/S259 WT peptide alone has no significant effect on the locomotor activity (Fig. 2B). Vertical counts and stereotypy are shown in figures 2C and 2D respectively. Similar to locomotor activity, vertical counts were higher in vehicle/AMPH and TAT-WT/AMPH groups compared to vehicle/saline or TAT-WT/saline group (Fig. 2C). The vertical activity of TAT-WT/saline group did not differ from that of vehicle/saline group (Fig. 2C). Rats in the vehicle/AMPH and TAT-WT/AMPH groups exhibited significantly higher stereotypy compared to rats in the vehicle/saline or TAT-WT/saline group (Fig. 2D). The stereotypy of TAT-WT/saline group did not differ from that of vehicle/saline group (Fig. 2D). The vertical activity (Fig. 2C) or the stereotypy (Fig. 2D) of TAT-WT/AMPH group did not differ from that of vehicle/AMPH group. Together, the results demonstrate that the AMPH-induced hyperactivity, but not the vertical activity or stereotypy, is significantly attenuated by intra-accumbal infusion of TAT-NET-T258/S259 WT peptide.

3.3. Intra-accumbal TAT-NET-T258A/S259A mutant or TAT-NET-T258/S259 scrambled peptide microinjections do not affect acute AMPH-induced hyperactivity – In order to check the specificity of TAT-NET-T258/S259 peptide strategy, we microinjected TAT-NET-T258A/S259A mutant peptide or TAT-NET-T258/S259 scrambled peptide prior to AMPH administration. The total distance traveled by the rats in vehicle/AMPH group or TAT-Mut/AMPH group was significantly higher compared to vehicle/saline group and there was no significant difference between these two groups (Fig. 3A). Similarly, the total distance traveled by the rats in vehicle/AMPH group or TAT-Scr/AMPH group was significantly higher compared to vehicle/saline group and there was no significant difference between these two groups (Fig.

3B). The results presented in figures 3A and 3B show that the TAT-NET-T258A/S259A mutant or the scrambled peptide is unable to prevent AMPH-induced hyperactivity.

3.4. Intra-accumbal TAT-NET-T258/S259-WT peptide, but not the mutant or scrambled peptide attenuates AMPH-induced locomotor sensitization – After a period of no drug-exposure following repeated AMPH administration, a low dose of AMPH challenge produces locomotor sensitization. As shown in figure 4, 0.5 mg/kg AMPH challenge elicited a robust hyperactivity, which is higher than the hyperactivity measured following acute 1 mg/kg AMPH administration (comparing veh/AMPH group in Fig. 4 with veh/AMPH group in Fig. 2B) indicating drug-induced locomotor sensitization. However, the locomotor sensitization observed in the TAT-WT/AMPH group was significantly lower when compared to that seen in the vehicle/AMPH group (Fig. 4). Interestingly, the locomotor sensitization observed in the TAT-Mut/AMPH group or TAT-Scr/AMPH group was not significantly different from that of vehicle/AMPH group (Fig. 4). These results demonstrate that TAT-NET-T258/S259 WT peptide, but not the TAT-NET-T258A/S259A mutant peptide or the scrambled peptide is able to attenuate AMPH-induced locomotor sensitization (Fig. 4).

3.5. Intra-accumbal TAT-NET-T258/S259-WT peptide, but not the mutant or scrambled peptide attenuates AMPH-induced CPP expression and reinstatement – As reported in literature (32-34), AMPH is very effective in inducing CPP in our rats at 1 mg/kg dose. Rats microinjected with TAT-NET-T258/S259 WT peptide prior to post-conditioning test exhibited lower CPP expression compared to rats microinjected with vehicle or TAT-NET-T258/S259 scrambled peptide (Fig. 5A). As reported in published studies (33,34), rats conditioned with AMPH exhibited CPP reinstatement in response to a priming injection of 0.5 mg/kg AMPH following complete extinction (Fig. 5B). The CPP reinstatement, observed in the rats microinjected with TAT-NET-T258/S259 WT peptide 15 min prior to AMPH challenge, was significantly lower compared to the CPP reinstatement observed in the rats microinjected with vehicle or the scrambled peptide (Fig. 5B).

4. Discussion

Psychostimulants enhance monoaminergic signaling by interfering with monoamine transporter function. Altered cell-surface expression of monoamine transporters is a critical mechanism for regulating amine transport, and trafficking of monoamine transporters including that of NET has been shown to occur under basal conditions and following treatment with transporter substrates and inhibitors (35). We have shown that AMPH downregulates NET function and surface expression via transporter T258/S259 trafficking motif. Our previous studies elucidated two distinct molecular mechanisms involved in the regulation of NET by psychostimulants, cocaine and AMPH (16,20). While Cocaine upregulates NET via p38 MAPK-dependent T30 phosphorylation (21), AMPH downregulates NET via T258/S259 trafficking motif (20). Utilizing TAT-peptide strategy that interferes with NET-T30 motif, we demonstrated that blocking T30-mediated NET upregulation effectively attenuates cocaine-mediated behaviors in the mice (36). Here we demonstrate that brain region specific intra-accumbal infusion of the TAT-peptide, which encompasses NET-T258/S259 trafficking motif, prevented AMPH-mediated NET inhibition and AMPH-induced behaviors. Studies have shown the involvement of CaMKII and PKC mediated DAT regulation in AMPH-mediated DA efflux (18,37-41), and that

TAT-conjugated peptide containing CaMKII binding domain of DAT act in a dominant-negative manner disrupting DAT-CaMKII interaction and AMPH-induced locomotor hyperactivity (42). We have previously demonstrated that T258/S259 motif in the NET is phosphorylated by PKC (16) and this motif is required for AMPH-induced NET downregulation (20). It is possible that by competing with T258/S259 motif in a dominant-negative fashion, TAT-NET-T258/S259 WT peptide prevents NET-T258/S259 phosphorylation and AMPH-mediated NET downregulation. The data reported here along with our published studies suggest that the T258/S259 mediated NET regulation plays a role in eliciting AMPH-induced behaviors.

The data presented here along with our published studies indicate a role for T258/S259 motif-dependent NET regulation in AMPH-induced behaviors. Similar TAT-peptide strategy has been utilized in studies examining psychostimulant-mediated behaviors, antidepressant- and anxiolytic- like behaviors and working memory (43-45). The blockade of NET inhibition was observed only in the TAT-NET-T258/S259 WT infused hemisphere, and not in the vehicle infused hemisphere of a rat. In addition, AMPH-mediated NET inhibition was apparent in both vehicle and scrambled peptide injected hemispheres of a rat. These observations within and across the subjects provide strong evidence for the specificity of TAT-peptide strategy and the utility of in-vivo interference of T258/S259 motif in the NET to study AMPH regulation in an animal model. Mesolimbic NAc palys a major role in psychomotor stimulation and drug-reward behaviors of psychomotor stimulant drugs. The effect of AMPH is attributed to its actions on the core, shell or both regions (46-52). Therefore, we have targeted whole NAc region in our approach for microinjections where TAT-peptides were infused just above the NAc as shown in the micrograph. The metabolism of AMPH in rodent brain is between 1-2 hrs. We used TAT-NET peptide infusions 15 min prior to AMPH administration based on our observation that TAT-peptides were highly stable for up to 2-6 hrs and starts to degrade after 24 hrs. Nevertheless, as shown in our results section, our approach ensured that intact TAT-peptides are present in the same duration as that of AMPH presence in the brain.

Drug-abuse and addiction involve many neuroadaptations and whole mesocorticolimbic circuitry is involved in drug-reward like behaviors (53). While the corticolimbic NAc plays a crucial role in psychostimulant reinforcement, the PFC is considered critical for the development and maintenance of drug-seeking behavior (54). It is important to note that DA clearance in the PFC is mediated by the NET (9,55-59), and the behavior at the transport level is linked to NET function (60). In addition, both DAT dependent and independent mechanisms contribute to drug-reward and anatomical and functional interactions exist between NE and DA systems (61). While a systemic AMPH elicits locomotor activation with a small increase in the extracelluar DA mediated by NE signaling in the PFC, no behavioral activation is noted despite a substantial increase in the extracelluar DA following intra-accumbal injection of AMPH (62). It is interesting to note that both AMPH-induced locomotor activaton and CPP are blocked by TAT-NET-T258/S259 WT peptide. Blockade of acute AMPH-induced NET downregulation by TAT-NET-T258/S259 WT peptide correlates with blockade of locomotor activity. In-vitro studies have shown that AMPH-mediated NET inhibition and down regulation occur with in minutes involving changes in the NET protein trafficking mechanisms (20). Measurement of AMPH-induced CPP expression is carried out on the day following 3-day drug-conditioning period and TAT-NET-T258/S259 WT peptide significantly attenuated this AMPH-induced CPP. Earlier studies have reported that chronic (3-day) treatment with AMPH downregulates NET, and

degradation of NET protein is postulated to be a likely mechanism (63). A systematic study examining NET following a long-term AMPH exposure is required to correlate the mechanisms underlying AMPH-induced catecholamine transport with drug-reinforcing behaviors such as CPP. The sequence surrounding T258/S259 motif is similar in NET and DAT and it is possible that TAT-NET-T258/S259 WT peptide infusion in the NAc might interfere with AMPH-mediated regulation of NET, or NET and DAT, resulting in the attenuation of AMPH-induced behaviors. Further studies utilizing NET and DAT k/o will help delineate the role of T258/S259 motif in a transporter-specific manner.

Our results demonstrate that a single bilateral intra-accumbal infusion of TAT-NET-T258/S259 WT peptide, prior to AMPH administration significantly attenuated acute AMPH-induced psychomotor stimulation as seen in the open-field activity monitoring. This effect is specific since TAT-NET-T258A/S259A mutant or the scrambled peptide infusion failed to attenuate AMPH-induced locomotor activation. Current findings show that acute AMPH locomotor activation is sensitive to interference of NET-T258/S259 trafficking motif, and together with our published study (20), demonstrate a role for T258/S259-dependent NET regulation in AMPH-mediated psychomotor activation. Repeated psychostimulant use leads to drug-sensitization and increased liability to drug-seeking behavior. The findings presented in this study showed the efficacy of TAT-NET peptide strategy in attenuating locomotor sensitization following a low dose AMPH challenge. Only T258/S259-WT peptide but not the mutant or the scrambled peptide was effective in attenuating locomotor sensitization in response to AMPH challenge. Our findings from the current study show that intra-accumbal microinjection of TAT-NET-T258/S259 WT peptide but not the scrambled peptide significantly attenuated both AMPH-induced CPP and drug-primed CPP reinstatement. It is possible that AMPH-mediated NET-T258/S259 phosphorylation/regulation is maintained throughout the drug-conditioning period and account for drug reinforcement as seen with AMPH-induced CPP. The AMPH-mediated NET-T258/S259 phosphorylation/regulation may either cease to exist following withdrawal and re-emerge following drug reinstatement. Both CPP and self-administration are valid experimental models to investigate the drug-seeking and drug-taking behaviors and are complementary to each other. The AMPH-mediated NET regulation following administration by an experimenter may or may not be different from that following self-administration. A systematic approach examining NET regulation during various stages of drug exposure (conditioning, withdrawal and reinstatement) is warranted to correlate NET regulation to different aspects of drug reward-like behaviors. Nonetheless, our study adds to existing evidence that support the importance of NE and NET in mediating addiction-associated behaviors (64,65), and accordingly, NE directed pharmacological treatments are promising venues in the drug-development for treating addiction (66-69).

Conclusions

Psychostimulant abuse continues to extract enormous socioeconomic resources, and there are no effective FDA-approved treatments. Both NET and DAT are known to mediate important aspects of psychostimulant-reinforcement, and AMPH downregulates both NET and DAT (13-16,37,42,70-72). We have identified that NET is downregulated following NK1R activation and AMPH treatment via T258/S259 trafficking motif (16,20). Here, we presented findings demonstrating that the AMPH-mediated behaviors are sensitive to interference of T258/S259 motif dependent NET regulation. While studies have shown that AMPH-mediated DAT

regulation is linked to PKC and CaMKII signaling pathway, as well as mechanisms involving transporter associated proteins (37,42,70-72), it is not known whether T258/S259 motif is involved. It is also important to note that NET and DAT share strong homology in their amino acid sequences, and the sequence surrounding the T258/S259 motif is similar in NET and DAT with only one amino acid difference. NET: LYFSLWKGVKTSGKVVWITATLP and DAT: LYFSLWKGVKTSGKVVWITATMP (differing residue underlined). Therefore, it is possible that TAT-NET-T258/S259 peptide might interfere with both NET and DAT, and T258/S259-dependent regulation of NET, DAT or both may play a role in AMPH-elicited behaviors. We have recently shown that a clinically used NK1 receptor antagonist, aprepitant attenuates psychostimulant-induced behaviors including AMPH-induced CPP in the C57BL/6J mice (73). Since DAT is also downregulated following NK1R activation (74), we extended the above study to examine the effect of aprepitant on AMPH-induced CPP in the NET- and DAT- KO mice. Aprepitant effectively blocked AMPH-induced CPP in the DAT-KO mice and in the WT littermates, but not in the NET-KO mice indicating the specificity of NK1R-mediated NET regulation in AMPH-induced behaviors (unpublished observations). We acknowledge that future studies using NET- and DAT- KO mice are warranted to distinguish the involvement of T258/S259 motif -dependent NET regulation in AMPH-induced behaviors. Nonetheless, the finding that the T258/S59 motif dependent catecholamine transport regulation exerts control over AMPH-mediated behaviors is unique and novel in that it directs our attention to transporter regulatory phenomenon as drug-abuse therapeutic targets.

Author Contribution

Padmanabhan Mannangatti conducted experiments and acquired data. Lankupalle Jayanthi conceived the project, planned the experiments, performed data analysis and drafted the manuscript. Sammanda Ramamoorthy critically reviewed the manuscript, and Lankupalle Jayanthi and Sammanda Ramamoorthy reviewed and revised the manuscript prior to submission.

Acknowledgements

This work was supported by the National Institute of Health grant DA039451 (LDJ).

Statement Of Conflict Of Interest

The authors express no conflict of interest.

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Figure Legends

Figure 1: *A. Intra-accumbal microinjection of TAT-T258/S259 WT peptide, but not the scrambled peptide blocks acute AMPH-mediated NET inhibition.* * indicates significant inhibition of NE uptake in rats receiving AMPH (1 mg/kg i.p.). Data represent means ± SEM. By one-way ANOVA Dunnett's multiple comparisons test, $F_{(3,8)} = 7.514$, * $p < 0.01$ (WT); $F_{(3,8)} = 8.035$, * $p < 0.$ 0.01 (Scr). *B. Stability of TAT-NET-T258/S259 peptide.* Picture of SDS-PAGE shows the stability of TAT-NET-T258/S259 peptide at indicated time periods following microinjections. *C & D. Cannula placement in the NAc.* Schematic and photo micrographic representation of the locations of injector tips and injection sites in the NAc, based on Paxinos and Watson's stereotaxic atlas of the rat brain (2005).

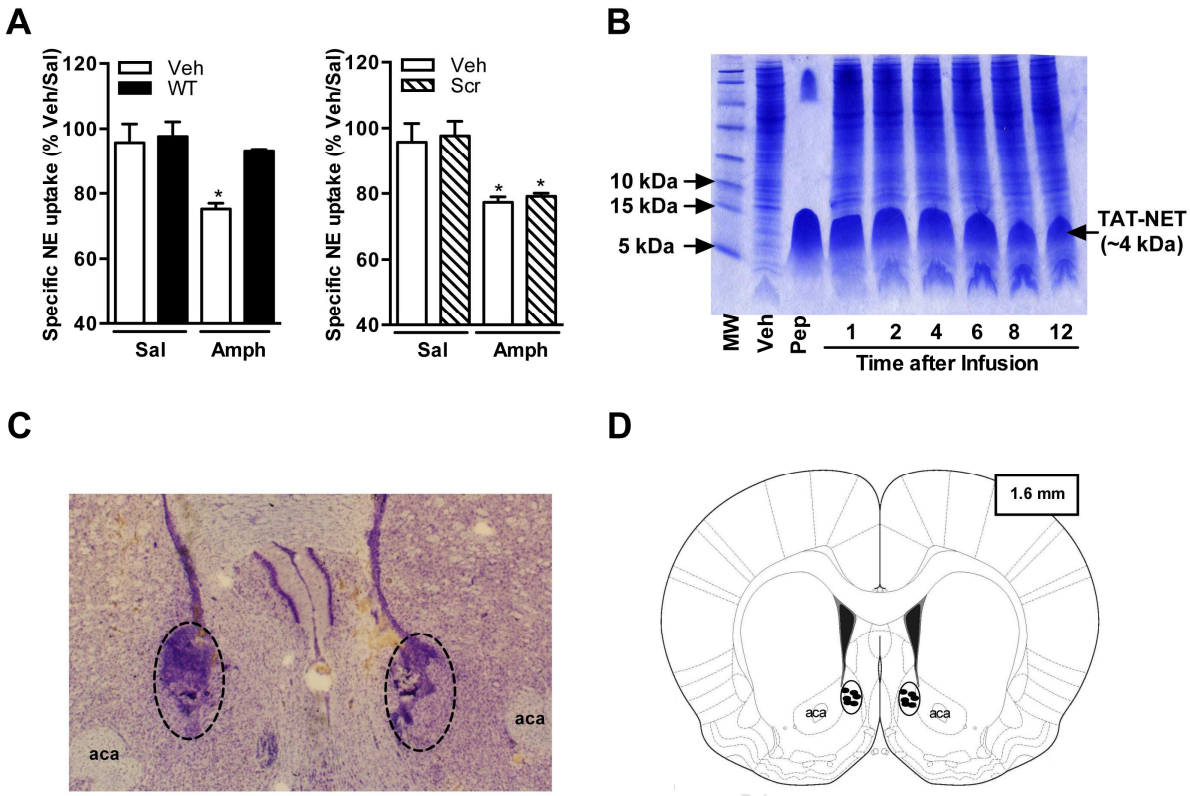
Figure 2: *Intra-accumbal microinjection of TAT-T258/S259 WT peptide 15 min prior to AMPH blocks acute AMPH-induced hyperactivity. A. Raw data of locomotor activity measured over a 90 min time period given as distance traveled in cm per minute. B. Bar graph shows total locomotor activity (horizontal) measured over a 90 min period.* Data represent means ± SEM. AMPH significantly increased the horizontal locomotor activity [F (3,22) = 25.11] by one-way analysis of variance; Tukey's multiple comparison test revealed *p < 0.001 when veh/AMPH compared with veh/sal or WT/sal; no significance when WT/AMPH compared with veh/sal or WT/sal; #p < 0.001 when WT/AMPH compared with veh/AMPH indicating significant

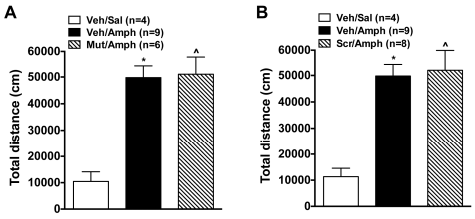
attenuation of AMPH-induced hyperactivity by TAT-NET-T258/S259 WT peptide; no significance when WT/sal compared with veh/sal (indicates no effect by TAT-NET-T258/S259 WT peptide on basal horizontal locomotor activity). Basal locomotor activity is indicated by a dotted line. *C. Bar graph shows total vertical counts measured over a 90 min period.* Data represent means ± SEM. AMPH significantly increased the vertical counts [F (3,22) = 17.02] by one-way analysis of variance; Tukey's multiple comparison test revealed test revealed *p < 0.001 when veh/AMPH compared with veh/sal or WT/sal; ^p < 0.01 when WT/AMPH compared with veh/sal or WT/sal; no significance when WT/sal compared with veh/sal or when WT/AMPH compared with veh/AMPH (indicates no effect by TAT-NET-T258/S259 WT peptide on basal or AMPH-induced vertical activity respectively). *D. Bar graph shows total stereotypy counts measured over a 90 min period.* Data represent means ± SEM. AMPH significantly increased stereotypy counts [F (3,22) = 26.99] by one-way analysis of variance; Tukey's multiple comparison test revealed *p < 0.001 when veh/AMPH compared with veh/sal or WT/sal; ^p < 0.005 when WT/AMPH compared with veh/sal or WT/sal; no significance when WT/sal compared with veh/sal or when WT/AMPH compared with veh/AMPH (indicates no effect by TAT-NET-WT peptide on basal or AMPH-induced stereotypy respectively).

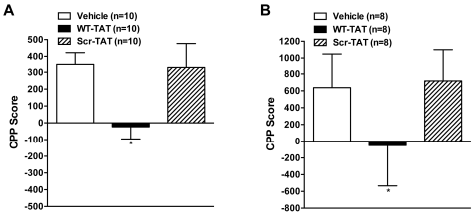
Figure 3: *Intra-accumbal microinjection of TAT-NET-T258A/S259A mutant or the scrambled peptide does not block acute AMPH-induced hyperactivity. A. TAT-NET-T258A/S259A mutant peptide.* AMPH significantly increased the locomotor activity [F (2,16) = 19.34] by one-way analysis of variance; Tukey's multiple comparison test revealed *p < 0.001 when veh/AMPH compared with veh/sal; ^p < 0.005 when Mut/AMPH compared with veh/sal; no significance when Mut/AMPH compared with veh/AMPH (indicates no effect by TAT-NET-T258A/S259A mutant peptide on AMPH-induced locomotor activation). *B. TAT-NET-T258/S259 scrambled peptide.* AMPH significantly increased the locomotor activity [F (2,18) = 20.75] by one-way analysis of variance; Tukey's multiple comparison test revealed test revealed *p < 0.005 when veh/AMPH compared with veh/sal; ^p < 0.005 when Scr/AMPH compared with veh/sal; no significance when Scr/AMPH compared with veh/AMPH (indicates no effect by TAT-NET-T258/S259 scrambled peptide on AMPH-induced locomotor activation).

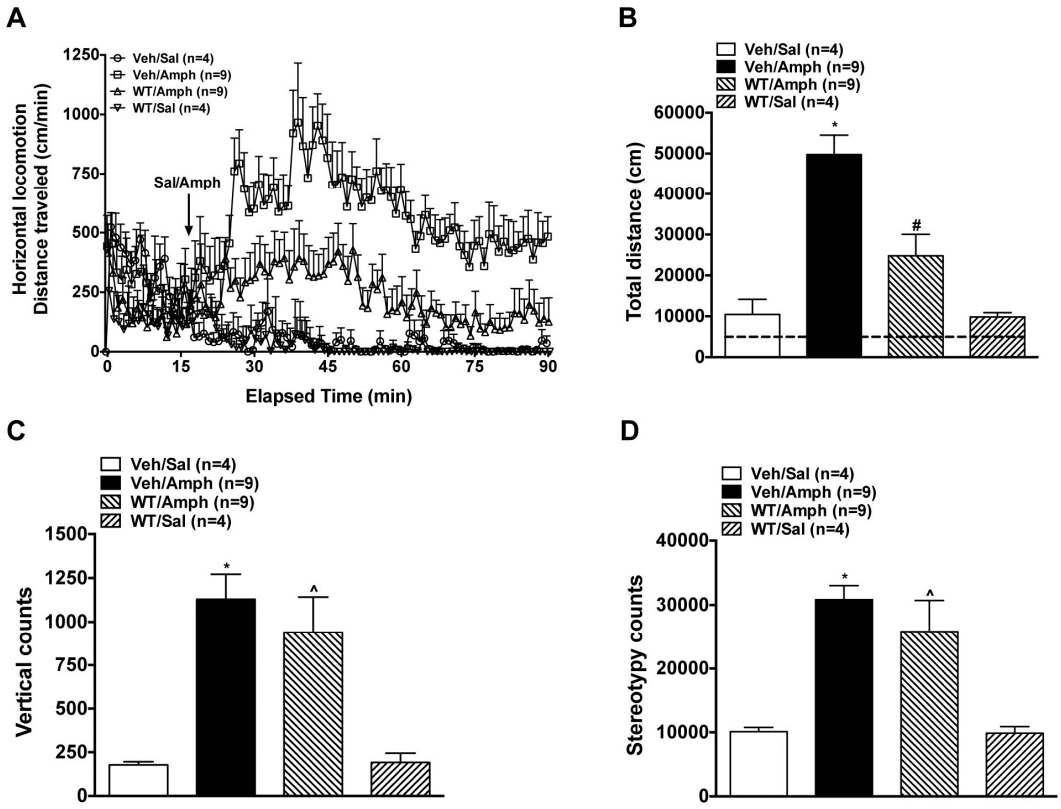
Figure 4: *Intra-accumbal microinjection of TAT-NET-T258/S259-WT, but not the mutant or scrambled peptide blocks AMPH-induced locomotor sensitization.* Bar graph shows total distance traveled (horizontal locomotor activity) measured over a 90 min period following AMPH challenge. Data represent means ± SEM. * indicates significant change ($p< 0.0001$) in the locomotor sensitization of rats microinjected with TAT-NET-T258/S259 WT peptide (One-way analysis of variance, Dunnett's multiple comparison test: Treatment: [F (3,34) = 15.81]. Basal locomotor activity is indicated by a dotted line.

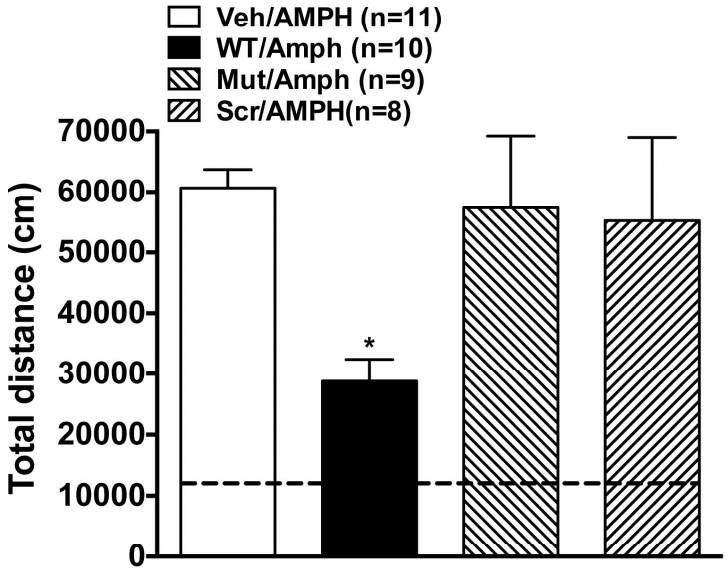
Figure 5: *Intra-accumbal infusion of TAT-NET-T258/S259 WT peptide, but not the scrambled blocks AMPH-induced CPP expression and reinstatement. A. CPP expression*. Data represent means ± SEM. * indicate significant change ($p< 0.0001$) in CPP expression in rats microinjected with TAT-NET-T258/S259 WT peptide (One-way analysis of variance, Dunnett's multiple comparison test: Treatment: F (2, 27) = 11.15. *B. CPP reinstatement.* * indicates significant change ($p=0.003$) in CPP reinstatement of rats microinjected with TAT-NET-T258/S259 WT peptide (One-way analysis of variance, Dunnett's multiple comparison test: Treatment: F (2, 21) = 7.79.










Veh/AMPH (n=11)
WT/Amph (n=10)
Mut/Amph (n=9)
Scr/AMPH(n=8)
Total distance (cm)
70000
60000
50000
40000
30000
20000
10000
0
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