Activation of α7 nicotinic acetylcholine receptor ameliorates HIV-associated neurology and neuropathology

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Abstract

HIV-associated neurocognitive disorders (HAND) in the era of combination antiretroviral therapy are primarily manifested as impaired behaviors, glial activation/neuroinflammation and compromised neuronal integrity, for which there are no effective treatments currently available. In the current study, we used doxycycline-inducible astrocyte-specific HIV Tat transgenic mice (iTat), a surrogate HAND model and determined effects of PNU-125096, a positive allosteric modulator of α7 nicotinic acetylcholine receptor (α7 nAChR) on Tat-induced behavioral impairments and neuropathologies. We showed that PNU-125096 treatment significantly improved locomotor, learning and memory deficits of iTat mice while inhibited glial activation and increased PSD-95 expression in the cortex and hippocampus of iTat mice. Using α7 nAChR knockout mice, we showed that α7 nAChR knockout eliminated the protective effects of PNU-125096 on iTat mice. In addition, we showed that inhibition of p38 phosphorylation by SB239063, a p38 MAPK-specific inhibitor exacerbated Tat neurotoxicity in iTat mice. Lastly, we used primary mouse cortical individual cultures and neuron-astrocytes co-cultures and in vivo staining of iTat mouse brain tissues and showed that glial activation was directly involved in the interplay among Tat neurotoxicity, α7 nAChR activation, and p38 MAPK signaling pathway. Taken together, these findings

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demonstrated for the first time that α7 nAChR activation led to protection against HAND and suggest that α7 nAChR modulator PNU-125096 hold significant promise for development of therapeutics for HAND.

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**Running title:** α7 nAChR activation ameliorated HAND symptoms

**Keywords:** α7 nAChR; PAM; HIV Tat; HAND; p38 MAPK
Abbreviations: cART, combination antiretroviral therapy; CORT, cortex; DMSO, dimethyl sulfoxide; Dox, doxycycline; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; HAND, HIV-associated neurocognitive disorders; HIP, hippocampus; Iba-1, ionized calcium-binding adaptor protein 1; iTat, doxycycline-inducible and astrocyte-specific HIV Tat transgenic mice; JNK, Jun N-terminal kinase; MAP2, microtubule-associated protein 2; MWZ, Morris Water Maze test; nAChR, nicotinic acetylcholine receptor; OPT, open field test; p38 MAPK, p38 mitogen-activated protein kinase; PAM, positive allosteric modulator; PSD-95, postsynaptic density protein 95; SYP, synaptophysin; Tat, HIV transactivator of transcription; Wt, wild-type

Introduction

Combination antiretroviral therapy (cART) has effectively suppressed HIV replication and improved immune function as well as prolonged the lifespan of HIV-infected people.\(^1\)-\(^5\) However, HIV-associated neurocognitive disorders (HAND) have become more prevalent. The neurological manifestations include slowed locomotor activity, impaired learning and memory, and the neuropathological hallmarks are astrocyte/microglia activation, chronic neuroinflammation, and comprised neuronal integrity.\(^6\)-\(^15\) None of antiretrovirals that are used in the current cART regimens have shown significant penetration into the CNS and have provided effective treatments for HAND.\(^16\)-\(^19\)
HIV viral protein Tat is a major pathogenic factor for HAND. It is secreted from HIV-infected microglia and astrocytes and taken up by neurons and detected in the brain of HIV-infected people who are cART naive and who are actively treated with cART. Tat expression activates glial fibrillary acidic protein (GFAP) expression in astrocytes through a cascade of transcription factors such as STAT3, early growth response 1 and p300 and causes astrocyte dysfunction and decreases neuron survival. In addition, Tat expression alters autophagy, endoplasmic reticulum stress, lysosomal exocytosis, neurite growth, and neurogenesis. Importantly, Tat expression in the brain of doxycycline-inducible astrocyte-specific HIV Tat transgenic mice (iTat) in the absence of HIV infection leads to locomotor, learning and memory deficits, and astrocyte/microglia activation, chronic neuroinflammation and loss of neuronal integrity, the consistent neurological and neuropathological hallmarks of HAND in the era of cART.

Nicotinic acetylcholine receptors (nAChR) are expressed throughout the peripheral and central nervous system, and they respond/bind to endogenous agonist neurotransmitter acetylcholine or exogenous agonist drug nicotine. One of the most abundant nAChR is homomeric α7 nAChR, which is both ionotropic and metabotropic and is predominantly expressed in neurons and glia of cortex and hippocampus. The ionotropic feature of α7 nAChR is the fast desensitization rate, characterized by very short agonist-induced onset duration and rapid decay with a long-lasting desensitized state. The metabotropic feature is related to M3-M4 intracellular loop, which involves multiple cellular signaling pathways including MAPK, PLC-RhoA, JAK2-STAT3 and PI3K-Akt and modulates synaptogenesis...
and growth in neural cells and inflammatory response in immune cells. α7 nAChR is involved in several neurological disorders such as Alzheimer’s disease, schizophrenia, drug addiction, depression and pain. It has been proposed as potential therapeutic targets for Alzheimer’s disease, addiction, schizophrenia, ischemic and traumatic brain injury. α7 nAChR has also been shown to be involved in HIV gp120 or gp41 neurotoxicity. However, little is known about its roles in HAND and its potential as a therapeutic target for treating HAND.

In the current study, we aimed to determine the roles of α7 nAChR in HAND. We took advantage of PNU-120596, a prototype-II and highly selective α7 nAChR positive allosteric modulator (PAM), and the surrogate HAND model iTat mice and determined if PAM treatment would lead to any changes in Tat-induced behavioral impairments and neuropathologies. In addition, we used α7 nAChR knockout mice and determined the direct roles of α7 nAChR in Tat neurotoxicity. Furthermore, we determined whether and which MAPK signaling pathway(s) were involved in the interplay between Tat neurotoxicity and α7 nAChR activation. Lastly, we used individual primary mouse cortical cultures and neuron-astrocytes co-cultures and assessed the relative contribution of neurons, astrocytes and microglia to the interplay among Tat neurotoxicity, α7 nAChR activation and p38 MAPK signaling pathway.

**Materials and methods**
**Mouse and drug administration:** Doxycycline (Dox)-inducible and astrocyte-specific HIV-1 Tat-transgenic mice (iTat) were generated as previously described.\textsuperscript{38} Wild-type (Wt, C57BL/6) and α7 nAChR knockout mice (α7\textsuperscript{−/−}, B6.129S7- Chrna\textsuperscript{7tm1Bay/J}) were purchased from the Jackson Laboratory (Bar Harbor, ME). All the animal procedures were approved by the Institutional Animal Care and Use Committee. Mice were housed with a 12-hour light and 12-hour dark photoperiod and provided water and food *ad libitum*. α7\textsuperscript{−/−} iTat mice were generated by cross breeding iTat mice with α7\textsuperscript{−/−} mice. α7\textsuperscript{−/−} genotyping was slightly modified with the JumpStart\textsuperscript{™} Taq DNA Polymerase-based PCR (JumpStart\textsuperscript{™} Taq DNA Polymerase, Sigma, catalog # D9307). Briefly, genomic DNA was extracted from the mouse tail and used a template for PCR with a program of 94°C for 1 min, 35 cycles of 94°C for 30 sec; 55°C for 30 sec, and 72°C for 1 min, and 72°C for 1 min using primers 5’-TTC CTG GTC CTG CTG TGT TA-3’ and 5’-ATC AGA TGT TGC TGG CAT GA-3’ for α7\textsuperscript{+/+} Wt mice, and 5’-TTC CTG GTC CTG CTG TGT TA-3’ and 5’-CCC TTT ATA GAT TCG CCC TTG-3’ for α7\textsuperscript{−/−} knockout mice). Mice of 10-14 weeks old with body weights of 20-35 grams were fed with Dox-containing diet (0.625g/kg, Envigo, catalog # TD.01306) as stated. PNU-120596 hydrate (Alomone, catalog # P-350) was dissolved in DMSO and s.c. injected (15 mg/kg/day, 40 µl for male mice with an average weight of 30 grams and 30 µl for female mice with an average weight of 22 grams to ensure minimal DMSO-associated toxicity).\textsuperscript{76} SB239063 (Tocris, catalog # 1962) was dissolved first in 100% DMSO and mixed with PEG400 (Sigma-Aldrich, catalog # 91893) and saline in a ratio of 0.8:32:67 and i.p. injected (15 mg/kg/day, 200 µl for male mice and 150 µl for female mice).\textsuperscript{77}
Behavioral tests: Open Field Test (OPT) and Morris Water Maze (MWZ) were performed sequentially using a computerized video tracking system (Anymaze, Stoelting) to determine the spontaneous locomotor activity and spatial learning and memory, respectively. For OPT, each mouse was allowed to move freely around a clear acrylic chamber (40.5 × 40.5 × 30.5 cm) for 10 min. Travel distance and maximum speed were determined by the AnyMaze software. For MWZ, a circle pool (1.2 m diameter) surrounded with a curtain was divided into four equal quadrants, and four signs with different shape were fixed onto the middle of each quadrant wall above the opaque water (24 ± 1°C). A hidden platform (1.5 cm below water surface) was put into a certain quadrant. Two stages consisting of 5-day training and probe test were carried out. In the training stage, four trials with a 15-20 min interval were conducted in the every day training, and for each trial mice were allowed to freely seek the platform within 90 sec right after they were randomly put into one quadrant facing to the pool wall. If the mice found the platform within 90 sec, 10 sec would be added to allow them staying on the platform for memorizing, however, if failed, they would be directed toward the platform and allowed to stay on it for 15 sec. Mice that were immobile or floating and unable to find the platform during the training stages were excluded from the experiments. Probe test was carried out on the next day following the 5-day training stage with a 60 sec trial, and the platform was removed to prevent the mice from climbing onto it during the testing. One day after the behavioral tests, mice were euthanized and the brains were harvested.
**Western blotting:** A RIPA buffer (50 mM Tris.HCl, pH 8.0, 280 mM NaCl, 0.5% NP-40, 1% C24H39NaO4, 0.2 mM EDTA, 2 mM EGTA and 10% glycerol) supplemented with protease inhibitors (Millipore-Sigma, catalog# S8830) and phosphatase inhibitors (Millipore-Sigma, catalog # 4906845001) was used to lyse the brain tissues and cultured cells with brief sonication on ice. Protein concentrations of the lysates were determined using a Bio-Rad DC protein assay kit (Bio-Rad, catalog # 5000111). Lysates were denatured in the SDS-PAGE loading buffer at 100°C for 10 min, then electrophoretically separated by 8-15% SDS-PAGE, blotted onto 0.45 µm polyvinylidene fluoride membrane (GE Healthcare Life Sciences, catalog # 10600023), and probed using appropriate antibodies against PSD-95 (Abcam, catalog # ab18258, and 1:2000 dilution), SYP (Abcam, catalog # ab8049, and 1:1000 dilution), Iba-1 (Wako, catalog # 016-20001, and 1:500 dilution) and GFAP (DAKO, catalog # z330s, and 1:2000 dilution), p-p38 (Santa Cruz, catalog # sc-166182, and 1:1000 dilution), p38 (Santa Cruz, catalog # sc-535, and 1:1000 dilution), p-JNK (Santa cruz, catalog # sc-6254, and 1:1000 dilution), JNK (Santa cruz, catalog # sc-571, and 1:1000 dilution), p-ERK (Santa cruz, catalog # sc-7383, and 1:1000 dilution), and ERK (Santa cruz, catalog # sc-514302, and 1:1000 dilution), α7 nAChR (Alomone, catalog # ANC-007, and 1:1000 dilution) and β-actin (Sigma-Aldrich, catalog # A1978, and 1:2000 dilution). A Bio-Rad ChemicDoc imaging system (Bio-Rad) and Image J were used for image capturing and analysis, respectively.

**3’-Diaminobenzidine (DAB) staining:** Mice were anesthetized using avertin
(tribromoethanol) and transcardially perfused with first phosphate-buffered saline (PBS) and then 4% paraformaldehyde (PFA). Then, the brains were dissected out, fixed in 4% PFA at 4°C overnight, dehydrated in 30% sucrose, embedded in OCT, sagittally sectioned (20 μm) using a cryostat, and preserved in a cryoprotectant containing 30% ethylene glycol, 30% glycerol, and 40% PBS. For staining, floating sections were permeabilized in 0.1% triton X-100 in PBS (PBST), blocked by 1% BSA in PBST, probed by Iba-1 antibody (Wako, catalog # 019-19741, and 1:800 dilution), inactivated endogenous peroxidases in 1% hydrogen peroxide, probed again by a goat anti-rabbit secondary antibody (Southern Biotech, catalog # 4030-05, and 1:200 dilution), and developed using a DAB kit (Abcam, catalog # ab103723). All images were taken using a Nikon Eclipse E800 microscope with a 20x objective and analyzed by the Cellprofiler program. Every section was averaged from three brain regions prefrontal, occipital and parietal cortex, and the average of the three sections was used to represent individual animals.

Preparation of primary mouse cortical neurons, microglia, astrocytes, neuron-astrocyte co-cultures: All primary cells were prepared from one-day old α7−/− pups and their isogenic Wt pups. Primary cortical neurons: One-day old pups were genotyped, the brains with desired genotypes were harvested, removed of meninges, and dissected out cortex. The cortex was minced in a cold HBSS buffer (Sigma-Aldrich, catalog # 55021C). HBSS buffer was replaced sequentially by 0.25% trypsin (Sigma-Aldrich, catalog # T4049) and 2 μg/ml deoxyribonuclease I (Sigma-Aldrich, catalog # D5025) (at 37°C for 20 min) and fetal bovine serum (2 min), and 2% B27
(Thermofisher, catalog # 17504044) neurobasal medium (Thermofisher, catalog # 21103049) and 1% GlutaMAX (Thermofisher, catalog # 35050061). Brief centrifugation (300 g, 1-5 min) was used to recover the tissues and cells. The tissues were then triturated using a 10 ml pipette, the disassociated cells were washed, seeded into a 24-well plate (0.25 M/well) which was either coated with or contained coverslips coated with 0.1% ploy-D-lysine (Sigma-Aldrich, catalog # P6407) in borate buffer (pH 8.5), cultured at a 37°C, 5% CO₂ incubator for 2 days, and treated with 2.5 µM cytosine β-D-arabinofuranoside (Ara-C, Sigma-Aldrich, catalog # C1768) to remove astrocytes. Medium change by 50% was performed every two days, the neurons were ready for use on day 12 with purity above 90%, estimated by MAP2 staining (Santa cruz, catalog # sc-32791, and 1:500 dilution).

**Primary cortical neuron-astrocytes cocultures:** A similar protocol was used to generate primary cortical neuron-astrocyte co-cultures except for omission of the Ara-C treatment from the protocol. The ratio of neurons to astrocytes in the co-cultures was estimated to be 1.5-2:1, estimated by MAP2 and GFAP staining (1:500 dilution with the same antibodies for Western blotting).

**Primary cortical microglia and astrocytes:** A similar protocol was used to generate primary microglia and astrocytes except for use of full DMEM (Corning, catalog # MT15013CM) in place of B27 neurobasal medium, which was used to allow glia to grow. All cells were seeded into a T-75 flask without coating. Once the cells reached confluence, which usually took 12-14 days for brain extraction of two pups, the flask was shaken around 200 rpm for two hours to dislodge microglia. The culture medium containing the microglia was transferred to a 12-well plate, incubated in a 37°C, 5% CO₂ incubator for 30 min to allow the microglia to attach to the bottom of the well, and
replaced by 20% LADMAC conditioned medium (see below) and 80% full DMEM and cultured for 3-4 days with medium change every other day. The leftover astrocytes attached to the T-75 flasks were trypsinized, seeded into a 12-well plate, and cultured in full DMEM for 3-4 days. The purity of microglia and astrocytes was greater than 99% as determined by staining for Iba-1 (the same antibody as the DAB staining) and GFAP, respectively.

Preparation of conditioned media and use in primary cell cultures:

**LADMAC conditioned medium:** LADMAC (ATCC, catalog # CRL-2420™) were cultured in a T-75 flask with EMEM (ATCC, catalog # 30-2003™), 10% FBS, and 1% penicillin and streptomycin for 5-7 days to reach confluence. Then, the culture medium was collected, removed cell debris by brief centrifugation (300 g, 5 min), and filtered (0.22 µm filter, SIMSII, catalog # S30PES022S), and saved as LADMAC conditioned medium.

**Tat-containing or control DMEM conditioned media:** 293T (ATCC, catalog # CRL-3216™) were seeded into a 10 cm dish, cultured in DMEM containing 10% FBS, 1% penicillin and streptomycin for 24 hr, transfected with 20 µg pcDNA3-Tat.Myc by calcium phosphate precipitation, cultured for 24 hr, changed to fresh DMEM medium, and cultured for additional 48 hr. Then, the culture medium was collected, removed cell debris by centrifugation (300 g, 5 min), followed by filtering through a 0.22 µm filter, and saved as Tat-containing conditioned medium (Tat-CM). pcDNA3 was also transfected as a control to generate the control condition medium (Ctrl-CM). To treat primary microglia, 70% Tat-containing or control conditioned medium, 20% fresh DMEM, 10% LADMAC conditioned medium were used. To treat primary astrocytes, 70% Tat-containing or control
conditioned medium and 30% fresh DMEM were used.  **Tat-containing or control neurobasal conditioned medium:** Tat-containing and control neurobasal conditioned medium was similarly prepared except for use of an increased number of seeding cells, 48 hr culturing after transfection, change to neurobasal medium with 1% GlutaMAX, and 24 hr after the medium change, and saved as Tat-containing neurobasal conditioned medium (Tat-CM) or control neurobasal conditioned medium (Ctrl-CM).  To treat primary neurons and primary cortical neuron-astrocyte co-cultures, 98% Tat-containing or control neurobasal conditioned medium and 2% B27 (50X) were used to replace 50% culture medium.  To activate α7 nAChR in cell cultures, 1μM PNU-120596 accompanied with 0.5 μM PNU-282987 (Sigma-Aldrich, catalog # P6499) (PAM+P2) was applied into conditioned medium.  Both of them were dissolved in DMSO at high concentration (PNU-120596: 100 mM, PNU-282987: 50 mM) as stocks.  A Nikon Eclipse TE2000-S microscope with a 10x objective was used to capture the images of microglia in the bright field, and the images were converted by the Ilastik program to visualize the cell morphology and quantitated by the Cellprofiler program.  Three images were randomly captured from each well.

**Immunofluorescence staining:** Brain sections (20 μm) were permeabilized in PBST, blocked in 1% BSA in PBST, and probed using appropriate primary antibodies against PSD-95 (1:500 dilution with the same western blotting antibody) or MAP2 (same to above) and secondary antibodies goat anti-rabbit 555 (Thermofisher, catalog # A21428, and 1:500 dilution) or goat anti-mouse 488 (Thermofisher, catalog # A11001, and 1:500 dilution).  For cultured cells, the cells were first fixed with 4% PFA for 10 min, and then proceeded using
the same procedures as for brain sections. All images from brain sections were taken using Olympus FV10i confocal microscope with a 60X oil objective. The entire cortex and hippocampus region were checked, and frontal cortex and CA1 were chosen as the representative regions to verify PSD-95 expression. All culture cell images were taken using a Nikon Eclipse 800 microscope with a 100X oil objective. Three to six individual neurons were randomly selected and averaged for each coverslip. PSD-95-positive puncta within the trunk of neuron dendrites (first order) was analyzed for primary cortical neurons, while PSD-95-positive puncta of the secondary dendrites (second order) were analyzed for neuron-astrocyte co-cultures. Image J was used to determine the percentage of PSD-95 positive area to the total MAP2-positive area.

**Data analysis:** Three-way repeated measures ANOVA was used in MWZ training stages, and all other experiments used either two-way or three-way ANOVA, whenever applicable. Bonferroni test was used for all post hoc analyses. All statistical analyses were performed using IBM SPSS 20. \( p < 0.05 \) was considered significant and marked as *, \( p < 0.01 \) and \( p < 0.001 \) were both considered highly significant and marked as ** and ***, respectively.

**Data availability:** All raw data that were presented or mentioned in the manuscript are available from the corresponding author upon request.

**Results**
PAM administration alleviated HIV Tat-induced behavioral deficits and neuropathologies

To determine if α7 nAChR is involved in HAND, iTat mice were fed with Dox-containing food pellets for 7 days, injected s.c. with a well-studied and highly specific positive allosteric modulator (PAM) PNU-120596 of α7 nAChR for 5 days, and then subjected to open field test (OPT) for locomotor activity on day 14, and Morris Water Maze test (MWZ) for learning and spatial memory on day 15-20, and euthanized to harvest tissues on day 21 (Fig. 1A). Dox-containing food pellet feeding and PAM injection continued during the next 7 days of behavioral tests. To minimize the effects of injection on behavioral tests, PAM was injected 5 hr after each behavioral test. Wild-type mice and solvent DMSO were included as controls for iTat mice and PAM in the experiments, respectively, mice were further grouped by sex, which gave rise to a total of 8 experimental groups for analyses (Table 1).

For OPT, the total travel distance and the maximum speed were measured. In DMSO treatments, iTat mice showed shorter travel distance than Wt mice for both male and female mice, and the difference between the female iTat mice and female Wt was much more than the difference between male iTat mice and female Wt mice (left panel, Fig. 1B). In comparison, PAM treatment led to improvement of the travel distance of iTat mice, some improvement of the travel distance of Wt mice. For maximum speed, there was no difference between iTat mice and Wt mice in the DMSO treatment group, and PAM treatments led to increases of the maximum speed of male Wt mice but not other mice (right
panel, Fig. 1B).

At the MWZ training stage, escape latency time (Escape latency) and cumulative travel distance (Cumulative distance) were measured. Compared to Wt mice treated with DMSO, iTat mice treated with DMSO showed longer escape latency time (upper left panel, Fig. 1C) and longer cumulative travel distance (lower panel, Fig. 1C) on all five days, with differences of cumulative travel distances in male mice and escape latency time for female mice on day 4. PAM treatment led to remarkable improvements in iTat mice, with differences of escape latency time in male iTat mice on day 4 and 5 and female iTat mice on day 2 and 4 (upper panels, Fig. 1C), and differences of cumulative travel times in male iTat mice on day 4 and female iTat mice on day 2 (lower panels, Fig. 1C). However, only some improvements on male Wt mice were found by PAM treatment. In addition, there was an increase in cumulative travel distance in PAM-treated female iTat mice on day 5 when compared with PAM-treated female Wt mice.

For the MWZ probe test, latency to platform, platform entries, time at target quadrant, distance to target quadrant, time at platform, and distance to platform were measured. Male iTat mice treated with DMSO showed less time at target quadrant and shorter distance to target quadrant, which were reversed by PAM treatment with the differences in distance to target quadrant (middle panels, Supplementary Fig. 1). Interesting to note is the longer latency to platform in male iTat mice treated with DMSO than male Wt mice-treated with DMSO, and decreases of latency to platform in both male iTat and Wt mice by PAM
treatment (left panel, Supplementary Fig. 1). For other indices including platform entries, time at platform, and distance to platform, there were no differences between iTat and Wt mice, DMSO- and PAM-treated mice, and male and female mice.

We next determined the changes of neuron presynaptic synaptic marker synaptophysin (SYP) and postsynaptic marker PSD-95, astrocyte marker GFAP and microglia marker Iba-1 in cortex (CORT) and hippocampus (HIP). In CORT, iTat mice treated with DMSO showed significantly higher GFAP and Iba-1 than Wt mice treated with DMSO (right panels, Fig. 1D; upper and left panels, Fig. 1E). PAM treatment led to significant increases of PSD-95 in Wt mice and even more in iTat mice but had decreased GFAP in iTat mice and decreased Iba-1 in both Wt and iTat mice. Meanwhile, SYP showed no changes between iTat mice and Wt mice, and between DMSO and PAM treatment (right panels, Fig. 1D; upper and left panels, Fig. 1E). In HIP, PAM treatment increased PSD-95 in both Wt and iTat mice, and iTat mice treated with DMSO showed more Iba-1 than Wt mice treated with DMSO (left panels, Fig. 1D; lower and left panels, Fig. 1E). There were no differences of SYP and GFAP between Wt mice and iTat mice, and between DMSO- and PAM-treated mice. PSD-95 expression and its location in all these tissues were further confirmed by double immunofluorescent staining for PSD-95 and MAP2 (Fig. 1F).

We next determined if and which MAPK signaling pathways were responsive to PAM neuroprotective effects against Tat-induced neuropathologies. Both CORT and HIP were analyzed for expression and phosphorylation of p38, JNK, and ERK. PAM treatment led to
increases of both p38 and p-p38α in both CORT and HIP, increases of JNK in CORT of Wt mice, increases of JNK in CORT of iTat mice, increases of JNK and p-JNK in HIP of all mice, and decreases of ERK in HIP of iTat mice (Fig. 1D; right panels, Fig. 1E). PAM treatment also led to increases of JNK and p-JNK in both CORT and HIP of Wt mice. There was a difference of JNK between PAM-treated iTat mice and PAM-treated Wt mice. But there were no differences of p-JNK in CORT of all mice and no differences of p-ERK in CORT and HIP of both iTat and Wt mice. Interestingly, iTat mice showed no changes of p38, JNK, ERK, and their phosphorylated counterparts compared to Wt mice, both in the absence and presence of PAM treatment. Furthermore, no differences were noted between female and male mice in all the molecular analyses, thus the data were pooled after normalization using Wt mice treated with DMSO as the reference.

Taken together, we showed that PAM treatment significantly increased locomotor activity, enhanced learning and memory processes, elevated PSD-95 expression, and inhibited GFAP and Iba-1 expression in iTat mice. We also showed that these changes were associated with increased levels of p38 and its phosphorylation, to a lesser extent JNK and its phosphorylation, but not ERK and its phosphorylation. All these changes were more pronounced in CORT of iTat mice than HIP of iTat mice. These findings demonstrated that PAM was neuroprotective against HIV Tat-induced behavioral impairments and neuropathologies and suggest that PAM-mediated activation of α7 nAChR is likely involved.

α7 nAChR knockout abrogated PAM-induced neuroprotective
function against Tat-induced behavioral impairments and neuropathologies

To ascertain the neuroprotective function of PAM and the roles of α7 nAChR in PAM neuroprotective function against Tat-induced behavioral impairments and neuropathologies, we cross bred α7 nAChR knockout mice (α7−/−) with iTat mice, generated α7−/−iTat mice, and used these mice in the subsequent three-way ANOVA design studies (α7 nAChR, iTat, and sex, Table 1), in which all eight groups of mice were fed with Dox-containing food pellets and s.c. injected with PAM in the same way as stated in Fig. 1A and subject to the same behavioral tests and analysis of SYP, PSD-95, GFAP, IBA-1 and MAPK signaling pathways.

In OPT, there were only differences of total travel distance and maximum speed between α7−/−Wt female mice and α7−/−iTat female mice (Fig. 2A). During the MWZ training stage, α7−/−iTat male mice showed longer escape latency than α7+/+ iTat male mice on all five days with differences on day 2 and 3 (upper left panel, Fig. 2B). α7−/−iTat male mice also had longer cumulative travel distance than α7+/+ iTat male mice on day 3 (lower left panel, Fig. 2B). All female mice showed no differences of escape latency and cumulative distance (right panels, Fig. 2B). In addition, compared with α7−/−WT mice, α7−/−iTat mice showed longer escape latency and longer cumulative distance on day 3 (male mice) and on day 4 (female mice), suggesting that Tat expression caused more severe learning impairment in α7−/− mice. Furthermore, α7−/−Wt had longer escape latency and longer cumulative distance than α7+/+ Wt on day 4 (male mice), and only longer escape latency on day 5 (female mice) and longer cumulative distance on all five days (female mice), indicating that α7 nAChR
knockout itself also had negative effects on the learning of the mice, particularly on female mice. In the probe test, $\alpha_7^{-/-}$iTat male mice had fewer platform entries, shorter time at target quadrant, time at platform, and distance to platform than $\alpha_7^{+/+}$Wt male mice (Supplementary Fig. 2). $\alpha_7^{-/-}$iTat male mice also showed shorter distance to target quadrant than $\alpha_7^{+/+}$iTat male mice. In female mice, iTat mice showed longer latency to platform and shorter time at target quadrant than Wt mice. No other differences were noted among different groups of mice.

Protein expression in CORT and HIP of these mice were also determined. In CORT, $\alpha_7^{+/+}$iTat mice had higher PSD-95 than $\alpha_7^{+/+}$Wt mice, while no differences of PSD-95 were detected between $\alpha_7^{-/-}$iTat mice and $\alpha_7^{-/-}$Wt mice (left panels, Fig. 2C; upper left panels, Fig. 2D). $\alpha_7^{-/-}$Wt mice had lower GFAP than $\alpha_7^{+/+}$Wt mice, and $\alpha_7^{-/-}$iTat mice had lower GFAP than $\alpha_7^{+/+}$iTat mice and $\alpha_7^{-/-}$Wt mice. $\alpha_7^{-/-}$iTat mice had higher Iba-1 than $\alpha_7^{-/-}$Wt mice and $\alpha_7^{+/+}$iTat mice. In HIP, $\alpha_7^{-/-}$iTat mice showed lower PSD-95 than $\alpha_7^{+/+}$iTat mice (right panels, Fig. 2C; lower left panels, Fig. 2D). $\alpha_7^{-/-}$Wt mice had higher Iba-1 than $\alpha_7^{+/+}$Wt mice and $\alpha_7^{-/-}$iTat mice. PSD-95 expression and its location were further confirmed by double immunofluorescent staining for PSD-95 and MAP2 (Fig. 2E). With regard to MAPK signaling pathways, in CORT, $\alpha_7^{-/-}$iTat mice showed lower p38 and p-p38$\alpha$ than $\alpha_7^{-/-}$Wt and $\alpha_7^{+/+}$iTat mice, while $\alpha_7^{-/-}$Wt mice showed higher p38 and p-p38$\alpha$ than $\alpha_7^{+/+}$Wt mice (left panels, Fig. 2C; upper and right panels, Fig. 2D), further suggesting possible interplays among $\alpha_7$ nAChR, HIV Tat neurotoxicity and p38 MAPK signaling pathway. In HIP, p38 and p-p38$\alpha$ showed similar trends but are lower only for p-p38$\alpha$ in $\alpha_7^{-/-}$iTat mice.
Only $\alpha^7$ iTat showed lower JNK than $\alpha^7$ Wt in CORT. $\alpha^7$ Wt had higher ERK and p-ERK than $\alpha^7$ Wt in CORT, while $\alpha^7$ iTat had higher p-ERK than $\alpha^7$ iTat in CORT. No other differences were noted with JNK, p-JNK, ERK, and p-ERK in HIP.

Taken together, we showed that $\alpha^7$ nAChR knockout abolished the neuroprotective effects of PAM against locomotor and learning and memory deficits of iTat mice. We also showed that $\alpha^7$ nAChR knockout specifically led to significant decreases of PSD-95 and p38$\alpha$ in both CORT and HIP, p-p38$\alpha$ in CORT and significant increases of Iba-1 in CORT of iTat mice. These findings confirm that $\alpha^7$ nAChR was directly involved in PAM neuroprotective function against Tat-induced behavioral impairments and neuropathologies.

**p38 inhibition aggravated Tat-induced behavioral impairments and neuropathologies**

The inverse correlation between Tat-induced behavioral impairments and neuropathologies and p38 expression and phosphorylation by PAM treatment (Fig. 1) and $\alpha^7$ nAChR knockout (Fig. 2) raised the possibility that p38 MAPK signaling pathway would be an important mediator of Tat neurotoxicity and PAM neuroprotective function against Tat neurotoxicity. To address this possibility, we took advantage of SB239063, a potent and selective p38 MAPK inhibitor and determined its effects on Tat-induced behavioral impairments and neuropathologies using the same experimental scheme as Fig. 1A except for i.p. injection of SB239063 in place of s.c. injection of PAM in these eight groups of mice (Table 1).
In OPT, iTat mice showed longer total travel distance than male Wt mice and shorter travel distance than female Wt mice and slower maximum speed than male and female Wt mice (Fig. 3A). SB239063 treatment led to no significant differences in travel distance and maximum speed between Wt and iTat mice, both male and female mice. Of note was that there was a difference of travel distance between SB239063-treated and vehicle-treated female mice, both Wt and iTat mice. In the MWZ training stage, male iTat mice showed longer escape latency than male Wt mice on all 5 days, while SB239063 treatment led to even longer escape latency than Wt mice, with differences on day 3 and 4 for male mice (upper and left panels, Fig. 3B). Female iTat mice had similar trends with difference on day 4 compared with female Wt mice (upper and right panels, Fig. 3B). Similar patterns of the results were obtained about the cumulative distance, with difference between vehicle-treated male iTat and male Wt mice on day 3 and difference between SB239063-treated male iTat and male Wt mice on day 2 and 3 and female iTat and Wt mice on day 4 (lower panels, Fig. 3B). In the MWZ probe test, in the vehicle treatment, iTat male mice had shorter time at target quadrant and distance to target quadrant than Wt male mice, while in the SB239063 treatment, male iTat mice had fewer platform entries, shorter distance to target quadrant and distance to platform than Wt male mice (Supplementary Fig. 3). Except for latency to platform in which SB239063-treated female iTat mice had longer latency to platform than vehicle-treated female iTat mice, all SB239063-treated female iTat mice had fewer platform entries, shorter time at target quadrant, distance to target quadrant, time at platform, and distance to platform than vehicle-treated female Wt and iTat mice and
SB239063-treated female Wt mice.

Similarly, CORT and HIP of both male and female mice were analyzed for protein expression, as they showed differential response to SB239063 treatment above (Fig. 3C & D; Supplementary Fig. 4A & B). In CORT and HIP of both male and female mice, PSD-95 showed an identical pattern: vehicle-treated iTat mice had higher PSD-95 than vehicle-treated Wt mice, and SB239063-treated iTat mice had lower PSD-95 than SB239063-treated Wt mice (Fig. 3C & D; Supplementary Fig. 4A & B). All changes in PSD-95 were further verified by immunofluorescent staining, also overlapped with MAP2 staining (Fig. 3E & Supplementary Fig. 4C). In both CORT and HIP of male mice, vehicle-treated iTat mice had higher SYP and GFAP than vehicle-treated Wt mice, but SB239063-treated iTat mice showed no differences of SYN and GFAP (except HIP) from SB239063-treated Wt mice (Fig. 3C & D). In both CORT and HIP of female mice, vehicle-treated iTat mice showed no differences of SYP, GFAP and Iba-1 from vehicle-treated Wt mice, but SB239063-treated iTat mice showed lower SYP, GFAP and Iba-1 with differences than SB239063-treated Wt mice (Supplementary Fig. 4A & B). There were other differences between individuals and groups, but with no consistent patterns.

Taken together, we showed that inhibition of p38 MAPK signaling pathway by SB239063 led to worsening learning and memory deficits in iTat mice and PSD-95 down-regulation and that SB239063 treatment showed no effects on the locomotor activity but showed sex-specific changes of protein expression of SYP and GFAP. These findings suggest
strong interplays between p38 MAPK signaling pathway and PSD-95 in Tat neurotoxicity.

**Differential response of neurons, microglia, astrocytes and neuron-astrocyte co-cultures to Tat and PAM**

To ascertain our findings obtained from iTat mice above and to elucidate relative roles of each type of brain cells in the interaction among Tat neurotoxicity, PAM/α7 nAChR, and p38 MAPK signaling pathway, we isolated primary cortical neurons, microglia, and astrocytes from Wt and α7 nAChR knockout mice, cultured them in the presence of Tat-containing conditioned medium (Tat-CM) or its control conditioned medium (Ctrl-CM) and in the presence of PAM and PNU-282987 (P2), or its solvent control DMSO for 24 hr, and determined expression levels of PSD-95, Iba-1, GFAP and p-p38 and p38 in these cells by Western blotting. PNU-282987 (P2) was used as an α7 nAChR agonist in these in vitro experiments.

In primary cortical neurons of Wt mice, treatment of Tat, PAM+P2, or Tat plus PAM+P2 all showed lower PSD-95, p-p38 and p38, than the control treatment, while primary cortical neurons of α7−/− mice showed similar effects, and treatments of these neurons with Tat, PAM+P2, or Tat plus PAM+P2 showed no further changes (**upper panels, Fig. 4A & B**). In microglia of Wt mice, PAM+P2 or Tat plus PAM+P2 showed lower Iba-1, p-p38, and p38, while Tat alone increased Iba-1; In microglia of α7−/− mice, only Tat (plus DMSO or PAM+P2) has higher Iba-1 than its control (plus DMSO or PAM+P2), and there were no differences of Iba-1 between DMSO and PAM+P2 (**middle panels, Fig. 4A & B**), which
were consistent with our findings in CORT (Fig. 2D & E). Moreover, primary microglia of Wt and α7<sup>−/−</sup> mice under different treatments were visualized for their morphology (Fig. 4E), skeletonized (Fig. 4F), and calculated for the total length of the branches, indicative of microglia activation (Fig. 4G). In microglia of Wt mice, Tat had shorter branches than its control, while PAM+P2 had longer branches in both Tat and its control treatments. In microglia of α7<sup>−/−</sup> mice, Tat had shorter branches than its control and there were no differences of the branch lengths between DMSO and PAM+P2. In astrocytes of Wt mice, Tat had higher GFAP, p-p38, and p38, while Tat plus PAM+P2 had lower GFAP, but maintained the higher level of p-p38 and p38; In astrocytes of α7<sup>−/−</sup> mice, only Tat (plus DMSO or PAM+P2) had lower GFAP and p-p38 than its control (plus DMSO or PAM+P2) and there were no differences of GFAP and p38 between DMSO and PAM+P2 (lower panels, Fig. 4A & B), which were also consistent with our findings in CORT (Fig. 2D & E).

In addition, we also performed immunofluorescence staining of primary cortical neurons for PSD-95 (Fig. 4C) and quantified PSD-95 expression. Consistent with the findings from Western blotting, Tat or PAM+P2 had lower PSD-95 than its control in Wt neurons, and only Tat (DMSO and PAM+P2) had lower PSD-95 than its control (DMSO and PAM+P2) in α7<sup>−/−</sup> neurons and there were no differences of PSD-95 between DMSO and PAM+P2 (Fig. 4D).

To determine whether neuron-astrocyte interaction would be important for the interaction among Tat neurotoxicity, PAM/α7 nAChR, and p38 MAPK signaling pathway, similar experiments were performed using primary cortical neurons-astrocyte co-cultures from Wt
and α7 nAChR knockout mice. In the neuron-astrocyte co-cultures of Wt mice, PAM+P2, Tat, or Tat plus PAM+P2 all had higher PSD-95, p-p38, and p38, while in the neuron-astrocyte co-cultures of α7+/− mice, only Tat (plus DMSO or PAM+P2) has lower PSD-95, p-p38 and p38 than its control (plus DMSO or PAM+P2) and there were no differences of PSD-95, p-p38 and p38 between DMSO and PAM+P2 (Fig. 5A & B).

Similar results were obtained for PSD-95 in neuron-astrocyte co-cultures (Fig. 5C & D).

Taken together, we showed that Tat treatment led to significant lower PSD-95 in neurons and higher PSD-95 in neuron-astrocyte co-cultures, higher Iba-1 in microglia, higher GFAP in astrocytes, while PAM treatment led to lower PSD-95 in neurons, higher PSD-95 in neuron-astrocyte co-cultures, lower Iba-1 in microglia, and lower GFAP in astrocytes, and α7 nAChR knockout plus Tat led to lower p-p38 and p38 with differences in neurons, microglia, astrocytes, and neuron-astrocyte co-cultures. PSD-95 staining and microglia morphology showed similar results. These findings are consistent with the in vivo findings in mouse CORT (Fig. 1E & F; Fig. 2D & E) and suggest that both microglia and astrocytes are important for the interaction among Tat neurotoxicity, PAM/α7 nAChR, and p38 MAPK signaling pathway.

**Microglia activation in mouse cortex in response to Tat, PAM and α7 nAChR knockout**

To further determine the relationship between microglia activation, Tat neurotoxicity, and PAM/α7 nAChR, we performed the immunohistochemistry staining of microglia in CORT of Wt and iTat mice treated with and without PAM (Fig. 1). Microglia were visualized for
their morphology (Fig. 6A), and skeletonized (Fig. 6B), and quantitated for the number of branches and endpoints (Fig. 6C). iTat mice had more branches and endpoints that its Wt control, PAM-treated iTat mice had fewer branches and endpoints than PAM-treated Wt mice and DMSO-treated iTat mice. Similar immunohistochemistry staining was also performed with CORT of α7+/+ Wt, α7+/+ iTat, α7−/− Wt, and α7−/− iTat mice in the presence of PAM (Fig. 2). α7−/− iTat mice had more branches and endpoints than α7−/− Wt mice and α7−/− iTat mice (Fig. 6D & E). These findings further confirmed that microglia activation was directly involved in Tat neurotoxicity and its interaction with PAM/α7 nAChR.

Discussion

In this study, we first showed that PAM treatment greatly improved locomotor activity, learning and memory of iTat mice and increased PSD-95 expression and decreased GFAP and Iba-1 expression in CORT of iTat mice. We next showed that knockout of α7 nAChR abolished PAM protection against Tat-induced neurotoxicity. α7 nAChR expression was detected in both cortex and hippocampus of iTat mice, although its expression was increased in cortex by either Tat expression or PAM treatment but only decreased in hippocampus by both Tat expression and PAM treatment (Supplementary Fig. 5A-C). In addition, PAM/P2 treatment led to decreased α7 nAChR expression in neurons but increased α7 nAChR expression in microglia and astrocytes, while Tat led to increased α7 nAChR expression in microglia and astrocytes but decreased α7 nAChR expression on neurons (Supplementary Fig. 5D & E). These results demonstrated that α7 nAChR expression was directly involved
in PAM neuroprotective activity against Tat neurotoxicity, but changes of α7 nAChR expression in response to Tat and PAM treatment was brain region- and cell type-dependent. Thus, the changes of α7 nAChR expression in response to Tat and PAM treatment alone could not account for the PAM neuroprotective effects in this study. Furthermore, it is quite reasonable to assume that α7 nAChR is expressed in the brain of other rodent HAND models, even though no studies about α7 nAChR expression in these models are currently available.

In addition, we showed that inhibition of p38 MAPK signaling pathway worsened Tat-induced learning and memory impairments and was associated with down-regulation of PSD-95 in both cortex and hippocampus. Inhibition of p38 MAPK signaling pathway has led to improved cognition, learning and memory in several neurological diseases. We indeed noted some beneficial effects in the Wt mice when treated with p38 MAPK inhibitor SB239063. On the other hand, PSD-95 up-regulation and improved behaviors were noted with PAM-treated iTat mice while little p38 MAPK was altered in iTat mice in the absence of PAM treatment. These results together suggest p38 MAPK regulation of PSD-95 expression is involved in PAM-activated α7 nAChR-mediated protection against Tat neurotoxicity and that p38 MAPK activation is not involved in Tat neurotoxicity, at least at the level of Tat expression in the brain of these iTat mice. In vitro studies about the involvement of p38 MAPK on Tat neurotoxicity are not consistent. For instance, inhibition of p38 by SB203580 failed to block Tat-induced neurite losses and cell death in striatal neurons, but prevented Tat-induced apoptosis in cerebellar granule neurons. Moreover, inhibition of p38 MAPK by a different inhibitor SB202190 decreased Tat-induced
inflammation and oxidation in hippocampal slice cultures. Several factors could contribute to these inconsistencies. First, the specificity of the inhibitors differs. SB203580 and SB202190 both have shown off-target effects. SB 239063, the inhibitor used in this study is the second generation of p38 MAPK inhibitor and is much more selective and more popular for neurological disease studies; Second, in vitro experimental systems differ. For example, different types of neuron cultures were used. Lastly and more plausibly, higher concentrations of Tat were often used in these in vitro experiments than that in the brain of the iTat mice, while Tat expression in the brain of iTat mice is more relevant to that in the HIV infected brain in the era of anti-retroviral therapy. Interestingly, we also noted that both female Wt and female iTat mice performed better in the OPT than male Wt and male iTat mice in response to SB239063, which was consistent with higher SYP in CORT of the female mice than male mice. These results suggest the unique roles of p38 MAPK signaling pathway in sex-dependent specific behavioral changes of iTat mice and that SYP expression in CORT is associated with the locomotor activity control.

We also determined neuropathological changes in response to PAM treatment and α7 nAChR knockout, by focusing on neuronal markers SYP and PSD-95, and microglia marker Iba-1 and astrocyte marker GFAP in two important brain regions CORT and HIP. SYP showed no significance changes in both CORT and HIP of iTat mice when treated with PAM in the presence and absence of α7 nAChR, which was further confirmed by immunofluorescent staining (Supplementary Fig. 6). PSD-95 showed increases in both CORT and HIP of Wt and iTat mice and PAM treatment led to more increases, while α7 nAChR led to decreases of
PSD-95 in both CORT and HIP of iTat mice in the presence of PAM. Similar results were obtained in our subsequent neuron-astrocyte co-cultures with and without $\alpha_7$ nAChR expression. Nevertheless, we and others have shown that Tat induces PSD-95 down-regulation when Tat-expressing conditioned medium from astrocytes was used to treat neurons or using recombinant Tat protein in a transwell setting of astrocytes and neurons without direct contact. These studies further support that astrocytes are the key regulator of Tat neurotoxicity and suggest that the cell-cell interaction between astrocytes and neurons is important for this regulatory role.

Besides SYP and PSD-95, we also showed significant changes of Iba-1 and GFAP in response to Tat, PAM/$\alpha_7$ nAChR and p38 MAPK signaling pathway. Tat expression led to increases of GFAP expression and PAM treatment completely reversed the increases in CORT and in vitro. Similar response was obtained with Iba-1. $\alpha_7$ nAChR knockout further abrogated PAM effects on Tat-induced Iba-1 expression but not GFAP expression, suggesting distinct roles of $\alpha_7$ nAChR in astrocytes. $\alpha_7$ nAChR expressed in these non-excitible glia cells is usually associated with its anti-inflammatory role. However, we failed to detect infiltrates of immune cells in Tat-expressing brain of the iTat mice (Supplementary Fig. 7). We also failed to detect significant changes of pro-inflammatory cytokines/chemokines such as TNF-$\alpha$ in the brain of iTat mice and in in vitro Tat-containing conditioned medium-treated primary microgialia and astrocytes (Supplementary Fig. 8), despite the fact that astrogliosis and microgliosis were clearly detected by the current study and our previous studies. Taken together, the results demonstrated that activation of
microglia and astrocytes were important links among Tat neurotoxicity, PAM/α7 nAChR interaction, and p38 MAPK signaling pathway and suggest that α7 nAChR anti-inflammatory property is not primarily responsible for PAM neuroprotective effects against Tat neurotoxicity. Of note were differences of GFAP and Iba-1 expression in CORT and HIP and their differential response to PAM/α7 nAChR activation, which may likely result from the cell heterogeneity in different brain regions and different expression of α7 nAChR in these cells.

Importantly, the findings from the current study raised the possibility that PSD-95 is the convergent link involved in the interplays between Tat neurotoxicity and α7 nAChR activation. PSD95 participates in synapse formation, maturation and plasticity and glutamate-mediated neurotoxicity, its loss leads to abnormal LTP, impairs learning and memory process, and is involved in several neurological and psychiatric diseases. Tat binds to LRP and induces LRP/PSD-95/NMDAR complex formation potentiates glutamate excitotoxicity and promotes neuronal apoptosis. Therapeutic approaches have been proposed to disrupt PSD-95/NMDAR complex to ameliorate pathological changes. Meanwhile, our results showed increased PSD-95 expression was closely associated with improved learning and memory and locomotor activity. It is possible that increased PSD-95 serves as a compensatory mechanism for its loss in formation of abnormal complexes and to enhance its synaptic function, as evidenced in studies in which increased PSD-95 correlates with its protective effects in AD and PD animal models.
In conclusion, we showed that PAM treatment led to significant protection against Tat-induced locomotor, learning, and memory impairments and astrocyte/microglia activation and neuronal injury. We also showed that $\alpha_7$ nAChR activation, followed by p38 MAPK-mediated PSD-95 expression contributed to PAM-induced neuroprotection against Tat neurotoxicity. These findings demonstrate for the first time that $\alpha_7$ nAChR and its PAM hold significant therapeutic promise for development of therapeutics for HAND.
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Competing interests

All authors have no competing financial interests in relation to the work.

Supplementary material

Supplementary material is available at Brain online.

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Figure legends

Figure 1. Effects of PAM on HIV-1 Tat-induced behavioral impairments and neuropathologies. Wt or iTat mice of 3-4 months old were fed with doxycycline (Dox)-containing food pellets and injected s.c. with PAM PNU-125096 (15 mg/kg/day) or its solvent DMSO control (A), and grouped by sex (n = 6-11/group, B). The mice were subject to Open Field Test (OPT, C) and Morris Water Maze (MWM) test (training stage, D), and the behavioral indices were determined by the Anymaze software. During the behavioral tests, Dox-containing food pellet feeding continued ad libitum, PNU-125096 was administrated within five hours following each behavioral test. On day 21, one day after the last behavioral test, all mice were euthanized, cortex (CORT) and hippocampus (HIP) were dissected out to determine expression of synaptophysin (SYP), PSD-95, GFAP and Iba-1, and total and phosphorylated p38, JNK, and ERK by Western blotting (E). Protein expression was quantified by densitometry and normalized to the loading control β-actin and calculated using Wt+DMSO as a reference, which was set at 1 (F) (n=6/group, three males and three females). Immunofluorescent staining was performed for PSD-95 and MAP2 expression (G, scale bars: 20 µm). Sub-regions in Frontal CORT and HIP CA1 were chosen as representative region for CORT and HIP, respectively. $p < 0.05$ was considered significant and marked as *, # or $ for comparisons among different groups; $p < 0.01$ and $p < 0.001$ were both considered highly significant and marked as ** and ***, respectively.

Figure 2. Effects of α7 nAChR knockout on PAM neuroprotection against Tat-induced behavioral impairments and neuropathologies. A similar experimental scheme was
performed as Fig. 1A except for using α7 nAChR knockout mice (α7-/−) and α7-/−iTat mice, which were obtained by cross breeding α7-/− mice with iTat mice and that the mice in all groups (A) were receiving Dox-containing food pellets and PAM injections (n = 6-11/group). Similar behavioral Open field Test (B), Morris Water Maze training test (training stage, C), and Western Blotting (D & E) were performed (n=6/group, three males and three females). Immunofluorescent staining was also performed for PSD-95 and MAP2 (F, scale bars: 20 µm). GT: genotype.  

Figure 3. Effects of p38 MAPK inhibitor SB239063 on Tat-induced behavioral impairments and neuropathologies. A similar experimental scheme was performed as Fig. 1A except for using i.p. injection of p38 MAPK inhibitor SB239063 (15 mg/kg/day) in place of s.c. injection of PAM PNU-125096 for mice in all groups (A, n = 7-12/group). Similar behavioral Open field Test (B), Morris Water Maze test (training stage, C), Western Blotting (D & E, n=6/group, only males; Results from females were shown in Supplementary Fig.4), and immunofluorescent staining was performed for PSD-95 and MAP2 (F, scale bars: 20 µm).  

Figure 4. Response of primary neurons, microglia, astrocytes, and neuron-astrocyte
**co-cultures to Tat and PAM.** Primary neurons, microglia, astrocytes were isolated from one-day old pups of Wt and α7⁻/⁻ mice, treated with the conditioned medium from pcDNA3-transfected cells (Ctrl-CM) or pcDNA3-Tat-transfected cells (Tat-CM) and PNU-125096 (PAM, 1 µM) and an α7 agonist PNU-282987 (P2, 0.5 µM) for 24 hr, and harvested to determine expression of PSD-95, Iba-1, or GFAP, or p-p38, p38 and β-actin by Western blotting (A). Protein expression was quantitated as Fig. 1E (B). Primary neurons were also double immunostained for PSD-95 and MAP2 (C). The positive staining area of PSD-95 puncta was quantitated by image J (D). Primary microglia were visualized for their morphologies by microscopy before harvesting for cell lysates (E) and then skeletonized (F). The line shaped branches, indicative of the ramified stage, and their total length were quantified in (G). The cells with shorter, non-completely formed or no line shaped branches were recognized as more amoeba-like phenotypes which presented more often in only Tat treated Wt group and α7- with Tat treatment groups (A). Multiple independent repeats were used for statistical analysis (n = 3/group for Western blotting; n = 8/group for immunofluorescent staining, n = 6/group for microglia morphology). p < 0.05 was considered significant and marked as *; p < 0.01 and p < 0.001 were both considered highly significant and marked as ** and ***, respectively. Scale bars: 10 µm (C) and 50 µm (E).

**Figure 5. Response of neuron-astrocyte co-cultures to Tat and PAM.** Primary cortical neurons-astrocytes were isolated from one-day old pups of Wt and α7⁻/⁻ mice, treated with the conditioned medium from pcDNA3-transfected cells (Ctrl-CM) or pcDNA3-Tat-transfected cells (Tat-CM) and PNU-125096 (PAM, 1 µM) and an α7 agonist PNU-282987 (P2, 0.5 µM)
for 24 hr, and harvested to determine expression of PSD-95, p-p38, and p38 and β-actin by Western blotting (A). Protein expression was quantitated (B). Primary neurons were also double immunostained for PSD-95 (red) and MAP2 (green) (C) and quantitated for PSD-95 (D). Multiple independent repeats were used for statistical analysis (n = 3/group for Western blotting). $p < 0.05$ was considered significant and marked as *; $p < 0.01$ and $p < 0.001$ were both considered highly significant and marked as ** and ***, respectively. Scale bars (C): 10 µm.

Figure 6. Microglia activation by PAM in CORT of iTat mice and α7⁻ iTat mice. The brains from Fig. 1 were dissected, fixed, sectioned and stained for Iba-1 (A). Microglia morphologies were skeletonized (B), and the total length and endpoints of branches in three brain regions including prefrontal, parietal and occipital CORT were quantified and averaged from three sections of each mouse (n = 6/group, 3 males, and 3 females) (C). Similarly, the brains from Fig. 2 were processed and stained for Iba-1 (D), skeletonized and quantitated (E). The Wt+DMSO group was used as a reference and set as 1. $p < 0.05$ was considered significant and marked as *; $p < 0.01$ and $p < 0.001$ were both considered highly significant and marked as ** and ***, respectively. Scale bars (A): 200 µm.
Figure 1

507x426mm (300 x 300 DPI)
Figure 2

507x410mm (300 x 300 DPI)
Figure 3

339x464mm (300 x 300 DPI)
Figure 4

482x331mm (300 x 300 DPI)
Figure 5

334x231mm (300 x 300 DPI)
Figure 6

244x236mm (300 x 300 DPI)