Appendix A. Supplementary methods

"Chronic HIV-1 Tat exposure alters anterior cingulate cortico-basal ganglia-thalamocortical synaptic circuitry, associated behavioral control, and immune regulation in male mice"

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The following is the Supplementary methods to this article:

Supplemental Methods

2.3 Spine density assessment

Dendritic length, branching, and spine density were assessed in tissues of Tat(–) and Tat(+) mice after 2 and 8 weeks of DOX exposure. Tissues were subjected to Golgi impregnations using the FD rapid GolgiStain^{TM} kit according to manufacturer's directions (FD Neurotechnologies, Columbia, MD). In brief, whole forebrains were harvested after 2 or 8 weeks DOX exposure, fixed and impregnated per the manufacturer's directions and using the proprietary solutions supplied, and then sectioned (185 μm-thick) in the coronal plane using a Leica VT1000s vibratome (Leica Biosystems, Wetzlar, Germany), and mounted on gelatin-coated glass slides. Sections were stained, dehydrated in graded ethanol solutions to remove water, and cleared in xylene, before being coverslipped with Permount. The number of spines/10 μm were counted on dendrites of fully impregnated neurons within three distinct regions of the anterior cingulate cortical (ACC)- basal ganglia BG-thalamocortical circuit, the ACC, striatum, and medial dorsal (MD) thalamus using the Allen Institute Mouse Brain Reference Atlas, Version 3 in accordance with previous methods (Hauser et al., 1989; Fitting et al., 2013; Hahn et al., 2015). Spine density on apical, oblique, and basal dendrites of layer 5 pyramidal neurons, and dendritic length and branching were quantified in the ACC. Dendritic spines were counted on tertiary dendrites on medium spiny neurons (MSNs) in the striatum and on stellate neurons in the MD thalamus.

2.4 Synaptic protein assessment

2.4.1 Immunoblotting

Immunoblotting was performed on PFC, striatal, and thalamic tissue of Tat(+) and Tat(−) mice to assess pre- and postsynaptic protein markers as previously described (Fitting et al., 2013).

After 8 weeks of DOX exposure, brains were grossly dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until assay. Due to the small size of the ACC, the whole PFC was used for immunoblotting assays. Intact tissue samples of the PFC, striatum, and thalamus were homogenized in Pierce IP lysis buffer (Thermo Fisher Scientific, Waltham, MA), containing Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific). The bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL) was performed to quantify protein concentrations according to manufacturer's instructions. Samples were boiled in 4× Laemmli buffer for 5 min and loaded (40 μg per well) into 4-20% Criterion TGX Stain-Free gels (Bio-Rad, Hercules, CA). Proteins were transferred to Immuno-Blot PVDF membranes (Bio-Rad) and incubated with primary antibodies to Syt2 (Mouse IgG2a SP 2/0, Zebrafish International Resource Center, Eugene, OR; 1:100), synapsin 1 (Mouse IgG1 106001, Synaptic Systems, Göttingen, Germany; 1:1000), VGLUT1 (Mouse IgG1 N28/9, NeuroMab, Davis, CA; 1:400), VGLUT2 (Mouse IgG1 N29/29, NeuroMab; 1:400), GAD67 (Mouse IgG2a MAB5406, Millipore, Burlington, MA; 1:6000), PSD-95 (Mouse IgG2a K28/43, NeuroMab; 1:3000) and gephyrin (Mouse IgG1 147011, Synaptic Systems; 1:1000) followed by HRP-conjugated secondary antibodies (Southern Biotech, Birmingham, AL 1:10000). Protein levels were normalized to GAPDH (rabbit polyclonal ab9485, Abcam; 1:2500). Immunoblotted proteins were detected with ChemiDoc MD imaging system and analyzed using Image Lab 5.2.1 (Bio-Rad).

2.4.2 Immunohistochemistry

Based on significant inhibitory synaptic marker changes in the whole PFC, possible inhibitory changes in layer II/III and V of the ACC were assessed by semiquantitative areal measures of immunofluorescence of Syt2 and gephyrin co-localization. After 8 weeks of DOX, mice were perfused with 4% paraformaldehyde (PFA) in neutral phosphate buffer. Whole forebrains were

removed and post-fixed in 4% PFA in neutral phosphate buffer overnight at 4 °C. Brains were washed in PBS, incubated in 10% sucrose followed by 20% sucrose for at least 24 h, embedded in Tissue-Tek O.C.T. compound on dry ice, and stored at −80 °C. Coronal sections (20 μm) were cut on a Leica CM1850 cryostat, mounted on SuperFrost Plus Gold slides (Thermo Fisher Scientific), and dried for 15 min. Slides were rinsed in PBS, 50% EtOH, 70% EtOH, 50% EtOH, and PBS for 15 min, respectively. Sections were incubated for 30 min in permeability solution (0.1% Triton X-100 and 0.1% bovine serum albumin (BSA) in PBS) followed by blocking solution (0.1% BSA and 0.1% normal goat serum in PBS). Primary antibodies Syt2 (mouse IgG2a, Zebrafish International Resource Center; 1:75), gephyrin (Rabbit IgG1 cat. no. 147018, Synaptic Systems; 1:500), and MAP2 (Guinea pig polyclonal, cat. no. 188004, Synaptic Systems; 1:200) were diluted in blocking buffer and applied to the sections overnight at 4 °C. Sections were rinsed in PBS and incubated with the secondary antibodies Alexa 594 (Donkey-anti-mouse, Invitrogen, Carlsbad, CA; 1:1000), Alexa 488 (Goat-anti-rabbit, Invitrogen; 1:1000), and Dylight 405 (goat-anti-guinea pig, cat. no. 106-475-003, Jackson ImmunoResearch, West Grove, PA; 1:800) for 1 h at room temperature. Tissue sections were again rinsed, then coverslipped in ProLong Gold Antifade reagent (Life Technologies, Grand Island, NY, USA). Sections were imaged using a Zeiss LSM 700 confocal microscope at 63× magnification (Zeiss, Oberkochen, Germany). Colocalization analysis was performed using the overlap coefficient in the ZEN 2010 Edition software (Carl Zeiss INC, Thornwood, NY). The software determined the overlap coefficient by computing the sum of the overlapping Syt2 and gephyrin immunoreactive pixels divided by the sum of the all Syt2 and gephyrin immunoreactive pixels regardless of colocalization. The value of the overlap coefficient ranges from 0 (no colocalization) to 1 (perfect colocalization).

2.5 Cytokine assessment

Bio-Plex Pro Mouse Cytokine 23-plex assay kits (Bio-Rad Laboratories, Inc., Hercules, CA) were used to assess chemokine/cytokine levels (i.e. CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1, G-CSF, GM-CSF, TNFα, IFN-γ, IL-2, IL-3, IL-6, IL-9, IL-12p40, IL-12p70, IL-17A, IL-4, IL-5, IL-10, IL-13) in different brain regions according to the manufacturer's directions as previously described (Gonek et al., 2018). Briefly, mice were exposed to Tat for 48 h, 2 weeks, or 8 weeks, after which the whole PFC, striatum, and thalamus were grossly dissected and homogenized in IP lysis buffer (Pierce Biotechnology, Rockford, IL, USA) with protease and phosphatase inhibitors (Roche, Mannheim, Germany). Consistent with immunoblotting, the whole PFC was used due to the small size of the ACC. Protein concentrations were determined using the BCA assay according to manufacturer instructions (Pierce Biotechnology). Samples at a concentration of 500 µg/ml and standards were incubated with fluorescent, antibody-tagged microspheres for 1 h at room temperature with shaking followed by streptavidin-phycoerythrin-labeled detection antibodies. Plates were read on a Bio-Plex® 200 System (Bio-Rad) and samples were fitted to respective standard curves to determine cytokine concentrations via Bio-Plex Manager 4.0 software (Bio-Rad). Data are reported as the mean cytokine concentration ± SEM; duplicate samples were assayed from each animal. No values were excluded for being above detection limits.

References

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