


# Restoration of KCC2 Membrane Localization in Striatal Dopamine D2 Receptor-Expressing Medium Spiny Neurons Rescues Locomotor Deficits in HIV Tat-Transgenic Mice

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## Abstract

People infected with HIV (PWH) are highly susceptible to striatal and hippocampal damage. Motor and memory impairments are common among these patients, likely as behavioral manifestations of damage to these brain regions. GABAergic dysfunction from HIV infection and viral proteins such as transactivator of transcription (Tat) have been well documented. We recently demonstrated that the neuron specific Cl<sup>-</sup> extruder, K<sup>+</sup> Cl<sup>-</sup> cotransporter 2 (KCC2), is diminished after exposure to HIV proteins, including Tat, resulting in disrupted GABA<sub>A</sub>R-mediated hyperpolarization and inhibition. Here, we utilized doxycycline (DOX)-inducible, GFAP-driven HIV-1 Tat transgenic mice to further explore this phenomenon. After two weeks of Tat expression, we found no changes in hippocampal KCC2 levels, but a significant decrease in the striatum that was associated with hyperlocomotion in the open field assay. We were able to restore KCC2 activity and baseline locomotion with the KCC2 enhancer, CLP290. Additionally, we found that CLP290, whose mechanism of action has yet to be described, acts to restore phosphorylation of serine 940 resulting in increased KCC2 membrane localization. We also examined neuronal subpopulation contributions to the noted effects and found significant differences. Dopamine D2 receptor-expressing medium spiny neurons (MSNs) were selectively vulnerable to Tat-induced KCC2 loss, with no changes observed in dopamine D1 receptor-expressing MSNs. These results suggest that disinhibition/diminished hyperpolarization of dopamine D2 receptor-expressing MSNs can manifest as increased locomotion in this context. They further suggest that KCC2 activity might be a therapeutic target to alleviate motor disturbances related to HIV.

## Keywords

dopamine D1 receptor, dopamine D2 receptor, HIV-associated neurocognitive disorders, KCC2, locomotor dysfunction, NKCC1

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While the advent of combined antiretroviral therapy (cART) has greatly improved the prognosis of HIV-1 infection, people infected with HIV (PWH) nevertheless have a diminished quality of life due to a high prevalence of HIV-associated neurocognitive disorders (HAND). In fact, about half of PWH experience detriments in varying neurocognitive domains including perceptual-motor function and learning and memory (Antinori et al., 2007; Heaton et al., 2010; Saylor et al., 2016). These cognitive and behavioral alterations are likely due to

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synaptodendritic damage and circuitry disruption caused by direct and secondary damage from HIV proteins and persistent neuroinflammation (Masliah et al., 1997; Brailoiu et al., 2008; Tavazzi et al., 2014; Alakkas et al., 2019). While neurons are not infected by HIV, microglia and astrocytes can be infected and release viral proteins and inflammatory factors that damage bystander neurons and alter neuronal excitability. Both the striatum and hippocampus seem to be particularly vulnerable to HIV, likely underlying the motor and memory deficits respectively, found in humans and in animal models of HAND (Gelman et al., 2006; Chang et al., 2008; Maki et al., 2009; Fitting et al., 2010, 2013; Nath, 2015; Marks et al., 2016; Alakkas et al., 2019).

The HIV-1 protein, transactivator of transcription (Tat), is a primary mediator of HIV-induced central nervous system (CNS) degeneration that is secreted by infected cells. Elevated Tat levels can be seen in the CNS of PWH even when receiving effective cART (Johnson et al., 2013; Henderson et al., 2019). The doxycycline (DOX)-inducible, glial fibrillary acidic protein (GFAP)-driven Tat transgenic mice correctly model many neuropathological features and behavioral abnormalities seen in HAND patients (Kim et al., 2003; Carey et al., 2012; Fitting et al., 2012, 2013; Paris et al., 2014b, Hahn et al., 2016; Marks et al., 2016; Nass et al., 2020). Tat is capable of inducing proinflammatory phenotypes in both astrocytes and microglia, and can directly injure neurons. Through activation of *N*-methyl-D-aspartate receptor (NMDAR),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), and L-type voltage gated  $\text{Ca}^{2+}$  channels, Tat can induce focal disruptions in  $\text{Ca}^{2+}$  homeostasis resulting in loss of synaptodendritic complexity, and creating excitatory-inhibitory imbalances (Chandra et al., 2005; Fitting et al., 2010; Hargus and Thayer, 2013; Fitting et al., 2014; Napier et al., 2014; Schier et al., 2017).

Hyperexcitability is a hallmark of the HIV-exposed CNS and excessive glutamate release and reduced clearance have been well-studied (Pappas et al., 1998; Z. Wang et al., 2003; Longordo et al., 2006; Musante et al., 2010). Accumulating evidence has also revealed the importance of GABAergic disruption in hyperexcitability. Human postmortem tissue from HAND patients as well as *in vivo* models of HAND display reduced GABAergic markers (Gelman et al., 2012; Fitting et al., 2013; Buzhdygan et al., 2016; Marks et al., 2016; Nass et al., 2020) and functionality (Xu et al., 2016; Barbour et al., 2020).  $\text{K}^+$ - $\text{Cl}^-$  cotransporter 2 (KCC2) is neuron-specific and functions to extrude  $\text{Cl}^-$  while  $\text{Na}^+$ - $\text{K}^+$ - $\text{Cl}^-$  cotransporter 1 (NKCC1) imports  $\text{Cl}^-$ . Under normal conditions, mature neurons express high levels of KCC2 and minimal NKCC1, resulting in a low

intracellular  $\text{Cl}^-$  concentration ( $[\text{Cl}^-]_i$ ) necessary for GABA<sub>A</sub>R-induced membrane hyperpolarization. The functionality of KCC2 is determined by its proximity to the cell membrane and regulated by multiple phosphorylation sites (Kahle and Delpire, 2016; Cordshagen et al., 2018). One well-studied residue, serine 940 (S940), can be phosphorylated by protein kinase C (PKC) to increase membrane stability and localization. Conversely, dephosphorylation by protein phosphatase 1 (PP1) increases rates of internalization (Lee et al., 2007, 2011). Thus, reductions in KCC2 expression, loss of KCC2 function due to its trafficking away from the cell membrane, or relative increases in NKCC1 expression can increase  $[\text{Cl}^-]_i$  and diminish GABA<sub>A</sub>R-mediated hyperpolarization/inhibition. In fact, altered KCC2 function is thought to contribute to several neurological disorders (Sen et al., 2007; Boulenguez et al., 2010; Arion and Lewis, 2011; L. Chen et al., 2017a; M. Chen et al., 2017b; F. Wang et al., 2017; Dargaei et al., 2018; Lizhnyak et al., 2019). Importantly, some neurological deficits have been rescued by enhancing KCC2 activity with CLP257 or its prodrug, CLP290 (M. Chen et al., 2017b; B. Chen et al., 2018; Lizhnyak et al., 2019), although the underlying mechanism by which KCC2 function is recovered remains elusive. We recently demonstrated that KCC2 is a target of the HIV proteins Tat and gp120 in primary human neurons *in vitro* and in an *in vivo* model of neuroHIV (Barbour et al., 2020). Thus, KCC2 has been implicated in neuronal dysfunction underlying HAND and this assertion is further tested in the present study.

Neurons in the striatum are composed primarily of dopamine D1 receptor (D1R)-expressing medium spiny neurons (MSNs) and dopamine D2 receptor (D2R)-expressing MSNs. This area is particularly vulnerable to HIV-induced damage in PWH (Gelman et al., 2006; Alakkas et al., 2019), and *in vivo* models of neuroHIV (Fitting et al., 2010, 2014; Schier et al., 2017). Striatal MSNs, particularly those expressing D2R (Schier et al., 2017) are vulnerable to Tat-induced spine reductions due to dendritic  $\text{Ca}^{2+}$  influx (Fitting et al., 2014). Given striatal vulnerability to Tat-induced KCC2 loss (Barbour et al., 2020) and clinical evidence demonstrating motor deficits in PWH, we explored whether striatal KCC2 loss might be mechanistically linked to motor dysfunction, if such effects were reversible by CLP290, and if there was a preferential vulnerability between D1R-expressing or D2R-expressing MSNs. We found a loss of KCC2 selectively in D2R-expressing MSNs of the striatum of mice expressing the Tat transgene (Tat+) versus those without the transgene (Tat-), associated with motor hyperactivity after two weeks DOX administration. Additionally, we were able to rescue

phosphorylation of S940-KCC2, membrane localization of KCC2, and motor dysfunction with CLP290 oral gavage.

## Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were performed in accordance with ethical guidelines defined by the National Institutes of Health.

### Subjects and Treatment

Mice used for behavioral and western blot experiments were male, doxycycline (DOX)-inducible, GFAP-driven, HIV-1 Tat-transgenic mice and littermate controls aged 10–12 weeks ( $N=118$ ) generated in the vivarium of Virginia Commonwealth University (Bruce-Keller et al., 2008; Hauser et al., 2009). Mice that expressed the *rtTA* and *tat* transgenes (Tat+) or mice that only expressed *rtTA* without *tat* (Tat–), were fed DOX-containing chow (6 mg/g, Harlan Indianapolis, IN) for 2 weeks prior to behavioral experimentation or 2, 4, or 8 weeks prior to tissue collection. This model has been verified to produce HIV-1 Tat protein in multiple brain regions, including striatum, by immunohistochemistry and western blotting (Bruce-Keller et al., 2008; Fitting et al., 2010).

Tat+ and Tat– mice were crossed with B6.Cg-Tg (Drd1a-tdTomato)6Calak/J line 6 mice (#016204; The Jackson Laboratory) ( $N=17$ ) or Drd2-eGFP (#036931 – UCD; Mutant Mouse Resource and Research Centers) mice ( $N=16$ ) (Ade et al., 2011) to detect D1R-expressing and D2R-expressing neuronal subpopulations, respectively, and were used for immunohistochemistry (IHC)/colocalization experiments.

All mice were housed two to five per cage with *ad libitum* access to food and water in a temperature-, humidity-controlled, AAALAC-accredited facility on a 12:12 h light–dark cycle.

### Drug Administration

Tat+ and Tat– mice were treated with either 50 mg/kg of the KCC2 enhancer, CLP290 (Aobious, Gloucester, MA), freshly suspended in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and 20% 2-hydroxypropyl- $\beta$ -cyclodextrin (HPCD; Tocris, Bristol, UK), or vehicle (20% HPCD in DMSO), once per day (morning, p.o., 200  $\mu$ L total volume per mouse) for seven days during the second week of DOX administration. The dose and route of administration was chosen based on prior reports demonstrating that CLP290 was highly efficacious at

this dose by oral gavage or intraperitoneal injection (Ferrini et al., 2017; B. Chen et al., 2018; Lizhnyak et al., 2019).

### Locomotor Behavior

Tat+ and Tat– mice were assayed for locomotor effects with the open field assay after two weeks total of DOX exposure and the drug administration paradigm described above. Mice were placed in the top left corner of a 40  $\times$  40  $\times$  35 cm Plexiglas box and allowed to freely engage in locomotor activity for 20 min. As a measure of motor and exploratory behavior, rearing responses were recorded when the mice broke the array of photo-beams 4.5 cm above the bottom of the cage, and total distance traveled (meters) was video-recorded and encoded using the ANY-maze behavioral tracking system (Stoelting Wood Dale, IL). Time spent in the center of the open field was also measured by ANY-maze software (Stoelting) to assess anxiety-like behavior (Crawley et al., 1997; Paris et al., 2016).

Mice were also assessed for evoked locomotion and sedation using the rotarod test. Mice were placed on an immobile rotarod (44.5 cm high, 3 cm in diameter; Columbus Instruments, Columbus, OH) prior to the initiation of an accelerating speed trial (0 RPM at start, acceleration step speed of 1 RPM, 7.5 s intervals). Latency to fall after the start of the acceleration protocol was recorded by Rotamex-5 software (Columbus Instruments). Decreased latency to fall is indicative of worsened motor coordination or sedation (Martin et al., 1993).

### Western Blot

KCC2 and NKCC1 were quantified by western blot of the striatum from Tat+ and Tat– mice. Freshly harvested whole striata and hippocampi were homogenized in RIPA buffer (Sigma-Aldrich, St. Louis, MO) with HALT™ Protease & Phosphatase Inhibitor Cocktail (Thermo Fisher, Waltham, MA) to prevent protein degradation. Lysates were separated and stored at  $-80^{\circ}$  C until assay. Protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL). Forty  $\mu$ g lysate per sample were loaded into 4–20% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to PVDF membranes (Bio-Rad) and probed with anti-KCC2 (rabbit polyclonal; 1:2000; RRID:AB\_10638485; Protein Tech Cat#: 19565-1-AP, Rosemont, IL), anti-phospho-S940 (pS940) KCC2 (rabbit polyclonal; 1:2000; RRID:AB\_2860553; Novus Bio Cat# NBP2-29513, Littleton, CO), or NKCC1 (rabbit polyclonal; 1:1000; RRID:AB944433; Abcam Cat# ab59791) and GAPDH (mouse

monoclonal; 1:2000; RRID:AB\_2107448; Abcam Cat# ab8245, Cambridge, UK) antibodies. KCC2, NKCC1, and GAPDH proteins were detected measuring fluorescent signal from Alexa Fluor 647 (goat anti-rabbit; 1:2000; RRID:AB\_2633282; Thermo Fisher Cat# A32733) and 488- (goat anti-mouse; 1:2000; RRID:AB\_2633275; Thermo Fisher Cat# A32723) conjugated secondary antibodies. pS940-KCC2 proteins were detected using pS940-KCC2 specific antibody, horseradish peroxidase (HRP; goat anti-rabbit; 1:10000; RRID:AB\_2687483; Southern Biotech Cat# 4030-05, Birmingham, AL) and SuperSignal<sup>TM</sup> West Femto Maximum Sensitivity Substrate (Thermo Fisher).

Membrane-bound KCC2 was examined by western blot by first performing membrane fractionation on striata from Tat transgenic mice. Briefly, tissue was homogenized in lysis buffer (4 mM HEPES (N-2-hydroxyethylpiperazine-N-3-ethane sulfonic acid; Thermo Fisher Scientific), 320 mM sucrose, 5 mM EDTA (ethylenediaminetetraacetic acid; Thermo Fisher Scientific), and HALT<sup>TM</sup> Protease & Phosphatase inhibitor cocktail (Thermo Fisher Scientific)), centrifuged at 100,000 x g, and the pellet containing the membrane fraction was resuspended in lysis buffer and stored at -80° C until assay. Protein concentration measurements and western blots for these samples were performed as above, except that a 20- $\mu$ g lysate load was used per sample lane. KCC2 was normalized to total lane protein detected by Revert<sup>TM</sup> 700 Total Protein Stain (LI-COR, Lincoln, NE), and Alexa Fluor 488-conjugated secondary antibodies (goat anti-rabbit; 1:2000; RRID:AB\_2633280; Thermo Fisher Cat# A32731) were used to detect KCC2.

### Immunohistochemistry

After two weeks of DOX administration, Drd1a-tdTomato/Drd2-eGFP X Tat+/Tat- mice were fixed by cardiac perfusion with 4% paraformaldehyde (PFA). Brains were then dissected, submerged in 4% PFA for 24 h, washed in PBS, and sequentially placed in 10%, 20%, and 30% sucrose for 24 h each before being embedded in Tissue-Tek<sup>®</sup> O.C.T. compound (Sakura Finetech, Torrance, CA). Serial 20  $\mu$ m sections were cut on a cryostat, thaw-mounted, dried, and stored frozen at -80° C. IHC was then performed by permeabilizing for 30 min (0.01% Triton-X100), blocking for 1 h, and overnight incubation with primary KCC2 antibody (rabbit polyclonal; 1:500; RRID:AB\_10638485; Protein Tech Cat#: 19565-1-AP) at 4° C. The following day, sections were washed and incubated for 1 h with 488 (goat anti-rabbit; 1:1000; RRID:AB\_2633280; Thermo Fisher Cat# A32731) and 594 (goat anti-rabbit; 1:1000; RRID:AB\_2762824; Thermo Fisher Cat# A32740) Alexa-Fluor-conjugated secondary antibodies, respectively, for

1 h. Hoechst 33342 (1:10000, Thermo Fisher) was used to identify nuclei and weighted coverslips were adhered with ProLong<sup>TM</sup> Gold Antifade Mountant (Thermo Fisher).

### Colocalization

To visualize KCC2 immunofluorescence and tdTomato or eGFP, Z-stack images were obtained with a Zeiss LSM 700 confocal module configured to an Axio Observer Z.1 with a 20x (0.8 NA) or 63x (1.40 NA) objective (Zeiss, Oberkochen, Germany) and Zen 2010 software (Carl Zeiss INC, Thornwood, NY). 3-D reconstruction and colocalization of KCC2 and tdTomato- (D1R-expressing MSNs) or eGFP- (D2R-expressing MSNs) containing voxels from 20x objective Z-stacks (0.31  $\mu$ m  $\times$  0.31  $\mu$ m  $\times$  0.79  $\mu$ m) was performed with Imaris software (Bitplane, South Windsor, CT). Colocalization thresholds were determined using secondary control (without primary antibody incubation) sections from the same mice. Thresholds were equivalent across all mice and set to < 1% colocalization between tdTomato/eGFP and KCC2 immunofluorescence for control slices. Colocalization results are displayed as the percentage of voxels with tdTomato or eGFP fluorescence colocalized with voxels containing KCC2 immunofluorescence. High magnification (63x objective) images were obtained to visualize KCC2 subcellular localization.

### Statistics

A two-tailed Student's *t* test was used to examine potential differences in KCC2 western blots between Tat+ and Tat- mice and for colocalization experiments. Two-way analysis of variance (ANOVA) was used to examine behavioral assays and western blots with drug (CLP290/Vehicle) and genotype (Tat+/Tat-) as factors. Interactions were examined using a Bonferroni *post hoc* test to determine group differences. Data are displayed as the mean value  $\pm$  standard error of the mean. Results were considered significant when *p* < .05. All statistical analyses were performed using R analytical software.

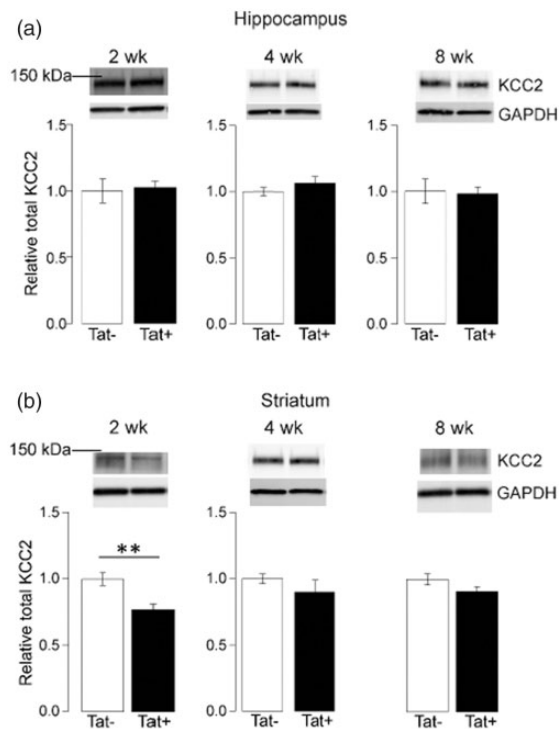
## Results

### Dynamics of Cl<sup>-</sup> Cotransporter Regulation in the Hippocampus and Striatum After DOX-Induced Tat Expression

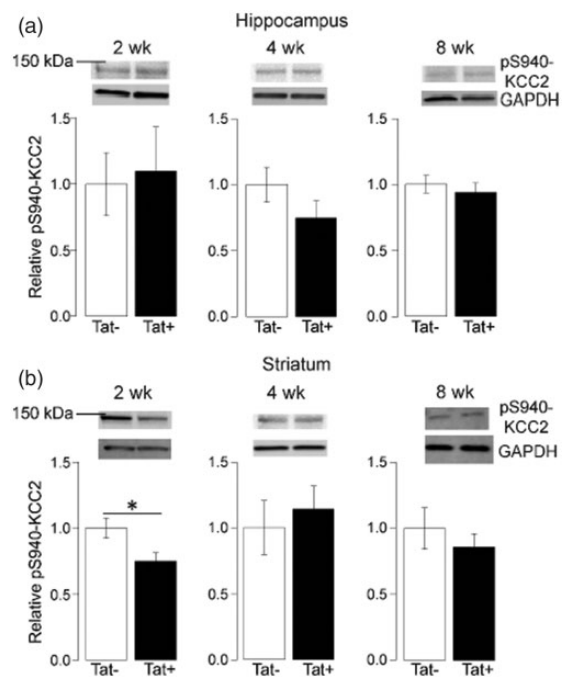
Clinical evidence and results from Tat-transgenic mice have demonstrated vulnerability of the hippocampus and striatum to HIV- and Tat-induced neuropathology. Expression dynamics between the Cl<sup>-</sup> symporters, KCC2 and NKCC1, determine the direction of Cl<sup>-</sup> flux upon GABA<sub>A</sub>R activation. Regulation of KCC2 membrane

localization and, therefore,  $\text{Cl}^-$  extrusion capacity, is mediated by several phosphorylation sites. Among these, phosphorylation of S940 is well known to increase membrane stability. Both HIV-induced neuropathology and pathological  $\text{Cl}^-$  transporter regulation are temporally dynamic (von Giesen et al., 2000; F. Wang et al., 2017; Lzhnyak et al., 2019). Thus, we examined total KCC2, pS940-KCC2, and NKCC1 by western blot in hippocampal and striatal tissue from Tat-transgenic mice after DOX administration for two, four, and eight weeks. There were no differences found in the hippocampus at any time point for total KCC2 (two week DOX:  $p = .82$ ,  $n = 6$ ; four week DOX:  $p = .51$ ,  $n = 6$ ; eight week DOX:  $p = .65$ ,  $n = 7$ ) (Figure 1a), pS940-KCC2 (two week DOX:  $p = .81$ ,  $n = 6$ ; four week DOX:  $p = .2$ ,  $n = 6$ ; eight week DOX:  $p = .54$ ,  $n = 6$ ) (Figure 2a), or NKCC1 (two week DOX:  $p = .56$ ,  $n = 6$ ; four week DOX:  $p = .89$ ,  $n = 6$ ; eight week DOX:  $p = .91$ ,  $n = 6$ ) (Figure 3a). Nor were significant differences found in the striatum after four ( $p = .28$ ,  $n = 6$ ) or eight ( $p = .12$ ,  $n = 6$ ) weeks of DOX-induced Tat exposure for either

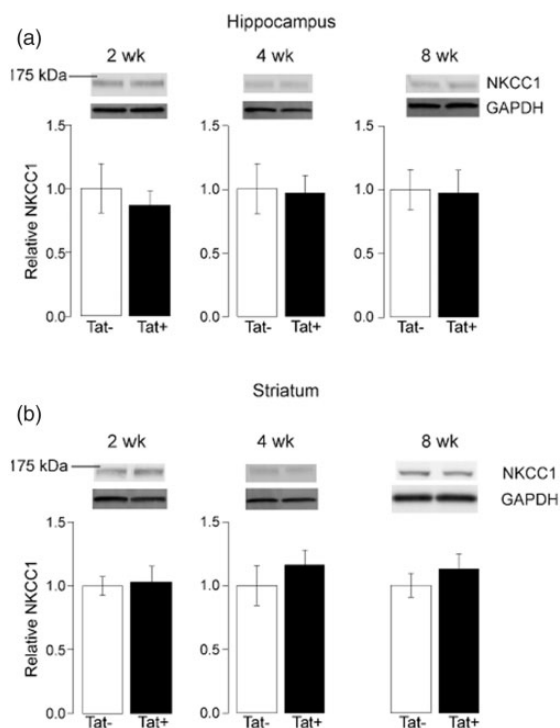
total (Figure 1b) or pS940-KCC2 (4 weeks:  $p = .61$ ,  $n = 6$ ; 8 weeks:  $p = .125$ ,  $n = 6$ ) (Figure 2b). However, two weeks of Tat expression induced a significant decrease in KCC2 ( $p < .01$ ,  $n = 6$ ), consistent with our previous studies (Barbour et al., 2020). Further investigation revealed a significant loss of pS940-KCC2 ( $p = .02$ ,  $n = 11-12$ ) in the striatum of Tat+ mice compared to Tat- mice on DOX for two weeks (Figure 2b). There were no significant changes in NKCC1 levels in the striatum following 2 ( $p = .83$ ,  $n = 10$ ), 4 ( $p = .43$ ,  $n = 6$ ), or 8 ( $p = .43$ ,  $n = 6$ ) weeks of DOX administration (Figure 3b). Overall, these results suggest that the striatum is differentially vulnerable to Tat-induced KCC2 loss via reduced phosphorylation of S940 in a biphasic, or possibly more complex manner. Thus, striatal circuitry may undergo time-dependent disruptions of  $[\text{Cl}^-]_i$  and dysfunction in  $\text{GABA}_A$ -mediated inhibition.



**Figure 1.** DOX-Induced Tat Effects on Total KCC2. Analysis of western blots from the hippocampus of mice administered DOX for two ( $p = .82$ ,  $n = 6$ ), four ( $p = .51$ ,  $n = 6$ ), and eight weeks ( $p = .65$ ,  $n = 7$ ) showed no significant differences between genotypes (a). Two weeks of Tat exposure in the striatum significantly reduced KCC2 (\*\* $p < .01$ ,  $n = 6$ ) while four ( $p = .28$ ,  $n = 6$ ) and eight ( $p = .125$ ,  $n = 6$ ) weeks of Tat exposure in the striatum showed no significant differences (b). All KCC2 western blots are represented as relative intensity to GAPDH normalized to Tat- groups. Data are presented as mean  $\pm$  SEM.



**Figure 2.** DOX-Induced Tat Expression Reduced Striatal pS940-KCC2 at Two Weeks. Analysis of western blots from the hippocampus of mice administered DOX for two ( $p = .82$ ,  $n = 6$ ), four ( $p = .2$ ,  $n = 6$ ), and eight weeks ( $p = .54$ ,  $n = 6$ ) show no significant differences between genotypes (a). Two weeks of DOX administration significantly reduced pS940-KCC2 in the striatum of Tat+ mice compared to Tat- mice (\* $p = .02$ ,  $n = 11-12$ ) while four weeks ( $p = .61$ ,  $n = 6$ ) or 8 weeks ( $p = .54$ ,  $n = 6$ ) of DOX administration resulted in no significant differences. Representative blots showing decreased pS940-KCC2 in Tat+ compared to Tat- mice after two weeks DOX treatment (b; top left). All pS940-KCC2 western blots are represented as relative intensity to GAPDH normalized to Tat- groups. Data are presented as mean  $\pm$  SEM.



**Figure 3.** Tat Expression Does Not Affect NKCC1 in the Hippocampus or Striatum at Any Time Point Examined. NKCC1 levels did not significantly differ between Tat+ and Tat- mice at two ( $p = .56$ ,  $n = 6$ ), four ( $p = .89$ ,  $n = 6$ ), or eight ( $p = .91$ ,  $n = 6$ ) weeks of DOX-induced Tat exposure in the hippocampus (a), nor at two ( $p = .83$ ,  $n = 10$ ), four ( $p = .43$ ,  $n = 6$ ), or eight ( $p = .43$ ,  $n = 6$ ) weeks of DOX-induced Tat exposure in the striatum (b). All NKCC1 western blots are represented as relative intensity to GAPDH normalized to Tat- groups. Data are presented as mean  $\pm$  SEM.

### Tat Expression Reduced Total KCC2 in the Striatum and CLP290 Rescued pS940-KCC2 and KCC2 Membrane Localization

To determine if striatal KCC2 loss could be reversed, we treated Tat+ and Tat- mice with 50 mg/kg CLP290 or vehicle by oral gavage once per day during the second week of DOX administration. Western blot analysis revealed a main effect of genotype with a reduction of total KCC2 in the striatum of Tat+ compared to Tat- mice without an interaction (main effect:  $p = .002$ ,  $n = 12-18$ ), suggesting that Tat induction diminished KCC2 levels and that CLP290 failed to rescue the effects of Tat (Figure 4a). Membrane localization of KCC2 and, thus, Cl<sup>-</sup> extrusion activity is promoted by phosphorylation of S940. Thus, we sought to determine whether CLP290 treatment was capable of reversing the deficits in pS940 caused by Tat. Analysis of western blots of striata harvested from Tat+ and Tat- mice treated with either CLP290 or vehicle for pS940-KCC2 revealed a drug-by-genotype interaction ( $p < .05$ ,  $n = 8-12$ ).

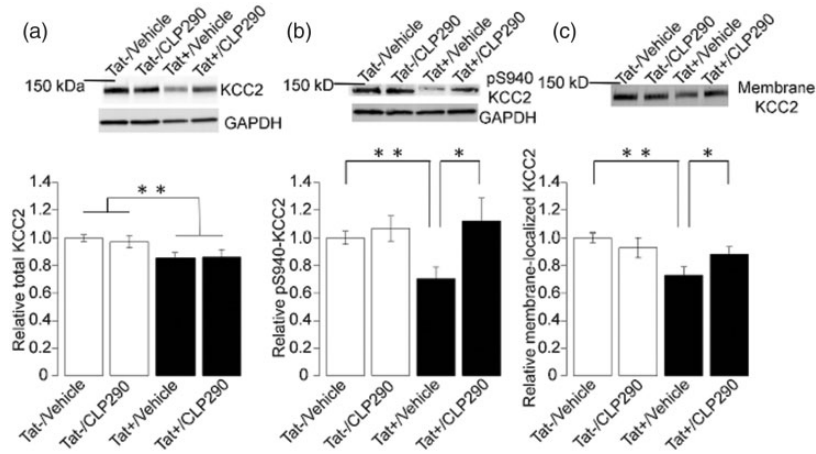
Investigation of group differences determined that two weeks of Tat expression reduced pS940-KCC2 and that administration of CLP290 rescued this effect ( $p < .05$ ;  $n = 8-12$ ) (Figure 4b), likely restoring membrane localization and Cl<sup>-</sup> extrusion capacity of KCC2. To confirm that CLP290 was affecting KCC2 trafficking to the cell membrane, we performed a western blot for KCC2 on pelleted membrane fractions. KCC2 levels were significantly reduced after two weeks of Tat exposure, and levels were restored by CLP290 administration ( $p < .05$ ,  $n = 8-9$ ) (Figure 4c). These data suggest that CLP290 can rescue the functional state of KCC2, and provide insight into a mechanism involving phosphorylation of S940 that may underlie the effects of CLP290.

### CLP290 Administration Reversed the Hyperactive Locomotion Displayed by Tat+ Mice After Two Weeks of Tat Exposure

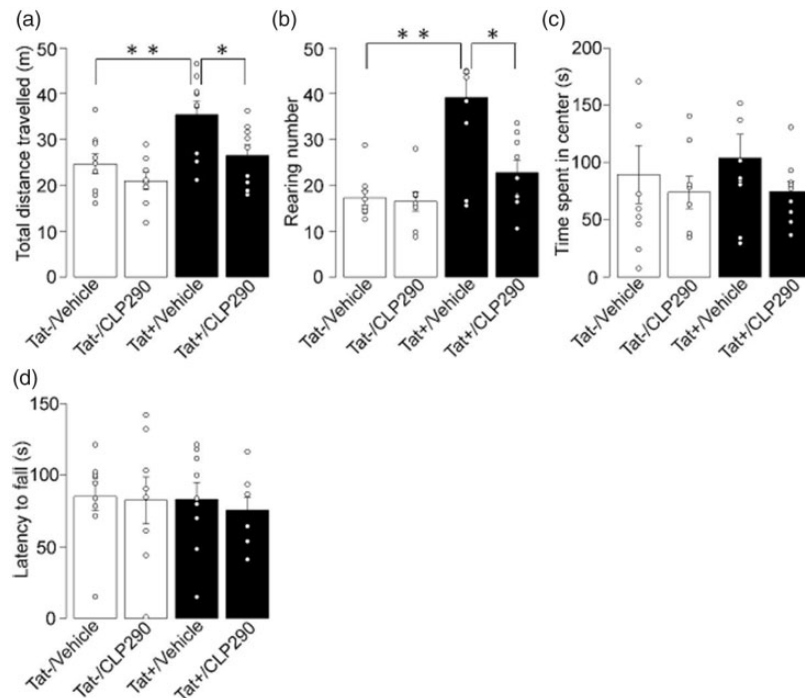
To determine whether Tat-induced disruptions in KCC2 phosphorylation/localization led to any motor deficits, we assayed the locomotor activity of Tat+ and Tat- mice treated with either CLP290 or vehicle in the open field and rotarod tests after two weeks of DOX administration. Motor hyperactivity was seen in the open field assay with significant increases in the distance travelled and number of times rearing in the Tat+/Vehicle group that were reversed in the Tat+/CLP290 group ( $p < .05$ ,  $n = 8-9$ ) (Figure 5a and b). These results suggest that restoration of KCC2 membrane localization, seemingly in the striatum, is sufficient to regulate locomotor activity. We also examined the time spent in the center zone in the open field assay as a measure of anxiety-like behavior and found no significant differences ( $n = 8-9$ ) (Figure 5c). Animals were also tested on the rotarod to determine if treatments affected motor coordination or sedation. No differences in latency to fall were found ( $n = 8-9$ ) (Figure 5d) suggesting that the motor deficits in Tat+ mice after 2 weeks of DOX exposure in the current study are specific to hyperactivity and do not involve a loss in coordination. Further, CLP290 ameliorates this hyperactivity in Tat+ animals without sedative effects. Overall, these results suggest that diminished KCC2 activity in the striatum, likely resulting in the collapse of the Cl<sup>-</sup> gradient and resulting neuronal disinhibition, led to excessive locomotion, and that restoring the subcellular location of KCC2 to the cell membrane with CLP290 was sufficient to return locomotor activity of Tat+ mice to that of Tat- controls.

### D2R-Expressing MSNs Show Enhanced KCC2 Loss in Response to Tat Induction Compared to Their D1R-Expressing Counterparts

Our previous work demonstrated a loss in dendritic spines selectively in D2R-expressing MSNs of Tat+



**Figure 4.** CLP290 Administration Restores Phosphorylation of S940 and Membrane Localization of KCC2 in the Striatum. Two-way analysis revealed a main effect of Tat expression for 2 weeks to reduce total KCC2 (\*\*,  $p < .01$ ,  $n = 12-18$ ) suggesting that CLP290 failed to rescue total KCC2 levels (a). Examination of phosphorylation of S940 revealed a significant decrease in the Tat+ /Vehicle group compared to control (\*\*,  $p < .01$ ,  $n = 8-12$ ) and rescue with CLP290 (\*,  $p < .05$ ,  $n = 8-12$ ) (b) suggesting that membrane-bound KCC2 may be reduced, which was confirmed by western blots on membrane fractions (\*\*,  $p < .01$ ; \*,  $p < .05$ ,  $n = 8-9$ ) (c). Blots for total and pS940-KCC2 are represented as intensity relative to GAPDH normalized to Tat- /Vehicle controls (a and b). Blots for membrane bound KCC2 are relative to total lane protein normalized to controls (c). Data are presented as mean  $\pm$  SEM.

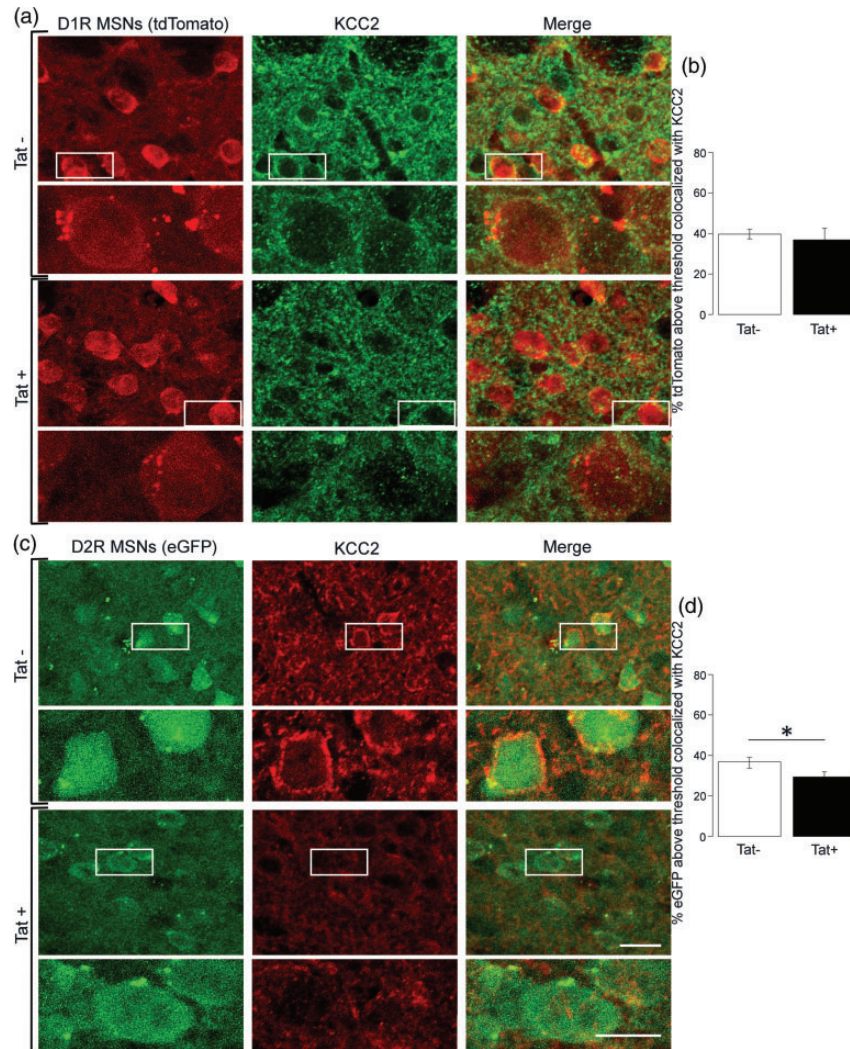


**Figure 5.** CLP290 Reverses Hyperactive Locomotion of Tat+ Mice in the Open Field Assay. Tat+ mice given vehicle showed increased motor activity compared to controls in the open field assay, measured by both total distance travelled (a) and rearing number (b) (\*\*,  $p < .01$ ,  $n = 8-9$ ) during the 20 min open field testing period. 50 mg/kg CLP290 administration significantly decreased total distance travelled and rearing number in Tat+ mice (\*,  $p < .05$ ,  $n = 8-9$ ), suggesting that restoration of KCC2 function was sufficient to rescue abnormal locomotor activity in these mice. We also examined time spent in the center zone during the open field assay as a measure of anxiety-like behavior and found no significant differences ( $n = 8-9$ ) (c). Data from rotarod testing showed no significant differences in latency to fall between any group ( $n = 8-9$ ), suggesting that Tat induction or CLP290 administration did not affect motor coordination or sedation (d). Data are presented as mean  $\pm$  SEM.

mice after two weeks of DOX-induced Tat exposure (Schier et al., 2017). Given the biochemical and behavioral results presented here, and the role KCC2 plays in dendritic spine maintenance (Li et al., 2007; Fiumelli et al., 2013), we hypothesized that D2R-expressing MSNs are selectively vulnerable to Tat-induced KCC2 loss, contributing to disrupted striatal circuitry manifesting as hyperlocomotion.

We crossed Tat<sup>+</sup> and Tat<sup>-</sup> mice with *Drd1a*-tdTomato and *Drd2*-eGFP mice to allow for independent

examination of D1R-expressing and D2R-expressing MSNs to determine the preferential vulnerability of D1R- or D2R-expressing MSNs to KCC2 loss induced by two weeks of Tat exposure. To visualize KCC2 colocalization with tdTomato (D1R-expressing MSNs) or EGFP (D2R-expressing MSNs), we performed IHC with antibodies specific for KCC2 and either 488- or 594-conjugated secondary antibodies, respectively. Z-stacks of striata were obtained and 3D reconstruction and subsequent analysis of colocalization was performed



**Figure 6.** Tat Selectively Reduces KCC2 and Alters Its Cellular Distribution in D2R-Expressing MSNs While D1R-Expressing MSNs Are Unaffected. Colocalization analyses revealed no change in KCC2 immunofluorescence-containing voxels with tdTomato-containing voxels between Tat<sup>+</sup> x *Drd1a*-tdTomato and Tat<sup>-</sup> x *Drd1a*-tdTomato mice ( $n = 3$  (Tat<sup>-</sup>), 12 (Tat<sup>+</sup>)) (a and b). Z-stack images showed no change in KCC2 immunofluorescence in D1R-expressing MSNs (colocalized with tdTomato). Higher magnification (imaged with 63X objective) reveals KCC2 immunofluorescence localized adjacent to the cell membrane in D1R-expressing MSNs (A; under lower magnification (20X) multi-neuron images; white box represents field of view for higher magnification images). Tat<sup>+</sup> x *Drd2*-eGFP mice showed decreased colocalization between KCC2 immunofluorescence-containing voxels and eGFP-containing voxels compared to Tat<sup>-</sup> x *Drd2*-eGFP mice ( $*p < .05$ ;  $n = 8$ ) (c and d). Z-stack images showed a strong colocalization in eGFP-containing voxels (D2R-expressing MSNs) and KCC2 immunofluorescence in Tat<sup>-</sup> mice, while Tat<sup>+</sup> mice showed decreased KCC2 immunofluorescence colocalized with eGFP (d). High magnification images reveal membrane-localized KCC2 immunofluorescence in Tat<sup>-</sup> x *Drd2*-eGFP mice. This pattern of subcellular localization is lost in Tat<sup>+</sup> x *Drd2*-eGFP mice (c). These results suggest a preferential vulnerability of D2R-expressing MSNs to Tat-induced KCC2 loss. Data are presented as mean  $\pm$  SEM. Scale bar = 20  $\mu$ m.



with Imaris software. We found a loss of KCC2 immunofluorescence-containing voxels colocalized with eGFP-containing voxels in Tat+ x Drd2-eGFP mice compared to Tat- x Drd2-eGFP mice ( $p < .05$ ,  $n = 8$ ) without significant changes in KCC2 colocalization between Tat+ or Tat- x Drd1a-tdTomato mice ( $p = .67$ ;  $n = 5-12$ ) (Figure 6). Particularly noticeable is that somatic KCC2 staining appears primarily around the cell membrane in D1R-expressing MSNs and Tat- x D2R-expressing MSNs. This localization was not evident in Tat+ x D2R-expressing MSNs (Figure 6), suggesting a loss of KCC2 function, and a resultant shift in reversal potential of Cl<sup>-</sup> and diminished hyperpolarization in this population of neurons. Overall, data from these experiments suggest a preferential vulnerability of D2R-expressing MSNs to Tat-induced KCC2 loss, which provides critical insight into the circuit disruption underlying the locomotor abnormalities found above.

## Discussion

These studies highlight a novel route by which HIV-1 Tat can disrupt neuronal circuitry leading to motor dysfunction. Loss of KCC2 detection was associated with motor hyperactivity without deficits in coordination after two weeks of DOX-induced Tat exposure, and we also documented a preferential vulnerability of D2R-expressing MSNs. Importantly, we were able to reestablish KCC2 membrane-localization and attenuate motor disturbances with the KCC2 activity enhancer, CLP290, thus demonstrating the potential efficacy of KCC2 as a therapeutic target for the treatment of HAND.

The classic striatal model of motor initiation involves a balance between activation of the D1R-expressing MSN-mediated 'Go-pathway' and the D2R-expressing MSN-mediated 'No Go-pathway' (Gerfen and Young, 1988; Kravitz et al., 2010). Based on this model, we would expect to have seen a preferential vulnerability of D1R-expressing MSNs to Tat-induced KCC2 loss, suggesting a loss of GABA<sub>A</sub>-mediated inhibition and, thus, overactivation of the D1R-mediated 'Go pathway' of motor initiation. Instead, our data align with the growing body of evidence refuting this model. Recent prevailing theories suggest a more subtle circuitry underlying striatal motor initiation and have demonstrated increased D1R- and D2R-expressing MSN activity during movement (Cui et al., 2013; Kupchik et al., 2015; Barbera et al., 2016; Klaus et al., 2017; Parker et al., 2018). Thus, reduced inhibition of either D1R- or D2R-expressing MSNs might lead to abnormal locomotion, as reflected by our findings suggesting that the collapse of the Cl<sup>-</sup> gradient in D2R-expressing MSNs induces motor hyperactivity in the open field assay.

Motor impairment has long been known to afflict a large portion of PWH (Navia et al., 1986) and has

persisted into the era of cART treatment (Robinson-Papp et al., 2008; Valcour et al., 2008; Heaton et al., 2010). Typically, HIV-induced motor deficits are associated with hypokinetic symptoms such as bradykinesia (Mirsattari et al., 1998; Valcour et al., 2008; Sullivan et al., 2011; Bhidayasiri and Tarsy, 2012), but hyperkinetic states like tremor, which can be caused by disinhibition of the striatum (Oran and Bar-Gad, 2018), can be seen as well and may occur with or without other signs of parkinsonism (Nath et al., 1987; Mirsattari et al., 1998; Cardoso, 2002). The motor hyperactivity observed here after Tat exposure (Figure 4a and b) and an associated reduction of membrane localization of KCC2 (Figure 3c) suggests a striatal mechanism that may result in HIV-induced tremor. Studies utilizing positron emission tomography to measure basal ganglia metabolism found that early stages of HIV-infection/HAND were associated with hyperactivity of the basal ganglia, followed by basal ganglia hypometabolism and bradykinesia as the disease progresses (von Giesen et al., 2000). Our results with two weeks DOX treatment may represent early disease stages, resulting in excessive striatal activity caused by hyperactivity in D2R-expressing MSNs of the striatum and increased locomotion. We found that KCC2 localization and S940 phosphorylation returned to baseline by 4 weeks of Tat induction (Figures 1 and 2), although this does not necessarily preclude ongoing deficits in striatal neuron structure or other aspects of striatal neuron function. Coincidentally, our previous work documented that 4 and 12 weeks of Tat expression in these mice *decreased* locomotion (Hahn et al., 2015; Paris et al., 2016). This suggests that the mice display a biphasic change in locomotor activity that mirrors the clinical results showing increased, then decreased, striatal activation (von Giesen et al., 2000).

Since HIV-1 Tat-induced motor deficits can be mitigated by maintaining levels of phosphorylated KCC2 and membrane localization, targeting this pathway may be beneficial for PWH who experience motor impairment. As a therapeutic target, KCC2 may be useful for correcting more than motor deficits in PWH, although this remains to be explored experimentally. Indeed, long-term cognitive changes involving KCC2 and hippocampal circuitry have been documented in multiple human diseases (M. Chen et al., 2017b; Dargaei et al., 2018; Kelley et al., 2018). While we found no overt changes in total KCC2 in the hippocampus, there may be more subtle alterations in specific neuronal subsets that are not detectable by western blot of the entire hippocampus and changes to KCC2 subcellular localization and/or function may be present and operative in memory deficits seen in Tat-transgenic mice and PWH.

It is important to note that this study only utilized male mice. Previous studies from our lab as well as others have demonstrated sex differences in

neuropathological and behavioral deficits in rodent models of HIV (Hahn et al., 2015; Paris et al., 2016; McLaurin et al., 2017). We plan to expand our examination of neuronal  $\text{Cl}^-$  transporter dynamics in response to Tat exposure to female mice. Our results should also be validated and expanded upon by examining the functional responses of D1R- and D2R-expressing MSNs by either gramicidin perforated patch clamp or tight-seal cell-attached current-clamp to measure  $\text{Cl}^-$  reversal and GABA-mediated postsynaptic potentials (Perkins, 2006), respectively. We hypothesize that D2R-expressing MSNs would preferentially show functional deficits based on our colocalization results demonstrating selective vulnerability of D2R-expressing MSNs to Tat-induced KCC2 loss. Previous studies demonstrated that D2R-expressing MSNs were selectively vulnerable to decreased dendritic spine density induced by Tat (Schier et al., 2017). Given the importance of KCC2 in dendritic spine stability (Li et al., 2007; Fiumelli et al., 2013), and D2R-expressing MSN vulnerability to both KCC2 and dendritic spine loss, future studies should examine whether maintenance of KCC2 is sufficient to restore dendritic spines. CLP290 or related treatments might thus find utility in restoring GABAergic function as well as excitatory circuitry. Curiously, there was no difference in the distance traveled, and a decrease in rearing behavior in the open field test after two weeks Tat exposure in a previous study (Schier et al., 2017). However, that study used Tat+ and Tat- mice crossed with D1-tdTomato and D2-eGFP mice for all experiments, while the behavioral assays here utilized the parent strain of Tat+ and Tat- mice. This suggests that there are likely strain differences in the effects of Tat on locomotion. In a previous study, D2-eGFP mice showed elevated motor activity, compared to C57Bl/6 control mice (Ade et al., 2011). Increased motor activity in the Tat- mice from our earlier study, including distance traveled and rearing time (Schier et al., 2017), compared to the Tat-/Vehicle mice in the current study may thus have been due to interbreeding of D2-eGFP and Tat-transgenic mice.

HIV infection comorbidities such as substance use disorder (SUD) or chronic medical conditions (diabetes, hepatitis, etc.) can affect the progression of HAND (Bell et al., 2002; Byrd et al., 2011; Canan et al., 2018; Denis et al., 2019; National Institute on Drug Abuse, 2019). PWH have also been shown to have disrupted reward processing, placing them at higher risk for adverse outcomes associated with SUD (Plessis et al., 2015; Anderson et al., 2016). Drugs of abuse have profound impact on striatal microcircuitry causing long-term epigenetic changes and altered synaptic connectivity/function resulting in dependency (Koob and Volkow, 2010, 2016; Browne et al., 2020; Serafini et al., 2020). Our previous work demonstrated that activation of  $\mu$ -opioid receptors by morphine can reduce KCC2 in

human neurons *in vitro* (Barbour et al., 2020) and previous studies have shown that opiate dependency can cause GABA to act in an excitatory rather than an inhibitory manner (Laviolette et al., 2004; Vargas-Perez et al., 2009). Fitting et al. (2010) also demonstrated that opiate exposure can exacerbate Tat-induced striatal dendritic pathology (Fitting et al., 2010). Given striatal vulnerability to disruption from both HIV and drugs of abuse, the experiments performed here should be expanded to examine whether drugs of abuse interact with Tat to further compromise striatal KCC2, GABAergic function, and associated behavioral dysfunction. Tat exposure also increased cocaine-induced motor hyperactivity (Harrod et al., 2008; Paris et al., 2014a), suggesting that these factors may converge to exacerbate hyperlocomotion through KCC2 dysregulation.

CLP290 had previously been utilized to restore normal motor (B. Chen et al., 2018) and somatosensory-related behaviors (Lizhnyak et al., 2019) in mice after traumatic spinal cord and brain injuries, as well as morphine-induced hyperalgesia in rats (Ferrini et al., 2017). Here, CLP290 restored levels of membrane-localized KCC2 and returned motor activity to baseline levels in an HIV transgenic model with CNS-specific production of HIV-1 Tat. While its efficacy has been well-documented, the mechanism(s) by which CLP290 reestablishes KCC2 activity remains elusive. Membrane localization and degradation of KCC2 are tightly regulated by several different phosphorylation events. Our results suggest that CLP290 rescues KCC2 activity by reestablishing phosphorylation of S940 to increase membrane stabilization of KCC2 and, therefore,  $\text{Cl}^-$  extrusion. Whether CLP290 directly interacts with KCC2 to maintain S940 phosphorylation, or if it enhances PKC or inhibits PP1 to increase or decrease S940 phosphorylation, respectively, remains to be investigated.

The findings here build on our previous *in vitro* work that documented dysregulation of KCC2 by HIV-1 and the HIV proteins Tat and gp120. They implicate KCC2 as a significant player in the motor deficits induced by CNS exposure to HIV-1 Tat. KCC2 is emerging as a key regulator of hyperexcitability in several neurological disorders and the use of CLP290 has shown promise for pharmacological restoration of KCC2 and rescue of physiological and behavioral deficits. Our work suggests a novel mechanism by which CLP290 reinstates KCC2 activity by controlling its membrane localization. KCC2 seems increasingly viable as a target for restoring aspects of the aberrant neurocircuitry seen in multiple CNS regions in neuroHIV, with potentially positive outcomes on both motor and cognitive disturbances. Our results particularly point to potential benefits for PWH who present with HAND, especially those with motor impairment.

## Summary Statement

CNS exposure to HIV-Tat for two weeks reduced striatal KCC2 in dopamine D2 receptor-expressing neurons and was associated with locomotor deficits. Motor abnormalities were rescued with the KCC2 enhancer, CLP290, by restoring phosphorylation of S940 and membrane localization of KCC2.




## Declaration of Conflicting Interests

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