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## Neonatal intrahippocampal HIV-1 protein Tat<sub>1-86</sub> injection: Neurobehavioral alterations in the absence of increased inflammatory cytokine activation

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### Abstract

Pediatric AIDS caused by human immunodeficiency virus type 1 (HIV-1) remains one of the leading worldwide causes of childhood morbidity and mortality. HIV-1 proteins, such as Tat and gp120, are believed to play a crucial role in the neurotoxicity of pediatric HIV-1 infection. Detrimental effects on development, behavior, and neuroanatomy follow neonatal exposure to the HIV-1 viral toxins Tat<sub>1-72</sub> and gp120. The present study investigated the neurobehavioral effects induced by the HIV-1 neurotoxic protein Tat<sub>1-86</sub>, which encodes the first and second exons of the Tat protein. In addition, the potential effects of HIV-1 toxic proteins Tat<sub>1-86</sub> and gp120 on inflammatory pathways were examined in neonatal brains. Vehicle, 25 µg Tat<sub>1-86</sub> or 100 ng gp120 was injected into the hippocampus of male Sprague-Dawley pups on postnatal day 1 (PD1). Tat<sub>1-86</sub> induced developmental neurotoxic effects, as witnessed by delays in eye opening, delays in early reflex development and alterations in prepulse inhibition (PPI) and between-session habituation of locomotor activity. Overall, the neurotoxic profile of Tat<sub>1-86</sub> appeared more profound in the developing nervous system *in vivo* relative to that seen with the first exon encoded Tat<sub>1-72</sub> (Fitting et al., 2008b), as noted on measures of eye opening, righting reflex, and PPI. Neither the direct PD1 CNS injection of the viral HIV-1 protein variant Tat<sub>1-86</sub>, nor the HIV-1 envelope protein gp120, at doses sufficient to induce neurotoxicity, necessarily induced significant expression of the inflammatory cytokine IL-1β or inflammatory factors NFκ-β and Iκ-β. The findings agree well with clinical observations that indicate delays in developmental milestones of pediatric HIV-1 patients, and suggest that activation of inflammatory pathways is not an obligatory response to viral protein-induced neurotoxicity that is detectable with behavioral assessments. Moreover, the amino acids encoded by the second *tat* exon may have unique actions on the developing hippocampus.

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## Keywords

Tat<sub>1-86</sub>; gp120; HIV-1; developmental delay; cytokines; neurotoxicity

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## 1. Introduction

Pediatric human immunodeficiency virus type 1 (HIV-1) infection is a matter of global concern, though it is not equally distributed around the world. As of 2012, ~3.3 million children (under age 15) have been reported to live with HIV-1, with most of these children living in Sub-Saharan Africa (~2.9 million) and less than 1% living in Northern America and Western and Central Europe (UNAIDS, 2013). With the introduction of antiretroviral therapies given to HIV-1 infected women during pregnancy, transmission rate of HIV-1 has decreased dramatically (Suksomboon et al., 2007; Volmink et al., 2007), although in developing countries, the mother-to-child transmission rate of HIV-1 remains very high, ranging from 23% to 34%, with an estimated 260,000 children newly infected in 2012 (UNAIDS, 2013). Because the developing brains of infants and children are extremely vulnerable to this debilitating disease (Mintz, 1994), pediatric HIV-1 is of special interest. The transition of HIV-1 from an acute, lethal disease to a subacute, chronic disease has major implications for the neurocognitive development of children. In the era of combination antiretroviral therapy (cART), vertically acquired HIV-1 infected children that survive to adulthood show high rates of asymptomatic neurocognitive impairment (Paramesparan et al., 2010). Among the neurological abnormalities most commonly seen in HIV-1 infected children on cART are delays in developmental milestones and poor neuropsychological functioning, including attentional, memory and motor dysfunction (Gavin and Yogev, 1999; Gupta et al., 2009; Jeremy et al., 2005; Keller et al., 2004; Le Doare et al., 2012; Paramesparan et al., 2010; Patel et al., 2008; 2009; Willen, 2006).

It is well-established that HIV-1 infected cells release neurotoxic viral proteins, including the transactivating protein Tat and the envelope protein glycoprotein 120 (gp120) (Bansal et al., 2000; Brenneman et al., 1988; Catani et al., 2003; Nath et al., 2000). The nonstructural regulatory protein Tat and the envelope gp120 are likely agents of the observed synaptodendritic alterations (Desplats et al., 2013; Ellis et al., 2007; Gelman and Nguyen, 2010; Moore et al., 2006) and neuronal loss (Del Valle et al., 2000; Jones et al., 2000; Nath et al., 2000) in the brains of HIV-1+ patients and have been studied extensively with *in vitro* and *in vivo* animal studies (Aksenov et al., 2001; 2003; 2006; Aksenova et al., 2005; 2006; Bertrand et al., 2013; 2014; Bruce-Keller et al., 2003; Cheng et al., 1998; Corasaniti, et al., 2001a, 2001b, 2001c; Fitting et al., 2006a; 2006b; 2007; 2008a; 2008b; 2010a; Lipton et al., 1995). Tat and gp120 exposure in rats reveals a similar pattern of synaptodendritic damage as seen in patients with HIV-1-associated neurocognitive disorders (HAND) (Tat, Fitting et al., 2010b; 2013; gp120, Gorantla et al., 2012; Kang et al., 2010; Toggas et al., 1994; Roscoe et al., 2014), lending support to the general idea that HIV-1 proteins are pathophysiologically relevant in HIV-1 related neurocognitive abnormalities. *In vitro* as well as *in vivo* studies have demonstrated that the HIV-1 Tat protein itself can increase chemokine/cytokine production, and cause astrogliosis, microgliosis and neuronal death (El-

Hage et al., 2006; 2008). Specifically, the HIV-1 Tat protein has been shown to increase expression of interleukin-1 $\beta$  in cultures of human fetal astrocytes (Nath et al., 1999).

Tat protein is encoded by two exons producing a clinical isolate with either 86- (Jeang, 1996) or, the more common clinical isolate, 101-amino acids (Jeang et al., 1999). The first 72 amino acids correspond to the first exon of *tat* and this sequence is essential for efficient viral replication (Frankel et al., 1989); however, the Tat<sub>1-72</sub> protein has never been observed *in vivo* (Campbell & Loret, 2009). The second *tat* exon encodes the C-terminus, defined by residues 73–101. Relative to the extensive study of the first exon functions in viral pathogenesis (Frankel et al., 1989; Romani et al., 2010), the physiological role of the second exon is less well-defined (Smith et al., 2003). The second exon encodes a RGD sequence in residues 77–79 (Watson and Edwards, 1999). RGD motifs are part of the recognition sequence for cell surface integrin binding (Barczyk et al., 2010), and integrins have a key role in neonatal hippocampal development (Gary et al., 2003; Murase et al., 2011; Wakselman et al., 2008). Further, as documented by *in vitro* studies, the uptake of extracellular Tat<sub>1-86</sub> is 10X more efficient relative to the first exon Tat<sub>1-72</sub>, suggesting that the region encoded by the second exon is important in mediating efficient Tat internalization (Ma and Nath, 1997). Thus, inclusion of the second *tat* exon encoded polypeptide, containing the RGD and internalization sequence, in the present studies of Tat<sub>1-86</sub> is anticipated to produce specific effects on hippocampal development.

Using extant preclinical models such as transgenic rats/mice, or direct CNS injection of virotoxins, it is possible to study the direct effects of the HIV-1 proteins independent of any secondary HIV-1 infections or the virus itself. Previous studies in our lab have established that both Tat<sub>1-72</sub> and gp120 produce adverse long-term effects on neurocognitive processes involved in sensorimotor gating when injected bilaterally into the hippocampus of postnatal day (PD) 1-old rats (Fitting et al., 2006a; 2006b), and that Tat<sub>1-72</sub>- and gp120-induced delays in early reflex development are correlated with alterations in hippocampal cell number (Fitting et al., 2008b). The hippocampus is of particular interest due to both the high viral load present in this region (Wiley et al., 1998), and evidence of synaptodendritic injury in the hippocampus in both HIV-1+ humans (Sa et al., 2004; Desplats et al., 2013) and in rats exposed to viral proteins (Fitting et al., 2013).

In addition to the release of different viral proteins by HIV-1 infected cells, elevation of a variety of cytokines may be detected. Cytokines and other inflammatory biomarkers have been clearly associated with HIV-1-associated dementia (HAD) in the pre-cART era (Gartner and Liu, 2002; Glass et al., 1993; Perrella et al. 1992), but they may also play a role in the development of less severe forms of HAND despite viral suppression due to cART. A recent longitudinal study reported significantly higher levels of Interferon- $\alpha$ -2b, IL-6, and sIL-2R in HIV-1+ individuals that became neurocognitively impaired, remained impaired, or worsened one year after the baseline study, compared to HIV-1+ individuals who had either no impairment or improved neurocognitive status (Cassol et al., 2013). Neurocognitive impairment has also been associated with elevated levels of cytokines in perinatally HIV-1-infected children (Foster et al. 2012). The inflammatory response initiated by the immune system in response to the viral presence can damage neurons and disrupt function (Ensoli et al., 1999; Miura et al., 2003; Yadav and Collman, 2009), and could likely

account for many clinical and pathological conditions seen in the nervous system in HIV-1+ individuals. Children with HIV-1 may be particularly vulnerable to neuronal damage related to inflammatory cytokine activation, given both an immature CNS and higher levels of cytokines compared to HIV-1+ adults (Jin et al., 2009; Ketlinskii et al., 1992). In particular, cytokines play an important role in hippocampal function (Arisi, 2014) and brain development (Bilbo and Schwarz, 2012).

In the present study, we injected Tat<sub>1-86</sub> into the neonatal rat hippocampus on PD1 and investigated two aspects: *First*, we examined the effects of the HIV-1 neurotoxic protein Tat<sub>1-86</sub> on neurobehavioral development. Several early developmental reflexes were used to assess different aspects of neurodevelopment in neonatal rats (i.e. righting reflex, negative geotaxis, eye opening). Preattentive processes were indexed by prepulse inhibition (PPI), a neurodevelopmental assessment of sensorimotor gating (Parisi & Ison, 1979). Locomotor activity measures were employed to determine the presence or absence of motor dysfunction. *Second*, we investigated the potential effects of Tat<sub>1-86</sub> and gp120 on inflammatory responses. The role of inflammatory processes may provide important insights into HIV-1 protein-induced neurotoxicity and is critical in determining the contribution of the viral proteins, Tat<sub>1-86</sub> and gp120, to the neurological and neuropsychiatric impairment consequent to early HIV-1 infection. In sum, using Tat<sub>1-86</sub>, which encodes both the first and second exons, we sought to ascertain HIV-1 viral protein-induced effects on neurobehavioral development and inflammatory activation.

## 2. Methods

### 2.1. Animals

Sprague-Dawley pregnant dams ( $n = 14$ ) were ordered from Harlan Laboratories, Inc. (Indianapolis, IN) and delivered to the vivarium before embryonic day seven. Dams were singly housed with food (Pro-Lab Rat, Mouse Hamster Chow #3000, NIH diet #31) and water available *ad libitum*. The day the dam gave birth was designated postnatal day 0 (PD0). The animal facility was maintained at  $20 \pm 2$  °C,  $50 \pm 10$  % relative humidity and had a 12-h light: 12-h dark cycle with lights on at 0700h (EST). The animals were maintained according to the National Institute of Health (NIH) guidelines in AAALAC-accredited facilities. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina, Columbia.

For the behavioral experiment, on PD1, litters ( $n = 8$ ) were culled to five males and five females. Two males from each litter were assigned to one of the two treatment conditions tested in the present study – vehicle vs. Tat treatment. At 22 days of age, animals were weaned and litters were culled to only treated rats. These two-rat housing arrangements were maintained for the remainder of the experiment.

For the inflammatory biomarker experiment, on PD1, six males from each litter ( $n = 9$ ) were randomly assigned to one of three treatment groups: vehicle, Tat or gp120. On PD3, 48 h after experimental treatment, the pups were sacrificed and brains were removed.

## 2.2. HIV-1 Proteins

Purified recombinant Tat<sub>1-86</sub> (LA1/Bru strain of HIV-1 clade B, Genbank accession no. K02013, Diatheva, Fano, Italy) and T-tropic gp120 (Protein Sciences Corp., Meriden, CT) were purchased and stored at  $-80^{\circ}\text{C}$  until used for intrahippocampal injections. Recombinant Tat and gp120 proteins were dissolved in sterile buffer to final concentrations of  $25\ \mu\text{g}/\mu\text{L}$  and  $100\ \text{ng}/\mu\text{L}$ , respectively.

## 2.3. Surgical Techniques and Protein Treatment

Standard stereotaxic surgery techniques, modified for neonates, were used for treatment injection. Individual pups were removed from the dam and cryogenically anesthetized (AVMA, 2001) before being placed in a modified stereotaxic holder for surgery of neonates (Kopf, Inc., Tujunga, CA), which included a chilled base to maintain cryogenic anesthesia. Rubber head bars held the skull in place while microinjections were made directly into the hippocampus using stereotaxic coordinates and a microsyringe [Hamilton Co., Nevada, USA (Microliter #701 RN,  $10\ \mu\text{L}$ )]. For both the behavioral and inflammatory biomarker studies, bilateral injections were conducted with silanized syringes. The set of coordinates used for the left and right hippocampus were:  $-0.65\ \text{mm}$  anterior to bregma,  $1.0\ \text{mm}$  lateral to bregma, and  $-2.2\ \text{mm}$  dorsal from dura (Fitting et al., 2008a; 2008b). Hippocampal injection order was alternated across animals, using the same set of coordinates. The injection volume was  $1.0\ \mu\text{L}$  released over two min, after a one-min resting period that allowed the tissue to return to its original conformation (Fitting et al., 2006a; 2006b; 2007). Animals were injected bilaterally with sterile buffer (vehicle (VEH);  $10\ \text{mM}$  Tris HCl,  $300\ \text{nM}$  NaCl,  $\text{pH} = 7.58$ , sterile), Tat  $25\ \mu\text{g}$ , or  $100\ \text{ng}$  gp120. These doses were selected on the basis of our previous developmental research (Fitting et al., 2006a; 2006b; 2007; 2008b; 2010a); doses at which no gross growth impairment would be anticipated. The injection needle was withdrawn over two min to prevent reflux. The piercings in the skin of the head were closed with surgical glue and the pups warmed on a heat pad ( $35^{\circ}\text{C}$ ) before being returned to the dam, where they were closely monitored for any indications of rejection. No pups were rejected or abused by the dam.

## 2.4. Behavioral Experimental Design

A randomized block design was employed, with litter as the blocking factor, in which both experimental treatments were represented. Thus, male rats were randomly assigned to one of two treatment groups [VEH ( $n = 8$ ), or Tat ( $n = 8$ )] that received bilateral hippocampal injections on PD1. Weight was taken on the day of surgery, PD1, and subsequent, concurrent weighing started on PD3. Behavioral assessment began on PD3. All rats were tested for (1) early reflex development by assessing righting reflex on PD3-5 and negative geotaxis on PD8-10; (2) developmental milestone by assessing eye opening on PD13-17; (3) preattentive processes by assessing PPI of the acoustic startle response (ASR) on PD18; and (4) motor function by assessing locomotor activity in post-weanling rats on PD26-28.

### 2.4.1. Health and Somatic Growth

**Body Weight:** Pups were weighed on a daily basis starting on PD3 for the remainder of the experiment.

**Eye opening:** Eye opening was examined as a developmental milestone on PD13-16. The first day any opening was witnessed was PD13; all animals' eyes were fully opened by PD16. Eye opening is a crucial development of sensory processes and vestibular plasticity (Grassi et al., 2004) that critically influences the cellular connectivity of the visual cortex (Heinen et al., 2004; Katz and Shatz, 1996). Eye opening was assessed separately for right and left eye between 0800–1000h (EST). Eye opening was assessed on a two-point scale, with a zero score indicating a closed eye and a score of 1 indicating an open eye.

#### 2.4.2. Early Reflex Development

**Righting Reflex:** The righting reflex was examined on PD3-5 to assess preweaning alterations in the sensory-motor system very early in development. The goal was to assess the development of the sensory-motor system at a time point when other developmental milestones were still underdeveloped and thus not confounded by secondary variables, such as eye opening and ear opening. The righting reflex is defined as the ability to assume an upright position when there has been a departure from it (Walton et al., 2005). Pups were tested each day between 1600–1800h (EST) on a metallic wire grid. A pup was placed on its back and time in seconds was recorded to return to the upright position by turning over onto its ventral surface.

**Negative Geotaxis:** Another test to determine the development of the sensory-motor system was the assessment of time in seconds to perform negative geotaxis on PD8-10. Negative geotaxis is defined as an orienting response and movement expressed in opposition to cues of a gravitational vector (Motz and Alberts, 2005). Pups were tested each day between 1600–1800h (EST). The test was conducted by placing the pup oriented facing head down on a 25° tilted plane with a metallic wire grid. The dependent variable was time in sec to turn 180° and orient toward the higher end of the plane.

#### 2.4.3. Sensorimotor function – Preattentive Process (PPI of the ASR)

**Apparatus:** The startle chamber (SR-Lab Startle Reflex System, San Diego Instruments, Inc.) was enclosed in a 10 cm thick double-walled, 81 × 81 × 116 cm isolation cabinet (external dimensions) (Industrial Acoustic Company, INC., Bronx, NY). Each animal was tested individually in the dark with a high-frequency loudspeaker, that produced a background white noise [70 dB(A)] and was mounted inside the chamber 31 cm above the Plexiglas cylinder. The startle chamber consisted of a Plexiglas cylinder 3.9 cm in internal diameter for preweaning rats. The Plexiglas cylinder was resting on a 12.5 × 20 cm Plexiglas stand. The startle stimulus was 100 dB(A) and the acoustic prepulse stimulus was 85 dB(A) in intensity. Both stimuli had a duration of 20 ms. The animal's response to the startle stimulus produced deflection of the Plexiglas cylinder, which was converted into an analog signal by a piezoelectric accelerometer. The signals were then digitized (12 bit A to D) and saved to a hard disk on a Pentium class computer. Acoustic stimulus intensities and response sensitivities were calibrated with a sound level meter (Extech Instruments: Waltham, MA) with the microphone placed inside the Plexiglas cylinder.

**Testing Procedures:** All rats were tested for approximately 20 min on PD18. Animals were first exposed to a five-min acclimation period of 70 dB(A) background of white noise,



followed by six single white noise stimuli of 100 dB(A) as adaptation trials, and 36 PPI trials with 0, 8, 40, 80, 120 and 4000 ms interstimulus intervals (ISIs), assigned by a Latin-square design. The stimulus duration was 20 ms. The PPI trials with the 0 and 4000 ms ISIs were defined as control trials in order to provide a reference baseline ASR within the PPI test. Within the constraints of the software, the ISI represents the time from the offset of the prepulse stimulus to the onset of the startle stimulus. For PPI the dependent measure analyzed was peak ASR amplitude.

**2.4.4. Motor Function – Locomotor Activity**—The activity monitors were square (40 cm × 40 cm) chambers (Flex-Field, San Diego Instruments, San Diego, CA) that detected free movement of animals by infrared photocell interruptions. This equipment used an infrared photocell grid (32 emitter/detector pairs) with locomotor activity being measured by assessing the number and type of photocell interruptions within a 60-min period. Testing occurred on PD26-28 between 1500–1700h (EST) under dim light conditions, in the absence of direct overhead lighting (<10 lx). The dependent variable was the animal's total ambulation (cm; the sum of x and y photocell interruptions). Total time (s) spent in the center and the periphery of the activity monitor was also recorded.

**2.4.5. Data Analyses**—The data were expressed as mean ( $\pm$  SEM) or median ( $\pm$  Interquartile Range), as appropriate. Continuous data were analyzed using ANOVA techniques (SYSTAT 11.0 for Windows, SYSTAT Inc.) including treatment (VEH or Tat<sub>1-86</sub>) as a between-subjects factor. For the within-subjects terms (e.g., test day, trial, ISI), potential violations of sphericity (Winer, 1971) were preferentially handled by the use of the orthogonal decomposition or, if necessary, the use of the Greenhouse-Geisser *df* correction factor (Greenhouse and Geisser, 1959). The orthogonal decomposition approach for the within-subjects terms also permitted a determination of the shape of the temporal functions (e.g., linear, quadratic, etc.) (Winer, 1971). Simple main effects of test day were also included where appropriate. The non-parametric Mann-Whitney U-Test was performed on the somatic growth index of eye opening. An alpha level of  $p = 0.05$  was considered significant for interpretation of all statistical tests.

## 2.5. Inflammatory Biomarker Experimental Design

**2.5.1. Tissue collection and cDNA library generation**—On PD3, 48 h after viral protein injections, pups were sacrificed by rapid decapitation and the brain was removed from the cranial vault for microdissection. This 48 h interval was selected on the basis of our prior study of Tat protein injection and the temporal relationship to reactive astrocytosis and protein oxidation (Aksenov et al., 2003) and early behavioral sequelae (Fitting et al., 2008b). After removal of obstructing meninges the midbrain was dissected out of the surrounding midbrain tissue to facilitate access to the hippocampus. Both left and right hippocampal tissue were collected from all 6 males of each litter, and pooled within the appropriate treatment groups to ensure sufficient tissue mass for RNA isolation. Total RNA was isolated using the RNAqueous-4PCR kit (Ambion, Austin, TX) and concentration was measured by the 260/280 nm absorbance ratio (DNA Calculator, GeneQuant, Piscataway, NJ). A cDNA library was generated by the reverse transcription PCR (RT-PCR) of 1  $\mu$ g purified RNA using 50 ng/ $\mu$ L random hexamer primers (Cloned AMV First-Strand cDNA Synthesis kit,

Invitrogen, Carlsbad, CA). An additional sample lacking the reverse transcription enzyme was included in the RT-PCR reaction to control for potential DNA contamination.

**2.5.2. MPCR**—Following the RT-PCR production of cDNAs the samples were probed for the expression of inflammatory cytokine IL-1 $\beta$ , inflammatory factors NF $\kappa$ - $\beta$  and I $\kappa$ - $\beta$ , and the internal control, GAPDH. The MPCR reaction was optimized for cycle number and DNA content, and samples were run in a cycle protocol of 94 °C melting, 64 °C annealing and 70 °C elongation. Cytokine primers, a PCR reaction buffer containing dNTPs and a positive control were provided in the MPCR kit (Rat Inflammatory Cytokines–Set 2, Maxim Biotech, San Francisco, CA). Optimal results were achieved with 4 % v/v RT-PCR mixture over 30 cycles at the aforementioned temperatures. Amplicons were resolved on a 10 % TBE gel for 1 h at 200 V. DNA molecular weight ladders, cDNA positive controls and a negative control were included on the gels to provide reference standards.

**2.5.3. Data Analysis**—PCR amplicons were separated on a 10 % TBE precast gel (Bio-Rad, Hercules, CA) at 200 V for one h, followed by a 30 min gel stain in SYBR Green (Molecular Probes, Carlsbad, CA). Bands were visualized on a ChemiDoc XRS Molecular Imager (Bio-Rad, Hercules, CA) and the data recorded and summarized with the accompanying Quantity One software. Data analysis was then conducted using ANOVA techniques, as with the behavioral data, with treatment as a between-subjects factor and inflammatory biomarker as a within-subjects factor (Winer, 1971). Again, an alpha level of  $p = 0.05$  was considered significant for interpretation of all statistical results.

### 3. Results

#### 3.1. Behavioral Data

##### 3.1.1. Health and Somatic Growth

**Body Weight:** Figure 1 illustrates the mean ( $\pm$  SEM) body weight data across test days for the VEH- and Tat-treated animals. Separate ANOVAs were conducted for each test set with treatment as the between-subjects factor and, where appropriate, test day as a within-subjects factor. Neither a main effect of treatment nor a treatment x day interaction was noted for any of the test sets. As illustrated in Figure 1, body weight increased with age and no detrimental effect of the Tat<sub>1-86</sub> protein was detected.

**Eye Opening:** Eye opening started at PD13 for VEH-treated animals, and PD14 for Tat<sub>1-86</sub>-treated animals. Eyes were fully open for all rats on PD16. Eye opening data were analyzed as ‘eye closed’ (coded as 0) and ‘eye open’ (coded as 1). Collapsing across PD14-15 and computing the sum of left and right eye scores, the maximum rank score was 2 and the minimum rank score was 0.

A Mann-Whitney U-Test on the combined data (collapsed across PD14-15), revealed a Tat-induced deficit in eye opening ( $U = 14.5$ ,  $p = 0.05$ , one-tailed). Separate U-tests conducted for each day suggested an effect of Tat on the second assessment day of eye opening, specifically (PD14:  $U = 16.0$ ,  $p = 0.06$ , one-tailed; Tat, median= 0, IQR=0; VEH, median=0.5, IQR=1). Thus, the analysis of daily observation data indicated significant



alterations on PD14 as a function of treatment. No significant treatment effect was noted on PD15.

### 3.1.2. Early Reflex Development

**Righting Reflex:** Figure 2A illustrates mean ( $\pm$  SEM) response time for righting reflex of VEH- and Tat-treated animals across the three test days (PD3–5). A 2 (treatment) x 3 (test days) mixed-model ANOVA conducted on the log transformed latency data (time in s) of each day revealed a significant treatment effect [ $F(1, 14) = 9.8, p = 0.01$ ]. Tat-treated animals were much slower in the righting reflex compared to the VEH-treated group. A significant day effect was found [ $F(2, 28) = 3.4, p = 0.05$ ] with a prominent linear component [ $F(1, 14) = 7.1, p = 0.05$ ], indicating that execution of the righting reflex improved across test days. Planned contrast analyses for each treatment indicated significant differences in the progression of the righting reflex across the three testing days. The overall significant day effect was due to a significant linear trend for a decrease in response time for the VEH group [ $F(1, 7) = 9.1, p = 0.05$ ], which was not evident for the Tat-treated group. Thus, the Tat<sub>1-86</sub> treatment appeared to disrupt maturation of the righting reflex across test days.

**Negative Geotaxis:** Figure 2B illustrates the mean ( $\pm$  SEM) response latency for negative geotaxis across the three test days (PD8–10) by treatment group. A 2 (treatment) x 3 (test days) mixed-model ANOVA on latency (time in s) revealed a significant treatment effect [ $F(1, 14) = 4.7, p = 0.05$ ], indicating overall slower response latencies for animals treated with Tat<sub>1-86</sub>. Further, the analysis revealed a test day effect [ $F(2, 28) = 8.3, p = 0.01$ ] with a prominent linear component [ $F(1, 14) = 4.7, p = 0.05$ ]. No significant treatment x test day interaction was detected, suggesting that both groups showed a similar rate of improvement across test days, approximating similar terminal levels of acquisition.

**3.1.3. Sensorimotor Function (PPI of the ASR)**—Figure 3 displays ( $\pm$  SEM) peak ASR amplitude for preweanlings (PD18) across ISI (0–4000 ms) by treatment group.

**Control Trials (0, 4000 ms ISI combined):** A one-way ANOVA on peak ASR amplitude revealed no significant treatment effects, suggesting that Tat<sub>1-86</sub> did not affect the animals' baseline startle response.

**PPI Trials (8–120 ms ISI):** Response amplitude for the PPI trials was decreased by 61.8 % compared to the ASR control trials, demonstrating effective sensorimotor gating across groups. A 2 (treatment) x 4 [ISI (8, 40, 80, 120 ms)] mixed-model ANOVA conducted on peak ASR amplitude demonstrated a significant ISI effect [ $F(3,42) = 10.3, p = 0.001$ ] with a most prominent quadratic component [ $F(1,14) = 36.1, p = 0.001$ ]. Although the treatment x ISI interaction failed to achieve statistical significance, the functional relationship describing the inhibition curve appeared to be altered as a function of treatment. Specifically, the ISI function for the VEH-treated animals was exclusively represented by a significant quadratic relationship [ $F(1,7) = 28.7, p = 0.001$ ], whereas the ISI function for the Tat-treated animals displayed both linear and quadratic components [ $F(1,7) = 6.2, p = 0.05$  and  $F(1,7) = 10.8, p$

0.01, respectively]. Both treatment groups displayed maximal prepulse inhibition at the 80 ms ISI.

**3.1.4. Motor Function (Locomotor Activity)**—Mean total ambulation over a 60-min period on PD26-28 is illustrated in Figure 4. A 2 (treatment) x 3 (test days) mixed-model ANOVA revealed a significant test day effect [ $F(2, 28) = 9.4, p = 0.002$ ] with significant linear and quadratic orthogonal components [ $F(1, 14) = 11.2, p = 0.005$  and  $F(1, 14) = 6.2, p = 0.03$ , respectively]. A significant test day effect was confirmed in the VEH-treated group [ $F(2,28)=7.68, p<0.002$ ], with a prominent linear component [ $F(1,14)=11.5, p = 0.005$ ], indicating that the VEH-treated animals displayed prominent consistent between-session habituation. In contrast, the significant test day effect for the Tat-treated group [ $F(2,28)=3.41, p<0.05$ ] contained only a significant quadratic component [ $F(1,14)=6.19, p<0.03$ ], suggesting a failure to habituate across the daily test sessions. Tests of simple main effects further confirmed that the Tat<sub>1-86</sub>-treated group demonstrated significantly greater ambulation on the third session compared to the VEH-treated group [ $F(1,14)=5.9, p = 0.05$ ]. Temporal measures of activity revealed a similar pattern of results with a parallel profile of statistically significant outcomes (data not shown).

### 3.2. Inflammatory Biomarker Data

Figure 5 illustrates the mean ( $\pm SEM$ ) inflammatory biomarker expression by treatment group. Expression of IL-1 $\beta$ , NF $\kappa$ - $\beta$ , and I $\kappa$ - $\beta$  was detected across all three treatment groups. Neither an overall ANOVA nor one-way ANOVAs for each of the three genes expressed revealed a significant treatment effect of Tat or gp120 on biomarker expression.

## 4. Discussion

Neurobehavioral assessments revealed significant, perhaps transient, adverse effects of hippocampal HIV-1 protein Tat<sub>1-86</sub> injection in male rats, whereas an absence of significant expression of inflammatory cytokine IL-1 $\beta$  and related factors NF $\kappa$ - $\beta$  and I $\kappa$ - $\beta$  was observed following Tat<sub>1-86</sub> or gp120 injection. Notably, no significant effects on body weight were detected, indicating that the Tat<sub>1-86</sub> dose used in the present study produced no general growth deficits, similar to previous findings when injecting Tat<sub>1-72</sub> (Fitting et al., 2008b). The protein variant, Tat<sub>1-86</sub>, has been shown to induce neuronal cell death *in vitro* (Pocernich et al., 2005), and its uptake is ten times more efficient relative to the first exon Tat<sub>1-72</sub> (Ma et al., 1997). The results of the present study provide greater relevance of this model by demonstrating the effects of the Tat<sub>1-86</sub>.

The significant Tat-induced alterations detectable during the neonatal period included measures of both somatic (eye opening) and reflex development. Our results suggest that the viral HIV-1 protein Tat<sub>1-86</sub> acts differently on the developing CNS compared to the short HIV-1 protein variant Tat<sub>1-72</sub>. Whereas Tat<sub>1-86</sub> delayed the onset of eye opening in the current study, alterations in eye opening following treatment with Tat<sub>1-72</sub> (same dose) were never large enough within a reasonable sample size to be confirmed as a delay in development (Fitting et al., 2008b). The two Tat proteins also appeared to induce a different profile of effects on the righting reflex, an early reflex developmental milestone; specifically, Tat<sub>1-86</sub> produced a significant overall impairment in the execution of the

righting reflex across test days, whereas Tat<sub>1-72</sub> produced a significant deficit only on PD3, with normal latency displayed on PD4 and 5 (Fitting et al., 2008b). In contrast, regarding negative geotaxis, the outcome from either Tat variant appeared quite similar. With Tat<sub>1-86</sub>, there was over a 100 % slower latency to right on P8; with Tat<sub>1-72</sub>, there was a 70 % slower latency to right on P8 (Fitting et al., 2008b); on P10, righting latencies for either Tat-treated group were not distinguishable from VEH-treated controls. Thus, negative geotaxis data suggested an approximate two-day delay in reflexive development, albeit not differentially as a function of Tat variant.

The preweaning/weanling age neurobehavioral assessments also supported the perspective that the two Tat proteins may produce a different profile of neurobehavioral alterations. With respect to the auditory startle response and PPI, the Tat<sub>1-86</sub> treatment appeared to alter the prepulse inhibition function relative to VEH controls whereas no significant effects of Tat<sub>1-72</sub> were detectable during the preweaning period (Fitting et al., 2008b). However, neonatal Tat<sub>1-72</sub> treatment-induced alterations in PPI were detectable once the animals reached adulthood (PD91). Regarding the weanling assessments of locomotor activity, both the Tat<sub>1-86</sub>- and the Tat<sub>1-72</sub>-treated (Fitting et al., 2008) animals displayed less between-session habituation across days relative to the VEH-treated controls. One possible explanation for the reduction in habituation is a Tat-induced altered response to novelty. Dopamine systems are particularly vulnerable to the neurotoxic effects of Tat (Aksenov et al. 2008; Ferris et al. 2009; Midde et al. 2013; Zhu et al. 2009; Zhu et al. 2011), and variations in dopamine systems have been associated with differences in locomotor responses to novel environments (Chefer et al., 2003; Hooks et al., 1992, 1994; Zhu et al., 2007). Based on the Tat<sub>1-86</sub>-induced delays in eye opening, delays in righting reflex development, and alterations in PPI, the present data suggest that Tat<sub>1-86</sub> is more neurotoxic in the developing nervous system *in vivo* compared to Tat<sub>1-72</sub>. The Tat<sub>1-72</sub> isolate protein has never been observed *in vivo*, whereas Tat<sub>1-86</sub> and Tat<sub>1-101</sub> represent clinical isolates (Campbell and Loret, 2009), and may represent more fully the physiological actions of the Tat protein.

The sequences of both Tat<sub>1-86</sub> and Tat<sub>1-72</sub> differ mainly in the presence of the second exon encoded region of the protein. Both versions of the HIV-1 Tat protein have been shown to induce synaptodendritic injury (Bertrand et al., 2013) and neuronal cell death *in vitro* (Pocernich et al., 2005); however, the uptake of extracellular Tat into brain cells by Tat<sub>1-86</sub> is 10 times more efficient relative to the first exon Tat<sub>1-72</sub> (Ma and Nath, 1997). Moreover, the second exon region of the Tat protein contains an RGD sequence; RGD motifs are part of the recognition sequence for cell surface integrin binding (Barczyk et al., 2010). Integrins have a key role in neonatal hippocampal development (Gary et al., 2003; Wakselman et al., 2008), perhaps regulating the survival of hippocampal neurons and the formation of appropriate connectivity (Murase et al., 2011). Thus, the presence of the second exon in Tat<sub>1-86</sub> protein might be expected to produce different effects on hippocampal development, relative to Tat<sub>1-72</sub>.

A design-based stereological study from our laboratory demonstrated long-term neurotoxicity in response to intrahippocampal injection of Tat<sub>1-72</sub> (Fitting et al., 2008a). Tat<sub>1-72</sub> decreased the number of neurons and increased glial cells in specific hippocampal

subfields, and these alterations were correlated with delays in early reflex development (Fitting et al., 2008b). Astrogliosis is frequently reported in pediatric HIV-1 infection (Saito et al., 1994; Tornatore et al., 1994; Vallat et al., 1998) and is suggested to occur only if the insult includes the activation of microglia and the production of inflammatory cytokines in the perinatal CNS (Corasaniti et al., 2001a). HIV-1 infection of microglia/monocytes has been shown to lead to CNS pathology through the release of toxic cytokines (Dickson et al., 1993; Nath et al., 1999; Philippon et al., 1994). Further, perinatal inflammatory cytokine challenges can result in distinct neurobehavioral alterations; e.g., IL-1 $\alpha$  treated rats displaying abnormal startle response and prepulse inhibition after puberty (Tohmi et al., 2004) and IL-6 overexpressing transgenic mice showing a progressive decline in avoidance learning (Heyser et al., 1997). The important role of cytokines in pediatric CNS disease following HIV-1 infection is suggested by previous research that reported significant elevation of inflammatory cytokines, including IL-1 $\beta$  and TNF- $\alpha$  (Foster et al., 2012; Jin et al., 2009; Ketlinskii et al., 1992; Torre et al., 1996). Neither the presence of HIV-1 protein Tat<sub>1-86</sub> nor gp120, at least at the doses our studies typically employ, was sufficient to induce significant gene expression of the inflammatory cytokine IL-1 $\beta$  or inflammatory factors NF $\kappa$ - $\beta$  and I $\kappa$ - $\beta$ . As such, it is unlikely that the induction of an inflammatory response by the HIV-1 Tat<sub>1-86</sub> protein provides any significant contribution to the observed neurobehavioral alterations seen in the present study. Overall, while increased cytokine production may be associated with diffuse gliosis in pediatric AIDS (Saito et al., 1994; Tornatore et al., 1994; Vallat et al., 1998), the present data suggest that activation of inflammatory pathways is not an obligatory response to viral protein induced neurotoxicity that is detectable with behavioral assessments. Nevertheless, the chronic overexpression of inflammatory cytokines in the mammalian CNS, as via the transgenic model approach, does suggest that expression of specific cytokines alone in the intact CNS results in unique neuropathological alterations and functional impairments (Heyser et al., 1997). Given that the present study examined a limited profile of inflammatory indicators at a specific time point, it remains possible that other cytokines might be elevated at different time points post-treatment. As the gene expression reported here does not directly correlate with protein expression, further experiments that include measures of cytokine protein expression may provide more information on inflammatory pathways that may be involved in HIV-1 infection.

In the absence of an overt inflammatory response, a likely cause of the observed developmental and neurobehavioral alterations is Tat-induced injury to synaptodendritic networks. Synaptic dysfunction has been reported in HIV-1+ patients with latent viral infection (Desplats et al., 2013; Gelman and Nguyen, 2010), and, along with dendritic injury (Ellis et al., 2007; Moore et al., 2006), has also been correlated with severity of neurocognitive decline (Everall et al., 1999; Masliah et al., 1997). Single protein (Tat or gp120) transgenic mice studies confirm decreased spine density and/or synaptic dysfunction (Tat, Fitting et al., 2010b; 2013; gp120, Gorantla et al., 2012; Kang et al., 2010); moreover, synaptic alterations are found in HIV-1 transgenic rats expressing both Tat and gp120 proteins throughout development (Roscoe et al., 2014). Tat-induced synaptodendritic damage is highly specific and dependent upon the presence of the cysteine region of the Tat protein (Bertrand et al., 2013). Moreover, Tat-induced synaptodendritic damage occurs prior to cell death, at very low concentrations, and may be reversible (Bertrand et al., 2014).

Another central mechanism of HIV-1 viral protein-induced neurotoxicity is dopamine transporter (DAT) impairment. *In vitro* studies have shown that DAT is targeted by Tat<sub>1-86</sub> (Aksenov et al. 2008; Ferris et al. 2009; Midde et al. 2013; Zhu et al. 2009; Zhu et al. 2011), which induces DAT impairment with direct protein-protein interactions (Zhu et al. 2009) involving an allosteric modulation of DAT by the Tat<sub>1-86</sub> protein (Zhu et al. 2011). In addition, DA-dependent signaling has been identified as a mechanism of HIV-1 protein neurotoxicity (Aksenova et al. 2006; Silvers et al. 2007; Wallace et al. 2006). DA systems are indeed particularly vulnerable to HIV-1-induced neurotoxic insults that may underlie HAND. Reduced DAT levels in the putamen and ventral striatum (Chang et al., 2008; Wang et al., 2004), reductions in DA levels in the substantia nigra (Kumar et al. 2011), and decreases in homovanillic acid (di Rocco et al. 2000) have been associated with neurocognitive deficits in HIV-1+ individuals.

Collectively, there were three main findings: (1) Direct PD1 CNS injection of the HIV-1 protein variant Tat<sub>1-86</sub> induced developmental neurotoxic effects, as witnessed by delays in eye opening, delays in early reflex development and alterations in PPI and between-session habituation of locomotor activity, in the absence of any gross growth deficits. (2) The neurotoxic profile of Tat<sub>1-86</sub> appeared more profound in the developing nervous system *in vivo* relative to that seen with Tat<sub>1-72</sub> (Fitting et al., 2008b) as noted on measures of eye opening, righting reflex, and PPI. (3) Neither the direct PD1 CNS injection of the full-length viral HIV-1 protein variant Tat<sub>1-86</sub>, nor the HIV-1 envelope protein gp120, at doses sufficient to induce neurotoxicity, induced significant expression of the inflammatory cytokine IL-1 $\beta$  or inflammatory factors NF $\kappa$ - $\beta$  and I $\kappa$ - $\beta$ . Additionally, it is of interest that Tat<sub>1-86</sub> contains integrin binding sequences, whereas Tat<sub>1-72</sub> does not, suggesting further investigation of the biological actions of the second exon-encoded polypeptide region of Tat on hippocampal development and plasticity is warranted.

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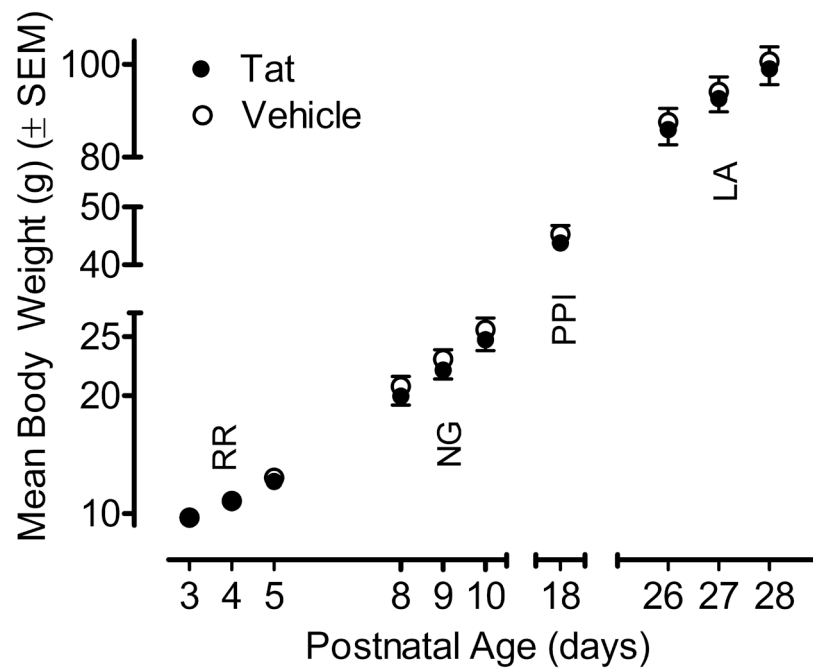
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### Highlights

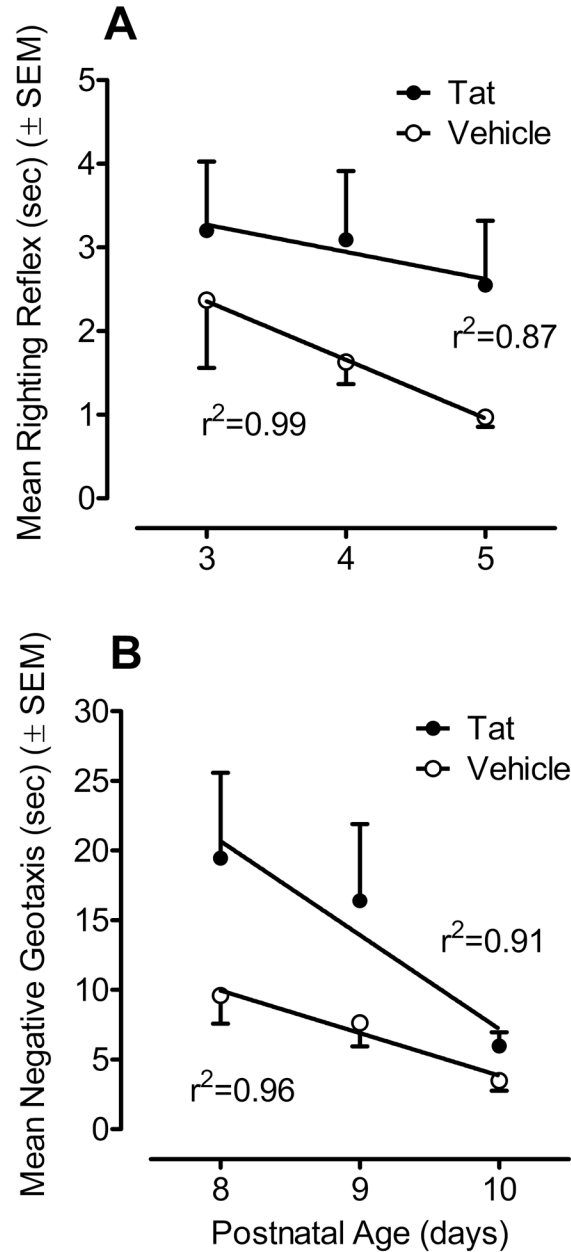
- The neurotoxic profile of Tat<sub>1-86</sub> appeared more profound relative to that of Tat<sub>1-72</sub>.
- Tat<sub>1-86</sub> did not induce significant expression of inflammatory biomarkers.
- The second *tat* exon may uniquely perturb hippocampal development.





**Figure 1.**

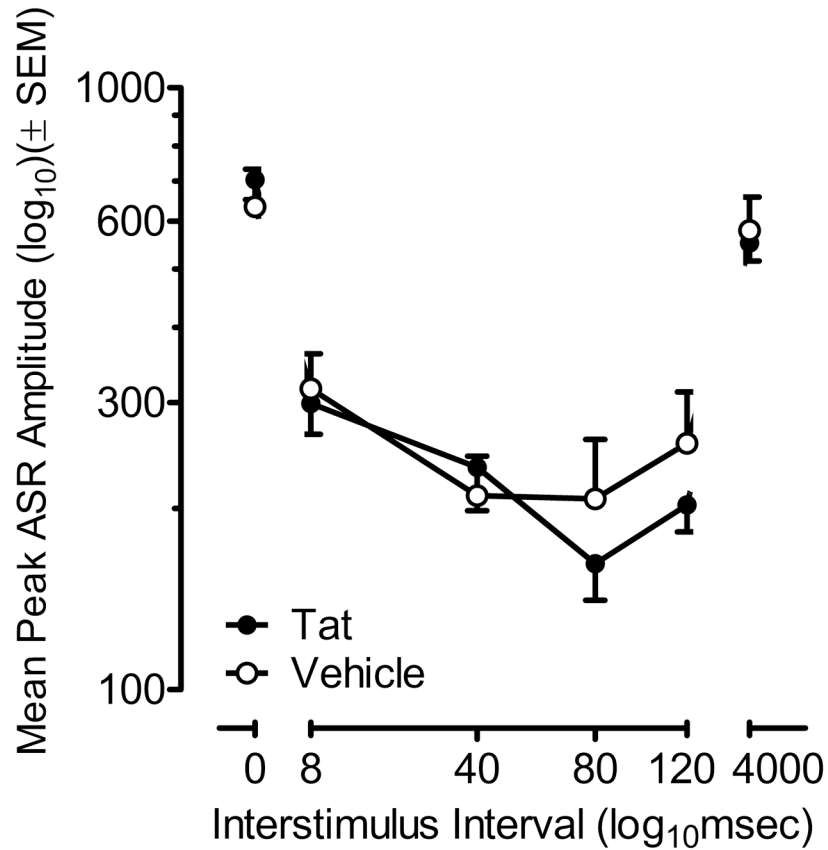
Mean ( $\pm$  SEM) body weight across the various test days by treatment. No significant treatment effect or interaction between treatment and day was noted. RR = righting reflex; NG = negative geotaxis; PPI = prepulse inhibition; LA= locomotor activity.



**Figure 2.**

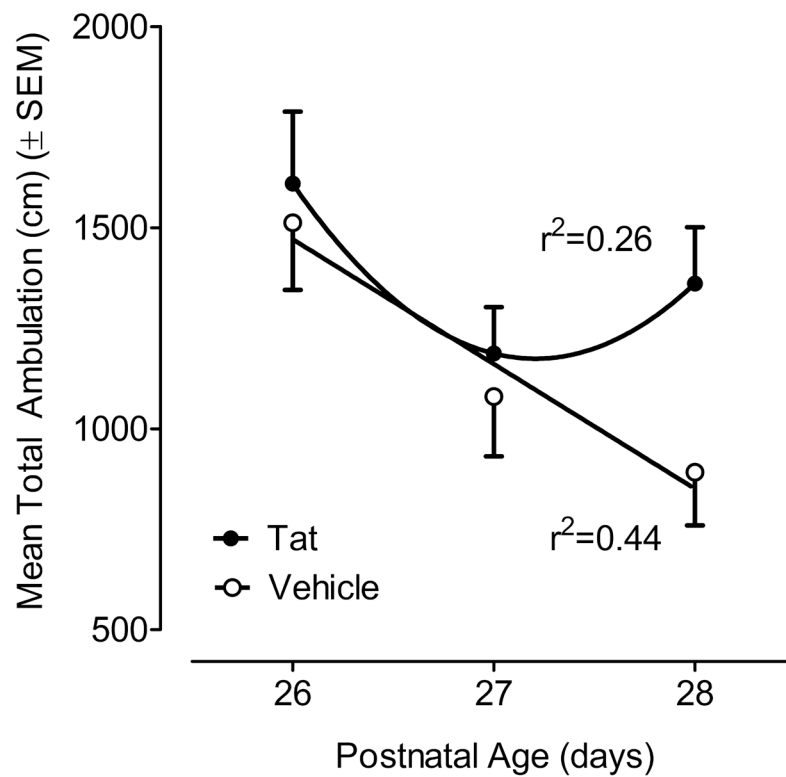
Mean ( $\pm$  SEM) response time with the best fit linear regression across three days for each treatment group. (A) A significant treatment effect was detected for righting reflex across all test days (PD3-5) ( $p < 0.05$ ); Tat<sub>1-86</sub>-treated animals were much slower in the righting reflex compared to the VEH-treated group. A significant test day effect was found ( $p < 0.05$ ) with a prominent linear component ( $p < 0.05$ ), indicating that execution of the righting reflex improved across test days. Planned contrast analyses for each treatment group revealed a significant linear component for the VEH-treated animals ( $p < 0.05$ ) which was not found in the Tat<sub>1-86</sub>-treated group. Thus, the Tat<sub>1-86</sub> treatment appeared to disrupt maturation of the righting reflex across test days. (B) A significant overall treatment effect

was detected for negative geotaxis across all test days (PD8-10) ( $p < 0.05$ ); Tat<sub>1-86</sub>-treated animals had overall slower response latencies compared to VEH-treated animals. A significant day effect was found ( $p < 0.05$ ) with a prominent linear component ( $p < 0.01$ ), indicating that execution of the righting reflex improved across test days. No treatment x test day interaction was detected, suggesting that both groups showed a similar rate of improvement across test days, approximating similar terminal levels of acquisition.

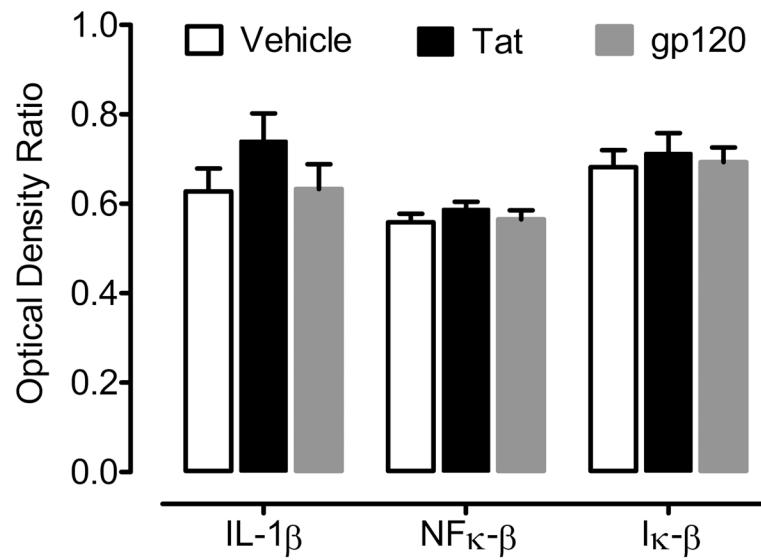


**Figure 3.**

Mean ( $\pm$  SEM) peak ASR amplitude by treatment across ISIs (0–4000 ms). No significant treatment effect was found on control trials (0 and 4000 ms ISIs), suggesting that Tat<sub>1-86</sub> did not affect the animals' baseline startle. Both groups displayed peak inhibition at the 80 ms ISI. The characteristic quadratic fit of the PPI ISI function for the VEH-treated animals was shifted in the Tat<sub>1-86</sub>-treated animals to also include a prominent linear component to the PPI ISI function.



**Figure 4.** Mean ( $\pm$  SEM) total ambulation for each treatment group across the three test days (PD26-28). Between-session habituation was confirmed by a significant effect of test day with a significant linear decrease across test day ( $p = 0.005$ ) for the VEH-treated animals; whereas the between-session habituation of the Tat-treated animals was best represented by a quadratic orthogonal component ( $p = 0.03$ ). The Tat<sub>1-86</sub>-treated group demonstrated significantly greater ambulation on the third session compared to the VEH-treated group ( $p = 0.05$ ).



**Figure 5.**

Mean ( $\pm$  SEM) optical density ratio values of biomarkers reflecting the potential activation of inflammatory pathways as a function of *in vivo* neonatal Tat or gp120 treatment. Statistical analysis failed to detect any significant increase in the expression of IL-1 $\beta$ , NF $\kappa$ - $\beta$ , or I $\kappa$ - $\beta$  in Tat<sub>1-86</sub>-treated (non-significant % increases of 17.9 %, 4.5 %, and 5.3 %, respectively) or gp120-treated (all values within 2 % of control) animals.